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Physical characterization and mechanistic studies of the
[Fe₄S₄]-siroheme catalytic apparatus in sulfite and nitrite reductase
enzymes

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The Ohio State University, 1994
Physical Characterization and Mechanistic Studies of the [Fe4S4]-Siroheme Catalytic Apparatus in Sulfite and Nitrite Reductase Enzymes

Dissertation

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

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June 1994

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DEDICATION

To My Beloved Parents

and

Three Younger Brothers
ACKNOWLEDGEMENTS

It is most humbly that I thank all of the many individuals who have contributed to this thesis. Thanks to former and present graduate students working in this project (Jian Tan, Bonnie Wolfe, Aileen Soriano, Wen Liang).

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FIELD OF STUDY

Chemistry
# TABLE OF CONTENTS

Dedication..................................................................................................................................................ii
Acknowledgements.........................................................................................................................................iii
Vitae................................................................................................................................................................v
Table of Contents........................................................................................................................................vi
List of Abbreviations..................................................................................................................................xii
List of Tables..................................................................................................................................................xiv
List of Figures..............................................................................................................................................xvi

Chapters

I. Overview
   1.1 Background and Significance
       Metalloenzymes.....................................................................................................................................1
       Sulfite Reductase.................................................................................................................................2
   1.2 Goals and Specific Aims of the Dissertation......................................................................................18
   1.3 Methodology.......................................................................................................................................21
   1.4 Dissertation Abstract.........................................................................................................................23

II. Bacterial Growth of *D. vulgaris* (Hildenborough) and Purification of SiR and DV
   Abstract....................................................................................................................................................26
   2.1 Introduction.........................................................................................................................................28
2.2 Materials and Methods
2.2.1 Bacterial Growth of *D. vulgaris* (Hildenborough) .....................31
2.2.2 Isolation and Purification of SiR and DV ...................................32
2.2.3 Chemical Characterization of Enzymes
   - Purity and Molecular Weight ....................................................37
   - Isoelectric Point .................................................................38
   - Electronic Absorption ..........................................................38
   - EPR Spectroscopy .................................................................38
   - Iron Quantitation .................................................................38
   - Sulfide Quantitation ..............................................................39

2.3 Results
Bacterial Growth of *D. vulgaris* (Hildenborough) ...............................39
Fractional Separation of Crude cell solution ........................................40
Purification and Characterization of Recombinant SiR .........................40
Purification and Characterization of DV ...............................................42
Quantitation of Prosthetic Centers in DV ............................................51
EPR Measurements of DV ...............................................................56

2.4 Discussion
Functional Expression of SiR Genes in *Desulfovibrio* species ..............56
Metallation or Demetallation of DV ? A Comparison with related work .........61
EPR Parameters for DV .....................................................................65

III. Kinetic and Mechanistic Studies of Enzymatic Reduction of Inorganic
     Anions by DV

Abstract .............................................................................................67

3.1 Introduction ......................................................................................69

3.2 Materials and Experimental Methods
   Stopped-Flow Kinetics ......................................................................76
   Kinetics of SO$_3^{2-}$ vs NO$_2^-$ Reduction .....................................81

3.3 Results and Discussion
3.3.1 Pre-Steady-State Kinetics Analysis and Mechanistic Implication
   Pre-Steady-State Kinetics .............................................................82
Conformational Gating and Implications for the Reaction Pathway .................................................................143
Putative Structural Mechanisms for Conformational Gating ..........148

V. Direct Electrochemical Studies of SiR and DV

Abstract .........................................................................................................................................................149

5.1 Introduction .............................................................................................................................................152

5.2 Materials and Experimental Methods

5.2.1 Reduction Potential Measurements of the Redox Centers
   Direct Electrochemistry ................................................................. 159
   Controlled Potential Coulometry................................................. 160

5.2.2 Systematic pH Titration Experiments of the [Fe₄S₄] Siroheme Prosthetic Center
   Electrochemical pH Titration Experiments ............................ 164
   Simulation of E° versus pH .......................................................... 167

5.2.3 Characterization of the Redox Thermodynamics of the Prosthetic Center
   Nonisothermal Electrochemistry ................................................ 169

5.3 Results

5.3.1 Reduction Potential Measurements of the Redox Centers
   Determination of Reduction Potentials .................................... 170
   Evidence for Direct Reversible Electrochemistry .................... 178
   Nernstian n .................................................................................. 182

5.3.2 Systematic pH Titration Experiments of the [Fe₄S₄] Siroheme Prosthetic Center
   Effect of pH on Reduction Potentials ....................................... 182

5.3.3 Characterization of the Redox Thermodynamics of the Prosthetic Center
   Evaluation of Redox Thermodynamic Parameters .................. 188
   Dependence of ΔS° on pH ............................................................... 193

5.4 Discussion

5.4.1 Reduction Potential Measurements of the Redox Centers
Comparison of Reduction Potentials for SiR and DV........193
Coupling of the [Fe₄S₄] Cluster and Siroheme ...............197
Reduction Potential of the Second Redox Couple..............197

5.4.2 Systematic pH Titration Experiments of the [Fe₄S₄]-Siroheme Prosthetic Center
pH-Dependence of Redox Potentials.................................200

5.4.3 Characterization of the Redox Thermodynamics of the Redox Centers
Evaluation of Redox Thermodynamic Parameters..............200
Dependence of ΔSO on pH..................................................201

Concluding Remarks..........................................................203

VI. Studies of Electronic/Magnetic Properties of the [Fe₄S₄]-Siroheme Prosthetic Center of SiR and DV by EPR Spectroscopy

Abstract..................................................................................204

6.1 Introduction.........................................................................206

6.2 Materials and Methods
EPR Instrumentations............................................................209
Preparation of Photoreduced Enzymes.................................209

6.3 EPR Theory
Basics Concepts.......................................................................210
g Factors..................................................................................213
Rhombicity..............................................................................215
Relaxation Time.......................................................................224
Power Saturation Measurements..........................................226
Spin Quantitations....................................................................226
Variable Temperature Measurements.................................227

6.4 Results
SiR in Different Oxidation States..........................................229
DV in Different Oxidation States..........................................233
Rhombicity.............................................................................239
Spin Quantitations..................................................................242
Power Saturation Measurements..........................................242
Variable Temperature Experiments.......................................251
6.5 Discussion
Comparison of EPR Spectra Exhibited by Sulfite Reductase Enzymes ................................................................. 260
Heterogeneity in the Power Saturation Levels of Rhombic Siroheme g-Tensor Components ........................................ 261
Exchange-Coupling between the Siroheme and Cluster in Oxidized and Reduced SiR and DV ........................................ 264
Coordination and Redox Properties ........................................ 269
Effect of Cyanide Binding on the Relative \( E^0 \)'s of DV .................................. 271
Concluding Remarks ................................................................. 272

6.6 Appendix .................................................................................. 275

VII. Optical Properties of SiR and DV
Abstract ......................................................................................... 278

7.1 Introduction ................................................................................. 280

7.2 Materials and Methods ........................................................... 284

7.3 Results
SiR in Different Oxidation States .................................................... 288
Ligand-Bound SiR in Different Oxidation States ................................ 290
DV in Different Oxidation States ..................................................... 296
Ligand-Bound DV in Different Oxidation States .............................. 298

7.4 Discussion
Trends in Optical Spectra for Sulfite/Nitrite Reducing Enzymes .............................................................................. 299
Coordination, Redox and Electronic Properties of SiR .......... 302
Coordination, Redox and Electronic Properties of DV ............. 303
Concluding Remarks ................................................................. 304

List of References ........................................................................... 306
LIST OF ABBREVIATIONS

aa  amino acid
ATCC  American Type Culture Collection
DEAE  diethylaminoethyl
D. vulgaris  Desulfovibrio vulgaris
SiR  assimilatory sulfite reductase
DV  desulfoviridin or dissimilatory sulfite reductase
EDTA  ethylenediaminetetraacetate
EPR  electron paramagnetic resonance
FPLC  fast protein liquid chromatography
IEF  isoelectric focussing
MeV+  methyl viologen radical, reduced form
Mr  molecular weight
KP  potassium phosphate
NH2OH  hydroxylamine
NMR  nuclear magnetic resonance
OD  optical density
PAGE  polyacrylamide gel electrophoresis
SDS  sodium dodecyl sulfate
<table>
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<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>APS reductase</td>
<td>adenylyl sulfate reductase or adenosine-5'-phosphosulfate</td>
</tr>
<tr>
<td>HA</td>
<td>hydroxylapatite</td>
</tr>
<tr>
<td>1, 5-IAEDANS</td>
<td>5-(((2-iodoacetyl)amino)ethyl)amino)-naphthalenesulfonic acid</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric pH</td>
</tr>
<tr>
<td>SWV</td>
<td>square wave voltammetry</td>
</tr>
<tr>
<td>PGE</td>
<td>pyrolytic graphite electrode</td>
</tr>
<tr>
<td>E°r</td>
<td>standard reduction potential vs NHE</td>
</tr>
<tr>
<td>Kₐ</td>
<td>association constant</td>
</tr>
<tr>
<td>DMF</td>
<td>dimethylformamide</td>
</tr>
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</table>
LIST OF TABLES

Table 1.1 Various oxidation states of nitrogen and sulfur atom .................................................. 5
Table 2.1 Composition of LS medium ............................................................................................. 33
Table 2.2 Composition of Baars medium .......................................................................................... 34
Table 2.3 Fractions from the first DEAE-52 column of cell solution from *D. vulgaris* (Hildenborough) .......................................................... 41
Table 2.4 Evaluation of iron equivalents per mole of enzyme for fractions isolated from FPLC purification of desulfoviridin ........................................................................ 47
Table 2.5 Characterization of desulfoviridin .................................................................................. 49
Table 2.6 Spin integrations for oxidized and reduced desulfoviridin .............................................. 60
Table 3.1 Pre-steady-state kinetics parameters for desulfoviridin .................................................. 74
Table 3.2 Summary of reaction rate constants at room temperature ............................................ 91
Table 3.3 Pre-steady-state bond cleavage data ................................................................................ 102
Table 3.4 Steady-state activation parameters ................................................................................. 105
Table 3.5 Break-down of steady-state activation parameters ......................................................... 106
Table 3.6 Summary of the factors contributing to the activation free energy .................................. 107
Table 4.1 Rate constants for redox-linked conformational changes .............................................. 134
Table 4.2 Second-order rate constants for direct redox chemistry on the prosthetic centers .................. 136
Table 4.3 Activation energies for redox-linked conformational changes ........................................ 141
Table 5.1  \( E^o \) values for the redox couples in SiR and DV from \textit{D. vulgaris} using SWV and CPC methods

Table 5.2  \( pK_a^{\text{ox}} \) and \( pK_a^{\text{red}} \) values obtained from pH titration studies at 25°C for SiR and DV using SWV electrochemical method

Table 5.3  Redox Thermodynamic Parameters at pH 7.0 and 25°C

Table 6.1  Characterization of different types of low-spin heme proteins by grouping according to rhombicity (V/A) versus Δ/L

Table 6.2  Comparison of EPR spectral data for low spin native oxidized SiR and Mb-N\textsuperscript{3-} adduct

Table 6.3.  Comparison of EPR spectral data for DV and related high spin sulfite reductases

Table 6.4.  Comparison of EPR g-values and power saturation behavior for the reduced Fe/S cluster in sulfite reductase enzymes

Table 6.5  Spin integrations for oxidized and reduced DV

Table 6.6.  Comparison of EPR g-values and power saturation behaviors

Table 7.1  Comparison of physicochemical data for a variety of sulfite and nitrite reductases

Table 7.2  Comparison of optical characteristics for oxidized and reduced sulfite reducing enzymes from prokaryotic and eukaryotic sources
LIST OF FIGURES

Figure 1.1 The biological sulfur cycle.................................................................4
Figure 1.2 The sulfate reduction pathway..........................................................8
Figure 1.3 Structures of different porphyrin rings in biological systems...........12
Figure 1.4 The proposed structure of the [Fe₄S₄]-siroheme prosthetic center...........................................................................................................13

Figure 2.1 EPR spectra of anaerobic solutions of 31 μM native oxidized sulfite reductase obtained in 50 KP buffer (pH 7.5) at 11.0 K.......................43
Figure 2.2 EPR spectra of anaerobic solutions of 31 μM reduced sulfite reductase (SiR) obtained in 50 KP buffer (pH 7.5) at 13.2 K...........44
Figure 2.3 Absorption spectrum of oxidized recombinant SiR taken with 9 μM enzyme in 100 mM potassium phosphate buffer, pH 7.6, at 298 K.................................45

Figure 2.4 FPLC chromatogram from the final purification step for desulfoviridin...........................................................................................46
Figure 2.5 Electronic absorption spectrum of the oxidized siroheme extracted from desulfoviridin in 0.1 M NaOH..............................................53
Figure 2.6 Electronic absorption spectrum of the pyridine complex with extracted chromophore obtained from desulfoviridin after: (A) acidic acetone extraction and addition of pyridine (to 25 %); and (B) alkaline extraction in 0.1 M NaOH / 25 % pyridine.....................54
Figure 2.7 EPR spectrum of an anaerobic solution of 0.11 mM native oxidized desulfoviridin obtained in 10 mM potassium phosphate buffer (pH 7.5) at 8 K.................................................................57
Figure 2.8  A spectrum of a desulfoviridin sample obtained after successive chromatography on hydroxy apatite, DE-52, G-200 sephadex, and a final DE-52 column prior to final FPLC purification is shown for comparison........................................................................................................58

Figure 2.9  Spectrum of fully reduced desulfoviridin obtained by deazaflavin photoreduction....................................................................................................................................................59

Figure 2.10 Diagram shows the exchangeable protons of siroheme in D_2O.....63

Figure 3.1 Summary of a working model for enzymatic reduction of oxidized sulfur and nitrogenous substrates catalyzed by [Fe_4S_4]-siroheme catalyzed centers...................................................................................................................................................73

Figure 3.2 Experimental setup for stopped-flow rapid kinetics measurements..................................................................................................................................................78

Figure 3.3  (A) Optical changes of DV during ligand binding to reduced form. (B) Optical changes of DV during redox change from reduced to oxidized forms..........................................................................................................79

Figure 3.4  Scheme outlines sulfate reduction................................................................................................................................................................................................................83

Figure 3.5 A typical fit to a rise-fall rate profile.............................................................................................................................................................................................................86

Figure 3.6 Stopped-flow kinetics data for substrates and ligands................................................................................................................................................................................................................88

Figure 3.7 EPR spectra observed during turnover of 14NO_2^- by desulfoviridin........................................................................................................................................................................................................93

Figure 3.8 Pre-steady-state data. Plot of R ln (k_rh/kT) versus 1/T for a variety of substrate molecules......................................................................................................................................................................................................99

Figure 3.9 General free energy profiles for substrate turnover showing the components of ΔG^*, ΔG_0^*, ΔG_d and ΔG_t^*........................................................................................................................................................................................................104

Figure 3.10 Isokinetic plot of ΔH^* versus ΔS^*........................................................................................................................................................................................................110

Figure 4.1 Structure of the fluorescence probe, 1,5-IAEDANS.........................................................................................................................................................................................................123

Figure 4.2 Emission profiles for free versus enzyme-bound 1,5-IAEDANS.........................................................................................................................................................................................................128
Figure 4.3  A typical fit to a one-exponential rate profile for the change in emission from 1,5-IAEDANS labeled desulfoviridin..................129

Figure 4.4  A typical fit to a one-exponential rate profile for the change in emission from the natural low-level fluorescence exhibited by desulfoviridin.................................................................131

Figure 4.5  Plot of $R \ln (k h/kT)$ versus $1/T$..........................................................138

Figure 4.6  Model for redox-linked gating of enzyme activity..............................145

Figure 5.1  Reaction entropies for selected transition metal and metalloprotein couples............................................................................................................................158

Figure 5.2  Electrochemical cell for controlled potential coulometry......................161

Figure 5.3  Plot of the reduction potential ($E^0'$) of a hypothetical redox center vs solution pH when the couple has $pK_{a_{ox}}$ and $pK_{a_{red}}$..........................166

Figure 5.4  Thermodynamic cycle for the coupled electron- and proton-transfer reactions displaying the potentiometric behavior illustrated in Figure 5.3.................................................................168

Figure 5.5  A typical square wave voltammogram obtained from a 10 μM DV solution with 8 mM Cr(NH$_3$)$_6^{3+}$ in 50 mM potassium phosphate/10 mM NaCl (298 K, pH 7.5) under Ar(g)....................172

Figure 5.6  A typical square wave voltammogram obtained from an 8 μM SiR solution with 8 mM Cr(NH$_3$)$_6^{3+}$ in 10 mM potassium phosphate/10 mM NaCl under Ar(g) (278 K, pH 7.5)....................173

Figure 5.7  Controlled Potential Coulometry data for DV. $[DV] = 450$ μM, $[mediator] = 50$ μM (DV : mediator = 9 : 1)..............................................175

Figure 5.8  Dependence of the peak current ($i_p$) on the square root of the frequency of the applied potential pulse ($v^{1/2}$) for the first redox couple in DV........................................................................179

Figure 5.9  Dependence of the peak current ($i_p$) on the square root of the frequency of the applied potential pulse ($v^{1/2}$) for the siroheme redox couple (A) and the cluster redox couple (B) in SiR..............180
Figure 5.10 Variation of $E^\circ$ (mV) with pH, showing a fit to the equation, $E_m = E^\circ + 59 \log \left( \frac{K_{a\text{red}} + [H^+]}{K_{a\text{ox}} + [H^+]} \right)$ for SiR: (A) siroheme signal, and (B) cluster signal. 

Figure 5.11 Variation of $E^\circ$ (mV) with pH, showing a fit to the equation, $E_m = E^\circ + 59 \log \left( \frac{K_{a\text{red}} + [H^+]}{K_{a\text{ox}} + [H^+]} \right)$ for the first redox couple (P') in DV. 

Figure 5.12 Temperature dependence of the first reduction potential in DV. 

Figure 5.13 Temperature dependence of the reduction potentials of the siroheme - cluster redox couples in SiR: (A) siroheme signal; and (B) cluster signal. 

Figure 5.14 pH dependence of $\Delta S^0$ for (A) the siroheme redox couple in SiR; (B) the cluster redox couple in SiR; and (C) the first redox couple in DV. 

Figure 5.15 Summary of redox reactions: (A) DV, and (B) SiR. 

Figure 5.16 pH dependence of $\Delta S^0$ for horse heart cytochrome c. 

Figure 6.1 Illustration of the Zeeman energies of electrons in an external magnetic field $H$. 

Figure 6.2 Representative EPR spectra of different systems: isotropic, axial, rhombic. 

Figure 6.3 Energy diagram for one-electron orbitals for $3d^5 e^-$ with axial distortion $A$ and rhombic distortion $V$. 

Figure 6.4 Crystal field correlation diagrams for low-spin ferric hemoproteins. 

Figure 6.5 Diagram shows the population of the three Kramers doublets in $S = 5/2$ at different temperatures. 

Figure 6.6 Stacked EPR spectra of SiR with 0, 1.2 and 2 electron equivalents added. 

Figure 6.7 Stacked EPR spectra of DV with 0, 1 and 2 electron equivalents added showing the simultaneous appearance of oxidized siroheme.
and reduced cluster of desulfoviridin after addition of one-electron equivalent

Figure 6.8 Representative power saturation data for each g-tensor component of the oxidized SiR

Figure 6.9 Representative power saturation data for each g-tensor component of DV

Figure 6.10 Comparison of the temperature dependence of the g-tensor components of the low spin hemes in (A) oxidized SiR, and (B) myoglobin + N3-

Figure 6.11 Temperature dependence of the g = 6.30 feature of the high spin siroheme signal in oxidized desulfoviridin

Figure 6.12 Plot of the fractional population of oxidized siroheme (foS) versus the number of electron equivalents taken up by the redox pair (x) to estimate AE° (ES° - EC°)

Figure 6.13 Stacked EPR spectra of fully reduced SiR with and without bound AsO2-

Figure 6.14 Schematic illustration of the influence of CN- binding on heme potentials, where AE° = ES°(free) - ES°(CN-)

Figure 7.1 Structure of 5'-deazaflavin synthesized for photoreduction

Figure 7.2 Absorption spectra of oxidized, 1 e- reduced, and 2 e- reduced SiR

Figure 7.3 Comparison of the absorption spectra of oxidized SiR in the native form and SiR-AsO2- adduct

Figure 7.4 Absorption spectra of SiR in fully reduced forms with and without AsO2-

Figure 7.5 Absorption spectra of oxidized and 2 e- reduced DV

Figure 7.6 Absorption spectra of DV in fully reduced forms with and without AsO2-
CHAPTER I

Overview

1.1 BACKGROUND AND SIGNIFICANCE

Metalloenzymes

Transition metal ions have been extensively utilized by nature in the design of metalloproteins and enzymes that catalyze redox/nonredox reactions, store or transfer important cellular substrates, and regulate the activities of other biological macromolecules. This reflects the diverse coordination chemistry (ligand preference, geometry, redox state, kinetic and thermodynamic stabilities and labilities) of the d-block elements, and it is not surprising that these metals occur in a wide variety of functional roles. Transition metals are most often associated with oxygen-binding and oxido-reductase proteins and enzymes. The latter function reflects the variable oxidation states available to these cations.

Electron-transfer reactions have been a continuing source of fascination to chemists and have proved central to the development of modern coordination chemistry. Redox proteins and enzymes are thus
the best characterized and most widely studied by inorganic biochemists. They exhibit interesting electronic and magnetic phenomena and display a variety of redox chemistry that raises many important and fundamental questions concerning reaction mechanisms.

Nature employs a large number of oxido-reductase enzymes, reflecting the necessarily complex biological environment in which they function. The polypeptide chains that make up the protein regulate both the physicochemical properties of the redox centers, and interactions of the protein with other biological molecules. Protein side chains may enhance the reactivity of a metalloprosthetic group. Despite the obvious complexities of these molecules there are a few simplifying features; many possess common prosthetic centers with related functional properties. In this dissertation, the prosthetic unit being studied is a combination of two redox centers, [Fe4S4]-siroheme couple, which is a catalytic apparatus for multielectron enzymatic reduction of sulfite and nitrite. It is common to a group of metalloenzymes named sulfite reductase.

**Sulfite Reductase**

Sulfur-containing compounds are essential components of the biosphere. Reduced sulfur in the form of thiols, disulfides, or thioethers in organic molecules accounts for up to 1% of cellular material. However, extracellular sulfur exists predominantly in the oxidized form and is commonly distributed as sulfates in soils, rocks, rivers, the oceans, or as sulfur oxides, a minor component of the atmosphere.
Sulfate must be reduced to the oxidation level of $S^{2-}$ before the sulfur atom can be utilized to form cysteine in a process called assimilatory sulfate reduction. When the sulfur atom is released from an organic skeleton it can be oxidized by soil organisms to its highest oxidation state ($SO_4^{2-}$). One may think of a sulfur cycle in nature much like the nitrogen cycle, since there are actually many similarities between the biochemistry of S and N. The sulfur atom, like N, exits in several inorganic forms. Table 1.1 shows the variety of oxidation numbers of S and N.

The biological reduction of sulfate is critically important for life processes and is of comparable importance to the more thoroughly investigated problem of nitrogen fixation. Sulfur, like all the other biological elements, is transformed and translocated in the biosphere by a combination of biological and chemical agencies. The biological sulfur cycle is depicted in Figure 1.1. Sulfate ($SO_4^{2-}$) is reduced to sulfide ($S^{2-}$) by dissimilatory sulfate-reducing bacteria and provides substrates for sulfide-oxidizing bacteria that convert it, through elemental sulfur ($S^0$), back to sulfate. In assimilatory sulfate reduction the sulfur of sulfate passes through the sulfide oxidation level and is used in the formation of amino acids (RSH) before incorporation into bacteria, plants or higher organisms. These are eaten by animals and the sulfur is eventually returned to the cycle as sulfide after the break down and putrefaction (by bacteria) of the dead organism.

Sulfite reduction is thus a step in the process of reducing sulfate to sulfide in both sulfate-reducing bacteria (Millet, 1955; Ishimoto and
Colored sulfur bacteria, *Thiobacillus*, etc.

Sulfur oxidation

Sulfur oxidation:
- Beggiatoa,
- Some thiobacilli,
- Colored sulfur bacteria, etc.

Sulfide oxidation:
- *Desulfiuromonas*,
- *Campylobacter*,
- *Desulfovibrio* etc.

Sulfur reduction:
- *Desulfovibrio*,
- *Desulfotomaculum*,
- *Desulfobacter*, etc.

Sulfate reduction:
- *Desulfovibrio*,
- *Desulfotomaculum*,
- *Desulfobacter*, etc.

Proteolysis

Protein synthesis

Yeast, bacteria, plants

Figure 1.1 The biological sulfur cycle (Postgate, 1984).
Table 1.1 Various oxidation state of nitrogen and sulfur atom.

<table>
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<tr>
<th>Oxidation Number</th>
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<th>Nitrogen</th>
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<tr>
<td>+6</td>
<td>$\text{SO}_4^{2-}$</td>
<td>-</td>
</tr>
<tr>
<td>+5</td>
<td>-</td>
<td>$\text{NO}_3^-$</td>
</tr>
<tr>
<td>+4</td>
<td>$\text{SO}_3^{2-}$</td>
<td>-</td>
</tr>
<tr>
<td>+3</td>
<td>-</td>
<td>$\text{NO}_2^-$</td>
</tr>
<tr>
<td>+2</td>
<td>$\text{S}_2\text{O}_3^{2-}$</td>
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</tbody>
</table>
Fujimoto, 1961) and sulfate-assimilating organisms (Wilson et al., 1961). In the latter organisms, the enzymes responsible for the reduction of sulfite to sulfide in the synthesis of sulfur-containing cell constituents have been obtained (Naiki, 1965; Yoshimoto et al., 1968; Kamin et al., 1967; Siegel et al., 1973; Yoshimoto and Sato, 1968; Asada et al., 1969) and characterized as single proteins, sulfite reductases [hydrogen-sulfide: NADP oxidoreductase, EC 1.8.1.2]. The dissimilatory sulfate-reducing bacteria comprise a specialized group of microorganisms that reduce sulfate to hydrogen sulfide as the terminal electron transport system for their respiratory pathways (Campbell and Postgate, 1965; Postgate, 1955; Postgate and Campbell, 1966), in contrast to conventional aerobic respiration which reduces oxygen to water. Except for the small amount of reduced sulfur assimilated by the organism, most sulfide is released into the external environment as sulfide ion, which is usually hydrolysed to free H$_2$S (Postgate, 1984).

As in the nitrogen cycle, some anaerobic bacteria such as *Desulfovibrio vulgaris* utilize sulfate as a terminal oxidizing agent in a similar fashion to those microorganisms that perform nitrate respiration. This is called dissimilatory sulfate reduction and H$_2$S is the reduced form of sulfur produced. Sulfite and nitrite reducing enzymes are essential for the biosynthesis of amino acids and nucleotides that contain reduced sulfur and nitrogen centers. Green plants, fungi, and many species of bacteria can utilize sulfate as their only sulfur source by taking sulfates from the fully oxidized to the fully reduced state, prior to incorporation into sulfur-containing amino acids or metabolites. In contrast, animals (from protozoa to man) do not perform assimilatory sulfate reduction
and ultimately depend on plants and/or microbes as a source of reduced sulfur. Therefore, the enzymatic reduction of sulfate (or sulfite) is as important as nitrogen fixation for life processes. Despite their fascinating properties, this group of microbes has been neglected in biological research because they are odorous, difficult to grow (strictly anaerobic), and many of the intrinsic enzymes and proteins are difficult to isolate and purify in high yield. However, they have been shown to exhibit novel chemistry and physiology by those who are persistent enough to work with them.

The sulfate-reducing bacteria reduce inorganic sulfate to sulfite through the intermediary formation of adenosine-5'-phosphosulfate (APS) (Peck, 1959; Peck and LeGall, 1982; Ishimoto and Fujimoto, 1959). The subsequent reduction of APS to AMP and sulfite is catalyzed by APS-reductase (Peck, 1961). Sulfite reductase catalyzes the six-electron reduction of sulfite to sulfide. Figure 1.2 illustrates the sulfate reduction pathway. The sulfate ion is first activated by ATP to form APS in a reaction catalysed by ATP sulfurylase (or called sulfate adenylyl transferase) (Peck, 1959 & 1962). Subsequently APS is reduced to sulfite ($SO_3^{2-}$) and AMP by APS reductase. Finally the six-electron reduction of sulfite to sulfide is catalyzed by a single enzyme called sulfite reductase (Peck and LeGall, 1982), which is the focus of this thesis.

Based on physiological function, sulfite reductases may be grouped into two general categories: (1) assimilatory sulfite reductases which are involved in the synthesis of sulfur-containing cell constituents; and (2) dissimilatory sulfite reductases which participate in the respiratory
Figure 1.2  The sulfate reduction pathway.
pathways of sulfate-reducing bacteria. One type of assimilatory enzyme is produced in green plants, fungi and some bacteria (Siegel et al., 1973; Yoshimoto and Sato, 1968; Asada et al., 1969), while the other type is found only in anaerobic bacteria (Methanosarcina barkeri, Desulfuromonas acetoxidans and Desulfovibrio vulgaris [Hildenborough]) (Lee et al., 1973b; Huynh et al., 1984; Moura et al., 1968). Several kinds of dissimilatory-type sulfite reductase have been identified: (1) desulfoviridin from Desulfovibrio gigas, D. salexigens, and D. vulgaris (Lee and Peck, 1971; Lee et al., 1973; Czechowski et al., 1986); (2) desulforubidin from D. desulfuricans (Norway strain) (Lee et al., 1973a); (3) P-582 from Desulfitomaculum (Dt.) ruminis and Dt. nigrificans (Trudinger, 1970; Akagi and Adams, 1973); and (4) desulfofuscidins from D. thermophilus (LeGall and Fauque, 1988) and Thermodesulfobacterium commune (Hatchikian and Zeikus, 1983). Dissimilatory-type sulfite reductases are also found in the photosynthetic bacteria (Schedel, 1979), Thiobacilli (Schedel, 1975) and acetoclastic methanogenic bacteria (Moura, 1982).

In the presence of reduced methyl viologen, purified assimilatory sulfite reductase can catalyze the six-electron reduction without formation of free intermediates (Siegel, 1968; Prabhakararao, 1969), while dissimilatory sulfite reductase reduces sulfite to sulfide with accumulation of trace amounts of trithionate and thiosulfate (Lee, 1971; Findley, 1969; Kobayashi, 1969, 1972). It has been suggested that sulfite is reduced by D. vulgaris to sulfide through a dissimilatory pathway involving thiosulfate, and possibly, trithionite. However, the production of trithionate and thiosulfate is an irreversible process, and there is evidence to suggest that this trithionate pathway may not occur in intact
cells (Akagi, 1981; Peck, 1982). Nevertheless, these observations suggest that there are intrinsic differences in the active site chemistry between the assimilatory and dissimilatory enzymes.

Sulfate (or sulfite) reduction is one example of a series of enzymatic redox reductions of gaseous molecules or small inorganic anions that are key reactions in numerous metabolic pathways. These oxido-reductase enzymes are the principal components of important respiratory pathways and play key roles in anabolic and catabolic reactions in cellular metabolism (LeGall & Postgate, 1973; LeGall & Fauque, 1988; Peck & LeGall, 1982). Important examples of reactions catalyzed by this class of enzyme include the conversion of $\text{N}_2 \rightarrow \text{NH}_3$ (nitrogenase), $\text{H}_2$ (hydrogenase), $\text{SO}_4^{2-} \rightarrow \text{SO}_3^{2-} \rightarrow \text{S}^2-$ (sulfate or sulfite reductases), $\text{NO}_3^- \rightarrow \text{NO}_2^- \rightarrow \text{NH}_3$ or $\text{N}_2$ (nitrate or nitrite reductases), and $\text{O}_2 \rightarrow \text{H}_2\text{O}$ (cytochrome c oxidase) (Higuchi et al., 1987; Huynh et al., 1984; Grande et al., 1983, Bastian et al., 1988; Wackett et al., 1988; Cowan and Sola, 1990; Tan and Cowan, 1991; Lee et al., 1973; Averill et al., 1982; Godden et al., 1991; Larsen, 1992; Shapleigh et al., 1992). Such enzymes possess at least one, and frequently several redox centers that serve as sites for the influx or efflux of electrons and/or the coordination of substrate molecules prior to oxidation or reduction (Cowan, 1993). They contain more than one prosthetic group in order to perform two functions: to store electrons efficiently, as well as to bind and activate substrates for the subsequent redox reactions. These basic redox units may occasionally be coupled together to form centers with distinct electronic and chemical properties. Such coupled centers are employed by this class of sulfite reductase.
The sulfite reductases isolated from various sources have been studied by a number of researchers, and it has been found that two redox centers (siroheme and [Fe₄S₄] cluster) are linked together by a bridging ligand to a prosthetic group. The redox center (siroheme) is the active site for sulfite binding and reduction (Siegel et al., 1982; Young & Siegel, 1988) in sulfite reductases. By various spectroscopic and chemical analyses, the siroheme structure has been found to be an iron porphyrin with the methyl groups attached to adjacent pyrrole rings of the porphyrin nucleus, called iron isobacteriochlorin (Murphy et al., 1973; Scoot et al., 1978). Figure 1.3 shows the structures of different porphyrin rings commonly found in biological systems. Because of the doubly reduced nature of two pyrrole rings, sirohemes possess higher electron densities on the macrocycle ring in comparison to other systems. Siroheme is connected to the [Fe₄S₄] cluster through the bridging ligand (X), while the cluster is attached to the protein polypeptide through cysteine by covalent bonding. Siroheme (through the negatively charged carboxylates on the macrocycle) probably forms numerous salt bridges to a cluster of positively charged residues in the active site. Figure 1.4 illustrates the proposed structure of this [Fe₄S₄]-siroheme prosthetic center.

The assimilatory sulfite reductase (NADPH-sulfite reductase) of E.coli is one the most extensively studied redox enzymes. It consists of two parts: the flavoprotein (α, SiR-FP) and the hemoprotein (β, SiR-HP). The enzyme is an oligomeric complex (α₈β₄). SiR-FP is an octamer containing identical 66-kDa peptides and 4 FAD, 4 FMN per octamer (Ostrowskiet al., 1989b). It catalyzes electron transfer from NADPH, the physiological reductant, to SiR-HP, which serves as the site of sulfite
Figure 1.3  Structures of several types of macrocycles commonly found in biology. The oxidized, reduced, and doubly reduced rings are termed porphyrin, chlorin, and bacteriochlorin or isobacteriochlorin, respectively. The latter two are distinguished by the relative positions of the reduced pyrrole rings [adjacent (cis) or opposite (trans)]. Siroheme falls in the category of an iron isobacteriochlorin.
assimilatory sulfite reductase (SiR)  
L = His, X = S^{2-}

dissimilatory sulfite reductase (DV)  
L = H_2O, or vacant, X = S^{2-}

**Figure 1.4** The proposed structure of the [Fe_4S_4]-siroheme prosthetic center in the active site of desulfoviridin and the low molecular weight assimilatory sulfite reductase. Desulfoviridin contains a pentacoordinate high-spin siroheme with no protein-derived ligand L. It is not clear if H_2O binds to the axial site in desulfoviridin. The axial ligand (L) in the case of the smaller assimilatory sulfite reductase is likely to be His (Cowan and Sola, 1990). In both cases the bridging ligand X is most likely S^{2-} or HS^{-}.
reduction (Siegel and Davis, 1974; Siegel et al., 1982). SiR-HP is a tetramer of identical 63 kDa peptides, each containing one siroheme coupled via a bridging ligand to an [Fe₄S₄] cluster (Ostrowski et al., 1989a). SiR-HP subunit can be isolated from the NADPH-sulfite reductase by chromatography (Siegel and Davis, 1974), and can then be studied as an isolated unit. It has been found that the SiR-HP subunit can catalyze both sulfite and nitrite reduction at high rates if supplied with a suitable artificial electron donor (Siegel et al., 1982).

Extensive spectroscopic characterizations have been performed on *E.coli* SiR-HP (Christner et al., 1983 a & b; Christner et al., 1984; Cline et al., 1986; Madden et al., 1989), and a preliminary 3 Å X-ray crystal structure in the oxidized state has been published (McRee et al., 1986). The X-ray data shows that the siroheme iron is located 4.4 Å from the corner iron of the [Fe₄S₄] cluster. These two iron atoms appear to be bridged by a common ligand, possibly the cysteine sulfur from the polypeptide chain. The [Fe₄S₄] cluster cubane is located at one face of the siroheme, and the groups are so close together that one of the cubane sulfur atoms is in van der Waals contact with the edge of the siroheme macrocycle. The sixth coordination site of the siroheme in oxidized SiR-HP appears to be unoccupied and is relatively exposed to solvent, which is consistent with the high-spin ferric heme assignment from optical and EPR studies (Siegel et al., 1982). ENDOR data also supports the five-coordinate nature of the siroheme Fe(III) with a cysteine (or serine) as the only axial ligand. Two strongly interacting protons were seen with coupling consistent with that expected for a dipolar interaction of the β-CH₂ and α-CH protons on a bridging cysteine (or serine) ligand bound to the siroheme iron. No
proton ENDOR couplings were seen at values expected for an H$_2$O ligand to the heme iron, and the only $^{14}$N couplings observed were those expected for the pyrrole/pyrrolline ligands of the siroheme macrocycle. Resonance Raman spectra on oxidized SiR-HP strongly suggest that a cysteine sulfur, rather than a serine oxygen, is in fact the ligand that bridges the two redox centers. It shows evidence for two sulfur isotope-sensitive Fe-S vibrations excited by laser irradiation in the siroheme Soret band region (Madden et al., 1989). These bands do not appear to arise from the [Fe$_4$S$_4$] cluster itself, and can be accounted for by the symmetric and anti-symmetric stretches of the putative Fe$_{heme}$-S-Fe$_{cluster}$ bridge.

Exchange-coupling between siroheme and cluster in oxidized SiR-HP is evidenced by Mossbauer and ENDOR studies of $^{57}$Fe-substituted SiR-HP (Christner et al., 1981; Cline et al., 1985). This exchange-coupling is maintained in reduced SiR-HP (Janick and Siegel, 1982 & 1983; Christner et al, 1983a; Cline et al., 1986), and in SiR-HP complexes with a variety of heme ligands (Christner et al., 1983b), indicating that close interaction between the siroheme and [Fe$_4$S$_4$] cluster is probably of significance during the catalytic cycle of the enzyme.

The dissimilatory sulfite reductases isolated from a number of sulfate-reducing bacteria are oligomeric with a molecular weight of ca. 200 kDa (Lee et al., 1973b). Desulforubidin, the dissimilatory enzyme from D.baculatus strain DSM, contains two siroheme and four [Fe$_4$S$_4$] clusters per molecule. Mossbauer measurements demonstrated that the sirohemes are high spin and each siroheme is exchange-coupled to a [Fe$_4$S$_4$] cluster (Moura et al., 1988). It is also shown, by Mossbauer
spectroscopy, that desulfoviridin from $D. gigas$ has an exchanged $[\text{Fe}_4\text{S}_4]$-siroheme couple (Moura et al., 1988).

A novel group of assimilatory-type sulfite reductases isolated from three anaerobic bacteria, $\text{Methanosarcina barkeri}$, $\text{Desulfuromonas acetoxidans}$, and $D. vulgaris$ (Hildenborough), have proven to be particularly useful for detailed studies (Moura et al., 1986; Tan and Cowan, 1991, Cowan and Solo, 1990; Tan et al., 1991). The bridging ligand in the enzyme from $D. vulgaris$ is found to be inorganic sulfide instead of a sulfur from a cysteine of the peptide chain (Tan and Cowan, 1991).

The assimilation of nitrate by higher plants and some microorganisms occurs in two steps: the two-electron reduction of nitrate to nitrite; and the six-electron reduction of nitrite to ammonia (Vega and Kamin, 1977). The second step is carried out by a ferredoxin-nitrite reductase that has been purified from higher plants (spinach and chlorella), algae, and fungi (Hucklesby et al., 1972; Zumft, 1972; Vega et al, 1975; Vega and Kamin, 1977). Lancaster et al. (1979) have demonstrated that spinach ferredoxin-nitrite reductase contains one siroheme and one $[\text{Fe}_4\text{S}_4]$ cluster. Spinach ferredoxin-sulfite reductase and nitrite reductase both catalyze either ferredoxin, or reduced methylviologen-dependent six-electron reduction of sulfite and nitrite (Krueger and Siegel, 1982a). EPR data demonstrates that exchange-coupling exists between siroheme and the $[\text{Fe}_4\text{S}_4]$ cluster in spinach sulfite reductase, and preliminary Mossbauer data suggests the possibility of such an interaction in the spinach ferredoxin-nitrite reductase (Krueger & Siegel, 1982b).
Based on these studies, the exchange-coupled \([\text{Fe}_4\text{S}_4]\)-siroheme prosthetic center appears to be common as the active site for all sulfite reducing enzymes (Krueger and Siegel, 1982b; Huynh et al., 1984; Moyra et al., 1986 & 1988; Ostrowski et al., 1989; Cowan and Solo, 1990; Wolfe et al., 1994) and several nitrite reductases (Murphy et al., 1974; Vega et al., 1975; Lancaster et al., 1979). Despite the differences in their reaction mechanisms, both types of sulfite reductase were shown to contain sirohemes and \([\text{Fe}_4\text{S}_4]\) clusters (Murphy, 1973; Liu, 1979; Hall, 1979).

Mossbauer studies of the hemoprotein subunit of the \(E.\ coli\) NADPH-sulfite reductase disclosed that the siroheme and the \([\text{Fe}_4\text{S}_4]\) clusters are exchange coupled (Christner, 1981, 1983, 1983). The elegant studies of Siegel and coworkers on \(E.\ coli\) sulfite reductase have provided a fairly clear picture of the coupled \([\text{Fe}_4\text{S}_4]\)-siroheme prosthetic site that is common to sulfite and many assimilatory nitrite reducing enzymes. However, recently, this proposal has been challenged by Hagen and coworkers who claimed the presence of a novel \([\text{Fe}_6\text{S}_6]\) cluster and the absence of coupling between the \([\text{Fe}_4\text{S}_4]\) cluster and siroheme in desulfoviridin from \(D.\ vulgaris\) (Hildenborough). Demetallion of the siroheme was also suggested. This controversy emphasizes the necessity for re-evaluation of the formulation of the prosthetic center (Wolfe, et al, 1994). Although this model has recently been questioned, studies in our own laboratory on sulfite reducing enzymes isolated from the bacterium \(Desulfovibrio vulgaris\) (Hildenborough), fully support the \([\text{Fe}_4\text{S}_4]\)-siroheme coupled unit as prosthetic center.

It is intriguing how these prosthetic groups interact with each other to carry out the desired reaction within an enzyme-substrate complex.
Despite many years of study the details of molecular mechanisms for metalloredox enzymes are only now beginning to emerge. In large part the difficulties of handling enzymes that are often membrane-bound and multimeric, and containing multiple prosthetic units (frequently oxygen-sensitive), have impeded rapid progress. As a result, a detailed mechanistic understanding of reaction pathways for these enzymes is still lacking. The recent availability of relatively high resolution X-ray structural data on nitrogenase and copper nitrite reductase has provided useful insight as to the disposition of prosthetic sites and possible orientations of bound substrate, and allows speculative mechanistic schemes to be proposed. However, relative to the accumulated wealth of spectroscopic data, little headway has been made in understanding the molecular mechanisms of these reactions.

For the class of redox enzymes that act on small molecular or ionic substrates, the broader issues of how the prosthetic center functions in concert with active site residues to bind substrate and catalyze reaction chemistry have scarcely been addressed in quantitative detail. With regards to the reaction pathway of sulfite reduction, there are few literature reports on the molecular details (Tan and Cowan, 1991; Lui et al, 1993 & 1994). Unraveling the underlying mechanisms of enzymatic pathways is helpful in understanding the choice of specific redox cofactors for a variety of enzymatic reactions (Lui et al, 1993 & 1994). A detailed mechanistic understanding of the molecular pathways underlying these reactions has proved elusive.

1.2 GOALS AND SPECIFIC AIMS OF THE DISSERTATION
The six-electron reduction of $\text{SO}_3^{2-}$ to $\text{S}^{2-}$ is catalyzed in the sulfate-reducing bacterium *Desulfovibrio vulgaris* (Hildenborough) by the assimilatory sulfite reductase in anabolic metabolism, or by the dissimilatory sulfite reductase in respiratory metabolism (termed hereafter as SiR and DV, respectively). These sulfite reductases are found to be also capable of reducing $\text{NO}_2^-$ to $\text{NH}_3$. The assimilatory sulfite reductase appears to belong to a family of low molecular weight enzymes that have been identified in several anaerobic bacteria and are characterized by possession of a low-spin hexacoordinate siroheme. The dissimilatory enzyme is usually called desulfovirdin, and has a higher molecular weight and belongs to the family with high-spin pentacoordinate sirohemes. The dissimilatory enzyme (desulfovirdin) is a multimeric enzyme that can be readily isolated in large quantities (up to 7% soluble protein). However, aside from preliminary reports of its isolation and characterization, and recent EPR results, the biochemistry of this enzyme had not been studied in detail. These two sulfite reductases from *Desulfovibrio vulgaris* (Hildenborough) are targeted to investigate not only the physicochemical properties of the prosthetic center and the molecular details of the reaction pathway, but also the details of the structural basis for substrate recognition, bond activation and catalysis. These enzymes are amenable to study by a variety of kinetics, spectroscopic and physicochemical methods.

The activation of inorganic anions or gaseous molecules by metalloenzymes differs in several important respects when compared to the more commonly studied examples of non-redox enzyme catalysis on organic substrates. Selective binding and activation of a small inorganic
anion imposes very distinct demands on the enzyme. Metalloredox centers play a unique role in the binding and catalytic activation of small neutral or anionic ligands. Elucidation of the coordination environment in the active site should serve as a prelude to understanding the mechanism of action. Detailed mechanistic studies that include an evaluation of the relative importance of the prosthetic center and active site pocket toward substrate binding and catalysis are targeted. The unique coordination chemistry of the coupled center has been investigated insofar as it relates to understanding the enzymatic reaction mechanism. These issues have been addressed by steady-state and pre-steady-state kinetics, electrochemical, EPR, optical and fluorescence investigations of native enzymes. Sulfate-reducing bacteria also provide excellent model systems to evaluate the function of more complex enzymes found on respiratory pathways that link electron transfer events to the generation of metabolic energy in the form of ATP. My research attempts to understand these metabolic pathways at the molecular level using the language of chemistry. Such an investigation might yield useful general insights on the recognition and activation mechanisms of low molecular weight substrate anions or gaseous molecules by enzymatic catalysis. No such understanding currently exists for any enzyme in this class.

To investigate the underlying principles of substrate binding and catalytic activation that control this type of redox process, a detailed study that focuses on mechanistic aspects of enzymatic sulfite/nitrite reduction chemistry has been carried out, along with physical characterization of the
prosthetic center and implications for functional chemistry. The following specific goals are summarized:

1. To elucidate the mechanistic pathway for six-electron reduction of sulfite and nitrite reduction, and understand the interplay between the prosthetic centers and active site environment that optimizes the efficiency of the reaction (Ch. III, Ch. IV).

2. To understand the structural influence of the protein backbone in controlling the catalysis of the coupled prosthetic center (Ch. IV).

3. To understand the significance of this type of prosthetic center (with unusual redox and coordination chemistry) with regard to its role as a catalyst for sulfite and nitrite reduction (Ch. III).

4. To study the redox and coordination chemistry of the unusual coupled [Fe₄S₄]-siroheme center identified in sulfite and nitrite reducing enzymes (Ch. II, Ch. VI, Ch.V).

5. To investigate the unique electronic and magnetic properties of this coupled prosthetic center (Ch. VI, VII).

1.3 METHODOLOGY

My approach to study the metal-activated redox chemistry of sulfite and nitrite reduction in biology has been to bring a knowledge of inorganic chemistry to bear on an important question at the interface of chemistry and biology. EPR, optical and fluorescence spectroscopies, direct bioelectrochemistry, stopped-flow techniques, and protein modifications have been used to obtain information on the electronic and
magnetic properties of the prosthetic center, and insight on the mechanistic reduction of sulfite to sulfide and nitrite to ammonia.

The bacterium is cultured and the sulfite-reducing enzymes are isolated and highly purified for spectroscopic and other analytical studies (Ch. II). Rapid stopped-flow methods have been used to perform pre-steady-state kinetics experiments on these sulfite-reducing enzymes to obtain detailed mechanistic information. Microscopic rate constants for binding ($k_2$) and reductive cleavage of bonds ($k_r$) during enzymatic reduction of $SO_3^{2-}$ and $NO_2^-$ have been determined. Activation barriers for reaction pathways have been estimated by variable temperature experiments (Ch. III).

Fluorescence spectroscopy and fluorescence stopped-flow techniques are used to determine the rates of redox-linked conformational changes that this enzyme demonstrates. Rates of conformational change are determined through kinetic measurements of enzyme that has been covalently modified with fluorophores. By comparing these results with data from pre-steady-state measurements of active site chemistry, structural contributions to the regulation of reaction chemistry can be rationally investigated. The role of the protein backbone in controlling the catalysis is examined (Ch. IV).

To probe the redox properties of the enzymes, direct bioelectrochemistry is used to determine the reduction potentials of redox centers of the enzymes. In addition, important intrinsic thermodynamic information, namely free energy terms associated with electron transfer ($\Delta H^o$ and $\Delta S^o$), can be obtained by examining the redox properties of
proteins as a function of temperature. These redox thermodynamic terms may yield important information as to how enzymes that contain redox centers have been able to optimize their catalytic activity and affords insight on enzyme function (Ch. V).

Electron paramagnetic resonance spectroscopy (EPR) is a very powerful technique to study metalloproteins with respect to quantitative identification of the types and redox states of transition metal centers, investigation of the local environments of the transition metal centers, and determination of thermodynamic or kinetic parameters of the metal centers by monitoring the intensity and/or shape of the EPR spectrum as a function of redox potential, substrate concentration, time, etc. The electronic and magnetic properties of the prosthetic center have been examined in different oxidation states and environments. The relative reduction potentials for siroheme and [Fe₄S₄] centers as a function of axial coordination have been examined (Ch. VI). Optical spectroscopy has been used to detect any change of the enzymes upon reduction, oxidation, addition of ligands, and structural perturbation (Ch. VII).

1.4 DISSE Y RATION ABSTRACT

Both the low molecular weight monomeric assimilatory sulfite reductase (SiR, Mᵣ ~ 23,500) and the larger hexameric (α₂β₂γ₂) dissimilatory enzyme desulfoviridin (DV, Mᵣ ~ 224,000) from the sulfate-reducing bacterium Desulfovibrio vulgaris (Hildenborough) have been targeted in my studies. Each possess the [Fe₄S₄]-siroheme prosthetic center that is ubiquitous to this class of enzyme (Figure 1.4), while the chemistry of the low spin hexacoordinate siroheme in SiR makes for an
interesting comparison with the pentacoordinate siroheme in DV. Efforts have also been directed toward mechanistic and kinetics studies of the native DV enzyme. The followings summarizes what has been achieved in my graduate study:

1. Modifications of the bacterial growth, isolation, and purification of the assimilatory and dissimilatory sulfite reductases from *D. vulgaris* (Hildenborough), and evaluation of the formulation of the prosthetic centers have been performed (Ch. II).

2. A working model for the reaction pathway of the sulfite and nitrite reducing activity of this class of enzyme has been devised from detailed mechanistic studies and comprehensive kinetics experiments with DV. A systematic strategy for the analysis of a multistep enzymatic reduction of an inorganic anion has been proposed. The energy profile of the reaction pathway has been mapped by quantitative evaluation of activation barriers for substrates and reaction intermediates from variable temperature experiments ($\Delta G_r^*$) (Ch. III). Insights on the recognition and activation mechanism of anion substrates have been obtained, leading to the understanding of the role of siroheme in controlling catalysis.

3. Conformational gating that is linked to redox changes at the prosthetic center has been probed by fluorescence stopped-flow measurements of surface-labeled DV. The rate constants defining these structural perturbations, induced after oxidation and reduction, have been determined by monitoring changes in both
the natural emission from desulfoviridin (DV), and the emission from a surface-bound fluorophore (1,5-IAEDANS) (Ch. IV).

4. Direct protein electrochemistry has been established to measure the reduction potential of sulfite reductases. Square-wave voltammetry (SWV) has been performed on both the dissimilatory hexameric sulfite reductase ($M_r \sim 224,000$), which is the largest protein ever studied by direct electrochemistry, and the smaller assimilatory sulfite reductase ($M_r \sim 23,500$). Redox thermodynamics and pH titration studies have offered insights on the active site chemistry related to catalysis (Ch. V).

5. Optical, EPR and electrochemical studies have elucidated some novel aspects of the electronic, magnetic, and coordination properties of the coupled prosthetic centers (Ch. VI, VII).
CHAPTER II

Bacterial Growth of *Desulfovibrio vulgaris* (Hildenborough)

and Purification of SiR and DV

ABSTRACT

This chapter describes modifications to published procedures for growth of *Desulfovibrio vulgaris* (Hildenborough), and isolation and purification of the assimilatory and dissimilatory sulfite reductases (SiR and DV, respectively). The best yields and highest purities for spectroscopic studies of these two enzymes are obtained by following the modifications described herein. Special effort was made to address the final critical purification step of DV to obtain highly purified enzyme that would be suitable for EPR spectroscopy.

The assimilatory sulfite reductase (SiR, Mr ~ 23,500) from *Desulfovibrio vulgaris* (Hildenborough) is obtained from an expression system in the natural host using the broad-host-range plasmid pDSK519, containing the SiR gene insert (Tan et al., 1991 & 1994). Production is increased greater than fifty-fold relative to natural expression levels. New purification steps have been established to obtain better yields and
purity. Recombinant SiR cannot be distinguished from the native enzyme on the basis of biochemical analysis, spectroscopic characteristics, or enzyme activity.

The dissimilatory sulfite reductase, usually called desulfoviridin (DV, \(M_r \approx 224,000\)), is obtained from the natural host *Desulfovibrio vulgaris* (Hildenborough). Fortunately, sufficient quantity is obtained from natural expression levels. However, purification steps are critical if one wishes to obtain spectroscopically clean samples. Conditions for the rigorous purification of DV have been established. A final purification by fast protein liquid chromatography (FPLC) yields at least three distinct bands, each exhibiting the characteristic absorption spectrum of desulfoviridin. Two of these have been extensively characterized by standard biochemical analysis. Each contains two pairs of \([\text{Fe}_4\text{S}_4]\) and siroheme units. These results stand in marked contrast to recent work (Pierik and Hagen, 1991) claiming significant demetallation of siroheme, excess iron content, and the presence of \([\text{Fe}_6\text{S}_6]\) clusters. These proposals are critically assessed in light of our results, and other published work. Highly purified samples of DV display an electron paramagnetic resonance spectrum characteristic of rhombic high spin ferric heme centers while the fully reduced enzyme shows EPR features typical of \([\text{Fe}_4\text{S}_4]\) clusters. A combination of EPR spin quantitation, optical spectroscopy and chemical analysis are consistent with two \([\text{Fe}_4\text{S}_4]\)-siroheme per DV. Detailed characterization and studies of the magnetic properties of the prosthetic centers by EPR spectroscopy will be discussed in chapter VI.
2.1 INTRODUCTION

Six-electron reduction of $\text{SO}_3^{2-}$ to $\text{S}^{2-}$ is catalyzed by assimilatory or dissimilatory sulfite reductases (Huynh et al., 1984). These sulfite reductases can also reduce $\text{NO}_2^-$ to $\text{NH}_3$ by a six-electron reduction. Several examples of low molecular mass (~ 25 kDa) assimilatory-type sulfite reductases have been isolated from the anaerobic bacteria *Methanosarcina barkeri*, *Desulfuromonas acetoxidans*, and *Desulfovibrio vulgaris* (Hildenborough) (Lee and Peck, 1973; Lee et al., 1973 a & b; Moura et al., 1986). These enzymes differ from the larger sulfite reductase subunits isolated from *E. coli*, *S. typhimurium*, and spinach by possession of a hexacoordinate low-spin siroheme rather than the high-spin pentacoordinate heme common to the latter. Our laboratory is studying the biochemistry of enzymatic sulfite reduction in *D. vulgaris* (Hildenborough) (Tan and Cowan, 1990; Cowan and Sola, 1990; Tan et al., 1991; Tan and Cowan, 1991; Lui et al., 1993 & 1994, Tan et al., 1994; Wolfe et al, 1994) and has shown these low $M_r$ sulfite reducing enzymes to be excellent model systems for detailed studies of enzymatic multielectron redox chemistry.

Although sulfite and nitrite reductases have been the subject of extensive spectroscopic examination (Cammack et al., 1978; Christner et al., 1981 & 1983 a, b; Cline et al., 1986; Cowan and Sola, 1990; Han et al., 1989; Janick et al., 1983; McRee et al., 1986; Siegel et al., 1982; Moura et al., 1988), a systematic study of structure/function relationships is lacking. This reflects the absence of both primary structure data for enzymes of this class, and cloned/expressed genes for systematic mutagenesis studies.
The primary protein sequences for spinach nitrite reductase (Back et al., 1988), and the assimilatory sulfite reductases from *Salmonella typhimurium*, *E. coli* B, and *D. vulgaris*, have only been reported recently (Tan et al., 1991; Ostrowki et al., 1989 a, b, c). Some sequence homology was noted in the region of putative cluster binding domains for the *vulgaris* sequence relative to the other examples (Tan et al., 1991). In an elegant study, Wu *et al.* have successfully over-expressed the *E. coli* sulfite reductase heme subunit holoenzyme in *E. coli* (Wu et al., 1991).

The low molecular mass assimilatory-type sulfite reductase from *D. vulgaris* (Hildenborough) is an excellent model for understanding both the structural basis for regulation of the physicochemical properties of prosthetic centers by a protein environment, and the mechanistic details of the reaction pathway for such a biological multielectron redox reaction (Tan et al., 1991). Previously, our laboratory has cloned and sequenced the gene encoding the assimilatory sulfite reductase (SIR) (Tan et al., 1991), and functionally expressed this gene in *D. vulgaris* (Hildenborough) and *D. desulfuricans* (Tan et al., 1994). This chapter describes modifications to published procedures for bacterial growth, isolation and purification of SIR to obtain the best yields and highest purity, especially in the column chromatography steps. This lays the groundwork for more detailed structure/function studies of the enzyme, physical inorganic studies of the coupled prosthetic center, and mechanistic studies of the reaction pathways since the natural expression level is too low to be practical for spectroscopic studies such as NMR and EPR.
The dissimilatory enzyme, commonly referred to as desulfoviridin (DV), is a multimeric enzyme that can be readily isolated in large quantities (up to 7% soluble protein; Peck and LeGall, 1982). Aside from preliminary reports of its isolation and purification (Lee et al., 1973 a, b), the enzyme has only recently been the subject of close scrutiny (Pierik and Hagen, 1991; Pierik et al., 1992). The work on DV will demonstrate that in common with other sulfite- and nitrite-reducing enzymes, it appears to contain siroheme and [Fe₄S₄] prosthetic centers (Huynh et al., 1984; Krueger and Siegel, 1982 a, b; Cline et al., 1986; Christner et al., 1983 & 1984; Madden et al., 1989; McRee et al., 1986; Moura et al., 1988; Vega and Kamin, 1977; Lancaster et al., 1979; Cammack et al., 1978; Hirasea et al., 1989). The assimilatory sulfite reductase (ASiR) from Escherichia coli is the most thoroughly characterized example of this class and has formed an important basis for studies on related enzymes (Christner et al., 1984; Siegel et al., 1973 & 1982; Siegel and Davis, 1974; Murphy et al., 1973; Janick et al., 1983; Janick and Siegel; 1983 a,b; Wilkerson et al., 1983).

There are, however, some important differences in the structural and spectroscopic characteristics of these enzymes that have not yet been adequately addressed. These topics will be covered in later chapters.

Previously Seki and coworkers separated desulfoviridin from strain Miyazaki into two forms by diethylaminoethyl (DEAE)-Sephadex chromatography (Seki and Ishimoto, 1979; Seki et al., 1979). The major and minor forms had isoelectric pHis of 4.4 and 4.5-4.6, respectively. By including an additional purification step by fast protein liquid chromatography (FPLC), we have been able to resolve the Hildenborough enzyme into at least three distinct forms (Wolfe et al., 1994). Two of these
components have been characterized in detail (Wolfe et al., 1994); viz, by quantitation of siroheme and Fe-S cluster prosthetic centers, amino acid analyses of subunits, determination of isoelectric point. In the absence of such careful final purification steps, multiple impurities are evident from EPR measurements that bear striking resemblance to published data (Pierik and Hagen, 1991). Recent work claiming significant demetallation of siroheme, excess iron content, and the presence of [Fe₆S₆] clusters is critically assessed (Pierik and Hagen, 1991; Moura et al, 1988; Moura et al., 1986; Lai et al., 1991).

2.2 MATERIALS AND METHODS

2.2.1 Bacterial Growth of *D.vulgaris* (Hildenborough)

**General Materials and Instruments.** Unless otherwise indicated, all chemicals were purchased from Sigma or Aldrich. Buffer salts were of molecular biology grade (Fisher or Aldrich Chemical Co.). DNase I and Kanamycin were purchased from BRL. Measurements of solution pH were made with an Accumet pH Meter 910 equipped with a Corning semi-micro combination pH electrode. Sephadex G-200 gel filtration material was obtained from Sigma, and DEAE-52 ion exchange resin from Whatman. The deionized water used in all procedures was obtained by filtering distilled water through a Barnstead nanopure system and exhibited a resistivity of 18 MΩ cm⁻¹. Liquid helium was purchased from Helium Technology.

**Bulk Culture Growth.** *Desulfovibrio vulgaris* was grown either in an enriched Baars medium [American Type Culture Collection medium
No. 1249], or LS medium (Rapp and Wall, 1987) in 4 x 50 L carboys and harvested after 50 h growth (32 °C) using an Amicon DC 20L concentrator. Media composition are listed in Table 2.1 and 2.2 (LS and Baars media, respectively). Small-scale inocula were typically deoxygenated by Ar(g) purging (20 min) prior to inoculation. Large volumes (50 L) were degassed by bubbling N₂(g) through the culture medium for ca. 1 hour prior to inoculation. In all media preparations, kanamycin (200 µg/mL) was added as an antibiotic selector. The cell paste obtained was stored at -80°C.

2.2.2 Isolation and Purification of SiR and DV

Initial workup of cell paste. The initial workup for recombinant cells or native cells followed literature procedures (Huynh et al., 1984). Approximately 250 g of cell paste was thawed and mixed with ca. 250 mL of 10 mM Tris-HCl (pH 7.5, 4°C) to give a cell suspension, which was then sonicated using a Fisher sonic dismembranator (model 300) at 90 % of its power. Sonication was carried out in a series of one min bursts followed by a two min cooling in ice. This sequence was repeated approximately five times. The temperature was maintained below 10°C by keeping the suspension in an ice bath. About 1 mg of DNase I (BRL) was mixed with the suspension at 4°C to decrease its viscosity. Sonication was continued until the suspension was flowing smoothly. The preparation was then treated with 5 g of streptomycin sulfate (dissolved in 5 mL of 10 mM Tris-HCl, pH 7.5), stirred at 4°C for 40 min, and then centrifuged at 13,200 x g in a Sorvall RC-5B refrigerated centrifuge for 1 hr. The pellet was washed with 10 mM Tris-HCl (pH 7.5, 4°C) and the suspension was
<table>
<thead>
<tr>
<th>Composition</th>
<th>Amount (per L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na Lactate (60 %)</td>
<td>12.5 g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>2 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>2 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>4 g</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.035 g</td>
</tr>
<tr>
<td>EDTA</td>
<td>0.002 g</td>
</tr>
<tr>
<td>cysteine·HCl</td>
<td>0.125 g</td>
</tr>
<tr>
<td>Yeast</td>
<td>1 g</td>
</tr>
<tr>
<td>Na₂S·9H₂O</td>
<td>0.25 g</td>
</tr>
</tbody>
</table>
Table 2.2 Composition of Baars medium.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Amount (per L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na Lactate (60 %)</td>
<td>5.84 g</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1 g</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>1 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.5 g</td>
</tr>
<tr>
<td>CaSO₄</td>
<td>1 g</td>
</tr>
<tr>
<td>Na citrate</td>
<td>5 g</td>
</tr>
<tr>
<td>Yeast</td>
<td>1 g</td>
</tr>
<tr>
<td>Fe(NH₄)₂(SO₄)₂·6H₂O</td>
<td>0.15 g</td>
</tr>
</tbody>
</table>
centrifuged again for 1 hr. The supernatant was combined and centrifuged in a Beckman L5-75B ultracentrifuge (144,000 x g, 90 min., 4°C)

**Isolation and Purification of SiR.** The initial workup for recombinant cells followed the procedure described in the previous section. Modifications were made in the column chromatography steps. After ultracentrifugation, the supernatant was collected and applied to a DEAE-52 (Whatman) column (6 x 30 cm) that had been equilibrated with 10 mM Tris-HCl (pH 7.5, 4°C). All purification procedures were carried out at 4°C and all buffers were adjusted to pH 7.5. The column was run with steps of increasing Tris-HCl concentration. First with 10 mM Tris-HCl to remove a greyish green band and unabsorbed red cytochromes; then with 50 mM Tris-HCl to remove all other orange red cytochromes completely. The buffer would then be stepped up to 100 mM Tris to collect the SiR fraction, which is eluted as a pale brown fraction containing hydrogenase as contaminant. Approximately 1 L of 100 mM Tris-HCl was applied to the column to get all the SiR fractions. This pale brown fraction was pooled together, concentrated and exchanged to 50 mM potassium phosphate (KP) in an amicon (10 K membrane). This concentrated brown fraction was then applied to a hydroxylapatite (HA) column (2 cm x 10 cm) (HA resin is synthesised according to literature procedures, Method in Enzymology Vol. XXI, p.97), which was equilibrated with 50 mM KP buffer. During loading at 50 mM KP, a pale brown band containing APS reductase and a trace amount of red cytochromes were immediately eluted from the HA column. The column was run with steps of increasing KP concentration (100mM) to remove a yellowish band containing hydrogenase, and then finally with 300 mM KP
to elute the greyish brown band containing SiR. This greyish brown fraction contained SiR as the major component. Occasionally, this band was already pure (homogenous by SDS-PAGE) and no further purification steps were needed. However, sometimes an additional FPLC step (a gradient of 0 mM NaCl to 500 mM NaCl in 10 mM Tris-HCl, pH 6.9 at 25°C) was needed. SiR was eluted at ca. 300 mM NaCl in 10 mM Tris-HCl.

The original literature procedure used the HA column before the DEAE column. In this case, FPLC is always needed to get pure SiR and the yield is much lower. The G-75 gel filtration column suggested in the literature was omitted.

**Isolation and Purification of DV.** The isolation and purification of DV was similar to the literature procedure (Lee et al., 1973 a, b) with the following modifications. The initial workup of the cell paste was the same as mentioned previously. After running the first DEAE column with an initial elution buffer of 100 mM Tris-HCl to remove fractions containing SiR, the column was then washed with 150 mM Tris-HCl to remove a brown band containing APS reductase. DV was then eluted with 250 mM Tris-HCl from the DEAE-52 column. All the green fractions eluted at 250 mM Tris-HCl were pooled together, concentrated and exchanged to 50 mM KP buffer in an amicon (100 K membrane). The concentrated green fraction was then applied to a second DEAE-52 column that had been pre-equilibrated with 50 mM KP buffer. A small brown band containing APS reductase was removed from DV by washing the column with 100 mM KP. The green band containing DV was eluted with 300 mM KP buffer. The green fractions were pooled together and precipitated by a 40 % saturated (NH₄)₂SO₄ solution. The solution was
centrifuged and the pellet of DV was suspended in a minimal volume of 50 mM KP (ca. 1-2 mL), and finally applied to a G-200 gel filtration column pre-equilibrated with 50 mM KP buffer. Further purification by FPLC on a Mono Q column (1 x 10 cm) using non-denaturing conditions resulted in resolution of at least three bands. Optimal separation of bands I and II was obtained by a gradient method, using two stocks of degassed potassium phosphate buffer, pH 7.5 [A: 50 mM, B: 500 mM]. The total running time was 40 min (5 min 11% B, 17 min 11 to 100% B, and 10 min 100% B). This final FPLC step was found to be essential to obtain DV of high purity.

2.2.3 Chemical Characterization of Enzymes

Purity and Molecular Weight. The purity and the molecular mass of the purified enzymes were examined by running sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a Phast electrophoresis system (Pharmacia/LKB) at 15°C using pre-made 20% homogeneous gels (Pharmacia/LKB). An aliquot of protein solution (4 μL) was treated with an SDS gel loading buffer solution (4 μL, prepared according to the manufacturers protocols) and boiled at 100°C for two minutes. Approximately 6 μg of protein (1 μL of sample) was applied to each lane of the gel using an automated gel loading applicator (Pharmacia/LKB). Running and staining (Coomassie) conditions followed recommended preprogrammed operating conditions. Molecular mass determinations were referenced to commercially available standards (BRL, high molecular mass range). The purified SiR ran as a single band
on the gel, and its molecular weight was estimated to be 24 kDa. The purified DV ran as three bands on the gel, containing three subunits.

**Isoelectric Point.** The isoelectric point was determined by running an analytical IEF gel (Pharmacia/LKB) on the Phast electrophoresis system using precast 20% homogeneous gel (pH 3.5 - 9.0) at 15°C. The isoelectric pH values were referenced to oxidized and reduced myoglobin (pI 8.1 and 7.4, respectively) (Cowan et al., 1989) and high-potential iron protein (*C. vinosum*) (pI 3.6) (Bartsch, 1971 & 1978). Typically 1 µL of a 0.5 µM protein solution was applied to each lane and electrophoresed under recommended preprogrammed operating conditions. The protein bands in the gel were visualized by coomassie blue staining.

**Electronic Absorption.** Electronic absorption spectra were measured on a Hewlett-Packard 8452A spectrophotometer (run by software from On-Line Instrument Systems). Sample concentrations were ca. 6 µM in 50 mM potassium phosphate buffer (pH 7.5). Data was obtained at 298 K.

**EPR Spectroscopy.** EPR spectra were recorded at X-band with a Bruker ESP 300 spectrometer equipped with an Oxford liquid helium cryostat and an internal integration program in the Bruker software package. The temperature was measured by use of a Au/Fe v. Cr thermocouple. Experimental parameters are listed in footnotes to the tables and figure legends. Samples were purged with Ar(g) prior to obtaining spectra.

**Iron Quantitation.** Two methods were employed to determine Fe content: (1) First, the protocol described by Moulis and Meyer was used
(Moulis and Meyer, 1982), however, an additional step was incorporated to remove possible trace iron contaminants from the enzyme solution: enzyme solution was concentrated in a centricon (Amicon Inc.), diluted with Na$_2$EDTA (10 mM in 50 mM phosphate, pH 7.5) solution, concentrated, and diluted with fresh buffer solution. A series of concentration/dilution steps were used to remove residual EDTA. Subsequently we demonstrated that the EDTA pre-treatment had little effect on the iron quantitation results, and so there are negligible amounts of adventitiously bound iron in these samples. The assay was performed on 3-5 nmole of protein. (2) Second, the method of Fish was employed (Fish, 1988). Established protocols were closely followed. The assay was performed on 4-10 nmole of protein.

Sulfide Quantitation. Sulfide concentrations were accurately determined by a spectrophotometric assay (methylene blue reaction) (Siegel, 1965). Results from analysis of enzyme samples (2-6 nmole) were quantitated against a calibration plot that was constructed from a series of measurements on freshly prepared solutions of known sulfide concentration.

2.3 RESULTS

Bacterial Growth of *D. vulgaris* (Hildenborough). *Desulfovibrio vulgaris* or *desulfurican* carrying plasmid pDSK519/SiR(S/E) showed at least a 50-fold increase in isolated enzyme relative to the yields of enzyme obtained from similar procedures on regular native *D. vulgaris* cells (Huynh et al., 1984). From 250 g of wet cell paste up to 50 mg of enzyme were obtained from a culture grown in LS medium. Yields obtained with
this medium were far superior to results obtained with modified Baars medium. The cell mass was free from FeS(s) debris which occurred in Baars medium. The yield of SiR was not affected, even in the absence of FeSO₄ salt, as the other components in the medium (e.g. yeast and phosphate salt) contained enough Fe content for the synthesis of siroheme and [Fe₄S₄] cluster. Cell growth was optimal if the 50-L carboys were N₂-purged before inoculation. Growth temperature (32-34°C) was found to be critical to obtain the best yields of cell mass.

**Fractional Separation of Crude Cell Solution.** The crude cell solution was applied to the first DEAE-52 column and separated into a number of fractions containing different enzymes from *D. vulgaris* (Table 2.3). Table 2.3 shows the general sequence of the eluted fractions. SiR elutes at ca. 75 - 100 mM Tris-HCl, while DV elutes at ca. 200 - 300 mM Tris HCl.

**Purification and Characterization of Recombinant SiR Holoenzyme.** The modified purification steps noted in previous sections give better yields and higher purity of SiR compared to published procedures. The recombinant enzyme was characterized by comparison of chemical analysis, spectral characteristics, and enzymatic assays (Tan et al., 1994). Iron and sulfide analyses (Tan et al., 1994), using the standard methods described in the experimental section, yielded 4.9 ± 0.3 iron centers and 5.1 ± 0.5 sulfides per mole of enzyme, in good agreement with previous data on native sulfite reductase (Huynh et al., 1984). The activity of the recombinant enzyme (Tan et al., 1994) showed satisfactory agreement with native enzyme when tested against a variety of substrate
Table 2.3 Fractions from the first DEAE-52 column of cell solution from *D. vulgaris* (Hildenborough).

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Possible Candidate</th>
<th>Tris-HCl buffer concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Greyish green</td>
<td>? (unknown)</td>
<td>10 mM</td>
</tr>
<tr>
<td>2. Reddish orange</td>
<td>cytochromes</td>
<td>10 mM</td>
</tr>
<tr>
<td>3. Orange</td>
<td>cytochromes</td>
<td>50 mM</td>
</tr>
<tr>
<td>4. Red</td>
<td>cytochromes</td>
<td>50 mM</td>
</tr>
<tr>
<td>5. Greenish brown</td>
<td>SiR + hydrogenase</td>
<td>75 to 100 mM</td>
</tr>
<tr>
<td>6. Bright yellow</td>
<td>flavodoxin + hydrogenase</td>
<td>100 mM</td>
</tr>
<tr>
<td>7. Light orange red</td>
<td>cytochromes</td>
<td>100 mM</td>
</tr>
<tr>
<td>8. Brown</td>
<td>APS reductase</td>
<td>100 to 150 mM</td>
</tr>
<tr>
<td>9. Green</td>
<td>DV + &quot;unknown&quot; protein that gives rise to the dirty EPR spectrum of DV</td>
<td>200 to 300 mM</td>
</tr>
<tr>
<td>10. Reddish brown</td>
<td>? (known)</td>
<td>300 to 400 mM</td>
</tr>
</tbody>
</table>
molecules. The EPR spectrum of oxidized recombinant enzyme in Figure 2.1 shows good agreement with published data (Huynh et al., 1984). Figure 2.2 also shows the spectrum of fully reduced enzyme, which previously had not been reported. Spin quantitations by EPR (details in Ch. VI) were carried out using the Vanngard equation (Fee, 1975), relative to the low-spin myoglobin:N₃⁻ complex and CuEDTA as standards. Quantitation data for oxidized low-spin siroheme and fully reduced cluster demonstrated 1.1 ± 0.2 siroheme/enzyme and 1.0 ± 0.2 cluster/enzyme; both consistent with the expected values. The electronic absorption (Figure 2.3) of the recombinant enzyme matched those obtained from the native enzyme (Cowan and Sola, 1990; Huynh et al., 1984), again indicating the incorporation of all prosthetic centers.

Purification and Characterization of DV. Desulfoviridin was isolated as previously described (Lee et al., 1973 a, b), but including the modifications mentioned in Section 2.2. Seki and coworkers had previously shown that desulfoviridin from *D. vulgaris* (Miyazaki) may be isolated as two discrete bands by DEAE-Sephadex chromatography (Seki et al., 1979 & 1981). By use of FPLC as an additional purification step, we have been able to resolve desulfoviridin from strain Hildenborough into three distinct forms (Figure 2.4), which we have designated DV-I, II, and III. Table 2.4 summarizes the results of iron analyses obtained from fractions (a) - (f) collected during a typical FPLC run. Prior to FPLC we find an average iron content of ~ 24 equivalents, in close agreement with published data on desulfoviridin (Pierik and Hagen, 1991). However, this arises from a significant contaminant band between peaks II and III of the FPLC trace. The first two major bands (I and II) could be isolated in
Figure 2.1 EPR spectra of anaerobic solutions of 31 μM native oxidized sulfite reductase (SiR) obtained in 50 KP buffer (pH 7.5) at 11.0 K. Spectral conditions: microwave frequency = 9.45 GHz; power = 10 mW; modulation frequency = 100 kHz; modulation amplitude = 10 Gauss; time constant = 10.2 ms; sweep width = 2200 Gauss.
Figure 2.2  EPR spectra of anaerobic solutions of 31 μM reduced sulfite reductase (SiR) obtained in 50 KP buffer (pH 7.5) at 13.2 K. Spectral conditions: microwave frequency = 9.45 GHz; power = 10 mW; modulation frequency = 100 kHz; modulation amplitude = 10 Gauss; time constant = 10.2 ms; sweep width = 2200 Gauss.
Figure 2.3  Absorption spectrum of oxidized recombinant SiR taken with 9 μM enzyme in 100 mM potassium phosphate buffer, pH 7.6, at 298 K.
Figure 2.4 FPLC chromatogram from the final purification step for desulfoviridin. Peaks are assigned DV-I to III in order of increasing buffer ionic strength. The buffer gradient (indicated by $\cdots\cdots\cdots$) runs from 100 mM to 500 mM buffer. Better resolution was obtained with potassium phosphate buffer (pH=7.6) than with Tris-HCl (pH=7.6). The fractions labeled (a) to (f) were analyzed for iron content per mole equivalent of enzyme (concentration evaluated from $A_{630nm}$) present in that fraction. These values are summarized in Table 2.4.
Table 2.4  Evaluation of iron equivalents per mole of enzyme for fractions isolated from FPLC purification of desulfoviridin.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Iron Equivalents</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>12.4</td>
</tr>
<tr>
<td>b</td>
<td>13.4</td>
</tr>
<tr>
<td>c</td>
<td>12.4</td>
</tr>
<tr>
<td>d</td>
<td>83</td>
</tr>
<tr>
<td>e</td>
<td>12.2</td>
</tr>
<tr>
<td>f</td>
<td>15.2</td>
</tr>
<tr>
<td>control</td>
<td>24</td>
</tr>
</tbody>
</table>

\[a\] The fractions selected are indicated in Figure 2.4.

\[b\] After one additional FPLC run these samples yielded iron analyses in the 10 - 11 equivalents range, as noted in Table 2.5.

\[c\] Control data was taken from a sample of desulfoviridin prior to FPLC treatment.
sufficient quantity and purity for further characterization after one
further injection. The activity of the purified enzyme was always greater
than or equal to that prior to FPLC, and is in favorable agreement with
previous determinations (Lee et al., 1973 a, b). Taking account of the fact
that $SO_3^{2-}$ is a six-electron reduction (while our data assumes two reaction
sites per enzyme), the relative activities (in equivalents of $H_2$ uptake per
minute per milligram of enzyme) are 0.5 and 0.26 for our enzyme
preparation and previous reports (Lee et al., 1973 a, b), respectively.

The results from the characterization experiments (Wolfe et al.,
1994) are detailed in Table 2.5. The resolution of these components by
FPLC was extremely dependent on the buffers and gradients employed to
effect the separation. Potassium phosphate proved superior to a Trizma
buffer system and the gradient shown in Figure 2.4 provided optimal
separation of bands I and II. To determine whether these discrete bands
represented various stages of enzyme degradation, purified samples of
each band were left at room temperature for 1 week. Significant
denaturation products were then removed by centrifugation. However,
the chromatogram following FPLC analysis contained one band at the
original elution position. Samples of purified or mixed peaks gave
reproducible chromatograms after storage at $-20^\circ$C for up to six months.
These discrete bands did not therefore appear to derive from an
equilibrium mixture of conformational isomers, nor from spontaneous
degradative products. It is possible that these bands might derive from
proteolytic loss of a few terminal amino acid residues during normal cell
metabolism or, possibly, during work-up. These fragments must be small
since there is no observable change in the molecular masses of either the
Table 2.5  Characterization of Desulfoviridin.

<table>
<thead>
<tr>
<th></th>
<th>DV-I</th>
<th>DV-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sirohemes / DV</td>
<td>2.0 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Iron ions / DV</td>
<td>11 ± 1</td>
<td>10 ± 1</td>
</tr>
<tr>
<td>Inorganic Sulfide / DV</td>
<td>10.0 ± 0.1</td>
<td>9.6 ± 0.6</td>
</tr>
<tr>
<td>pH</td>
<td>4.3 ± 0.1</td>
<td>4.5 ± 0.1</td>
</tr>
<tr>
<td>solvent exposed sulfhydryls&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5 ± 0.4</td>
<td>3.4 ± 0.4</td>
</tr>
<tr>
<td>solvent exposed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>disulfide bonds&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Total cysteine content&lt;sup&gt;b&lt;/sup&gt;</td>
<td>47</td>
<td>45</td>
</tr>
<tr>
<td>α2 subunit</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>β2 subunit</td>
<td>23</td>
<td>21</td>
</tr>
<tr>
<td>γ2 subunit</td>
<td>9</td>
<td>8</td>
</tr>
</tbody>
</table>

<sup>a</sup> 2 subunit

<sup>b</sup> 32 subunit
Under denaturing conditions a larger number of sulfhydryls (20 - 30) and disulfides (15 - 22) are determined. The exact number depends on the denaturant employed (urea or guanidine thiocyanate). The lower estimates of sulfhydryl and disulfide content determined under normal solution conditions suggests that most cysteine and cystine are internal and not solvent exposed (Robyt and White, 1990).

Values are approximate. Recently the γ-subunit has been cloned and sequenced, revealing a total of 6 cysteine per dimer (Karkhoff-Schweizer, 1993).
α or β subunits as determined by SDS-PAGE gel electrophoresis. This would readily explain the small difference noted in the isoelectric pH's for each pair of subunits. Within experimental error, both the amino acid quantitations and kinetic parameters (Wolfe et al., 1994) for DV-I and DV-II were similar. It is interesting to note that the ratio of DV-I, DV-II, and DV-III isolated from each batch of cell paste was found to be approximately the same. At this time we are unable to say whether these forms represent natural isomeric forms or products from proteolytic loss of terminal residues. In any event, the kinetic behavior of DV-I and DV-II were similar (Wolfe et al., 1994), which argues against significant perturbation of the active site environment. Enzyme purified in this rigorous manner was found to give three bands in SDS-PAGE gels corresponding to the α, β, and γ subunits (Pierik et al., 1992). Subsequent reinjection of an isolated band provided material with a clean EPR spectrum.

Quantitation of Prosthetic Centers in DV. Quantitative determination of sulfide, siroheme and iron content by a variety of chemical methods are given in Table 2.5. The results are self consistent and point toward a pair of [Fe₄S₄]-siroheme centers per enzyme.

Sulfide Quantitation: On the basis of the sulfide determination (Wolfe et al., 1994) there is sufficient ligand available to provide for a bridging sulfide. We have previously argued, however, that while the chemical identity of this ligand (sulfide or cysteine) is certainly of interest for any given enzyme, it is unlikely to be crucial with regard to discussion of reaction mechanism (Tan and Cowan, 1991).
Siroheme Quantitation: Siroheme quantitations (Wolfe et al., 1994) based on extraction procedures that employ acid conditions should be regarded with caution. Facile loss of heme iron has been demonstrated (see for example Figure 2.5) (Wolfe et al., 1994). To avoid these problems alkaline conditions were employed, using several standard tests commonly used for heme quantitation, based on the pyridine hemochrome test (Tan and Cowan, 1990). Formation of the CN⁻ adduct of siroheme provided a spectrum (Figure 2.5 C) (Wolfe et al., 1994) that was similar to that previously published by Kang et al. (1987) for the CN⁻ complexes of siroheme isolated from desulforubidin and assimilatory sulfite reductase, both of which are accepted to contain fully-metallated siroheme (Moura et al., 1988; Kang et al., 1987). Using the absorbance at ~ 595 nm yielded a heme content of 1.9 ± 0.1 per mole of desulfoviridin. Siroheme was also extracted under basic conditions to form the pyridine adduct (spectra of the oxidized and reduced adducts are shown in Figure 2.5 B) (Wolfe et al., 1994). The spectrum of both the enzyme and the reduced siroheme-pyridine adduct are similar to those previously reported by Lee et al. (1973 a, b). The pyridine complex from both DV and the assimilatory reductase (SiR) (Lee et al., 1973 a, b) show variations from Siegel's published data and may reflect differences in solvent conditions (Siegel et al., 1978). Siroheme spectra are known to be particularly dependent on solvent and pH (Kang et al., 1987). The spectrum of the pyridine-siroheme adduct, obtained following extraction in acidic acetone, yields a spectrum (Figure 2.6 A) that is very similar to that of the chromophore isolated from D. gigas desulfoviridin (Moura et al., 1988), but stands in contrast to the spectrum obtained when acid-free conditions are employed. Using acidic
Figure 2.5  Electronic absorption spectrum of the oxidized siroheme extracted from desulfoviridin in 0.1 M NaOH. Taken with ca 2.9 µM enzyme at room temperature. As follows: (A) reference spectrum showing oxidized enzyme, (B) in 25 % pyridine, (C) in 0.4 M cyanide. The latter is similar to the CN⁻ complexes of siroheme isolated from desulfurubidin and assimilatory sulfite reductase, both of which contain fully-metallated siroheme (Moura et al., 1988; Kang et al., 1987). For the cyanide complex (C), $A_{595} = 0.085$ ($e = 1.5 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$); that is, the concentration of siroheme cyanide complex is 5.7 µM. This yields a siroheme:enzyme ratio of 1.95 : 1
Figure 2.6  Electronic absorption spectrum of the pyridine complex with extracted chromophore obtained from desulfoviridin after: (A) acidic acetone extraction and addition of pyridine (to 25 %); and (B) alkaline extraction in 0.1 M NaOH / 25 % pyridine. The spectrum is little affected by dithionite reduction. The enzyme concentration used in (A) was approximately three times that used in (B) since material was lost in the precipitate formed in the acidic acetone.
conditions, we observed precipitation of denatured protein that can occlude some of the released chromophore.

If in fact there were additional sirohydrochlorin in the enzyme sample, the optical methods would quantitate the overall [siroheme] and [sirohydrochlorin] content as greater than 2, since both chromophores absorb in similar regions of the visible spectrum, which should lead to a larger estimate of siroheme content, contrary to our observations. In fact we find 2 hemes to be the upper limit and under no circumstance was a ratio greater than 2 obtained. Our data therefore eliminates the possibility of significant numbers of metal-free sirohydrochlorin in this enzyme.

Iron quantitation: Iron quantitations were performed by two independent colorimetric tests (Moulis and Meyer, 1982; Fish, 1988). Satisfactory agreement was obtained between measurements (Table 2.5). Table 2.4 lists data obtained from the fractions collected during FPLC purification (Figure 2.4). Note that the average value of ~ 24 mole equivalents of iron per enzyme obtained prior to the FPLC step is consistent with the excessive iron contents previously reported for this class of enzyme (Pierik and Hagen, 1991; Moura et al., 1988). However, the data in Table 2.4 clearly show that these abnormally high iron quantitations result from contaminants that appear to be eluted from the FPLC column between bands II and III (Figure 2.4). After one additional FPLC run, the stoichiometry of iron : enzyme content was fixed at ~ 10 : 1 (Table 2.5). Ten irons may be accommodated by two [Fe₄S₄] clusters and two sirohemes. While this data does not directly demonstrate coupling, it does support the siroheme and [Fe₄S₄] prosthetic center content, in full
agreement with the model originally developed by Siegel and coworkers for this class of enzyme (Janick and Sigel, 1983 a, b).

**EPR Measurements of DV.** Desulfoviridin was recently reported to possess an extremely complex EPR spectrum with g-values ranging from 1.63 to 17 (Pierik and Hagen, 1991). A spin quantitation of ca. 0.2 hemes/half mole of enzyme was also reported. By use of rigorous purification procedures we have eliminated these spurious signals to yield the standard rhombic high-spin heme spectrum shown in Figure 2.7. Highly purified samples of native oxidized desulfoviridin shows a typical rhombic high-spin ferric heme EPR spectrum \(S = \frac{5}{2}, g = 6.30, 5.63, 2.00\). Figure 2.8 shows an EPR spectrum of DV prior to FPLC, resembling the published report by Hagen and coworkers (Pierik and Hagen, 1991). Figure 2.7 and 8 show the importance of the FPLC purification step to obtain highly pure DV. Figure 2.9 shows the EPR spectrum of reduced DV, exhibiting the typical \([Fe_4S_4]\) cluster signal around "\(g = 1.94\)", which disproves the proposal of the presence of \(Fe_6S_6\) cluster in DV (Pierik and Hagen, 1991). Detailed EPR characterization of DV is detailed in Ch. VI. Table 2.6 lists the spin quantitation data for DV from our work and reference (Pierik and Hagen, 1991). The concern about the demetallation of siroheme and the presence of \([Fe_6S_6]\) cluster in DV will be presented later in the Discussion section.

### 2.4 DISCUSSION

**Functional Expression of SiR Genes in Desulfovibrio Species.** The successful application of the triparental conjugation method (Voordouw et al., 1990) by Tan and Cowan (Tan et al., 1991 & 1994) led us to achieve
Figure 2.7  EPR spectrum of an anaerobic solution of 0.11 mM native oxidized desulfoviridin obtained in 10 mM potassium phosphate buffer (pH 7.5) at 8 K. * = background cavity signal. Spectral conditions: microwave frequency = 9.53 GHz; power = 0.40 mW; modulation frequency = 100 kHz; modulation amplitude = 10G; time constant = 20.5 ms; sweep width = 3904 G
Figure 2.8  A spectrum of a desulfoviridin sample obtained after successive chromatography on hydroxy apatite, DE-52, G-200 sephadex, and a final DE-52 column prior to final FPLC purification is shown for comparison. Experimental conditions same as Figure 2.7.
Figure 2.9  Spectrum of fully reduced desulfoviridin obtained by deazaflavin photoreduction (Massey and Hemmerich, 1978). This spectrum was obtained at 13.2 K. Experimental conditions same as Figure 2.7.
Table 2.6  Spin integrations for oxidized and reduced desulfoviridina.

<table>
<thead>
<tr>
<th>redox state</th>
<th>our work</th>
<th>referenceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxidized (heme equiv.)</td>
<td>0.6±0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>fully reduced (cluster equiv.)</td>
<td>0.5±0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

aData is quoted for the enzyme, rather than half-enzyme equivalents. Spin quantitations were carried out using the Aasa-Vanngard equation (see text in Ch. VI), relative to myoglobin or CuEDTA (Aasa and Vanngard, 1975; Fee, 1975).

bFrom Pierik and Hagen, 1991.
functional expression of the SiR gene in *Desulfovibrio* species and obtain high yield of SiR for spectroscopic studies. The functional expression system for the low molecular mass assimilatory sulfite reductase from *D. vulgaris* (Hildenborough) now affords the opportunity for mutagenesis experiments, while the increased yield of enzyme allows for detailed mechanistic and spectroscopic studies. Modification of the purification steps further improves the yield. By virtue of its size and accessibility by a variety of kinetic and physicochemical methods, this enzyme system provides a valuable model for understanding the chemistry of multielectron redox reactions of small inorganic substrates.

**Metallation or Demetallation of DV? A Comparison with Related Work.** For both assimilatory sulfite reductase and desulfoviridin, we have found an additional FPLC step to be essential for obtaining enzyme samples of sufficient purity for kinetic and spectroscopic studies. Our iron, sulfide, and siroheme quantitations are fully consistent with the presence of two pairs of [Fe₄S₄] and siroheme centers. Significant demetallation has been claimed for a number of dissimilatory sulfite reductases from *Desulfovibrio vulgaris* and *gigas* (Pierik and Hagen, 1991; Moura et al., 1988 & 1986; Lai et al., 1991; Arendsen et al., 1993). In the case of desulfoviridin from *D. vulgaris*, we have demonstrated the evidence for demetallated siroheme to be flawed, while additional peaks noted in EPR spectra are artefactual, arising from impurities that can be removed by careful FPLC purification. Since these issues have been the topic of intense debate, we outline below the arguments against claims for excessive iron content, demetallation, and novel cluster systems. Clearly,
the question of prosthetic center composition is central to a discussion of reaction mechanism.

(1) Resonance Raman studies of desulfoviridin demonstrate significant shifts for core-marker bands in D$_2$O (Lai et al., 1991). It has been argued that this demonstrates protonation of the pyrrolic, and pyrroline nitrogens, and so the heme must be demetallated. This analysis is flawed, however, since Strauss and coworkers have previously demonstrated facile exchange of the pyrroline CH and CH$_2$ protons by $^1$H NMR studies in D$_2$O (Sullivan et al., 1991). Figure 2.10 shows the exchangeable protons of siroheme in D$_2$O. The D$_2$O effects claimed to demonstrate demetallation may therefore be rationalized in terms of the normal siroheme model.

(2) Optical spectra of acid-extracted chromophore are purported to demonstrate the presence of demetallated siroheme in desulfoviridin. We have demonstrated that acid conditions may demetallate siroheme during the extraction procedure. The data in Figure 2.6 compares the spectra of heme isolated under acidic and basic conditions. In both cases the final spectra of the products were obtained under basic conditions. Demetallation of siroheme following acid extraction is clearly evident.

(3) Fractions (a) - (f) in the FPLC trace shown in Figure 2.4 were analyzed for iron content and the results summarized noted in Table 2.4. The high iron content of published preparations arises in part from contaminants in the preparations studied. Previously, the iron content for the dissimilatory enzyme had been evaluated at ~ 22
Figure 2.10  Diagram shows the exchangeable protons of siroheme in D$_2$O.
mole equivalents of iron per enzyme (Pierik and Hagen, 1991; Moura
et al., 1988). This is in good agreement with our average value of ~
24 obtained prior to the FPLC step. The data in Table 2.4 clearly
shows that these abnormally high iron quantitations result from
contaminants that appear to be eluted from the FPLC column
between bands II and III (Figure 2.4). After one additional FPLC run,
the stoichiometry of iron : enzyme content was fixed at 10 : 1 (Table
2.5).

The EPR spectrum shown in Figure 2.8 was obtained from DV prior
to FPLC purification. The spectrum is very similar to published data
(Pierik and Hagen, 1991). The spurious peaks at low field were taken
as evidence for a [Fe₆S₆] cluster. They have reported three different
spectrum of DV from different protein preparations which contained
different extents of contaminants. While such signals may very well
arise from a novel and interesting cluster system, this is not located
in the dissimilatory sulfite reductase, but rather from an as yet
unpurified component in the preparation. It is unlikely that the
putative [Fe₆S₆] clusters decompose during work up since we find
that the activity of enzyme typically increases (and is never lower)
after the FPLC step, and is greater than published values. Perhaps
the clearest evidence against the occurrence of high nuclearity
clusters in these enzymes derives from the fully reduced enzyme,
which is most easily obtained by the photoreduction method of
Massey & Hemmerich (1978). The spectrum in Figure 2.9 is typical of
a reduced [Fe₄S₄] center. Previous evidence for a high nuclearity
cluster has rested on the appearance of unusual signals with large g-
values obtained on the native oxidized enzyme. We have shown that these signals do not derive from the dissimilatory sulfite reductase. The contaminants in the pre-FPLC sample do not appear to be significantly inhibitory of enzyme activity, based on quantification of enzyme by monitoring the 630 nm band. The EPR spectra of purified DV (oxidized and reduced, Figure 2.7 and 2.9 respectively) are those of typical high spin ferric hemes and regular [Fe₄S₄] centers, although our studies do not directly address the issue of whether or not these centers are coupled by a bridging ligand in the dissimilatory sulfite reductase.

**EPR parameters for DV.** The body of evidence presented above argues convincingly for the [Fe₄S₄] and siroheme prosthetic center content of this class of dissimilatory enzyme. The zero-field parameter $D \approx 6.5 \pm 1.5$ cm$^{-1}$ (details in Ch. VI) is lower than the value of 10 cm$^{-1}$ commonly found for high spin ferric hemes, while for the *E. coli* sulfite reductase, a $D \approx 8$ cm$^{-1}$ has been reported. Table 2.6 also clearly demonstrates the loss of spin intensity in both the oxidized and reduced state. The reasons for this are unclear at present, although the data suggest partial spin population of EPR silent states. EPR silence of fully-metallated sirohemes has previously been observed for the sulfite adduct of *E. coli* sulfite reductase. Possible origins of this effect have been extensively discussed by Young & Siegel (1988) and Day et al. (1988). More recent reports on the dissimilatory sulfite reductase from *Desulfosarcina variabilis* (an enzyme accepted to possess a full complement of two metallated siroheme) also records a significantly lower spin count for the
high-spin hemes (Arendsen et al., 1993). Additional spin density was observed from a set of signals ascribed to a mixture of low spin hemes. However, we have shown that the analogous signals in the \textit{D. vulgaris} enzyme arise from impurities and disappear after careful chromatography.

In summary, several reports have recently appeared on the family of dissimilatory sulfite reductases isolated from a variety of sulfate reducing bacteria. Unusual results have prompted a reexamination of the long-held model of the prosthetic site in these enzymes. In this chapter, it has been demonstrated that the purification and study of dissimilatory sulfite reductase enzyme is a non-trivial exercise. Careful purification and chemical quantitation of cofactors offers firm evidence for two pairs of $[\text{Fe}_4\text{S}_4]$ and siroheme cofactors. It is clear that there are interesting magnetic properties associated with these enzymes that should fuel further investigation. With regard to the issue of a bridging contact, we have previously argued that, from the point-of-view of the chemistry, a bridge is not required to effect electronic communication (Tan and Cowan, 1991). Certainly for desulfoviridin, this point remains moot. However, at this time there is no substantive evidence that argues convincingly against the model of Siegel and coworkers. It also appears unlikely that the enzyme contains high nuclearity clusters. The unusual EPR signatures noted most certainly arise from an as yet uncharacterized species.
CHAPTER III

Kinetic and Mechanistic Studies of

Enzymatic Reduction of Inorganic Anions by DV

ABSTRACT

This chapter will focus on kinetics studies performed on the dissimilatory sulfite reductase, desulfoviridin (DV) from Desulfovibrio vulgaris (Hildenborough), as an illustration of a systematic approach to unravel the underlying mechanisms of the enzymatic multielectron redox reactions of an inorganic anion. This chapter is divided into two parts: (I) Pre-steady-state kinetics analysis and mechanistics implications; and (II) Variable-temperature kinetics experiments (to map the energy profile of the multielectron redox reaction).

In Part I pre-steady-state experiments have been performed on DV and the microscopic rate constants for binding ($k_2$) and reductive cleavage of bonds ($k_r$) during enzymatic reduction of SO$_3^{2-}$ and NO$_2^-$ have been determined. For NO$_2^-$ reduction the reactivity of reaction intermediates have also been measured and a mechanistic scheme has been devised.
Second order rate constants for ligand association \([k_2(\text{AsO}_2^-) \sim 3 \times 10^3 \text{M}^{-1}\text{s}^{-1}; k_2(\text{HS}^-) \sim 1.8 \text{M}^{-1}\text{s}^{-1}]\) are consistent with the dominance of \(\pi\)-acceptor or \(\sigma\)-donor properties, respectively, of substrate molecules. A systematic strategy for the analysis of a multistep enzymatic reduction of an inorganic anion is described.

In part II variable temperature pre-steady-state kinetics experiments carried out on DV are described, and a detailed analysis of the total energy profile is made by considering results from variable temperature steady-state kinetics experiments (Lui et al., 1994). Activation free energies for reductive bond cleavage (\(\Delta G_{\text{r}*}\)) in \(\text{SO}_3^{2-}, \text{NO}_2^-, \text{NO},\) and \(\text{NH}_2\text{OH}\) substrates have been evaluated from variable temperature pre-steady-state kinetics data. Also, ground state (\(\Delta G_{\text{d}}\)) and transition state (\(\Delta G_{\text{t}*}\)) contributions to the overall activation free energy (\(\Delta G^*\)) from steady-state experiments have been examined. The choice of siroheme cofactor for this class of enzyme most likely reflects two factors underlying a preference for \(\pi\)-acceptor ligands. First, strong binding of substrate and weaker binding of product is promoted by the dominance of \(\pi\)-back-bonding. Second, population of an antibonding orbital in \(\pi\)-acceptor substrates lowers the transition state contribution to the activation free energy and serves to weaken the chemical bond that is to be reductively cleaved. These conclusions are supported by quantitative evaluation of activation barriers for substrates and reaction intermediates.
3.1 INTRODUCTION

The field of mechanistic inorganic biochemistry remains at an early stage of development. Oxidoreductase reactions (enzyme-catalyzed electron-transfer reactions) of inorganic anions and gaseous molecules are key steps in respiratory and anabolic pathways in cellular metabolism, and represent an area of intense investigation (Stiefel et al., 1988; Voucouvanis, 1988; Orme-Johnson, 1985; Seefeldt et al., 1992; Madden et al., 1991; Kim and Rees, 1992; Higuchi et al., 1987; Huynh et al., 1984 a, b; Grande et al., 1983; Bastian et al., 1988; Wackett et al., 1988; Tan and Cowan, 1991; Moura et al., 1986 & 1988; Pierik and Hagen, 1991; Averill and Tiejde, 1982; Godden et al., 1991; Vega et al., 1977; Henry and Bessieres, 1984; Petratos et al., 1986; McRee et al., 1986; Christner et al., 1983; Janick et al., 1983; Larsen et al., 1992; Chan and Li, 1990; Shapleigh et al., 1992). Typically these reactions occur in a step-wise manner via a series of partially reduced intermediates. Mechanisms for selective binding and activation of inorganic anions or gaseous molecules by metalloenzymes may differ in several important respects relative to common examples of non-redox enzymatic catalysis on organic substrates. The metallo-redox prosthetic center both binds and catalytically activates the substrate while neighboring protein side chains may regulate and optimize the electronic and coordination properties of the prosthetic metal sites toward these functions. Ionizable residues may further contribute to substrate recognition, binding and activation either by serving as proton donors or providing electrostatic stabilization of charged substrates or intermediates.
With these issues in mind, our laboratory has targeted the [Fe₄S₄]-
siroheme containing sulfite reducing enzymes (SO₃²⁻ —> HS⁻) from
Desulfovibrio vulgaris (Hildenborough) for detailed study. This enzyme
class also catalyzes the six-electron reduction of NO₂⁻ —> NH₃,
although a larger range of prosthetic redox centers and pathways are
available for the reduction of nitrite: [Fe₄S₄]-siroheme (NO₂⁻ —> NH₃)
(Cammack et al., 1978), hexaheme (NO₂⁻ —> NH₃)(Moura et al., 1986),
copper ion (NO₂⁻ —> N₂, N₂O) (Averill and Tiejde, 1982; Godden et al.,
1991; Vega and Kamenn, 1977; Henry and Bessieres, 1984; Petratos et al.,
1986). The chemistry of the electron rich iron isobacteriochlorin
(siroheme) has been addressed in model studies (Ricjardson et al., 1979;
Chang and Fajer, 1980; Barkigia et al., 1982; Chang et al., 1981). Inasmuch
as the NO₂⁻ reduction pathway proceeds via intermediates that can be
studied as discrete substrates, this method provides a useful probe of the
general mechanistic features associated with this class of enzyme.
Previous studies have focussed extensively on structural issues with
emphasis on elucidation of the coordination details of the prosthetic
redox centers (Averill and Tiejde, 1982; Godden et al., 1991; Vega and
Kamen, 1977; Henry and Bessieres, 1984; Petratos et al., 1986; MvRee et al.,
1986; Christner et al., 1983; Janick et al., 1983). Mechanistic schemes have
been proposed only for the copper-containing dissimilatory nitrite
reducing enzymes from denitrifying bacteria following use of isotopically-
labeled substrates to elucidate reaction pathways (Averill and Tiejde, 1982;
Godden et al., 1991; Vega and Kamen, 1977; Henry and Bessieres, 1984;
Petratos et al., 1986; Weeg-Aerssens et al., 1988; Scott et al., 1989). To our
knowledge such schemes have not been tested by kinetics studies.
Although investigations of steady-state kinetics are of great importance to the enzymologist, they have severe limitations. They allow the calculation of the kinetic constants $K_m$ and $k_{cat}$, but the meaning of these constants depends on the assumptions made, such as the number of intermediates involved and the rates of their interconversion. Also, in the case of multielectron redox reactions involving sulfite and nitrite reduction, the intimate details of the stepwise mechanistic pathway are lost. In Part I of Ch. III, stopped-flow rapid-mixing methods are used to follow individual two-electron reduction steps by using substrate and intermediate analogues for each step, e.g. $\text{NO}_2^-$, NO and $\text{NH}_2\text{OH}$ for nitrite reduction. The kinetic mechanism of this enzymatic multielectron redox reaction can then be investigated in more detail by this pre-steady state kinetics method, enabling individual rate constants to be calculated.

To increase the mechanistic understanding of such reactions a series of studies to elucidate the molecular details of the chemistry underlying enzymatic nitrite and sulfite reduction has been initiated. Part I describes a systematic strategy to investigate individual two-electron reduction steps of a multielectron redox reaction. This methodology is of importance in application to other related systems. Results from pre-steady-state kinetics experiments of enzymatic reduction of sulfite and nitrite catalyzed by DV are reported. The enzyme contains the coupled $[\text{Fe}_4\text{S}_4]$-siroheme prosthetic center common to this class of sulfite and nitrite reductases (Cowan and Sola, 1990; Christner et al., 1983). As mentioned earlier, sulfite-reducing enzymes are capable of reducing nitrogenous substrates ($\text{NO}_2^-$ or $\text{NH}_2\text{OH}$ to $\text{NH}_3$), which are often more
convenient substrates for detailed mechanistic studies since NH$_2$OH is an intermediate state of the NO$_2^-$ reduction pathway (NO$_2^-$ $\rightarrow$ $\rightarrow$ NH$_2$OH $\rightarrow$ NH$_3$). From the pre-steady-state kinetics analysis of the reductive pathway for DV-catalyzed reduction of both SO$_3^{2-}$, NO$_2^-$ and putative reaction intermediates, microscopic rate constants for ligand binding ($k_2$) and bond cleavage steps ($k_r$) obtained are measured. Mechanistic implications from this pre-steady-state kinetics analysis are discussed in light of the role of the siroheme prosthetic center in controlling catalysis.

The working model for the reaction pathway studies is summarized in Figure 3.1, which shows enzymatic reduction arising through a sequence of three two-electron reductive cleavages of X-O bonds (X = S or N). By comparing the steady-state $k_{cat}$ values (Wolfe et al., 1994) with pre-steady-state rate constants for bond cleavage reactions ($k_r$) (from Part I), it is clear that only in the case of the intermediate species NH$_2$OH does $k_{cat} \sim k_r$ (Table 3.2), suggesting rate-limiting bond cleavage in NH$_2$OH. The structural and stereoelectronic factors that control this reaction, differentiate substrates and intermediates, and promote substrate binding and catalytic activation are unclear. This lack of detailed mechanistic insight is generally valid for the broad spectrum of oxido-reductase enzymes required for electron transfer to substrate anions and gaseous molecules.

Biochemical processes can only proceed spontaneously in such a direction that the free energy of the system, i.e. the energy that can be used to perform work, decreases. However, even energetically-favourable
Figure 3.1 Summary of a working model for enzymatic reduction of oxidized sulfur and nitrogenous substrates catalyzed by [Fe4S4]-siroheme catalyzed centers. (A) Profile for the first step of SO3^2- reduction. (B) Proposed reaction scheme for NO2^- reduction. (C) Reaction scheme for reduction of nitric oxide radical by the two-electron reduced enzyme.
Table 3.1 Pre-steady-state kinetics parameters for desulfoviridin.\textsuperscript{a}

<table>
<thead>
<tr>
<th>substrate/ligand</th>
<th>$k_2$ (M(^{-1}) s(^{-1}))</th>
<th>$k_r$ (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO(_3^{2-})</td>
<td>$4.3 \times 10^3$ (1')</td>
<td>12 (2')</td>
</tr>
<tr>
<td>NO(_2^{-})</td>
<td>$3.6 \times 10^3$ (1)</td>
<td>14 (2)</td>
</tr>
<tr>
<td>NO</td>
<td>$7 \times 10^5$ (3)</td>
<td>6.5 (4)</td>
</tr>
<tr>
<td>NH(_2)OH</td>
<td>24 (5)</td>
<td>9 (6)</td>
</tr>
<tr>
<td>AsO(_2^{2-})</td>
<td>$3 \times 10^3$</td>
<td>-</td>
</tr>
<tr>
<td>HS(^{-})</td>
<td>1.8</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Stopped-flow kinetics data for substrates and ligands were obtained as described in the legend to Figure 3.5 with the following solution conditions [ligand, $\lambda_{obs}$(nm), concentration range(mM)]: NO\(_2^{-}\), 438 nm, 1-200 mM; NO, 438 nm, 0.1-1 mM; NH\(_2\)OH, 438 nm, 1-250 mM; SO\(_3^{2-}\), 438 nm, 10-250 mM; AsO\(_2^{2-}\), 554 and 438 nm, 0.3-10 mM; HS\(^{-}\), 438 nm, 10-1000 mM]. Errors in each measurement are estimated to be on the order of ± 50%.

\textsuperscript{b} Numbers in parentheses ([#]) after rate constants correspond to the reaction step indicated in Figure 3.6.
chemical reactions have to overcome a potential-barrier, known as the activation energy, before the reaction can take place. This is explained by the need to form unstable transition-states. Catalysts, including enzymes, act by allowing the formation of different, more stable, transition-states and, thus, reduce the activation energy. The position of chemical equilibrium is unchanged but is reached much faster than in the equivalent uncatalyzed reaction. Enzymes react with substrates to form enzyme-substrate (ES) complexes; these are quite distinct from the transition-states which also occur as part of the process of enzyme catalysis. Figure 3.9 illustrates the course of reaction for a general enzyme catalysed reaction. Part II of Chapter III will concentrate on unraveling this energy profile for the enzymatic reduction carried by DV.

In addition to the bond cleavage step, the activation barriers for each step of the reaction reflect contributions from binding, steric barriers from protein sidechains, and conformational motion of the protein backbone. Evaluation of these discrete contributions to the total activation energy and understanding their relative magnitudes for a variety of substrate molecules represents a significant challenge. The availability of kinetics methods to monitor both steady- and pre-steady-state rate profiles for a number of substrates and reaction intermediates afforded us the opportunity to address this problem in quantitative detail. Accordingly, Part II has concentrated on evaluating kinetic activation energy barriers to map the energy profile of the enzymatic multielectron redox reaction by DV and rationalizing the underlying mechanistic implications. Results from variable temperature experiments conducted under steady-state (Lui et al., 1994) and pre-steady-state conditions that
account for the activation energies at each stage of the multi-step reduction reaction are reported. The free energy profiles resulting from this analysis offer insight on the catalytic mechanism and the importance of the special siroheme cofactor for mediating this reaction pathway. The systematic approach presented in this chapter serves as an illustration of how to unravel detailed mechanistic pathways for enzymatic multielectron reactions and can be adapted to study other related systems.

3.2 MATERIALS AND EXPERIMENTAL METHODS

General Materials. Buffer salts were of molecular biology grade (Fisher or Aldrich Chemical Co). Measurements of solution pH were made with an Accumet pH Meter 910 equipped with a Corning semi-micro combination pH electrode. Sephadex G-200 gel filtration material were obtained from Sigma, and DEAE-52 ion exchange resin from Whatman. Deazaflavin was synthesized by literature methods (Janda & Hemmerich, 1976). All water used was purified with a Barnstead nanopure system and exhibited a resistivity of 18 MΩ cm⁻¹.

Bacterial Growth, Isolation and Purification of DV. *D. vulgaris* (Hildenborough, NC1B 8303) possessing a broad-host range expression vector (pDSK519) with the SiR gene and promoter cloned into the multiple cloning site (Tan et al., 1994) was grown in a lactate-sulfate medium. The purification details were referred to Ch. II.

Stopped-Flow Kinetics. In order to investigate the formation (ligand binding) and breakdown of the enzyme-substrate complex (here this is equivalent to the reductive cleavage of an X-O bond), it is necessary
to use techniques capable of detecting changes taking place over time scales on the order of magnitude of milliseconds. Stopped-flow techniques have been adopted in this chapter to study the pre-steady state kinetics of enzymatic sulfite/nitrite reduction. Solutions of enzymes and substrates are rapidly injected together into an observation chamber and the course of reaction monitored continuously. Figure 3.2 shows the experimental setup for the stopped-flow rapid kinetics experiments.

**Preparation of photoreduced enzyme:** A 10 ml volume of a solution containing 60 μM enzyme, 120 μM deazaflavin and 15 mM EDTA in potassium phosphate buffer (50 mM, pH 7.6) was deaerated in a 10 ml pear-shaped flask by purging the surface of the stirred solution for 30 min with O₂-free Ar(g). A gas-tight Hamilton syringe was pre-flushed with Ar(g) and loaded with the Ar(g)-purged solution under positive pressure. The syringe mouth was fitted with a small serum stopper to prevent O₂ diffusion and subsequently immersed in ice water and irradiated (1000 W lamp, 90% power) for 20 min to promote deazaflavin photoreduction of DV (Massey and Hemmerich, 1978; Janda and Hemmerich, 1976; Yoneda et al., 1976). During preliminary experiments, reduction was monitored by electronic absorption spectroscopy and irradiation was continued until no further change was observed in the optical spectrum. A second gas-tight syringe was loaded in the same manner with the appropriate argon-purged substrate solution (see footnote to tables), without subsequent irradiation. The stopped-flow apparatus was flushed with Ar(g)-purged buffer prior to each experiment. Reactants in the drive syringes were pre-equilibrated at the appropriate temperature prior to mixing, and absorption changes were typically monitored at 438 nm. Figure 3.3 shows
Figure 3.2  Experimental setup for stopped-flow rapid kinetics measurements. Reproduced from Burgess, 1978.
Figure 3.3  (A) Optical changes of DV during ligand binding to reduced form. Spectra were taken in a 1 cm pathlength optical cuvette with 6 μM enzyme, 10 mM AsO$_2^-$ in 50 mM potassium phosphate buffer, pH 7.5, at 298 K.

(B) Optical changes of DV during redox change from reduced to oxidized forms. Spectra were taken in a 1 cm pathlength optical cuvette with 6 μM enzyme in 50 mM potassium phosphate buffer, pH 7.5, at 298 K.
the optical changes of DV during ligand binding to reduced form and the change from reduced form to oxidized form. The optical changes at 438 nm are found to be maximal to be followed during reaction. During the course of the experiment the gas-tight Hamilton syringe containing the enzyme was occasionally irradiated in situ for 2 min. to ensure retention of a high fraction of the two-electron reduced enzyme during the prolonged experiments. A water filter was placed between the syringe and the lamp. **Instrumentation and methods:** Data were obtained with an OLIS (On-Line Instrument Systems, Inc) stopped-flow apparatus. A broad band 75 watt xenon arc lamp source (Ischio) powered by an OLIS XL150 power supply was filtered through a monochromator (model H10 by Instruments Sa.) with a resolving power of 8 nm/mm. A photomultiplier tube (Homatsu) usable between 185 and 900 nm was mounted linearly from the source to detect at 438 nm. The piston gas (nitrogen) was delivered at a rate between 9 to 14 ml/sec of the reaction solution. Rate constants were determined by use of the OLIS Operating System software (version 12.05) by fitting to proprietary software for rise-fall kinetics.

**Kinetics of SO$_3^{2-}$ vs NO$_2^-$ Reduction.** Since many nitrite reductases possess the same siroheme and [Fe$_4$S$_4$] prosthetic center, and inasmuch as the NO$_2^-$ reduction pathway proceeds via intermediates that can be studied as discrete substrates, this method provides a useful probe of the general mechanistic features associated with this class of enzyme. Nitrogenous substrates are in fact excellent tools for mechanistic investigations. Actually, it is found that the sulfite reductases can also perform the nitrite reduction (Wolfe et al., 1994). The relevance of using nitrite versus sulfite as a substrate may be questioned. Since the [Fe$_4$S$_4$]-
siroheme prosthetic center is common to both sulfite and nitrite reductase enzymes, comparative studies of NO$_2^-$/SO$_3^{2-}$ chemistry appears reasonable. This situation should be contrasted with the copper and heme cd containing nitrite reductases where there is no sulfite reductase analog. The use of substrate analogs is an often used strategy in enzymology while there are additional reasons for detailed investigations of NO$_2^-$ reduction; namely, the availability of reduced intermediates that can be further employed in mechanistic studies. The utility of this strategy have been previously demonstrated (Wolfe et al., 1994). So in this chapter, effort will be focussed on the analysis of the nitrite reduction kinetics. Similar arguments can be applied to sulfite reduction kinetics. The reaction scheme previously outlined for SO$_3^{2-}$ reduction to HS$^-$ is shown in Figure 3.4.

3.3 RESULTS and DISCUSSION

3.3.1 Pre-Steady-State Kinetics Analysis and Mechanistic Implication

A program of experiments to evaluate the detailed molecular mechanism for enzymatic reduction of inorganic substrates has been initiated. Here the reaction pathways for reduction of substrate molecules possessing N-O and S-O bonds under pre-steady-state conditions are examined, and substrate binding and bond cleavage chemistry are evaluated.

Pre-Steady-State Kinetics. Although investigations of steady-state kinetics are of great importance to the enzymologist, they have severe
Figure 3.4  Scheme outlines sulfite reduction.
limitations. They allow the calculation of the kinetic constants $K_m$ and $k_{cat}$, but the meaning of these constants depends on the assumptions made, such as the number of intermediates involved and the rates of their interconversion. Also, in the multielectron redox reaction (like sulfite and nitrite reduction), the detail in the mechanistic pathway is lost. In this chapter, stopped-flow rapid-mixing methods are used to follow individual two-electron reduction steps by using substrate and intermediate analogues for each step; e.g., NO$_2^-$, NO and NH$_2$OH for nitrite reduction. The kinetic mechanism of this enzymatic multielectron redox reaction can then be investigated in detail by pre-steady state measurements.

The six-electron reduction can be viewed and investigated in three subsequent reductive steps. Each one can be represented by the following reaction scheme:

$$\begin{align*}
   &E_{\text{red}} + S &\rightarrow &E_{\text{red}} \cdot S &\rightarrow &E_{\text{ox}} \cdot P_1 \\
\end{align*}$$

where $E_{\text{red}}$ represents the two-electron reduced enzyme; $S$ represents the substrate; $E_{\text{ox}}$ represents the native oxidized enzyme; $P_1$ represents the intermediate formed after the first reductive cleavage of a X-O bond by transferring the two electrons from $E_{\text{red}}$ to $S$. 
After the first two-electron reductive bond cleavage, the enzyme is not supplied with any additional electron source, thus the reaction is quenched at this point. The reaction being monitored hence represents the individual reductive step (including bimolecular binding and unimolecular bond cleavage). In a separate experiment, another substrate (intermediate analog) can be used and the reaction being monitored will represent the corresponding reductive step. In this manner, each step can be easily investigated separately to get more detailed mechanistic information.

**Stopped-Flow Experiments.** Absorbance wavelengths used in stopped-flow experiments give rise to prominent changes following redox chemistry or ligand binding, and so both reaction steps were directly monitored (Figure 3.5, Table 3.1). Relative to oxidized DV, there is a pronounced decrease in the absorbance tail from the Soret region (~ 400 nm) that extends to the Q-band region (~ 450-630 nm) for reduced enzyme (either with or without exogenous ligand) (Lui et al., submitted). This affords a useful spectral domain to probe kinetics of both oxidation/reduction and ligand binding by optical methods.

Deazaflavin photoreduction of 60 μM desulfoviridin yielded the two-electron reduced enzyme (Massey and Hemmerich, 1978; Janda and Hemmerich, 1976; Yoneda et al., 1976). Substrate binding and subsequent reaction to regenerate oxidized siroheme results in absorbance changes that were used to monitor the two-electron reduction of SO\(_3^{2-}\), NO\(_2^-\) and intermediates. Excess ligand was reacted with reduced enzyme in the mixing chamber of a stopped-flow instrument under pseudo-first order
Figure 3.5  A typical fit to a rise-fall rate profile. The data shown was taken for a final [DV] = 30 μM, [NO$_2^-$] = 100 mM.
conditions. Ligand concentrations are detailed in Table 3.1. For substrates, both the binding ($k_2$) and reductive ($k_r$) steps were directly monitored and the resulting optical traces fit by rise-fall kinetics profiles (Figure 3.5). One of the rate constants, obtained by fitting the trace with rise-fall kinetics, is found to dependent on the concentration of the ligand/substrate concentrations. This was assigned to be the bimolecular binding rate constant ($k_2$). The other rate constant is independent on the ligand/substrate concentration and is assigned to be the unimolecular bond cleavage rate constant ($k_r$). Second-order rate constants ($k_2$) were evaluated by variation of ligand/substrate (L) concentration ($k_{obs} = k_2[L]$).

**Binding and Reaction Rates.** Figure 3.6 summarizes the stopped-flow kinetics data of the working model mentioned earlier. The kinetic parameters detailed in Table 3.1 suggest that substrate binding to reduced enzyme is generally rapid and not rate limiting. An exception is found with lower concentrations of NH$_2$OH. The dependence of on-rates ($k_2$) on ligand nucleophilicity suggests that binding to the siroheme is associative in character: $k_2$(SO$_3^{2-}$) $\sim 4.3 \times 10^3$ M$^{-1}$s$^{-1}$, $k_2$(NO$_2^-$) $\sim 3.6 \times 10^3$ M$^{-1}$s$^{-1}$, $k_2$(NO) $\sim 7 \times 10^5$ M$^{-1}$s$^{-1}$, $k_2$(NH$_2$OH) $\sim 24$ M$^{-1}$s$^{-1}$. Moreover, if H$_2$O is bound to the axial site of the "pentacoordinate" high spin siroheme, its release is not rate limiting. On-rates for AsO$_2^-$ and HS$^-$ [$k_2$(AsO$_2^-$) $\sim 3 \times 10^3$ M$^{-1}$s$^{-1}$, $k_2$(HS$^-$) $\sim 1.8$ M$^{-1}$s$^{-1}$] are consistent with the dominance of $\pi$-acceptor or $\sigma$-donor properties, respectively, of substrate molecules. Although AsO$_2^-$ has the ability to both $\sigma$-donate and $\pi$-accept, the latter appears to dominate. This observation is consistent with the coordination behavior of other potential $\sigma$-donor/$\pi$-acceptor ligands such
Figure 3.6  Stopped-flow kinetics data for substrates and ligands were obtained as described in Figure 3.5 and Table 3.1. Only the bridge iron of the cluster is shown. Formal valencies on the iron centers are to aid in electron counting. It is possible that initial oxidation results by electron loss from the ring, rather than the ferrous ion of siroheme. Alternatively, both electrons may initially derive from the metal and the organic chromophore of siroheme. The detailed timing of proton transfer steps, relative to substrate binding and electron transfer, is not clear at this time. The bridging ligand X is most likely HS⁻ or S²⁻. (A) Rate profile for the first step of SO₃²⁻ reduction. (B) Proposed reaction scheme for NO₂⁻ reduction. (C) Reduction of nitric oxide radical by the two-electron reduced enzyme most likely proceeds by a modified pathway (via a radical cation) that is mechanistically consistent with the proposed pathways for NO₂⁻ and SO₃²⁻ reduction. The numeral adjacent to certain steps on the reaction pathways can be cross-referenced with the rate data in Table 3.1.
as CN−, and CO. The electron rich siroheme ring promotes π-backbonding through the d-orbitals of the ferrous ion. It is likely, in fact, that π-backbonding of nitrite and sulfite plays a crucial role in the molecular mechanism of catalysis: promoting tight binding of substrate relative to product, while population of antibonding N-O or S-O orbitals results in a weakening of the bonds that are to be reductively cleaved. The large value of \( k_2(\text{NO}) \approx 10^5 \text{M}^{-1}\text{s}^{-1} \) for NO binding is consistent with on-rates determined for other heme proteins (hemoglobin (α/β-subunits) \( \approx 3 \times 10^7 \text{M}^{-1}\text{s}^{-1} \)) (Olson, 1981).

Steady-state kinetics parameters for NO\(_2^-\) and NH\(_2\)OH and other substrates have been previously measured (Wolfe et al., 1994; Lui et al., 1994). Table 3.2 summarizes the reaction rate constants for pre-steady-state and steady-state kinetics at room temperature. With the exception of NH\(_2\)OH, reaction rates \( (k_r) \) show minimal correlation with steady-state turnover, \( k_{\text{cat}} \): \( k_r(\text{SO}_3^{2-}) \approx 12 \text{s}^{-1}, k_{\text{cat}}(\text{SO}_3^{2-}) \approx 0.3 \text{s}^{-1}; k_r(\text{NO}_2^-) \approx 14 \text{s}^{-1}, k_{\text{cat}}(\text{NO}_2^-) \approx 0.04 \text{s}^{-1}; k_r(\text{NH}_2\text{OH}) \approx 9 \text{s}^{-1}, k_{\text{cat}}(\text{NH}_2\text{OH}) \approx 30 \text{s}^{-1} \) (Wolfe et al., 1994), suggesting that bond cleavage steps are not rate limiting in the early stages of the reaction\(^1\). For hydroxylamine, \( k_r \approx k_{\text{cat}} \) and the enzyme saturates when \([\text{NH}_2\text{OH}] > 250 \text{mM} \) \( (K_m \approx 46 \text{mM}) \) (Wolfe et al., 1994). Both observations are consistent with the second order binding constant, \( k_2 \approx 24 \text{M}^{-1}\text{s}^{-1} \).

---

\(^1\) Rapid-quench EPR experiments yielded \( k_r(\text{SO}_3^{2-}) \approx 95 \text{s}^{-1}, k_r(\text{NO}_2^-) \approx 115 \text{s}^{-1} \) for \( \text{E.coli} \) sulfite reductase (Janick et al., 1983).
Table 3.2  Summary of reaction rate constants at room temperature.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\text{SO}_3^{2-}$</th>
<th>$\text{NO}_2^-$</th>
<th>$\text{NO}$</th>
<th>$\text{NH}_2\text{OH}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_r \ (s^{-1})$</td>
<td>12</td>
<td>14</td>
<td>6.5</td>
<td>9</td>
</tr>
<tr>
<td>$k_{cat} \ (s^{-1})$</td>
<td>0.3</td>
<td>0.04</td>
<td>-</td>
<td>30</td>
</tr>
</tbody>
</table>

Reproduced from Lui et al., 1994.
Implications for Enzyme Mechanism and a Molecular Understanding of the Reaction Pathway. In the context of the reaction scheme previously outlined for \( \mathrm{SO}_3^{2-} \) reduction to \( \mathrm{HS}^- \) (Tan and Cowan, 1991), Figure 3.1 illustrates the corresponding model for reduction of \( \mathrm{NO}_2^- \). That each of the substrates employed (\( \mathrm{NO}_2^- \), \( \mathrm{NO} \), \( \mathrm{NH}_2\mathrm{OH} \)) reacts readily lends credence to this mechanistic interpretation of the results. The reaction is viewed as arising through three 2-electron reductive cleavages of \( \mathrm{N-O} \) (or \( \mathrm{S-O} \)) bonds (Tan and Cowan, 1991). As detailed later, there is a requirement for a number of distinct proton transfer steps. The reaction of nitrosyl radical most likely reflects the availability of an additional reducing equivalent in the isobacteriochlorin ring (Figure 3.1 C). Nevertheless, a transition siroheme (\( \text{Fe}^{2+}\text{-NO} \)) adduct can be detected by EPR (Figure 3.7) after addition of \( \mathrm{NO}_2^- \) to reduced DV (characteristics coupling patterns are observed with \( ^{14}\text{NO}_2^- \) and \( ^{15}\text{NO}_2^- \)) (Janick et al., 1983). In related enzymes this evidence has been used to argue for nitric oxide as an obligatory intermediate (Janick et al., 1983), however, this would require only a one-electron reduction. Subsequent reductive steps would require either a further three-electron reduction to produce \( \mathrm{NH}_2\mathrm{OH} \), or a series of one-electron and/or two-electron transfers. Although this cannot be discounted a priori, we find this less appealing inasmuch as it is difficult to rationalize the need for one-electron reductive steps.
Figure 3.7  EPR spectrum observed upon addition of $^{14}\text{NO}_2^-$ to 2e$^-$ reduced desulfoviridin.
Scheme 1 illustrates an alternative explanation for formation of this nitrosyl complex by invoking back-electron transfer from "[NO-]" after the initial reductive addition. No additional electrons are added to push the reaction forward, and so the thermodynamically most stable form of the intermediate is adopted.

**Role of Proton Transfer.** The mechanism formulated in this chapter implicitly demands participation by ionizable residues in substrate binding and proton delivery. Clearly, reduction of either SO$_3^{2-}$ or NO$_2^-$ (summarized below, assuming a reaction pH of 7.6) requires an efficient pathway for transfer

\[
SO_3^{2-} + 6e^- + 7H^+ \rightarrow HS^- + 3H_2O
\]
NO$_2^-$ + 6e$^-$ + 8H$^+$ $\rightarrow$ NH$_4^+$ + 2H$_2$O

of proton equivalents to the active site. In this regard resonance Raman studies of *E. coli* sulfite reductase have suggested hydrogen bond formation to enzyme-bound CN$^-$ (Han et al., 1989). There are, however, two significant differences in the reactions represented by these equations. First, the formal reaction products are formally regarded as an acid (H$_2$S) or a base (NH$_3$), respectively. Second, the number of protons that must be delivered to the Fe-bound N or S atom (at pH 7.6) is distinct [one for SO$_3^{2-}$ and 3 (or 4) for NO$_2^-$]. This is a simple consequence of the inability of nitrogen, unlike sulfur, to accommodate these additional electron lone pairs. We have found the turnover number ($k_{cat}$) for NO$_2^-$ and NH$_2$OH to be essentially independent of pH over the range 5 - 10. This contrasts with steady-state kinetics data for *E. coli* sulfite reductase that demonstrated pH optima of 7.9, 8.6 and 9.5 for SO$_3^{2-}$, NO$_2^-$, and NH$_2$OH, respectively (Siegel et al., 1974). The dependence of the optimum pH on substrate suggests that for that enzyme the data is not tracking ionization of an amino acid side-chain. In addition the results show no obvious correlation with the pKa values listed below for these molecules. For desulfoviridin proton transfers are apparently non-rate-limiting. Regular solution pKa's for substrate and product molecules are as follows: H$_2$SO$_3$ (pK$_1$ 1.8, pK$_2$ 6.9); HNO$_2$ (pK 3.4); NH$_3$OH$^+$ (pK 6.0); H$_2$S (pK$_1$ 12.0, pK$_2$ 7.0); NH$_4^+$ (pK 9.3) (C. R. C., 1990). Accordingly, the protonation states of substrate anions and intermediates shown in Figure 3.1 and 3.6 are appropriate for the pH of 7.6 employed in these experiments, and simply
represent book-keeping. The precise timing of the proton transfer steps indicated in Figure 3.1 and 3.6 (relative to substrate binding and electron transfer) remains unclear at this time. For example, does SO$_3^{2-}$ or HSO$_3^-$ bind initially, is two-electron reductive bond cleavage facilitated by further protonation of the oxygen atom to produce H$_2$O or is HO$^-$ released? Clearly these points require further evaluation.

In summary, a pre-steady-state analysis of an enzymatic multielectron redox reaction of an inorganic anion has been established and a preliminary mechanistic model that rationalizes these and other published data is outlined. The structural and stereoelectronic features of the catalytic apparatus that promote this reaction and the rate-limiting factors in the early stages of sulfite and nitrite reduction are unclear at this time but form the basis for future investigations.

3.3.2 Variable Temperature Kinetics Experiments to Map the Energy Profile of the Multielectron Redox Reaction.

A small apparent $k_{cat}$ for SO$_3^{2-}$ and NO$_2^-$ relative to NH$_2$OH reduction has been previously demonstrated (Wolfe et al., 1994) (Table 3.2), suggesting that bond cleavage steps in the early stages of the reaction might be rate-limiting. This would follow expectations based on the relative S-O and N-O bond energies for each substrate. That is, the resonance stabilized sulfite and nitrite anions react more slowly than hydroxylamine. However, this hypothesis is not supported by the similarity in rate constants for bond cleavage determined from pre-steady-state kinetic measurements for two-electron reduction of SO$_3^{2-}$, NO$_2^-$, NO and NH$_2$OH ($k_r$(SO$_3^{2-}$) $\sim$ 12 s$^{-1}$, $k_r$(NO$_2^-$) $\sim$ 14 s$^{-1}$, $k_r$(NO) $\sim$ 6.5 s$^{-1}$ and
$k_T(NH_2OH) \sim 9 \text{s}^{-1}$} [Part I]. This dichotomy can be explained if the $\pi$-acid ligands $SO_3^{2-}$ and $NO_2^-$ should exhibit weaker S-O and N-O bonds, respectively, after binding to the electron rich siroheme as a result of $\pi$-back-bonding and population of the antibonding S-O and N-O orbitals. In this part a series of experiments that lend quantitative support to this hypothesis is described, and a free energy profile for the catalytic reduction of $NO_2^-$ to $NH_3$ is constructed. This provides considerable insight on the factors underlying the enzymatic reduction of these inorganic species.

To delineate the energy profile for the multi-step redox reaction catalyzed by the dissimilatory sulfite reductase, the temperature dependence of steady-state (Lui et al., 1994) and pre-steady-state kinetic rate constants have been systematically examined. The latter principally reflect bond cleavage chemistry, while the former also reflect additional activation barriers on the reaction pathway. Moreover, only for the pre-steady-state study (and also the steady state kinetics of hydroxylamine reduction) can individual two-electron reductive bond cleavage steps along the reaction pathway be investigated. That is, steady-state turnover data obtained for $SO_3^{2-}$ or $NO_2^-$ reduction includes the complete sequence of three two-electron reductive steps.

**Pre-Steady-State Variable Temperature Data.** The temperature dependence of the Arrhenius rate constant $k_T$ is given by equation (1), which can be rewritten in a form (2) that is of greater utility for variable temperature experiments since it accounts for the inherent temperature
dependence of $\Delta G_r^*$. This form of the rate equation has been used to analyze pre-steady-state rate data. Figure 3.8 illustrates typical plots obtained from variable temperature stopped-flow data using equation (2). The activation free energy ($\Delta G_r^*$) is calculated directly from equation (1), and the enthalpic ($\Delta H_r^*$) and entropic ($\Delta S_r^*$) components from the temperature dependence of $k_r$ defined by equation (2). The results from these measurements are given in Table 3.3. The data for the family of nitrogenous substrates will be considered before commenting later on the result for sulfite. The free activation barrier ($\Delta G_r^*$) for two-electron reduction of nitrite (16.2 kcal mole$^{-1}$) is similar to that for nitrous oxide (16.0 kcal mole$^{-1}$), but differs from the value for hydroxylamine (14.9 kcal mole$^{-1}$). There is, however, a more systematic change in the enthalpic and entropic components, which is discussed in a later section. In particular a decrease in the magnitude of the enthalpic barrier for the more reduced substrates is observed, which may correlate with substrate bond energy, and also an increase in the entropic barrier.
Figure 3.8  Pre-steady-state data. Plot of $R \ln \left( \frac{k_r h}{kT} \right)$ versus $1/T$ for a variety of substrate molecules. Enthalpic and entropic components ($\Delta H_r^*$ and $\Delta S_r^*$) were obtained from the slope and intercept according to equation (2).
Table 3.3  Pre-steady-state bond cleavage data.

<table>
<thead>
<tr>
<th>substrate</th>
<th>$\Delta H_f^*$ (kcal mole$^{-1}$)</th>
<th>$\Delta S_f^*$ (cal K$^{-1}$ mole$^{-1}$)</th>
<th>$\Delta G_f^*$ (kcal mole$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$SO_3^{2-}$</td>
<td>12.2</td>
<td>-11.4</td>
<td>15.6</td>
</tr>
<tr>
<td>$NO_2^-$</td>
<td>17.4</td>
<td>4.0</td>
<td>16.2</td>
</tr>
<tr>
<td>NO</td>
<td>12.5</td>
<td>-11.9</td>
<td>16.0</td>
</tr>
<tr>
<td>$NH_2OH$</td>
<td>5.0</td>
<td>-33.1</td>
<td>14.9</td>
</tr>
</tbody>
</table>

Stopped-flow kinetics data for substrates were obtained as previously described by monitoring the change in absorbance at 438 nm (Lui et al., 1993). The solution conditions are detailed as follows: $[DV] = 30 \mu$M, $[SO_3^{2-}] = 50$ mM, $[NO_2^-] = 50$ mM, $[NO] = 1$ mM, $[NH_2OH] = 100$ mM. Parameters were determined from equations (1-2) presented in the text. Samples were incubated for 15 minutes at the appropriate temperature over the range 8 to 45 °C prior to mixing. Errors in each measurement are estimated to be on the order of $\pm 0.3$ kcal mole$^{-1}$ for $\Delta H_f^*$, $\pm 1.5$ cal K$^{-1}$ mole$^{-1}$ for $\Delta S_f^*$, and $\pm 0.4$ kcal mole$^{-1}$ for $\Delta G_f^*$. 
Steady-State Variable Temperature Data (Lui et al., 1994). Initial studies (Wolfe et al., 1994) have shown that the steady state kinetics of the reaction can be reasonably considered in terms of a Michaelis-Menten model. In this model $k_{cat}$ reflects transition state energies and $K_m$ reflects ground state binding energies (Fersht, 1985). The assumption that $K_m \sim K_d$ (to a reasonable approximation) appears justified inasmuch as the $K_m$'s determined for substrates are very similar to $K_d$'s evaluated for inhibitor analoges (CN$^-$ and HS$^-$), while the likely error range would influence neither the discussion nor conclusions presented below (see Appendix B). For steady-state turnover, several simple relationships between standard parameters ($k_{cat}$ and $K_m$) and activation energies are readily derived, where $\Delta G_d$ and $\Delta G^*_t$ are the ground and transition state contributions to the activation free energy ($\Delta G^*$) (Figure 3.9). Detailed description of the steady-state derivations are provided in appendix. The steady-state variable temperature data (Lui et al., 1994) were added to obtain more understanding of the energy profile. These profiles can be developed more thoroughly by including the analysis of the temperature dependence of pre-steady-state rates since each discrete bond breaking step can be independently analyzed.

The activation parameters thereby determined are listed in Tables 3.4 to 3.6. Overall, there is a general decrease in the magnitude of $\Delta G^*$ from SO$_3^{2-}$ and NO$_2^-$ to NH$_2$OH. This arises not through a decrease in the magnitude of $\Delta G^*_t$, which in fact increases over this series, but rather through a decrease in the magnitude of $\Delta G_d$, reflecting stronger binding by the $\pi$-acceptor ligands NO$_2^-$ and SO$_3^{2-}$. The lower $\Delta G^*_t$ for these $\pi$-acceptor ligands presumably arises through population of the antibonding
Figure 3.9  General free energy profiles for substrate turnover showing the components of $\Delta G_r^*$, $\Delta G_{of}^*$, $\Delta G_d$ and $\Delta G_t^*$. Specific data is listed in Tables 3.3 to 3.6.
Table 3.4  Steady-state activation parameters.

<table>
<thead>
<tr>
<th>substrate</th>
<th>$\Delta H^\circ$ (kcal mole$^{-1}$)</th>
<th>$\Delta S^\circ$ (cal K$^{-1}$ mole$^{-1}$)</th>
<th>$\Delta G^\circ$ (kcal mole$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO$_3^{2-}$</td>
<td>2.6</td>
<td>-51.7</td>
<td>18.0</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>-6.8</td>
<td>-79.8</td>
<td>17.0</td>
</tr>
<tr>
<td>NH$_2$OH</td>
<td>3.1</td>
<td>-41.3</td>
<td>15.4</td>
</tr>
</tbody>
</table>

Kinetics data were obtained as previously described using saturating concentrations of substrate and MeV$^+$. Samples were incubated for 15 minutes at the appropriate temperature, over the range 10 to 45 °C, before the addition of MeV$^+$. Reaction conditions were as follows: [DV] = 140 nM, [SO$_3^{2-}$] = 1mM, [NO$_2^-$] = 200 mM, [NH$_2$OH] = 150 mM. The activation free energy at 298 K was determined directly from equation (4), and the enthalpic and entropic components were determined from equation (5). Errors in each measurement are estimated to be on the order of ± 0.5 kcal mole$^{-1}$ for $\Delta H^\circ$, ± 2 K$^{-1}$ cal mole$^{-1}$ for $\Delta S^\circ$, and ± 0.2 kcal mole$^{-1}$ for $\Delta G^\circ$. Any temperature dependence of the constant $K_m$ was accounted for over the temperature range employed. Nitric oxide proved to be unsuitable as a substrate for steady-state kinetics experiments.

Reproduced from Lui et al., 1994.
### Table 3.5 Break-down of steady-state activation parameters.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\Delta H_t^*$ (kcal mole$^{-1}$)</th>
<th>$\Delta S_t^*$ (cal K$^{-1}$ mole$^{-1}$)</th>
<th>$\Delta G_t^*$ (kcal mole$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO$_3^{2-}$</td>
<td>-5.8</td>
<td>-60.5</td>
<td>12.2</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>-5.6</td>
<td>-53.8</td>
<td>10.4</td>
</tr>
<tr>
<td>NH$_2$OH</td>
<td>12.6</td>
<td>-3.9</td>
<td>13.8</td>
</tr>
</tbody>
</table>

Kinetics data were obtained as described in the legend to Table 3.4. The activation free energy at 298 K was determined directly from equation (6a), and the enthalpic and entropic components were determined from equation (6b). Errors in each measurement are estimated to be on the order of $\pm 0.5$ kcal mole$^{-1}$ for $\Delta H_t^*$, $\pm 2$ cal K$^{-1}$ mole$^{-1}$ for $\Delta S_t^*$, and $\pm 0.2$ kcal mole$^{-1}$ for $\Delta G_t^*$.

Reproduced from Lui et al., 1994.
Table 3.6 Summary of the factors contributing to the activation free energy.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\Delta G_r^*$</th>
<th>$\Delta G_{of}^*$</th>
<th>$\Delta G^*$</th>
<th>$\Delta G_t^*$</th>
<th>$\Delta G_d$ (kcal mole$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SO$_3^{2-}$</td>
<td>15.6</td>
<td>2.4</td>
<td>18.0</td>
<td>12.2</td>
<td>5.8</td>
</tr>
<tr>
<td>NO$_2^-$</td>
<td>16.2</td>
<td>0.8</td>
<td>17.0</td>
<td>10.4</td>
<td>6.6</td>
</tr>
<tr>
<td>NO</td>
<td>16.0</td>
<td>-</td>
<td>$\leq$ 17.0</td>
<td>$\sim$ 1.9 (est)</td>
<td>$\sim$ 14.1 (est)</td>
</tr>
<tr>
<td>NH$_2$OH</td>
<td>14.9</td>
<td>0.5</td>
<td>15.4</td>
<td>13.8</td>
<td>1.6</td>
</tr>
</tbody>
</table>

All data is in units of kcal mole$^{-1}$. Kinetics parameters were determined from equations (1 - 8) and using the data from Tables 3.3 -3.5. Errors in each measurement are estimated to be on the order of $\pm$ 0.6 kcal mole$^{-1}$. Other than $\Delta G_r^*$, data for NO has been estimated (see Appendix C) owing to the problems associated with steady-state turnover of this reactive substrate.
N-O (or S-O) orbitals, which weakens the bond toward reductive cleavage (Jolly, 1984).

**Activation Parameters.** For \( \text{NO}_2^- \), \( \text{SO}_3^{2-} \) and NO reduction, through pre-steady-state kinetic measurements, it has been demonstrated that \( k_{\text{cat}} \) does not reflect reductive bond cleavage [Part I], and so the activation parameters noted in Figure 3.9 for \( \text{NO}_2^- \) reduction include other factors (\( \Delta G_{\text{of}}^* \)) that so far remain ill-defined. That is, the \( \Delta G^*(\text{NO}_2^-) \) indicated in Figure 3.9 contains contributions from both \( \Delta G_r^* \) and \( \Delta G_{\text{of}}^* \) (equation 3).

\[
\Delta G^*(\text{NO}_2^-) = \Delta G_r^*(\text{NO}_2^-) + \Delta G_{\text{of}}^*(\text{NO}_2^-)
\]  

The contribution to \( \Delta G^* \) from bond cleavage (\( \Delta G_r^* \)) can be independently determined from the pre-steady-state variable temperature data for the two-electron reduction of \( \text{NO}_2^- \), \( \text{SO}_3^{2-} \), and NO. Using the \( \Delta G^* \) values determined from steady-state rate measurements, the additional contributions (\( \Delta G_{\text{of}}^* \)) from enzyme conformational changes etc. can be determined (Table 3.6). These results demonstrate that bond cleavage gives rise to the dominant barrier for enzymatic substrate reduction (that is, \( \Delta G_r^* \gg \Delta G_{\text{of}}^* \)). In the specific case of hydroxylamine we might expect \( \Delta G_{\text{of}}^*(\text{NH}_2\text{OH}) \approx 0 \) since \( k_{\text{cat}} \approx k_r \). It is therefore reassuring to note that this is indeed experimentally verified [\( \Delta G^*(\text{NH}_2\text{OH}) \approx 15.4 \text{ kcal mole}^{-1} \); \( \Delta G_r^*(\text{NH}_2\text{OH}) \approx 14.9 \text{ kcal mole}^{-1} \); \( \Delta G_{\text{of}}^*(\text{NH}_2\text{OH}) \approx 0.5 \text{ kcal mole}^{-1} \)], confirming the conclusions reached earlier that bond-cleavage is the
dominant rate-limiting step for this substrate. For earlier reaction intermediates there is apparently a significant contribution from $\Delta G_{of}^*$ (but much smaller than $\Delta G_r^*$). The origin of this barrier remains unclear at this time, but may involve conformational changes of the protein or siroheme ring. Figure 3.9, and Tables 3.3 to 3.6 summarize our current evaluation of the free energy profiles that can be constructed from this data.

For a series of similar reactions there can exist a linear relationship between the activation enthalpies and entropies (Isaacs, 1987). Writing the free energy change ($\Delta G^*$) in a revised form ($\Delta H^* = T \Delta S^* + \Delta G^*$) it is seen that a linear plot of $\Delta H^*$ versus $\Delta S^*$ (obtained from variable temperature studies on a family of related reactions) yields a temperature $T$ (isokinetic temperature) at which the Arrhenius plots for each data set would intersect. In theory such a linear relationship suggests the dominance of one stereoelectronic parameter in controlling the relative rates of a number of substrates (Isaacs, 1987). The isokinetic relationship also reflects a balancing of the change in one activation parameter by another. That is, a more negative enthalpic component is offset by a more negative entropic component, and vice versa. Figure 3.10 shows the isokinetic plot obtained from the variation of $\Delta H_r^*$ and $\Delta S_r^*$, which yielded an isokinetic temperature of 334K (correlation coefficient of 0.998). As noted earlier, there is a general decrease in the magnitude of $\Delta H_r^*$, which most likely reflects the difference in bond energies. Since related plots for $\Delta H^*$ versus $\Delta S^*$, and $\Delta H_t^*$ versus $\Delta S_t^*$ are obtained over a narrower range of values, the deviations from experimental error are
Figure 3.10 Isokinetic plot of $\Delta H_r^*$ versus $\Delta S_r^*$. The gradient yields an isokinetic temperature of 334K (correlation coefficient of 0.998). Data for $SO_3^{2-}$, $NO_2^-$, NO, and NH$_2$OH was taken from Table 3.3.
more pronounced, but the general trends are similar.

Implications for the Understanding of Reaction Mechanism. In the discussion that follows, emphasis will be placed on the enzymatic reduction of NO$_2^-$ to NH$_3$ rather than the reaction of SO$_3^{2-}$. This simply reflects the availability of reaction intermediates and substrate analogues for the former with which to carry out detailed kinetics studies. It is reasonable to assume that the arguments and conclusions presented below are equally valid for SO$_3^{2-}$ reduction. It is also noted that there is an implicit assumption that the magnitude of $\Delta G^*$ for any given substrate mainly reflects the activation barrier for the first two-electron reductive cleavage. That is $\Delta G^*$ for NO$_2^-$ reflects the first two-electron step for NO$_2^-$ $\rightarrow$ "NO$^{-}\"$ etc, rather than reduction of a later intermediate. This is justified inasmuch as there is a decrease in the magnitude of $\Delta G^*$ moving from NO$_2^-$ to NH$_2$OH as substrate (see Appendix C for the special case of nitric oxide).

For nitric oxide only the magnitude of $\Delta G_{r}^*$ could be determined experimentally. However, estimates of $\Delta G^*$, $\Delta G_{t}^*$, and $\Delta G_d$ could be readily evaluated as described in Appendix C. The following trends in activation free energies may be noted from the data in Tables 3.4 to 3.6. First, the magnitude of $\Delta G^*$ decreases as SO$_3^{2-}$ $\rightarrow$ NO$_2^-$ $\sim$ NO $\rightarrow$ NH$_2$OH. Second, $\Delta G_{t}^*$ increases, with SO$_3^{2-}$ and NO$_2^-$ $<$ NH$_2$OH. Third, the binding affinity $\Delta G_d$ varies as SO$_3^{2-}$ and NO$_2^-$ $>$ NH$_2$OH. Note that NO yields anomalous $\Delta G_{t}^*$ and $\Delta G_d$ values as a result of the unusually strong binding to siroheme (see Appendix C). The discussion that follows will show how the observed trends in free energy components lend
considerable insight on the design of the catalytic site, and in particular the choice of siroheme as the catalytic cofactor.

The Role of the Siroheme Cofactor. The general decrease in $\Delta G_d$ over the series $SO_3^{2-}$ and $NO_2^-$ to $NH_2OH$ reflects the change in bonding as we progress from the strong $\pi$-acceptors $SO_3^{2-}$ and $NO_2^-$ to the purely $\sigma$-donor $NH_2OH$. It has been noted in Part I how the electron rich siroheme ring has a particularly high affinity for $\pi$-acceptor ligands (Lui et al., 1993). Now it will be shown that the bonding mode that results in tight binding by $NO_2^-$ is also responsible for the reversal in the relative magnitudes of $\Delta G_t^*$ along the same series. Population of the antibonding $S-O$ and $N-O$ orbitals as a result of $\pi$-back-bonding weakens the chemical bonds that are to be reductively cleaved (Jolly, 1984). This is reflected in the smaller values of $\Delta G_t^*$ for $SO_3^{2-}$ and $NO_2^-$ relative to the corresponding parameter for $NH_2OH$ (Table 3.6). It is then seen that the relative magnitudes of the $\Delta G_d$ and $\Delta G_t^*$ components are in opposition with regard to defining the overall activation barrier $\Delta G^*$. Although the absolute magnitude of the $\Delta G_t^*$ component is substantially greater than that of $\Delta G_d$, the relative change in the magnitude of $\Delta G_d$, comparing substrates and reaction intermediate, is larger than the corresponding change in the $\Delta G_t^*$ term. The observed trend in the magnitude of the $\Delta G^*$ values therefore reflects the dominance of the binding term ($\Delta G_d$). The design of the prosthetic center therefore accommodates two important requirements.

(1) Strong binding of substrate and weaker binding of product is promoted by the dominance of $\pi$-back-bonding. These factors are also
manifest by the kinetic rate constants for release of substrate and product (Lui et al., 1993; Christner et al., 1983). DV does show a relatively high $K_m$ for nitrite (Wolfe et al., 1994). The structural or electronic reasons for this selectivity are presently not clear. Assuming that $K_m$ is proportional to binding affinity, the increase in $K_m$ for NH$_2$OH relative to NO$_2^-$ reflects the weaker binding of intermediates and product relative to the $\pi$-acceptor substrate anion (NO$_2^-$). This represents part of the enzymes product release mechanism. The product species (NH$_3$) is a $\sigma$-donor ligand and is bound weakly by the reduced siroheme.

(2) The aforementioned scheme serves to weaken the chemical bond that is to be reductively cleaved by populating an antibonding orbital in $\pi$-acceptor substrates, thereby lowering the transition state contribution to the activation free energy (Jolly, 1984). This is carried to an extreme in the case of nitric oxide (Appendix C), although the intermediate (HNO) to be expected during nitrite turnover would most likely fit in with the overall trends noted above for $\Delta G^*$, $\Delta G_{d}$, and $\Delta G_{t}^*$ in the series NO$_2^-$ $\rightarrow$ HNO $\rightarrow$ NH$_2$OH $\rightarrow$ NH$_3$. It is noted that the contributions of proton transfer steps is not discussed which appear to be rapid and do not contribute significantly to $\Delta G^*$.

**Summary.** In Part II the component activation energies for each step of the catalytic reduction of an inorganic anion by variable temperature experiments conducted under steady-state and pre-steady-state conditions have been evaluated in detail. The free energy profiles resulting from this analysis offer insight on the catalytic mechanism and the importance of the special siroheme cofactor for mediating this
reaction pathway. This strategy should be of general value for the analysis of multistep enzymatic reductions of other inorganic substrates. This represents part II of a detailed study of the structural and electronic features of the substrate and enzyme that control ground and transition state energies for enzyme-catalyzed reduction of inorganic anions.

3.4 APPENDIX

(A) The magnitude of $\Delta G^*$ can be directly determined at any temperature from equation (4), which can be rewritten in the form (5), where $k$, $R$, and $h$ are the Boltzmann, gas, and Planck constants, respectively. The latter equation was used to evaluate the values of $\Delta H^*$ and $\Delta S^*$ (Table 3.4) from the temperature dependence of $k_{cat}$.

$$ k_{cat} = \left( \frac{kT}{h} \right) \exp\left( \frac{\Delta G^*}{RT} \right) $$

(4)

$$ R \ln\left( \frac{k_{cat}h}{kT} \right) = \Delta S^* - \frac{\Delta H^*}{T} $$

(5)

The transition state contribution ($\Delta G_t^*$) to the activation free energy ($\Delta G^*$) can be estimated in a similar fashion from $\ln(k_{cat}/K_m)$, $\ln(kT/h)$ (equation 6a), and the enthalpic and entropic components from the temperature dependence defined by equation (6b). For each substrate the temperature dependence of $K_m$ was evaluated and the appropriate value was used. Estimates of $\Delta G_d$ are made from relationship (8), assuming $K_m \sim K_d$. 
\[ RT \ln \left( \frac{k_{\text{cat}}}{K_m} \right) = RT \ln \left( \frac{kT}{h} \right) - \Delta G_t^* \] (6a)

\[ R \ln \left[ \frac{(k_{\text{cat}} h)/(K_m kT)}{kT} \right] = \Delta S_t^* - \frac{\Delta H_t^*}{T} \] (6b)

\[ \Delta G^* = \Delta G_t^* + \Delta G_d \] (7)

\[ \Delta G_d = -RT \ln K_m \] (8)

(B) Substrate or ligand anions of similar bonding capabilities appear to have similar binding affinities. For example, compare \( K_m(SO_3^{2-}) \sim 59 \text{ mM} \) with \( K_d(CN^-) \sim 200 \text{ mM} \), and \( K_m(NH_2OH) \sim 59 \text{ mM} \) with \( K_d(HS^-) \sim 21 \text{ mM} \) (Liang & Cowan, unpublished results). Minor systematic errors in \( K_d \) will have no effect on the conclusions reached in our discussion of the data, while major discrepancies are unlikely. A ten-fold error in \( K_d \) introduces an error of 1.4 kcal mole\(^{-1}\) in \( \Delta G_d \). Since \( K_d \leq K_m \), only if the magnitude of \( K_d \) for NH\(_2OH\) is underestimated by \( \sim 4 \text{ kcal mole}^{-1} \) (that is, by 10\(^3\) fold) are the general conclusions compromised. The on-rate for NH\(_2OH\) has previously been determined (\( \sim 24 \text{ M}^{-1} \text{ s}^{-1} \)) (Lui et al., 1993) and so an off-rate of \( \sim 1.2 \times 10^{-3} \text{ s}^{-1} \) would be required for a binding
affinity in the 50 mM range. This is inconsistent with estimates of off-rates for related ligands \( k_{\text{off}}(\text{NH}_3) \geq 30 \text{ s}^{-1} \) and \( k_{\text{off}}(\text{HS}^-) \geq 0.3 \text{ s}^{-1} \); Liang & Cowan, unpublished results, and Lui et al., 1993), and suggests that \( K_d \) for \( \text{NH}_2\text{OH} \) will not be significantly smaller than \( K_m(\text{NH}_2\text{OH}) \).

(C) The binding energy \( K_d \) may be estimated from \( k_{\text{off}}/k_{\text{on}} \). We previously determined \( k_{\text{on}}(\text{NO}) \sim 7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1} \) (Lui et al., 1993). We have no estimate of \( k_{\text{off}} \) for the NO complex of desulfoviridin; however, Olson has reported \( k_{\text{off}} \sim 3 \times 10^{-5} \text{ s}^{-1} \) for binding to heme in the \( \alpha \) and \( \beta \)-subunits of hemoglobin (Olson, 1981). Using this as an estimate yields \( K_d \sim 4.3 \times 10^{-11} \text{ M} \) (or \( \Delta G_d \sim 14.1 \text{ kcal mole}^{-1} \) at 298 K). We have determined \( \Delta G_r^* \sim 16 \text{ kcal mole}^{-1} \). For \( \pi \)-accepting nitrogenous species, \( \Delta G_{\text{of}}^* \) typically falls in the range of \( \sim 1 \text{ kcal mole}^{-1} \) (Table 3.6). Use of the relevant forms of equations (3) and (7) puts an upper limit of ca 17 kcal mole\(^{-1}\) for \( \Delta G^* \), and so if \( \Delta G_d \sim 14.1 \text{ kcal mole}^{-1} \) then \( \Delta G_t^* \sim 1.9 \text{ kcal mole}^{-1} \).
CHAPTER IV

Conformational Gating of the Dissimilatory Sulfite Reductase

ABSTRACT

The siroheme prosthetic center in the dissimilatory sulfite reductase (desulfoviridin) from Desulfovibrio vulgaris (Hildenborough) readily binds exogenous ligands in the reduced state, but does not do so in the oxidized state. In contrast, free oxidized siroheme in solution is observed to bind ligands rapidly. This can only be explained by a structural barrier that precludes ligand binding to the enzyme in the oxidized state, but is removed after reduction. These observations suggest a redox-linked structural transformation that provides a gating mechanism for enzyme activation. This chapter will demonstrate the strategy to probe this conformational gating in this class of enzymes. Experiments have been performed on DV as an illustration. The rate constants defining these structural perturbations, from oxidized → reduced and reduced → oxidized, have been determined by monitoring changes in both the natural emission from desulfoviridin and the emission from a surface-bound fluorophore (1,5-IAEDANS). Consistent results were obtained by these two independent methods (at 25 °C: \( k_{\text{ox} \rightarrow \text{red}} \approx 8 \text{ s}^{-1} \), \( k_{\text{red} \rightarrow \text{ox}} \approx 0.05 \text{ s}^{-1} \)). Activation energies for each transition have been
determined from Arrhenius plots. These data are used to further develop a functional model previously proposed for this class of enzyme in Ch. III. The data is consistent with a two-state hypothesis for enzyme activity: an active form (DVA) that binds and catalyzes substrate reduction and an inactive form (DVI) for the resting enzyme. Only the active form of the enzyme need be considered during steady-state turnover. Finally, second-order rate constants have been determined for reduction [at 25 ºC: Ti³⁺, k₂ ~ 18 M⁻¹ s⁻¹; MeV⁺, k₂ ~ 6.5 x 10⁴ M⁻¹ s⁻¹] and oxidation [at 25 ºC: W(CN)₆⁴⁻, k₂ ~ 200 M⁻¹ s⁻¹] of the siroheme prosthetic center.
4.1 INTRODUCTION

Many electron-transfer proteins (and heme proteins in general) undergo local or global structural changes following oxidation or reduction. Cytochrome c and hemoglobin are well characterized examples (Dickerson & Geis, 1983; Northrup & McCammon, 1984; Takano & Richardson, 1981a,b). Such redox proteins can be said to display the property of conformational gating; albeit in the simplest possible terms. In the case of oxido-reductase enzymes that couple electron-transfer to atom-transfer chemistry, conformational gating may play a more important role in the regulation of enzyme function. Examples of the latter include the α2 dimeric subunit of nitrogenase, which couples MgATP/MgADP binding to electron transfer from the [Fe₄S₄] ferredoxin cluster (Georgiadis et al., 1992). Moreover, ATP hydrolysis regulates binding of the α2 dimer (Fe-protein) and the α₂β₂ MoFe protein that contains the catalytic prosthetic center (Kim & Rees, 1992). Cytochrome c oxidase also functions by way of a conformationally gated proton-pumping mechanism (Chan & Li, 1990; Larsen et al., 1992; Shapleigh et al., 1992). In this case the structure of the enzyme is dependent on both the oxidation and coordination states of the component copper and heme redox centers.

The molecular mechanisms of the class of sulfite or nitrite reducing enzymes that possess a coupled [Fe₄S₄]-siroheme complex as the active redox prosthetic center has been investigated by kinetics studies in Ch. III. This chapter is a further investigation of the mechanism in another aspect, conformational gating. In both enzymes (SiR and DV) it has been found that the siroheme (which is the normal coordination site for substrate and other
exogenous ligands) is extremely inert toward binding when the enzyme is in the oxidized state, but readily binds exogenous ligands in the reduced state. This result is consistent with previous observations made for the oxidized state of the \textit{E. coli} enzyme (Janick et al., 1983). Inasmuch as free siroheme in solution binds exogenous ligand rapidly in either the oxidized or reduced state (estimated $k_2 \gg 200 \text{ M}^{-1} \text{ s}^{-1}$)\textsuperscript{1} these results suggest that for most, if not all, enzymes in this class, the switch from slow to rapid binding following reduction is gated by a redox-linked conformational change. Note that this need not correspond to a global change in enzyme structure, and may be manifest as a simple change in orientation of a single amino acid side-chain. The approach to probe the conformational gating is presented. DV is the enzyme being studied in this chapter. To determine the rates of these structural transitions we have surface-labeled desulfoviridin with the fluorescent probe 1,5-IAEDANS, which has been extensively used, both as a structural probe and in studies of protein dynamics (Park et al., 1991; Gardner & Matthews, 1991; Kawata & Hamaguchi, 1991; Gorman et al., 1987). This chapter reports results from rapid-stopped-flow kinetics experiments that quantitatively evaluate the rate constants for the structural changes induced after switching from the oxidized to the reduced state (ox $\rightarrow$ red), and also from the reduced to the oxidized state (red $\rightarrow$ ox). The results demonstrate that these changes are bona fide intramolecular pathways and do not simply correlate with second-order oxidation or reduction of the

\footnote{During siroheme extractions from sulfite reductase enzymes, exogenous ligands (CN$^-$ and pyridine) are found to bind to the oxidized heme in fractions of a second. Estimates of second-order rate constants from alkaline solutions of known cyanide concentration place a conservative lower limit of 200 M$^{-1}$ s$^{-1}$ on the association rate constants for CN$^-$ binding to free oxidized siroheme in solution.}
prosthetic center. Variable temperature experiments have been performed to determine the magnitude of the activation barriers for these transformations. By comparing these results with data previously obtained from pre-steady-state and steady-state measurements of active site chemistry (Ch. III, Lui et al., 1993, 1994), the structural contributions to the regulation of reaction chemistry have been rationally investigated. The kinetic rate constants and activation barriers evaluated herein are used to further develop the catalytic model described previously in Ch. III. The proposed structural change is consistent with a two-state model for enzyme activity: with a transition between an active form (DVa) that binds and catalyzes substrate reduction and an inactive form (DV1) for the resting enzyme.

4.2 MATERIALS and METHODS

General Materials and Instruments. Buffer salts were of molecular biology grade (Fisher or Aldrich Chemical Co). Measurements of solution pH were made with an Accumet pH Meter 910 equipped with a Corning semi-micro combination pH electrode. Sephadex G-25 gel filtration material was obtained from Sigma, and DEAE-52 ion exchange resin from Whatman. The fluorophore 1,5-IAEDANS was purchased from Molecular Probes Inc. Titanium(III) chloride was purchased from Aldrich Chemical Co. All water used was purified with a Barnstead nanopure system and exhibited a resistivity of 18 MΩ cm⁻¹. Deazaflavin was synthesized by literature methods (Janda & Hemmerich, 1976).

Culture Growth / Protein Isolation and Purification. Desulfoviridin was obtained from Desulfovibrio vulgaris (Hildenborough, NCIB 8303), grown in a lactate-sulfate medium. The purification details of and DV were
described to Ch. II. For DV, band I from FPLC chromatography is typically used in experiments as no differences were observed between DV-I and DV-II.

Preparation and Characterization of 1,5-IAEDANS Labeled Desulfoviridin. Desulfoviridin was labeled with 1,5-IAEDANS by incubating a 1:22 mixture of the enzyme (73.6 \( \mu \text{M} \)) and 1,5-IAEDANS (1.6 mM) in the dark for 16 hours at 4 °C in 50 mM phosphate buffer (pH 7.5). The solution was then dialyzed against several changes of the same buffer before gel filtration chromatography on a Sephadex G-25 column equilibrated with the 50 mM phosphate buffer. The protein, so obtained, was absorbed onto a DEAE-52 column equilibrated with 50 mM phosphate (pH 7.5) and eluted by a gradient made up of 1 liter each of 50 mM phosphate and 350 mM phosphate (pH 7.5). Final purification was achieved by FPLC as described for native enzyme. To avoid photodecomposition of the fluorophore, the surface-labeled protein was protected from light. The average stoichiometry of 1,5-IAEDANS attached to each enzyme was determined by comparing the absorbance at 336 nm and 630 nm [\( \epsilon \) in units of M\(^{-1}\) cm\(^{-1}\): desulfoviridin, \( \epsilon_{336 \text{ nm}} = 8.35 \times 10^4 \) and \( \epsilon_{630 \text{ nm}} = 5.3 \times 10^4 \); 1,5-IAEDANS, \( \epsilon_{336 \text{ nm}} = 5.7 \times 10^3 \) and \( \epsilon_{630 \text{ nm}} = 0 \) (Haugland, 1992)]. Figure 4.1 shows the structure of the fluorescent probe molecule, 1,5-IAEDANS.

Fluorescence Measurements. Steady state fluorescence measurements were performed using a Perkin Elmer luminescence spectrometer, model LS50B. Instrument parameters were as follows: slit width = 7, scan range of the emission = 300 - 600 nm, excitation wavelength = 336 nm. All fluorescence measurements were conducted at 25 °C using the
Figure 4.1 Structure of the fluorescence probe, IAEDANS.
reagent concentrations described in the legends to Figures 4.3 and 4.4. 1,5-IAEDANS displayed an emission band with $\lambda_{\text{max}} \sim 480$ nm.

Oxidation and Reduction of Desulfoviridin for Stopped-Flow Kinetics Studies. For studies of redox-linked conformational gating, the ox $\rightarrow$ red change was brought about by reduction with titanium(III) citrate ($E^0 = -480$ mV vs NHE at pH 7.5; Strubl, 1938). In control studies with reduced methyl viologen (MeV$^+$), the radical cation was generated by zinc reduction as described elsewhere (Lui et al., 1994). The red $\rightarrow$ ox change was carried out with $K_4W(CN)_8$ ($E^0 = +510$ mV vs NHE; Baadsgaard & Treadwell, 1955). Titanium(III) citrate was made by a modified literature procedure (Zehnder et al., 1976). A 7.9 mL volume of buffer solution containing 200 mM sodium citrate and 50 mM potassium phosphate at pH 8.0 was purged for 20 min with O$_2$-free Ar(g). A 100 μL volume of titanium(III) chloride (in 20-30 wt % of hydrochloric acid) was taken up with an argon-flushed gas-tight Hamilton syringe and then quickly transferred to the argon-purged buffer solution. The pH of the resulting titanium(III) citrate solution was adjusted to 7.5 by slow dropwise addition of NaOH (from a 2M stock) with vigorous stirring under an argon atmosphere. The pH-adjusted titanium(III) citrate solution was stored under an argon atmosphere during experiments. Deazaflavin was synthesized by literature methods (Janda & Hemmerich, 1976). Potassium octacyanotungstate was obtained as a gift from Dr. R. L. McCreery (The Ohio State University).

Stopped-flow Instrumentation and Methods. Data were obtained with an OLIS (On-Line Instrument Systems, Inc) stopped-flow apparatus. A broadband 75 watt xenon arc lamp source (Ischio) powered by an OLIS XL150
power supply was filtered through a monochromator (model H10 by Instruments Sa.) with a resolving power of 8 nm/mm. A photomultiplier tube (Homatsu) with a detection range between 185 and 900 nm was mounted perpendicular from the source relative to the sample chamber. The piston gas (nitrogen) was delivered at a rate between 9 to 14 ml/sec of the reaction solution. When rates were monitored through the fluorophore 1,5-IAEDANS, the excitation wavelength was 336 nm, and the fluorescence emission was monitored by use of a visible long-pass colored glass filter with a cut-on wavelength of 420 nm (Oriel Corporation, filter # 51280). When rates were monitored by following the natural fluorescence of the enzyme, the excitation wavelength was 280 nm and a long pass filter with a cut-on wavelength of 299 nm (Oriel Corporation, filter # 51225) was used.

All solutions were in 50 mM potassium phosphate buffer (pH 7.5). Reactants were pre-equilibrated at the appropriate temperature in a water bath, and the stopped-flow apparatus flushed with an Ar(g)-purged buffer prior to mixing. A volume of an enzyme solution (27 μM) was deaerated by purging the surface of the stirred solution for 30 min with O2-free Ar(g). Two gas tight syringes (Hamilton) were loaded with 5 mL each of Ar(g)-purged reactants. For studies of the conformational change accompanying the transition from ox → red enzyme, one gas tight syringe was loaded with an enzyme solution while the other was loaded with titanium(III) citrate solution. For studies of the conformational change accompanying the transition from red → ox enzyme, one gas tight syringe was loaded with a photoreduced enzyme solution, while the other was loaded with air-saturated K₄W(CN)₈ solution. To effect deazaflavin photoreduction (Massey & Hemmerich, 1978), a 5 ml volume of a solution containing 15.7 μM enzyme,
120 μM deazaflavin and 15 mM EDTA in potassium phosphate buffer (50 mM, pH 7.5) was argon-purged for 30 min and loaded into a gas-tight Hamilton syringe that had been pre-flushed with Ar(g). The syringe mouth was fitted with a small serum stopper to prevent O₂ diffusion and subsequently immersed in ice water and irradiated (1000 W lamp, 90 % power) for 20 min to promote deazaflavin photoreduction of DV. During the course of the experiment the gas-tight Hamilton syringe containing the enzyme was irradiated for 2 min to insure retention of a high fraction of the two-electron reduced enzyme during the prolonged experiments. A water filter was placed between the syringe and the lamp. Rate constants were determined by use of the OLIS Operating System software (version 12.05) by fitting to proprietary software.

4.3 RESULTS

Surface Labeling. Desulfoviridin was labeled with 1,5-IAEDANS by stirring an approximate 22-fold excess of the modifying agent with the enzyme for 16 h at 4 °C. The reaction between 1,5-IAEDANS and the enzyme is a simple nucleophilic substitution of the iodo functional group by a cysteine thiol group on the enzyme (Gorman et al., 1987). The stoichiometry of the modification was found to be ~ 2.4 mole of 1,5-IAEDANS per mole of DV, as determined from the relative absorbances of enzyme at 336 and 630 nm, and fluorophore at 336 nm. The ratio was significantly lower if the reaction was carried out for shorter periods of time, but no more than 3 equivalents of label were taken up by the enzyme, irrespective of reaction time. This is consistent with our previous estimates (Wolfe et. al., 1994) of surface accessible cysteines (2 to 4) determined by the
Ellman reaction (Roby & White, 1990). The pI value for the modified enzyme is similar to that of native (pI ~ 4.4), and so the surface-modified enzyme demonstrated similar elution profiles to native enzyme during purification by ion-exchange chromatography. The elution profile following the final FPLC purification was similar, irrespective of whether the enzyme peak was monitored by siroheme absorption or IAEDANS emission. When the labeled enzyme was stored in 50 mM phosphate at pH 7.5 at -20 °C, its fluorescence characteristics remained unchanged for months.

**Characterization of Modified Desulfoviridin.** The absorption maxima and extinction coefficients of the surface-labeled enzyme are similar to those of the unmodified enzyme. The concentration of enzyme was conveniently measured at 630 nm, where IAEDANS shows no absorbance. Figure 4.2 shows the emission profile for similar concentrations of IAEDANS in the free and enzyme-bound forms. The IAEDANS emission peak from the modified enzyme at 480 nm is only 2 nm different from the signal from the free fluorescent probe; however, the relative emission intensity of the surface-label is approximately 17% that of the fluorescent probe, most likely as a result of energy transfer to siroheme. The steady-state turnover rate (k\text{cat} ~ 20 \text{NH}_2\text{OH s}^{-1} \text{heme}^{-1}) of the labeled desulfoviridin is similar to that of native enzyme (k\text{cat} ~ 29 \text{NH}_2\text{OH s}^{-1} \text{heme}^{-1}) (Wolfe et. al., 1994).

**Kinetics Studies.** To evaluate the rate of conformational change arising after electron transfer to or from the redox prosthetic centers, the change in fluorescence emission both from a fluorophore (1,5-IAEDANS) attached to surface residues (Figure 4.3) and also from a weak natural emission (presumably from Trp or Tyr residues) (Figure 4.4) have been
Figure 4.2  Emission profiles for free (——) versus enzyme-bound (- - - -) 1,5-IAEDANS. In each case [IAEDANS] ~ 132 μM. The enzyme concentration for the bound form is ~ 60 μM.
Figure 4.3  A typical fit to a one-exponential rate profile for the change in emission from 1,5-IAEDANS labeled desulfoviridin. (A) Oxidized $\rightarrow$ reduced. The data shown was taken for a final $[DV] = 35 \, \mu M$, $[Ti^{3+}] = 10.5 \, \text{mM}$ at a temperature of $36 \, ^\circ\text{C}$, where $k_{\text{ox} \rightarrow \text{red}} \sim 13 \, \text{s}^{-1}$. (B) Reduced $\rightarrow$ oxidized. The data shown was taken for a final $[DV] = 15.7 \, \mu M$, $[W(CN)_{8}^{4+}] = 100 \, \mu M$ at a temperature of $39 \, ^\circ\text{C}$, where $k_{\text{red} \rightarrow \text{ox}} \sim 0.14 \, \text{s}^{-1}$. 
Figure 4.4  A typical fit to a one-exponential rate profile for the change in emission from the natural low-level fluorescence exhibited by desulfoviridin. (A) Oxidized $\rightarrow$ reduced. The data shown was taken for a final $[DV] = 60 \mu M$, $[Ti^{3+}] = 11 \text{mM}$ at a temperature of 9 °C, where $k_{\text{ox} \rightarrow \text{red}} \sim 1.1 \text{s}^{-1}$. (B) Reduced $\rightarrow$ oxidized. The data shown was taken for a final $[DV] = 44 \mu M$, $[W(CN)_8^{4-}] = 220 \mu M$ at a temperature of 25 °C, where $k_{\text{red} \rightarrow \text{ox}} \sim 0.05 \text{s}^{-1}$. 
monitored. The results obtained with each method are summarized in Table 4.1 and, as detailed below, were found to be entirely self-consistent. Both the ox → red and red → ox transformations have been examined. Appropriate control experiments to demonstrate that the emission changes observed do not arise through simple oxidation or reduction of the redox chromophores have been carried out.

Oxidized → Reduced. A 300-fold excess of titanium citrate was reacted with an argon-purged solution of oxidized labeled desulfoviridin in the mixing chamber of a stopped-flow instrument under pseudo-first order conditions. The resulting change in emission was directly monitored and the optical trace fit by a first-order kinetics profile (Figure 4.3 A) to yield an observed rate constant of ~ 8 s⁻¹. To eliminate the possibility that the change in emission intensity monitored during the stopped-flow experiment was actually arising from a change in oxidation state at the siroheme, we examined the dependence of the measured rate constant against the concentration of reductant. No dependence was found over a 7.7 mM to 17.6 mM range⁲, and so the change in emission (reflected in Figure 4.3) presumably results from a first-order structural change following reduction. This conclusion is also supported by further studies on the native enzyme since desulfoviridin possesses a weak intrinsic fluorescence that could be detected by the photomultiplier tube on our stopped-flow instrument (Figure 4.4 A). The rate of change of the emission intensity for native enzyme was evaluated following reduction of the enzyme in much the same way as

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² The concentration range of reductant that could be usefully employed was limited by too high an absorbance at higher concentration of titanium (III), and problems arising from partial oxidation of the reduced enzyme at lower concentrations.
Table 4.1 Rate constants for redox-linked conformational changes.\textsuperscript{a, b}

<table>
<thead>
<tr>
<th>enzyme</th>
<th>$k_{\text{ox} \rightarrow \text{red}}$ (s\textsuperscript{-1})</th>
<th>$k_{\text{red} \rightarrow \text{ox}}$ (s\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>surface-labeled DV</td>
<td>8.0</td>
<td>0.044</td>
</tr>
<tr>
<td>unmodified DV</td>
<td>8.5</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Data determined at 25 °C. Rate profiles are shown in Figures 4.3 and 4.4.

\textsuperscript{b} Errors are at least ± 50 % on all values.
previously described for surface-modified desulfoviridin. The first-order rate constant evaluated from these measurements \( (k \approx 8.5 \, \text{s}^{-1} \text{ at } 25 \, ^\circ\text{C}) \) was in close agreement with the result obtained with the labeled enzyme \( (k \approx 8 \, \text{s}^{-1} \text{ at } 25 \, ^\circ\text{C}) \). Note also that the relative increase and decrease of emission intensity in Figures 4.3 A or 4.4 A, and Figures 4.3 B or 4.4 B, respectively, are entirely self-consistent, with an increase in emission intensity for the reduced enzyme.

As a further control, the rate constant for chemical reduction of siroheme with titanium(III) citrate was independently determined under the conditions employed during the experiment by monitoring the siroheme absorbance change at 438 nm. The observed rate constant was found to vary with the concentration of reductant, and a second-order rate constant \( k_2 = 18 \, \text{M}^{-1} \, \text{s}^{-1} \) was determined at 25 °C (Table 4.2) while the rate of conformational change during reduction is 8 s\(^{-1}\). Clearly the emission from the fluorophore is not directly responsive to the redox state of the prosthetic centers. Inasmuch as normal steady-state turnover experiments use MeV\(^+-\) as an electron donor, the second order rate constant for reduction of siroheme was also evaluated (Table 4.2) as a further control. The \( k_2 \approx 6.5 \times 10^4 \, \text{M}^{-1} \, \text{s}^{-1} \) determined for MeV\(^+-\) is more useful for comparative discussions with results from steady state kinetics experiments made later in the text. Also, these numbers allow the interesting comparison of reduction with a negatively-charged complex \([\text{Ti(citrate)}_3]^2-\), and the positively-charged radical cation \([\text{MeV}^+-]\). Note that methyl viologen could not be used in fluorescence experiments since the radical cation served as an efficient quencher.
Table 4.2  Second-order rate constants for direct redox chemistry on the prosthetic centers.$^a$

<table>
<thead>
<tr>
<th>reductant</th>
<th>$k_{2(\text{ox} \to \text{red})}$ (M$^{-1}$ s$^{-1}$)</th>
<th>oxidant</th>
<th>$k_{2(\text{red} \to \text{ox})}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>titanium citrate</td>
<td>18</td>
<td>$K_4W(CN)_8$</td>
<td>200</td>
</tr>
<tr>
<td>MeV$^+$</td>
<td>6.5 x 10$^4$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$  Errors are at least ± 50% on all values.
**Reduced → Oxidized.** In a related series of experiments, the rate constant for the conformational change accompanying oxidation of the deazaflavin photoreduced enzyme was determined. An excess of $K_4W(CN)_8$ oxidant was reacted with deazaflavin photoreduced desulfoviridin in the mixing chamber of a stopped-flow instrument under pseudo-first order conditions. A first-order decrease in fluorescence intensity was observed (Figure 4.3 B) by monitoring the emission from surface-bound fluorophore. The rate constant $k_{ox} \sim 0.044 \text{ s}^{-1}$ obtained from studies of the labeled enzyme was again confirmed by monitoring the natural fluorescence from native desulfoviridin ($k_{ox} \sim 0.05 \text{ s}^{-1}$, Figure 4.4 B). In both cases the observed rate constants were independent of the $K_4W(CN)_8$ concentration. Also, in a series of control experiments the rate constant for chemical oxidation of the siroheme with $K_4W(CN)_8$ was independently determined under the conditions employed during the experiment by monitoring the siroheme absorbance change at 438 nm. The observed rate constant was found to vary with the concentration of oxidant and a second-order rate constant $k_2 = 200 \text{ M}^{-1} \text{ s}^{-1}$ was determined at 25 °C (Table 4.2). Again, this demonstrates that the emission from the fluorophore is not directly responsive to the redox state of the prosthetic centers.

**Variable Temperature Studies of the Rates of Conformational Change Arising from Reduction or Oxidation of Desulfoviridin.** The temperature dependence of the first-order rate constant arising from the structural change following reduction or oxidation of the prosthetic redox centers in desulfoviridin was examined using the Arrhenius rate equation (1 or 2), where $k$, $R$, and $h$ are the Boltzmann, gas, and Planck constants,
Figure 4.5  Plot of \( R \ln \left( \frac{k \ h}{kT} \right) \) versus \( 1/T \) for: (A) oxidized \( \rightarrow \) reduced; and (B) reduced \( \rightarrow \) oxidized. Enthalpic and entropic components (\( \Delta H^\circ \) and \( \Delta S^\circ \)) were obtained from the slope and intercept according to equation (2).
respectively. Figure 4.5 shows the Arrhenius plots obtained from variable temperature stopped-flow studies of the ox → red and red → ox transitions. The activation free energy (ΔG*) is calculated from the enthalpic (ΔH*) and entropic (ΔS*) components. The results from these measurements are given in Table 4.3, and in each case a substantial entropic component was observed (-43.8 cal K⁻¹ mole⁻¹ and -26.6 cal K⁻¹ mole⁻¹, respectively).

4.4 DISCUSSION

Properties of Surface-Bound 1,5-IAEDANS. Desulfoviridin possesses approximately 4 solvent exposed cysteines, of which an enzyme labeled with 2 to 3 equivalents of probe molecules per mole of enzyme has been isolated and purified. IAEDANS-labeled desulfoviridin has been used in stopped flow experiments to monitor the kinetics of structural changes resulting from reduction and oxidation of the enzyme (Figure 4.3). Since neither the primary nor tertiary structures are known, no attempt is made to identify sites of surface binding. However, IAEDANS is known to show high selectively for cysteine residues (Gorman et al., 1987; Andley & Clark, 1988; Lakowicz, 1986). The results obtained do not depend on the number of surface labels up to the maximum of three (average) noted above. Moreover, since a conformational change that is likely to occur with a unique rate
Table 4.3  Activation energies for redox-linked conformational changes.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Transformation</th>
<th>$\Delta H^*$ (kcal mol\textsuperscript{-1})</th>
<th>$\Delta S^*$ (cal K\textsuperscript{-1} mol\textsuperscript{-1})</th>
<th>$\Delta G^*$ (kcal mol\textsuperscript{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxidized $\rightarrow$ reduced\textsuperscript{b}</td>
<td>3.5</td>
<td>-43.8</td>
<td>16.5</td>
</tr>
<tr>
<td>reduced $\rightarrow$ oxidized\textsuperscript{c}</td>
<td>11.3</td>
<td>-26.6</td>
<td>19.2</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Errors in each measurement are estimated to be on the order of $\pm 0.5$ kcal mol\textsuperscript{-1} for $\Delta H^*$, $\pm 2$ cal K\textsuperscript{-1} mol\textsuperscript{-1} for $\Delta S^*$, and $\pm 0.6$ kcal mol\textsuperscript{-1} for $\Delta G^*$.

\textsuperscript{b} Titanium(III) citrate is the reductant.

\textsuperscript{c} $K_4W(CN)_8$ is the oxidant.
constant is being measured, a first-order response that is independent of probe position is expected (and observed). This stands in contrast to the multiphasic behavior that is commonly observed in transient lifetime measurements of excited states in multiply-labeled enzymes (Andley & Clark, 1988; Lakowicz, 1986).

**Activation Barriers and Reaction Energetics.** The energy profiles for reduction of a number of substrate anions and putative reaction intermediates have been previously mapped out in Ch. III. (Lui et al., 1994). Both steady-state and pre-steady state variable temperature kinetics experiments were carried out to evaluate the component energies summarized in equation (3). The overall activation energy ($\Delta G^*$) for a variety

$$\Delta G^* = \Delta G_r^* + \Delta G_{of}^*$$

of substrates has been determined from steady-state turnover experiments. The major contributor to $\Delta G^*$ is the the free energy of activation for bond cleavage ($\Delta G_r^*$), determined from variable-temperature pre-steady-state experiments, while the term ($\Delta G_{of}^*$) represents other ill-defined activation barriers. Previously it has been shown that the magnitudes of $\Delta G_{of}^*$ are typically small (less than 2.4 kcal mole$^{-1}$) and ascribed these principally to local (or global) structural changes that might accompany redox chemistry at the prosthetic sites (Lui et al., 1994). To determine if the $\Delta G_{of}^*$ contribution could in fact be accounted for by the conformational gating mechanism described above, we evaluated the free energy of activation for both the $\text{ox} \rightarrow$
red and red → ox transformations (Figure 4.5). Table 4.3 shows that in each case the activation free energies (16.5 kcal mol⁻¹ and 19.2 kcal mol⁻¹) were significantly larger than the maximum value of 2.4 kcal mol⁻¹ estimated from turnover experiments (Lui et al., 1994). In both cases the barrier we measure has a large entropic component (Table 4.3) as might be expected for a perturbation to protein structure (Cantor & Schimmel, 1980). We conclude that the proposed structural transitions of desulfoviridin are not observed during normal steady-state turnover, and so the activation barriers determined for these transitions are not manifest in results obtained from previous steady-state experiments (Lui et al., 1994). These results led us to formulate a hypothesis that there might be two conformational states of the enzyme: both active and inactive forms. The rational for this, and important mechanistic implications are developed more fully in the following section.

Conformational Gating and Implications for the Reaction Pathway.

It has been found desulfoviridin is remarkably inert toward ligand binding in the fully oxidized state. There appears to be no binding of CN⁻, AsO₂⁻, or N₃⁻ to the oxidized siroheme of desulfoviridin. This is consistent with the rather slow coordination rates observed for cyanide binding to the siroheme of E. coli sulfite reductase and spinach nitrite reductase (Janick et al., 1983). In contrast, in free solution, siroheme does rapidly bind ligands such as cyanide and pyridine to produce a stable hexacoordinate species. After reduction, we have also found ligand binding to the reduced enzyme

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3 EPR studies show that the penta-coordinate siroheme in native desulfoviridin remains high-spin (S = 5/2) even after the addition of exogenous ligand in Ch. VI. Studies on free siroheme show that the six-coordinate complex is low-spin (S = 1/2) (Kang et al., 1987).
prosthetic site to be extremely facile [for example, $k_{on}(\text{SO}_3^{2-}) = 4.3 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$ and $k_{on}(\text{AsO}_2^{-}) = 3.0 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$] (Lui et al., 1993). We conclude that the kinetic barriers for ligand binding to enzyme-bound siroheme are structural in origin and that ligand binding in the reduced form of the enzyme is promoted by a conformational change that allows easier access of the ligand to the siroheme. Substrate binding is precluded for oxidized enzyme, but access to the active site is possible after reduction. The results and observations reported in the results section can be understood in terms of the working model shown in Figure 4.6. Briefly, the enzyme can exist in two structural states: (1) an active form (DV$a$) that binds and catalyzes substrate reduction; and (2) an inactive form (DV$i$) for the resting enzyme. Only the active form of the enzyme need be considered during steady-state turnover. As noted earlier, these two structural forms do not necessarily differ markedly in global conformation and may differ only in the orientation of one protein side-chain. The distinction at this point is of no relevance for the remainder of the discussion. After reduction, the enzyme changes to the active form (red-DV$a$) at which point it may bind substrate. It is important to note that a structural change is the only reasonable explanation that accounts for the inability of the enzyme-bound siroheme to bind ligands in the oxidized form, while free siroheme in solution does so quite readily. The first-order rate constant ($k \sim 8 \text{ s}^{-1}$) obtained at ambient temperature for the

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4 Also, while it is possible that substrate or ligand binding may produce further changes in conformation, there is little direct evidence for this at present. Efforts to measure the rates of such conformational change were unsuccessful; either they are insignificant, or they produce a negligibly small change in the fluorescence of the surface label. In any event, given the rate constants determined in this chapter, and reported elsewhere in Ch. III (Lui et al., 1993, 1994), such changes are required to be rapid and not rate-limiting.
Figure 4.6 Model for redox-linked gating of enzyme activity. The enzyme can exist in inactive (DV\textsuperscript{i}) and active (DV\textsuperscript{a}) states. Substrate binding is precluded from binding to DV\textsuperscript{i}, but access to the active site is possible after reduction and a conformational transition to the active form, DV\textsuperscript{a}. The rate constants determined for each of these transformations is noted. During step (a), substrates bind to red-DV\textsuperscript{a} and are reduced to yield product molecules. The rate of oxidation for red-DV\textsuperscript{a} —► ox-DV\textsuperscript{a} is fairly rapid (k\textsubscript{2} ~ 200 M\textsuperscript{-1} s\textsuperscript{-1}). Both steady-state, and pre-steady-state rate constants have been reported previously for oxidation by reaction with a variety of substrate anions and molecules (Lui et al., 1993, 1994). For step (b), reduction of the oxidized prosthetic centers by exogenous reductants occurs readily (Table 4.2); especially for MeV\textsuperscript{+}, the exogenous reductant employed during steady-state turnover. The relatively slow transformation from ox-DV\textsuperscript{a} —► ox-DV\textsuperscript{i} suggests that the enzyme remains in an active conformation during steady-state turnover.
conformational change (red-DV^i \rightarrow \text{red-DV}^a) resulting from reduction of the redox prosthetic centers is greater than or approximately equal to the turnover rates \( k_{\text{cat}} \) for a range of substrate molecules determined from steady-state kinetics studies. That is, the structural change is not rate-limiting. We will, however, present an argument below that this activation step is unlikely to be involved once steady-state turnover has been achieved.

When bound to the active reduced form of the enzyme, bound substrate undergoes a reductive transformation and the enzyme prosthetic center is oxidized. At this point during normal turnover, the enzyme remains in the active conformation, since the rate constant for the ox-DV^a \rightarrow ox-DV^i transformation is small \( (0.05 \text{s}^{-1}) \). Further reducing equivalents are added from an exogenous electron donor (typically MeV^+; in our steady-state turnover experiments). It has been previously shown that the steady-state turnover rate is independent of MeV^+ concentration (the exogenous electron donor) over a concentration range from 0.05 to 0.25 mM. Consideration of steady-state turnover rates places a lower limit of \( k_2 > 2.5 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \) on the second order rate constant for the reverse step (b) in Figure 4.6, for ox-DV^a \rightarrow \text{red-DV}^a. That is, \( 2.5 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \) is the minimum value to support turnover at the observed rates by the exogenous electron donor under the conditions employed in a typical steady-state kinetics experiment.\(^5\) This is

\(^5\) Steady-state experiments on typical substrates generally yield a turnover rate \( k_{\text{cat}} \) \(< 1 \text{s}^{-1} \), with \([\text{MeV}^+] \sim 0.2 \text{mM} \). Taking into account the fact that each enzyme possesses two active centers, an estimate of the rate of electron delivery can be made using the relationship, \( k_{\text{cat}} = 2 \times (k_2 \cdot [\text{MeV}^+]^1) \). From this, a minimal value of \( k_2 > 2.5 \times 10^3 \text{ M}^{-1} \text{s}^{-1} \) can be determined. Maximal turnover rates of up to 30 s^{-1} are observed for NH$_2$OH, however, within error our experimental value of \( k_2 \sim 6.5 \times 10^4 \) can accommodate this.
higher than the corresponding rate constant \((k_2 \sim 18 \text{ M}^{-1} \text{s}^{-1})\) for the ox-DV\textsuperscript{i} \(\rightarrow\) red-DV\textsuperscript{i} transformation determined for reduction by titanium citrate. Since this rate constant is insufficient to support the observed turnover rates with MeV\textsuperscript{+} as electron donor (Lui et al., 1994), it is necessary to determine the rate constant for this reductant. Table 4.2 compares the relative rate constants for reduction with titanium(III) and methyl viologen \((18 \text{ M}^{-1} \text{s}^{-1} \text{ and } 6.5 \times 10^4 \text{ M}^{-1} \text{s}^{-1})\), respectively. Assuming similar rate constants for the ox-DV\textsuperscript{a} \(\rightarrow\) red-DV\textsuperscript{a} and ox-DV\textsuperscript{i} \(\rightarrow\) red-DV\textsuperscript{i} redox reactions (Figure 4.6),\textsuperscript{6} the data for MeV\textsuperscript{+} satisfies the minimal requirements for steady-state turnover. The difference in magnitudes of the rate constants for titanium citrate and methyl viologen most likely reflects the different electrostatic terms (the enzyme has a pI of \(\sim 4.4\), and will carry a net negative charge at neutral pH), and the differing hydrophobicities of the two reductants, which may allow better penetration by MeV\textsuperscript{+} at the enzyme surface. This observation is in accord with the Marcus theory of electron transfer (Marcus & Sutin, 1985).

Given the relative rate constants summarized in Figure 4.6, steady-state turnover apparently must arise from the red-DV\textsuperscript{a} and ox-DV\textsuperscript{a} couple. The rate constants for the transformation ox-DV\textsuperscript{a} \(\rightarrow\) ox-DV\textsuperscript{i} is small \((\sim 0.05 \text{ s}^{-1})\) and is not relevant for studies of steady-state turnover. The relatively slow rate of return to the inactive form most likely serves to preserve the enzyme in an active state. The red-DV\textsuperscript{i} and ox-DV\textsuperscript{i} forms can only be detected, or implicated, under the fast-kinetic conditions employed in these

\textsuperscript{6} This appears reasonable since a minor structural change is unlikely to influence the rate of electron-transfer to the prosthetic center to a significant extent.
studies and in the absence of substrate molecules. Why the activity of this enzyme should be gated in this way is an intriguing question and one that will be difficult to address in the absence of a more detailed understanding of the physiological chemistry of the enzyme in vivo.

**Putative Structural Mechanisms for Conformational Gating.** Protein structural changes resting from reduction of the prosthetic redox center may result from the influence of siroheme redox state on the pucker of the siroheme ring. Crystallographic studies have clearly demonstrated the flexibility of the reduced isobacteriochlorin ring, and the propensity for structural change accompanying redox chemistry at a metalloisobacteriochlorin center (Strauss et al., 1983; Cruse et al., 1982; Barkigia et al., 1982). Such changes of the ring conformation will influence the salt bridges (siroheme carboxylates to positively-charged residues) that electrostatically hold the siroheme to the enzyme. Modulation of the electrostatic attraction of the siroheme and protein sidechains through these salt bridge contacts provide an obvious mechanism for communicating a change in redox state of the heme to motion of the enzyme. Similar arguments can be made for the [Fe₄S₄] cluster, and the cysteine residues that bind to the iron ions, however, clusters typically show only minor changes in core size and geometry.
CHAPTER V

Direct Electrochemical Studies of SiR and DV

ABSTRACT

This chapter describes the characterizations of the redox properties of the [Fe₄S₄]-siroheme prosthetic center in SiR and DV by direct electrochemical methods. It involves three parts: (I) reduction potential measurements of the redox centers; (II) systematic pH titration experiments of the [Fe₄S₄]-siroheme prosthetic center; and (III) characterization of the redox thermodynamics of the prosthetic center. This series of systematic electrochemical investigations provides insight on active site chemistry.

In part I, direct electrochemical studies have been performed on DV (Mr ~ 224,000) and SiR (Mr ~ 23,500), by use of square-wave voltammetry, with an edge pyrolytic graphite electrode (PGE) and redox inactive Cr(NH₃)₆³⁺ promoter. Diffusion controlled reversible heterogeneous electron transfer is observed for µM enzyme concentrations of either enzyme. The reduction potential for the first redox couple of the [Fe₄S₄]-siroheme prosthetic center in DV has been determined as E°′(25°C, pH 7.5) ~ -310 mV versus NHE. The half-height peak width is 122 mV, in excellent agreement with the theoretical value of 126 mV expected for a reversible redox couple.
one-electron transfer. Uptake of a second electron occurs at a reduction potential that is too negative to be detected over the range allowed with PGE. The second reduction potential of ~ -620 mV vs NHE was measured with a Hg(l) pool electrode by controlled potential coulometry (CPC). The reduction potentials for the first and second redox couples of the [Fe₄S₄]−siroheme prosthetic center in SiR have been determined as E₁°* ~ -21 mV (siroheme) and E₂°* ~ -303 mV (cluster) vs NHE at pH 7.5 and 25 °C. The half-height peak width of 134 mV for the first redox couple (siroheme) is again in excellent agreement with the theoretical value of 126 mV expected for a reversible redox couple involving one-electron transfer, however, the half-height peak width for the second redox couple ([Fe₄S₄] cluster) is only 84 mV, corresponding to less than one electron transfer.

In part II, systematic pH-titration studies have provided insight on active site chemistry, including evidence for an ionizable ligand bound to the prosthetic redox center. These results support direct coupling of the siroheme and [Fe₄S₄] cluster by the bridging ligand.

In part III, variable temperature electrochemical experiments have been performed on SiR and DV by use of square-wave voltammetry with an edge pyrolytic graphite electrode and redox inactive Cr(NH₃)₆³⁺ promoter. Enthalpic and entropic contributions to E°' values have been determined. Comparison is made with the redox thermodynamic parameters of cyt c (horse), myoglobin, and high potential iron protein. The pH-dependence of these parameters has been examined and implications for active site chemistry are discussed. For DV, both enthalpic and entropic terms are independent of pH over the range from 3 to 10; however, for SiR the entropic
component shows a pH-dependence with an estimated pH of \(\approx 7.0\). This is particularly pronounced for the siroheme redox couple and is proposed to originate from release of an axial histidine residue following reduction.
5.1 INTRODUCTION

Thermodynamics (free energy change, entropy change, enthalpy change and reduction potential, etc.) provides the core of bioenergetics. The driving force for a reaction in an isolated system is an increase in entropy, which may broadly related to the degree of disorder of the system. Oxidation-reduction events in biology usually involve a sequence of reactions in which electrons are transferred from one component to another. While many of these components simply gain one or more electrons in going from the oxidized to the reduced form, in others the gain of electrons induces an increase in the pK of one or more ionizable groups, with the result that reduction is accompanied by the gain of one or more protons. For example, cytochrome c undergoes a one-electron reduction, NAD+ undergoes a two-electron reduction and gains one proton, ubiquinone (UQ) undergoes a two-electron reduction followed by the addition of two protons.

Metalloproteins bearing redox prosthetic centers can be divided into several functionally distinct classes. These include electron-carrier proteins (proteins involved in the binding and transport of small anions or gaseous molecules), and oxido-reductase enzymes. According to Adman (1979), electron transport proteins are generally defined by five functional characteristics: (a) possession of a cofactor that serves as an electron sink; (b) placement of the cofactor close enough to the protein surface to allow facile electron exchange; (c) a hydrophobic pocket that is adjacent to, but does not necessarily surround the cofactor; (d) relatively small structural changes accompanying electron transfer (to minimize the inner-sphere reorganizational energy); and (e) a flexible architecture that permits
expansion or contraction in certain preferred directions following electron transfer. A fundamental question in this field concerns how a simple prosthetic group, such as a heme or siroheme, or the \([\text{Fe}_4\text{S}_4]\) cluster in ferredoxin's or HiPIP's, can exhibit such a wide range of reduction potentials and functional diversity. Metalloproteins that function as electron transferases typically place their prosthetic groups in a hydrophobic environment and provide occasional hydrogen bonds to stabilize one or other of the available metal oxidation states, while severe inner-sphere reorganizational barriers are apparently avoided by dictating a coordination environment that is a compromise between that favored by the oxidized and reduced metal ions (Mayo et al.,). It is clear that a detailed evaluation of the thermodynamic parameters associated with the redox chemistry of specific metalloredox proteins will provide insight on the structural mechanisms used to regulate their functional chemistry.

Quite often, metalloproteins contain more than one redox center to perform biological functions. An intriguing reason for studying multisite metalloproteins (polynuclear transition metal complexes) is the possibility that the chemical reactivity associated with a metal center is significantly altered by the presence of two or more metal ions per molecule. Such a possibility becomes especially interesting if reactivity changes are not simply additive. Most of the presently known redox metalloenzymes (eg. cytochrome \(\text{c}\) oxidase, nitrogenase) typically contain, in addition to a substrate binding site consisting of one or more redox-active metal ions, additional redox cofactors that function as intramolecular electron transfer agents. The presence of multiple redox centers in enzymes catalyzing reactions requiring two or more electrons in proceeding from reactants to
products may prevent the formation of radical substrate intermediates that would be produced by single electron-transfer. Thus, protein-bound, multi-metal redox centers might be particularly advantageous in the reactions of small molecules such as dioxygen, dinitrogen, or small ions such as sulfite or nitrite.

Knowledge of the relative reduction potentials of redox prosthetic centers in metalloenzymes is an important factor in the evaluation of enzyme function and mechanism. Direct determination of reduction potentials has been demonstrated for redox active sites in low molecular weight electron-carrier proteins (5-20 kDa) (Armstrong et al., 1988; Hill, 1993; Sucheta et al., 1992 & 1993; Armstrong, 1990; Heller, 1990; Willner et al., 1992; Yeh and Kuwana, 1977; Eddowes and Hill, 1977; Armstrong et al., 1984; Butt et al., 1991; Stankovich and Bard, 1978; Varfolomez and Berezen, 1978; Guo et al., 1989 & 1990; Bagby et al., 1990; Taniguchi et al., 1982), however, the methodology is not routine and requires extensive experimentation with solution conditions and electrode materials to obtain tractable data. EPR or optical methods of monitoring mediator titration experiments are more commonly used, although such techniques are of limited scope for studies that require systematic variation of solution pH or temperature. These potentiometric titration methods often suffer interference from the mediators employed, and so tedious background subtraction is needed. This chapter describes the use of square-wave voltammetry (SWV) with an edge pyrolytic graphite electrode (PGE) to perform direct electrochemistry on the 224 kDa DV and the 23.5 kDa SiR. SWV enjoys several advantages over cyclic voltammetry and differential pulse voltammetry; which include greater speed and sensitivity in analysis, lower consumption of electroactive
species, and fewer problems arising from blockage of the electrode surface (O'Dea et al., 1981; Osteryoung and Osteryoung, 1985; Smith and Feinberg, 1990; Ramaley and Krause, 1969; Smith et al., 1991). We have found this method to be very well suited to studies of many redox proteins and enzymes, requiring limited (μM) concentrations of sample. These two metallo redox centers in SiR and DV serve as a catalytic apparatus for the enzymatic multielectron reduction of SO₃²⁻ to S²⁻, and also NO₂⁻ to NH₃. In Ch. III results from steady-state and pre-steady state kinetics measurements directed toward elucidation of mechanistic issues pertaining to this class of enzyme have been described (Lui et al., 1993 & 1994). A more thorough understanding of the mechanism of sulfite reductase requires evaluation of the thermodynamic properties of the two redox sites that constitute the prosthetic center. Thermodynamic constraints offer insight on factors controlling redox changes at the redox metal centers, and mechanistic information can be deduced.

In part I the use of square-wave voltammetry (SWV) with an edge pyrolytic graphite electrode (PGE) to perform direct electrochemistry on SiR and DV will be described. The prosthetic redox center from this class of enzyme most likely contains a [Fe₄S₄] cluster connected to a siroheme through a bridging S²⁻ ligand (Tan and Cowan, 1991; Wolfe et al., 1994). The presence of the [Fe₄S₄] cluster and siroheme prosthetic centers in both DV and SiR has been confirmed (Ch. II), in full agreement with the general formulation originally proposed by Siegel and coworkers for this enzyme class (Wolfe et al., 1994). Figure 1.4 shows the prosthetic centers. The electrochemical results also provide insight on active site chemistry, and support direct coupling of the siroheme and [Fe₄S₄] cluster to form a
coupled redox pair (P) (that apparently takes up two electrons in consecutive steps (P → P⁺ → P²⁻)). These studies form a basis for further investigations of the redox chemistry of the coupled [Fe₄S₄]-siroheme prosthetic center.

Variable temperature experiments and systematic pH-titration that provide further insight on redox-linked active site chemistry are described in part II and III.

In part II a series of experiments that systematically explore the pH-dependence of \( E^0 \) will be described. The results provide insight on active site chemistry, including an ionizable ligand bound to the prosthetic redox center, and support direct coupling of the siroheme and [Fe₄S₄] cluster to form a coupled redox pair (P) that apparently takes up two electrons in consecutive steps (P → P⁺ → P²⁻). Sulfite-reducing enzymes have been found to contain an active site containing an [Fe₄S₄] cluster coupled to a siroheme by a bridge, either an inorganic S²⁻ bridge in the sulfite reductase from *Desulfovibrio vulgaris* (Tan and Cowan, 1991) or possibly a cysteine bridge in the *E. coli* enzyme (McRee et al., 1986; Cline et al. 1985; Madden, 1989). Our experiments suggest that the dominant ionization site is the bridging ligand that couples the cluster and siroheme.

In Part III, a detailed characterization of the redox thermodynamics of these two enzymes will be presented. The temperature dependence of the reduction potentials for the siroheme and cluster in SiR and the first redox couple of DV has been monitored and key thermodynamic parameters have been evaluated. Electrostatic charge effects and specific ligand solvation appear to dominate the reaction entropies exhibites by transition metal complexes (Yee et al., 1979; Wawrousek, 1974; Borchard, 1984 & 1982;
Negatively charged redox couples tend to have negative reaction entropies and positively charged couples display positive reaction entropies, shown in Figure 5.1. Bulky, hydrophobic ligands (phen and bipy, for example) depress the magnitude of the observed $\Delta S_{\text{RCO}}^\circ$. Metalloprotein couples (Taniguchi et al., 1980 & 1982 a, b; Reid et al., 1982; Crutchley et al., 1985; Margalit and Schejter, 1970), on the other hand, tend to have negative reaction entropies that do not correlate with protein charge. A knowledge of the thermodynamics of electron transfer reactions is needed in order to adequately understand the reactions.

5.2 MATERIALS AND EXPERIMENTAL METHODS

General Materials. Buffer salts were of molecular biology grade (Fisher or Aldrich Chemical Co). Sepadex G-200 gel filtration material was obtained from Sigma and DEAE-52 ion exchange resin from Whatman. All water used was purified with a Barnstead nanopure system and exhibited a resistivity of 18 M$\Omega$ cm$^{-1}$. Chromium hexaammine trichloride was synthesised according to a literature procedure (Angelici, 1986). The Ag/AgCl reference electrode was purchased from BAS, and the Pt counter electrode was purchased from Alfa. Pyrolytic graphite was obtained as a gift from Dr. R. L. McCreery at The Ohio State University.

Bacterial Growth, Isolation and Purification of SiR and DV. 

*D. vulgaris* (Hildenborough, NC1B 8303) possessing a broad-host range expression vector (pDSK519) with the SiR gene and promoter cloned into the multiple cloning site, (Tan et al., 1994) was grown in a lactate-sulfate medium. The purification details were described to Ch. II. For DV, band I
Figure 5.1  Reaction entropies for selected transition metal and metalloprotein couples. (Values taken from Yee and Weaver, 1980; Hanania et al., 1967; Taniguchi et al., 1980 & 1982; Crutchley et al., 1985)
from FPLC chromatography is typically used in experiments as no differences were observed between DV-I and DV-II.

5.2.1 Reduction Potential Measurements of the Redox Centers

**Direct Electrochemistry. Configuration of the Electrochemical Cell and Sample Preparation:** A standard three-electrode configuration was used. Direct electrochemical measurements were performed with an edge pyrolytic graphite electrode (PGE) and redox inactive Cr(NH₃)₆³⁺ as promoter. The PGE was made by embedding a strip of pyrolytic graphite with an exposed edge of surface dimensions 1 mm x 1 mm in an electrochemically-clean epoxy resin (Eccobond, Johnson-Matthey). A fresh PGE surface was cut for each experiment, followed by sonication in nanopure water to remove residual impurities. The reference electrode was a saturated Ag/AgCl electrode purchased from BAS. A length of platinium wire (4 cm x 1 mm) was used as the counter-electrode. The electrochemical cell was arranged in a nonisothermal configuration (Taniguchi et al., 1980 a, b & 1982 a, b; Reid et al., 1982; Crutchley et al., 1985). The reference electrode was held at constant temperature, while the temperature of the solution containing the redox couple of interest was set at the value of interest. The electrochemical cell was double-walled with a water jacket maintained at a constant temperature by a thermostatted circulating water bath. The temperature was recorded with an OMEGA HH 81 digital thermometer. All electrochemical experiments were performed with a positive pressure of O₂-free Ar(g) purging the surface of a 2 mL working solution. To prevent solvent loss over the course of several experiments, O₂-free Ar(g) was bubbled through water to achieve water-saturation before purging the
sample solution. No reduction in sample volume from evaporation was observed during the course of the experiment. The 2 mL volume of solution contained 8 μM enzyme with 8 mM Cr(NH$_3$)$_6^{3+}$ as promoter, and 10 mM NaCl as supporting electrolyte in 10 mM potassium phosphate (buffered at the appropriate pH), and was purged with O$_2$-free, water-saturated Ar(g) for 15 min prior to running each experiment. The sample solution was stirred during purging, but not during data acquisition.

*Electrochemical Data Acquisition and Analysis:* Square wave voltammograms (SWV) were obtained on a computer-interfaced PARC 263 potentiostat/galvanostat. Experimental conditions are noted in the figure legends. Before each voltammogram, the potential was poised at 0 mV for 10 s to remove fouling products from the electrode surface. Following this procedure, voltammograms could be reproducibly obtained under the same experimental conditions. Reduction potentials were determined from the point at which the maximum net peak current was observed, and are reported relative to the NHE. The number of electrons transferred per redox site (n) were determined by dividing the theoretically expected value of 126 mV, for a reversible redox couple monitored by SWV, by the observed peak width at half height (Smith and Feinberg, 1990; Brumleve et al., 1981).

**Controlled Potential Coulometry. Configuration of the Electrochemical Cell:** The configuration of the CPC electrochemical cell for the basic coulometric technique has been described by Watt and coworkers (Watt, 1979; Watt et al., 1980). A three-electrode microelectrochemical cell of 60 μL capacity was made according to Smith and Adams (1993). Figure 5.2 shows the experimental design for the CPC electrochemical cell. The
Figure 5.2  Electrochemical cell for Controlled Potential Coulometry (cross-section view). Cell components are identified as (a) pyrex cell body; (b) inverted Hg(l) pool working electrode; (c) Ag/AgCl reference electrode; (d) platinum counter electrode; (e) silicon septum; (f) swagelok fitting; and (g) O-rings. See text for details.
reference electrode is a saturated Ag/AgCl electrode purchased from BAS and the counter-electrode is a piece of Pt wire (4 cm x 1 mm) feathered and sealed in borosilicate tubing of 7 mm outer diameter. The working electrode is a Hg(l) pool of virgin grade purchased from Aldrich, and further purified by filtering the mercury through a perforated filter paper (pinholed) to remove surface scum and oxides. Following Sawyer and Roberts (1974), the mercury pool working electrode was constructed from a plastic support with a cup for a Hg pool at one end. A carbon paste electrode (without carbon paste in the cup) was purchased from BAS to serve as the electrode support to hold the mercury pool. All three electrodes were equipped with an o-ring to make a tight seal to the cell body. The volume of the sample was kept to a minimum (a drop of 60 µL) so that complete electrolysis at each potential is achieved without any stirring facility. The three electrodes were immersed inside the drop of enzyme sample. The electrodes were adjusted to be separated by about 2 mm from each other. The set-up was analogous to the one employed by Su and Heineman (1981) who used a thin layer cell (OTTLE design) for coulometry measurements. The relatively small distances between the electrodes minimizes IR drop. The set-up was successfully tested with methyl viologen, ferricyanide and myoglobin, which yielded results (within experimental error) that were consistent with literature data.

Preparation of Samples for Coulometry: A 500 µL volume of enzyme solution of concentration 0.5 mM was stirred and purged with O₂-free Ar(g) in a 1 mL pear-shaped flask for 15 min. A second 1 mL pear-shaped flask containing a 500 µL volume of a mixed mediator solution was purged in a similar fashion. The mediators used were: anthraquinone-2-sulfate (E°_{NHE} = -225 mV); benzyl viologen dichloride (E°_{NHE} = -440 mV); ethyl viologen
(E°NHE = -480 mV); and 2-amino-4-pteridone (E°NHE = -660 mV). Each mediator had a concentration of 0.5 mM in the final mixture. The three electrodes were screwed into the microelectrochemical cell with the o-rings inside swagelok fittings to provide a good seal. The microelectrochemical cell was fitted with the three electrodes and flushed with O2-free Ar(g) for 15 min, as were the experimental sample solutions. A gas-tight syringe (Hamilton) was flushed with O2-free Ar(g) several times prior to withdrawing samples under a positive pressure of Ar(g). A 6 µL volume of mediator mixture and 54 µL of enzyme solution were withdrawn consecutively into a 100 µL gas tight syringe. The 60 µL sample solution was then injected into the preflushed microelectrochemical cell through a rubber septum. Care was taken to avoid displacing the Hg(l)-pool from the top of the working electrode. The rubber septum was then sealed with grease to prevent O2 leakage. Final adjustment of the positions of the three electrodes was made at this point so that each electrode was immersed inside the sample drop and maintained at ~ 2 mm distance from each other to avoid direct contact. The Ar(g) purging was discontinued after adjusting the positions of electrodes. Final concentrations were 450 µM for the enzyme and 50 µM for the mediators (enzyme : mediator = 9:1). The buffer was 10 mM potassium phosphate solution at pH 7.5, containing 100 mM NaCl as supporting electrolyte. For each controlled potential coulometrical measurement, an identical control measurement was performed on a solution containing only mediators and buffer as a background reference. The corrected sample data for the enzyme solution was obtained by subtracting the background data from the raw sample data. For each measurement the potential was held until the current reached a small but
constant level. The potential was then stepped to the next value and the procedure repeated. The time for each CPC measurement was kept at a constant value of 100 s.

**Data Acquisition and Analysis:** A controlled potential was supplied by a PAR 263 potentiostat equipped with a built-in electronic integrator. A modified headstart software from EG & G PARC was used to calculate the charge transferred at each potential. The potential was held until a complete decay curve was observed. The area under the decay curve was integrated to determine the charge transferred at that potential. A plot of charge vs potential was obtained and redox potentials were evaluated by fitting the data to equation (1) (Siegel et al., 1982), where \( n \) is the number of electrons taken up per half-molecule of DV (each molecule contains two [Fe\(_4S_4\)]-siroheme prosthetic centers) at a solution potential \( E \), where \( E_1 \) and \( E_2 \) are the redox potentials of the two redox couples in the prosthetic center of DV.

\[
n = \frac{1}{1 + 10(E-E_1)/0.059} + \frac{1}{1 + 10(E-E_2)/0.059}
\]  

5.2.2 Systematic pH Titration Experiments of the [Fe\(_4S_4\)]-Siroheme prosthetic Center

Electrochemical pH Titration Experiments. Electron-exchange at protein-bound redox centers is frequently accompanied by ion (especially proton) uptake or release. Under such circumstances the reduction potential
of the protein can be shown to vary with pH, and can be quantified by use of the Nernst equation. For the redox reaction (2), which couples electron and proton transfer at 25°C, the Nernst equation (3) can be rearranged to equation (4). The latter equation indicates that the reduction potential will

\[
E_{\text{red}H} \iff E_{\text{ox}} + e^- + H^+ \tag{2}
\]

\[
E = E^0 + (0.059)*\log \left( \frac{[E_{\text{ox}}][H^+]}{[E_{\text{red}H}]} \right) \tag{3}
\]

\[
E = E^0 + (0.059)*\log \left( \frac{[E_{\text{ox}}]}{[E_{\text{red}H}]} \right) - (0.059)*pH \tag{4}
\]

shift -59 mV for every unit increase in pH. This reflects the fact that the oxidized and reduced form of a redox center may have very different proton affinities. For most redox couples, proton transfer is limited by two pK_a's; one for the oxidized form (pK_{aox}), and the other, at higher pH, for the reduced form (pK_{ared}). At alkaline pH, a coordinated water molecule may also deprotonate. Figure 5.3 illustrates a hypothetical plot of E^0 vs pH (Cowan, 1993). The pK_a's are revealed because, above and below the pK_a's, proton transfer becomes insignificant and the value of E^0 loses its dependence on the solution pH. Three distinct regions
Figure 5.3  Plot of the reduction potential ($E^0'$) of a hypothetical redox center vs solution pH when the couple has $pK_{a^{ox}}$ and $pK_{a^{red}}$. Three regions are illustrated: $pH < pK_{a^{ox}}$, $pK_{a^{ox}} < pH < pK_{a^{red}}$, where $E^0'$ decreases by 59 mV per pH unit increase; and $pH > pK_{a^{red}}$. Dashed lines extrapolate to the respective $pK_{as}$ (Reproduced from Cowan, 1993).
occur in the plot. At intermediate pH's ($\text{pK}_a^{\text{ox}} < \text{pH} < \text{pK}_a^{\text{red}}$), proton uptake is coupled to the redox reaction described by equation 3. Above and below the pK$_a$'s, stoichiometric proton uptake no longer accompanies reduction. At pH $>>$ pK$_a^{\text{red}}$, the predominant redox reaction is defined by (5).

$$E_{\text{red}}^- \leftrightarrow E_{\text{ox}} + e^- \tag{5}$$

However, at pH $<<$ pK$_a^{\text{ox}}$, the reaction will become defined by (6).

$$E_{\text{redH}} \leftrightarrow E_{\text{oxH}^+} + e^- \tag{6}$$

Simulation of $E^0$ versus pH. The change in reduction potential with pH should follow a sigmoidal curve reflecting the pK$_a^{\text{ox}}$ and pK$_a^{\text{red}}$ of the ionizable residue or ligand near the redox center, where the pK$_a$'s are the equilibrium constants for the proton association of the ionizable ligand or residue for the oxidized and reduced forms of the protein (E). The equilibria are represented as shown by Figure 5.4, which shows a thermodynamic cycle for the coupled electron- and proton-transfer reactions displaying a potentiometric behavior illustrated in Figure 5.3.

Potential versus pH plots may be analyzed using the methods outlined by Clark (1960) in order to obtain pK$_a$'s by nonlinear least squares
Figure 5.4  Thermodynamic cycle for the coupled electron- and proton-transfer reactions displaying the potentiometric behavior illustrated in Figure 5.3.
fits to the data. In our studies the pH data were fit by the standard equation (7), where $E_m$ is the mid-point potential at different pH values, and $E^\circ$ is the standard reduction potential.

$$E_m = E^\circ + 59.2 \log \left( \frac{K_{a,\text{red}}}{K_{a,\text{ox}}} + \frac{[H^+]}{[H^+]} \right) \text{ mV}$$

(7)

5.2.3 Characterization of the Redox Thermodynamics of the Prosthetic Center

Nonisothermal Electrochemistry: Weaver and coworkers have noted that the use of nonisothermal cells avoids certain problems associated with the reference electrode for an isothermal cell configuration. If the temperature coefficients of thermal junction potentials can be made either negligible or constant relative to the overall temperature coefficient of the nonisothermal cell ($\frac{dE^\circ}{dT}$), then this method provides a direct measure of the entropy change for half-cell reactions: $\Delta S^\circ_{rc} = S^\circ_{\text{red}} - S^\circ_{\text{ox}} = nF \frac{dE^\circ}{dT}$. When the magnitude of ($\frac{dE^\circ}{dT}$) is greater than 0.2 mV/deg (de Bethune et al., 1959), errors arising from temperature gradients are small and can be neglected. The net entropy change for the complete cell reaction (referenced to NHE) is given by $\Delta S^\circ_{\text{cell}} = \Delta S^\circ_{rc} + (nS^\circ_{H^+} - \frac{1}{2} n S^\circ_{H_2O})$, where the $S^\circ$ terms represent partial molal entropies. The entropy difference due to the reference electrode may be separated from that due to the redox couple of interest by use of the third law of thermodynamics (Smith, 1977). The overall cell reaction entropy
\( \Delta S_{\text{cell}}^0 \) for a one-electron redox processes can be written in the form of equation (8), when taking 31.2 eu for the partial molal entropy of \( \text{H}_2 \), and

\[
\Delta S_{\text{cell}}^0 = \Delta S_{\text{rc}}^0 - 15.6 \text{ eu}
\]  

Latimer's convention of zero for \( S_{\text{H}_2}^0 \) (Latimer, 1952). Thus the entropy change for the overall cell reaction \( \Delta S_{\text{cell}}^0 \) (obtainable via the isothermal experiments) is equal to the reaction entropy \( \Delta S_{\text{rc}}^0 \) (obtainable via the nonisothermal experiment) minus 15.6 eu. The value of \( \Delta S_{\text{rc}}^0 \) is determined from the slope of a plot of potential versus temperature \( [\Delta S^0 = nF \left( \frac{\partial \Delta E^0}{\partial T} \right) ] \) and \( \Delta S_{\text{cell}}^0 \) from equation (8). The value of \( \Delta G^0 \) can be determined from the relationship \( \Delta G^0 = -nF\Delta E^0 \). After obtaining \( \Delta S_{\text{cell}}^0 \) and \( \Delta G^0 \) from the variable temperature reduction potential measurements, \( \Delta H^0 \) can be evaluated by standard thermodynamic relationships \( (\Delta G^0 = \Delta H - T\Delta S^0) \).

5.3 RESULTS

5.3.1 Reduction Potential Measurements of the Redox Centers

**Determination of Reduction Potentials.** *Dissimilatory sulfite reductase (DV):* With the high sensitivity of SWV, we were able to obtain an electrode response from a micro-PGE (1 mm x 1 mm) at \( \mu \text{M} \) concentrations of enzyme. Typically a scan range from 0 mV to -600 mV vs NHE was used. The square wave conditions used in specific experiments are noted in the figure legends. The reduction potential for the first redox couple of the \([\text{Fe}_4\text{S}_4]\)-siroheme prosthetic center has been determined to be \( E^0(25^\circ C, \text{pH 7.5}) \approx -310 \text{ mV vs NHE} \), and corresponds to one-electron transfer to the redox
site (Figure 5.5). Repulsive interactions between oxygen-containing functional groups on the graphite surface and acidic residues on desulfoviridin (pI ~ 4.4) were electrostatically neutralized by adding high valent Cr(NH$_3$)$_6^{3+}$ as a promoter (Armstrong et al., 1987 & 1989). At higher pH, increased concentrations of Cr(NH$_3$)$_6^{3+}$ were required to obtain an electrode response from DV (2 mM at pH 3.0 vs 8 mM at pH 8.5). Presumably there is more extensive electrostatic repulsion as a result of deprotonation of additional functional groups on both the enzyme and the electrode.

**Assimilatory sulfite reductase (SiR):** Similarly, two discrete electron-transfer steps were readily seen in direct electrochemical measurements (Figure 5.6) at an edge PGE with Cr(NH$_3$)$_6^{3+}$ as a redox promoter. Previous EPR and optical data had suggested that the enzyme undergoes two discrete reduction steps, with the first electron adding to the siroheme followed by the addition of a second electron equivalent to the cluster (Ch. VI & VII). The reduction potential for the siroheme and cluster were determined to be -21 mV and -303 mV vs NHE at pH 7.5, 25 °C. The more positive peak was assigned to the siroheme redox couple on the basis of EPR results for partially reduced enzyme (Ch. VI).

The difference in reduction potentials between siroheme and cluster in SiR was measured to be 280 mV by the SWV method. In contrast, only one peak at -310 mV was obtained for DV in the SWV scan from +200 mV to -500 mV vs NHE (Figure 5.5). Scans to more negative potentials (up to -800 mV) were attempted, but no second peak was observed. Previous EPR studies on photoreduced intermediates of DV have shown that the [Fe$_4$S$_4$] cluster and
Figure 5.5  A typical square wave voltammogram obtained from a 10 μM DV solution with 8 mM Cr(NH₃)₆³⁺ in 50 mM potassium phosphate/10 mM NaCl (298 K, pH 7.5) under Ar(g). SWV conditions: pulse potential = 25 mV; step potential = 1 mV; frequency of the applied potential = 80 Hz. The dotted line shows a Gaussian fit of the data.
Figure 5.6 A typical square wave voltammogram obtained from an 8 μM SiR solution with 8 mM Cr(NH₃)₆³⁺ in 10 mM potassium phosphate/10 mM NaCl under Ar(g) (278 K, pH 7.5). SWV conditions: pulse potential (Eₚ) = 50 mV; step potential (Eₛ) = 1 mV; frequency of the applied potential = 140 Hz. The solid line shows a Gaussian fit of the data.
siroheme have similar reduction potentials, and that a single electron is
shared by the siroheme and [Fe₄S₄] cluster (Ch. VI). That is, the oxidized
siroheme EPR signal decreases by half with the appearance of half of the
reduced cluster signal. Upon one-electron reduction of DV, approximately
one-half of the molecules contain reduced siroheme and the other half
contain reduced cluster (Ch. VI). This contrasts with the two distinct
potentials found for the assimilatory sulfite reductase enzymes from D.
*vulgaris* and *E. coli* (Ch.VI). We postulate that the siroheme and cluster in DV
form a bridged redox pair that apparently takes up two electrons in
consecutive steps (P → P⁻ → P²⁻). The potential reported here (-310 mV)
corresponds to the first redox couple (P → P⁻). Uptake of a second electron
occurs at a reduction potential that is too negative to be detected over the
range available to PGE in aqueous solution (Miller and Zittel, 1963) and so
controlled potential coulometry (CPC) was performed to obtain the second
reduction potential for DV. The coulometry data was obtained with a Hg(I)-
pool working electrode. Two potentials were detected at ~ -320 mV and -620
mV vs NHE (Figure 5.7). The data was corrected by substracting the
background data from raw data. Within experimental error, the first redox
potential (-310 mV) obtained from SWV shows good general agreement with
the potential of -320 mV determined by the CPC method. The difference in
redox potentials between the first and second redox couples was estimated to
be 300 mV by the CPC method. Table 5.1 summarizes the redox potentials
for SiR and DV from *D. vulgaris*, and for comparison also lists data for the
assimilatory sulfite reductase from *E. coli*, cytochrome c (horse), myoglobin
and hemoglobin.
Figure 5.7  Controlled Potential Coulometry data for DV. \([\text{DV}] = 450 \ \mu\text{M},\)
\([\text{mediator}] = 50 \ \mu\text{M} \ (\text{DV} : \text{mediator} = 9 : 1).\) Detailed
experimental conditions was described in the text. Data was
corrected by substracting background data (with mediator
only) from the the raw data. Simulation (the dotted line)
was done by fitting the data with equation (1).
Table 5.1  \( E^0' \) values for the redox couples in SiR and DV from \textit{D. vulgaris} using SWV and CPC methods.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Redox Couple</th>
<th>( E^0' / \text{mV vs NHE} )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiR (25°C pH 7.5)(^b)</td>
<td>siroheme</td>
<td>-21</td>
<td>this work</td>
</tr>
<tr>
<td></td>
<td>cluster</td>
<td>-303</td>
<td>this work</td>
</tr>
<tr>
<td>DV (25°C pH 7.5)(^c)</td>
<td>1st redox couple (P(^-))</td>
<td>-310</td>
<td>this work</td>
</tr>
<tr>
<td></td>
<td>2nd redox couple (P(^2-))</td>
<td>-620(^c)</td>
<td>this work</td>
</tr>
<tr>
<td>\textit{E. coli} sulfite reductase(^b, d)</td>
<td>siroheme</td>
<td>-340</td>
<td>Siegel et al.</td>
</tr>
<tr>
<td></td>
<td>cluster</td>
<td>-405</td>
<td>1982.</td>
</tr>
<tr>
<td>cyt c (horse)</td>
<td>heme</td>
<td>+250</td>
<td>Moore, 1976.</td>
</tr>
<tr>
<td>myoglobin</td>
<td>heme</td>
<td>+50</td>
<td>Moore, 1976.</td>
</tr>
<tr>
<td>hemoglobin</td>
<td>heme</td>
<td>+170</td>
<td>Moore, 1976.</td>
</tr>
</tbody>
</table>
Table 5.1 (Continued)

a All $E^{o'}$ values were determined at pH 7.5 and 25 °C with respect to the NHE.

b For SiR and *E. coli* sulfite reductase, $E_{1}^{o'}$ represents the reduction potential for the siroheme, while $E_{2}^{o'}$ represents the reduction potential for the [Fe$_4$S$_4$] cluster.

c For DV, $E_{1}^{o'}$ represents the reduction potential for the first redox couple. $E_{2}^{o'}$ has not been determined by direct electrochemistry but has been estimated as ~ -620 mV by controlled potential coulometry.

d For *E.coli* sulfite reductase, the reduction potentials were determined by EPR potentiometric titrations.
Evidence for Direct Reversible Electrochemistry. In a reversible redox process, the concentration of species at the electrode surface depends only on thermodynamic considerations; that is, according to the equilibrium established by the applied potential at the electrode surface. The current-time response under the appropriate applied potential then follows the Cottrell equation (9),

\[
i = \frac{nFAD^{1/2}C^*}{\pi^{1/2}t^{1/2}}
\]  

(9)

where \(n\) is the number of electrons transferred/redox site/molecule, \(F\) is the Faraday constant, \(D\) is the diffusion coefficient of the redox species, and \(C^*\) is the bulk concentration of the redox species. When applying the Cottrell equation to SWV, a plot of \(i_p\) vs \(v^{1/2}\) is linear if diffusion control and rapid equilibrium conditions exist, and so such a plot provides an indicator of the extent to which the electrode kinetics influences the equilibrium at the electrode (Smith and Feinberg, 1990).

In Figures 5.8 and 5.9 it is shown that the peak current \(i_p\) is directly proportional to the square-root of the frequency of the applied potential for each of the signals observed by the SWV method; that is, the reduction potentials determined for the siroheme and cluster couples in SiR, and the first redox couple in DV. The linearity of the plots of \(i_p\) vs \(v^{1/2}\) for the aforementioned data sets indicates that the current is diffusion controlled for each of these redox couples, suggesting that direct electron transfer between
Figure 5.8 Dependence of the peak current ($i_p$) on the square root of the frequency of the applied potential pulse ($v^{1/2}$) for the first redox couple in DV. The linearity indicates that the electroactive species is under diffusion control and that the enzyme interacts reversibly with the electrode surface.
Figure 5.9  Dependence of the peak current ($i_p$) on the square root of the frequency of the applied potential pulse ($v^{1/2}$) for the siroheme redox couple (A) and the cluster redox couple (B) in SiR. The linearity indicates that the electroactive species is under diffusion control and that the enzyme interacts reversibly with the electrode surface.
the electrode and the sulfite reductase enzymes from *D. vulgaris* was indeed taking place, and that diffusion control and rapid equilibrium conditions existed during the electrochemical measurement.

**Nernstian n.** Theoretically, the peak width at half-height ($W_{1/2}$) in SWV is $\frac{126}{n}$ mV for a reversible redox couple (Smith and Feinberg, 1990). For SiR, the magnitude of $W_{1/2}$ was found to be 134 mV for the siroheme signal and 84 mV for the cluster signal, equivalent to a value of 0.94 electrons transferred for the siroheme site and 1.5 electron equivalents transferred for the cluster site. This slightly lower than expected value most likely reflects the oxidative instability of this highly reduced form. For DV, the $W_{1/2}$ value for the first redox couple was 122 mV (corresponding to the transfer of 1.03 electron equivalents); again in excellent agreement with the theoretical value expected for a reversible redox couple involving one-electron transfer. From the controlled potential coulometrical measurements performed on DV, the number of electron equivalents transferred ($n$) was estimated to be 1.06 and 1.12 for the first and second redox couples ($P^-$ and $P^{2-}$) respectively.

### 5.3.2 Systematic pH Titration Experiments of the [Fe4S4]-siroheme Prosthetic Center

**Effect of pH on Reduction Potentials.** Systematic pH-titration studies of SiR and DV were performed at 25 °C. Figure 5.10 and 5.11 show the trends observed for SiR and DV, respectively. For DV, the pH-dependence of $E^\circ$ (Figure 5.11) was examined, and from the gradient in the central region of the plot ($\Delta E_m/\Delta pH = -62$ mV/pH unit) we estimate an $e^- /H^+$ transfer ratio of one. One-electron exchange is also supported by
Figure 5.10  Variation of $E^0'$ (mV) with pH (at 25°C), showing a fit to the equation, $E_m = E^0' + 59 \times \log\left(\frac{K_{a_{\text{red}}} + [H^+]}{K_{a_{\text{ox}}} + [H^+]}\right)$ for SIR: (A) siroheme signal, and (B) cluster signal. All data were taken under the experimental conditions described in the legend to Figure 5.6.
Figure 5.11  Variation of $E^o'$ (mV) with pH (at 20°C), showing a fit to the equation, $E_m = E^o' + 59 \cdot \log((K_{a\,\text{red}} + [H^+])/(K_{a\,\text{ox}} + [H^+]))$ for the first redox couple ($P^-$) in DV. All data were taken under the experimental condition described in the legend to Figure 5.5.
analysis of the signal response (Figure 5.5). The pH data were fit by the
standard equation (7) (Clark, 1960) to yield two pKₐ values (pKₐ°ₓ = 3.2 and
pKₐred = 9.0). The separation of pKₐred - pKₐ°ₓ ~ 6 units is large in
comparison to the difference typically obtained for ionizable residues
neighboring a redox site, however, such a separation has previously been
observed for met-Mb, which possesses a bound water molecule (Antonini
and Brunori, 1971). It is possible, therefore, that the response obtained
derives from either a siroheme-bound H₂O, or a bridging sulfide ligand (Tan
and Cowan, 1991). Further investigations were obviously required to
confirm the identity of this species, and are described below. Clearly,
however, redox chemistry at the prosthetic site is intrinsically coupled to an
influx of protons that may play role in active turnover. It is most likely of
functional significance given the fact that sulfite or nitrite reduction requires
uptake of at least seven proton equivalents.

Figure 5.10 shows the pH dependence of the siroheme and cluster
reduction potentials of SiR. The gradients (ΔEₚ/ΔpH) from the central
region of the plots were determined as follows: SiR siroheme, ΔEₚ = -64
mV/pH unit; SiR cluster, ΔEₚ = -52 mV/pH unit. We estimate an e⁻/H⁺
transfer ratio of approximately one for each redox couple observed. The pH
data were fit by the standard equation described in the experimental section
to yield two pKₐ values (pKₐ°ₓ and pKₐred) that are summarized in Table
5.2.

5.3.3 Characterization of the Redox Thermodynamics of the Prosthetic
Center
Table 5.2  

$p_{K_{a}^{ox}}$ and $p_{K_{a}^{red}}$ values obtained from pH titration studies at 25°C for SiR and DV using SWV electrochemical method.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Redox couple</th>
<th>$p_{K_{a}^{ox}}$</th>
<th>$p_{K_{a}^{red}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiR</td>
<td>siroheme</td>
<td>3.0</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>cluster</td>
<td>2.9</td>
<td>8.7</td>
</tr>
<tr>
<td>DV</td>
<td>1st redox couple (P-)</td>
<td>3.2</td>
<td>9.0</td>
</tr>
</tbody>
</table>

Experimental conditions were the same as in the legend to Figure 5.6 for SiR and Figure 5.5 for DV.
**Evaluation of Redox Thermodynamic Parameters.** Results obtained for the temperature dependence of the first reduction potential of DV, and the two reduction steps of SiR are illustrated in Figures 5.12 and 5.13, respectively. The solid line is a linear least-squares fit to the data. The redox thermodynamic parameters (25 °C, pH 7.0) derived from these nonisothermal experiments are summarized in Table 5.3.

It is significant that the $\Delta S^0$ values are all negative in magnitude and are large in comparison with related data obtained from horse cytochrome c, which is a low-spin heme ($\Delta S^0' = -28.5 \text{ eu}$) (Taniguchi et al., 1982 a, b), but smaller than the high-spin heme in myoglobin ($\Delta S^0' = -38 \text{ eu}$) (Crutchley et al., 1985). Interestingly, the $\Delta S^0'$ value for low-spin SiR shows more similarity to the value reported for high-spin Mb, while the $\Delta S^0'$ value for high-spin DV shows more similarity to the value reported for low-spin cytochrome c. There seems to be no relationship between the spin state and the magnitude of $\Delta S^0'$ values. The relevance of these findings for an understanding of active site chemistry will be a topic of discussion later in the chapter. It is also noteworthy that the $\Delta H^0$ value for the siroheme in SiR (-11.8 kcal mol$^{-1}$) is significantly more negative in comparison to $\Delta H^0$ values for the cluster in SiR and the first redox couple in DV (-4.5 and -3.0 kcal mol$^{-1}$, respectively), but shows more similarity to the $\Delta H^0$ values for cyt c and Mb (-14.5 and -14 kcal mol$^{-1}$). The $\Delta H^0$ values for the redox couples listed in Table 5.3 are all negative in magnitude. The more negative $\Delta H^0$ in SiR siroheme, cyt c and Mb, most likely reflects the favorable metal to ligand $\pi$-back bonding (Fe(II) heme/siroheme with axial ligand), which tends to stabilize ferrous ion.
Figure 5.12  Temperature dependence of the first reduction potential in DV. The entropic terms ($\Delta S^0$) were determined from the slope of $E^0$ versus $T$ plot. Variable temperature reduction potential measurements were done at pH 7.0 and 25°C.
Figure 5.13 Temperature dependence of the reduction potentials of the siroheme - cluster redox couples in SiR: (A) siroheme signal; and (B) cluster signal. The entropic terms (ΔS°) were determined from the slope of E° versus T plot. Variable temperature reduction potential measurements were done at pH 7.0 and 25°C.
### Table 5.3  Redox Thermodynamic Parameters at pH 7.0 and 25 °C.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta S^0$</th>
<th>$\Delta S_{\text{TC}}^0$</th>
<th>$\Delta H^0$</th>
<th>$\Delta G^0$</th>
<th>$E^0$ (V vs NHE)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>eu</td>
<td>eu</td>
<td>kcal mol$^{-1}$</td>
<td>kcal mol$^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SiR</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Siroheme</td>
<td>-36.4</td>
<td>-20.8</td>
<td>-11.8</td>
<td>+0.97</td>
<td>-0.042</td>
<td>this work</td>
</tr>
<tr>
<td>Cluster</td>
<td>-34.7</td>
<td>-19.1</td>
<td>-4.5</td>
<td>+5.8</td>
<td>-0.251</td>
<td>this work</td>
</tr>
<tr>
<td><strong>DV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>-31.3</td>
<td>-15.7</td>
<td>-3.0</td>
<td>+6.3</td>
<td>-0.272</td>
<td>this work</td>
</tr>
<tr>
<td><strong>Redox Couple</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cyt c (horse)</td>
<td>-28.5</td>
<td>-12.9</td>
<td>-14.5</td>
<td>-6.0</td>
<td>+0.260</td>
<td>Taniguchi et al., 1982.</td>
</tr>
<tr>
<td>Mb</td>
<td>-38.0</td>
<td>-22.4</td>
<td>-14.0</td>
<td>-2.8</td>
<td>+0.120</td>
<td>ibid</td>
</tr>
</tbody>
</table>
Dependence of $\Delta S^o'$ on pH. Figure 5.14 A shows the pH-dependence of $\Delta S^o'$ for the siroheme signal in SiR. It was observed to have a sigmoidal curvature with a $pK_a \sim 7$. This behavior was not observed in either the cluster signal in SiR, nor the first redox couple (P-) in DV (Figure 5.14 B & C). Instead, for the cluster signal of SiR the $\Delta S^o'$ was found to have a maximum around pH 7. At either low pH or high pH, the $\Delta S^o'$ values were measured to be smaller compared to the value at intermediate pH. This effect may be due to nearby ionizable residues (not directly bind to the cluster redox center), affecting the reaction entropy change of the cluster. At extreme pHs, the enzyme is predominantly in protonated form at low pH, and in deprotonated form at high pH. At intermediate pH, there is a equilibrium between these two forms, thus greater entropy change occurs at intermediate pH.

5.4 DISCUSSION

5.4.1 Reduction Potential Measurements of the Redox Centers

Comparison of Reduction Potentials for SiR and DV. Table 5.1 summarizes the electrochemically-determined reduction potentials of the redox couples in the prosthetic centers of SiR and DV from *D. vulgaris*, and makes the comparison with published data on the *E. coli* assimilatory sulfite reductase and other common heme proteins. The reduction potentials of each of the first redox couples of [Fe₄S₄]-siroheme prosthetic centers lie in the negative range of $E^o$ values, while the hemes in myoglobin, cyt c and hemoglobin have positive $E^o$ values (+50 mV, +250 mV, and +170 mV, respectively (Moore, 1976). The principal feature that distinguishes the two
Figure 5.14 pH dependence of $\Delta S^\circ$ for (A) the siroheme redox couple in SiR; (B) the cluster redox couple in SiR; and (C) the first redox couple in DV. Experimental conditions were the same as Figure 5.5 and 5.6 for DV and SiR, respectively.
classes of heme center in these enzymes (siroheme and heme) lies in the
degree of saturation of the porphyrin-derived macrocycle. In the former case
the elements of methane (CH\textsubscript{4}) have been added across two pyrrolic rings
(Figure 1.3). The additional electron density on the siroheme ring tends to
stabilize the Fe(III) state, resulting in more negative E\textsuperscript{o} values in comparison
to the heme in Mb, Hb and cyt c. This is also reflected by the ease of
oxidation of the siroheme ring, which in some circumstances may be more
facile than oxidation of the ferrous ion in reduced siroheme. It was noted
that the first redox couple in SiR (corresponding to siroheme reduction) has a
less negative E\textsuperscript{o} value in comparison to DV and \textit{E. coli} sulfite reductase. This
may well arise from the presence of a sixth ligand (proposed as His) (Cowan
and Sola, 1990) that can stabilize the reduced form of siroheme, leading to a
more positive potential. Both DV and \textit{E. coli} sulfite reductase contain high-
spin pentacoordinate siroheme. Alternatively, the increase in the E\textsuperscript{o} value
for siroheme in SiR may arise from a more hydrophobic environment for
siroheme inside the protein pocket. Inasmuch as there are no side-products
produced during turnover of SO\textsubscript{3}\textsuperscript{2-}, such as those observed for DV (Lee et al.,
1973), we have previously argued that the prosthetic center in SiR is likely to
be less solvent exposed, or that the entry to the active site is more hindered.
Lower polarity (or a more hydrophobic environment) will lead to a more
positive heme redox potential (Moore, 1976) : for example, the heme proteins
Hb, Mb, and cyt c all have the redox active site (heme) buried inside a very
hydrophobic medium. Finally, the less negative E\textsuperscript{o} value for the heme center
in SiR may also reflect the fact that there is no change in spin state upon
reduction of the low-spin siroheme in this enzyme. A change of spin state
upon reduction leads to more negative E\textsuperscript{o} values, since this typically results
in a significant redistribution of electrons in orbitals, influencing metal-
ligand geometries and promoting substantial activation barriers that impede
reduction. *E. coli* sulfite reductase has been observed to undergo a change of
spin state from high spin \((S = 5/2)\) to an intermediate spin \((S = 1)\) after one-
electron reduction (Janick and Siegel, 1982) while oxidized DV also contains
a high-spin siroheme \((S = 5/2)\) and is also likely to show a change of spin
state following reduction.

**Coupling of the \([Fe_4S_4]\) Cluster and Siroheme.** The generality of the
coupled cluster-siroheme model for the class of enzyme has recently been
questioned (Peirik and Hagen, 1991). Previously published NMR data on SiR
strongly suggests direct coupling of the two redox centers (Cowan and Sola,
1990) following the original proposal of Siegel and coworkers for the *E. coli*
enzyme (Christner et al., 1982 & 1983 & 1984; Janick and Siegel, 1982; Janick
et al., 1983; McRee et al., 1986; Cline et al., 1985; Madden et al., 1989; Siegel et
NHE for DV. Since only one peak is observed over the pH range 3 - 10, these
results also support direct coupling of the siroheme and \([Fe_4S_4] \) cluster,
forming a bridged redox pair \((P)\) that apparently takes up two electrons in
consecutive steps \((P \rightarrow P^- \rightarrow P^{2-})\). The potential reported here corresponds to
the first redox couple \((P \rightarrow P^-)\). Uptake of a second electron occurs at a
reduction potential that is too negative to be detected over the range
available to PGE in aqueous solution, while the \(Cr(NH)_6^{3+}\) promoter is
reduced (Armstrong et al., 1987; Miler and Zittel, 1963).

**Reduction Potential of the Second Redox Couple.** The \([Fe_4S_4]\) cluster
in SiR has a less negative \(E^0\) value (-300 mV) compared to the \(E^0\) value of the
second redox couple in DV (-620 mV) and the \([\text{Fe}_4\text{S}_4]\) cluster in \textit{E. coli} sulfite reductase (-405 mV) (Siegel et al., 1982) This must reflect variations in their peptide environment surrounding the prosthetic centers in each enzyme. Perhaps most noteworthy is the rather negative (~ -620 mV) potential for the second redox couple of DV. This potential is not accessible with typical physiological reductants. We might expect that this potential would shift to less negative values after substrate (or ligand) binding following one-electron reduction. We have not yet been able to determine potentials for ligand-bound enzyme species to test this hypothesis, although this is now a focus of effort in our laboratory.

The EPR data in Ch. VI demonstrates that the potentials of the cluster and siroheme in DV are similar. Consider the cluster/ siroheme pair to be a single redox center that can take up two electrons, but in a stepwise manner. One can imagine the first electron residing on either the cluster or siroheme. There is simultaneous reduction of cluster and siroheme over the ensemble of centers, but not in the same pair. After addition of the first electron, addition of a second electron requires a more negative potential. We have summarized the sequence of reduction steps for DV in Figure 5.15 A. However, for SiR, EPR data (Ch. VI) demonstrates that the potentials of the cluster and siroheme are quite different. After addition of the first electron, the electron will localize at the siroheme redox center which has a more positive potential (possibly stablized by the sixth ligand histidine). The addition of second electron equivalent will then go to the cluster redox center. The sequence of reduction steps in SiR is summarized in Figure 5.15 B.
Figure 5.15  Summary of redox reactions: (A) DV, and (B) SiR.
5.4.2 Systematic pH Titration Experiments of the \([\text{Fe}_4\text{S}_4]\)-siroheme Prosthetic Center

**pH-Dependence of Redox Potentials.** The pH titration curves for the redox couples in DV and SiR were found to have similar broad features (Figure 5.10 & 5.11). The ligand or residue affecting the change of \(E^\circ\) value with pH values is not a neighboring amino acid as the pH titration curve will not show such a broad feature. We postulated that this ligand must be directly bound to the redox center. In DV it can be either the bound \(\text{H}_2\text{O}\) at the sixth coordination site to the siroheme, or the bridging \(\text{S}^2-\) ligand. The \(pK_a\) value for the first ionization of \(\text{H}_2\text{S}\) is 6.97, while that of \(\text{H}_2\text{O}\) is 7.0 (CRC, 1990). If the pH behavior is due to the bound \(\text{H}_2\text{O}\), it should not be observed in SiR, which only possesses a His at the axial site. The redox centers in SiR and DV showed similar broad pH dependence except there was an additional residue with \(pK_a\sim 7\) buried under the broad pH titration curve at the siroheme signal of SiR. This might be due to the axial His. The broad pH titration behavior is most likely due to the bridging sulfide directly bound to the redox centers in both SiR and DV as there is no siroheme-bound \(\text{H}_2\text{O}\) in SiR.

5.4.3 Characterization of the Redox Thermodynamics of the Prosthetic Center

**Evaluation of Redox Thermodynamic Parameters.** The redox thermodynamic parameters (25 °C, pH 7.0) derived from these non-isothermal experiments are summarized in Table 5.3.
Entropy change can be related to the kinetics of electron transfer. For example, a study of the kinetics of electron transfer between cyt c' and flavin semiquinones (Meyer et al., 1986) concluded that these cyt c' are ca. three times more reactive than mitochondrial cyt c. This reactivity difference was attributed to a greater solvent exposure of the heme in the c' type cytochromes. Another interpretation of these data is that cyt c' exhibit somewhat lower reorganization energies. ($\Delta S^0_{RC}$ for cyt c', cyt c are -8.1 and -12.9 eu, respectively). From Table 5.3, SiR exhibits higher $\Delta S^0_{RC}$ for both siroheme and cluster redox couples (-20.8 and -19.1 eu, respectively). These imply that the redox centers in SiR are less solvent exposure (consistent with no other byproduct formation during turnover) or the redox centers exhibit higher reorganization energies compared to the redox couple in DV.

Dependence of $\Delta S^0$ on pH. The pH-dependence of $\Delta S^0$ for the siroheme signal in SiR (Figure 5.14 A) was observed to have a sigmoidal curvature with a $pK_a \sim 7$. This behavior was not observed in either the cluster signal in SiR, nor the first redox couple ($P^-$) in DV (Figure 5.14 B & C). Instead, for the cluster signal of SiR the $\Delta S^0$ was found to have a maximum around pH 7. At either low pH or high pH, the $\Delta S^0$ values were measured to be smaller compared to the value at intermediate pH.

Figure 5.16 shows the pH dependence of $\Delta S^0$ for horse heart cytochrome c, which exhibits an alkaline transition with a $pK_a \sim 9.1$ (Taniguchi et al., 1982). This transition is believed to be associated with the dissociation of Met-80. As the pH increases, $\Delta S^0$ drastically decreases, as expected for a situation in which reduction is accompanied by a large change in molecular geometry (Sutin et al., 1980).
Figure 5.16  pH dependence of $\Delta S^0$ for horse heart cytochrome c
(Reproduced from Taniguchi et al, 1982).
Concluding Remarks. The application of direct electrochemical methods to measure reduction potentials for [Fe₄S₄]-siroheme prosthetic centers in sulfite-reducing enzymes lay the groundwork for more detailed investigation of the redox chemistry of the coupled center common to this class. Characterization of the redox thermodynamics, pH-titration studies and catalytic reactions at the electrode of these enzymes are now feasible.

Although the discussion here has focussed extensively on the bridge site it is questionable whether this site is actually protonated during active turnover. One might think that the proton is ultimately trapped by intermediate species formed by reduction of substrate. Our results do, however, demonstrate that a pathway exists which facilitates movement of protons from the bulk medium to the active site pocket, and that this proton flux is indeed coupled to oxidation and reduction of the catalytic prosthetic centers. Indeed it is interesting to speculate that the movement of protons might be related in some way to the redox-linked conformational gating mechanism recently demonstrated for at least one member of this class of enzyme. This connection remains to be established.
CHAPTER VI

Studies of Electronic/Magnetic Properties of the [Fe₄S₄]-Siroheme Prosthetic Center of SiR and DV by EPR Spectroscopy

ABSTRACT

EPR characterizations (g-values, rhombicity, spin quantitation, power saturation and variable temperature measurements) have been performed on SiR and DV in both oxidized and reduced forms. Highly purified samples of the enzymes display standard EPR characteristics with the purity especially critical in DV (Ch. II). EPR spectra of both enzymes at different stages of reduction offers insights on the redox and electronic properties of the [Fe₄S₄]-siroheme prosthetic centers and allow the estimation of the difference of the reduction potentials of the siroheme and cluster as a function of axial ligands. Power saturation behavior gives evidence for the coupling of siroheme and cluster in the prosthetic center.

Oxidized SiR shows a rhombic low-spin hexacoordinate siroheme (S = 1/2, g = 2.45, 2.38, 1.78), and the reduced enzymes show characteristic typical ferredoxin-like [Fe₄S₄] EPR spectra (S = 1/2, g = 2.04, 1.93) that differ from spectra obtained with E. coli sulfite reductase (Siegel et al., 1982). EPR spectra of SiR in different oxidation states (SiR⁰/-1/-2) have
demonstrated the stepwise reduction of siroheme and cluster in a manner consistent with the optical changes observed during reduction (Ch. VII). The signal from the oxidized siroheme disappeared completely before the appearance of the signal arising from the reduced cluster; that is, the one-electron reduced enzyme is EPR silent. This implies that siroheme and cluster in SiR have very different reduction potentials.

Highly purified native oxidized DV exhibits a typical rhombic high spin siroheme system ($S = 5/2, g = 6.30, 5.63, 2.00$) in contrast to the published result by Hagen and coworkers (1991), who have reported a very complicated EPR spectrum for DV and attributed this to the presence of a [Fe$_6$S$_6$] cluster. There is no evidence for demetallated siroheme or clusters of higher nuclearity in our highly purified DV samples (discussed in Ch. II). EPR spectra for DV in different oxidation states (DV$^{0/-1/-2}$) show different behavior from SiR. DV$^{-2}$, similar to SiR$^{-2}$, displays typical [Fe$_4$S$_4$] cluster signal ($g = 2.07, 1.94, 1.89$) though more rhombic. However, DV$^{-1}$ shows distinct EPR spectrum which displays half oxidized siroheme and half reduced cluster signal, in contrast to the EPR-silent SiR$^{-1}$. This implies that the difference in reduction potentials of the siroheme and cluster in DV are quite similar, thus no clear stepwise reduction step, like SiR, is observed during the course of reduction of DV. The EPR-active DV$^{-1}$ contains half of the molecules with reduced sirohemes and the other half with reduced clusters.
6.1 INTRODUCTION

The enzymes involved in electron transport and oxidation-reduction reactions characteristically contain many of the transition-group "trace metals" that are essential microconstituents of cells (Bastian et al., 1988; Godden et al., 1991; Shapleigh et al., 1992). In many cases trace metals participate in enzymatic reactions involving the transfer of single electron. Therefore unpaired electrons in the atomic orbitals of the metal constituents may also contribute to the EPR spectra of these enzymes allowing studies of the active site chemistry by EPR techniques. EPR can be used as a probe of the oxidation state and chemical environment of various transition metals in biology (e.g. Fe$^{3+}$, Mo$^{5+}$, Cu$^{2+}$, Mn$^{2+}$, Co$^{2+}$, Ni$^{3+}$ and multinuclear centers having half-integral electron spin like [Fe$_4$S$_4$]$^+$ cluster) (Cowan, 1993).

The two enzymes being investigated, SiR and DV, both contain two metal redox centers: siroheme and cluster that serve as sites for influx or efflux of electrons and/or coordination of substrate molecules prior to reduction. Siroheme is EPR active in the oxidized form: ferric iron either in high spin ($S = 5/2$) or low spin ($S = 1/2$) depending on the coordination environment. Reduced siroheme is EPR silent: ferrous iron either in low spin ($S = 0$, diamagnetic) or high/intermediate spins ($S = 2$ or $1$, though paramagnetic, generally unobservable in normal EPR operating conditions) (Palmer, 1979). The [Fe$_4$S$_4$] cluster is EPR inactive in the oxidized form ($S = 0$) but EPR active in the reduced form ($S = 1/2$) (Bertini, 1993). The two metal redox centers are found to be coupled together and function as a whole catalytic unit for the enzymatic reduction of sulfite.
and nitrite (Cowan and Sola, 1990; Tan and Cowan, 1991; Tan et al., 1991 & 1994; Wolfe et al., 1994; Tan and Cowan, 1990; Lui et al., 1993 & 1994). The structure, surrounding environment, electronic, magnetic, and redox properties of the active site in these enzymes are important pieces of information to understand the catalytic mechanism. Results from extensive chemical analysis (Ch. II) demonstrate that DV contains two siroheme and two [Fe₄S₄] centers, while SiR possesses one pair only (Ch. II, Tan et al., 1994; Wolfe et al., 1994). Though the enzymes contain the basic catalytic unit, the spectral characteristics are very different, owing to differences in the coordination geometry and protein environment at the active site.

This chapter will concentrate on the elucidation of the electronic, magnetic and redox properties of the novel [Fe₄S₄]-siroheme prosthetic centers in SiR and DV by EPR techniques. The goals of this chapter are to: (1) quantitively and qualitatively identify, by comparison with EPR spectra of known proteins or model compounds, which transition metals or metal clusters are present in our proteins and what their redox state is; (2) quantitatively determine the stoichiometry of these metals or clusters by integration using spin standards; (3) qualitatively determine themodynamic redox properties of the metal centers by monitoring the EPR spectrum intensity as a function of ligand or reducing equivalents; and (4) understand the role of the metals in the biological functioning of these metalloproteins. Detailed EPR characterizations of both enzymes in different oxidation states have been performed. Implications on the coordination environment, electronic/magnetic/redox properties of the [Fe₄S₄]-siroheme prosthetic center are discussed. Evidence for exchange-
coupling in the [Fe₄S₄]-siroheme unit in both enzymes is observed through power saturation studies, in contrast to the recent proposal of a non-coupled unit in DV (Pierik and Hagen, 1991).

The distinguishable differences in EPR characteristics observed in SiR, DV, and the E. coli enzyme suggest that the electronic properties of the coupled [Fe₄S₄]-siroheme redox center common to both nitrite and sulfite reducing enzymes (Siegel et al., 1982; Christner et al., 1983; Cammack et al., 1978) are apparently strongly dependent on the environment generated by protein sidechains.

6.2 MATERIALS AND METHODS

General Materials. Buffer salts were of molecular biology grade (Fisher or Aldrich Chemical Co). Measurements of solution pH were made with an Accumet pH Meter 910 equipped with a Corning semimicro combination pH electrode. Sephadex G-200 gel filtration material was obtained from Sigma, and DEAE-52 ion exchange resin from Whatman. Deazaflavin was synthesized by literature methods (Janda & Hemmerich, 1976). All water used was purified with a Barnstead nanopure system and exhibited a resistivity of 18 MΩ cm⁻¹. Liquid helium was purchased from Helium Technology. Myoglobin was available in our laboratory.

Bacterial Growth, Isolation and Purification of SiR and DV. 
_D. vulgaris_ (Hildenborough, NC1B 8303) possessing a broad-host range expression vector (pDSK519) with the SiR gene and promoter cloned into the multiple cloning site (Tan et al., 1994) was grown in a lactate-sulfate
medium. The purification details of SiR and DV were described in Ch. II. For DV, band I from FPLC chromatography is typically used in experiments as no differences were observed between DV-I and DV-II.

**EPR Instrumentation.** EPR spectra were recorded at X-band with a Bruker ESP 300 spectrometer equipped with an Oxford liquid helium cryostat. Temperature was measured by use of a Au/Fe v. Cr. thermocouple. General spectrometer settings were as follows: microwave frequency = 9.53 GHz; modulation frequency = 100 kHz; modulation amplitude = 10 G; time constant = 10.2 ms; microwave power = 1 mW; sweep width = 2200 G. EPR signals from the oxidized siroheme in SiR and DV were optimally detected at 11.0K and 4.6 K, respectively. EPR signals from the reduced cluster in SiR and DV were optimally detected at 13.2 K. Specific experimental parameters are listed in footnotes to the tables and figure legends. Samples were purged with Ar gas and frozen in liquid N2 prior to obtaining spectra.

**Preparation of Photoreduced Enzyme.** A 250 µl volume of a solution containing 100 µM enzyme, 100 µM deazaflavin and 10 mM EDTA was degassed with O2-free Ar(g) in a 1 ml pear-shaped flask with stirring for 20 min. EPR tubes were fitted with a serum stopper and purged with O2-free Ar(g) for 5 min. Gas tight syringes (Hamilton) with teflon needles were flushed with Ar(g) several times prior to withdrawing samples under a positive pressure of Ar(g). Samples were injected into Ar(g) purged EPR tubes and the septum sealed with grease to inhibit O2 diffusion. Samples were reduced using the deazaflavin/EDTA photoreduction method of Massey and Hemerich (1978). The sealed EPR
tube was transferred to a glass beaker filled with ice-water and irradiated (1000W lamp, 90% power) for 5-30 min, depending on the sample requirements. Reduction was monitored by electronic absorption and irradiation was continued until no further change was observed in the optical spectrum to obtain a fully reduced enzyme. Solutions of deazaflavin/EDTA and enzyme (with ligand) were also prepared and reduced in the same way. Subsequently the samples were frozen in liquid nitrogen.

6.3 EPR Theory

Basics Concepts. EPR takes advantage of the fact that only the paramagnetic molecules in a sample can be made to interact with a suitable external magnetic field. The magnetic dipole of an electron may be thought of as deriving from the fact that the electron is a charged particle and that a charge in motion creates a magnetic field. Two types of magnetic dipoles are possible: (1) the orbital magnetic dipole (from the motion of electron about the nucleus of an atom); and (2) the spin magnetic dipole (from the spinning of the electron about an axis through its center). In the absence of an external field, the free electron and its spin magnetic moments are randomly oriented and are in the same average energy state. If an external magnetic field is applied, the electronic magnets will become aligned. The electronic magnets can either direct themselves parallel to the field (which is a more stable energy condition) or opposed to it (antiparallel). Thus the applied magnetic field segregates the system of equally energetic paramagnets into two subsets with a small energy difference (ΔE). Figure 6.1 shows the energy diagram of an electron
\[ E^+ = +1/2 \, g\beta H; \quad E^- = -1/2 \, g\beta H \]
\[ \Delta E = E^+ - E^- = g\beta H. \]

Figure 6.1 Illustration of the Zeeman energies of electrons in an external magnetic field $H$. 
in the presence of an external magnetic field. Energy transitions are now possible in which the orientation of the electron spins will change. Electron spins can resonate with the external radiation field: they can absorb energy from it and give energy to it. Some parallel magnetic dipoles will absorb and make themselves jump to a higher excited state, and some antiparallel magnetic dipoles will flip over to a lower parallel state, thus releasing energy to the electromagnetic field. Particles always populate the lower state more than the upper level, thus at resonance there will be more parallel than antiparallel electrons. Hence the resonance phenomenon will give rise to a net absorption of energy from the field. It is this net absorption of electromagnetic energy at resonance that is detected and amplified to provide the sample signal in EPR spectroscopy.

The energies of electron-spin magnetic moments in a magnetic field are given by:

\[ \Delta E = g\beta H \]

\[ E = h\nu = g\beta H M_s = \pm \frac{1}{2} g\beta H \]

where \( g \) = a constant of proportionality for a given system

\( \beta = \text{electron magneton} = \frac{e\hbar}{4\pi mc} \)

\( H = \text{magnetic field} \)

\( M_s = \text{spin quantum number, } \pm \frac{1}{2} \text{ for an } e^- \)
E is called electron Zeeman energy while $\Delta E$ is the energy gap between the two energy levels. The selection rule for an allowed EPR transition is $\Delta M_S = \pm 1$. For technical reasons, it is more convenient to vary the magnetic field strength $H$ than the microwave frequency. The effect is to vary the energy separating the Zeeman energy states rather than the excitation energy itself. Generally, resonance at magnetic field $H_r$ can be represented by:

$$H_r \text{ (in Gauss)} = \frac{\hbar \nu}{g \beta} = \frac{714.484 \times \nu \text{ (in GHz)}}{g}$$

$g$ Factors. The $g$ factor in the above equation is a universal constant and characteristic of the electron ($g_e = 2.00232$), provided that $H_r$ is the magnetic field at the electron. The application of an external magnetic field, however, may generate an internal magnetic field in the sample which add to or subtract from the external field. Any local magnetic fields are accounted for by allowing the $g$ factor to vary; i.e.

$$g_{eff} = \frac{\hbar \nu}{\beta H_r}$$

where $H_r$ is defined as the external magnetic field at resonance. Thus $g$ factor can be considered as a quantity characteristic of the molecule in which the unpaired electrons are located. The measurement of the $g$
factor for an unknown signal can then be a valuable aid in the identification of the signal origin.

For electrons constrained in atomic or molecular orbitals, orbital as well as spin momentum is possible. So the principal source of the local magnetic fields, which cause $g$ to deviate from the free-electron value $g_e$, is an orbital magnetic moment introduced by a mixing of excited states with ground states. For most molecules the mixture of excited states is not isotropic (orientation independent) but is anisotropic (orientation dependent), i.e. the magnitude of the induced local field (hence the deviations of the $g$ factor from $g_e$) depends on the orientation of the molecule with respect to the external magnetic field, in regular structure e.g. octahedron, tetrahedron, or cube, then $g_x = g_y = g_z$. In such a case, the $g$ factor is isotropic and can be represented by a single value. This is also true if the paramagnetic entity is in a solution of low viscosity, such as liquid water, where molecular tumbling causes all the $g$-factors to be averaged out. If the molecule contains a three-fold or higher axis of symmetry along the $Z$ direction, then $X$ and $Y$ are equivalent. This is called axial symmetry and $g_x = g_y \neq g_z$. For molecules that contain no threefold or higher axis of symmetry, all three principal $g$ factors are different. This is called a rhombic system. For most organic free radicals, spin and orbital angular momenta are uncoupled and $g$ is approximately 2. With most transition metal complexes, however, the extent of spin-orbit coupling is often significant, and $g$ may vary between 1 and 9. Furthermore, for an asymmetric molecule, the extent of spin-orbit coupling is generally dependent upon the orientation of the molecule in the magnetic field. For a randomly oriented sample imbedded in a solid
(e.g. a frozen liquid), up to three \( g \) values, corresponding to the three principal axes of an orthorhombic coordinate system, may be required to describe the observed EPR signal. Figure 6.2 illustrates the isotropic, axial and rhombic systems.

**Rhombicity.** The characteristics of the EPR spectrum may be used to describe the symmetry of the heme. For metalloproteins, the characteristics of the EPR spectra are sensitive to conformational changes of the protein moiety, which in turn produce geometrical changes at the metal site (Peisach and Blumberg, 1971 & 1969). Ferric heme embedded in protein matrices may exhibit departures from this tetragonal symmetry, hence the protein environment may impose constraints on the heme which destroy this tetragonal symmetry. The departure from tetragonality may be brought about by mechanical distortion of the heme, by perturbations of the \( \pi \)-electron distribution of the heme system, or through \( \pi \) electron binding to iron at the fifth and sixth ligand positions of the heme iron.

**Low Spin Hemoproteins** (\( S = 1/2 \)): The three \( t_2g \) orbitals (\( d_{xy}, d_{xz}, d_{yz} \)) are completely isolated from the remaining 3d orbitals so that the five valence electrons are located in these \( t_2g \) orbitals (\( t_2g^5, S = 1/2 \)). These ligand field orbitals are subjected to an asymmetric ligand field. In \( D_{2h} \) symmetry the nonspherical part of the ligand fields can be decomposed into a large axial component \( \Delta \) (proportional to the field strength along \( z \)) and a smaller rhombic component \( V \) (proportional to the difference in ligand field strength along \( x \) and \( y \)). The component \( \Delta \) is a stretching or compression effect along the \( z \) axis while \( V \) is an in-plane distortion.
Figure 6.2  Representative EPR spectra of different systems: isotropic, axial, rhombic.
resulting in the inequivalency of $d_{xz}$ and $d_{yz}$ and is never larger than $2/3$ $\Delta$ (because when the separation between each of the three orbitals is equal, one has maximum rhombicity) (Palmer, 1979). Figure 6.3 shows the energy levels of $t^2_g$. With $V/\Delta = 2/3$ the system is said to be 100% rhombic. The unpaired electron in $t^2_g$ is not confined to $d_{yz}$ but is located in a linear combination of $d_{yz}$, $d_{xz}$ and $d_{xy}$. The physical wavefunction ($\psi$) can be expressed as the followings (Weisbluth, 1973; Taylor, 1977; Bohan, 1977):

$$
|\psi^+> = a |dyz> + b |dxy> + c |dxz>
$$

$$
|\psi> = a |dyz> + b |dxy> + c |dxz>
$$

where $a$, $b$, $c$ are real, signed numbers and + and - are the electron spin.

After a series of mathematical derivations (Palmer, 1979), the quantities $a$, $b$, $c$, $V$, $\Delta$ can be obtained with the following expressions:

$$
a = \frac{(gz + gy)}{\sqrt{8 (gz + gy - gx)^1/2}}
$$

$$
b = \frac{(gz - gx)}{\sqrt{8 (gz + gy - gx)^1/2}}
$$
Figure 6.3  Energy diagram for one-electron orbitals for 3d⁵ e⁻ with axial distortion Δ and rhombic distortion V.
\[ c = \frac{(g_y - gx)}{[8(g_z + g_y - gx)]^{1/2}} \]

\[ V = \frac{gx}{\lambda (g_z + g_y)} + \frac{gy}{g_z - gx} \]

\[ \Delta = \frac{gx}{\lambda (g_z + g_y)} + \frac{gz}{g_y - gx} - \frac{1}{2} \frac{V}{\lambda} \]

where \( V \) and \( \Delta \) are given in units of the spin-orbit coupling constant \( \lambda \). It is also found that the quantity

\[ g_z + g_y - gx = 2(a + b + c)^2 \]

and Bohan points out the normalization condition \( a^2 + b^2 + c^2 = 1 \) leads to the quadratic relationship

\[ g_x^2 + g_y^2 + g_z^2 - g_x g_z - g_y g_x + g_x g_y - 4 g_x - 4 g_y + 4 g_z = 0 \]

This expression thus allows one to compute any \( g \) value knowing the other two, which is very useful when one \( g \) value is in a very high field and can never be seen in an EPR spectrum.

The origin of the rhombicity in the low-spin \( g \) values is not fully understood. It is generally believed to arise from asymmetry in the \( \pi \)-bonding between \( d_{yz}, d_{xz} \) and \( \pi \) orbitals on the axial ligands, particularly in the proximal histidine. Blumberg and Peisach (Chevion et al., 1977;
Brautigan et al., 1977; Blumberg and Peisach) discovered that the EPR spectra of low-spin heme protein fell into five relatively well-defined groups that could be characterized by similar g values (Table 6.1) and hence by comparable values for the ligand field V and Δ. Figure 6.4 shows a plot of V/Δ versus Δ/λ. The abcissa is a function of the electron donation to the iron atom so that compounds located along the same vertical line will suffer the same electrostatic charge (but be of variable geometry). The ordinate measures the distortion at the iron atom, thus points falling along the same horizontal line are subject to the same geometrical distortions but with varying charge. Species present in zones C, B, H, O have histidine as the fifth ligand, and thus infers that the overall symmetry is imposed by the four pyrrole nitrogens plus the imidazole N and that the effect of the sixth ligand is to add varying amounts of total charge without changing the overall geometry significantly. Typical low-field g values for heme proteins having histidine as the proximal ligand as a function of the sixth ligand are 3.5 (CN⁻), 3.3 (amine), 3.2 (methionine sulfur), 3.0 (N₃imidazole, N₁H), 2.8 (N₃imidazole, N₁⁻), 2.5 (OH⁻), 2.4 (RS⁻). Zones B and H have different sets of g values though they contain the same ligand, di-histidine derivatives. It was proposed that members of the B and H groups posses the distal histidine with N-1 protonated and unprotonated, respectively.

High Spin Hemoproteins (S = 5/2): The incorporation of hemin into a protein constrains the heme in such a manner that there is a departure from tetragonal symmetry toward rhombic (gₓ ≠ gᵧ). In these cases, the resonance absorption derivative near g = 6 is either broadened or split into
Table 6.1  Characterization of different types of low-spin heme proteins by grouping according to rhombicity (V/Δ) versus Δ/L.

<table>
<thead>
<tr>
<th>Class</th>
<th>Example</th>
<th>Average g values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gz</td>
</tr>
<tr>
<td>C</td>
<td>Cytochrome c</td>
<td>3.05</td>
</tr>
<tr>
<td>B</td>
<td>Cytochrome b</td>
<td>2.95</td>
</tr>
<tr>
<td>H</td>
<td>Hemoglobin histidine</td>
<td>2.80</td>
</tr>
<tr>
<td>O</td>
<td>Hemoglobin hydroxide</td>
<td>2.55</td>
</tr>
<tr>
<td>P</td>
<td>Cytochrome P-450</td>
<td>2.41</td>
</tr>
</tbody>
</table>

Figure 6.4 Crystal field correlation diagrams for low-spin ferric hemoproteins (Reproduced from Blumberg and Peisach).
two resolvable g values dependent upon the symmetry of the heme and the interaction of the protein with the heme. The greater the constraint on the heme imposed by the protein and the greater the asymmetry of the heme, the greater will be the departure from tetragonality. Thus the EPR of high spin heme proteins can be used as a sensitive protein conformation probe. In high spin ferric heme proteins, such changes may be detected at levels far below that which would effect either the optical spectrum or the magnetic susceptibility. The structure and conformation of various high spin ferric heme proteins can be sensed at the heme and small changes of conformation can effect the immediate environment of the heme (Peisach and Blumberg, 1971). In high spin ferric heme proteins these departures from tetragonality, which in turn are measures of differences caused by variations in protein constraints, are measured by differences in EPR spectra. A physical description of rhombicity of heme systems has been published (Blumberg et al., 1968). To first order, the splitting at $g \sim 6$ is directly related to departures from tetragonality of the heme by $E/D = \Delta g / 48$ where $E$ and $D$ are the coefficients of the second rank rhombic and axial spin operators in the spin Hamiltonian and $\Delta g$ is the absolute difference in g values between the two components near $g = 6$. Since $E/D$ has maxium value of $1/3$, one may express the rhombicity as a percentage of the total difference between a completely tetragonal and completely rhombic field (Blumberg, 1967):

$$R = \left( \frac{\Delta g}{16} \right) \times 100 \%$$
where $R$ is the percentage of rhombicity. These departures from tetragonality are sensitive measures of direct effects of protein environments on the heme.

**Relaxation Time.** The amount of energy that is absorbed is proportional to the intensity of the incident radiation as long as the lifetime of the state is sufficiently short so that the electrons return to the ground state faster than the irradiation raises them to the excited state. The electrons in the excited state relax back to the ground state within a relaxation time $\tau$. In optical spectroscopy the relaxation time is usually sufficiently short ($t \sim 10^{-8}$ s) so that the relaxation process does not impede the absorption rate. In radiofrequency spectroscopy, on the other hand, typical relaxation times amount to a millisecond or longer, and the spins do not have time to relax completely. An excess population of spins may be built up in the excited states, thereby slowing down the rate at which energy can be absorbed. Such a situation is referred to as "saturation".

From Einstein theory of transition probabilities, the ratio of the probability of spontaneous emission to that of induced absorption is proportional to the cube of the frequency, so saturation becomes much more prevalent as the frequency decreases. This is why NMR relaxation times tend to be longer than their ESR counterparts. The spin-spin relaxation time $T_2$ is a measure of the rate at which magnetic energy can be distributed within the spin system, and the spin-lattice relaxation time $T_1$ measures the rate of transfer of energy from the spins to the lattice.
The term $\Delta t$ that results from the transition rate (between electron spin states) will yield a linewidth termed "inherent". This $\Delta t$ is related to the time $T_1$. The inherent linewidth is given by

$$\Delta H_i = \frac{\hbar}{g \beta 2 T_1}$$

The observed linewidth is defined in terms of a relaxation time $T_2$ such that

$$\Delta H_0 = \frac{\hbar}{g \beta 2 T_2}$$

where $\frac{1}{T_2} = \frac{1}{T_2'} + \frac{1}{2 T_1}$.

The relaxation time $T_2$, called the spin-spin relaxation time, encompasses all additional sources of broadening. The relaxation time $T_2$ is simply a function of the observed linewidth. $T_1$ varies strongly with temperature, almost always decreasing as the temperature increases. Many mechanisms contribute to $T_1$; one of the most important of these is the interaction between the spin and orbital motion of the electron. If the "spin-orbit coupling" is strong, $T_1$ may be so short that the line is many thousands of gauss wide and virtually undetectable. This is the case for many transition metal ions. The temperature of the solution is lowered to lengthen $T_1$. In the studies of SiR and DV, liquid helium was used to carry out the EPR measurements at low temperature ($\approx 4.6 \text{ K} - 14 \text{ K}$).
Power Saturation Measurements. Relaxation times were shown to be extremely sensitive to influences from paramagnetic ions. Although people always refer to the use of proton spin relaxation, electron spin relaxation should lend itself to similar applications in appropriate biological systems. In EPR, a power saturation measurement is usually carried out to compare relative relaxation times of different spin systems.

In EPR, power saturation behaviors are usually examined to compare relaxation properties of different spin systems. Saturation with microwave power was determined by measuring signal size versus different microwave energies. The measured signal height (S) was divided by the square root of the microwave power (P^2) and log (S/P^2) was plotted against log P^2 (Beinert and Orme-Johnson, 1967; Rupp et al., 1978). This results in straight lines parallel to the abscissa when no saturation is obtained. The lines will bend toward the abscissa in the case of saturation. The power at half saturation (P_1/2) is given by the intersect of the extrapolated straight line portions (broken lines) of these curves. The position of P_1/2 with respect to the abscissa is then a measure of the saturation of the particular species. The faster the relaxation, the higher the P_1/2 value. Theoretically, an isolated spin system will have homogenous saturation behavior (similar P_1/2 values) on the g-tensors.

Spin Quantitations. Spin quantitations were carried out under non-saturating conditions. The standards for oxidized siroheme in SiR and reduced clusters in SiR and DV were either the azide adduct of sperm whale myoglobin (low spin) or CuEDTA as S=1/2 standards (Fee, 1975; Assa and Vanngard, 1975). The standards for oxidized siroheme in DV
was sperm whale myoglobin in native form (high spin) as $S = 5/2$

standards. Data for both samples and standards were obtained using the
same buffer conditions. EPR spin quantitation was performed using the
Aasa-Vanngard equation (1975) below,

$$N_u = N_s \cdot (H_{ms}/H_{mu}) \cdot (T_s/T_u) \cdot (P_s/P_u)^{1/2} \cdot (f_s/f_u)^2 \cdot (G_s/G_u) \cdot (S_u/S_s) \cdot (g_{ps}/g_{pu}) \cdot (SC_u/SC_s)^2$$

where $u$ or $s$ represent the unknown species and the standard,
respectively, and $N$ is the spin concentration, $H_m$ is the modulation
amplitude, $T$ is the temperature, $P$ is the applied microwave power, $f$ is
the diameter of the EPR tube, $G$ is the receiver gain, $S$ is the integral over
the entire absorption envelope (corrected for the Boltzmann distribution),
$SC$ is the field scan of the spectrum, and $g_p$ is defined by

$$g_p = \frac{2/3[(g_x)^2 + (g_y)^2 + (g_z)^2]/3]^{1/2} + [g_x + g_y + g_z]/9$$

**Variable Temperature Measurements.** For high spin ferric iron
proteins, the four ligands contributed by the porphyrin of the heme are
constrained to lie nearly in a plane, and the EPR spectrum is thus
required to show at most only small departures from axial symmetry
(Peisach and Blumberg, 1971). Usually, the second rank interactions in the spin Hamiltonian dominate the fourth rank interactions (the cubic field) and the latter can be neglected. In these cases, the magnetic energy levels for iron in the absence of a magnetic field comprise three Kramers doublets, lying at 0, 2D, and 6D in energy, where D is the secondary rank axial coefficient in the spin Hamiltonian (Bleany, 1953). Energy levels in the three principal directions will each be split to a different extent, but the separations between Kramers doublets are the same. The EPR spectrum observed at X-band for high spin heme iron extends from approximately g = 6 to g = 2 and arises only from the lowest Kramers doublet (Peisach and Blumberg, 1971). Meaningful absolute quantitation of high spin ferric EPR spectra cannot be made other than from experiments at very low temperature or from a knowledge of the zero field splitting, D. For S = 5/2 spin system,
where \( D = \) axial zero field splitting

\( E = \) rhombic zero field splitting

Zero-field splitting parameters were evaluated by use of standard theory,

\[
\frac{N_T}{N_O} = \frac{(IT)/(IT)_0}{1 + \exp(-2D/kT) + \exp(-6D/kT)}^{-1}
\]

where \( I \) is the signal amplitude of the derivative spectrum, \( T \) is the temperature, and \( (IT)_0 \) is a limiting value close to absolute zero. This describes the fraction of centers in the lowest Kramers doublet \( (m_S = \pm 1/2) \). Figure 6.5 shows the population of each doublet at different temperatures.

6.4 RESULTS

SiR in Different Oxidation States. Oxidized SiR shows similar \( g \) values as reported by Huynh (1984). Partially and fully reduced SiR, which have not been reported in the literature, are characterized by EPR spectroscopy in this chapter. Detailed EPR characterization has been performed on SiR in different oxidized states with and without ligands. Figure 6.6 shows the EPR spectra of oxidized, partially reduced (1.2 e\(^-\)) and fully reduced (2 e\(^-\)) SiR. The oxidized SiR shows a rhombic low spin hexacoordinate siroheme \((S=1/2, g = 2.45, 2.38, 1.78)\) EPR spectrum. One-electron reduced SiR \((\text{SiR}^{-1})\) is EPR silent. The cluster signal from fully reduced SiR \((\text{SiR}^{-2})\) \((S = 1/2, g = 2.04, 1.93)\) is characteristic of a reduced ferredoxin-like \([\text{Fe}_4\text{S}_4]\) center, which differs from the \textit{E.coli} sulfite
Figure 6.5  Diagram shows the population of the three Kramers doublets in $S = 5/2$ at different temperatures.
Figure 6.6  Stacked EPR spectra of SiR with 0, 1.2 and 2 electron equivalents added. Spectra were taken of an anaerobic solution of 100 µM SiR in 50 mM potassium phosphate, pH 7.5, containing 100 µM deazaflavin and 10 mM EDTA at 11 K and 13.2K for siroheme and cluster signals respectively. Spectral conditions: microwave frequency = 9.53 GHz; modulation frequency = 100 kHz; modulation amplitude = 10 G; time constant = 10.2 ms; sweep width = 2200 G; microwave power = 1 mW.
reductase (a mixture of different spins which have been ascribed to exchange coupling between siroheme and [Fe₄S₄] cluster) (Siegel et al., 1982). The g-values for the oxidized siroheme and reduced cluster in SiR are listed in Table 6.2 and 6.4, respectively.

Figure 6.6 shows EPR spectra following the uptake of 0, 1.2 and 2 electron equivalents by SiR. The data supports a large difference in the reduction potentials of siroheme and cluster inasmuch as the electron density is localized on the siroheme after one-electron addition, and there is no evidence for the presence of oxidized siroheme and reduced cluster in the same EPR spectrum during partial reduction. This is in accord with the results obtained by monitoring reduction through optical spectra with consecutive uptakes of electron equivalents by the two redox sites in the prosthetic center (Ch. VII). Two distinct optical spectra are observed for SiR⁻¹ and SiR⁻². Both optical and EPR characteristics suggest there is a stepwise reduction step in SiR, two stages of electron uptake by the two redox sites in the prosthetic center, which will be discussed later in the Discussion section.

**DV in Different Oxidation States.** EPR parameters of oxidized DV have been measured, but differed from the recent report by Hagen and coworkers (1991), who have claimed their complex EPR spectrum is due to the presence of [Fe₆S₆] cluster in DV and absence of [Fe₄S₄]. Ch. II has described how highly purified samples of native oxidized DV show a typical rhombic high-spin ferric siroheme EPR spectrum (S = 5/2, g = 6.30, 5.63, 2.00) in contrast to the published result. There is no evidence for demetallated siroheme or cluster of higher nuclearity. Figure 6.7 shows
Table 6.2  
Comparison of EPR spectral data for low spin native oxidized SiR and Mb-N₃⁻ adduct.

<table>
<thead>
<tr>
<th></th>
<th>SiR (D. vulgaris)ᵇ</th>
<th>Mb-N₃⁻ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td>gz</td>
<td>2.45</td>
<td>2.80</td>
</tr>
<tr>
<td>g values</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gy</td>
<td>2.38</td>
<td>2.22</td>
</tr>
<tr>
<td>gx</td>
<td>1.78</td>
<td>1.74</td>
</tr>
<tr>
<td>V/Δ (rhombicity)ᵃ</td>
<td>1.57</td>
<td>0.49</td>
</tr>
<tr>
<td>V/λ</td>
<td>3.92</td>
<td>2.44</td>
</tr>
<tr>
<td>Δ/λ</td>
<td>2.49</td>
<td>4.96</td>
</tr>
<tr>
<td>Class</td>
<td>C</td>
<td>O</td>
</tr>
</tbody>
</table>

ᵃ From Palmer, 1979, the usual convention used for heme system is that the heme normal establishes the z axis and the heme equator defines the xy plane; g max = gz; gy = g mid and gx = g min.

ᵇ Both enzymes were measured under the same EPR experimental conditions (refer to the legends in Figure 6.6).
Table 6.4. Comparison of EPR g-values and power saturation behavior for the reduced Fe/S cluster in sulfite reductase enzymes.

<table>
<thead>
<tr>
<th>reduced enzyme</th>
<th>g-tensor</th>
<th>P_{1/2} (mW)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiR</td>
<td>2.04</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>1.93</td>
<td>3.4</td>
</tr>
<tr>
<td>SiR + AsO_{2^-}</td>
<td>2.06</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>1.93</td>
<td>2.0</td>
</tr>
<tr>
<td>DV</td>
<td>2.07</td>
<td>5.35</td>
</tr>
<tr>
<td></td>
<td>1.93</td>
<td>3.14</td>
</tr>
<tr>
<td></td>
<td>1.89</td>
<td>4.78</td>
</tr>
<tr>
<td>DV + AsO_{2^-}^b</td>
<td>2.07</td>
<td>5.01</td>
</tr>
<tr>
<td></td>
<td>1.93</td>
<td>3.79</td>
</tr>
<tr>
<td></td>
<td>1.90</td>
<td>4.73</td>
</tr>
<tr>
<td>E. coli sulfite reductase^c</td>
<td>4.86</td>
<td>.d</td>
</tr>
<tr>
<td></td>
<td>2.29</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>1.93</td>
<td>20</td>
</tr>
</tbody>
</table>

^a Data taken at 13.5 K. See footnote to Table 6.6.

^b N_3^-, CO, CN^-, and S^{2-} gave similar g-values and power saturation parameters for DV.

^c Data taken from Janick & Siegel, 1982.

^d The rapid relaxation of this unusual "S = 3/2" signal (Janick and Siegel, 1982) prohibited measurement of P_{1/2} (Christner et al., 1981, 1983 & 1984; Cline et al., 1986). Refer to Christner et al., for a more detailed discussion of this signal.
Figure 6.7 Stacked EPR spectra of DV with 0, 1 and 2 electron equivalents added showing the simultaneous appearance of oxidized siroheme and reduced cluster of desulfoviridin after addition of one-electron equivalent. The number of electron equivalents is determined per coupled cofactor unit. Spectra were taken of an anaerobic solution of native oxidized desulfoviridin (150 µM) in 50 mM potassium phosphate, pH 7.5, containing 50 µM deazaflavin and 15 mM EDTA. Spectral conditions: microwave frequency = 9.53 GHz; modulation frequency = 100 kHz; modulation amplitude = 10 G; time constant = 20.5 ms; sweep width = 3904 G. Since each signal has different saturation characteristics they are optimally observed at distinct temperatures [13.3 K for Fe₄S₄(red) and 4.5 K for siroheme (ox)].
magnetic field strength (Gauss)
the EPR spectra of oxidized, one-electron reduced and two-electron reduced DV. The siroheme of DV (high spin) is different from the siroheme of SiR (low spin) which is likely enforced by hexacoordination (previous NMR studies from our laboratory suggest histidine as a possible axial ligand) (Cowan and Sola, 1990). The reduced cluster EPR signal of reduced DV, which has not been reported in literature, is characteristic of a reduced ferredoxin-like [Fe₄S₄] center \((g = 2.07, 1.93, 1.89)\), in contrast to the proposal of a [Fe₆S₆] cluster (Pierik and Hagen, 1991). The reduced cluster signal of DV is similar to that in SiR, though it is more rhombic in DV. Both SiR and DV exhibit typical reduced [Fe₄S₄] cluster EPR signals, different from E.coli enzyme which contains a mixture of \(S = 1/2, 3/2\) spins (Siegel et al., 1982; Janick and Siegel, 1982). Most interestingly, in contrast to SiR\(^{-1}\), DV\(^{-1}\) is not EPR-silent. Following limited photoirradiation, features from both the oxidized siroheme and the reduced cluster are observed (Figure 6.7, middle trace). With one electron equivalent addition to DV, half of the sirohemes are reduced and half of the clusters are reduced. To explain this phenomenon one might argue that there exists a non-equilibrium distribution of electrons immediately after photoreduction; that is, two-electron reduction of the redox centers in some enzyme molecules and no reduction in others. In this case one would predict that over a period of time the electrons would re-equilibrate according to the relative reduction potentials of each redox site, resulting in a decrease in the signals from both the reduced cluster and oxidized siroheme. However, a time-course plot of intensity vs time showed no significant variation in relative peak intensities over a time scale of 20 min to 5 hours. An alternative explanation is that this
represents an equilibrium distribution of added electrons over the two redox prosthetic centers according to their relative reduction potentials. The characteristic EPR spectrum of partial reduced enzymes \((0 < \# \text{ of } e^- < 2)\) suggests the reduction potentials of siroheme and cluster are similar in DV. There is no clear stepwise reductive step during the course of reduction of DV, consistent with the absence of the distinct optical spectra in one- and two-electron reductions. This EPR characteristic of the partially reduced DV allows us to estimate the \(\Delta E^0 (\Delta E^0 = E^0_s - E^0_c)\) which is mentioned later in this chapter. The \(g\) values for the oxidized siroheme and reduced cluster for DV are listed in Table 6.3 and 6.4, respectively.

The "one-electron reduced" spectrum in Figure 6.7 show additional minor features at 750, 1500, and 2700 Gauss. The 1500 G feature arises from the background "junk iron" signal that is more prominent here as a result of the greater amplification of the y-axis scale. The 2700 G feature is characteristic of a minor impurity that is found in various preparations according to the rigor of purification. The identity of the 750 G signal is currently uncertain, however, EPR studies of the \(E. coli\) sulfite reductase have shown unusual signals for reduced samples as a result of coupling between the cluster and siroheme. Such features would not be entirely unexpected. Currently there is no good theoretical understanding of the spin physics for this enzyme class, and so this issue is not discussed further.

**Rhombicity. Low Spin SiR:** Table 6.2 shows the rhombicity parameters for the low spin SiR and Mb-N₃⁻ adduct. The usual
Table 6.3. Comparison of EPR spectral data for DV and related high spin sulfite reductases; ASiR (assimilatory sulfite reductase in *E. coli*) and DSiR (dissimilatory sulfite reductase in *D. gigas*)

<table>
<thead>
<tr>
<th></th>
<th>DV (D. vulgaris)</th>
<th>ASiR (E. Coli)</th>
<th>DSiR (D. gigas)</th>
</tr>
</thead>
<tbody>
<tr>
<td>gx</td>
<td>6.30</td>
<td>6.63</td>
<td>7.20</td>
</tr>
<tr>
<td>g values</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gy</td>
<td>5.63</td>
<td>5.24</td>
<td>4.95</td>
</tr>
<tr>
<td>gz</td>
<td>2.00</td>
<td>1.98</td>
<td>1.93</td>
</tr>
<tr>
<td>D (cm⁻¹)</td>
<td>6.5±1.5*</td>
<td>8.0</td>
<td>-</td>
</tr>
<tr>
<td>(rhombicity) %</td>
<td>4.19</td>
<td>8.69</td>
<td>14.06</td>
</tr>
</tbody>
</table>

* A value of D = 9 cm⁻¹ has been reported in Pierik and Hagen, 1991.
convention for g values (noted in the footnote in Table 6.2) in heme systems conforms to Blumberg’s definition of a "proper" coordination system (Blumberg, 1968). In Blumberg's definition, the coordinate system has z equal to the axis of maximum distortion from cubic symmetry and x and y chosen so that $V/D < 2/3$. The rhombicities ($V/D$) calculated for low spin SiR and Mb-N3 adduct are 1.57 and 0.49, respectively. The rhombicity for SiR is much higher than Mb-N3 adduct, actually even higher than maximal value, 2/3. This is because the increased geometric stresses make $V > 2/3 D$ in the original set of coordinates; this implies that the axis of maximum distortion has changed. So the axis of maximum distortion in SiR is not on the z axis, but is probably on the x-y plane (i.e. the siroheme plane). This implies that the siroheme ring in SiR may be an asymmetric doubly reduced porphyrin ring (i.e. cis-isobacteriochlorin), or possibly the protein asserts such a great strain on the siroheme that it suffers a lot of distortion on the heme plane. Figure 1.3 shows different types of porphyrin rings.

**High Spin DV:** Table 6.3 shows the rhombicity values for high spin DV and assimilatory sulfite reductase in *E.coli* and dissimilatory sulfite reductase in *D.gigas*. Here the rhombicity calculated for the high spin system is based on the difference in $g_x$ and $g_y$ (see Theory), so the rhombicity value reflects the departure from tetragonality. DV exhibits the smallest departure from tetragonality compared to *E.coli* and *D.gigas* enzymes, implying that the siroheme in DV is under a more symmetric environment, or itself is a more symmetric siroheme, i.e. trans-isobacteriochlorin (Figure 1.3). At this point we cannot tell which contributes to the small rhombicity in DV siroheme.
Spin Quantitations. The spin quantitation using Cu(ETDA) and Mb-N$_3^-$ adduct as the standards for low spin siroheme in oxidized SiR is 0.94 and 0.96 spin per molecule, respectively. It is consistent with one siroheme and one cluster per SiR molecule. DV was recently reported to possess an extremely complex EPR spectrum with g-values ranging from 1.63 to 17 (Pierik and Hagen, 1991). A spin quantitation of ca. 0.2 hemes/half mole of enzyme was also reported. By use of a rigorous purification procedure we eliminated these spurious signals to yield the standard rhombic high-spin heme spectrum shown in Figure 6.7 (upper trace). The spin quantitations of siroheme and cluster in DV are listed in Table 6.5. Table 6.5 compares these with the previous report on DV (Pierik and Hagen, 1991). Integrations of the high spin siroheme in oxidized enzyme were made relative to high spin Mb (S = 5/2), while measurements on the reduced cluster in fully reduced enzyme were made relative to a copper-EDTA standard (S = 1/2). The results of our quantitations in Table 6.5 have been corrected for the fractional population according to the Boltzmann distribution. As previously observed (Pierik and Hagen, 1991), there is an apparent loss of spin. The significance of this observation has been discussed in Ch. II.

Power Saturation Measurements. Relaxation phenomena often provide a sensitive probe of the micro-environment of metal centers in biological molecules (Beinert and Orme-Johnson, 1967; Rupp et al., 1978; Makinen and Wells, 1987). Systematic power saturation studies have been carried out on both oxidized and fully reduced SiR and DV. In part this was guided by our interest in comparing the spectral characteristics of the two sirohemes (differing in coordination geometry) for SiR (low-spin
### Table 6.5  Spin integrations for oxidized and reduced DV\(^{a}\).

<table>
<thead>
<tr>
<th>redox state</th>
<th>our work</th>
<th>reference(^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxidized (heme equiv.)</td>
<td>0.6±0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>fully reduced (cluster equiv.)</td>
<td>0.5±0.2</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^{a}\) Data is quoted for the enzyme, rather than half-enzyme equivalents. Spin quantitations were carried out using the Aasa-Vanngard equation (see text), relative to myoglobin (\(S = 5/2\)) or CuEDTA (\(S = 1/2\)) (Fee, 1975; Aasa and Vanngard, 1975).

\(^{b}\) From Pierik and Hagen, 1991.
hexacoordinate) and DV (high-spin pentacoordinate). \( P_{1/2} \) values of each g values were measured according to the procedures described in Section 6.3 (EPR Theory). Unusual heterogeneities have been identified in the power saturation behaviors of the rhombic g-tensor components from siroheme in native oxidized SiR and especially DV, that contain the bridged siroheme-[Fe\(_4\)S\(_4\)] prosthetic centers common to this class of enzyme. Other than the inherent interest of the effect, our observation provides support for direct coupling of the siroheme-[Fe\(_4\)S\(_4\)] centers in DV, in contrast to a recent report (Pierik and Hagen, 1991), and also in SiR.

### A. Oxidized Enzymes (DV\(^0\) and SiR\(^0\)):

Half saturation values \( (P_{1/2}) \) for each of the rhombic g-component of the oxidized siroheme in SiR and DV are summarized in Table 6.6. Data for Mb and Mb-N\(_3^-\) adduct have also been measured under the same experimental conditions for comparison. The studies have identified heterogeneity in the power saturation behavior of the rhombic g-tensor components from oxidized siroheme in both native sulfite reductases (DV and SiR). Figure 6.8 and 6.9 show the result of half-power saturation values \( (P_{1/2}) \) for each of the rhombic g-tensors of oxidized SiR and DV. The \( g_z \) signal shows a \( (P_{1/2})_z \) that is significantly smaller than the \( (P_{1/2})_{x,y} \) values obtained for \( g_x \) or \( g_y \). This stands in contrast to either the high spin (native) or low spin (N\(_3^-\) adduct) heme center in myoglobin, which has no direct linkage to other prosthetic groups. All the g-tensors in myoglobin (both high spin native form and low spin N\(_3^-\) adduct) have similar \( P_{1/2} \) values for all g-components which are usual for an isolated spin system (Beinert and Orme-Johnson, 1967). The power saturation behavior of the oxidized
Figure 6.8 Representative power saturation data for each g-tensor component of the oxidized SiR. EPR spectra of an anaerobic solution of native oxidized enzyme (100 μM) were obtained in 50 mM potassium phosphate buffer (pH 7.5) at 11 K. Spectral conditions: microwave frequency = 9.53 GHz; modulation frequency = 100 kHz; modulation amplitude = 10 G; time constant = 10.2 ms; sweep width = 2200 G.
Figure 6.9  Representative power saturation data for each g-tensor component of DV. EPR spectra of an anaerobic solution of native oxidized enzyme (110 μM) were obtained in 50 mM potassium phosphate buffer (pH 7.5) at 8 K. Spectral conditions: microwave frequency = 9.53 GHz; modulation frequency = 100 kHz; modulation amplitude = 10 G; time constant = 20.5 ms; sweep width = 3904 G.
Table 6.6. Comparison of EPR g-values and power saturation behavior for the low-spin ferric heme in the assimilatory sulfite reductase (SiR) from *D. vulgaris* and sperm-whale myoglobin-N$_3^-$ adduct and high spin ferric heme in dissimilatory sulfite reductase (DV) from *D. vulgaris* and sperm-whale myoglobin.

<table>
<thead>
<tr>
<th>oxidized enzyme</th>
<th>g-tensor</th>
<th>P$_{1/2}$ (mW)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiR ($S = 1/2$)</td>
<td>2.45</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>2.38</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>1.78</td>
<td>1.4</td>
</tr>
<tr>
<td>myoglobin + N$_3^-$ ($S = 1/2$)</td>
<td>2.80</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td>2.22</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>1.74</td>
<td>12.7</td>
</tr>
<tr>
<td>DV ($S = 5/2$)</td>
<td>6.30</td>
<td>14.4</td>
</tr>
<tr>
<td></td>
<td>5.63</td>
<td>12.0</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>1.6</td>
</tr>
<tr>
<td>myoglobin ($S = 5/2$)</td>
<td>5.91</td>
<td>58.0</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>61.6</td>
</tr>
<tr>
<td>hemoglobin ($S = 5/2$)</td>
<td>5.87</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>2.0</td>
</tr>
</tbody>
</table>

$^a$ Determined by use of the equation $S=(P)^{1/2}/[1+ (P/P_{1/2})^{1/2}$, and a plot of log ($S/P_{1/2}^{1/2}$) vs log $P$; where $S$ is the signal amplitude of the derivative spectrum, $P$ is the power, and $P_{1/2}$ is the half-saturation power (Beinert and Orme-Johnson, 1967). Experimental conditions are given in the legend to Figure 6.6, and 6.7 for SiR and DV, respectively.
sulfite reductase from E. coli has not been reported and so no direct comparison can be made. However, the results are suprisingly similar in SiR and DV (Table 6.6), both possess a direct linkage between siroheme and cluster. This observation cannot be readily explained by either cross-relaxation with iron centers in the [Fe₄S₄] clusters also located in this enzyme, or overlapping signals from sirohemes in distinct environments or conformational substates (Beinert and Orme-Johnson, 1967; Antanaitas and Moss, 1975; Riedel et al., 1975; Bloembergen et al., 1959). Two possible explanations may be either the influence of siroheme asymmetry or a distinct mechanism that requires a significant cluster contribution to the siroheme spin manifold along one of the molecular axes. The lower P₁/₂ for the g₂ component can be rationalized by coupling of the siroheme to the [Fe₄S₄]²⁺ cluster along the z-axis (molecular axes), which results in a significant cluster contribution to the siroheme spin manifold. Normal cross-relaxation mechanisms cannot explain the observed decrease in power saturation for g₂ (slow relaxation). These results confirm a direct coupling between the siroheme and cluster in the prosthetic unit in SiR and DV. This aspect will be addressed in in Discussion section.

B. Reduced Enzymes (SiR⁻², DV⁻²): Half saturation values (P₁/₂) for each of the g-components of the reduced cluster in SiR and DV are summarized in Table 6.4. Janick and Siegel (Siegel et al., 1982; Janick and Siegel, 1982 & 1983) have previously reported anomalous power saturation data for the fully reduced E. coli sulfite reductase heme subunit (Table 6.4). However, in that work the signals that yielded distinct P₁/₂ values were taken from three distinct spin states of the reduced [Fe₄S₄] cluster. In contrast, our P₁/₂ values refer to a discrete spin state (S = 1/2).
In contrast to the sirohemes in oxidized SiR and DV, the clusters in reduced SiR and DV show no heterogeneity in the g-tensor components (Table 6.4). Also, the EPR spectrum of the reduced SiR and DV is a typical ferredoxin-like [Fe₄S₄] cluster signal ("g=1.94"), and this phenomenon is different from the fully reduced E.coli sulfite reductase heme subunit (Janick and Siegel, 1982). The latter shows a complicated mixture of spin states ($S = 3/2, 1/2$) which is assigned to be due to the exchange coupling between the reduced siroheme and the reduced cluster. The reduced SiR only exhibits a normal "$g = 1.94$" classical ferredoxin EPR signal, while the reduced cluster shows no heterogeneity in the $P_{1/2}$ values of the g-tensors. This may imply that there is no or weak exchange-coupling between the siroheme and cluster in the fully reduced SiR. There is no information about the coupling in the partially reduced SiR. The power saturation characteristics remained similar to the data obtained for the reduced cluster without ligand (that is, there is no heterogeneity), Table 6.4.

Variable Temperature Experiments. Variable temperature experiments have been performed on oxidized siroheme signal of both SiR and DV. These results are compared with two heme proteins (myoglobin and hemoglobin).

A. Oxidized SiR: The variable temperature profile obtained for the low spin siroheme of SiR compares favorably with that for the low spin heme in Mb.N₃⁻ (Figure 6.10). Deviations from Curie behavior at lower temperatures result from the onset of saturation with the power levels employed.
Figure 6.10  Comparison of the temperature dependence of the g-tensor components of the low spin hemes in (A) oxidized SiR, and (B) myoglobin + N3-. Other spectral parameters were noted in the legend to Figure 6.6.
B. Oxidized DV: The oxidized siroheme signal of DV shows an exponential curve upon increasing temperature. Figure 6.11 shows the variable temperature plot for g = 6.30 feature of DV. By fitting the data with the theoretical equation mentioned in Section 6.3, the zero-field parameter D ~ 6.5 cm⁻¹, which is lower than the value of 10 cm⁻¹ commonly found for high spin ferric hemes, while for the *E.coli* sulfite reductase a D ~ 8 cm⁻¹ has been reported. Table 6.3 shows the D values for DV and *E.coli* sulfite reductase.

Estimation of the Difference Between Siroheme and Cluster Reduction Potentials by EPR Techniques. The partially reduced DV exhibits EPR spectrum containing both oxidized DV and reduced cluster signals, these characteristic EPR spectra have been used to define the relative reduction potentials for the siroheme (E₅⁰) and [Fe₄S₄] centers (E₇⁰) (ΔE⁰ = E₅⁰ - E₇⁰) as a function of axial coordination to the siroheme in DV. Following limited photoirradiation, features from both oxidized siroheme and reduced cluster are observed (Figure 6.7, middle). As mentioned earlier, the explanation for this phenomenon during reduction of DV can be explained by the equilibrium distribution of added electrons over the two redox centers according to their relative reduction potentials. Similar observations had previously been made for *E. coli* sulfite reductase, however, the trends that we observe are very different from those obtained with the aforementioned enzyme. We also develop in the discussion that follows (and in appendix) a mathematical formalism to quantitatively account for this behavior that is distinct from that presented in earlier work, and which we believe to be of greater utility for data analysis.
Figure 6.11  Temperature dependence of the g = 6.30 feature of the high spin siroheme signal in oxidized desulfoviridin. The plot shown was obtained by taking the area of the peak (I), multiplied by the temperature T(K). A similar result was obtained by estimating the value of N_u (see text) by double integration, with an N_t obtained from the limiting value of data taken around 4 K. Other spectral parameters were noted in the legend to Figure 6.8. The data was fit to the equation given in the text, and gave D = 6.5 ± 1.5 cm⁻¹ for the S = 5/2 system.
The fractional population of oxidized siroheme or reduced cluster was estimated from \( I/I_0 \) ratios, where \( I_0 \) represents the maximal peak intensity of either fully oxidized siroheme or fully reduced cluster, and \( I \) is the EPR signal intensity of this particular feature after introducing \( x \) electron equivalents. The difference in reduction potentials was then easily evaluated in terms of the Nernst equation (1),

\[
\Delta E^\circ = E_c^\circ - E_s^\circ = (RT/nF) \ln \left( f_c^s \cdot f_r^s \right) / \left( f_c^c \cdot f_0^s \right)
\]  

(1)

where \( f_s \) and \( f_c \) represent the fractional concentrations of siroheme and cluster, respectively. Note that \( f_r^s \) is determined from \( f_r^s = 1 - \left( I_s^s / I_0^s \right) \) since only the oxidized form of siroheme is EPR detectable. Figure 6.12 shows how potential differences for ligated and ligand-free enzyme may be estimated from comparisons of experimental data and theoretical plots of \( f_0^s \) versus \( x \) (the number of electron equivalents taken up by the redox pair). The theoretical relationship (2) between \( f_0^s \) and \( x \), taking into account \( \Delta E^\circ \), is derived in appendix, where \( y = \exp \left( nF \Delta E^\circ / RT \right) \). The absolute number of electrons taken up by each coupled redox pair (which in principle could vary from 0

\[
x = (1 - f_0^s)(2y + f_0^s(1 - y))/(f_0^s + y - f_0^s y)
\]  

(2)
Figure 6.12  Plot of the fractional population of oxidized siroheme ($f_{O^5}$) versus the number of electron equivalents taken up by the redox pair ($x$) to estimate $\Delta E^0$ ($E_{O^5}^0 - E_{C^0}^0$). The theoretical lines shown correspond to $\Delta E^0$ (mV) values (from top to bottom) of: -100, -50, -40, -30, -20, -10, 0, 10, 20, 30, 40, 50, 100, respectively. Symbols represent no added ligand (▲); $\text{AsO}_2^-$ (●); $\text{CN}^-$ (■); CO (▲); $\text{N}_3^-$ (○); $\text{S}_2^-$ (□).
to 2 when averaged over the ensemble of protein molecules) was estimated from the fractional ratio of $f_r^a$ and $f_r^c$. Estimates for $\Delta E^\circ$ could therefore be made from the best fit of several individual data sets (Figure 6.12) representing various stages of reduction (the average number of electrons taken up is accounted for by $x$). For the $S^2$-ligated center the additional electron density (following one-electron reduction) was mostly located on the cluster (86%) relative to the siroheme (14%), reflecting destabilization of the reduced state of the siroheme. However, for ligand-free desulfoviridin, the ratio was 69% and 31%, respectively. The difference in potential ($\Delta E^\circ$) for either free or ligated enzyme typically lies in a range from -10 to -50 mV. The difference in potential was found to be less negative for strongly $\pi$-accepting ligands. For ligands with good $\sigma$-donor properties, such as $S^2^-$, the reduced siroheme is destabilized to a greater extent. Experimental errors preclude a more precise determination, however, the most significant aspect of this data is the notable contrast with results obtained for the E. coli sulfite reductase (and related enzymes from S. typhymurium and spinach), where $\Delta E^\circ$ is always large and positive (varying between +65 and +335 mV).

**EPR Characteristics of SiR and DV with Ligands.** There is no evidence from EPR that the ligand which binds to DV in the oxidized form as the siroheme is still in high spin after adding ligand. Reduced DV, with the addition of ligands gives a similar reduced cluster EPR signal with similar relaxation behavior as reduced DV without ligands. EPR characterizations of the ligand-enzyme adducts in reduced forms are also described. In oxidized form, there is no evidence for the binding of ligands to SiR and DV from EPR technique as there is no change in EPR
spectra of DV and SiR after the addition of ligands for several hours. The cluster signals obtained from fully reduced SiR with CN\(^-\) or N\(_3\)\(^-\) bound to siroheme were found to be similar to those previously described for free reduced enzyme. However, when AsO\(_2\)\(^-\) was bound to reduced SiR the low field g-value of the reduced cluster shifted from \(g = 2.04\) to \(g = 2.06\) (Table 6.4, Figure 6.13). The cluster signal is more rhombic in the reduced SiR with AsO\(_2\)\(^-\) then without AsO\(_2\)\(^-\). Following oxidation of the AsO\(_2\)\(^-\)-bound enzyme a novel set of EPR features characteristics of a spin 3/2 species with g values around 4. Previous results obtained with the CN\(^-\) adduct of the reduced E. coli enzyme suggest that hexacoordinate siroheme need not necessarily be low spin (in the case of the E. coli SiR, a \(S = 1\) species was noted for the CN\(^-\) adduct). The \(S = 3/2\) species might then arise from a \(S = 3/2\) ferric siroheme center, or an antiferromagnetically coupled ferrous \((S = 2)\) siroheme cation radical. The possibility of a ferromagnetically coupled ferrous \((S = 1)\) siroheme cation radical is less likely. Similar features are also found following oxidation of the reduced AsO\(_2\)\(^-\) adduct of the dissimilatory enzyme, and similar reasoning for the origin of these signals is valid.

The EPR signals of the reduced cluster in DV with different ligands such as AsO\(_2\)\(^-\), N\(_3\)\(^-\), CO are similar to that without ligands. There appears to be weaker coupling between the cluster and siroheme in reduced DV in comparison with \(E. coli\) sulfite reductase reflected by the similarity in g-values and in the \(P_{1/2}\) values for the reduced cluster g-components with and without added ligands, Table 6.4.
Figure 6.13  Stacked EPR spectra of fully reduced SiR with and without bound AsO$_2^-$: Spectra were taken of an anaerobic solution of 100 μM SiR in 50 mM potassium phosphate, pH 7.5, containing 100 μM deazaflavin and 10 mM EDTA at 11 K and 13.2K for siroheme and cluster signals respectively. Ligand concentration was 10 mM. Spectral conditions: microwave frequency = 9.53 GHz; modulation frequency = 100 kHz; modulation amplitude = 10 G; time constant = 10.2 ms; sweep width = 2200 G; microwave power = 1 mW.
Comparison of EPR Spectra Exhibited by Sulfite Reductase Enzymes. In contrast to desulfoviridin, *E. coli* sulfite reductase displays unusual EPR g-values that are distinct from typical ferric heme/reduced cluster signatures (Siegel et al., 1973; Janick and Siegel, 1982 & 1983; Janick et al., 1983). These have been ascribed to coupling between the [Fe₄S₄] cluster and siroheme centers. A measure of the cluster-heme-ligand coupling interaction was obtained from EPR potentiometric titrations. The potential of the cluster was found to be 70 mV more negative for the CN⁻ siroheme adduct than for the corresponding CO complex. With bound ligands, the EPR signals of the reduced cluster are converted quantitatively to the classical "g=1.94" type signal that is typical of S=1/2 [Fe₄S₄] centers. However, the exact line shapes and g-values differ markedly when different ligands are bound to the heme, indicative that the siroheme-[Fe₄S₄] interaction persists even when the heme Fe is bound to exogenous ligands. These data contrast with the situation for DV and SiR isolated from *D. vulgaris*. In the reduced form of SiR and DV, the cluster exhibits only the classical "g=1.94" cluster signal. No significant change in line shape or g-values were observed in the presence of exogenous ligands. Moreover, the relaxation properties of the reduced cluster were similar in the presence and absence of ligands (Table 6.4). However, we observe other distinct behaviors for SiR and DV, which support the coupling of the siroheme and cluster in the oxidized forms in the following section.
Heterogeneity in the Power Saturation Levels of Rhombic Siroheme g-Tensor Components. Half-power saturation values \( (P_{1/2}) \) for each of the rhombic g-components of oxidized siroheme in SiR and DV are summarized in Table 6.6. The \( g_z \) signal shows a \( (P_{1/2})_z \) that is significantly smaller than the \( (P_{1/2})_{x,y} \) values obtained for \( g_x \) or \( g_y \). This stands in contrast to either the high spin (native) or low spin (\( N_3^- \) adduct) heme center in myoglobin, which has no direct linkage to other prosthetic groups. As mentioned earlier, two possible explanations include the influence of siroheme asymmetry, or a distinct mechanism that requires a significant cluster contribution to the siroheme spin manifold along one of the molecular axes. Each is discussed in detail below.

Previous observations of such heterogeneity have been explained either by the overlap of signals from discrete paramagnetic centers (Beinert and Orme-Johnson, 1967; Antanaitas and Moss, 1975) or the alignment of paramagnetic centers in ordered layers (e.g., the Rieske center in either cytochrome b6f or cytochrome bcl). Riedel et al. (1991) have described three models that explain the observation of heterogeneous relaxation. Only two are relevant to our studies. First (of relevance to DV), a "dimer model" covers the possibility of distinct relaxation behavior for each of the two paramagnetic sirohemes in our enzyme. Second, the possibility of conformationally distinct substates also requires the overlap of signals from clusters in discrete environments. We have no biochemical or kinetic evidence for such a distinction, while the \( g_x \) or \( g_y \) components show similar power saturation behavior. Janick and Siegel have previously reported anomalous power saturation data for
the fully reduced *E. coli* sulfite reductase heme subunit (Table 6.4) (Janick and Siegel, 1982). However, in that work the signals that yielded distinct \( P_{1/2} \) values were taken from three distinct spin states of the reduced [Fe\(_4\)S\(_4\)] cluster. In contrast, our \( P_{1/2} \) values refer to a discrete spin state. The power saturation behavior of the oxidized reductase from *E. coli* has not been reported and so no direct comparison can be made. We have considered the possibility of cross relaxation with the bridging iron center in the cluster,\(^1\) but such a mechanism would tend to enhance the relaxation rate of the z-component rather than lower it (Bloembergen et al., 1959).

Brill and coworkers have previously reported heterogeneity in the ratios of spin-lattice relaxation times and intrinsic linewidths for the g-components of hemoglobin (Brill et al., 1975). In this case, the explanation rests simply with the intrinsic asymmetry of the ligand set. Table 6.6 notes \( P_{1/2} \) data for the two siroheme systems discussed in this chapter, and the two protoheme proteins Mb and Hb. The absence of heterogeneity in our experimental values of \( P_{1/2} \) (Table 6.6) for myoglobin in both high and low spin states demonstrates that asymmetry need not necessarily result in heterogeneous power saturation behavior. We also note here the out-of-plane distance for the ferric ion relative to the heme plane (Fe-Ph)\(^2\). For met-Mb (Fe-Ph) the distance is \(-0.4\), while for met-Hb (Fe-Ph) \(-0.07\) or \(-0.21\) for the -\(\alpha\) or \(\beta\)-subunits, respectively (Takano, 1977; Ladner et al.,

---

\(^{1}\) Overall, the cluster is diamagnetic due to coupling of the individual paramagnetic iron centers.

\(^{2}\) In effect this corresponds to the vertical distance between the iron ion and the center of the heme plane.
Taking these parameters as a criterion of heme asymmetry, it is apparent that there is a larger heterogeneity in $P_{1/2}$ for those cases where the iron center lies closer to the heme plane. Based on the examples for Hb and Mb described above one would expect SiR to show greater heterogeneity than DV. The former contains a hexa-coordinate siroheme that will lie closer to the heme plane than the pentacoordinate iron in DV (for typical values of Fe-Ph refer to the Hb review by Perutz (Perutz et al., 1987). In fact this is not the case (Table 6.6). It appears likely that there is an additional factor that must be considered in attempting to fully explain the saturation behavior observed in our studies. One possible mechanism for the lower value of $(P_{1/2})_z$ rests with a significant cluster contribution to the siroheme spin manifold, mediated through a bridging ligand along the $z$-axis. In the event of coupling to the cluster, the relaxation of the $g_z$-tensor component from siroheme will be governed by the relaxation properties of both cluster and siroheme. While we cannot ignore the basic influence of local symmetry in defining the power saturation behavior of the $x$, $y$, and $z$-components of the siroheme centers in our sulfite reducing enzymes, other arguments can be used that indicate, to some extent, the influence of coupling between the cluster/siroheme redox sites in defining the relaxation properties of each. To further test this possibility we examined the power saturation behavior of the $g$-tensor components from the reduced cluster in fully reduced enzyme. Ferredoxin-like [Fe$_4$S$_4$] centers exhibit low values of $P_{1/2}$ due to long relaxation times. Typically $P_{1/2}$ for [Fe$_4$S$_4$]$^+$ centers lie in the range 0.5-1.6 mW (Rupp et al., 1978 & 1979). The high $P_{1/2}$ values for the $g$-tensor components of the reduced clusters (Table 6.4) lend support to this idea. Significantly, the reduced
cluster of SiR, which shows the least perturbation in $P_{1/2}$ values for the siroheme $g$-tensor components, falls closest to this range. DV, which we had argued from data obtained from studies on the oxidized enzyme to show significant perturbations through coupling, also displays a significant deviation in the power saturation parameters of the reduced cluster relative to standard ferredoxins. The effect is seen more spectacularly in the published data from Siegel and coworkers on *E. coli* sulfite reductase (noted in Table 6.4). For the fully reduced enzyme, we must also take into account of the adjacent paramagnetic siroheme (most likely $S = 2$ for DV and *E. coli* sulfite reductase). This could result in enhancement of the cluster relaxation, thereby increasing $P_{1/2}$. Again, however, such an effect demands a close proximity (through-bond coupling) of the cluster and siroheme, especially for the *E. coli* enzyme.

Knowing that the intrinsic asymmetry of these coupled siroheme systems most likely contribute to the observed deviations in power saturation behavior for the oxidized siroheme, we have presented spectroscopic evidence from both the fully oxidized and fully reduced enzyme for a significant contribution from coupling of the cluster/siroheme redox centers. Additionally we have justified this in the context of observations from the standard heme systems in Hb and Mb. These observations may provide additional experimental input in the design and testing of spin coupling models for this unique class of prosthetic sites.

**Exchange-Coupling between Siroheme and Cluster in Oxidized and Reduced SiR and DV.** Huynh and coworkers have shown evidence for an exchange-coupled siroheme-[Fe$_4$S$_4$] unit in oxidized SiR by Mossbauer spectroscopy (Huynh et al., 1984). In general the [Fe$_4$S$_4$] cluster in the 2+
state is diamagnetic \((S = 0)\). They have observed a magnetic hyperfine interaction in the \([\text{Fe}_{4}\text{S}_{4}]^{2+}\) cluster, which can only be inferred through the siroheme. In other words, the siroheme and the \([\text{Fe}_{4}\text{S}_{4}]\) cluster are exchange coupled. Thus, Mossbauer spectroscopy suggests that the \([\text{Fe}_{4}\text{S}_{4}]\) cluster possesses a paramagnetic nature and the four iron sites in the cluster are pairwise equivalent. The two subsites have opposite sign in magnetic hyperfine constants, indicating that the spin coupling nature of the cluster is antiferromagnetic. One pair has spin-up and the other has spin-down, but the magnitude is different with the spin-down being larger.

In oxidized SiR the siroheme is \(S = 1/2\) and the cluster is effectively \(S = 0\). However, the heterogeneity in the power saturation behaviour of \(g\)-tensors from the oxidized siroheme EPR signal indicates that there is exchange-coupling between the oxidized siroheme and oxidized cluster, lowering the \(P_{1/2}\) value in the \(g_z\) tensor. This heterogeneity in \(P_{1/2}\) values of \(g\)-tensors requires a significant cluster contribution to the siroheme spin manifold along one of the molecular axes. The electronic configuration of the low spin heme \(\text{Fe(III)}\) is shown as follows:

\[
\begin{array}{c}
\downarrow \\
\end{array}
\begin{array}{c}
\uparrow \\
\end{array}
\begin{array}{c}
\downarrow \\
\end{array}
\begin{array}{c}
\uparrow \\
\end{array}
\begin{array}{c}
- \quad - \\
\end{array}
\begin{array}{c}
z^2 \\
 \end{array}
\begin{array}{c}
x^2-y^2 \\
 \end{array}
\begin{array}{c}
xz \\
yz \\
xy \\
\end{array}
\begin{array}{c}
S=1/2 \\
\end{array}
\end{array}
\]
In the oxidized Fe₄S₄ cluster a ladder of levels with S'>0 above the S'=0 ground state is obtained. Residual paramagnetism is observed in the oxidized cluster in a variable temperature ¹H NMR study (Cowan and Sola, 1990). This is supported by the Mössbauer measurement on oxidized SiR, which indicates paramagnetic nature of the [Fe₄S₄] cluster (Huynh et al., 1984). The paramagnetic nature of the [Fe₄S₄] cluster provides a mechanism for a cluster contribution to the siroheme manifold. Microscopically, we can think in terms of molecular orbitals. The metal orbitals dₓz and dᵧz can mix with siroheme e (π) orbitals. So the unpaired electron resides in a π-orbital. All four Fe's in the Fe₄S₄ cluster are high spin, no matter if it is in 2+ or 3+ state (Bertini, 1993). Thus the Fe atom at the corner connected to the siroheme through the bridge will possess a magnetic moment. The z-component of the magnetic moment associated with this Fe interacts through the unpaired electrons of the sulfur orbital in the S²⁻ bridge with the z-component of the magnetic moment of the π-orbital of siroheme. The magnetic moment of the cluster corner Fe is presumably in a direction opposite to that of siroheme; that is, antiferromagnetically coupled. This perturbation in the magnetization of siroheme in the z-direction by the cluster gives rise to a change in the relaxation property of its g₂ tensor. Magnetization M₂ is related to T₁ by the following equation:

\[
\frac{dM_z}{dt} = -\frac{(M_z-M_z^0)}{T_1}
\]
where $M_{20}$ is a new equilibrium value when a sudden change in the magnetic field is applied to the z direction, and $T_1$ is the time required for $M_z$ to rise to within a fraction $1/e$ of the value $M_{z0}$. Since $M_z$ is the sum of all $m_z$ components per unit volume, $M_z$ can change only if some of the dipoles change their spin state corresponding to a change in $M_S$ (Bloch 1964). That is, a change of magnetization in the z-direction ($M_z$) will change $T_1$ (characterization of the mean lifetime of a given spin state). Therefore, the sulfur bridge provides an efficient mechanism for the z-component of the magnetic susceptibility of the cluster "Fe" and the siroheme to interact. This is analogous to the case for oxidized Fe$_2$S$_2$ proteins where the singly occupied d-orbitals of the Fe ions interact through the paired electrons of the sulfur orbitals (Bertini, 1993).

In the fully reduced SiR, only a typical "g=1.94" ferredoxin-like Fe$_4$S$_4$ cluster signal was observed, and no heterogeneity was noted in the power saturation behavior of the g-tensors of the reduced cluster. These results suggest that the exchange-coupling in the oxidized SiR is destroyed or diminished upon 2-e$^{-}$ reduction. The reduced siroheme in SiR is EPR
silent, which implies \( S = 0 \) (low spin) or 1 (intermediate spin) or 2 (high spin). The reduced cluster is \( S = 1/2 \), behaving as an isolated \( S = 1/2 \) spin system, unlike the case in *E. coli* sulfite reductase which shows a mixture of spin states \( S = 3/2, 1/2 \) upon reduction (Janick and Siegel, 1982).

Regarding the absence of the \( S = 3/2 \) spin state and heterogeneity in \( P_{1/2} \) values of g-tensors for the reduced cluster, we postulate that the reduced siroheme is unlikely to be high spin \( S = 2 \), in which case strong exchange-coupling between reduced siroheme and cluster is expected. The reduced siroheme is likely to be low spin diamagnetic \( S = 0 \) or intermediate spin state \( S = 1 \). Proposed electronic configurations for the reduced siroheme in fully reduced SiR are shown as follows:

\[
\begin{array}{c}
\_ & _{x^2-y^2} & _{\_} & _{x^2-y^2} \\
\_ & _{z^2} & _{\_} & _{z^2} \\
\| & \| & _{xz, yz} & \_ & _{xy} \\
\| & _{xy} & \_ & \| & \| & _{xz, yz}
\end{array}
\]

\( S=0 \quad \quad S=1 \)

The binding of one nitrogen and one sulfur axial atom to the siroheme in model complexes can produce low spin diamagnetic species (Bertini, 1993). Further investigation of the spin state of the reduced siroheme by Mossbauer and magnetic susceptibility measurements are needed in order
to understand the exchange-coupling mechanism of siroheme and the Fe₄S₄ cluster in SiR.

In oxidized DV, the siroheme is $S = 5/2$. However, there are no Mossbauer measurements on oxidized DV to investigate the paramagnetism of the oxidized cluster. Most probably, the cluster also has residual paramagnetism like oxidized SiR, allowing coupling between the siroheme and cluster in DV. This causes the heterogeneity in the power saturation behavior of the oxidized siroheme in DV. The coupling mechanism can be similar to SiR, although the extent may be stronger as the spin of oxidized DV is high spin ($5/2$). In reduced DV, there may be weak or no coupling as the cluster exhibits a typical Fe₄S₄ signal. In this case no distinct spins are observed and only a small perturbation in $P_{1/2}$ values of the cluster is seen (Table 6.4). The effect is an increase in $P_{1/2}$ values compared to normal [Fe₄S₄]. The increase in DV is greater than that observed for SiR since the spin of oxidized DV is $5/2$ which presumably leads to a greater interaction.

**Coordination and Redox Properties.** SiR: Characterization of the siroheme in the assimilatory enzyme as a low-spin ferric heme complex ($S=1/2$) is obvious from both the characteristic EPR spectra and the absence of a ligand to metal charge-transfer band at ~ 700 nm in the optical spectrum of the oxidized SiR. The axial ligand at the sixth site (Figure 1.4) stabilizes the reduced siroheme sufficiently to promote full reduction of this site prior to cluster reduction. We have tentatively assigned this ligand as histidine (Cowan & Sola, 1990). Although it has been argued that this might be a $S^2$- adventitiously bound and isolated as
this adduct during purification (Kaufman et al., 1993), this explanation is unlikely inasmuch as the spectrum is retained even following turnover of substrate NO$_2^-$ to NH$_3$ during which the non-protein ligand would be displaced.

Stepwise reduction of siroheme and cluster has been observed from EPR and optical spectroscopies (Ch. VII). It is likely that the reduced siroheme in one-electron reduced SiR is stabilized by an axial $\pi$-accepting ligand (Sola & Cowan, 1990). There is no evidence for delocalization of electron between siroheme and cluster during partial reduction, which has been observed during reduction of DV. The redox potential will be discussed in Ch.V.

**DV**: There is no evidence for ligand binding to the oxidized siroheme, even over a period of one week. The optical spectrum of the reduced enzyme (in any coordination state) is relatively insensitive to ligand binding and only slight changes are observed in the absorption characteristics of the enzyme. The appearance of the optical spectra suggest minimal "$\pi$-d" orbital overlap for the siroheme. The ligand-metal charge-transfer band [~ 702(sh) nm], characteristic of high-spin heme, disappears after two-electron reduction. EPR spectra show no evidence of features at unusual g-values for either oxidized or reduced enzymes.

Both optical and EPR data support essentially non-specific reduction of [Fe$_4$S$_4$] and siroheme redox centers in the ligand-free and ligand bound states. The limits of $\Delta E^o$ values suggested by the plot in Figure 6.12 [typically $\Delta E^o \leq -50$ mV] stand in sharp contrast to similar
estimates for the *E. coli* enzyme (ΔE° ranging from +65 mV to +335 mV (Janick and Siegel, 1982). Clearly, for desulfoviridin, π-acceptor ligands do not appear to significantly stabilize the reduced siroheme relative to the cluster, while σ-donor ligands tend to destabilize the reduced siroheme.

The ΔE° values appear to increase with bound ligand [ΔE° = (free); < (CN-) < (S²-).] In contrast to DV and SiR, *E. coli* sulfite reductase displays unusual EPR g-values that are distinct from typical ferric heme/reduced cluster signatures. These have been ascribed to coupling between the [Fe₄S₄] cluster and siroheme centers.

**Effect of Cyanide Binding on the Relative E°’s of DV.** EPR data previously reported for the *E. coli* sulfite reductase fully support the changes in reduction potential (ΔE° = E°S - E°C) suggested by optical measurements (Janick & Siegel, 1982). The ΔE° values appear to increase with bound ligand [ΔE° (free) = +65 mV, ΔE° (CN-) = +335 mV]. In contrast, desulfoviridin shows no such stabilization of the reduced siroheme after binding ligands (π-acceptors or σ-donors). In this respect, desulfoviridin exhibits normal behavior inasmuch as ligands such as CN⁻ typically stabilize the ferric complex in heme chemistry. For example, after binding CN⁻ the heme b potential in myoglobin changes from 46 mV to -315 mV (King et al., 1993), while the heme c potential in a cytochrome from *D. vulgaris* changes from -59 mV to -263 mV (Tan & Cowan, 1990). The negative E°’s observed for *E. coli* (and other) sulfite reducing enzymes most likely arise from the electron rich sirohydrochlorin ring. Stabilization of reduced *E. coli* SiR by π-accepting ligands results from π-backbonding from the ligand, which clearly has a
greater influence than the electrostatic charge interactions that normally dominate (illustrated by the data listed above for CN⁻ binding). For desulfoviridin, differences in bonding between the electron rich siroheme and central iron ion apparently result in a decreased stabilization of the reduced form, relative to the E. coli enzyme, with the result that the potential (relative to the cluster) is more negative. For comparative purposes these trends are outlined more clearly in schematic fashion in Figure 6.14.

**Concluding Remarks.** There are obvious differences in both the electronic and coordination states of the siroheme-[Fe₄S₄] prosthetic site in this class of enzyme, and so no one enzyme can be used as a satisfactory paradigm for understanding the electronic character of the rather unique redox center in this class. The following trends and conclusions have been outlined:

1. Assimilatory and dissimilatory enzymes each give rise to distinct sets of optical characteristics that are independent of the coordination state of the siroheme.

2. Desulfoviridin gives rise to optical characteristics that are relatively insensitive to both the oxidation and coordination state of the siroheme.

3. The relative reduction potentials for siroheme (Eₛ⁰) and [Fe₄S₄] centers (Eₐ⁰) \( \Delta E^\circ = E_S^\circ - E_C^\circ \) in native enzyme vary from a value of \( \Delta E^\circ \geq +100 \text{ mV} \) for SiR, to \( \sim +65 \text{ mV} \) for the E. coli enzyme, and \( \sim -20 \text{ mV} \) for DV. These differences do not originate from the distinct coordination
Figure 6.14  Schematic illustration of the influence of CN⁻ binding on heme potentials, where $\Delta E^0 = E_{S^0}(\text{free}) - E_{S^0}(\text{CN}^-)$. Mb = myoglobin (King et al., 1993); Cyt = cytochrome from *D. vulgaris* (Tian & Cowan, 1990); DV = desulfoviridin (*D. vulgaris*); ESiR = assimilatory sulfite reductase (*E. coli*) (Janick & Siegel, 1982).
and spin states of the two enzymes since by those criteria, desulfoviridin should more resemble the *E. coli* sulfite reductase. The range of variation is more dramatic (from +65 mV to +335 mV) depending on the axial ligand. Clearly the physicochemical properties of this coupled siroheme-[Fe₄S₄] redox unit are tuned by the local protein environment (in a manner that has yet to be established).

(4) Without exception, all purified sulfite reductases of different species contain siroheme and [Fe₄S₄] cluster. Mossbauer and EPR studies of the hemoprotein subunit of *E. coli* sulfite reductase reveal that the siroheme and the [Fe₄S₄] cluster are exchange-coupled and operate as a functional unit (Christner, 1981, 1983, 1983). The present study indicates that sulfite reductases from *D. vulgaris* also contain an exchanged-coupled siroheme-[Fe₄S₄] center. These observations strongly suggest that the siroheme-[Fe₄S₄] unit is a common prosthetic group for sulfite reductases. Recently, evidence for interaction between the siroheme and the [Fe₄S₄] cluster in spinach nitrite reductase has also been reported (Wilkerson, 1983).

Clearly the physicochemical properties (optical spectrum, coordination chemistry, redox/electronic/magnetic properties) of this coupled siroheme-[Fe₄S₄] redox unit are varied by the local protein environment (in a manner that has yet to be established). Future investigations will be directed toward the molecular details of how the rather substantial differences in optical redox properties arise and how the implications for understanding their catalytic function will be determined.
6.6 APPENDIX.

A general equation that relates fractional populations of oxidized or reduced forms of two redox centers to the difference in their redox potentials can be determined as follows. We take $A =$ siroheme and $B =$ [Fe$_4$S$_4$] cluster. At equilibrium $\Delta E = 0 \text{ mV}$, and so

$$\Delta E^o = (RT/nF) \log \left( \frac{[\text{ox}]^A [\text{red}]^B}{[\text{red}]^A [\text{ox}]^B} \right) \tag{3}$$

If $f_{0A}$ is the fraction of $A$ oxidized (etc. for $f_{0B}$, ...) and we denote the concentration of fully oxidized $A$ by $A^o$ then,

$$[\text{ox}]^A = f_{0A} A^o, \quad [\text{red}]^A = f_{rA} A^o, \quad [\text{ox}]^B = f_{0B} B^o, \quad [\text{red}]^B = f_{rB} B^o \tag{4}$$

$$[\text{ox}]^A + [\text{red}]^A = A^o, \quad [\text{ox}]^B + [\text{red}]^B = B^o = A^o \tag{5}$$

For simplicity we can take the total concentration of each redox center to be unity. That is,

$$f_{rA} + f_{rB} = x/A^o, \quad f_{0A} + f_{0B} = (2-x)/A^o \tag{6}$$
where \( x \) is the number of electron equivalents added. We can rewrite (3) in the form of (8),

\[
\exp(nF\Delta E^0/RT) = ([\text{ox}]^A [\text{red}]^B/[\text{red}]^A [\text{ox}]^B)
\]

which can be further developed by sequential use of (4) - (7) to yield (9).

\[
\exp(nF\Delta E^0/RT) = (f_0^A f_r^B)/(f_r^A f_0^B)
\]

\[
= (f_0^A [x/A^o - f_r^A]/f_r^A [(2-x)/A^o - f_0^A])
\]

\[
= (f_0^A [(x/A^o)(1 - f_0^A)]/(1 - f_0^A)[(2-x/A^o)-f_0^A])
\]

Assuming \( A^o = 1 \) and taking \( f_0^A = f \), we can rewrite (9) in the form of (10), where \( y = \exp(nF\Delta E^0/RT) \).

\[
y = f\{x-(1-f)\}/(1-f)[2-x]-f\}
\]

(10)
Solving for $x$ yields (11).

\[
x = \frac{(1-f)(2y+f(1-y))/(f+y-f-1)}{(f+y-f-1)}
\] (11)

Related equations may be deduced in terms of $f_r^A$, $f_0^B$, $f_r^B$, depending on which center is most appropriate to follow. For example, equation (13) can be written for the case of reduced cluster (where $f = f_r^B$).

\[
x = f \left( \frac{2+(f-1)(1-y)}{(f+y-f-1)} \right)
\] (12)

Since the redox pair is able to accept up to two electrons, it is implicitly assumed that $\Delta E^\circ$ is the same for the fully oxidized and one-electron reduced systems.
CHAPTER VII

Optical Properties of SiR and DV and Implications for the Electronic Properties of [Fe4S4]Siroheme Prosthetic Centers

ABSTRACT

The assimilatory (SiR) and dissimilatory sulfite reductase (desulfoviridin, DV) from the sulfate-reducing bacterium Desulfovibrio vulgaris (Hildenborough) displays distinct optical and redox characteristics relative to the heme subunit of Escherichia coli assimilatory sulfite reductase.

For low-spin hexacoordinate SiR, two well-resolved changes in optical spectra of SiR are observed following the addition of the first and second electron equivalents. These two distinct changes in optical spectra upon reduction of SiR correlate to stepwise reduction of siroheme and cluster (determined from the corresponding EPR spectra, in Ch. VI). The one-electron reduced SiR showed a general decrease in the optical absorbance, with retention of all features. After addition of the second electron equivalent, the broad absorbance at ~ 400 nm in the oxidized spectrum was observed to split and more resemble the spectral features of
desulfoviridin. The AsO$_2^-$ adduct of SiR in both oxidized and reduced forms displays distinctive optical characteristics.

For high-spin pentacoordinate DV, there is minimal change in the absorbance of the oxidized chromophores both after reduction or following addition of exogenous ligands. A LMCT band ~ 702 nm, characteristic of high spin Fe$^{3+}$, is observed in both the oxidized and one-electron reduced enzymes. The optical characteristics of SiR is more reminiscent of that of the assimilatory sulfite reductase from *E. coli*, though the latter has a high-spin siroheme. The electronic properties of the coupled [Fe$_4$S$_4$]-siroheme redox center common to both nitrite and sulfite reducing enzymes are apparently strongly dependent on the environment generated by protein sidechains.
7.1 INTRODUCTION

Oxido-reductase enzymes usually possess at least one, and frequently, several redox centers that serve as sites for the influx or efflux of electrons and/or the coordination of substrate molecules prior to oxidation or reduction (Cowan, 1993). These basic redox units may occasionally be coupled together to form centers with distinct electronic and chemical properties. The assimilatory (SiR, Mr ~ 23500) and dissimilatory sulfite reductases (desulfoviridin, DV, Mr ~ 224,000) from Desulfovibrio vulgaris (Hildenborough), currently under study in our laboratory, are found to employ an exchanged-coupled siroheme-[Fe₄S₄] cofactors. A coupled [Fe₄S₄]-siroheme redox centers (Figure 1.4) has been shown to form the active prosthetic center in both sulfite and certain nitrite reducing enzymes from bacteria (aerobic and anaerobic) and higher organisms (spinach) (Siegel et al., 1982; Christner et al., 1983; Cammack, et al., 1978). This model has recently been challenged by Hagen and coworkers (Pierik and Hagen, 1991), though, we have demonstrated their results to be in error [Ch. II, VI].

The electronic spectral characteristics of the assimilatory sulfite reductase from Escherichia coli have been extensively characterized, and similar optical spectra have been reported for the sulfite and nitrite reducing enzymes from S. typhymurium and spinach, respectively (Janick and Siegel, 1983; Siegel et al., 1973, Murphy et al., 1974). There is substantial homology in amino acid sequence data for each of the enzymes from E. coli, S. typhymurium, and spinach (Ostrowski et al., 1989 a, b, c). Several examples of low molecular mass (~ 25 kDa) assimilatory-
type sulfite reductases have been isolated from the anaerobic bacteria *Methanosarcina barkeri*, *Desulfuromonas acetoxidans*, and *Desulfovibrio vulgaris* (Hildenborough) (Lee and Peck, 1973; Lee et al., 1973; Moura et al., 1986). These enzymes differ from the larger sulfite reductase subunits isolated from *E. coli*, *S. typhimurium*, and spinach by possession of a hexacoordinate low-spin siroheme rather than the high-spin pentacoordinate heme common to the latter. Our laboratory is studying the biochemistry of enzymatic sulfite reduction in *D. vulgaris* and has shown this low molecular mass sulfite reducing enzyme to be an excellent model system for detailed studies of enzymatic multielectron redox chemistry (Tan and Cowan, 1991; Tan et al., 1991 & 1994; Tan and Cowan, 1990; Cowan and Sola, 1990).

The assimilatory sulfite reductase (ASiR) from *Escherichia coli* is the most thoroughly characterized example of this class and has formed an important basis for studies on related enzymes (Christner et al., 1984; Janick and Siegel, 1983 a, b; Janick et al., 1983; Siegel et al., 1973 & 1982; Siegel and Davis, 1974; Murphy et al., 1973; Wilkerson et al., 1983). However, there are some important differences in the structural and spectroscopic characteristics of these enzymes that have not yet been adequately addressed. The structural and spectroscopic differences alluded to above may be summarized under the following headings: (1) the status of bridging links between the siroheme and Fe-S cluster centers in the active site, and the identity of putative bridging ligands; (2) the coordination state of the siroheme (penta or hexa) and the influence on spin state; (3) the electronic absorption spectra; (4) redox properties of the siroheme and cluster. How these differences might be manifest in the
reactivity of these enzymes is an interesting point for consideration. A few of these topics have been noted or discussed in previous reports from this and other laboratories (Huynh et al., 1984; Tan and Cowan, 1990; Tan and Cowan, 1991; Wolfe et al., 1994; Moura et al., 1986) and some of the relevant parameters are compared in Table 7.1 (Wolfe et al., 1994). Both the $E. coli$ heme subunit and desulfoviridin contain pentacoordinate high-spin siroheme, in contrast to the low molecular mass assimilatory enzyme from $D. vulgaris$ that possesses a low-spin hexacoordinate center. However, the electronic absorption spectra show greater similarity between the two assimilatory enzymes from $E. coli$ and $D. vulgaris$, while that for desulfoviridin is quite distinct (Huynh et al., 1984; Lee et al., 1973; Janick et al., 1983).

In this chapter, optical properties of SiR and DV in the oxidized, partially reduced and fully reduced states with or without ligand bound at the axial position are characterized, and implications for the electronic properties of the coupled $[Fe_4S_4]$-siroheme prosthetic centers are discussed. Moreover, comparison and contrast of the electronic properties of the two sulfite reductases (SiR and DV) from the sulfate-reducing bacterium $Desulfovibrio vulgaris$ (Hildenborough) with published work on related enzymes, and $E. coli$ sulfite reductase in particular, have been made. The coupled redox centers in such enzymes displays a remarkable diversity of optical features and redox chemistry, and so no single enzyme can be used as a satisfactory paradigm for understanding the electronic character of the rather unique redox center in this class. The influence of protein side chains on heme spectra is well documented in the literature of heme proteins (Cowan and Gray, 1989;
Table 7.1 Comparison of physicochemical data for a variety of sulfite and nitrite reductases.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Spin State</th>
<th>(λ&lt;sub&gt;max&lt;/sub&gt; nm, ε M&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>desulfoviridin (D. v.)</td>
<td>SO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;, NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, NH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>S&lt;sup&gt;2-&lt;/sup&gt;, (S&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;3&lt;/sub&gt;)&lt;sup&gt;-&lt;/sup&gt;, Lia&lt;sub&gt;222&lt;/sub&gt;</td>
<td>200 penta/H&lt;sub&gt;5&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>408 (1.54 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>680 (2.2 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>630 (5.3 x 10&lt;sup&gt;4&lt;/sup&gt;)</td>
</tr>
<tr>
<td>sulfite reductase (D. v.)</td>
<td>SO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;, NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, NH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>S&lt;sup&gt;2-&lt;/sup&gt;, NH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>23 6 hexa/L&lt;sub&gt;S&lt;/sub&gt;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>405 (6.3 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td></td>
<td></td>
<td></td>
<td>545 (1.9 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td></td>
<td></td>
<td></td>
<td>590 (2.2 x 10&lt;sup&gt;4&lt;/sup&gt;)</td>
</tr>
<tr>
<td>sulfite reductase (E. c.)</td>
<td>SO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;, NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, NH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>S&lt;sup&gt;2-&lt;/sup&gt;, NH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>o814</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>386 (6.56 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td></td>
<td></td>
<td></td>
<td>591 (1.8 x 10&lt;sup&gt;4&lt;/sup&gt;)</td>
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<td></td>
<td></td>
<td></td>
<td>714 (5.6 x 10&lt;sup&gt;4&lt;/sup&gt;)</td>
</tr>
<tr>
<td>spinach sulfite reductase</td>
<td>SO&lt;sub&gt;3&lt;/sub&gt;&lt;sup&gt;2-&lt;/sup&gt;, NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, NH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>S&lt;sup&gt;2-&lt;/sup&gt;, NH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>a2 61 penta/H&lt;sub&gt;5&lt;/sub&gt;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>384 (5.87 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
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<td></td>
<td></td>
<td></td>
<td>540 (1.23 x 10&lt;sup&gt;6&lt;/sup&gt;)</td>
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<td></td>
<td></td>
<td></td>
<td>587 (1.76 x 10&lt;sup&gt;6&lt;/sup&gt;)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>712 (5.87 x 10&lt;sup&gt;5&lt;/sup&gt;)</td>
</tr>
<tr>
<td>spinach nitrite reductase</td>
<td>NO&lt;sub&gt;2&lt;/sub&gt;&lt;sup&gt;-&lt;/sup&gt;, NH&lt;sub&gt;2&lt;/sub&gt;OH</td>
<td>NH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>98 69 penta/H&lt;sub&gt;5&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>386 (3.97 x 10&lt;sup&gt;6&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>573.690</td>
</tr>
</tbody>
</table>
DiFeo and Addison, 1991; Smith and Williams, 1970) and it is likely that the observed differences in spectral behavior and redox potentials are induced by variations in the protein environment surrounding each coupled center. The implications of this observation for the comparison of functional activity have yet to be resolved. This smaller enzyme (Mr ~ 23,500) displayed no strong amino acid homology with the *E. coli* class of enzyme, and also possessed distinct optical characteristics (Huynh et al., 1984) Also, the optical spectrum could be distinguished from the larger dissimilatory enzyme also isolated from *D. vulgaris* (Lee and Peck, 1973; Lee et al., 1973). In each of these enzymes there is strong evidence for one or more of an identical set of coupled [Fe₄S₄]-siroheme units (Cowan and Sola, 1990; Tan and Cowan, 1991; Tan et al., 1991 & 1994; Wolfe et al., 1994; Tan and Cowan, 1990; Siegel et al., 1982; Christner et al., 1983), however, there are substantial variations in electronic properties over the series.

### 7.2 MATERIALS AND METHODS

**General Materials.** Buffer salts were of molecular biology grade (Fisher or Aldrich Chemical Co). Measurements of solution pH were made with an Accumet pH Meter 910 equipped with a Corning semimicro combination pH electrode. Sephadex G-200 gel filtration material were obtained from Sigma, and DEAE-52 ion exchange resin from Whatman. Deazaflavin was synthesized by literature methods (Janda and Hemmerich, 1976). Figure 7.1 shows the structure of 5'-deazaflavin used in photoreduction experiments (ε₃₉₆nm = 12,000 M⁻¹cm⁻¹, Mr = 229). All water used was purified with a Barnstead nanopure system and exhibited a resistivity of 18 MΩ cm⁻¹.
Figure 7.1  Structure of 5'-deazaflavin synthesized for photoreduction.
Bacterial Growth, Isolation and Purification of SiR and DV.

*D. vulgaris* (Hildenborough, NC1B 8303), possessing a broad-host range expression vector (pDSK519) with the SiR gene and promoter cloned into the multiple cloning site (Tan and Cowan, 1990; Tan et al., 1991 & 1994) was grown in a lactate-sulfate medium. The purification details were described in Ch. II. For DV, band I from FPLC chromatography was typically used in experiments as no optical differences, chemical analysis, or activities were observed between DV-I and DV-II (Wolfe et al., 1994).

Electronic Absorption Spectra. Optical spectra were measured on a Hewlett-Packard 8452A spectrophotometer (run by software from On-Line Instrument Systems) using a 1-cm path length glass cuvette. The concentration of SiR and DV was \( \sim 10 \, \mu M \) in 50 mM KP buffer, pH 7.5, at 298 K. The ligand concentration was 10 mM in 50 mM KP buffer, pH 7.5, at 298 K. Exact conditions are listed under appropriate figure legends. In samples that were to be photoreduced, the spectrum of oxidized and reduced deazaflavin control spectra were subtracted from the spectrum of oxidized and reduced enzyme solutions, respectively. It is assumed that the absorbance of the reduced deazaflavin in the enzyme sample will not differ significantly from the control sample. In each case the concentration of EDTA was in great excess [EDTA/enzyme ratio \( \sim 1000-3000 \)].

Preparation of photoreduced enzyme. Due to limited solubility in water, deazaflavin was first dissolved in dimethylformamide (DMF) to make a stock solution of 8 mM. Small aliquots (several \( \mu L \)) were added to the aqueous reaction mixture to make a final concentration of 10 \( \mu M \). The
final solution contained 10 mM EDTA and a deazaflavin : enzyme ratio of 1:1. A 1 ml volume of a solution containing ca. 10 μM enzyme, 10 μM deazaflavin and 10 mM EDTA in 50 mM KP buffer (pH 7.5, 298K) was degassed with O2-free Ar(g) in a 1 ml quartz cuvette fitted with a serum stopper sealed with grease to inhibit O2 diffusion. The solution was degassed with stirring for 20 min. Samples were reduced using the deazaflavin/EDTA photoreduction method of Massey and Hemerich (1978). The cuvette was transferred to a glass beaker filled with ice-water and irradiated (1000W lamp, 90% power) for 5-30 min, depending on the sample requirements. Reduction was monitored by electronic absorption and irradiation was continued until no further change was observed in the optical spectrum. Control solutions of deazaflavin/EDTA and enzyme (without ligand) were also prepared and reduced in the same way.

Background to Optical Characteristics of Ferric and Ferrous Isobacteriochlorins. In contrast to porphyrin and heme spectra, the optical characteristics of reduced porphyrin rings have been less thoroughly documented. The oxidized, reduced, and doubly reduced rings are termed porphyrin, chlorin, and bacteriochlorin or isobacteriochlorin, respectively, Figure 1.3. The latter two are distinguished by the relative positions of the reduced pyrrole rings [adjacent (cis) or opposite (trans)]. Siroheme falls in the category of an iron isobacterchlorin. Several workers have published extensively on model complexes of these reduced porphyrin macrocycles, and examined both optical and redox properties (Stolzenberg et al., 1980 & 1981; Strauss and Holm, 1982; Procyk and Bocian, 1991; Chang and Fajer, 1980; Barkiggai et al., 1982; Chang et al., 1981).
7.3 RESULTS

**SiR in Different Oxidation States (SiR\(^{0/-1/-2}\)).** Two well-resolved changes were observed following the addition of the first and second electron equivalents to SiR (Figure 7.2) (determined by examining the EPR spectrum of reduced intermediates generated by photoreduction, which was described in Ch. VI\(^1\)). After a one-electron reduction (SiR\(^{-1}\)), the absorbances of all bands were observed to decrease and the 590 nm band was broadened and red shifted to 594 nm, the 550 nm band was also red-shifted to 554 nm, while the Soret band at \(\sim 402\) nm was blue-shifted to 388 nm. After addition of a second electron equivalent (SiR\(^{-2}\)), the broad Soret absorbance at \(\sim 400\) nm, common to both the oxidized and one-electron reduced enzyme, was observed to increase in intensity and split into two peaks (at 388 and 404 nm), to more resemble the spectral appearance of oxidized or reduced DV. The Q-band region, upon the second electron reduction, also demonstrated spectral changes. Both the 554 and 594 nm bands in the one-electron reduced form experienced a slight red-shift to 556 and 596 nm, respectively. As more electron equivalents were added to SiR, the Q-band exhibited a larger red-shift. It may be due to the filling of the antibonding orbital of the siroheme, destabilizing the electronic ground state. Thus the electronic transition is smaller in energy. There was no LMCT band ca. 700 nm, which is usually observed for high spin Fe\(^{3+}\). The optical characteristics of oxidized, one-electron and fully reduced SiR are summarized in Table 7.2. The

\(^1\) Reference spectra were obtained from EPR samples and judged to correspond to one-electron and two-electron reduced SiR (see Figure 6.7 in Ch. VI).
Figure 7.2  Absorption spectra of oxidized, 1 e⁻ reduced, and 2 e⁻ reduced SiR. Spectra were taken in a 1 cm pathlength optical cuvette with 13.6 µM enzyme in 50 mM potassium phosphate buffer, pH 7.5, at 298 K.
absorption spectra for both the assimilatory sulfite reductases from *E. coli* and *D. vulgaris* bear a strong resemblance to spectra obtained in a number of model metalloisobacteriochlorins, including the appearance of the first significant absorption band in the region around 590-600 nm (Stolzenberg et al., 1980 & 1981; Strauss & Holm 1982; Procyk and Bocian, 1991; Chang & Fajer, 1980; Barkigia et al., 1982; Chang et al., 1981; Richardson et al., 1979; Sullivan et al., 1991; Melamed et al., 1991; Strauss and Pawlick, 1986). The sharp spectral features of the latter are very characteristic of the \( \pi \)-\( \pi \) ligand absorption bands that are common to porphyrinic materials, indicating an absence of significant charge-transfer interactions with the ferrous heme center.

**Ligand-Bound SiR in Different Oxidation States.** There was no evidence for AsO\(_2^-\) binding directly to SiR in the oxidized form since the optical spectrum remained unchanged even after several hours. This result is consistent with previous observations by Siegel and coworkers (Janick and Siegel, 1983) for the oxidized state of the *E. coli* enzyme. The reduced SiR-AsO\(_2^-\) adduct can be formed by reducing SiR in the presence of AsO\(_2^-\). The oxidized SiR-AsO\(_2^-\) adduct can only be obtained by oxidizing the reduced SiR-AsO\(_2^-\) adduct. Air oxidation of the reduced SiR-AsO\(_2^-\) complex yielded a spectrum that was different from oxidized SiR in the native form, indicating that AsO\(_2^-\) was still tightly bound to the oxidized siroheme (Figure 7.3). Relative to native oxidized enzyme, the Soret band in the oxidized SiR-AsO\(_2^-\) complex is blue-shifted from ~402 nm to 388 nm, while the 550 nm band was blue-shifted to 546 nm. The spectrum of AsO\(_2^-\) bound to fully reduced SiR was similar to that obtained for the reduced enzyme alone (Figure 7.4). The 402 nm Soret
Figure 7.3 Comparasion of the absorption spectra of oxidized SiR in the native form and SiR-AsO₂⁻ adduct. Spectra were taken in a 1 cm pathlength optical cuvette with 13.6 μM enzyme, 10 mM AsO₂⁻ in 50 mM potassium phosphate buffer, pH 7.5, at 298 K.
Figure 7.4 Absorption spectra of SiR in fully reduced forms with and without AsO$_2^-$ Spectra were taken in a 1 cm pathlength optical cuvette with 13.6 μM enzyme, 10 mM AsO$_2^-$ in 50 mM potassium phosphate buffer, pH 7.5, at 298 K.
Table 7.2  Comparison of optical characteristics for oxidized and reduced sulfite reducing enzymes from prokaryotic and eukaryotic Sources.

<table>
<thead>
<tr>
<th>Enzyme (Source)a</th>
<th>λ(nm) [ε (mM^{-1}cm^{-1})]</th>
<th>ref/f'note</th>
</tr>
</thead>
<tbody>
<tr>
<td>SiR (D. vulgaris) ox</td>
<td>402 [61], 550 [18], 590 [22]</td>
<td>this work</td>
</tr>
<tr>
<td>1 e⁻ red</td>
<td>388 [58], 404 [54], 554 [17], 594 [19]</td>
<td>this work</td>
</tr>
<tr>
<td>2 e⁻ red</td>
<td>388 [60], 404 [63], 556 [18], 596 [20]</td>
<td>this work</td>
</tr>
<tr>
<td>SiR-AsO₂⁻ ox</td>
<td>388 [65], 546 [21], 590 [22]</td>
<td>this work</td>
</tr>
<tr>
<td>2 e⁻ red</td>
<td>386 [60], 404 [63], 554 [20], 596 [21]</td>
<td>this work</td>
</tr>
<tr>
<td>DV (D. vulgaris) ox</td>
<td>392 [130], 410 [140], 584 [33], 632 [56], 702 [11]</td>
<td>this work</td>
</tr>
<tr>
<td>red</td>
<td>382(sh)b [120], 390 [120], 410 [130], 498 [31], 540 [25], 584 [29], 632 [56]</td>
<td>this work</td>
</tr>
<tr>
<td>DV-AsO₂⁻ red</td>
<td>382 [120], 392 [120], 410 [150], 498 [31], 540 [28], 584 [22], 632 [53]</td>
<td>this work</td>
</tr>
<tr>
<td>DV-CN⁻ red</td>
<td>392 [115], 410 [128], 498 [33], 588 [30], 632 [55]</td>
<td>this work</td>
</tr>
<tr>
<td>DV-Cl⁻ red</td>
<td>382 [120], 392 [130], 410 [140], 498 [31], 588 [29], 632 [57]</td>
<td>this workc</td>
</tr>
<tr>
<td>DV-I⁻ red</td>
<td>382 [120], 392 [120], 410 [120], 498 [34], 588 [32], 632 [55]</td>
<td>this work</td>
</tr>
<tr>
<td>Oxidase (Organism)</td>
<td>Oxidative Form</td>
<td>Reductive Form</td>
</tr>
<tr>
<td>-------------------</td>
<td>----------------</td>
<td>---------------</td>
</tr>
<tr>
<td>DV (D. gigas)</td>
<td>ox 380(sh) [172], 390 [182], 408 [200], 583 [42], 628 [84]</td>
<td>data unavailable</td>
</tr>
<tr>
<td>SiR-CN⁻ (E. coli)</td>
<td>ox 386 [66], 549 [12], 596 [18], 714 [6]</td>
<td>401 [48], 416 [49], 544 [39], 578(sh) [38]</td>
</tr>
<tr>
<td>NiR (spinach)</td>
<td>ox 276 [72], 386 [40], 573 [10], 640(sh) [5], 690 [4]</td>
<td>394 [8], 557 [2], 588 [2]</td>
</tr>
</tbody>
</table>
Table 7.2  (Continued)

a  DV = dissimilatory sulfite reductase; SiR = assimilatory sulfite reductase.

b  (sh) = shoulder.

c  With the exception of I\(^-\) the other halides (Br\(^-\), F\(^-\)) gave similar absorbance results. These ligands are unlikely to bind to siroheme.

d  Data interpolated from reported absorbance ratio's in Murphy & Siegel (1973).

e  Data interpolated from spectra and reported absorbance ratio's in Krueger & Siegel (1977).

f  Data interpolated from spectra in Wilkerson et al. (1982) and Vega & Kamen (1977).
band in the native oxidized SiR split to give components at 386 and 404 nm after reduction. Bands at longer wavelength also experienced a red-shift similar to that noted for the native enzyme alone. We were unable to trap the one-electron reduced form of the AsO$_2^-$ adduct. After the formation of the reduced SiR-AsO$_2^-$ adduct, the sample was re-oxidized by air to obtain the adduct in oxidized form. Optical characteristics of AsO$_2^-$ adduct to the oxidized and fully reduced SiR are listed in Table 7.2.

**DV in Different Oxidation States (DV$^{0/-1/-2}$).** For DV$^0$, in addition to the primary absorbance bands around 380-420 nm and 580-630 nm, Figure 7.5 (upper) shows that DV exhibits a weak absorbance at ~702 nm (ε = 11 mM$^{-1}$ cm$^{-1}$). By analogy with *E. coli* sulfite reductase, we ascribe this to a ligand-metal charge transfer (Janick and Siegel, 1982). This LMCT band is characteristic of high spin Fe$^{3+}$. This shoulder disappears after two-electron reduction but is still prominent (ε = 7.8 mM$^{-1}$ cm$^{-1}$) in the one-electron reduced enzyme$^2$. A low intensity band is seen around 498 nm as a shoulder on the absorbance tail from the Soret region. As described in more detail later, this band became more prominent after reduction shown in Table 7.2 and Figure 7.5 (lower). A very weak absorption at 540 nm also appears after reduction. The 498 and 540 nm bands most likely correspond to weaker components from the Q-band absorption envelope (Figure 7.5, upper) (Gouterman and Pawlick, 1986; Adar, 1978).

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2 Reference spectra were obtained from EPR samples and judged to be one-electron reduced (see Figure 6.8 in Ch. VI).
Figure 7.5  Absorption spectra of oxidized and 2 e\textsuperscript{-} reduced DV. Spectra were taken in a 1 cm pathlength optical cuvette with 6 μM enzyme in 50 mM potassium phosphate buffer, pH 7.5, at 298 K.
Reduction results in several notable changes in the optical spectra that include the disappearance of the shoulder at 702 nm (charge-transfer band), shown in Figure 7.5 (lower). The absorbance bands in the 380-420 nm region also decrease in intensity relative to the reduced native enzyme. In particular, relative to the oxidized enzyme, the reduced form shows a very pronounced decrease in the absorbance tail from the Soret region that extends to the Q-bands (either with or without exogenous ligand). This is a particularly useful region to probe the kinetics of oxidation/reduction and ligand binding by optical methods [Lui et al., 1993 & 1994; Ch. III]. The absorbance of the 580 nm Q-band is reduced by 19% and is broadened in the reduced form. The Q-bands noted previously as shoulders on the Soret absorbance also decrease slightly in prominence.

**Ligand-Bound DV in Different Oxidation States.** There is minimal change in the absorbance of the oxidized chromophores after the addition of exogenous ligands (NO$_2^-$, SO$_3^{2-}$, SeO$_3^{2-}$, N$_3^-$, Cl$^-$, CN$^-$, AsO$_2^{2-}$, Br$^-$, I$^-$, NCS$^-$). Relatively small reductions (< 2%) in the absorbance of all bands (including the weaker charge transfer and Q-bands) were noted. EPR measurements demonstrate that these perturbations in optical spectra do not arise from axial ligation to the free coordination site (Figure 1.4) since the siroheme remains high spin, which is discussed in Ch. VI. Accordingly, we attribute these facts to random weak binding to the surface that result in minor conformational perturbations in the vicinity of the siroheme. Desulfoviridin was fully reduced within 10 min of irradiation (1000 W lamp, 90% power) in the presence of 10 µM deazaflavin/10 mM EDTA. Although the optical and EPR (discussed in
Ch. VI) spectra of oxidized enzyme were essentially unchanged over a period of several hours in the presence of the ligands noted above, rapid ligand binding has been observed in the reduced form (Lui et al., 1993). Binding of AsO$_2^-$ to DV resulted in several dramatic deviations from standard behavior (Table 7.2 and Figure 7.6, lower). In Figure 7.6, binding to the reduced form is accompanied by a 16 % increase in the A$_{410}$/A$_{392}$ ratio relative to reduced native enzyme. Also, a 14 % increase in intensity is observed for the 540 nm band. Overall, the changes reflect increased $\pi$-$\pi$ character in the transitions. Such changes are commonly observed in heme spectra: for example, in contrasting the optical spectra from ferric myoglobin with the ferrous derivative and ferrous-CO adduct, there is a prominent loss of charge-transfer character with $\pi$-$\pi$ transitions from the heme ligand dominating (Gouterman and Pawlick, 1986; Adar, 1978).

7.4 DISCUSSION

Trends in Optical Spectra for Sulfite/Nitrite Reducing Enzymes. Comparison of the data in Table 7.2 illustrates the sharp contrasts in optical characteristics for a variety of enzymes that carry the coupled [Fe$_4$S$_4$]-siroheme prosthetic center. The family of enzymes represented by the *E. coli* enzyme show significant spectral changes when comparing oxidized vs reduced, and ligated vs unligated enzyme (Janick and Siegel, 1982 & 1983; Siegel et al., 1973; Janick et al., 1983). The broad lines and uncharacteristic absorption bands (relative to the metal free isobacteriochlorin) reflect substantial $\pi$-$d$ (ligand-metal) orbital interactions and charge-transfer character. There have been some reports that certain enzymes in this class may contain demetallated siroheme
Figure 7.6  Absorption spectra of DV in fully reduced forms with and without AsO$_2^-$ . Spectra were taken in a 1 cm pathlength optical cuvette with 6 μM enzyme, 10 mM AsO$_2^-$ in 50 mM potassium phosphate buffer, pH 7.5, at 298 K.
(sirohydrochlorin) (Pierik and Hagen, 1991; Moura et al., 1988), though, work from our laboratory would suggest that these conclusions are flawed [Ch. II and VI]. We restrict our attention in this discussion to those cases where it is known through the work of others and ourselves that the enzyme contains fully-metallated siroheme. This includes desulfoviridin and sulfite reductase isolated from D. vulgaris (Hildenborough), D. gigas, E. coli and S. typhymurium sulfite reductase, spinach sulfite and nitrite reductase. The spectrum of enzyme-free siroheme displays prominent changes with pH and ligands (Kang et al., 1987). These variations are not obvious for the enzyme-bound complex since the bridging axial ligand on one face is fixed (sulfide or cysteinate), while the peripheral carboxylates are undoubtedly involved in the formation of salt bridges (Figure 1.4). In E.coli sulfite reductase, reduction results in significant changes in the absorption bands (Janick and Siegel, 1982), although without added ligand there is no sharp distinction between the one- and two-electron reduced samples. Discrete changes are observed for ligated samples, reflecting the change in reduction potentials for each. In contrast to E. coli, DV is relatively insensitive to both oxidation and coordination states.

The optical spectrum of the assimilatory reductase (SiR) from D. vulgaris shows the greatest similarity with the corresponding assimilatory enzymes isolated from E. coli, S. typhymurium, and spinach, despite the fact that SiR possesses a low-spin six-coordinate siroheme and the others are high-spin five-coordinate (Huynh et al., 1984; Janick and Siegel, 1982). All show broadened optical transitions with ill-resolved shoulders. The spectra of these assimilatory enzymes stand in contrast to those observed for dissimilatory enzymes, of which desulfoviridin is a
typical example. Again, these variations do not result from distinct coordination state inasmuch as almost all assimilatory and dissimilatory enzymes possess a five-coordinate siroheme.

**Coordination, Redox and Electronic Properties of SiR.**
Characterization of the siroheme in the assimilatory enzyme as a low-spin ferric heme complex \((S = 1/2)\) is obvious from both the characteristic EPR spectra (Ch. VI) and the absence of a ligand metal charge transfer band at \(\sim 702\) nm in the optical spectrum of the oxidized SiR. The axial ligand at the sixth site (Figure 1.4) stabilizes the reduced siroheme sufficiently to promote full reduction of this site prior to cluster reduction. We have tentatively assigned this ligand as histidine (Cowan and Sola, 1990). Although it has been argued that this might be a \(S^2^-\) adventitiously bound and isolated as this adduct during purification (Kaufman et al., 1993), this explanation is unlikely inasmuch as the spectrum is retained even following turnover of substrate \(\text{NO}_2^-\) to \(\text{NH}_3\), during which the non-protein ligand would be displaced.

The assimilatory sulfite reductase (SiR) from *D. vulgaris* shows the greatest similarity in the optical spectrum with the corresponding assimilatory enzymes isolated from *E. coli*, *S. typhymurium*, and spinach, despite the fact that SiR possesses a low-spin six-coordinate siroheme and the others are high-spin five-coordinate. All show broadened optical transitions with ill-resolved shoulders. The optical spectra of these assimilatory enzymes stand in contrast to those observed for dissimilatory enzymes. In each case the absorption bands are broadened relative to what might be expected for transitions that were significantly \(\pi-\pi^*\) in
origin. There is a suggestion of significant charge-transfer character in these bands as a result of \( \pi-d \) overlap between siroheme and sixth ligand.

One of the most interesting features of reduced SiR lies with the appearance of the "desulfoviridin-like" splitting of the Soret region after addition of the second electron equivalent. Interestingly, this has also been observed for the CN\(^{-}\) adduct of the \textit{E. coli} enzyme (Janick and Siegel, 1982). In that case the splitting arises even after addition of only one electron equivalent since CN\(^{-}\) stabilizes the reduced siroheme sufficiently to localize the electron on that chromophore. Since we have already demonstrated initial reduction of the siroheme, the change in the optical spectrum of the siroheme after addition of the second electron to the cluster provides evidence for a coupling mechanism (which may be either electronic or structural in origin) between these two redox centers. The possibility that the change arises from loss of the axial ligand to siroheme is currently being tested by high field NMR experiments.

**Coordination, Redox and Electronic Properties of DV.** There is no evidence for ligand binding to the oxidized siroheme, even over a period of one week. The optical spectrum of the reduced enzyme (in any coordination state) is relatively insensitive to ligand binding and only slight changes are observed in the absorption characteristics of the enzyme. The appearance of the optical spectra suggest minimal "\( \pi-d \)" orbital overlap for the siroheme. The ligand-metal charge-transfer band [\( \sim 702(\text{sh}) \text{ nm} \)], characteristic of high-spin heme, disappears after two-electron reduction. EPR spectra show no evidence of features at unusual \( g \)-values for either oxidized or reduced enzymes (Ch. VI).
Both optical and EPR data (shown in Ch. VI) support essentially non-specific reduction of $[\text{Fe}_4\text{S}_4]$ and siroheme redox centers in the ligand-free and ligand bound states. Clearly, for desulfoviridin $\pi$-acceptor ligands do not appear to significantly stabilize the reduced siroheme relative to the cluster, while $\sigma$-donor ligands tend to destabilize the reduced siroheme.

**Concluding Remarks.** There are obvious differences in both the electronic and coordination states of the siroheme-$[\text{Fe}_4\text{S}_4]$ prosthetic site in this class of enzyme, and so no one enzyme can be used as a satisfactory paradigm for understanding the electronic character of the rather unique redox center in this class. In this chapter the following trends and conclusions have been outlined:

1. Assimilatory and dissimilatory enzymes each give rise to distinct sets of optical characteristics that are independent of the coordination state of the siroheme.

2. Desulfoviridin gives rise to optical characteristics that are relatively insensitive to both the oxidation and coordination state of the siroheme.

3. Assimilatory enzymes which possess the axial ligand stabilizing the reduced form of siroheme, give rise to distinct optical characteristics in different oxidation states.

Clearly the physicochemical properties of this coupled siroheme-$[\text{Fe}_4\text{S}_4]$ redox unit are varied by the local protein environment (in a manner that has yet to be established). Future investigations will be
directed toward the molecular details of how the rather substantial differences in optical redox properties arise, and the implications for understanding their catalytic function.
LIST OF REFERENCES


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