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FE-S PROTEINS: CLUSTER ASSEMBLY AND DEGRADATION

DISSERTATION

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

By
Shumin Bian, M. S.

The Ohio State University 1998

Dissertation Committee:
Professor James A. Cowan, Adviser
Professor Russ Hille
Professor Claudia Turro

Approved by
Professor James A. Cowan, Adviser
Biophysics Program
ABSTRACT

High potential iron protein (HiPIP) from Chromatium vinosum has been used as a model in studies of [4Fe-4S] cluster assembly and disassembly, as it correlates with protein folding and degradation. Cluster stability was found to depend on the hydrophobic environment provided by the aromatic residues. An oxidative degradation pathway through a [3Fe-4S] cluster intermediate has been characterized in Phe66 mutants. Multiple kinetic intermediates were detected in the processes of cluster assembly and degradation by time-resolved fluorescence and UV-vis spectroscopy. The identities of these intermediates were further investigated by freeze-quench EPR techniques.

In the cluster stability studies, a number of point mutations of the conserved aromatic residue phenylalanine 66 (Phe66Tyr, Asn, Cys, Ser) in C. vinosum HiPIP have been examined with the aim of understanding the functional role of this residue. Non-conservative replacements with polar residues have a minimal effect on the midpoint potential of the [Fe_4S_4]^{3+/2+} cluster, typically < +25 mV, with a maximum change of +40 mV for Phe66Asn. With the exception of the Phe66Tyr mutant, the oxidized state was found to be unstable relative to the recombinant native, with regeneration of the reduced state. The pathway for this transformation involves degradation of the cluster in a fraction of the sample, which provides the reducing equivalents required to bring about...
reduction of the remainder of the sample. This degradative reaction proceeds through a transient \([\text{Fe}_3\text{S}_4]^+\) intermediate that is characterized by typical \(g\) values and power saturation behavior in EPR experiments, and is prompted by the increased solvent accessibility of the cluster core as evidenced by \(^1\text{H}-^{15}\text{N}\) HMQC NMR experiments. These results are consistent with a model where the critical role of the aromatic residues in the high potential iron proteins is to protect the cluster from hydrolytic degradation in the oxidized state.

In vitro Fe-S cluster assembly and protein folding were studied both in the absence and in the presence of inorganic sulfide. In the absence of \(S^{2-}\), adding Fe to apo-protein with excess amount of reducing agent DTT gave rise to an intermediate with fair stability. Several other kinetic phases were detected on the way to this intermediate by time-resolved fluorescence. The rates of formation of each kinetic intermediate were found to decrease by over one order of magnitude. Fast kinetic phases show a clear dependence on DTT concentration, suggesting a requirement for exogenous thiol to promote formation of the initial intermediates by providing additional coordination to the iron center(s). Subsequent steps are not dependent on DTT concentration. In the slower phases, changes in fluorescence likely reflect further protein conformational changes in accommodating the newly formed cluster. The rate constants of early steps increase with increasing Fe\(^{2+}\) concentration. In the slow reaction step, the rate does not depend on Fe\(^{2+}\) concentration. Changes in fluorescence may reflect internal ligand rearrangements. Samples from freeze-quench preparations show a very broad EPR signal after oxidation, indicative of spin-spin interaction between closely located Fe centers.
Full 4Fe-4S cluster assembly was studied by freeze-quench EPR techniques and time-resolved fluorescence. Fluorescence experiments detected at least three kinetic intermediates during cluster assembly. Freeze-quench EPR experiments identified a series of iron cluster intermediates, in which two important ones were well characterized as X and Y species. Based on a set of control and reference experiments, including seleno-substituted reconstitution, a working model for the mechanism of 4Fe-4S cluster assembly in HiPIP has been proposed. Iron binding to protein on cysteine residues is most likely the first event in cluster assembly. This step is probably diffusion-limited. DTT and possibly solvent provide necessary coordination for these single iron centers. In the presence of sulfide, the two adjacent single iron centers are rapidly (estimated rate constant $> 100 \text{ s}^{-1}$) converted to a 2Fe center bridged by inorganic sulfide(s), giving rise to an intermediate species X. Oxidized X has a distinct EPR signal with these g values: $g_1 = 1.96, g_2 = 2.00, g_3 = 2.08$. Formation of short bridges by sulfide ions in X promotes protein folding and the formation of another diiron center with a rate constant of $\sim 25 \text{ s}^{-1}$. Y species then forms when these two di-iron centers are brought close enough to have anti-ferromagnetic coupling interaction. The formation of Y markes the beginning of a series of slow structural rearrangements, a kinetically biphasic process, with rate constants around $0.3 \text{ s}^{-1}$ and $0.002 \text{ s}^{-1}$. These rearrangements must include the final replacement of any thiol coordination from DTT, and the sharing of sulfide bridge with three irons (two in the case of X and Y), as well as the oxidation of each of the diiron centers. These are all required for $[4\text{Fe-4S}]^{2+}$ cluster formation. Some of these processes are presumably the rate-limiting steps.
The disassembly of the 4Fe-4S cluster in HiPIP represents the other side of the cluster biochemistry, which is relevant to Fe regulation, and sensory functions, as well as many other biological processes. Cluster degradation monitored by UV-vis spectroscopy gave consistent kinetic results with that monitored by fluorescence. Based on kinetic analysis and spectroscopic characterization, a cluster disassembly model has been proposed. Full protonation of the four inorganic sulfide ions is required for the formation of the transition state complex. Multiple guanidine hydrochloride molecules are needed in the disassembly reaction for unfolding of the protein. Protonation occurs very likely in a step-wise manner, although it happens in a very rapid phase with an apparent rate constant of 168 s\(^{-1}\). Further protonation of the sulfide ions causes the release of HS\(^-\) /H\(_2\)S and the release of Fe ions in much slower kinetic phases. Change in the oxidation state of the cluster changes the observed rate constants, but does not change the general mechanism of cluster disassembly. Under the conditions used in this work, the disassembly reaction is largely irreversible.
Dedicated to my wife
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VITA

October 20, 1964...............................Born, Henan, China, P. R.

1984 .........................................B.S. Physics, Wuhan University

1987 .........................................M.S. Physics, Wuhan University

1987-1992 ...................................Instrumental Analyst, Wuhan University

1992-1998 ...................................Graduate Teaching and Research Associate,
                        The Ohio State University

PUBLICATIONS

Research Publication


FIELDS OF STUDY

Major Field: Biophysics
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LISTS OF SYMBOLS/ABBREVIATIONS

1D NMR.....One Dimensional NMR
2D NMR.....Two Dimensional NMR

C.v........Chromatium vinosum
COSY.......Correlation Spectroscopy
DTT.........Dithiotreitol
EDTA........EthyleneDiamineTetraAcetic acid
EPR........Electron Paramagnetic Resonance
GuHCl.......Guanidine Hydrochloride
HiPIP......High Potential Iron-sulfur Protein
HSQC.......Hetemuclear Single-Quantum Coherence (NMR Spectroscopy)
MLCT.......Metal-Ligand Charge Transfer
NMR.......Nuclear Magnetic Resonance
TCA........Trichloroacetic Acid
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CHAPTER 1

OVERVIEW

1.1 IRON-SULFUR PROTEINS

1.1.1 Introduction to Iron-Sulfur Proteins

Iron-sulfur proteins are proteins containing iron, which is at least partially coordinated by sulfur. They were not recognized as distinct from other non-heme iron-containing proteins until the early 1960s, when analytical techniques, particularly electron paramagnetic resonance (EPR), were applied to these proteins (Hagen, 1992). In an application of then newly developed EPR spectroscopy to metalloproteins, biochemist H. Beinert at the University of Wisconsin and physicist Sands at the University of Michigan studied mitochondrial membranes, in which they made the historical detection of what has long become a hallmark of iron-sulfur clusters, "the g = 1.94 signal" (Beinert and Sands, 1960; Sands and Beinert, 1960; Beinert, 1973). While the nature of this signal and the identity of the [2Fe-2S] cluster were being discussed in the following decade, other iron-sulfur clusters were found in a variety of species with different constitutions (Jensen, 1986). In 1962, Mortenson et al. (Mortenson, et al., 1962) reported a small molecular weight iron protein from the bacterium Clostridium pasteurianum that was shown to be essential in nitrogen fixation. They named the protein ferredoxin (Fd)
because of its iron content and role in redox reactions. In 1965 Lovenberg and Sobel (Lovenberg and Sobel, 1965) observed a red protein while isolating Fd from *C. pasteurianum*. They found it to be a low molecular weight iron protein (~6 k Daltons) that could substitute for Fd as an electron carrier in several reactions mediated by extracts from *C. pasteurianum*. They named it rubredoxin (Rd). By 1967, about a dozen ferredoxins and other iron-sulfur enzymes had been identified (Malkin and Rabinowitz, 1967).

After extensive studies by various chemical methods and physical techniques, such as EPR, Mössbauer spectroscopy, extended X-ray absorption fine Structure (EXAFS), X-ray crystallography, Raman spectroscopy, and nuclear magnetic resonance (NMR), it became evident that these and many other proteins containing iron and sulfur represented a new class, the iron-sulfur proteins. All members of the class have one or more Fe atoms per molecule, and all except the Rds and desulforedoxin (Dx) subclasses have "acid-labile" or inorganic sulfur as well. Analytical determination for iron (Stookey, 1970; Beinert, 1978; Fish, 1988) and sulfide (Siegel, 1965; Chen and Mortenson, 1977; Sakurai, et al., 1982; Moulis and Meyer, 1982; Beinert, 1983) is generally not very reliable in individual measurement. However, cumulative measurements together with spectroscopic and/or structural determinations have established the chemical composition of different iron-sulfur clusters as distinguished from other nonheme iron proteins. Now there have been found well over 100 iron-sulfur proteins (Cammack, 1992), and the number is rising steadily as more types of iron-sulfur cluster have been found, and as more spectroscopic signals have been observed in biological systems. More and more gene sequence information has also provided clues to new Fe-S proteins.
1.1.2 Diversified Molecular Structure of Fe-S Clusters

As more complicated types of iron-sulfur clusters are being identified, classification of Fe-S proteins becomes increasingly difficult. Nonetheless, we can still classify iron-sulfur proteins into several main types according to the type of their Fe-S cluster (Cammack, 1992). Table 1.1 lists some of the common Fe-S cluster types, the name of related proteins, and typical range of redox potential of the cluster. Most of the listed references are related to structural determination of Fe-S clusters. The reduction potential \( E_m \) of the same type of cluster changes from species to species.

The most common types of iron-sulfur clusters are presented in Figure 1.1 (Beinert, 1990). The rubredoxin type cluster has only one central iron coordinated to four cysteinyl thiolates. There is no acid-labile or organic sulfide. It is still considered iron-sulfur cluster because of the coordination. A variant of the [2Fe-2S] cluster ferredoxins (Knaff and Hirasawa, 1991) is the Rieske protein, named after Professor J. S. Rieske, of The Ohio State University (Rieske et al., 1964). Rieske proteins contain unique [2Fe-2S] clusters with two histidine nitrogen ligands instead of cysteines (Gurbriel, et al., 1989). They were first isolated from mitochondrial complex III, but similar proteins are found in other types of membrane electron transfer chains. [3Fe-4S] ferredoxins have been found in several bacteria strains, for example, \( D. \ gigas \) (Kissinger et al., 1991) and \( S. \ griseolus \) (O'Keefe et al., 1991). [4Fe-4S] clusters were found not only in low potential ferredoxins, but also in high potential iron proteins (HiPIP) (Dunham, et al., 1991).
Figure 1.1: Schematic representation of structures for the most basic building block of Fe-S clusters
<table>
<thead>
<tr>
<th>Type</th>
<th>Protein</th>
<th>$E_m$ range (mV)</th>
<th>Selected Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[\text{Fe}]^{3+/2+}$</td>
<td>Rubredoxins</td>
<td>+20 to -60</td>
<td>Stenkamp, 1990</td>
</tr>
<tr>
<td>$[\text{2Fe-2S}]^{2+/1+}$</td>
<td>Ferredoxins</td>
<td>-240 to -460</td>
<td>Rypniewski, 1991</td>
</tr>
<tr>
<td>$[\text{Fe}_2\text{S}_2(\text{RS})_2\text{N}_2]^{0/-1}$</td>
<td>Rieske proteins</td>
<td>+300 to -155</td>
<td>Gurbriel, 1989</td>
</tr>
<tr>
<td>$[\text{3Fe-4S}]^{1+/0}$</td>
<td>Ferredoxins, etc.</td>
<td>-50 to -420</td>
<td>Kissinger, 1991</td>
</tr>
<tr>
<td>$[\text{4Fe-4S}]^{2+/1+}$</td>
<td>Bacterial ferredoxins</td>
<td>0 to -645</td>
<td>Fukuyama, 1989</td>
</tr>
<tr>
<td>$[\text{4Fe-4S}]^{3+/2+}$</td>
<td>HiPIP</td>
<td>50 to 450</td>
<td>Freer, 1975</td>
</tr>
<tr>
<td>$[\text{6Fe-6S}]$</td>
<td>$D. \text{vulgaris}$ protein</td>
<td></td>
<td>Hagen, 1989</td>
</tr>
<tr>
<td>H-cluster</td>
<td>Hydrogenase</td>
<td></td>
<td>Adams, 1990</td>
</tr>
<tr>
<td>P-cluster</td>
<td>Nitrogenase protein I</td>
<td></td>
<td>Burgess, 1990</td>
</tr>
<tr>
<td>Fe-Mo cofactor</td>
<td>Mo Nitrogenase</td>
<td></td>
<td>Lindahl, 1985</td>
</tr>
<tr>
<td>Fe-V cofactor</td>
<td>V nitrogenase</td>
<td></td>
<td>Eady, 1987</td>
</tr>
<tr>
<td>Fe-Ni cluster</td>
<td>CO dehydrogenase</td>
<td></td>
<td>Eggen, 1991</td>
</tr>
</tbody>
</table>

**Table 1.1:** Types of Fe-S Clusters
More complex iron-sulfur clusters are known besides these widespread common types. Complexity is introduced by increasing the size of the cluster. [6Fe-6S] clusters are proposed in the [6Fe-6S] protein of D. vulgaris (Hagen, et al., 1989) and carbon monoxide dehydrogenase (Jetten, et al., 1991). The P-cluster in nitrogenase αβ components of several bacterial strains comprises eight iron and seven labile sulfide atoms (Burgess, 1990; Bolin, et al., 1990; Crane, et al., 1995). The H-cluster in Fe hydrogenase of C. pasteurianum and D. gigas is proposed to have six iron atoms (Adams, et al., 1989; van Dijk, et al., 1979; Adams, 1990). Further complexity exists for the so-called mixed-type clusters in which Fe-S clusters are bonded to other cofactors in an enzyme. Both the molybdenum-iron protein in nitrogenase and the "alternative" vanadium-iron nitrogenase have a mixed-metal cluster. In the Mo nitrogenase, the Fe-Mo cofactor was shown to have a composition of Fe₇MoS₉ (Chan, et al., 1993; Peters, et al., 1997). In the V nitrogenase the composition is Fe₅₋₇VS₄₊₆ (Eady, et al., 1987). There is also an iron-only nitrogenase (Chisnell, et al., 1988), which also contains a P-cluster like the other two types of nitrogenases. In carbon monoxide dehydrogenases, a Ni-Fe cluster with a composition of NiFe₃₋₄S₂₄ has been studied (Ragsdale, et al., 1990; Abbanat and Ferry, 1990).

In addition to the diversity of the Fe-S clusters, the diversity of iron-sulfur proteins comes through in yet another way. That is, many iron-sulfur proteins contain more than one Fe-S cluster of the same or different types, or contain one Fe-S cluster and a different cofactor. They work together in a concerted way to accomplish the physiological requirements. Most of the mechanisms for exactly how they are related have yet to be revealed. The well-known 8Fe ferredoxin in C. pasteurianum has two

1.1.3 Diversified Biological Functions in Nature

The functions of iron-sulfur proteins were first recognized in ferredoxins as electron transfer proteins. These proteins are essential to many of the fundamental processes of life including photosynthesis, respiration, and nitrogen fixation. Now the functions of iron-sulfur proteins have expanded to include the following categories: (1) catalysis (redox or nonredox); (2) stabilization of protein structure; (3) regulation of metabolic pathways; (4) biological sensor of iron, O₂, and O₂⁻. There are also iron-sulfur proteins with unknown functions (Cammack, 1992; Flint and Allen, 1996).
**Ferredoxins:** Various ferredoxins are the main group of electron transfer iron-sulfur proteins. Rubredoxins (Rd) are the simplest, with the Fe ligated entirely by cysteinyl sulfurs. One of the few known functions of this protein is as intermediate electron carrier in o-hydroxylation of fatty acids, for which a unique rubredoxin with two rubredoxin domains is used (Lode and Coon, 1971). [2Fe-2S] ferredoxins were identified as photosynthetic electron transfer proteins in chloroplasts of plants, such as spinach, and in cyanobacteria. The chloroplast [2Fe-2S] ferredoxins act as electron donors to several different enzymes, such as nitrite reductase and glutamate synthase (Knaff and Hirasawa, 1991). [2Fe-2S] ferredoxins were also found in kidney, adrenal glands and other animal tissues, acting as electron donor to cytochrome P-450. Like many other eukaryotic proteins, adrenal ferredoxin undergoes phosphorylation, which modulate its activity (Monnier, et al., 1991; Nemani, et al., 1989). [3Fe-4S] ferredoxins were found in *Streptomyces griseolus* with only three available cysteines for ligation (O'Keefe, et al., 1991). The [3Fe-4S] ferredoxin II of *Desulfovibrio gigas* was later considered to be the oxidation product of the *D. gigas* [4Fe-4S] ferredoxin I. 2[4Fe-4S] ferredoxins have almost twofold symmetry, and may be the precursors of the [4Fe-4S][3Fe-4S], the [4Fe-4S], and the [3Fe-4S] cluster ferredoxin (Fukuyama, et al., 1988). They are primarily found in bacteria, though a similar 2[4Fe-4S] ferredoxin was found in *Entamoeba histolytica* (Reeves, et al., 1980). Different organisms use the 2[4Fe-4S] ferredoxins for different purposes, as low-potential electron carriers, for example, in anaerobic metabolism of *C. pasteurianum* (Graves, et al., 1985).

**Membrane-bound Electron Carrier Proteins:** The first group of this category contains membrane-bound electron carrier Fe-S proteins that interact with quinones in the
respiratory chain of mitochondria, chloroplasts and bacteria. These include hydrogenase, formate dehydrogenase, and succinate dehydrogenase (Ohnishi, 1987), and on the oxidizing side, nitrate reductase (Johnson, et al., 1985), and fumarate reductase. All of these proteins have a similar construction, consisting of a membrane anchor subunit or subunits that act as the binding site for quinone, and soluble subunits on the membrane surface that react with the soluble substrates. The iron-sulfur clusters, which act as intermediate electron carriers, are situated between these two components (Cammack, 1992). Rieske proteins with histidine ligated [2Fe-2S] clusters have relatively high reduction potentials (0-300 mV). They were first isolated from mitochondrial respiratory chain complex III (ubiquinol: cytochrome c reductase) and later in other types of membrane electron transfer chains. For example, they were found in aromatic dioxygenases of bacteria *P. putida* (Kuila et al., 1987; Zylstra and Gibson, 1989), cytochrome b6f complex of chloroplasts and cyanobacteria, and in respiratory and photosynthetic bacteria (Trumpower, 1990; Davidson and Daldal, 1987). The second group of the membrane-bound category is the photosynthetic electron transfer chain of plants and cyanobacteria. In photosystem I, the primary electron acceptor complex comprises a large, membrane-bound complex in which electrons from the primary donor chlorophyll are transferred through iron-sulfur clusters to ferredoxin and ultimately NADP (Lagoutte and Mathis, 1989; Evans and Bredenkamp, 1990). One of the three iron-sulfur clusters in this complex, cluster X, is proposed to be a [4Fe-4S] Cluster bound between the two subunits PS1A1 and PS1A2 (products of the psaA and psaB genes) (Golbeck and Bryant, 1991). The terminal electron acceptors of photosystem I are iron-sulfur clusters A and B. Both of them have a 9-kDa subunit encoded by the psaC gene,
which shows a strong homology with the bacterial 2[4Fe-4S] ferredoxins (Oh-oka, et al., 1991).

**Soluble Electron-Carrying Protein with Other Cofactors:** The iron-sulfur proteins in this category interact with other electron-carrying center such as heme or non-heme iron, molybdenum, nickel, and some organic cofactors, such as flavin, quinone, or thiamin diphosphate. In sulfite and nitrite reductases of plants and bacteria, the [4Fe-4S] cluster was bridged with a specialized chlorin group known as siroheme by a sulfide, which is likely from cysteine (McRee, et al., 1986, Ida and Mikami, 1986). Molybdenum-containing iron-sulfur proteins generally have multiple Fe-S clusters and a molybdopterin cofactor (Rajagopalan, 1985; Bray, 1988) with the exception of Mo nitrogenase. These include oxidoreductases such as nitrate and formate reductases (Adams and Mortenson, 1985), and the molybdenum hydroxylases, such as xanthine dehydrogenase and aldehyde oxidase (Wootton, et al., 1991). The iron-sulfur clusters in nickel-containing hydrogenases serve to transfer electrons between the nickel site and donors or acceptors such as ferredoxin, NAD, deazaflavin, quinone, or cytochrome c (Lancaster, 1988; Voordouw, 1992). Flavoproteins such as ferredoxin:NADP reductase are the intermediaries for iron-sulfur protein to interact with NAD or NADP (Cammack, 1983).

In monooxygenases (Berg, et al., 1976) and dioxygenases (Batie, et al., 1991), iron-sulfur protein exists as a separate ferredoxin, or as part of an iron-sulfur flavoprotein.

**Iron-Sulfur Proteins as Redox Enzymes:** Iron-sulfur proteins having redox catalytic functions include carbon monoxide dehydrogenases (carbon monoxide oxidoreductases), iron hydrogenases, and nitrogenases. Carbon monoxide dehydrogenases catalyze the reversible oxidation of CO to CO₂. In aerobic bacteria a
molybdenum-containing iron-sulfur protein with [2Fe-2S] clusters catalyzes the oxidation of CO (Meyer, et al., 1986). All three types of nitrogenases (Mo, V, and Fe) have the so-called P-cluster, while the reduction of N₂ to ammonium probably happens at the Fe-X (X=Mo, V, or Fe) cofactor site (Pau, 1989). The nitrogenase reductase, or the iron protein, is the second essential component of the nitrogenase system, serving as a low-potential, ATP-dependent electron donor to the dinitrogen-reducing protein (Moffat, 1990). It has one [4Fe-4S] cluster connecting the dimer of two subunits. The Fe hydrogenases, which catalyze the production and consumption of hydrogen gas as in FeNi hydrogenases, also contain a special iron-sulfur cluster, the H-cluster.

**Iron-Sulfur Proteins as Nonredox Enzymes:** There is a large group of iron-sulfur proteins with nonredox functions, some of which catalyze hydration/dehydration of double bonds. The most famous one, aconitase, belongs to the hydro-lyase family (Kennedy and Stout, 1992). The inactive enzyme contains a [3Fe-4S] cluster that is converted to a catalytically active [4Fe-4S] cluster (Beinert and Kennedy, 1989). Other enzymes in this family, such as fumarate dehydratase (or fumarase) A and B from *E. coli* (Ueda, et al., 1991; Flint, et al., 1992) and dihydroxy-acid dehydratase of *E. coli* (Flint, et al, 1993) also contain a catalytic [4Fe-4S]^{2+/-1+} cluster with incomplete cysteine ligation. Spinach dihydroxy-acid dehydratase has a [2Fe-2S]^{2+/-1+} cluster with one non-cysteine ligation (Flint and Emptage, 1988).

Recent studies have demonstrated some novel functions of iron-sulfur clusters. These include transcriptional regulatory function of iron regulatory protein (apo cytosolic aconitase), oxygen and/or superoxide sensory proteins SoxR and FNR, and structural regulation as proposed in glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase...
and in Endonuclease III (Flint and Allen, 1992). More details will be discussed in the next section.

1.2 DIVERSE FUNCTIONS RELATED TO IRON-SULFUR CLUSTER ASSEMBLY AND DISASSEMBLY

1.2.1 Remarkable Structure Versatility of Fe-S Clusters

The almost universal presence of iron-sulfur clusters and the numerous functions for iron-sulfur proteins are based on its remarkable facility for conversion and interconversion in both the free and protein-bound conditions. In the synthetic field, various cluster types have been synthesized successfully. These include the mononuclear \([\text{Fe}(\text{SR})_4]\), binuclear \([\text{Fe}_2\text{S}_2(\text{SR})_4]\) and cubane clusters \([\text{Fe}_4\text{S}_4(\text{SPh})_4]\) (Berg and Holm, 1982; Holm, 1977; Hagen, et al., 1981; Kurtz, et al., 1984), the hexanuclear cluster \([\text{Fe}_6\text{S}_6(\text{SR})_6]\) (Christou, et al., 1981), and the cuboidal trinuclear cluster \([\text{Fe}_3\text{S}_4(\text{SR})_3]\), (Zhou, et al., 1996). Cluster conversion was also studied. In the early 80s, Hagen and coworkers defined reaction sequences resulting in self-assembly of \([\text{Fe}_4\text{S}_4(\text{SR})_4]\) clusters from simple reactants, in which they demonstrated elaboration of a tetranuclear cluster through mononuclear and then binuclear intermediates (Hagen, K. S., et al. 1981). Zhou, et al. in their synthetic studies have shown that the cuboidal trinuclear cluster \([\text{Fe}_3\text{S}_4(\text{SR})_3]\) has special versatility (Zhou, et al., 1992; Zhou, et al., 1997). It can be converted to several other cluster types, including the linear form trinuclear cluster \([3\text{Fe}\text{S}_4(\text{SR})_4]\) in the presence of thiol, and the cubane cluster when thiol and Fe\(^{2+}\) are
added, and the mixed-type cubane cluster \([\text{Mfe3S4(SR)4}]\) when a second metal such as Cu, Mn, or Cd is added.

The versatility of iron-sulfur clusters was recognized just as speedily in biological systems as in synthetic studies. Fe-S cluster conversion and interconversion have been found in many systems in the studies of cluster assembly and disassembly. Early in 1979, Christou, et al., reported a conversion of \textit{Clostridium pasteurianum} rubredoxin, which has a mononuclear Fe center, into a four-iron ferredoxin (Christou, et al., 1979). A molecular variant of \textit{Clostridium pasteurianum} rubredoxin was shown to be able to assemble a binuclear \([2\text{Fe}-2\text{S}]^{2+}\) cluster (Meyer, et al., 1997). Cluster conversion from \([2\text{Fe}-2\text{S}]\) to \([4\text{Fe}-4\text{S}]\) was observed in \textit{E. coli} biotin synthase (Duin, et al., 1997) when the isolated inactive protein was reduced with dithionite anaerobically to form the active protein. The reverse process, \([4\text{Fe}-4\text{S}]\) cluster to \([2\text{Fe}-2\text{S}]\) cluster, was observed in the iron protein of \textit{Azotobacter vinelandii} nitrogenase (Anderson, et al., 1984; Fu, et al., 1991). More recently, an almost quantitative conversion of \([4\text{Fe}-4\text{S}]\) cluster to \([2\text{Fe}-2\text{S}]\) cluster was observed on exposure of the FNR (fumarate nitrate reduction) protein of \textit{E. coli} to dioxygen (Khoroshilova, et al., 1997). Parallel to the synthetic discoveries, the cuboidal cluster in iron-sulfur proteins demonstrated remarkable facility in converting to other cluster types (Moura, I., et al., 1986; Finnegan, et al., 1995; Butt, et al., 1991). In \textit{Desulfovibrio gigas} ferredoxin II (Moura, et al., 1982) and the enzyme aconitase (Kent, et al., 1982), facile interconversion between the cubane \([\text{Fe}_4\text{S}_4]^{2+}\) and the cuboidal \([\text{Fe}_3\text{S}_4]^{1+}\) structures occurs. Aconitase is inactive in the \(\text{Fe}_3\text{S}_4\) state (Kent, et al., 1985). The spontaneous cluster reconstruction is the iron-dependent self-activation of aconitase, restoring the catalytic center with one substitutionally labile iron subsite where substrate
binds. Even more fascinating, cytosolic aconitase can be interconverted between [4Fe-4S] cluster and apo-protein, which is the iron regulatory protein 1, demonstrated a completely new area of iron regulatory function of the iron-sulfur proteins.

1.2.2 Cluster Assembly and Disassembly: Iron-Sulfur Proteins as Regulatory Proteins, Biosensors of Oxidants and Iron.

Iron-sulfur proteins are receiving new attention in recent years since the finding that they serve as sensors of iron, dioxygen, superoxide ion (O2-), and possibly nitric oxide (Rouault and Klausner, 1996; Beinert, et al., 1997). This is clearly demonstrated in three proteins: the iron regulatory protein 1 (IRP1) (Hentz and Kühn, 1996; Beinert, et al., 1996), SoxR protein in *E. coli* (Hidalgo, et al., 1995; Gaudu and Weiss, 1996), and the FNR (fumarate nitrate reductase) protein in *E. coli* (Khoroshilova, et al., 1995; Lazazzera, et al., 1996; Khoroshilova, et al., 1997).

One of these proteins, SoxR, is a transcriptional activator that senses superoxide and nitric oxide stress in *E. coli*. When post-translationally activated, it stimulates transcription of a second regulatory gene, soxS, by binding to its promoter DNA (Nunoshiba et al., 1992; Wu and Weiss, 1992). The product of the soxS gene, SoxS protein, then triggers expression of over 10 genes (including the gene for SOD) involved in defence against oxidative damage (Li and Demple, 1994) and antibiotic resistance (Chou et al., 1993; Ma et al., 1996). SoxR contains two [2Fe-2S] clusters, one in each monomer of this dimeric FeS protein (Hidalgo and Demple, 1994; Wu et al., 1995). It has been shown that SoxR FeS clusters are essential for promoting open-complex formation
by RNA polymerase and triggering expression of soxS gene, although they are not required for DNA binding (Hidalgo et al., 1995). Under reducing conditions, for example, as purified using thiol-containing buffer, the FeS cluster undergoes disassembly to form apoprotein with loss of activity. However, full transcriptional activity of SoxR can be restored by in vitro assembly of iron-sulfur clusters into the protein either noncatalytically or enzymatically with NifS (Hidalgo and Demple, 1996).

Iron regulatory protein 1 (IRP1) is another example where cluster assembly and disassembly provide the signals for activation of a regulatory mechanism responding to bioavailability of iron and oxidative stress (Beinert, et al., 1997). The control of intracellular iron levels depends critically on the function of two proteins: the transferrin receptor, which recognizes the iron-loaded transport protein transferrin and ushers it into cells, and the storage protein ferritin, which provides for reversible and safe storage of excess iron. The concentration of these proteins is regulated at the translational level by the IRP. The mRNAs for transferrin receptor and for ferritin contain so-called iron-responsive elements (IREs). These IREs bind with high affinity to IRP, which is found in all higher forms of life from mollusks to insects and vertebrates. The active form of IRP does not have an iron-sulfur cluster. In fact, the active IRP1 is the apo form of the iron-sulfur protein cytosolic aconitase (Rouault, et al, 1992, Haile, et al., 1992). IRP is activated during periods of iron deficiency, and as a result of cytosolic aconitase losing its cluster. IRP is inactivated when iron available for complexation is plentiful, giving rise to the reconstruction of the [4Fe-4S] cluster and activation of aconitase. At low iron level, IRP binds to the IRE in the 5' region of ferritin mRNA, resulting in the blocking of translation. Active IRP also binds to the IREs in the 3' region of tranferrin receptor.
mRNA, resulting in protection of the mRNA from being degraded by nucleases. Thus the interplay of the [4Fe-4S] cluster assembly and disassembly regulates the levels of transferrin receptor and ferritin in an opposing sense, as required either for acquisition of iron for use or storage.

1.3 HIGH POTENTIAL IRON-SULFUR PROTEIN (CHROMATIUM VINOSUM)

1.3.1 Introduction

HiPIPs (high potential iron-sulfur proteins) are a class of proteins found in purple photosynthetic bacteria, containing a cubane [4Fe-4S] cluster (Bartsch, 1978; Meyer et al., 1983; Meyer, 1994). They have been isolated from many species, such as the photosynthetic purple sulfur bacteria Chromatium vinosum (Bartsch, 1963) and Thiocapsa pfenigii (Meyer, 1970), the photosynthetic purple bacteria Rhodopseudomonas gelatinosa (DeKlerk and Kamen, 1966) and Rhodoferax fermentans (Hochkoeppler, et al., 1995), and denitrifying bacterium Micrococcus species (Hori, 1961). HiPIPs are peculiar among other iron-sulfur proteins because of the high redox potential at which the cluster exchanges electrons (from +50 mV to +450 mV) (Meyer, et al., 1983). A much larger number of ferredoxins show a lower redox potential, ranging from -250 to -650 mV (Armstrong, et al., 1988). Although the chemical and biophysical properties of HiPIPs are well defined (Bertini, et al., 1995; Heering et al., 1995), their biological functional role is still uncertain. Their function as electron transfer proteins in photosynthetic reactions has been suggested (Kennel, et al., 1972; Hochkoeppler, et al., 1995), but direct evidence on this area is still needed.
**Chromatium vinosum** HiPIP has been the subject of extensive characterization. It is a relatively small soluble protein with a molecular weight about 10 kDa. Iron and labile sulfide analysis give the 4Fe4S cluster type. Redox potential $E_{m,7} = 350$ mV.

Isoelectric point $pI (0^0)$ is 3.88 for oxidized protein and 3.68 for reduced (Dus, et al., 1967). UV-visible absorption spectra of both oxidized and reduced HiPIP (C.v.) have two major peaks: one at 282 nm, the other at 388 nm for reduced protein, and a shoulder around 450 for oxidized protein. The extinction coefficients for reduced protein are $\varepsilon_{mM}(282) = 41.3$, $\varepsilon_{mM}(388) = 16.1$. Oxidized protein has the same $\varepsilon_{mM}$ at 282 nm, but $\varepsilon_{mM}$ at 388 is larger, near 21. At 480 nm, the difference between these two, $\Delta\varepsilon_{mM (ox-rd)}$ is 10. The ratio $A_{282}:A_{388}$ in the absorbance spectra was often used as a purity index, with near or below 2.5 being considered pure (Bartsch, 1978).

The amino acid sequence of HiPIP (C.v.) is known (Dus, et al., 1973). There are 85 amino acids in this protein. There are only four cysteine residues in this protein, which are all coordinated to the cluster. The three dimensional structure has been solved by crystallography (Carter, et al., 1974a,b). An interesting feature for the [4Fe-4S] cluster is that it is surrounded by aromatic residues: Tyr19, Phe48, Phe66, Trp60, Trp76, and Trp80. The closest point of approach of the cluster to solvent appears to be two of the inorganic sulfur atoms, which are about 4.5 Å from the surface. Recently, three-dimensional structures of both the reduced (Banci, et al., 1994, 1995) and the oxidized (Bertini, et al., 1996) HiPIP (C.v.) were also determined by NMR techniques. There is no significant deviation between the solid state and solution structures.

Other physiochemical studies on HiPIP (C.v.) have provided more information on the [4Fe-4S] cluster. EPR (electron paramagnetic resonance) spectroscopy, since
introduced by Beinert and Sands (Beinert and Sands, 1960), has been the most versatile
method for characterizing iron-sulfur proteins (Hagen, 1992; Cammack and Cooper,
1993). Different types of iron-sulfur clusters, or different oxidation states of the same
cluster, usually give rise to different EPR signals (Pilbrow and Hanson, 1993). The
oxidized cluster $[4\text{Fe}-4\text{S}]^{2+}$ has a ground spin state of $S=1/2$, and therefore is
paramagnetic and is EPR active. EPR spectrum of HiPIP (C.v.) gives a characteristic,
mostly axial signal with $g_{\parallel}=2.11$, and $g_{\perp}=2.03$. Minor components at around $g=2.08$
were interpreted to be from possible interactions of a HiPIP dimer at high concentrations
(Dunham et al., 1991). Reduced HiPIP (C.v.) has a ground spin state $S=0$, and is EPR
silent. However, partial paramagnetic properties in the reduced protein were detected by
nuclear magnetic resonance (NMR) spectroscopy (Bertini, et al., 1991). Both the reduced
and oxidized protein were extensively characterized by NMR spectroscopy (Gaillard, et
al., 1992; Cowan and Sola, 1990). The NMR peaks of $^{15}$N-labeled HiPIP (C.v.) were also
assigned (Li, et al., 1995). All of these provided a generous platform on which further
structure/function and assembly/disassembly studies can be performed in a systematic
way.

1.3.2 Factors Contributing to the High Potential of HiPIP

The large difference in the redox potentials of HiPIP and other ferredoxins has
been a puzzle for scientists until now. Various theories have been proposed to give an
explanation from a specific point of view. Now it seems that the high potential arises
from a combination of many structural factors, which include the state of redox couples,
surface charges, solvent accessibility, backbone dipole contributions, and other factors (Cowan and Lui, 1998).

Carter's three-state hypothesis (Charter, 1974) was the first effort in explaining the huge difference in redox potentials between HiPIPs and low-potential ferredoxins. This theory can be summarized as the following presentation:

\[
\begin{align*}
[4\text{Fe}-4\text{S}]^{1+} & \quad \Longleftrightarrow \quad [4\text{Fe}-4\text{S}]^{2+} & \quad \Longleftrightarrow \quad [4\text{Fe}-4\text{S}]^{3+} \\
\text{Fd's} & \quad \text{Em} < 0 \text{ mV} & \quad \text{HiPIP's} & \quad \text{Em} > 0 \text{ mV}
\end{align*}
\]

In this phenomenal interpretation, HiPIP clusters have a redox state of \([4\text{Fe}-4\text{S}]^{3+/2+}\), while other ferredoxins have a redox state of \([4\text{Fe}-4\text{S}]^{2+/1+}\). The reduced HiPIP clusters have the same oxidation state as the oxidized Fds. Although there is no explanation for the reasons of choice from the proteins, this theory does point out that it is the packing of the protein about the iron-sulfur cluster, not the cluster itself that is responsible for the redox potentials of HiPIPs and Fds.

The second theory that received much attention was proposed by Warshel and coworkers (Langen, R., et al., 1992; Lee, et al., 1993; Jensen, et al, 1994; Stephens, et al., 1996). After examined the variation in the midpoint potential of several structurally well-characterized Fe-S proteins, they investigated the control mechanism for the redox potential of Fe-S clusters by its protein environment. They included electrostatic interactions, orientation of amide groups in the neighborhood of a cluster, solvation energy differences, hydrogen bonds, and other environmental factors contributing to the redox properties of clusters. By using the "protein dipoles Langevin dipoles (PDL)"
approach (Warshel and Russell, 1984; Churg and Warshel, 1986), Warshel and coworkers were able to model the protein control of the [4Fe-4S] cluster redox potentials. Calculations of the differences in redox potential of several ferredoxins were qualitatively consistent with experimental results (Jensen, et al., 1994) with the exception of HiPIP (C. v.). This theory is obviously not complete. However, it is supported by many other observations on control of reduction potential by protein matrix (Zhou, 1997; Capozzi, et al., 1998; Bertini, et al., 1997; Mauk and Moore, 1997).

1.3.3 Cluster Stability. Fe-S Cluster Assembly and Disassembly

In the searching for structural factors contributing to the high redox potential of HiPIP (C.v.), our lab focused on the role of aromatic residues, which form a hydrophobic pocket around the cluster (Figure 1.2), as demonstrated by the three-dimensional structure. A synthetic HiPIP gene sequence was constructed according to the known protein sequence and the codon usage of E. coli. The gene was cloned into E. coli and over-expressed (Agarwal, et al., 1993), paving the way for site-directed mutagenesis, NMR, EPR, and other spectroscopic and biochemical investigations.

The subsequent research results have not shown a significant influence by the aromatic side chains on the redox potential of HiPIP. Instead, mounting evidence has revealed the importance of the aromatic residues in stabilizing the [4Fe-4S] cluster, and the importance of the integrated [4Fe-4S] cluster to the protein (Agarwal, et al., 1996; Li, et al., 1996). The non-conservative oxidized Y19 mutants (Agarwal, et al, 1995) showed clear oxidative degradation, which is attributed to the increased solvent access to the
Figure 1.2: Backbone structure of *C. Vinosum* HiPIP showing the placement of key aromatic side chains that form a hydrophobic pocket for the \([\text{Fe}_4\text{S}_4]\) cluster.
oxidized cluster in the mutants, leading to hydrolytic degradation (O'Sullivan and Millar, 1985). Phe66 mutants (Bian et al., 1996), Phe48 mutants (Soriano et al., 1996) and Trp 80 mutants (Chen and Cowan, 1998) have demonstrated similar properties. C77 mutants, on the other hand, were not expressed except C77S, which is considered the closest to native in terms of cluster coordination. This indicated the importance of cluster assembly in the expression of the native protein. Another interesting phenomenon was observed in Phe66 mutants, where auto-reduction of the oxidized protein occurred, and a [3Fe-4S] cluster was identified as an intermediate in the cluster disassembly pathway.

In a reconstitution experiment, a rather stable intermediate species is observed with only Fe bound to the protein in the presence of DTT, but no sulfide. The intermediate species was shown to have a tertiary structure close to the native HiPIP by HSQC NMR spectroscopy (Natarajan and Cowan, 1997). These results, and the intriguing aspects of the regulatory functions through cluster assembly/disassembly in iron-sulfur proteins, prompted us in pursuing the mechanism and intermediates in the processes of cluster assembly/disassembly, using HiPIP (C.v.) as a protein model.

1.4 SIGNIFICANCE OF THIS WORK

HiPIP from Chromatium vinosum has been extensively characterized with regard to its molecular biology, structural biophysics, redox chemistry, and cluster reconstitution. It provides an excellent choice as a protein model for systematic investigation of cluster assembly and disassembly, which have demonstrated to be key
steps and essential mechanisms in nature’s iron and oxidative regulation. Using HiPIP as a model has definite advantages: the protein provides a real cluster environment as compared to model compound studies; availability of large quantity of purified proteins for physiochemical studies; a simplified system compared to complicated systems, such as FNR. To this end, we have for the first time:

1. Studied [4Fe-4S] cluster disassembly processes by using HiPIP (C.v.) as a model. Kinetic analysis revealed that protonation on four sulfides facilitates cluster disassembly. Structural changes are necessary for protonation and cluster disassembly. Cluster disassembly is also affected by its oxidation state. These results provided insights into the cluster disassembly mechanism for other regulatory iron-sulfur proteins. (see Chapter 2)

2. Discovered an autoreduction pathway in mutant HiPIPs as a natural path for cluster disassembly. Oxidized [4Fe-4S]^{2+} clusters were found unstable in mutants. They went through a [3Fe-4S] intermediate in the process of degradation, which then provided reducing equivalents for other oxidized proteins. (Chapter 3)

3. In cluster assembly studies in the absence of sulfide, several kinetic intermediates were detected. Cluster assembly leads to a rather stable intermediate, in which coordination of irons by cysteinyl sulfur (and exogenous thiol groups) causes the protein backbone to fold into an intermediate tertiary structure. This demonstrated the thermodynamic relevance of cluster assembly to the protein folding in iron-sulfur proteins. (Chapter 4)

4. Identified biological relevant iron-sulfur cluster intermediates in complete [4Fe-4S] cluster assembly. The proposed intermediates include mononuclear Fe centers,
binuclear [2Fe-2S] clusters, and others toward the formation of native [4Fe-4S] cluster. A working mechanism for [4Fe-4S] cluster assembly has been proposed, which is consistent with experimental results, and with synthetic compound studies, as well as examples in other biological systems. (Chapter 5)
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CHAPTER 2

IRON-SULFUR CLUSTER DISASSEMBLY

2.1 INTRODUCTION

Disassembly of iron-sulfur clusters is of fundamental relevance to biological functions of many iron-sulfur proteins. A set of novel functions of iron-sulfur proteins, such as transcriptional regulation and iron and/or oxygen sensing properties, are found to be closely related to the disassembly and reconstitution of iron-sulfur clusters. Iron-sulfur proteins have been recognized as one of nature’s modular (Helmut Beinert et al., 1997), and iron-sulfur cluster interconversion represents a powerful mechanism for different organisms to deal with external stresses.

A number of iron-sulfur proteins experience iron-sulfur cluster disassembly under oxidative stress. A group of enzymes involve in the homolytic cleavage of C-C and C-H bonds by radical (5'-deoxyadenosyl) mechanisms that require S-adenosylmethionine. These include biotin synthase (Duin et al., 1997; Jestin et al., 1996), pyruvate formate lyase (Broderick et al., 1997; Knappe and Sawers, 1990), and lysine 2,3-aminomutase (Petrovich et al., 1992; Frey and Reed, 1993). A subunit-bridging [4Fe-4S]$^{2+/1+}$ cluster in the active enzyme that can undergo oxidative degradation to form [2Fe-2S]$^{2+}$ clusters is proposed to be a common feature of these Fe-S enzymes. It is further suggested that
oxidative cluster conversion to \([2\text{Fe-2S}]^{2+}\) clusters may play a physiological role in these radical enzymes by providing a method of regulating enzyme activity in response to oxidative stress, without irreversible cluster degradation (Duin et al., 1997).

A similar process was found in FNR (fumarate and nitrate reduction) protein of *Escherichia coli*. FNR in *E. coli* functions as a transcription regulator that regulates a set of genes that provide alternative pathways for energy generation when oxygen becomes scarce (Khoroshilova et al., 1995). A \([4\text{Fe-4S}]\) cluster contained in FNR mediates the sensitivity of this transcription factor to oxygen, thus limiting FNR activity to anaerobic conditions. Active FNR with integrated \([4\text{Fe-4S}]\) cluster tends to dimerize and shows specific DNA binding activity (Lazazzera et al., 1996). Upon exposing to oxygen, \([4\text{Fe-4S}]\) cluster disassembles to form \([2\text{Fe-2S}]\) cluster in about 60% yield with loss of DNA-binding ability. It was further demonstrated that DNA-binding and the absorption spectrum characteristics of the \([4\text{Fe-4S}]^{2+}\) cluster could be largely restored from the \([2\text{Fe-2S}]^{2+}\) form when Cys, Fe, DTT, and the NifS protein were added. This finding leads to the suggestion that the form of FNR containing the \([2\text{Fe-2S}]^{2+}\) cluster may be an *in vivo* intermediate that is more rapidly converted to the active form than the apoprotein (Khoroshilova et al., 1997).

The most interesting example in cluster disassembly is the iron regulatory protein 1 (IRP 1). Cellular iron homeostasis is modulated and maintained through changes in synthesis of proteins involved in the uptake (transferin receptor), storage (ferritin), and utilization (erythroid 5-aminoevvulinate synthase or eALAS and other Fe proteins) of this essential mineral. It has been established that synthesis of these proteins is post-transcriptionally regulated through the action of cytosolic iron regulatory protein 1.
or IRP 1 (Haile et al., 1992a,b; Rouault et al., 1992; Hirling et al., 1994; Hentze and Kühn, 1996; Schalinske et al., 1997). IRP 1 is the apo form of cytoplasmic aconitase, an iron-sulfur protein catalyze the reaction of citrate to iso-citrate. When iron is abundant, aconitase has an intact [4Fe-4S] cluster and catalytic activity. When the iron level is low, the [4Fe-4S] cluster disassembles, which is accompanied by structural changes in the protein (Klausner et al., 1996). IRP 1 (apo-aconitase) then binds to iron responsive elements (IRE) which are stem-loop structures present in the 5' untranslated region (UTR) of ferritin and eALAS mRNAs and in the 3' UTR of transferrin mRNA. Formation of the IRP-IRE complex results in the repression of translation of ferritin as well as the simultaneous stabilization of transferrin mRNA and consequent increase in transferrin synthesis. Thus disassembly (and assembly) of the iron-sulfur cluster is the regulatory step (Beinert and Kiley, 1996).

Many other iron sulfur proteins exhibit oxidative disassembly of their [4Fe-4S] cluster, for example, *Desulfovibrio africanus* ferredoxin III (Busch et al., 1997), mammalian aconitase (Flint et al., 1993a), and the hydro-lyase class in *E.coli* (dihydroxy-acid dehydratase, fumarase A, fumarase B). Unlike superoxide inactivation in aconitase and the 8Fe ferredoxin III, which leads to formation of [3Fe-4S] clusters, oxidative degradation appeared to lead to a complete breakdown of the Fe-S clusters in the hydro-lyase class (Flint et al., 1993b).

While there is mounting evidence that Fe-S cluster disassembly plays an important role in the function of Fe-S proteins, the mechanism and pathways of Fe-S cluster disassembly, and the correlation between protein conformational change and cluster disassembly are areas requiring further exploration. Model cluster compounds
Fe₄S₄(SR)₄ (R = alkyl) have been found to represent the base species of week acids of pKa comparable to that of carboxylic acids. The acid species Fe₄S₄(SR)₄H⁺ is prone to reaction with O₂ and to acid-catalyzed dissolution, while the base species readily undergoes ligand exchange (Bruice et al., 1975; Job and Bruice, 1975). Kinetic studies indicated that there were likely three protonation steps before the rate-limiting step, in which iron and sulfide were finally released. Chromatium vinosum HiPIP and ferredoxins were also used to study the kinetics of acid-catalyzed disassembly (Maskiewicz et al., 1975; Maskiewicz and Bruice 1977a,b). In all these studies, protonation and cluster dissolution were achieved by directly adding acid to the protein solution. Our experiments showed that simultaneous cloudiness and precipitation were inevitable in this process, and consequently, interfere with the observed kinetic events.

In this study, we explored a modified approach in cluster disassembly, by using 0 to 2 M guanidine hydrochloride in addition to less than 1% trichloroacetic acid. These changes make the whole process of cluster disassembly transparent, suitable for spectroscopic monitoring (UV-visible and fluorescence). Moreover, cluster species were monitored by freeze-quench EPR techniques. The fast disappearance of the 4Fe-4S cluster signal and the slow evolution of the single iron signal in EPR experiments provide direct probes for cluster identities, and shed further insight into the mechanism of iron-sulfur cluster disassembly.

2.2 MATERIALS AND METHODS

Proteins, chemical reagents: HiPIP (C.v.) was purified according to previously published procedure (Agarwal, et al, 1993). The purity index A₂₈₂/A₃₈₆ is 2.5. HiPIP as
purified is in the reduced state, which can be stored at -20°C in 100mM Tris (pH 8.0) for up to 6 months without significant degradation. Oxidized HiPIP, made by either K₃Fe(CN)₆ or K₂IrCl₆ oxidation, is less stable in air. After passage through a G25 column, the oxidized protein was concentrated on a 5K amicon membrane against 50 mM Tris buffer (pH 8.0), and used immediately.

Guanidine hydrochloride (GuHCl) was either purchased from Sigma as an 8 M solution or in solid form from Fisher-Acros. The solid was re-crystallized with hot ethanol and filtered. All other chemicals were purchased from the best available source without further purification.

**Absobance Spectroscopy.** The degradation of the 4Fe-4S cluster in HiPIP (C.v.) was monitored on a HP 8452A diode array spectrophotometer at 386 nm, where the protein has a signature absorption peak in the reduced state from metal-ligand charge transfer (MLCT). For oxidized protein, instead of a peak, a shoulder appears extending to about 420 nm. The 386 nm absorbance was also used for oxidized protein for convenience. The decrease in absorption upon cluster degradation was assayed under different pseudo-first order conditions. Protein was added last, and care was taken to ensure that the mixture was homogeneous before and after adding the protein. The curve was best fitted to a single exponential process to obtain the apparent rate constant $k_{obs}$.

**Fluorescence Spectroscopy.** Fluorescence assays on cluster degradation was done on a Perkin Elmer LS50B luminescence spectrometer in the fluorescence mode. The excitation wavelength was 280 nm for the three tryptophan residues as part of the hydrophobic pocket around the cluster (Carter, et al., 1974). The emission wavelength was chosen at 352 nm, at the maximum fluorescence under the experimental conditions
when the pocket completely opens up. The wavelength resolution is 4 nm. Protein was added last, and care was taken to make sure the mixture was homogeneous before and after adding the protein. The increase in fluorescence upon cluster degradation was best fitted to a single exponential process to get the apparent rate constant $k_{obs}$.

Stopped-Flow Fluorescence and Temperature Dependence

Stopped-flow measurements were made on an OLIS 1000 RSM apparatus with a NESLAB RTE-111 temperature control unit. Temperature measurements were also checked by a digital thermometer with its thermal probe near the mixing cell. The temperature measurement was accurate to ± 0.5°C. Excitation was at 280 nm, and the emission was collected in a 90 degree fluorescence configuration using a 300-400 nm band-pass filter. A special slow response circuit was built into the PMT to increase the signal to noise ratio. One syringe was filled with 0.05 mM reduced HiPIP in 50 mM Tris (pH 8.0), while the other syringe contained 4 M GuHCl with 2% TCA and 0.4 mM EDTA. The pH after mixing was around 1.5. A signal exponential fitting was also used to find the apparent rate constant.

Freeze-Quench EPR Experiments

Freeze-quench preparations were made on a Model 715 system from Update Instrument Inc. with a ServoDisc DC Ram. Freeze-quench was achieved by shooting the mixed sample to a funnel filled with cooled isopentane (-150 °C). A homemade isopentane bath was used, which can hold eight funnels simultaneously, making the sample-making efficiency at least four times higher than the one provided with the system. A three-syringe serial flow system was setup in preparing Freeze-Quench EPR samples, in which the syringe containing HiPIP was first mixed with the syringe containing GuHCl/TCA. The mixture went through an incubation reactor, and was then oxidized by $K_2IrCl_6$ contained in the third syringe during the
Figure 2.1: 4Fe-4S cluster degradation dependence on the [TCA]. 0.035 mM HiPIP was added last to a solution of 2.0 M GuHCl with 0.2 mM EDTA and various amount of TCA in 50 mM tris pH 8.0. Single exponential fit gives observed rate constants $k_{obs}$. 

second mixing before the mixture was shot into the cooled isopentane. The frozen powder was packed into the bottom section of a 4 mm EPR tube attached to the funnel by heat-shrink tubing. After packing, this section (about 4 cm long) was detached and reconnected back to the upper section of an EPR tube. The sample part was kept in liquid nitrogen during this process.

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 vs. Cr thermocouple. Experimental parameters are listed in footnotes to the tables and figure legends. Relative spin quantitation was carried out by spin integration using native HiPIP as a standard.

2.3 EXPERIMENTAL RESULTS

2.3.1 Kinetic Assays by UV-vis Spectroscopy

Purified HiPIP has a distinct absorption peak around 386 nm (a shoulder in the case of oxidized protein) in its UV/Vis spectrum, due to a MLCT band. This can be used as a probe to investigate the integrity of the 4Fe-4S cluster. Upon addition of 1% TCA with 2 M GuHCl, this peak disappears rapidly, indicating the disappearance of the metal-ligand charge transfer process, which in turn indicates the destruction of the 4Fe-4S cluster in HiPIP.

This acid-facilitated cluster degradation is dependent on both the concentration of TCA and the concentration of GuHCl. Figure 2.1 shows the assay curves for cluster
degradation with different TCA concentration in the presence of 2 M GuHCl. When no TCA was added, 2 M GuHCl causes almost no change in $A_{386}$ within one minute. Addition of 0.5% TCA causes the absorption at 386 nm to disappear almost completely within one minute. Changing the concentration of GuHCl while holding the TCA concentration at 1% gives similar curves (not shown). When no GuHCl was added, 1% TCA cause little decrease in $A_{386}$ within one minute. Addition of 1 M GuHCl makes $A_{386}$ go to zero within one minute.

These kinetic traces can be best fitted into a single exponential process according to the following equation:

$$A_{386}(t) = A_{N386}\exp(-k_{obs}t)$$

(2.1)

where $A_{N386}$ is the absorbance of integrated 4Fe-4S cluster at 386 nm. $k_{obs}$ is the apparent rate constant.

2.3.2 Fluorescence Spectroscopy

There are three tryptophan residues in HiPIP, and they are all in the vicinity of the 4Fe-4S cluster as part of the hydrophobic pocket. These residues provide a sensitive fluorescent probe in cluster studies. We have previously utilized this probe in detecting kinetic intermediates in cluster assembly pathways (Bian and Cowan, 1998). When the cluster degrades, iron and sulfide ions are released from the pocket. The fluorescence increases as the tryptophan residues are freed from the quenching effect of ions.

Figure 2 shows the kinetic traces of fluorescence upon cluster degradation. Excitation was at 280 nm, and the emission wavelength was 352 nm, where apo-HiPIP
Figure 2.2: Protein unfolding and cluster degradation. Dependence on [GuHCl]. 0.01 mM HiPIP(C.v.) was added last to a solution of 1% TCA with 0.2 mM EDTA and various amount of GuHCl in 50 mM tris pH 8.0 (Final pH=1.5). [GuHCl] was labeled in molar on the graph. Fluorescence excitation at 280 nm, emission at 352 nm. Fitting to single exponential gives observed rate constant $k_{obs}$. 
<table>
<thead>
<tr>
<th>%TCA</th>
<th>1</th>
<th>0.75</th>
<th>0.5</th>
<th>0.4</th>
<th>0.35</th>
<th>0.3</th>
<th>0.25</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{obs}(s^{-1})$</td>
<td>0.350</td>
<td>0.197</td>
<td>0.071</td>
<td>0.029</td>
<td>0.007</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta k_{obs} \times 10^2$</td>
<td>± 1.32</td>
<td>± 0.44</td>
<td>± 0.01</td>
<td>± 0.02</td>
<td>± 0.01</td>
<td>± 0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$k'_{obs}(s^{-1})$</td>
<td>0.157</td>
<td>0.056</td>
<td>0.016</td>
<td>0.008</td>
<td>0.003</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta k'_{obs} \times 10^2$</td>
<td>± 0.14</td>
<td>± 0.02</td>
<td>± 0.01</td>
<td>± 0.01</td>
<td>± 0.02</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[H^+] \times 10^2(M)$</td>
<td>4.045</td>
<td>3.084</td>
<td>2.123</td>
<td>1.738</td>
<td>1.546</td>
<td>1.354</td>
<td>1.161</td>
<td>0.969</td>
</tr>
</tbody>
</table>

†. Error in least square fitting.
‡. From calibrated pH values.

**Table 2.1:** Observed rate constants vs. $[H^+]$ with 2 M GuHCl.

<table>
<thead>
<tr>
<th><a href="M">GuHCl</a></th>
<th>2</th>
<th>1.75</th>
<th>1.5</th>
<th>1.25</th>
<th>1</th>
<th>0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{obs}(s^{-1})$</td>
<td>0.218</td>
<td>0.125</td>
<td>0.059</td>
<td>0.018</td>
<td>0.006</td>
<td>0.001</td>
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<tr>
<td>$\Delta k_{obs} \times 10^2$</td>
<td>± 0.78</td>
<td>± 0.13</td>
<td>± 0.07</td>
<td>± 0.01</td>
<td>± 0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>$k'_{obs}(s^{-1})$</td>
<td>0.157</td>
<td>0.080</td>
<td>0.020</td>
<td>0.006</td>
<td>0.002</td>
<td>± 0.001</td>
</tr>
<tr>
<td>$\Delta k'_{obs} \times 10^2$</td>
<td>± 0.14</td>
<td>± 0.03</td>
<td>± 0.01</td>
<td>± 0.01</td>
<td>± 0.02</td>
<td>± 0.01</td>
</tr>
</tbody>
</table>

†. Error in least square fitting.

**Table 2.2:** Observed rate constant vs. [GuHCl] with 1% TCA.
fluoresces. The rate of degradation is shown to depend on both GuHCl and TCA (curves not shown) concentrations, as in UV/Vis studies. The curves are by and large single exponential processes with the observed rate constant $k_{\text{obs}}$, which has the format of the following equation:

$$I_{\text{obs}} = I_{\text{apo}} \left[ 1 - \exp(-k_{\text{obs}} t) \right] \quad (2.2)$$

$I_{\text{apo}}$ is the fluorescence intensity after degradation of the 4Fe-4S cluster, and the protein is assumed to be in the apo state.

The rate constants from fluorescence measurements are generally very close to those from absorbance measurements, with the later slightly higher. Table 2.1 and Table 2.2 summarize these constants from fits performed according to equations (2.1) and (2.2).

It is clear from the concentration dependence of the observed rate constants that the action of GuHCl and TCA were both needed for cluster degradation. Assuming the holo-protein acts like a Lewis base B, a simplified pathway can be illustrated as the following scheme:

$$\text{Scheme 2.1:} \quad B^{-} + mG + nH^{+} \xrightleftharpoons[K]{k_{1}} BGmH^{+n} \xrightleftharpoons[H^{+}k_{2}]{\text{Product}}$$

G represents GuHCl; K is the dissociation constant of the intermediate complex $BGmH^{+n}$; m, n are coefficients; k1 and k2 are the simulated rate constants (as opposed to apparent rate constants) in two parallel processes to the falling apart of the cluster.

From

$$K = \frac{[B^{-}][G]^{m}[H^{+}]^{n}}{[BGmH^{+n}]}$$

and

$$[B] = [B'] + [BGmH^{+n}]$$
we have

\[ k_{\text{obs}} = \frac{[G]^m[H^+]^n(k1 + k2[H^+])}{K + [H^+]^n[G]^m} \]  

(2.3)

Fitting \( k_{\text{obs}} \) vs. \( H^+ \) concentration \([H^+]\) or GuHCl concentration \([G]\) according to equation (2.3) gives us values of the parameters that help us understand the mechanism. Figure

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>m</th>
<th>( k1 ) (s(^{-1}))</th>
<th>( k2 ) (s(^{-1}))</th>
<th>K (mol(^{n+m}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>4.001</td>
<td>8.769</td>
<td>0.0846</td>
<td>6.118</td>
<td>6.475E-4</td>
</tr>
<tr>
<td>sd(( \pm ))</td>
<td>0.002</td>
<td>0.209</td>
<td>0.0116</td>
<td>0.3130</td>
<td>0.829E-4</td>
</tr>
</tbody>
</table>

a: standard deviation

Table 2.3: Parameters in Scheme 2.1.

2.3 and figure 2.4 (A) show fitting of experimental data under four different conditions according to equation 2.3. Resulting parameters were averaged and listed in Table 2.3.

It is very interesting to notice that coefficient \( n \) has a value of 4, the same as the number of \( S^2^- \) ions in the 4Fe-4S cluster. This indicates not only the number of sites of protonation needed, but also the possible location of protonation that leads to dissociation of the cluster. \( m \) is more than twice as many as \( n \). \( k2 \) is 6.12 s\(^{-1}\), nearly seventy fold larger than \( k1 \), indicating the preference of cluster degradation over these two parallel pathways.
Figure 2.3: Dependence of cluster degradation on proton concentration. The observed rate constants from Table 1 were plotted vs. [H⁺]. 0.035mM HiPIP added to 2M GuHCl and 0.2 mM EDTA with different amount of TCA.
Figure 2.4: HiPIP cluster degradation. Dependence on guanidine hydrochloride concentration [GuHCl]. (A) Observed rate constants from Table 2 were plotted vs. [GuHCl]. (B) $\ln k_{\text{obs}}$ vs. [GuHCl]. 0.035 mM HiPIP (C.v.) was added to 1% TCA and 0.2mM EDTA.
Figure 2.5: Degradation of HiPIP—fluence of [EDTA]. 0.035 mM reduced HiPIP was added to a solution of 0.4% TCA with 2 MGuHCl and EDTA in 50 mM tris pH8.0. Absorbance of MLCT at 386 nm was monitored over time. [EDTA] changed from 0 mM to 1 mM.

Figure 2.6: Cluster degradation monitored by UV-visible spectroscopy. Dependence of rate constants on cluster oxidation state and [H+].
The equilibrium constant $K$, reflecting the backward dissociation of the intermediate complex, is almost negligible.

In order to further investigate the reversibility of this reaction, the influence of EDTA on cluster degradation was monitored using UV/Vis assays. EDTA was used in all the cluster disassembly experiments in order to chelate the released Fe ions to prevent the reverse reaction. Figure 2.5 showed that when the concentration of EDTA was changed from 1 mM to 0mM, the degradation reactions followed the same trace within experimental error. This tells us that cluster dissociation under these conditions are basically non-reversible, consistent with a very small $K$ value.

The effect of the cluster charge on the degradation process was studied by comparing the oxidized cluster and the reduced cluster. Figure 2.6 shows that when all other conditions are the same, the observed rate constant for the oxidized cluster is smaller than that for the reduced. The extra positive charge on the oxidized cluster obviously hinders the multiple protonation process, therefore slows down the rate of degradation.

<table>
<thead>
<tr>
<th></th>
<th>$k_1$(s$^{-1}$)</th>
<th>$k_2$(s$^{-1}$)</th>
<th>$n$</th>
<th>$K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
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<td>17.2</td>
<td>3.79</td>
<td>8E-7</td>
</tr>
<tr>
<td>Ox</td>
<td>0</td>
<td>10.6</td>
<td>4.05</td>
<td>3E-7</td>
</tr>
</tbody>
</table>

Table 2.4: Fitting results according to equation 2.3 for the same set of experiments: oxidized cluster vs. reduced cluster.
2.3.3 Variable Temperature Stopped-flow Fluorescence

Variable temperature stopped-flow fluorescence measurements were carried out to study the temperature dependence of the observed rate constants on cluster degradation and to determine the activation energy for cluster degradation. The insert in Figure 2.7 shows one of the traces in the temperature dependence experiments. The rate constants were obtained by fitting the trace to a single exponential. The reaction rate was found to increases as temperature increases. The intensity of the intrinsic tryptophan fluorescence strongly depends on temperature, but the rate of reaction should not be affected assuming the intensity change is homogeneous over time. The apparent rate constant \( k \) was obtained by fitting the kinetic trace to equation 2.4.

According to the Arrhenius equation,

\[
 k = A \cdot \exp(-E_a/RT)
\]

and

\[
 \ln k = (-E_a/R) \cdot 1/T + \ln A \quad (2.4)
\]

where \( k \) is the rate constant, \( A \) the proportional constant, \( E_a \) the activation energy, \( R \) the gas constant 1.987 cal\( \cdot \)K\(^{-1}\)\cdot mol\(^{-1}\), \( T \) is the absolute temperature (in K). The Arrhenius plot, \( \ln k \) vs. \( 1/T \), should be a straight line, as shown in Figure 2.7. From the slop one can calculate the activation energy \( E_a \). Here, \( E_a = 33.08 \) kcal\( \cdot \)mol\(^{-1}\).

Compared with the \( E_a \) of rubredoxin from \textit{Pyrococcus furiosus} (Cavagnero et al), which has a value around 60 kcal\( \cdot \)mol\(^{-1}\), the activation energy for the degradation of the 4Fe-4S cluster in HiPIP is much smaller.
Figure 2.7: Arrhenius plot for HiPIP degradation at pH2, monitored by stopped-flow fluorescence. 0.05mM HiPIP in 50 mM tris pH8.0 was mixed with 4M GuHCl, 2% TCA and 0.4mM EDTA in tris. Ex.280nm, Emission was collected by using a 300nm-400nm band pass filter. $k = A \exp(-E_a/RT)$. $R = 1.987 \text{ cal K}^{-1} \text{ mol}^{-1}$; $E_a = 33.08 \text{ kcal mol}^{-1}$. Insert: Fluorescence trace at 40.1°C, fitting to a single exponential.
2.3.4. Kinetic Studies by Freeze-Quench EPR.

Both electronic and fluorescence spectroscopy are sensitive probes for studying cluster disassembly. However, they are unable to detect changes in the electromagnetic properties of the cluster in the early stages of the reaction. Freeze-Quench electron paramagnetic resonance (EPR) is a very powerful technique for probing alterations of magnetic properties in fast phases. X-band EPR is especially informative in Fe-S cluster studies. The EPR signatures of the holo-HiPIP and that of the final degradation product, free aqueous Fe species, are all known. Therefore, the degradation process can be easily seen as starting from the oxidized HiPIP signal then turning into the isolated iron signal.

Figure 2.8 shows some snap shots into the cluster degradation process using the freeze-quench EPR technique. From the bottom spectrum to the top one, oxidized EPR signals in the time course of cluster degradation are clearly shown. The \( g = 2.01 \) signal is the signature for the integrated HiPIP 4Fe-4S cluster (Dunham, et al, 1991). It disappeared very rapidly after the protein was mixed with GuHCl and TCA. The 4Fe-4S cluster was converted into an almost EPR-silent state within about 80 milliseconds. This intermediate state has a very small amount of spin integral. Approximately 4 seconds after the degradation started, the characteristic single iron EPR signal at \( g = 4.3 \) (Hagen, 1992) started to grow in, indicating the release of single iron ions out of the protein pocket into the solution, and were chelated by EDTA and solvent molecules. The release of single iron ions was comparatively much slower than the previous processes, finishing 10 minutes after the degradation started.
Figure 2.8: Freeze-Quench EPR for HiPIP [Fe4S4] cluster degradation. 0.8 mM HiPIP was added to 4M GuHCl with 1% TCA and 4 mM EDTA. After incubation (time marked on the graph), the mixture was oxidized with 24 mM Ir^3+ before freezing. EPR parameters: microwave frequency 9.46 GHz, microwave power 2 mw; modulation frequency 100 kHz, amplitude 2.53 G; receiver gain 10^4; temperature 5K.
Plotting the integrated signal intensity vs. time enables us to fit the kinetic traces, as shown in Figure 2.9. The decrease of the $g = 2.01$ signal is a single exponential process, which can be fit according to equation 2.5. The increase of the $g = 4.3$ signal is biphasic, and were fit into two exponentials according to equation 2.6.

$$\text{Int}_{g=2} - \text{Int}_{\text{interm}} = A_1 \cdot \exp(-k_1 t) \quad (2.5)$$

$$\text{Int}_\infty - \text{Int}_{g=4.3} = A_2 \cdot \exp[-k_2(t-t_0)] + A_3 \cdot \exp[-k_3(t-t_0)] \quad (2.6)$$

$\text{Int}_{g=2}$ is the intensity of 4Fe-4S cluster signal; $\text{Int}_{\text{interm}}$ is the intensity of the intermediate state, which is close to zero; $A_1$ and $k_1$ are the magnitude and apparent rate constant in this decreasing phase. $\text{Int}_\infty$ and $\text{Int}_{g=4.3}$ are the increasing and the final intensity of single iron species; $A_2$ and $k_2$ are the magnitude and apparent rate constant of the first increasing phase, $A_3$ and $k_3$ corresponding to the second phase, $t_0 = 0.5$ seconds is the starting time of the $g = 4.3$ signal. All parameters are summarized in Table 2.5.

<table>
<thead>
<tr>
<th>$k_1$ (s$^{-1}$)</th>
<th>$A_1$</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$A_2$</th>
<th>$k_3$ (s$^{-1}$)</th>
<th>$A_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$168 \pm 7^a$</td>
<td>$75 \pm 1$</td>
<td>$0.13 \pm 0.11$</td>
<td>$18 \pm 5$</td>
<td>$0.002 \pm 0.001$</td>
<td>$32 \pm 12$</td>
</tr>
</tbody>
</table>

$a$: error in least square fitting.

**Table 2.5**: Kinetic parameters (observed rate constant and corresponding amplitude) from Freeze-Quench EPR experiments
Figure 2.9: HiPIP [Fe4S4] cluster degradation by freeze-quench EPR as monitored by the decrease of the g = 2.01 signal (A) and the increase of single Fe g = 4.3 signal (B). EPR conditions same as the legends in Figure 2.8
2.4 DISCUSSION:

Cluster degradation studied by UV/vis and fluorescence demonstrated the dependence of the observed rate constants on both the proton concentration and the GuHCl concentration. A minimum mechanism for these observations is proposed in Scheme 2.1. Multiple binding of GuHCl and multiple protonation were required for transition state formation. There are possibly two parallel pathways from the transition state to the final state (either the disappearance of the cluster absorption at 386 nm, or the fluorescence at 352 nm reaching the apo level). One direct pathway has a rate constant k1; the second pathway is characterized by an extra protonation, and its rate constant is k2. Scheme 2.1 leads to an apparent rate constant k_{obs} as expressed by equation 2.3.

2.4.1 Acid-Facilitated Cluster Degradation.

Table 2.3 lists simulated parameters for Scheme 2.1 according to equation 2.3. Parameter n, the number of protonation steps required to form the transition state complex, is 4.00, almost an exact number in all cases. This result provides a direct indication of the location of protonation, that is, either on the four inorganic sulfide ions of the cluster, or on the four cysteinyl sulfide that ligate the cluster. Bruice et al. in their studies of model 4Fe-4S clusters and of Fe-S proteins proposed two major pathways for acid facilitated cluster degradation. In one pathway (Bruice, et al., 1975), a single protonation through a hydrogen-bridged structure involving the filled d-orbitals above a face of the cluster provides a conjugate acid of the cluster. It then opens spontaneously and reversibly by scission of two Fe-S bonds that form adjacent edges of the cluster to
provide a new compound A. In turn A reversibly associates with a proton to provide its conjugate acid AH, which then spontaneously unfolds, in a reversible manner similar to the first scission, yielding species B. Compound B is then destroyed in rate-determining steps by a spontaneous and acid-catalyzed process. The second pathway involves protonation on the ligated cysteiny1 sulfur. In their kinetic studies, they also indicated that a single protonation (for oxidized HiPIP), two protonations (for reduced HiPIP (Maskiewicz and Bruice, 1977), or three protonations (for synthetic 4Fe-4S cluster in organic solvent (Bruice, et al., 1975) lead to the final disassembly of the 4Fe-4S cluster.

The model in Scheme 2.1 does not exclude step-wise protonations in the early stages, but the kinetic analysis presented here provides evidence for the first time that four protons are required for the formation of the transition state. It is important to notice that guanidine hydrochloride is included in our experiments. Therefore the degradation pathways are not expected to be the same.

2.4.2 Reversibility of cluster degradation reaction.

The backwards dissociation constant $K$ in Scheme 2.1 is extremely small, on the order of ten thousands. Since $K = k_r/k_f$, a very small $K$ means a very slow backwards decomposition compared with the fast forward formation of the transition state complex, which in turn indicates a largely non-reversible degradation reaction.

The final rate limiting steps in cluster degradation should be non-reversible also. The result of the EDTA dependence studies supports this conclusion. Within experimental error, no dependence on EDTA concentration was observed for the apparent rate constants.
Activation energy measurements provided further information for the feasibility of cluster degradation under the conditions used in the experiments. No other activation energy for the degradation of 4Fe-4S cluster has been reported so far. Few examples are found in other Fe cluster systems. Silvia Cavagnero et al. reported the unfolding activation energy of rubredoxin from *Pyrococcus furiosus* (Cavagnero, et al., 1997). Their reported $E_a$ is about 60 kcal mol$^{-1}$, twice as large as the one we observed. A lower activation energy could be the result of a less buried cluster in the case of HiPIP compared with rubredoxin, or because of the use of different degradation conditions. In any case, a lower activation energy for cluster degradation may be a major cause for less reversibility for HiPIP.

2.4.3 Stability of the Oxidized Cluster vs. the Reduced Cluster

If cluster degradation involves protonation, the charge on the cluster will have an influence on the rate of degradation. Figure 2.6 shows that oxidized clusters generally degrade slower as compared to reduced clusters under the same condition. This can be easily understood because there is an extra positive charge on the oxidized cluster, which makes it more difficult for protonation to occur, thus slowing down the reaction rate.

Interestingly, after fitting $k_{obs}$ vs. proton concentration according to equation 2.3, it is found that oxidation does not alter the general pathway of cluster degradation described in Scheme 2.1. The required number of protons is the same for both reduced and oxidized cluster within experimental error. The parameters reflecting the reaction rate, $k_2$, $k_1$ and $K$, do differ between these two, as seen in Table 2.4. Therefore, the
requirement of full protonation and the action of guanidine hydrochloride are both needed regardless of the oxidation state.

2.4.4 Contribution from Protein Backbone to the Cluster Stability

In previous studies of Fe-S cluster assembly, 16% trichloroacetic acid (TCA) was used, followed by redissolving the precipitates and dialysis, in order to make apo-protein for reconstitution (Cowan, et al, 1989). The 4Fe-4S cluster falls easily from the protein under this condition. However, the resulting solution turns cloudy spontaneously because of the aggregation of degraded protein, making this approach inappropriate for cluster disassembly studies by any optical techniques. This method was revised later to include 2 M guanidine hydrochloride (GuHCl). Consequently, TCA can be decreased to less than 1% for complete cluster degradation in several minutes. It turns out to be a great way for making apo-HiPIP in terms of convenience and efficiency. Also, by including GuHCl with TCA, the degradation intermediates and products are all clear in solution. Therefore, the process can now be monitored by using UV/vis, fluorescence, and other optical spectroscopic methods. GuHCl has been shown to cause some structural changes to the protein (Bertini, et al, 1997). However, we have found that GuHCl itself was unable to disassemble the cluster in a short time even at 8 M concentration.

The dependence of the observed rate constants on guanidine hydrochloride (Figure 2.4) points out the importance of protein structural changes in assisting cluster degradation. GuHCl is a widely used denaturant in protein unfolding experiments. The action is presumably due to its strong polarity, which can interfere with the hydrogen-
bonding network of the protein. The number of GuHCl molecules required for transition state complex formation is found to be about 9 (m = 9±2). It should be noticed that in the experiment GuHCl and TCA were added together. Scheme 2.1 also shows that GuHCl and TCA work together in a concerted manner. However it is possible that some structural change was first caused by GuHCl (Bertini, et al, 1997), which paves the way for cluster degradation by acid catalysis. Attempt to detect such changes by fluorescence was complicated by the fact that GuHCl quenches the protein fluorescence, which otherwise would increase upon partilly unfolding.

In Figure 2.4B, the natural log of the observed rate constants was plotted against GuHCl concentration. The increase in reaction rate with increasing guanidine hydrochloride can be attributed to a large extent to the opening up of the cluster pocket, making it easier for solvent molecules, including H_3O^+, to approach the 4Fe-4S cluster. These will decrease K and increase k1 and k2 in Scheme 2.1 at the same time, resulting in a larger k_{obs}. A generally linear form is found in other unfolding processes. The bending down at the high GuHCl concentration end is probably due to an approaching to saturation.

Our results demonstrate that in lowering the concentration of acid, GuHCl becomes necessary in cluster degradation. Neither of these is likely used in vivo. However, the requirement of partial protein unfolding for cluster disassembly indicates the involvement of enzymes in cluster degradation processes.
2.4.5 Events in the Cluster Degradation Pathway.

What happens to the cluster in the early stages in forming the transition state? Does the cluster fall apart in a single step from the transition state? Scheme 2.1 established a general kinetic frame for cluster degradation without specifically addressing these questions. Freeze-quench EPR experiments, on the other hand, reveal more about the events happened during the course of degradation. The disappearance of the 4Fe-4S cluster signal occurs within 0.1 seconds (Figure 2.8). The observed rate constant for this process is 168 s\(^{-1}\) (\(k_1\) in Table 2.5). This can not be related to the degradation process observed by UV-vis or fluorescence, which has an observed rate constant in the order of 0.1 s\(^{-1}\). The disappearance of the g=2.1 signal indicates a rapid change in the magnetic property of the cluster. In the light of later slow release of Fe ions (the appearance of the g=4.3 signal), and of much smaller observed degradation rate (Table 2.1 and 2.2), this fast process can only be attributed to the protonation stages, in forming the transition state complex.

Although the transition state complex requires four protons, it is likely that stepwise protonation occurred in this fast stage. The binding of one proton either to a sulfide or to a cysteiny1 sulfur will disrupt the integrity of the 4Fe-4S cluster and/or change the magnetic property of the cluster, thus causing the disappearance of the g=2.1 signal.

The slow release of Fe ions is another indication of the sites of protonation. Protonation can happen either on the ligated cysteiny1 sulfur or on the inorganic sulfide. Both will lead to the final release of H\(_2\)S under acidic condition as observed during apo preparation. However, protonation on the cysteiny1 sulfide would cause the cluster to lose
one important anchoring ligand, and a labile iron would argue for early release of single Fe ions before the formation of a fully protonated transition state complex. This is not consistent with experimental results. Therefore, we propose that under these experimental conditions, cluster degradation starts with structural relaxation caused by guanidine hydrochloride and TCA, followed by rapid step-wise protonations on the four inorganic sulfide ions of the 4Fe-4S cluster. The protonated cluster was then further exposed to solvent molecules including $\text{H}_2\text{O}^+$ with the help of guanidine hydrochloride, causing the release of $\text{HS}^-$ or $\text{H}_2\text{S}$ and the release of single Fe species. The simulated rate constant $k_1$ and $k_2$ in Scheme 2.1 may reflect the evolving of $\text{HS}^{-1}$ and $\text{H}_2\text{S}$, since an extra protonation is required in the $k_2$ pathway. Consistent with this model, the observed rate constants in UV-vis and fluorescence measurements are slightly larger than the observed rate constants for iron release obtained by freeze-quench EPR. There are two phases in the increase of single Fe signal. This indicates that iron release is also a step-wise process, and means that the 4Fe-4S cluster falls apart from the transition state complex in a step-wise manner.

2.5 SUMMARY

In this work, we have investigated the disassembly of the 4Fe-4S cluster in HiPIP using UV-vis, fluorescence, and freeze-quench EPR techniques. The results show that full protonation of the inorganic sulfide ions is required for the formation of the transition state, as the action of GuHCl on the protein structure in causing it to open up. Protonation happens very rapidly, and is likely to be in a step-wise manner. The collapse of the
transition state complex occurs in a much slower rate, with the release of HS'/H₂S and Fe ions. The release of single iron species appears to have two kinetic stages. Cluster oxidation state affect the rate of degradation. Under conditions used in our experiments, cluster degradation is largely irreversible.

REFERENCES:


Maskiewicz, R., and Bruice, T. C., *Biochemistry* 16, 3024 (1977)


CHAPTER 3

CLUSTER STABILIZATION BY AROMATIC RESIDUES.

THE AUTO REDUCTION PATHWAY IN PHE 66 MUTANTS

3.1 INTRODUCTION. THE ROLE OF THE AROMATIC RESIDUES AROUND THE 4FE-4S CLUSTER

High-potential iron proteins (HiPIP's) are a structurally well-defined class of iron-sulfur cluster proteins that afford a useful paradigm for structure-function studies of the role of the protein matrix in defining the chemistry and functional properties of the [Fe₄S₄]^{3+/2+} prosthetic unit (Carter et al., 1974a; Carter, 1977; Rayment et al., 1992), including the stability, redox chemistry, and metal lability of the 4Fe center, and a clear understanding of the molecular mechanisms (Agarwal et al., 1995; Li et al., 1996a,b). Several proposals have been made for the importance of the aromatic residues around the cluster, which include fine tuning of the unusually high reduction potential of HiPIP as a result of favorable bonding interactions with the cluster, maintenance of a low dielectric solvent-free environment, or involvement in electron exchange pathways (Carter et al., 1974b; Bertini et al., 1993; Mizrahi et al., 1980; Jensen et al., 1994). Residue Phe66 is of particular interest since it lies in close proximity to the cluster and is a nonconserved residue relative to the HiPIP from the bacterium *Rhodocyclus tenuis* (Rayment et al., 1996).
1992), which shows an isoleucine residue at that site. For this protein the midpoint potential is 50 mV lower than that of C. Vinosum HIPPI (Przysiecki et al., 1985). In this chapter, we characterize several conservative and nonconservative Phe66 mutants of C. Vinosum HIPPI. In spite of some perturbations of signals in the NMR and EPR spectra, the reduction potentials of these mutants display only minor perturbations relative to recombinant native. More importantly, we demonstrate a requirement for the aromatic ring to maintain the stability of the iron-sulfur cluster in the oxidized state. The oxidative instability observed for many of the mutant proteins is a consequence of the increased solvent accessibility of the cluster core. In the course of these studies, a novel HIPPI degradation intermediate has been identified and characterized as a Fe$_3$S$_4$ cluster. While the formation of [Fe$_3$S$_4$] centers has been reported for low-potential ferredoxin-like centers, arising from ferricyanide or air oxidation of the cluster (Thomson et al., 1981; Johnson et al., 1982; Bell et al., 1982; Moura et al., 1982), or from Cys mutations (Rothery & Weiner, 1991; Kowal et al., 1995), this is only the second reported example of the transient formation of a 3Fe cluster in a high-potential iron-sulfur protein and differs from the degradative intermediate previously reported from our laboratory for the Cys77Ser mutant (Agarwal et al., 1996) which involved a directly coordinating residue.

3.2 EXPERIMENTAL METHODS

3.2.1 Enzymes, Chemicals, and Bacterial Strains.

Restriction and modifying enzymes were purchased from BRL (Life Technologies, MD), and DTT and IPTG were obtained from Boehringer Mannheim (Indianapolis, IN). Escherichia coli strain XL-1 blue was used for routine site-directed
mutagenesis and plasmid storage, strain RZ1032 was used for preparation of single-strand DNA for mutagenesis, and strain BL21 (DE3) plys S served as a host for protein expression. Helper phage VCS M13 for rapid single-strand rescue of DNA from the pET21d(+) phagemid was obtained from Stratagene (La Jolla, CA). General molecular biology procedures were followed from Sambrook et al. (1989).

3.2.2 Plasmid Construction, Site Directed Mutagenesis, and Nucleotide Sequencing.

Construction of the plasmid pET21d-(+)-HIPIP, encoding the entire gene region for *C. Vinosum* HIPIP, has been described elsewhere (Agarwal et al., 1993). Mutagenesis was performed according to the method of Kunkel et al. (1987). The oligonucleotide primer for mutagenesis was 27-mer in length and was designed with redundancies at the mutation site to allow isolation of discrete plasmids bearing point mutations. Specifically, TTC (Phe) was engineered to TAC (Tyr), AGC (Ser), TGC (Cys), AAC (Asn), CGC (Arg), and CAC (His); however, only plasmids bearing the first four mutations listed were identified during exhaustive screening. Uracil-enriched template pET21d(+)-HIPIP was obtained from the dut(-) ung(-) *E. coli* strain RZ1032 and grown in 2 x TY medium with the helper phage VCSM13. All mutations were finally characterized and confirmed by DNA sequencing according to the Sequenase protocol (United States Biochemical Corp., Cleveland, OH).
3.2.3 Recombinant Protein Purification

Cultures were grown, expression was induced by IPTG, and protein was purified as previously described for recombinant native (Agarwal et al., 1993). When necessary, further purification was carried out by FPLC on a Mono Q column (1 x 5 cm) using nondenaturing conditions. Optimal separation of bands was obtained by a gradient method, using two stocks of degassed phosphate buffer, at pH 7.5 (A, 10 mM phosphate; B, 10 mM phosphate, 500 mM NaCl). The total running time was 20 min (3 min with 0% B and 17 min from 0 to 100% B).

3.2.4 Electronic Spectra of the Phe66 Mutant Protein

Kinetic data were obtained at an ambient temperature of 295 K by monitoring the decrease in absorbance at 500 nm for oxidized mutant proteins by use of a Hewlett-Packard 8452A diode array spectrophotometer. Typical experimental conditions included a solution of 20 μM HiPIP in 10 mM sodium phosphate buffer with 0.1 M NaCl (pH 6.0). The protein concentration was determined by use of the extinction coefficients for the reduced native protein, \( \varepsilon_{382} = 41.3 \text{ mM}^{-1} \text{cm}^{-1} \) and \( \varepsilon_{388} = 16.1 \text{ mM}^{-1} \text{cm}^{-1} \). This is justified by the observation that the A282/A388 ratio was similar for the native and mutant HiPIP’s (Table 3.1). Approximately 100 μ M oxidant was added from a concentrated stock solution and the assay started immediately after mixing by inversion. The trace of an assay (OD decay at 500 nm) was then fit to a single-exponential curve and the first-order rate constant was obtained from the fitting. Rate constants are summarized in Table 3.2.
3.2.5 Electrochemistry of Phe66 Mutants

Protein reduction potentials were determined by a spectrochemical titration method (Dutton, 1978). To a quartz cuvette was added 1 μL of a protein stock solution, 100 μL of Fe(CN)$_4^{2-}$ (100 mM stock), and 800 μL of 50 mM potassium phosphate buffer (pH 7.0). Optical spectra were measured on a Hewlett-Packard 84522A spectrophotometer (run by software from On-Line-Instrument Systems). The solution absorbance at 500 nm was typically ca 0.1, which was then equilibrated for at least 15 min at the desired temperature. Neither Fe(CN)$_4^{2-}$ nor Fe(CN)$_6^{3-}$ absorb significantly at 500 nm, even at very high concentration. After measuring the absorbance of the solution, it was then titrated with 1 μL increments of Fe(CN)$_6^{3-}$ (200 mM stock solution) with subsequent reading of the absorbance at 500 nm after each addition of oxidant. After addition of at least 15 increments of oxidant, 10 μL increments of Fe(CN)$_6^{3-}$ were added until the fully oxidized form of the protein was reached, when no further change in absorbance was noted at 500 nm. The solution potentials were determined after completion of the experiment in order to minimize the time available for protein degradation. Volume changes after each addition were corrected for and the relative concentrations of oxidized and reduced protein were estimated from the limiting absorbance for fully reduced (A$_{red}$) and fully oxidized (A$_{ox}$) HiPIP and published extinction coefficients (Bartsch, 1978). The solution potential ($E_s$) was calculated from the Nernst equation and the known [ferricyanide]/[ferrocyanide] ratio. The influence of ionic strength, pH, and temperature on the ferro-/ferricyanide couple were accounted for according to the literature (O’Reilly, 1973). The midpoint reduction potential ($E_m$) for
the protein was determined from a Nernst plot of the solution potential \( (E_s) \) and the absorbance readings using the equation 
\[
E_s = E_m + \left( \frac{RT}{nF} \right) \ln\left( \frac{A_{\text{red}} - A_{\text{obs}}}{A_{\text{obs}} - A_{\text{ox}}} \right)
\]
(Dutton, 1978), where all other symbols have their usual meanings.

3.2.6 NMR Spectroscopic Characterization of Mutants

1D and 2D COSY NMR spectra of Phe66 mutants were acquired as previously described (Agarwal et al., 1995; Li et al., 1996). 1D spectra were acquired using a SUWEFT pulse sequence to optimize signal intensities and resolution of the paramagnetically shifted resonances (Inubushi & Becker, 1983), while effective solvent suppression was maintained. Samples dissolved in H\(_2\)O contained 10% (v/v) D\(_2\)O for the lock. 2D COSY experiments were acquired using the standard pulse program COSYPR with solvent suppression.

For the \(^1\text{H} / ^2\text{H}\) exchange experiments (Li et al., 1996b), the \(^{15}\text{N}\)-labeled native and mutant HiPIP samples were lyophilized and then redissolved in D\(_2\)O immediately before NMR data acquisition. The \(^1\text{H} / ^2\text{H}\) exchange experiments were performed on a Bruker DMX 600 instrument using a standard \(^1\text{H} - ^{15}\text{N}\) HMQC pulse sequence (Palmer et al., 1991; Kay et al., 1992; Schleucher et al., 1993). Each 2D HMQC data set was collected in a time frame of less than 12 min for a spectrum defined by a 2K \((t2)\) x 256 \((t1)\) data matrix, with four scans for each \(t1\) increment and an initial 16 dummy scans sufficing for the entire experiment. As a result of the large memory and digital filter capabilities of the DMX instrument, the time typically spent on collecting dummy scans before \(t1\) data collection and on saving each \(t1\) data set was eliminated. The delay time that distinguished each experiment was calculated from the time when the lyophilized
sample was dissolved in D$_2$O to the midpoint of each experiment. Cross-peak intensities were found to diminish with increasing delay time.

3.2.7 Electron Transfer Rate Determination by 2D EXSY

The use of 2D NMR for kinetic studies was first proposed by Jeener et al. (1979). The main feature of a quantitative 2D EXSY experiment is the relationship between the intensity of a crosspeak and the rate constants defining the chemical exchange. Based on the work of Macura and Ernst (1980), the integrated intensities of the two-dimensional absorption peak can be related to chemical exchange rate constants through a rate (or relaxation) matrix $R$, where the off-diagonal elements are $R_{ij} = -k_{ji}$, the diagonal elements are $R_{ii} = T_{1,i}^{-1} + \Sigma k_{ii}$, and $T_{1,i}$ is the spin-lattice relaxation time of nuclei in site $i$ in the absence of chemical exchange. The rate matrix $R$ can be solved to obtain the rate constants for chemical exchange (Perrin et al., 1984, 1990). The cross-peak intensities can be measured from the volume integral under the cross-peaks if the spectra were obtained in the phase-sensitive mode.

\[
\begin{align*}
    k_2 & \\
    \text{ox}_2 + \text{red}_1 & = \text{red}_2 + \text{ox}_1 \\
    k_2
\end{align*}
\]  
\hspace{1cm} (3.1)

\[
\begin{align*}
    k_f & \\
    \text{ox}(A) & = \text{red}(B) \\
    k_f
\end{align*}
\]  
\hspace{1cm} (3.2)

For a self-exchange reaction (1), the second-order rate constants $k_2$ and $k_{-2}$ can be estimated by assuming a pseudo-first-order reaction (3.2), where the forward rate
constant $k_f = k_2[\text{red1}]$, and the reverse rate constant $k_r = k_3[\text{ox1}]$. The first-order rate constants $k_f$ and $k_r$ are estimated from the experiment, and $k_2$ and $k_3$ are evaluated from the variation of $k_f$ and $k_r$ with the concentration of redox species.

3.2.8 EPR Characterization of Mutants

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 vs Cr thermocouple. Experimental parameters are listed in footnotes to the tables and figure legends. Spin quantitations were carried out under nonsaturating conditions using native oxidized HiPIP as a reference. Data for both samples and standard were obtained using the same buffer conditions. Spectra were obtained with an anaerobic solution of 0.7 mM protein in 10 mM sodium phosphate buffer (pH 7.0) at a variety of temperatures and power levels. EPR spin quantitation of intermediates produced during decomposition of the oxidized mutant HiPIP's (such as that shown in Figure 3.9) was performed using the Aasa-Vanngard equation (Aasa & Vanngard, 1975; Fee et al., 1975), relative to the standard oxidized form, and half-power saturation levels ($P1/2$) were estimated by use of the standard empirical equation

$$S = K[P/(1 + P/P_{1/2})^b]^{1/2} \quad (3.3)$$
FIGURE 3.1: Optical spectra of native and mutant derivatives of reduced C. Vinosum HiPIP. Spectra were taken in 10 mM potassium phosphate buffer containing 100 mM NaCl (pH 7). The spectra in order of decreasing intensity at 388 nm correspond to rec-native, Phe66Cys, Phe66Asn, Phe66Tyr, and Phe66Ser.
where $S$ is the signal amplitude, $P$ is the applied power, $K$ is a proportionality factor, and $b$ is the inhomogeneity parameter (Innes & Brudvig, 1989). The latter typically has a value between 1 and 3 and can be approximated as 1 in the limit of inhomogeneously broadened lines.

3.3 RESULTS

3.3.1 Protein Expression and Spectroscopic Characterization of Mutants.

A 27-mer oligonucleotide mutagenic primer, 5'-CAGTTTACCGGG-(TC)(AGT)AAGTTGGCAACC-3', was designed with redundancies to provide a range of aromatic, hydrophobic, hydrophilic, and charged sidechain residues. Four mutants (Phe66Ser, Cys, Tyr, Asn) were identified and expressed in *E. coli* as holoproteins and isolated in the reduced form following standard protocols (Agarwal et al., 1993). Expression yields for mutants (~10 mg/L) were considerably lower than for recombinant native protein (~30 mg/L). In the reduced state, the mutants were stable and could be maintained as frozen stocks (-20 °C) for many weeks.

The optical spectra for reduced native and mutant proteins are similar, with two absorbance bands at 282 and 388 nm (Table 3.1 and Figure 3.1). Following addition of a 5-fold excess of potassium ferricyanide, the mutant proteins formed brown solutions characteristic of the oxidized form (Bartsch, 1978).
Table 3.1: Ratios of Extinction Coefficients for Reduced Rec-native and Mutant HiPIP’s

<table>
<thead>
<tr>
<th></th>
<th>rec-native</th>
<th>Phe66Tyr</th>
<th>Phe66Cys</th>
<th>Phe66Ser</th>
<th>Phe66Asn</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{282 \text{ nm}}$</td>
<td>2.36</td>
<td>2.88</td>
<td>2.69</td>
<td>2.76</td>
<td>2.71</td>
</tr>
<tr>
<td>$A_{388 \text{ nm}}$</td>
<td>$^{a}$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^{a}$ For the reduced rec-native protein, $\varepsilon_{282} \sim 41.3 \text{ mM}^{-1} \text{ cm}^{-1}$ and $\varepsilon_{388} \sim 16.1 \text{ mM}^{-1} \text{ cm}^{-1}$ (Bartsch, 1978).

3.3.2 The Auto Reduction Phenomena

For oxidized protein, after rapid removal of excess oxidant by passage through a short G-25 column (0.8 cm x 2.5 cm), the optical spectra are generally found to be similar to oxidized native with a shoulder on the principal cluster absorbance at around 420-450 nm; however, with the exception of the Phe66Tyr derivative, the spectra for the oxidized mutants are observed to revert to the reduced form over a period of 20 min to 2 h (Figure 2) and formation of some reduced Phe66Asn mutant is usually observed immediately following gel filtration chromatography. The Phe66Tyr mutant slowly returns to the reduced state in a single-exponential process with a first-order rate constant of 0.002 min$^{-1}$. The Phe66Cys and Phe66Ser mutants undergo autoreduction more rapidly with first-order rate constants of 0.03 and 0.09 min$^{-1}$, respectively. The Phe66Asn mutant demonstrates the most rapid autoreduction rate of $\sim 0.17 \text{ min}^{-1}$.
FIGURE 3.2: Kinetic decay trace of oxidized mutant HiPIP (Phe66Ser) in the presence of a 10-fold excess of $K_2IrCl_6$ oxidant. The protein was dissolved in 10 mM potassium phosphate buffer containing 100 mM NaCl, pH 7. (a) Optical spectral changes as a function of time from the oxidation event showing the change from oxidized (top) to reduced (bottom) protein. (b) Change in the optical density at 500 nm as a function of time.
Table 3.2: Autoreduction Rate Constants for Oxidized HiPIP at 295 K

Table 3.2 shows that for either oxidant the autoreduction of the mutant proteins follows the same trend, with Phe66Asn being the most unstable and Phe66Tyr the most stable. Although minor differences are observed in the rate constants for the reactions initiated by the two oxidants used in these studies, the results obtained for each lie in the same range. In the presence of hexachloroiridate a “lag phase” is seen at the beginning of the autoreduction process, which is not obvious in the case of ferricyanide oxidation. The origin of this “lag phase” is not yet clearly defined but most likely reflects oxidation, by the more powerful IrCl$_6^{2-}$ oxidant, of reducing equivalents that are released during the initial stages of cluster degradation.

In the oxidized state, the mutant proteins were generally unstable over a time frame of 1-2 h, following a stability order Phe66Tyr >> Ser > Cys > Asn. The origins of this instability are described later. A number of control experiments were carried out to characterize the origin of the autoreduction phenomenon. The influence of oxidant potential was examined by comparing the effect of two species, [Fe(CN)$_6$]$^{3-/4-}$ ($Em = 420$
mV) and \([\text{IrCl}_6]^{2-}\) \((E_m=890\text{ mV})\), on the stability of oxidized HiPIP Phe66Ser, Phe66Cys, and Phe66Asn mutants. Autoreduction was observed with either oxidant, even in the presence of up to 20-fold excess of reagent. Given the tendency of the protein to undergo reduction, even in the presence of excess oxidant, we considered the possibility that the reducing equivalent might originate from an internal source. As described later, the reduction potentials for the mutant proteins are close to that of native, and so the absence of such an internal oxidation for the native protein would have to be ascribed to kinetic factors. We regarded it as unlikely that the electron donor would be the residue at position 66, since one might expect the Phe66Cys mutant to show a faster autoreduction rate as a result of the more effective thiol reductant.

A third possibility, and one that appears consistent with the available data, follows from the apparent hydrolytic instability of the oxidized cluster in most of the mutant HiPIP's. The release of ferrous ion and sulfide provides reducing equivalents for the remaining oxidized protein, giving rise to an apparent autoreduction pathway. The evidence for this proposal will be presented later in this chapter.

3.3.3 Reduction Potentials

The potentials for each of the mutants were found to be higher than that of the native. Mutant Phe66Tyr has a potential very close to native, while Phe66Ser and Phe66Cys are moderately \((\sim 15\text{ mV})\) higher. The most significant change was found for the Phe66Asn mutant with an increase of \(~ 40\text{ mV}\) relative to the native protein.
3.3.4 1D and 2D NMR Studies of Mutants

In 1D NMR spectra of mutants, resonances from hyperfine-shifted protons in the reduced (Figure 3.3) and oxidized (Figure 3.4) mutants show some variation relative to the native spectrum, although shifts in resonance positions were typically <1 ppm. The most prominent change observed in these spectra includes the upfield shift of the $\tilde{\alpha}$-CH resonance for Cys77 in the Phe66Tyr mutant from 12.73 to 10.95 ppm. Relative to the magnitude of the chemical shift changes for other resonances, this shift indicates a specific perturbation to Cys77, either in the form of a minor change of dihedral angle of the H-C-S-Fe bond, as recently defined by Bertini et al. (1995a) and/or by formation of a hydrogen bond from the Tyr hydroxyl to the sulfur center. A structural analysis by computer graphics indicates that the latter is a reasonable possibility, although it is difficult to distinguish between these two options. Perturbation of the remaining hyperfine-shifted signals suggests that most of these shifts arise from minor perturbations to the H-C-S-Fe dihedral angles (Bertini et al., 1995a). There appears to be a minimal perturbation of the electronic structure of the cluster, and changes in the spectrum arise from local steric effects and polarity changes that are specific for individual Cys protons. The temperature dependence of the shifted signals was examined from 5 to 35 °C and was found in all cases to be similar to that previously reported for native HiPIP with each resonance showing anti-Curie behavior (Bertini et al., 1992; Banci et al., 1993).

Over a period of 1-2 h, the spectra of oxidized mutant HiPIPs (Phe66Cys, Phe66Ser, Phe66Asn) were observed to change to the reduced form. No other hyperfine-shifted signals were observed, although new signals were observed in the diamagnetic
Figure 3.3: 1D NMR spectra of reduced HiPIP Phe66 mutants in D$_2$O.
Figure 3.4: 1D NMR spectra of oxidized HiPIP Phe66 Mutants in D$_2$O.
region that are reminiscent of apo-HiPIP. Integration of the shifted resonances indicated that a substantial fraction of the holoprotein (~ 20%) had degraded. The extent of degradation depended on both the delay time and the amount of oxidant added. More extensive degradation was observed after longer times and in the presence of larger amounts of oxidant.

Cross-peak patterns for the NH-C$_{\alpha}$H fingerprint and aromatic regions of standard 2D COSY maps taken in the diamagnetic region for Phe66 mutants were similar to those for native HiPIP (Figure 3.5). Minor changes in chemical shift were evident for some resonances in the fingerprint region, although the aromatic region shows a very similar pattern of cross-peaks. Spectral perturbations were more prominent for the Phe66Ser and Phe66Asn mutants. This affords some insight on the origin of the instability of the oxidized cluster inasmuch as the structural perturbations appear to follow the order Phe66Asn > Phe66Ser > Phe66Cys > Phe66Tyr ~ rec-native, which is consistent with the kinetic data on the stability of the oxidized mutants.

3.3.6 HSQC NMR Spectra Reveal Changes in Solvent Accessibility

Figure 3.6 shows a collection of $^1$H-$^{15}$N HSQC spectra obtained over a series of time intervals for each mutant and native HiPIP. The time (in minutes) marked on each spectrum corresponds to the midpoint time for the acquisition of that spectrum (each spectrum takes ~ 10 min to acquire) after the $^2$H/$^1$H exchange was initiated. Crosspeak assignments have previously been made for native HiPIP (Li et al., 1995; Banci et al., 1995). While there is some ambiguity for assignment of the mutant proteins
Figure 3.5: 2D COSY NMR spectra of reduced HiPIP Phe66 mutants in D$_2$O.
by inspection, a simple qualitative comparison yields significant insight on structural variations over this series of protein derivatives.

Both the native and Phe66Tyr proteins show significantly slower exchange of backbone amide protons relative to the Phe66Ser, Phe66Cys, and Phe66Asn mutants. Approximately 15 min after the exchange reaction has begun, more than half of the total NH protons still remain in the native and Phe66Tyr spectra, while less than a quarter of the cross-peak signals appear in the other three mutants. The cross-peaks observed in the 15 min spectra of the Phe66Ser, Phe66Cys, and Phe66Asn mutants are mostly those that remain in the 172 min spectra of the native and Phe66Tyr proteins. It is clear, therefore, that mutation of Phe66 to Ser, Cys, or Asn leads to an increase in solvent accessibility of the protein, especially for residues in the neighborhood of the amino terminus.

Three tryptophan residues are located in the aromatic pocket for the iron-sulfur cluster. It is noted for the Phe66Ser, Phe66Asn, and Phe66Cys mutants that one of the N \(^{1}H\) signals lying at about (10, 129) ppm for either Trp60 or Trp76 (Morgan et al., 1984) and the N \(^{1}H\) signal of Trp80 at (10.6, 132) ppm in the Phe66Cys mutant do not show up even in the 15 min spectra, indicating that these protons are in fast exchange.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>rec-native</th>
<th>Phe66Tyr</th>
<th>Phe66Cys</th>
<th>Phe66Ser</th>
<th>Phe66Asn</th>
</tr>
</thead>
<tbody>
<tr>
<td>(g_{\perp})</td>
<td>2.125</td>
<td>2.126</td>
<td>2.118</td>
<td>2.119</td>
<td>2.120</td>
</tr>
<tr>
<td>(A_{\perp}(G))</td>
<td>15</td>
<td>14</td>
<td>15</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>(g_{//})</td>
<td>2.038</td>
<td>2.038</td>
<td>2.034</td>
<td>2.033</td>
<td>2.034</td>
</tr>
<tr>
<td>(A_{//}(G))</td>
<td>18</td>
<td>18</td>
<td>16</td>
<td>14</td>
<td>16</td>
</tr>
</tbody>
</table>

*Parameters were obtained by simulation as shown in Figure 5.*

**Table 3.3:** EPR Parameters for the Major Components of oxidized Native and Phe66 Mutants
FIGURE 3.6: $^1$H-$^{15}$N HSQC spectra of rec-native and mutant HiPIP's. Spectra are taken at three time intervals after the addition of D$_2$O to a sample that has been lyophilized from a buffered H$_2$O solution. The time lapse to the middle of the 10 min acquisition period is given immediately after the descriptor for the spectrum.
Figure 3.6
FIGURE 3.7: EPR spectra of oxidized rec-native and mutant HiPIP obtained with 0.7 mM protein in 20 mM potassium phosphate buffer (pH 7). The samples were frozen in liquid nitrogen immediately following oxidation with ferricyanide ion. Experimental conditions were as follows: microwave frequency 9.45 GHz; modulation frequency 100 kHz; modulation amplitude 1 G; microwave power 1.2 mW; temperature 15 K.
3.3.7 EPR of Mutant and Deuterium-Labeled Proteins

In common with the native protein, reduced Phe66 HiPIP mutants are EPR inactive. However, immediately after oxidation, these derivatives display EPR features (Figure 3.7) that are similar to the native protein, with a major axial component \( g_{\parallel} = 2.03 \), \( g_{\perp} = 2.12 \) and evidence of a minor component with \( g = 2.07 \) that has previously been ascribed to HiPIP solution species that differ in either their aggregation state or electronic configuration (Bertini et al., 1992; Banci et al., 1993; Antanaitas & Moss, 1975; Dunham et al., 1991). When ferricyanide was used as an oxidant, the Phe66Cys and Phe66Ser mutants demonstrated additional fine features near \( g_{\perp} = 2.12 \), which could be simulated by including an additional minor component (Table 3.3 and Figure 3.8). These features disappeared gradually after oxidation. When hexachloroiridate was used as oxidant, the line width of the \( g_{\perp} \) signal was found to be narrower than that obtained by ferricyanide oxidation, while additional fine features were also observed near \( g_{\parallel} = 2.03 \) for native protein and the Phe66Tyr mutants. For native and mutant proteins, the EPR signals were not observable at temperatures above 60 K.

With the exception of the Phe66Tyr mutant, which proved to be fairly stable, the oxidized mutant EPR signals were found to disappear when the sample was maintained at ambient temperatures after oxidation. However, a new axial signal, almost isotropic with \( g \sim 2.01 \) (\( g_{\parallel} = 2.00 \), \( g_{\perp} = 2.02 \)), was observed to grow in for the Phe66Cys, Phe66Ser, and Phe66Asn mutants (Table 3.4 and Figure 3.9). Subsequently, this signal was also observed to decay. The time frame for appearance of the signal was shorter than 90
that for formation of the autoreduced protein for each mutant. This signal was weaker than that for the starting oxidized form and integrated to ~ 13% of the latter. The signal could not be detected at temperatures above 30 K. We had earlier speculated on the possibility of an internal reduction pathway to rationalize the autoreduction reaction. It seemed possible that this $g = 2.01$ signal might be related to such a process since it is similar to the signal obtained for a cysteiny1 radical species formed in *Azotobacter Vinelandii* ferredoxin oxidation, (Hu et al., 1994). If this was indeed the case, it would most likely be one of the cysteine ligands to the $[\text{Fe}_4\text{S}_4]^\text{2-}$ cluster since it appears in all three of the Phe66Cys, -Ser, and -Asn mutants. However, organic radical EPR signals can be seen at higher temperatures, and in the case of the cysteiny1 radical species identified in Fd I, the signal was observed at temperatures up to 77 K (Morgan et al., 1984, 1985). In contrast, the signal that is reported here, and shown in Figure 3.9, was not observed at temperatures greater than 30 K. Furthermore, no significant line-broadening was observed in cysteine-$d_2$-labeled mutant HiPIP's (Figure 3.8), expected of a cysteiny1 radical, and so other possibilities were considered. In particular, the $g = 2.01$ signal is very similar to the $[\text{Fe}_3\text{S}_4]^\text{2+}$ cluster signal observed in oxidized ferredoxin (Emptage et al., 1980; Papaefthymiou et al., 1987) and mutants of DMSO reductase (Rothery & Weiner, 1991). Figure 3.10 shows typical EPR power saturation plots for oxidized mutant HiPIP taken immediately after oxidation, corresponding to $[\text{Fe}_4\text{S}_4]^\text{3+}$, and 48 h after oxidation (incubating at 4 °C) to allow a buildup of the intermediate species. The signal intensity, $S$, was plotted against the power, $P$, and the data fit by use of eq 3.3 (Beinert & Orme-Johnson, 1967; Innes & Brudvig, 1989). The half-power saturation, $P_{1/2}$, is defined by the
FIGURE 3.8: (a) EPR spectrum of Phe66Ser HiPIP obtained immediately after oxidation. The mutant protein was labeled with deuterated Cys-d2, however, deuteration is not the origin of the fine splitting on the lower field signal. Sample preparation and conditions were similar to that described in the legend to Figure 4. (b) Computer simulation of the spectrum (see Table 3 for the simulation parameters). The EPR spectrum was obtained with a microwave frequency of 9.458 GHz, a modulation frequency of 100 kHz, a modulation amplitude of 4 G, a microwave power of 1.3 mW, and a temperature of 15 K.
turning point of the curve, and power saturation parameters for the fitted data are summarized in Table 3.5. Optimal fits were obtained with a $b$ value close to 1, corresponding to the inhomogeneous limit often observed for signals from Fe-S centers. A $P_{1/2}$ of 16 mW was determined for the signal observed from the immediately oxidized HiPIP, while the intermediate decay signal shows very different power saturation behavior. The lowest power used in this experiment is very close to the saturation limit of the autoreduced signal, which is below 400 $\mu$W and is again characteristic of oxidized Fe$_3$S$_4$ clusters (Rothery & Weiner, 1991; Rupp et al., 1978, 1979).

<table>
<thead>
<tr>
<th></th>
<th>Phe66Cys</th>
<th>Phe66Ser</th>
<th>Phe66Asn</th>
</tr>
</thead>
<tbody>
<tr>
<td>$g_{\perp}$</td>
<td>2.026</td>
<td>2.028</td>
<td>2.030</td>
</tr>
<tr>
<td>$A_{\perp}$ (G)</td>
<td>10</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>$g_{\parallel}$</td>
<td>2.002</td>
<td>2.006</td>
<td>2.008</td>
</tr>
<tr>
<td>$A_{\parallel}$ (G)</td>
<td>23</td>
<td>24</td>
<td>21</td>
</tr>
</tbody>
</table>

*Parameters were obtained by simulation as shown in Figure 6.*

**Table 3.4:** EPR Parameters for the Intermediate Observed during Decay of Oxidized Ph66 Mutants$^a$

In a valence-localized model we may assume loss of the ferrous ion, yielding an oxidized [Fe$_3$S$_4$]$^{1+}$ core with $S = 1/2$. This proposal explains the appearance of the Fe$_3$S$_4$ cluster signal but does not account for the remainder of the sample, since only ~ 13% of the 3Fe cluster is transiently formed on the basis of EPR integration. Also, there
FIGURE 3.9: (a) EPR spectrum of the intermediate formed following oxidation of Phe66Cys HiPIP. The sample, containing 0.7 mM protein in 20 mM potassium phosphate buffer (pH 7), was allowed to remain at room temperature for several hours following oxidation with ferricyanide before freezing in liquid nitrogen. (b) Computer simulation of the spectrum (see Table 3.4 for the simulation parameters). The EPR spectrum was obtained with a microwave frequency of 9.456 GHz, a modulation frequency of 100 kHz, a modulation amplitude of 1 G, a microwave power of 1.2 mW, and a temperature of 15 K.
FIGURE 3.10: (a) EPR power saturation study on the signals from Phe66Asn HIPIP immediately after oxidation. The EPR spectrum was obtained with a microwave frequency of 9.455 GHz, a modulation frequency of 100 kHz, a modulation amplitude of 0.5 mT, a receiver gain of $10^4$, and a temperature of 15 K. (b) EPR power saturation study on the signals from Phe66Asn HIPIP after formation of the decay immediate following oxidation. The EPR spectrum was obtained with a microwave frequency of 9.457 GHz, a modulation frequency of 100 kHz, a modulation amplitude of 5 G, a receiver gain of $10^4$, and a temperature of 15 K.
is no evidence for the presence of a [Fe$_3$S$_4$] cluster protein intermediate in the NMR spectra of the autoreduced sample, consistent with our interpretation of this center as a transient intermediate, which forms and subsequently decays. Minor peaks are observed at earlier stages of the reduction pathway; however, these are too weak to be characterized with any certainty. These observations are fully consistent with the reaction pathway summarized in Scheme 3.1, where degradation of the cluster in step 1 provides

**Scheme 3.1:**

\[
\begin{align*}
\text{HP}_{\text{ox}}[\text{Fe}_3\text{S}_4]^{3-} & \xrightarrow{-\text{Fe}^{2-}} \text{HP}_{\text{ox}}[\text{Fe}_3\text{S}_4]^{-} \\
\text{HP}_{\text{apo}} + 3\text{Fe}^{3-} + 4\text{S}^{2-} & \quad (1) \\
\text{HP}_{\text{ox}}[\text{Fe}_4\text{S}_4]^{3-} & \xrightarrow{\text{Fe}^{2-}/\text{S}^{2-}} \text{HP}_{\text{red}}[\text{Fe}_4\text{S}_4]^{2+} \quad (2)
\end{align*}
\]

the reducing equivalents (S$^{2-}$ and Fe$^{3+}$ ions) that result in reduction of the remainder of the sample in step 2. The relative rates of these reaction steps remain to be defined. Cluster degradation to form apoprotein was quantitated by comparison of integrations of 1D proton NMR spectra, and by the appearance of NMR resonance from apo-HiPIP, and appears to proceed by way of the [Fe$_3$S$_4$]$^+$ center.

### 3.4 DISCUSSION

#### 3.4.1 Influence of Mutations on the Electromagnetic Properties of the Cluster

A variety of nonconservative mutations have been made at residue Phe66 of *C. vinosum* HiPIP, a paramagnetic iron-sulfur protein. These mutations show only a small influence on the cluster reduction potential and result in a general increase in $Em$. The
Table 3.5: EPR Power Saturation Parameters for the Major Component EPR Signals of Oxidized Phe66Asn Mutant

<table>
<thead>
<tr>
<th></th>
<th>initial signal</th>
<th>intermed signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_{1/2}$ (mW)</td>
<td>16</td>
<td>0.4</td>
</tr>
<tr>
<td>$K$ (mW$^{1/2}$)</td>
<td>$1.36 \times 10^4$</td>
<td>$3.3 \times 10^5$</td>
</tr>
<tr>
<td>$b$</td>
<td>1.0</td>
<td>1.2</td>
</tr>
</tbody>
</table>

$^a$ Parameters were obtained by fitting to the standard equation, $S = K[P/(1 + P/P_{1/2})^b]^{1/2}$.

The largest change was observed following replacement of Phe with the amide side chain of Asn (Soriano et al., 1996). Significant perturbations were also observed for the shifted resonances in the $^1$H NMR spectrum of this mutant, although these changes were specific to the Cys63 residue coordinated to the cluster and did not indicate a general perturbation of the electronic structure of the cluster. It is likely that the larger than usual ($\sim 40$ mV) increase in $E_m$ for Phe66Asn reflects either minor structural changes in the active site and/or the influence of the amide dipole. Although the Phe66Asn mutant shows the most significant increase in solvent accessibility, this is unlikely to underlie the observed change in $E_m$ since the change in solvation for Phe66 mutants is less than we have previously observed for Tyr19 mutants, many of which showed smaller changes in $E_m$.

These results support a general conclusion from recent work in our laboratory (Agarwal et al., 1995, 1996), and other groups (Shen et al., 1993, 1994; Babini et al., 1996), that the side chains forming the cluster pocket serve little role in the definition of gross reduction potential. Our results, however, do indicate a general and important role of these residues in maintaining cluster stability. In view of these results, it is likely
that the shift of ~ -50 mV in the opposite direction observed for the *R. tenuis* HiPIP relative to the *C. vinosum* protein does not arise from the replacement of a Phe with Ile in the former, with the consequent change in local polarity in the cluster pocket or loss of binding interactions between the aromatic side chain and the cluster. Since there is unlikely to be a significant change in solvent accessibility (the oxidized cluster in *R. tenuis* HiPIP is stable), the difference in Em most likely reflects a global perturbation of the net dipole from the backbone amides (Jensen et al., 1994).

3.4.2 The Role of Aromatic Residues on the Cluster Stability

Non-conservative mutations of residue Phe66 have been found to introduce significant instability to the prosthetic Fe₄S₄ cluster. This instability appears to stem from the increase in solvent accessibility to the cluster core. This is made evident by comparison of the fingerprint region of 2D COSY spectra and ¹H-¹⁵N HSQC spectra (Figure 3.6) where a significant number of cross-peaks are absent relative to the native protein. Other prominent losses include the cross-peaks from ring NH protons located on Trp residues in the cluster cavity. The extent of solvent accessibility was found to be significantly lower than that exhibited by Tyr19 mutants. The latter have been extensively described in recent reports from our laboratory (Agarwal et al., 1995; Li et al., 1996b). In brief, Tyr19 is located at an entrance cleft to the hydrophobic pocket and serves to maintain a hydrophobic barrier for exclusion of water from the cluster cavity. Tyr19 mutant proteins demonstrate, to varying extents, the ability of water molecules to exchange with ionizable protons in the cluster binding pocket, and solvent accessibility resulted in facile oxidation of the cluster by atmospheric oxygen, with subsequent rapid
hydrolysis of the \([\text{Fe}_4\text{S}_4]^{3+}\) core. Such a decrease in cluster stability in the presence of a polar solvent is consistent with previous studies of model complexes that demonstrate sensitivity of the oxidized \([\text{Fe}_4\text{S}_4]^{3+}\) core to solvolytic decomposition (O’Sullivan & Millar, 1985; Maskiewicz & Bruice, 1977; Roth & Jordanov, 1992; Blonk et al., 1991; Nakamoto et al., 1988).

We have observed that solvent accessibility to the cluster pocket is diminished for Phe66 mutants relative to those for Tyr19, and so degradation of the cluster occurs at a slower rate. The consequences of reduced solvent accessibility for the Phe66 mutants are two-fold. First, reducing equivalents provided by the disintegrating cluster (one ferrous ion and four sulfides) may serve to reduce the remainder of the oxidized protein. This results in an apparent autoreduction pathway. Such a pathway is supported by comparison of the relative integrations from 1D NMR spectra obtained for reduced mutant proteins before oxidation and following auto-reduction. Integration of the paramagnetically shifted resonances shows that ~20% of the starting protein is lost, presumably reverting to apoprotein, as evidenced by changes in the diamagnetic region indicative of apo-HiPIP. As expected, the extent of degradation increases with the concentration of oxidant, which can either reoxidize the autoreduced protein or consume the reducing equivalents made available by cluster degradation. There are no additional hyperfine shifted peaks in the spectrum of the final autoreduced sample that would indicate a stable iron-containing species. Such an autoreduction pathway has precedent in small molecule analog chemistry.
3.4.3 The Auto Reduction Pathway

The second consequence of the slower decomposition rate is the appearance of an apparent intermediate in the decomposition pathway. This is cleanly detected by low-temperature EPR spectroscopy after the sample is held at ambient temperature for ~ 20 min to 1 h, depending on the mutant and the ambient temperature (varying from 4 to 25 °C). Relative to the oxidized protein, this species represents ~ 13% of the starting sample, as determined by integration of the EPR signals. Over time this signal also diminishes, consistent with its assignment as a degradation intermediate, although the microscopic rate constants for formation and degradation of this intermediate have not yet been evaluated. Amino acid radical centers were considered; however, with the exception of cysteine radical, the observed EPR features are unlike those observed for other residue-centered radicals, including Gly, Phe, and Tyr. The possibility of a cysteine radical was excluded by labeling with Cys-d2: no splitting or broadening was observed in the EPR bands. As an alternative we considered the possibility of an [Fe₃S₄]^+ center. The appearance of the spectrum, and the temperature and power dependence of the spectral features are consistent with previous observations on such metallocluster centers. There is also extensive evidence from model studies for oxidative conversion of a [Fe₄S₄]^3+ core to a [Fe₃S₄]^+ center and subsequent degradation in nucleo-phlic solvents (Roth & Jordanov, 1992). In enzyme systems, oxidative conversion to a [Fe₃S₄]^+ center was first reported for mammalian aconitase (Kennedy et al., 1983) and proposed for succinate-ubiquinone reductase (complex II) (Ackrell et al., 1984), although in the latter case the 3Fe center is now known to be the naturally occurring form of the cluster in the succinate-fumarate oxidoreductase enzymes (reviewed in Matsubara & Saeki, 1992).
This data again demonstrates a quite distinct role from that previously postulated for the aromatic core residues of high-potential iron proteins. In the oxidized state, the cluster is sensitive toward hydrolytic decomposition. The hydrophobic aromatic core residues apparently maintain a barrier toward exclusion of solvent. This idea was developed by Stout in earlier comparisons of the [Fe₄S₄] cluster environment in high- and low-potential ferredoxins (Stout, 1982). Our results for both Tyr19 and Phe66 mutants indicate that such residues have little role in defining the reduction potential of the cluster. Relative to the influence of Tyr19 mutants the increase in solvent accessibility is less pronounced for the Phe66 derivatives and is consistent with the positions of each residue. Tyr19 forms a barrier between the cluster pocket and bulk solvent, while Phe66 lies within the cluster and mediates its effect less directly. Two possible mechanisms for solvent access in the Phe66 mutants include the introduction of a general conformational flexibility that facilitates solvent exchange or the leakage of solvent at a specific location. Finally, it may be noted that core aromatic residues have been frequently cited as important mediators of electron-transfer reactions. Such a role is distinct from the model described above and is not supported by measurements of electron self-exchange rates for Phe66, Phe48, and Tyr19 mutants (Soriano et al., 1996).
REFERENCES


CHAPTER 4

CLUSTER ASSEMBLY IN THE ABSENCE OF SULFIDE.

PATHWAY TO A STABLE INTERMEDIATE

4.1 INTRODUCTION

Correct assembly of iron-sulfur clusters is a requirement for the normal biological function of many iron-sulfur proteins, which display a remarkable diversity of functional chemistry. These include electron-transfer (Knaff and Hirasawa, 1991; Beinert and Munck, 1997), catalysis (Flint and Allen, 1996; Emptage, et al., 1988; Holm, et al., 1996), stabilization of protein structure (Thayer, et al., 1995), and oxidative sensor (Hidalgo, et al., 1995; Gaudu and Weiss, 1996). The mechanism of assembly of the Fe-S prosthetic center is also of functional relevance in iron regulatory proteins (Hentze and Kuhn, 1996). Protein folding and prosthetic cluster assembly are of increasing research interests, promising to help us to understand protein functions, enzymatic mechanisms, gene regulation, protein/drug interactions and many other areas of applications.

Although a lot of work has been done with model compounds (Holm, 1977; Zhou et al., 1992, 1996, 1997), few details are known for the mechanisms of protein-bound cluster assembly. Less has been studied on how cluster assembly is coupled together with protein folding in metalloprotein production. There is still a long way to go in mapping out the intermediates in the process of cluster assembly.
HiPIP (*C. vinosum*) is a structurally well defined small protein (10 kDa) containing a [Fe₄S₄]^{3+/-2+} cluster. It has been cloned and over-expressed in this lab (Agarwal, et al., 1993). It has three tryptophan residues, all part of the hydrophobic pocket around the Fe₄S₄ cluster. HiPIP gives a strong intrinsic fluorescence when the cluster is lost, making HiPIP a very good model for studying Fe-S protein assembly by *in vitro* reconstitution. Previously, we described an approach to follow Fe-S cluster assembly and backbone folding for native *Chromatium vinosum* high potential iron protein (HiPIP), using ¹H-¹⁵N heteronuclear single quantum coherence (HSQC) and matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry experiments (Natarajan and Cowan, 1997). These studies suggest that the [Fe₄S₄] cluster assembles through a key structural intermediate (I, Scheme 4.1) that already possesses the tertiary structural elements associated with the native protein.

\[
\begin{align*}
\text{Fe/DTT} & \quad \text{apo} \quad [\text{Fe}_4]\text{int} \quad S^- \quad [\text{Fe}_4\text{S}_4]^{n+} \\
\text{observable species, I} & \quad \text{Scheme 4.1}
\end{align*}
\]

Fluorescence spectroscopy has been extensively applied to the study of protein folding pathways, either by use of the intrinsic protein fluorescence (Clark, et al., 1996; Kotik, M. et al., 1995) or proteins labeled with fluorescent labels (Agoshe, et al., 1995). However, in these examples the assembly of a metalloprosthetic center was not involved in the protein folding process. In this work, we further develop our model (described in Scheme 4.1) for FeS cluster formation by identifying additional kinetic intermediates and...
define kinetic rate constants for several steps of the reaction pathway by use of intrinsic fluorescence and freeze-quench EPR techniques.

4.2 MATERIALS AND METHODS

4.2.1 HiPIP and Chemicals

Recombinant native HiPIP was isolated from an *Escherichia coli* host following expression from a pET-21d(+) expression vector, as described previously (Agarwal et al., 1993). FeCl₂ and Na₂S·4H₂O were Baker’s Analytical Reagent grade; Dithiothreitol was from Aldrich; β-mercaptoethanol and Tris base were purchased from Sigma Chemicals. Guanidine Hydrochloride (GuHCl) was either purchased from Sigma as an 8M solution or as a solid from Acros. The solid was recrystallized with ethanol and filtered. All other chemicals were purchased from the best available sources without further purification.

4.2.2 Making Apo HiPIP

Initially the procedure used by Sola et al (Sola et al., 1989) was followed in making apo HiPIP. Apo HiPIP made in this way gave good reconstitution results, but sometimes the reconstitution failed in a unpredictable way, possibly because of damaged apoprotein due to the very acidic conditions used in the procedure. Since guanidinium can cause some structural perturbation on the protein (Bertini, et al., 1997), most apo HiPIP was made using a modified procedure described as the following. To 25 ml GuHCl (8.0 M) slowly added 3 ml HiPIP recombinant native (about 60 mg) with stirring, followed by 0.6 ml 200 mM EDTA (to a final concentration of 4 mM). Then TCA was
added drop-wise until the green color bleaches away. The final TCA concentration was around 0.7%. There might be a little insoluble material floating on the surface while adding TCA. It would disappear after several minutes of stirring. A very faint pink color might also develop. This could be regarded as normal, and would go away during dialysis. All the addition was done in a hood in order to facilitate the evolution of H2S gas. The solution was put on ice with stirring for about 1 hour after addition, then dialyzed (4 × 3 L) at 4 °C against 0.1M tris buffer pH 8.0 supplemented with 5 mM β-mercaptoethanol or DTT. A Spectrapor #1 dialysis membrane tubing (molecular weight cut off 7,000) was used. Concentration was done on an amicon with a 5 K membrane, and the concentrated apo protein (final concentration is about 2 mM in tris buffer) was supplemented again with 5mM DTT and stored away in – 20 °C refrigerator.

The advantage of this procedure is, first of all, convenience. Acid precipitation, washing, and centrifugation steps are eliminated, resulting in a higher yield, normally 80%. Less acidity can presumably protect the protein from being damaged in the process, resulting in more reproducible reconstitution reactions. Another advantage is that the whole process was transparent, making it suitable to be monitored using spectroscopic methods (see chapter 2).

The quality of apo HiPIP was tested by in vitro full reconstitution: 0.1 mM apo and about 7 mM DTT were incubated in tris buffer pH 8.0 for 30 minutes at room temperature. A final concentration of 1 mM of Fe²⁺ was added to the mixture followed by 20 minutes incubation. Finally, a final concentration of 1 mM of sulfide was added to the mixture. After another 20 minutes incubation, the mixture was passed through a G25 size exclusion column (0.8 cm × 10 cm). The reconstituted HiPIP was green-colored, which
elutes out first. The UV-visible spectrum was taken and was used as the criteria in judging the quality of the apoprotein.

4.2.3 Fluorescence Spectroscopy

Cluster assembly in the slow phases of partial reconstitution was monitored by fluorescence on a PE LS50B luminescence spectrometer in the fluorescence mode, with FLWINLab software. About 3.3 μM apo HiPIP in 0.1 M tris pH 7.8 with 0.44 mM DTT was used in both static and kinetic spectra. 30 minutes of preincubation was allowed before adding 33 μM Fe²⁺ to the apo solution in kinetic studies. The excitation wavelength was chosen at 280 nm for the three tryptophan residues as part of the hydrophobic pocket around the cluster (Carter, 1977). The emission wavelength was at 352 nm, which was the emission maximum when the pocket was completely open. The excitation slit width was 10 nm, and the emission 4 nm. The resolution was 4 nm. Kinetic assays lasted from 30 minutes to about 3 hours, with a time interval from 1 second to 30 seconds. All kinetic traces were fit into a consecutive, pseudo-first order reaction model as stated in the Kinetic Date Analysis section. Fitting was done by using the Origin graphic program from Microcal.

4.2.4 Stopped-Flow Fluorescence

Stopped-flow fluorescence was used to explore the initial fast phases lost in the mixing time in routine fluorescence experiments. This was done on an Olis RSM 1000 Rapid-Scanning Monochromator in the fluorescence mode. A 150 W Xenon lamp was used as light source with an LPS 220 Lamp Power Supply from Photon Technology
International. A pair of 600-line gratings with a blazing wavelength of 300 nm were used in the monochromator. The excitation wavelength was 280 nm, and emission fluorescence was collected using a 10 nm band pass filter centered around 355 nm. One syringe was filled with degassed 20 μM apo-HiPIP in 10 mM tris pH 8.0 and 0.9 mM DTT, the other syringe with degassed 0.2 mM FeCl₂ in the same buffer. A special slow-response circuit was built into the sample PMT to increase the signal to noise ratio. Argon purge through the light paths was found useful in getting higher light intensity toward UV region. Apparent rate constants were obtained from a consecutive first order reaction model. Samples without apo HiPIP or using buffer in place of Fe were used as controls.

4.2.5 Freeze-Quench EPR Experiments

Intermediate species from partial cluster assembly may be EPR active, which can provide further information to the identity of the intermediate. Freeze-quench preparations were made on a Model 715 system from Update Instrument Inc. with a ServoDisc DC Ram. Freeze-quench was achieved by shooting sample to a pre-cooled funnel filled with cooled isopentane (-150 °C). A two-syringe flow system was used for partial reconstitution without oxidation. A three-syringe serial flow system was used for partial reconstitution followed by oxidation, in which a syringe having 1.2 mM HiPIP in tris buffer with 60 mM DTT was mixed with the second syringe having 12 mM Fe²⁺ solution in the same buffer. The mixture went through an incubation reactor, and was then oxidized by K₂IrCl₆ contained in the third syringe in the second mixing before it
shot out of the nozzle to the cooled isopentane. The frozen powder was packed into the bottom section of a 4 mm EPR tube attached to the funnel by heat-shrink tubing, then connected back to the upper section of the EPR tube after packing. The part of the tube with sample was kept in liquid nitrogen during this process. For preparations with incubation reactors smaller than 200 ms, the reactor after the first mixer and the half-length delay reactor for the third syringe were changed correspondingly for different incubation length. For preparations with incubation times longer than 200 ms, both the 200 ms incubation reactor and the 100 ms delay reactor were fixed to ensure all solutions came to the second mixer at the same time. The delay time in the push-push-push function was changed instead to achieve different incubation times. The three-push operation avoided the waste of large amounts of sample, and was normally good to produce enough frozen particles for packing one EPR sample. The 2 ml syringes were used, and the ram velocity was 1.25 cm per second. Reactors and mixers were thoroughly rinsed between each sample with three times of de-ionized water and then one time of 95% ethanol using two aspirators.

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 WinEPR. The temperature was measured by use of an Au/Fe vs. Cr thermocouple. Experimental parameters are listed in footnotes to the tables and figure legends. Relative spin quantitation was carried out by using native HiPIP as a standard according to Aasa-Vanngard equation (Aasa and Vanngard, 1975):

\[
\frac{N_s}{N_0} = \frac{H_m \cdot H_m}{H_m \cdot H_m} \left( \frac{T_o \cdot T_s}{P_o \cdot P_s} \right)^{1/2} \left( \frac{f_o \cdot f_s}{g_o \cdot g_s} \right)^{1/2} \left( \frac{G_o \cdot G_s}{S_o \cdot S_s} \right) \left( \frac{g_{ps} \cdot g_{ps}}{S_{ps} \cdot S_{ps}} \right) \left( \frac{S_{ps} \cdot S_{ps}}{S_{ps} \cdot S_{ps}} \right) \tag{4.1}
\]
where \( s \) and \( o \) represent the sample and the standard respectively, \( N \) 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 Boltzmann distribution), \( SC \) is the field scan of the spectrum, and \( g_p \) is defined by

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

(4.2)

4.3 RESULTS

4.3.1 Quality of Apo HiPIP in Full Reconstitution. UV-visible Spectra

A typical UV-visible spectrum of apo HiPIP is shown in Figure 4.1. The main feature is the peak at 280 nm from aromatic (mostly tryptophan) absorbance. The absorbance is practically zero above 300 nm, consistent with the literature (Moulis, et al., 1988). Figure 4.1 also shows the spectra of WT HiPIP and fully reconstituted HiPIP by adding \( \text{Fe}^{2+} \) and \( \text{S}^{2-} \) to apo protein in the presence of excess reducing reagent DTT. Both of the spectra have another absorbance peak at 386 nm beside the one at 280 nm. This is from a metal to ligand charge transfer (MLCT) band (Noodleman and Baerends, 1984). Fully reconstituted HiPIP has almost the same \( A_{280}/A_{386} \) ratio with the WT protein (about 2.5), although the trough at around 345 nm was slightly shallower than WT HiPIP. In apo HiPIP, the 4Fe-4S cluster was obviously lost from the absence of the MLCT band and the bleaching of the characteristic green color.

From Figure 4.1 one can see that partially reconstituted HiPIP, made by adding \( \text{Fe}^{2+} \) to apo in the absence of \( \text{S}^{2-} \), shows no peak at 386 nm. However, a shoulder above
Figure 4.1: UV-visible spectra of Apo HiPIP (doted line), partially reconstituted (short-dashed line), fully reconstituted HiPIP (dashed line), and WT HiPIP (solid line). Full reconstitution conditions: 0.1 mM apo HiPIP and 7 mM DTT were incubated in tris buffer pH 8.0 for 30 minutes. Then 1 mM of Fe$^{2+}$ was added to the mixture followed by 20 minutes incubation. Finally, a final concentration of 1 mM of sulfide was added to the mixture. After another 20 minutes incubation, the mixture was passed through a G25 size exclusion column (0.8 cm × 10 cm). partially reconstituted sample was obtained in the same way without adding sulfide.
300 nm, extended to about 400nm, indicates formation of an intermediate type of Fe center different from the WT 4Fe-4S cluster (Bartsch, 1978). It was found that the GuHCl/TCA method in making apo HiPIP is quite reliable.

4.3.2 Fluorescence Spectra of the Apo, Stable Intermediate, and WT HiPIP

Figure 4.2 shows the emission spectra obtained from the holo and apo forms of HiPIP, which have not previously been reported, as well as from the stable intermediate I. HiPIP contains three Trp residues and one Tyr residue (Carter, 1974). Tyr emission at ~300 nm is efficiently quenched by neighboring Trp residues (Freifelder, 1982) and is not observed in the HiPIP fluorescence for either native or apo protein. The emission maximum at 350 nm for free Trp in aqueous solution at pH 7 contrasts with the band at 340 nm observed for most globular proteins. For HiPIP, the Trp residues show an emission maximum at > 350 nm for apo and intermediate states at pH 8, and there is evidence of overlapping bands that arise from the Trp’s in the protein. Control experiments taken in the absence of protein show no significant fluorescence in the observation range.

Addition of Fe$^{2+}$ to apo HiPIP with excess DTT causes a significant decrease in fluorescence intensity. The intermediate formed at the end of this process, corresponding to the one observed by Natarajan et al by HSQC NMR, has a defined tertiary structure close to that of the native. However, the fluorescence intensity level was very different from the native. The Fe species formed must also be different since sulfide, a necessary component for native cluster formation, was not added. The characteristic
Figure 4.2: Fluorescence spectra for WT (dotted line), apo (solid line), and intermediate (dashed line) HiPIP species recorded from 300 nm to 400 nm with excitation at 280 nm. The emission peak for each species was marked on the corresponding spectrum. Solution conditions: 3.3 μM apo HiPIP in 0.1M tris pH 7.8 with 0.44 mM DTT for the apo protein spectrum; 30 minutes after adding 33 μM Fe$^{2+}$ to the apo solution for the intermediate spectrum; 3.3 μM WT HiPIP in 0.1M tris pH 7.8 with 0.44 mM DTT for the holo protein spectrum.
fluorescence spectra of different species provide a distinct way of studying the kinetics of Fe cluster formation.

4.3.3 Kinetic Phases in Partial Reconstitution as Monitored by Fluorescence

By use of the distinct fluorescence emission characteristics of HiPIP, the kinetic profile for partial cluster assembly has been evaluated by time-resolved fluorescence methods (Figure 4.3). There is a large initial drop in fluorescence, which is lost in the mixing time of routine fluorescence experiments. This time regime was further explored by stopped-flow experiments. Following the addition of Fe^{2+} to apo HiPIP, there is an initial rapid quenching of the Trp fluorescence. This fast kinetic phase happens within 200 milliseconds. Further slower decrease in fluorescence intensity is observed in both stopped-flow and routine fluorescence measurements. At a certain point of time, the decrease ceases, and a subsequent increase in intensity is accompanied by a 2 nm red shift of the fluorescence band (Figure 4.2). Finally, fluorescence intensity levels off, reaching a stable intermediate state.

Since the total concentration of protein does not change during partial reconstitution, and all the species contribute to the total fluorescence at a given time, one can assume that the cluster assembly and the partial reconstitution follow a pseudo-first order consecutive kinetic model (Rodiguín and Rodiguina, 1964).
Figure 4.3: Time-dependent fluorescence traces for partial reconstitution of HiPIP Rate constants $k_1$, $k_2$, $k_3$, and $k_4$ (from the fastest phase to the slowest phase) were obtained by fitting the traces to a model for a series of consecutive reactions. Stopped-flow measurements were made on an Olis 1000 RSM apparatus with excitation at 280 nm, and the emission was collected using a 10-nm band-pass filter centered at 355 nm. One syringe was filled with 20 μM apo-HiPIP in 10 mM Tris (pH 8.0) and 0.9 mM DTT, while the other syringe contained 0.2 mM FeCl₂ in the same buffer. Routine fluorescence was done as described in the legend for Figure 1, with solution conditions as used for the stopped-flow experiment and detection of the emission wavelength at 352 nm.
4.3.4 The Consecutive Pseudo-First Order Kinetic Model

Consider a consecutive irreversible first order reaction with four stages. The initial substance \( A_0 \) (here it is the apo protein) has an initial concentration \( C_0^{(0)} \). The reagent B (here it would be Fe) which effects the reaction has a concentration \( Z \). This value is assumed to remain constant through all stages of the reaction.

In the first step, substance \( A_0 \) interacts with substance B, forming substance \( A_1 \); in the second stage, \( A_1 \) is converted to \( A_2 \); and then \( A_2 \) is transformed into \( A_3 \). The rate constants for these reactions are, \( k_1, k_2, k_3 \), respectively, and the course of the reaction is shown in the following scheme:

\[
\begin{array}{c}
A_0 \xrightarrow{k_1} A_1 \xrightarrow{k_2} A_2 \xrightarrow{k_3} A_3 \\
C_0 \xrightarrow{C_1} C_2 \xrightarrow{C_3}
\end{array}
\]

\( C_0, C_1, C_2, \) and \( C_3 \) are the concentrations of reaction products at any time \( t \).

\[
\frac{dC_0}{dt} = -k_1 C_0;
\]

\[
\frac{dC_1}{dt} = k_1 C_0 - k_2 C_1;
\]

\[
\frac{dC_2}{dt} = k_2 C_1 - k_3 C_2;
\]

\[
\frac{dC_3}{dt} = k_3 C_2;
\]

Here \( k_1 = k_1 Z; \quad k_2 = k_2 Z; \quad k_3 = k_3 Z; \)
\(k_i', k_2', k_3'\) are specific (second order) rate constants.

Solving these differential equations, notice that:

\[
C_0 (t) = C_0 + C_1 + C_2 + C_3
\]

we have,

\[
C_0 = C_0 (0) e^{-kt_0};
\]

\[
C_1 = C_0 (0) \left[ \frac{k_1}{k_2 - k_1} e^{-kt_1} + \frac{k_1}{k_1 - k_2} e^{-kt_2} \right];
\]

\[
C_2 = C_0 (0) \left[ \frac{k_1 k_2}{(k_2 - k_1)(k_3 - k_1)} e^{-kt_1} + \frac{k_1 k_2}{(k_1 - k_2)(k_3 - k_2)} e^{-kt_2} + \frac{k_1 k_2}{(k_1 - k_3)(k_2 - k_3)} e^{-kt_3} \right];
\]

\[
C_3 = C_0 (0) \left[ 1 - \frac{k_2 k_3}{(k_2 - k_1)(k_3 - k_1)} e^{-kt_1} - \frac{k_1 k_3}{(k_1 - k_2)(k_3 - k_2)} e^{-kt_2} - \frac{k_1 k_2}{(k_1 - k_3)(k_2 - k_3)} e^{-kt_3} \right].
\]

Assuming \(a_0, a_1, a_2,\) and \(a_3\) are the coefficients for each species in fluorescence intensity, and the fluorescence intensity is the linear sum of all the species. That is

\[
FL = a_0 C_0 + a_1 C_1 + a_2 C_2 + a_3 C_3;
\]

Apply equation 4.3 to equation 4.4,

\[
FL = C_0 (0) \left[ a_3 + \frac{a_0 (k_2 - k_1)(k_3 - k_1) + a_1 k_1 (k_3 - k_1) + a_2 k_1 k_2 - a_3 k_2 k_3 e^{-kt_1}}{(k_2 - k_1)(k_3 - k_1)}
+ \frac{a_1 k_1 (k_3 - k_2) + a_2 k_1 k_2 - a_3 k_2 k_3 e^{-kt_2}}{(k_1 - k_2)(k_3 - k_2)}
+ \frac{a_2 k_1 k_2 - a_3 k_1 k_2}{(k_1 - k_3)(k_2 - k_3)} e^{-kt_3} \right].
\]
In a case of only three substance consecutive reaction,

\[ FL = a_0 C_0 + a_1 C_1 + a_2 C_2; \]

\[ = C_0^{(0)} \left[ a_2 + \frac{a_0 (k_2 - k_1)}{(k_2 - k_1)} e^{-k_2 t} + \frac{a_1 k_1 - a_2 k_1}{(k_1 - k_2)} e^{-k_1 t} \right] \]  

(4.6)

Equation 4.5 and 4.6 predict that the fluorescence traces can be fit into a model of first-order consecutive reactions, as a sum of multiple exponentials, where \( k_1, k_2, k_3 \) and \( k_4 \) represent the rate constants from the fastest phase to the slowest phase, with \( a_1, a_2, a_3, a_4 \) being corresponding coefficients. Figure 4.3 shows a typical fit. The stopped-flow part is fit to a three-substance consecutive first order reaction, and the routine fluorescence part is fit to a four-substance consecutive first order reaction. Since the second phase in the stopped-flow experiments is the same as the first phase in the routine fluorescence experiments, there are a total of four kinetic phases in the partial reconstitution reaction, described by rate constant \( k_1, k_2, k_3 \) and \( k_4 \), from the fastest phase to the slowest phase. The average kinetic rate constants are summarized in Table 4.1.

<table>
<thead>
<tr>
<th>( k_1(\text{s}^{-1}) )</th>
<th>( k_2(\text{s}^{-1}) )</th>
<th>( k_3(\text{s}^{-1}) )</th>
<th>( k_4(\text{s}^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.1</td>
<td>0.01</td>
<td>0.002</td>
</tr>
</tbody>
</table>

\[ \Delta k^3 \]

\[ \pm 8 \quad \pm 0.02 \quad \pm 0.002 \quad \pm 0.0004 \]

a: Average deviation of three sets of experiments.

Table 4.1: Rate Constants in Partial Reconstitution
Table 4.1 shows that the rate constants in the kinetic events in partial cluster assembly are well spread, having an order of magnitude difference. The amplitudes in the phases described by \( k_1 \) and \( k_2 \) are much larger than those of \( k_3 \) and \( k_4 \).

### 4.3.5 Dependence of Rate Constants on Fe and DTT Concentration

In order to gain a deeper insight into the partial cluster assembly and partial reconstitution, it is necessary to study the dependence of the rate constants on the concentration of \( \text{Fe}^{2+} \) and/or DTT. It can be expected that some of the rate constants are \([\text{Fe}^{2+}]\) and/or \([\text{DTT}]\) dependent.

Table 4.2 and Figure 4.4 summarize the dependence of rate constants on Fe concentration. It can be seen from Figure 4.4 that both \( k_1 \) and \( k_2 \) have obvious \([\text{Fe}^{2+}]\) dependence: increase proportionally with the increase of iron concentration. As iron concentration changes from 100 nM to 400 nM, \( k_1 \) increases about 4 times, from 25 s\(^{-1}\) to near 100 s\(^{-1}\), and \( k_2 \) increases from 0.06 to 0.15 s\(^{-1}\). \( k_3 \) also depends on Fe concentration, although to a lesser extent. \( k_4 \) is very small compared with the other rate constants. It does not have an iron concentration dependence as seen from Table 4.2.

When the concentration of DTT changes, only \( k_1 \) and \( k_2 \) were found to be concentration-dependent (see Table 4.3 and Figure 4.5). As DTT concentration increase, so do the rate constants. \( k_3 \) and \( k_4 \) do not depend on DTT concentration in the range of 10 to 100 fold excess compared with apo HIPIP.

It was noticed in some experiments, especially with lower Fe concentration, that the fitting to the data was not very good according to our first order consecutive model.
<table>
<thead>
<tr>
<th>[Fe^{2+}] (μM)</th>
<th>(k_1(\text{s}^{-1})^a)</th>
<th>(k_2(\text{s}^{-1}))</th>
<th>(k_3(\text{s}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>21</td>
<td>0.0395</td>
<td>0.0064</td>
</tr>
<tr>
<td>120</td>
<td>44</td>
<td>0.0613</td>
<td>0.0081</td>
</tr>
<tr>
<td>160</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>180</td>
<td></td>
<td>0.0766</td>
<td>0.0147</td>
</tr>
<tr>
<td>200</td>
<td>43</td>
<td>0.1009</td>
<td>0.0168</td>
</tr>
<tr>
<td>240</td>
<td>55</td>
<td>0.0825</td>
<td>0.0228</td>
</tr>
<tr>
<td>280</td>
<td></td>
<td>0.1359</td>
<td>0.0325</td>
</tr>
<tr>
<td>320</td>
<td>108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>101</td>
<td>0.1369</td>
<td>0.0345</td>
</tr>
<tr>
<td>480</td>
<td>115</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a: Rate constants are the average of two or three sets of experiments.

Table 4.2: Dependence of rate constants on Fe concentration in partial reconstitution.
Figure 4.4: Dependence of rate constants on [Fe(II)]. (•)k₁; (○)k₂; (△)k₃. Experimental conditions are the same as described in the legend for Fig. 4.3 except that Fe²⁺ concentration varies.
<table>
<thead>
<tr>
<th>[DTT] (uM)</th>
<th>$k_1$ (s$^{-1}$)$^a$</th>
<th>$k_2$ (s$^{-1}$)</th>
<th>$k_3$ (s$^{-1}$)</th>
<th>$k_4$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>20</td>
<td>0.0138</td>
<td>0.0054</td>
<td>0.0024</td>
</tr>
<tr>
<td>300</td>
<td>16</td>
<td>0.0336</td>
<td>0.00465</td>
<td>0.00118</td>
</tr>
<tr>
<td>400</td>
<td>25</td>
<td>0.0564</td>
<td>0.00354</td>
<td>0.00127</td>
</tr>
<tr>
<td>500</td>
<td>20</td>
<td>0.0743</td>
<td>0.00397</td>
<td>0.00204</td>
</tr>
<tr>
<td>600</td>
<td>26</td>
<td>0.0678</td>
<td>0.0042</td>
<td>0.00241</td>
</tr>
<tr>
<td>700</td>
<td>23</td>
<td>0.0849</td>
<td>0.00442</td>
<td>0.00284</td>
</tr>
<tr>
<td>800</td>
<td>33</td>
<td>0.102</td>
<td>0.012</td>
<td>0.00226</td>
</tr>
<tr>
<td>900</td>
<td>31</td>
<td>0.0929</td>
<td>0.00948</td>
<td>0.00257</td>
</tr>
</tbody>
</table>

$a$: Rate constants are the average of two or three sets of experiments.

**Table 4.3**: Dependence of rate constants on DTT concentration in partial reconstitution.
Figure 4.5: Dependence of rate constants on [DTT]. (●)$k_i$; (○)$k_2$; (Δ)$k_3$; (□)$k_4$

Experimental conditions are the same as described in the legend for Fig. 4.3 except that DTT concentration varies.
The reason for that might be a deviation from the pseudo-first order assumption. However, the dependence of rate constants on Fe and DTT concentration seems to be generally valid.

4.3.6 EPR Characteristics of the Intermediate

Freeze-quench EPR techniques were used to investigate partial reconstitution by adding Fe$^{2+}$ to a solution of apo HiPIP and DTT. Without external oxidant, this process was found to be EPR silent by X-band EPR spectroscopy (data not shown). Trace amount of signal could be attributed to background buffer and Fe solution. However, when K$_2$IrCl$_6$ was used to oxidize the mixture of apo and DTT after incubation, two sets of EPR signals appeared as shown in Figure 4.6. One set is the high spin Fe$^{3+}$ signal at g=4.3 (Hagen, 1992). This signal does not change much in intensity, apparently because of the presence of excess iron in all preparations. The other signal has a g values near 1.8. It is extraordinarily broadened, and grow in rapidly after mixing.

The time dependence on the intensity of the broad EPR signal near g=1.8 shows basically two kinetic processes as shown in Figure 4.7. The first phase is very fast, with an observed rate constant of 11.7 s$^{-1}$. The second phase is much slower, having a rate constant of near 0.1 s$^{-1}$. A two-phase consecutive model gives a reasonably good fit, though not perfect.

The broad signal looks like an overlap of two or more signals. In an attempt to separate any overlapped signals, both temperature dependence and power saturation studies were carried out to deconvolute this broad signal. Surprisingly, it was found that the signal changed uniformly when either the temperature or the microwave power were
Figure 4.6: Partial cluster reconstitution as seen in freeze-quench EPR experiments. Apo-HiPIP and DTT in Tris buffer pH 8.0 was mixed with Fe$^{2+}$, and then oxidized by Ir$^{3+}$ before freeze-quench. EPR conditions: microwave power, 2 mW, frequency, 9.456 GHz, modulation frequency, 100 MHz, modulation amplitude, 2.5 G, temperature, 5 K.
Figure 4.7: Development of the $g=1.80$ oxidized EPR signal during reconstitution of apo-HiPIP in the absence of $S^2$. Conditions for freeze-quench experiments: 1.2 mM apo-HiPIP with 84 mM DTT in 50 mM tris pH 8.0 was mixed with 12 mM Fe$^{2+}$ in tris. After The mixture was then oxidized by 160 mM K$_2$IrCl$_6$ in tris after incubation. EPR conditions: microwave frequency, 9.456 GHz, modulation frequency, 100 KHz, microwave power, 2 mW, temperature 5K.
changed. This tells us that the apparently different features in this signal derive from the same species.

EPR temperature and power saturation studies of this broad signal were shown in Figure 4.8, indicating a fast-relaxing resonance. The intensity of this signal decreases dramatically as temperature increases, and disappears with temperatures higher than 30K. This property clearly excludes the possibility of a radical species, which generally persist above 70K.

4.4 DISCUSSION

4.4.1 Cluster Assembly and Protein Folding

Intrinsic protein fluorescence is a very sensitive and convenient probe for use in protein folding studies. In our case, since the metal cluster is closely surrounded by three tryptophan residues, changes in cluster construction will immediately affect the fluorescence. Thus, the intrinsic Trp fluorescence of HiPIP becomes a unique probe in our studies of cluster assembly.

Figure 4.3 reflects the consequences of partial cluster formation on protein fluorescence. Binding of iron to apo-HiPIP (probably to one or more cysteine residues) results in an initial rapid quenching of the Trp fluorescence. Further reductions in fluorescence intensity most likely reflect the binding of additional iron centers and intramolecular quenching mechanisms arising from structural changes as protein residues begin to form a more compact structure (Itzhaki, et al., 1994). A subsequent increase in
intensity is attributed to the exclusion of water molecules as the cluster intermediate develops and the protein folds around it (Freifelder, 1982) thereby establishing tertiary structure (Natarajan and Cowan, 1997).

4.4.2 Kinetic Pathway in Partial Cluster Assembly

Following addition of Fe$^{2+}$ and exogenous thiol (DTT) to apo-HiPIP, the fluorescence traces could best be fit to a model of consecutive reactions (summarized in Scheme 4.2) (Rodiguin and Rodiguina, 1964), where $k_1$, $k_2$, $k_3$, and $k_4$ represent the rate constants from the fastest phase to the slowest phase. This model, and the kinetics of intermediate formation measured by these fluorescence experiments, is consistent with the folding pathway to intermediate I previously characterized by NMR and mass spectrometric measurements. It is not possible to discount reversibility in early steps of the pathway, and so, the reported $k$'s are apparent rate constants.

Scheme 4.2:

$$
\text{apo/DTT} + \text{Fe}^{2+} \xrightarrow{k_1 \ 30 \text{ s}^{-1}} [\text{Fe}_x]_{\text{Int}A} \xrightarrow{k_2 \ 0.1 \text{ s}^{-1}} [\text{Fe}_2]_{\text{Int}C} \xrightarrow{k_4 \ 0.002 \text{ s}^{-1}} [\text{Fe}_4]_{\text{Int}} \xrightarrow{S^{2-}} [\text{Fe}_4\text{S}_4]^{n^+}
$$

A plausible reaction mechanism, that is consistent with earlier observations by Rabinowitz et al (Sweeney and Rabinowitz, 1980), is illustrated in Scheme 4.2, which shows an elaborated version of the assembly pathway described in Scheme 4.1.
Figure 4.8: (A) Power saturation curve for the $g = 1.80$ EPR signal formed during partial reconstitution of HiPIP in the absence of $S^2^-$. EPR: microwave frequency 9.456 GHz, modulation amplitude 2.5 G, modulation frequency 100 kHz, temperature 5K. (B) Temperature dependence of the $g = 1.8$ signal. Microwave power 2 mw.
Consistent with our previous study, steps $k_1$ and $k_2$ in the fast kinetic phase show a clear dependence on [DTT] (Figure 4.5), suggesting a requirement for exogenous thiol to promote formation of the initial intermediates $[\text{Fe}x]_{\text{intA}}$ and $[\text{Fe}y]_{\text{intB}}$ by providing additional coordination to the iron center(s). Later steps are not dependent on [DTT]. In later slower phases, changes in fluorescence reflect further protein conformational changes in accommodating the newly formed cluster. As expected, the rate constants $k_1$, $k_2$, and $k_3$ increase with increasing $[\text{Fe}^{2+}]$ (Figure 4.4), since iron is obviously involved in promoting the formation of cluster intermediates. The possibility of reversibility for these steps precludes a detailed assessment of iron stoichiometry in each step. The reaction step ($k_4$) does not depend on $[\text{Fe}^{3+}]$ and may reflect internal ligand rearrangement.

Martin et al. has reported a cluster-driven protein rearrangement for $A.\text{vinelandii}$ Ferredoxin I (Martin et al., 1990). Our time-resolved fluorescence study described here indicates that protein folding is also driven by cluster assembly. Compared with the relatively rapid protein folding without a cluster (Clark, et al., 1996; Kotik, et al., 1995; Denton, et al., 1994; Agoshe, et al., 1995; Kim, et al., 1994), cluster assembly appears to be the rate-limiting process, reflecting the greater complexity of the assembly reaction.

### 4.4.3 Intermediate I Formed in Partial cluster Assembly

Intermediate I has some interesting characteristics. It is formed by adding iron to apo-HiPIP incubated with DTT in the absence of inorganic sulfide. UV-visible spectrum of I lacks the MLCT absorption peak at 386 nm in the WT HiPIP. Only a small shoulder appears between 300 nm to 400 nm (Figure 4.1). Since there is no other
chromophore present, this peak most likely arises from a MLCT also. However, the metal complex has a lower extinction coefficient for the MLCT band, and a different ligation as indicated by shift in the peak position. A blue shift has previously been seen in other systems when sulfur ligation is (partially) changed to oxygen ligation (Cheng, et al., 1994; Xia, et al., 1996; Meyer et al., 1994; Meyer, et al., 1997). It is likely that iron atoms are partially ligated to the oxygen on DTT, H$_2$O, or even Tris buffer.

The fluorescence coefficient for the intermediate species was found to be much lower than for apo-HiPIP, yet significantly higher than the WT as seen from Figure 4.2. This implies that the intermediate species has an intermediate compactness in terms of protein backbone structure around the cluster, and an intermediate cluster species that has a lower quenching efficiency than the fully formed 4Fe4S center. The intermediate cluster may be less tightly packed, leading to an increased distance between Trp residues and iron atoms. The fluorescence peak position is close to the apo protein, indicating that in the intermediate species, Trp residues are exposed to polar residues (Freifelder, 1982). This may come from DTT, H$_2$O or even buffer molecules. The dependence of rate constants on DTT concentration, and the previous study in our laboratory is consistent with this interpretation.

More information about the intermediate cluster is provided by freeze-quench EPR experiments. First, in the absence of external oxidant, no intermediate step in partial reconstitution gives rise to an EPR signal in X-band. This tells us that all of the species formed in partial reconstitution either have no unpaired spin (EPR silent), or have even spin (difficult to observe with x-band perpendicular mode) (Hagen, 1992). When the partial reconstitution was followed by Ir$^{4+}$ oxidation, we see a very broad, complex-
looking signal at about \( g = 1.8 \) growing in rapidly in about 0.1 second. No other signals were observed except the high spin single \( \text{Fe}^{3+} \) signal at \( g = 4.3 \) (Figure 4.6).

The temperature dependence and power saturation studies on the broad signal strongly argue for the presence of a single species or identical species, in spite of the complex appearance. The low \( g \) value may suggest oxygen or nitrogen ligation to the Fe (Meyer, et al., 1997), which is supported by UV-visible and fluorescence spectroscopy as discussed earlier. In this case, oxygen ligation from DTT or \( \text{H}_2\text{O} \) molecules is more likely. The unusually broad feature of this signal suggests magnetic coupling of two iron centers (Dewitt, et al., 1991; Davydov, R. M., et al., 1997; Davydov, A., et al., 1997), which is also supported by temperature dependence and power saturation studies. The bridge is most likely DTT-derived, although it may also come from water or dioxygen. It was found that the level of oxygen exposure in preparation can sometimes affect this signal (Kshama Natarajan, unpublished results).

4.5 SUMMARY

Cluster assembly in partial reconstitution of HiPIP has been studied by UV-visible, time-resolved fluorescence spectroscopy, and freeze-quench EPR techniques. In this work, we have detected several kinetic intermediates and found their relative rates of formation to decrease stepwise by over 1 order of magnitude. Early intermediate species show clear iron and DTT dependence. The slower kinetic phases most likely reflect structural rearrangements of the protein backbone. The resulting cluster intermediates species may have partial oxygen ligation from \( \text{H}_2\text{O} \), or possibly DTT, besides cysteiny
thiolate. DTT or solvent ligation makes the intermediate iron cluster less compact than the native 4Fe-4S cluster, although the overall tertiary structure is close to that of the native protein.

REFERENCES:


CHAPTER 5

4Fe-4S CLUSTER ASSEMBLY IN HIPIP

5.1 INTRODUCTION

In chapter 4, we have looked into partial reconstitution in vitro, in which iron was added to apo-HiPIP in the presence of DTT. The resulting partially folded protein has a tertiary structure very close to that of the native, and a Fe center ligated by cysteines, DTT and possibly, solvent molecules. This type of Fe center has biological analogues in bacteria rubredoxin and may also exist in other species when a dysfunction in sulfide transportation occurs. In vivo, glutathione is a likely replacement for DTT.

There are also many biological functions related to iron sulfur cluster assembly that require sulfur to be involved. An integrated 4Fe-4S cluster is required for the iron regulatory protein (IRP) to function as aconitase (Rouault, et al., 1992; Beinert, et al., 1996) in catalyzing the reaction from citrate to isocitrate. The same is true for transcription factor FNR (fumarate nitrate reductase) and many other proteins that have 4Fe4S clusters (Khoroshilova, et al., 1995, 1997; Lazazzera, et al., 1996). In fact, integrated 4Fe-4S cluster assembly in vivo involves multiple protein components working together in ways that are not fully understood. NifS protein has been identified
as a sulfur transferase, involved in FeS cluster formation and the biological function of nitrogenase systems together with other gene products like NifU (Zheng et al, 1993, 1994). Surprisingly, people have discovered that NifS- and NifU-like proteins exist throughout a broad realm of living systems, from *E. coli*, *Azotobacter vinelandii*, to yeast and mammals (Flint, 1996; Hwang, et al., 1996). Beside the NifUS system, a second set of gene cluster, known as iscSUA-hscBA-fdx, has been identified in *A. vinelandii* (Zheng, et al., 1998). This gene cluster produces multiple enzymes apparently functioning in iron sulfur cluster assembly (the formation or repair). IscS has the same L-cysteine desulfurization activity as NifS, and iscU is a homolog to nifU, whose product NifU is involved in iron mobilization for nitrogenase-specific iron-sulfur cluster formation.

Similar to the nif system, a cysE-like sequence (produces O-Acetylserine synthase, which catalyzes cysteine biosynthesis) is found directly upstream of the isc (stands for iron sulfur cluster) gene, and a hscA-like gene (produces heat-shock chaperonins) is located downstream of iscSUA gene. It has been proposed that this complicated system is involved in Fe-S cluster assembly *in vivo* for proteins other than the nif system.

*In vitro* iron sulfur protein reconstitution with sulfur transferases has been carried out in the presence of externally added iron and reducing agent, such as DTT (Cheng, et al., 1994; Busch, et al., 1996), β-mercaptoethanol (Yamaguchi and Fujisawa, 1981). Rhodanese and NifS are the primary choice for providing sulfide (Pagani, et al., 1984; Zheng and Dean, 1994; Hidalgo and Demple, 1996). A regulatory function has been proposed for rhodanese in the aerobic energy metabolism of liver cells and of *E. coli* by interaction with FeS clusters of the electron transport chain (Marguerite, 1996).
Although *in vivo* iron sulfur cluster assembly studies have been advanced by fast-growing molecular biological techniques, the understanding of kinetics and structural intermediates in cluster assembly processes were severely limited by the complexity of the system. Crude extract was used (Flint, et al., 1996a) because it is apparently difficult to study iron sulfur cluster assembly *in vitro* with all the necessary biological components around. Some of them may not be even known at this time, especially with iron transportation enzymes. Even *in vitro* enzymatic reconstitution studies have difficulty in identify cluster intermediates by biophysical approaches because of interference from the enzymes used and/or the lack of them. These facts have motivated us to study iron sulfur cluster assembly *in vitro* using HiPIP as a protein model. In this work, 4Fe-4S cluster assembly and HiPIP reconstitution have been studied by UV-visible, fluorescence spectroscopy, and by freeze-quench EPR techniques. Complementary results were obtained for the kinetics of cluster assembly. Several cluster intermediates have been identified and characterized, a mechanistic model for 4Fe-4S cluster assembly has been proposed.

5.2 MATERIALS AND METHODS

*HiPIP Purification, Making Apo-HiPIP, Chemicals and Reagents.* See the "MATERIALS AND METHODS" part of Chapter 4.

*Fluorescence Spectroscopy, Stopped-flow and Freeze-quench Experiments.* These are similar to the "MATERIALS AND METHODS" part of Chapter 4, Except for the addition of S\(^2^-\) or Se\(^2^-\), which will be described in figure or table legends.
Reconstitution of Seleno-substituted HiPIP: In previous studies, fresh selenide solution used in 4Fe-4Se cluster reconstitution was prepared by reducing selenite with molar concentration of DTT (Sola and Cowan, 1989). Since DTT was also used in apo HiPIP solution, the final concentration of DTT in freeze-quench experiments would exceed 200 mM. Taking into consideration the ten-fold excess of Fe^{2+} over apo HiPIP, this makes oxidation by K_{2}IrCl_{6} very difficult, since the oxidant is not very soluble at high concentrations. In our preparation, fresh Se^{2-} was made by anaerobically adding degassed H_{2}O to solid sodium selenite (Aldrich Co.) and sodium hydrosulfite (Acros Co.). The final stock solution is 0.1 M for Na_{2}SeO_{3} and 0.3 M for Na_{2}S_{2}O_{4}.

5.3 RESULTS

5.3.1 4Fe-4S Cluster Assembly Studied by Freeze-Quench EPR

Freeze-quench/chemical-quench EPR techniques have been used in identifying intermediate species in fast reactions, provided that the species generated is EPR active or can become EPR active by further oxidation or reduction. Since iron is involved in cluster generation, it is very possible to have EPR active cluster intermediate species that can be studied by freeze-quench techniques. Further oxidation or reduction may also reveal important information about the kinetics and mechanism of FeS cluster assembly.
Figure 5.1: Full reconstitution of 4Fe4S cluster monitored by freeze-quench EPR. 1.2 mM Apo HiPIP with 70 mM DTT and 12 mM S²⁻ in 50 mM tris pH 8.0 was mixed with 12 mM Fe²⁺, then oxidized by K₂IrCl₆ after incubation, followed by freezing. EPR conditions: microwave power 2 mw, microwave frequency, 9.456 GHz, modulation amplitude, 2.5 G, modulation frequency, 100 MHz, temperature 5 K.
Full HIPPIP reconstitution was carried out by adding Fe$^{2+}$ to a solution of apo-HiPIP with DTT and inorganic sulfide. Control experiments were run in the absence of apo protein or iron. Figure 5.1 shows some snap shots of 4Fe-4S cluster formation followed by the freeze-quench EPR method. Each EPR spectrum was obtained from a particular sample with a specific incubation time. The incubation time is marked on the spectrum.

There are three sets of EPR active signals. The first set is almost isotropic, having a major g value of 4.3 at about 1570 gauss. A minor g component is sometimes seen with a g value near 8.6, which may or may not belong to this same species. This set is from a single Fe$^{3+}$ species (Hagen, 1992). The EPR intensity of this signal does not change much, since there is a ten fold excess iron added in every sample. The second set of signal is rhombic, having three g components: $g_1 = 1.96, g_2 = 2.00, g_3 = 2.08$. It appears in the early stages of cluster assembly, then decreases quickly. Therefore it represents an early intermediate species along the cluster assembly pathway. The third set of signal grows in slowly as the incubation time prolongs after mixing Fe with apo-HiPIP and sulfide in the presence of DTT. It is mostly an axial signal, having two major components at $g_\parallel = 2.03, g_\perp = 2.11$ and minor components with g values at $g = 2.08$ and $g = 1.99$. This is the wild type oxidized 4Fe-4S cluster signal seen in chapter 3. The small component with $g = 2.08$ was attributed to possible dimer interactions (Dunham, et al., 1991). Another minor signal at $g = 1.99$ has been seen previously following Ir$^{4+}$ oxidation. The g values for these signals are summarized in Table 5.1.
Figure 5.2: A blow-up of the high-field region in Figure 5.1.
Table 5.1: EPR Signals in 4Fe-4S Cluster Assembly Signal

<table>
<thead>
<tr>
<th></th>
<th>Fe$^{3+}$</th>
<th>X</th>
<th>Y</th>
<th>4Fe-4S$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>g₁</td>
<td>4.30</td>
<td>1.96</td>
<td></td>
<td>2.03</td>
</tr>
<tr>
<td>g₂</td>
<td>4.30</td>
<td>2.00</td>
<td>2.00</td>
<td>2.03</td>
</tr>
<tr>
<td>g₃</td>
<td>4.30</td>
<td>2.08</td>
<td>2.08</td>
<td>2.11</td>
</tr>
</tbody>
</table>

a: a minor component sometimes appears at g = 8.6.
b: minor components appear at g = 2.08 and g = 1.99.

Figure 5.2 presents a closer look at the changing signals in cluster assembly. Shortly after adding Fe to apo/S²⁻/DTT solution, at around 16 millisecond, an intermediate species develops quickly, which when oxidized, gives an EPR signal with three g components: g₁ = 1.96, g₂ = 2.00, g₃ = 2.08. This species, designated as X, then changes to another species (Figure 5.2, Figure 5.4), as evidenced by the fast disappearance of the g₁ = 1.96 component, and the decrease of the overall EPR intensity. The second intermediate species, designated as Y, shows a weak EPR signal similar to X, but lacking the g₁ = 1.96 component. It is almost EPR silent (Figure 5.4, the 4 s spectrum). Out of this second intermediate species the wild type HiPIP starts to form, several seconds after mixing. It does not look exactly like the WT signal at the beginning. Rather, it becomes more and more like the WT signal as the cluster assembly proceeded. This indicates that in this period of time, the second intermediate Y goes through some structural rearrangements to form the mature 4Fe-4S cluster. The mature
Figure 5.3: Temperature dependence of the $g=2.00$ signal from species X during reconstitution of HiPIP in the presence of $S^2$. EPR conditions: microwave frequency 9.456 GHz, modulation amplitude 2.5 G, temperature 5K.
4Fe-4S cluster forms much more slowly than previous intermediates. It takes around 10 minutes after mixing Fe with apo/DTT/S\(^2\) to see native-like 4Fe-4S cluster signal.

It should be mentioned that another set of freeze-quench EPR experiments were performed in the same way, with no oxidation after mixing Fe\(^{2+}\) with apo HiPIP and S\(^2\) in the presence of excess DTT. These experiments were carried out in order to see if any other EPR active species could be observed in the reconstitution. The results were surprising: no EPR active species were detected in the X-band. This indicates that all the cluster intermediate species including mature 4Fe-4S cluster have a zero or even ground spin state (Hagen, 1992). The reduced 4Fe-4S cluster in HiPIP has a ground spin state S = 0 because of the antiferromagnetic coupling of two S = 9/2 systems. The oxidized 4Fe-4S cluster has a ground spin state S = 1/2, which gives rise to the axial WT signal.

5.3.2 The g = 2.00 EPR Signal

Species X gives rise to the first oxidized EPR signal in milliseconds after mixing excess iron with apo HiPIP and S\(^2\) in the presence of excess DTT. The g value is very close to 2, and there is no other Fe center reported in the literature that has the same kind of g values, except the super-reduced iron bisnitrosyl species (Kennedy, et al., 1997), which in our case is very unlikely because of the oxidant used. Figure 5.3 shows the temperature dependence of this signal. As the temperature increases, the intensity drops rapidly. Above 70K there is essentially no more signal. This temperature dependence indicates that this signal does not arise from a radical species. In addition, it does not
Figure 5.4: Development of the oxidized 4Fe4S signal during reconstitution of HiPIP with the intermediate. Freeze-quench conditions: 1.2 mM apo-HiPIP with 84 mM DTT and 12 mM Fe$^{3+}$ in 50 mM Tris was added to 12 mM S$^{2-}$ in Tris before oxidation by 160 mM K$_2$IrCl$_6$. EPR: X-band frequency 9.456 GHz, modulation frequency 100 KHz, modulation amplitude 2.5G, microwave power 2 mW, temperature 5K.
appear in the control where there is no Fe added. Therefore, this early transient signal comes from protein-bound iron intermediate species.

By adding Fe first, the order of adding iron and sulfide was also studied for the identification of species X. Figure 5.4 shows the freeze-quench EPR spectra after adding $S^{2-}$ to a solution of apo HiPIP incubated with DTT and Fe$^{2+}$. (The complete experiment included a 1200 second sample, which was lost during storage). The signal from X species shows up in the first spectrum, 16 milliseconds after mixing $S^{2-}$ with apo/DTT/Fe$^{2+}$. The broad signal from Int I mentioned in Chapter 4 (where no $S^{2-}$ was added) does not appear. This is one of the evidences indicating the involvement of sulfide in the formation of this intermediate species X. The X signal decreases, and Y forms, which is almost EPR silent. This is similar to the previous case, where sulfide was added before iron. Notice that the time frame for the formation of Y, is several tens of seconds, and is longer compared with the case where Fe was added after sulfide. The development of WT 4Fe-4S cluster signal is also slower. After about 600 seconds, the $g_\parallel$ component is obvious, but the $g_\perp$ component is hardly seen.

In order to determine the role of sulfide in the formation of this species, selenide-substituted reconstitution experiments were carried out (Figure 5.5). 16 milliseconds after adding Se$^{2-}$ to a solution of apo/DTT/Fe$^{2+}$, no X-like signals had appeared. This is the second evidence indicating that $S^{2-}$ was involved in X formation. An X-like signal does grow up afterwards, as shown from the 160-millisecond sample. Compared with the signal from the X species, this signal has the $g = 2.00$ and $g = 2.08$ components, but lacks the $g_1 = 1.96$ component. It is unlikely to be a Y-like species for
Figure 5.5: Freeze-quench for Se\textsuperscript{2-} HiPIP reconstitution. 1.2 mM apo HiPIP was incubated anaerobically with 84 mM DTT and 12 mM Fe\textsuperscript{2+} for 15 minutes, and then mixed with 12 mM Se\textsuperscript{2-}; The mixture was then incubated for a period of time, oxidized by 160 mM Ir\textsuperscript{4+} before freezing. EPR parameters: microwave frequency, 9.46GHz, microwave power, 2mw; modulation frequency, 100 kHz, modulation amplitude, 2.53 G; receiver gain, 10\textsuperscript{4}; temperature 5 K.
two reasons: the order of appearance, and the sharpness of its $g_2$ component. The X-like signal reaches its maximum intensity in about one second after mixing Se$^{2-}$ with apo/DTT/Fe$^{2+}$, then decreases and changes to a broader signal, which is likely an Y-like species. Afterward, a 4Fe-4Se -like signal starts to grow slowly. The 4Fe-4Se cluster has two major $g$ components similar to 4Fe-4S cluster, with different $g$ values: $g_{\|} = 2.04$, $g_{\perp} = 2.16$. Two smaller components can be seen between $g = 2.08$ and $g = 2.12$ (Moulis, et al., 1988). Similar to the case in Figure 5.2, the Y-like signal does not quite disappear even 600 seconds after mixing.

When Fe$^{2+}$ was added to apo/DTT/Se$^{2-}$, the X-like signal appears in the shortest possible incubation time—16 milliseconds (data not shown), as found for the S$^{2-}$ case. The signal has $g = 2.00$ and $g = 2.08$ components, but lacks the $g_1 = 1.96$ in the X species. These are the same features of the X-like signal observed when adding Se$^{2-}$ to apo/DTT/Fe$^{2+}$. The difference brought by reversing the order of addition of Se$^{2-}$ and Fe$^{2+}$ will be discussed later.

Some other aspects about the X species were also studied. Particularly, the effect of oxidation by K$_3$Fe(CN)$_6$ instead of K$_2$IrCl$_6$, and the power saturation behavior were investigated. Figure 5.6 shows freeze-quench EPR spectra from samples oxidized by K$_3$Fe(CN)$_6$. K$_3$Fe(CN)$_6$ gives rise to a very broad intense EPR signal around $g = 3$ in the control experiments without apo HiPIP. This background leaves the left side of the spectra with a slope. The X signal, especially the $g = 1.96$ and $g = 2.00$ component, still can be seen, even though it is heavily covered by this background. As with Ir$^{4+}$ oxidation, the intensity of X signal decreases, and the WT 4Fe-4S cluster grows up slowly. The
Figure 5.6: Full reconstitution of 4Fe4S cluster in freeze-quench EPR experiments. Fe$^{2+}$ was added to a solution of apo-HiPIP with DTT and S$^{2-}$ in Tris buffer pH 8.0, then oxidized by K$_3$Fe(CN)$_6$ after incubation; EPR conditions: microwave power, 2 mW, microwave frequency, 9.456 GHz; temperature, 5K, modulation frequency, 100 kHz, modulation amplitude, 2.5 G.
Fe³⁺ 4Fe-4S X

<table>
<thead>
<tr>
<th></th>
<th>Fe³⁺</th>
<th>4Fe-4S</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₁/₂</td>
<td>7.93±1.69</td>
<td>1.57±0.14</td>
<td>0.25±0.06</td>
</tr>
<tr>
<td>K</td>
<td>17232±498</td>
<td>5416±101</td>
<td>12522±928</td>
</tr>
<tr>
<td>b</td>
<td>1.19±0.09</td>
<td>1.06±0.02</td>
<td>0.97±0.03</td>
</tr>
</tbody>
</table>

α: half-power saturation levels (P₁/₂) were estimated by use of the standard empirical equation: \( S = K\left[\frac{P}{1+P/P₁/₂^b}\right]^{1/2} \), where \( S \) is the signal amplitude, \( P \) is the applied power, \( K \) is a proportionality factor, and \( b \) is the inhomogeneity parameter (Innes & Brudvig, 1989).

Table 5.2: EPR Power Saturation Parameters of Several Iron Signals

development of 4Fe-4S cluster signal is slightly faster in this case, but still falls in the range of 100 seconds.

Figure 5.7 shows the power saturation behavior of this X signal. Unlike the other signals observed, the half-saturation power is 0.25 mW, and is much lower than the Fe³⁺ signal and the 4Fe-4S cluster signal (Table 5.2).

5.3.3 Fluorescence Traces in WT HiPIP Reconstitution

Freeze-quench EPR has been a very fruitful technique for directly monitoring iron cluster assembly. Since the environment of the three Trp residues are constantly altered by the formation of different cluster intermediates, the intrinsic fluorescence of HiPIP could also serve as a very sensitive probe to the changes of cluster species. In Chapter 4, this has been used in defining the kinetics of partial cluster assembly in the
absence of sulfide. It would therefore be interesting to compare that with the present study of full 4Fe-4S cluster assembly monitored by fluorescence.

The early kinetic phases looked similar to that in partial cluster assembly. Figure 5.8 shows the early fluorescence traces after adding Fe²⁺ to a solution of apo HiPIP with DTT and sulfide. Two kinetic phases were detected within a second after mixing. Since the cell dimension and electronics on stopped-flow instrument are very different from that on the fluorescence spectrometer, it is necessary to normalize its intensity. This was done by assuming the end point intensity at 5 seconds on stopped-flow is the same to the beginning point of fluorescence spectrometer, which has a dead time of about 5 to 10 seconds.

Although the early decrease in fluorescence intensity can be explained by the quenching from Fe and so called "hydrophobic collapse" of the aromatic core, the slower kinetic phases looked quite different now from the partial assembly case discussed in Chapter 4. Followed by the decrease in fluorescence intensity, the turning point and the subsequent increase in fluorescence in the absence of sulfide are lacking now in the presence of sulfide. This is an indication that the kinetic pathway for partial cluster assembly and that for full cluster assembly may be different, at least in the slow steps.

Freeze-quench EPR studies have already shown that later kinetic phases correspond to the slow formation of the 4Fe-4S cluster. Compared to the intermediate cluster I discussed in Chapter 4, which may be partially coordinated by DTT and solvents, integrated 4Fe-4S cluster leads to a much larger quenching effect, and polar solvent and DTT molecules are excluded completely from the aromatic residues. These may be the factors causing the elimination of the turning point and subsequent rising up in Trp
Figure 5.7: Power saturation curve for the $g=2.01$ signal during reconstitution of HiPIP in the presence of $S^2$. EPR spin quantitation was performed using the Aasa-Vannard equation, relative to the standard oxidized form, and half-power saturation levels ($P_{1/2}$) were estimated by use of the standard empirical equation: $S = K\left[\frac{P}{(1+P/P_{1/2})^{b}}\right]^{1/2}$, where $S$ is the signal amplitude, $P$ is the applied power, $K$ is a proportionality factor, and $b$ is the inhomogeneity parameter (Innes & Brudvig, 1989).
fluorescence in the process of 4Fe-4S cluster assembly. Sulfide and selenide have similar kinetic profile if Fe$^{2+}$ was added to a solution of apo HiPIP with S$^{2-}$ or Se$^{2-}$ in the presence of excess DTT. Figure 5.9 shows fluorescence traces in slow phases after anaerobically adding 0.1 mM Fe$^{2+}$ to 10 μM apo HiPIP with 0.1M S$^{2-}$ (bottom trace) or Se$^{2-}$ (upper trace) in the presence of 1.0 mM DTT. Both of them have two kinetic phases with approximately same rate constants. Under the same condition, the final fluorescence in Se$^{2-}$ reconstitution is always higher than that in S$^{2-}$ reconstitution. This is likely due to the fact that reconstitution is more complete with sulfide than with selenide. Therefore, less apo HiPIP was left in the final solution with S$^{2-}$, making the final fluorescence lower.

5.3.4 Kinetic Data Analysis in 4Fe4S Cluster Assembly

Kinetic data analysis provides additional information on the cluster assembly mechanism. It was found that most of the processes, both from freeze-quench EPR measurements and from fluorescence measurements, could be simulated according to the consecutive first-order reaction model proposed in Chapter 4.

**EPR Measurements.** Figure 5.10 (A) is typical for the decrease of X (first phase and Y species (second phase) studied by freeze-quench EPR. One exception is in the case where Se$^{2-}$ was added to apo HiPIP with DTT and Fe$^{2+}$ (Figure 5.11(A)). The data suggests that the formation of X involves both iron and sulfide. There are two transient phases for X. There is a very rapid phase of formation only seen in the case shown with Se$^{2-}$ in Figure 5.11(A), where the process was slowed down apparently because of Se$^{2-}$.
Figure 5.8: Fluorescence traces after adding 0.1 mM Fe$^{3+}$ to 10 μM apo-HiPIP in 10 mM Tris pH8.0 with 0.6 mM DTT and 0.1 mM S$^{2-}$. The upper-left side panel is from stopped-flow experiment using OLIS-1000 RSM in fluorescence mode. Its intensity was normalized to match the routine fluorescence. The lower-right panel is the routine fluorescence done on PE LS50B fluorescence spectrometer, with excitation at 282 nm, slit width 12 nm, emission at 353 nm, slit width 0 nm. Symbols represent experimental data, lines are fitting curves.
Figure 5.9: $S^2-$ vs. $Se^2-$ in reconstitution monitored by routine fluorescence. 10μM apo HiPIP in 50 mM tris pH 8.0 with 1 mM DTT and 100 μM FeCl₂; Add 100 μM $Se^2-$ (top), or $S^2-$ (bot) anaerobically. Excitation wavelength, 280nm, emission, 352nm. Entrance slit width, 4 nm, exit slit, 4 nm.
Figure 5.10: (A) Decrease of the X and Y signals at $g = 2.01$ and (B) Development of HiPIP oxidized EPR signal during reconstitution of apo-HiPIP in the PRESENCE of $S^{2-}$. 1.2mM apo-HiPIP with 84 mM DTT and 12 mM $S^{2-}$ in 50 mM tris pH 8.0 was mixed with 12 mM $Fe^{2+}$ in tris. After incubation for some time, the mixture was oxidation by 160mM $K_{2}IrCl_{6}$ in tris. EPR: microwave frequency, 9.456 GHz, modulation frequency, 100 KHz, microwave power, 2 mW, temperature, 5K.
Figure 5.11: Decrease of the X and Y signals at $g = 2.01$ during the reconstitution of apo-HiPIP in the PRESENCE of $S^2$. Same as Figure 5.10, but fit X and Y seperately.
Figure 5.12: Seleno-HiPIP 4Fe-4Se cluster assembly monitored by freeze-quench EPR experiment. To 1.2 mM apo HiPIP with 84 mM DTT and 12 mM Fe$^{2+}$ add 12 mM Se$^{2-}$. The mixture was incubated for some time before oxidized by 160 mM Ir$^{4+}$ followed by freezing. EPR parameters: microwave frequency, 9.46 GHz, power 2 mw; MF= 100 kHz, MA= 2.53 G, RG=10$^4$, T=5K.
The observed rate constant of this phase for S\(^2^-\), \(k_0\), is estimated to be over 100 s\(^{-1}\). This is followed by one fast decreasing phase, which has a rate constant \(k_1\) around 25 s\(^{-1}\) with S\(^2^-\), leading to the formation of Y. The slower decrease of Y has a rate constant \(k_2\). In the case where Fe\(^{2+}\) was added to apo/DTT/S\(^2^-\), the decrease of Y goes parallel with the second phase in the formation of 4Fe-4S cluster. In the case where S\(^2^-\) was added to apo/DTT/Fe\(^{2+}\), however, it goes with the first phase in the formation of 4Fe-4S cluster. In all cases with sulfide, regardless of the oxidant used (Ir\(^{4+}\) or Fe\(^{3+}\)), and regardless of the order of Fe\(^{2+}\) and S\(^2^-\) added, the appearance of X is rapid, well within 16 ms. (Figure 5.10(A)). It becomes much slower with selenide (Figure 5.11(A)). The number of data points was not enough to do a good fit, but the apparent rate constant for X-like signal was estimated to be around 10 s\(^{-1}\). Another difference with selenide is that the Y-like signal decreases slowly, with an estimated rate constant of about 0.2 s\(^{-1}\). With sulfide, the decrease of X is faster. Observed rate constants are summarized in Table 5.3.

### Table 5.3: Kinetic Rate Constants from Freeze-quench EPR Measurements

<table>
<thead>
<tr>
<th></th>
<th>(k_0) (s(^{-1}))</th>
<th>(k_1) (s(^{-1}))</th>
<th>(k_2) (s(^{-1}))</th>
<th>(k_3) (s(^{-1}))</th>
<th>(k_4) (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo/S+Fe2</td>
<td>&gt;100</td>
<td>16.2±6.9</td>
<td>0.0013±0.0001</td>
<td>0.63±0.17</td>
<td>0.0015±0.0005</td>
</tr>
<tr>
<td>Apo/S+Fe(^2+)</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td>0.79±0.08</td>
<td>0.001±0.0004</td>
</tr>
<tr>
<td>Apo/Fe2+S</td>
<td>&gt;100</td>
<td>31.9±8.5</td>
<td>0.47±0.17</td>
<td>0.36±0.04</td>
<td>0.0012±0.0003</td>
</tr>
<tr>
<td>Apo/Fe2+Se</td>
<td>10</td>
<td></td>
<td>= 0.2</td>
<td>0.73±0.19</td>
<td>0.002±0.0005</td>
</tr>
</tbody>
</table>

\(a\): Fe\(^{3+}\) was used as oxidant; in all other sets Ir\(^{4+}\) was used.
Figure 5.11(B) is a typical case for the development of the mature 4Fe-4S(Se)
cluster. It appears to be biphasic with either sulfide or with selenide, although in the case
where Fe$^{2+}$ was added to apo HiPIP with DTT and S$^{2-}$, the first phase was not very
obvious. The rate constants are also similar for S$^{2-}$ and for Se$^{2-}$.

*Fluorescence Measurements.* The intrinsic fluorescence from tryptophan
residues changes as the protein backbone folds around the iron sulfur cluster. Therefore,
it is used here to give complementary results on the kinetics of cluster assembly. Rate
constants were obtained from fitting fluorescence traces to a pseudo-first-order
consecutive reaction model (Rodiguin and Rodiguina, 1964). Figure 5.8 shows typical
fitting curves. The fast phases from stopped-flow measurement were fitted separately
from the slow phases from routine fluorescence measurement. There are totally four
kinetic phases. The rate constants are summarized in Table 5.4.

<table>
<thead>
<tr>
<th></th>
<th>$k_1^*$</th>
<th>$k_2^*$</th>
<th>$k_3^*$</th>
<th>$k_4^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apo/S+Fe</td>
<td>39.0±4.9</td>
<td>0.99±0.32</td>
<td>0.02±0.0004</td>
<td>0.002±0.0001</td>
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<tr>
<td>Apo/Fe + S</td>
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<td>0.026±0.0003</td>
<td>0.0036±0.0001</td>
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<tr>
<td>Apo/Fe+Se</td>
<td></td>
<td></td>
<td>0.044±0.0005</td>
<td>0.0073±0.0002</td>
</tr>
</tbody>
</table>

*Table 5.4:* Rate Constants in 4Fe-4S Cluster Assembly by Fluorescence Measurements
5.4 DISCUSSION

5.4.1 The Identity of X.

Intermediate species X in 4Fe-4S cluster assembly was characterized by its special EPR features in the oxidized state: $g_1 = 1.96$, $g_2 = 2.00$, $g_3 = 2.08$. It is EPR silent in the reduced state. X grows in rapidly after addition of iron to a solution of apo HiPIP with sulfide and excess DTT. The EPR signal intensity of X decreases to about zero at temperatures above 60K. The signal doesn’t appear in control samples that have no added Fe. It formed slower in selenide-substituted reconstitution. All of these indicate that X is an iron sulfur cluster intermediate in the early stage of 4Fe-4S cluster assembly.

Moreover, we propose that it is a di-ferrous iron center with sulfide and DTT bridges. Single Fe$^{3+}$ species give a distinct EPR signal at $g = 4.3$ that is nearly isotropic. Rubredoxins also have single iron centers, with a high-spin $S = 5/2$ state, giving rise to a signal at about $g = 4$ (Cammack and Cooper, 1993). Oxygen-coordinated single iron centers in dioxygenases and other systems generally give rhombic EPR signals with $g$ value between 4.5 and 3.5 (Cammack and Cooper, 1993). There is a predominate signal at $g = 4.3$ in our oxidized preparations presumably because of the excess Fe$^{3+}$ added. Besides the difference in EPR signals, it is very unlikely for X to be a single iron species because of the involvement of inorganic sulfide. Iron coordination with cysteiny1 sulfide is almost certain in the light of other iron sulfur proteins. It is difficult for a single sulfide or disulfide to coordinate to a single iron. Di-nitrosyl iron species do give rise to a similar EPR signal (Kennedy, et al., 1997) as X, however, it is not likely in our case since no
obvious NO source present. Reduced 2Fe-2S ferredoxins (Cammack, et al., 1985) and the semi-met form of hemerythrin-type di-iron centers (Pearce, et al., 1987) are spin 1/2 systems. They all have rhombic EPR signals with a predominant \( g_z \) component, which is not present in X. Three-iron centers in proteins usually give rise to a near axial signal close to \( g = 2.01 \) with a large \( g_z \) component. On the other hand, a diferrous center has a ground spin zero. Without further oxidation it is EPR silent. In one of its oxidized states \([\text{Fe}^{2+}\text{Fe}^{3+}]\), it has spin 1/2 because of antiferromagnetic coupling similar to the reduced 2Fe-2S ferredoxins. This can give rise to an EPR signal with \( g \) value around 2. However, the fact that the X signal does not look like that observed in 2Fe-2S ferredoxins most likely indicates differences in coordination of the iron centers.

It should be pointed out that the binding of iron to the cysteinyl sulfide is likely the very first event in cluster assembly. There are only four cysteines in HiPIP. Our study in Chapter 4 indicated a clear dependence of rate constants in the rapid phases on the concentration of DTT, which indicates the requirement of exogenous thiol groups in providing extra coordination to the irons bound to the protein cysteines. This step is too fast to be detected in freeze-quench EPR experiments, in which high concentrations (~mM) of protein and reagents were used. It may also have escaped detection in fluorescence measurements, forming during the dead time, although it is possible that the first rapid phase in fluorescence reflects these events, noticing that the concentration is about 100 times lower (10 \( \mu \)M) in the fluorescence experiments.

Since initially single irons were coordinated to cysteine and DTT molecules, it takes sulfide to replace DTT coordination, and to bring the single irons together to form di-iron centers. The di-iron center may contain, to a certain extent, residual DTT
coordination after forming one sulfide bridge. It is very likely that this special coordination system, when oxidized, gives rise to the X EPR signal.

5.4.2 Intermediate Species Y

Y has a g value about 2.00. It is formed after X. The EPR signal from Y species is very weak (Figure 5.4, 4s). Its decrease in intensity parallels with the increase of the signal from the 4Fe-4S cluster.

From these features of Y, we propose that Y is a loose association of two antiferromagnetically coupled X clusters, possibly with structural rearrangements. This is also consistent with the necessity of a WT 4Fe-4S cluster formation during the next step. The extent of antiferromagnetic coupling may depend on the order of Fe$^{2+}$/S$^{2-}$, and S$^{2-}$ vs. Se$^{2-}$ as discussed in the next section. This explains the difference in $k_2$ (Table 5.3).

5.4.3 Sulfide vs. Selenide and the Order of Adding Fe$^{2+}$/S$^{2-}$

Selenide-substituted HiPIP has a 4Fe-4Se cluster. The mature cluster does not have too much difference in terms of the cubane frame (Moulis, et al., 1988), and the electromagnetic properties are not very different (Sola, et al., 1989;). However, the process of initial intermediate formation may differ significantly because of the strong selective preference of Fe on S$^{2-}$ over Se$^{2-}$ and of the difference in their ionic radii. When Fe$^{2+}$ was added to apo-HiPIP in the presence of excess DTT, an intermediate cluster formed, in which iron centers were partially coordinated by cysteinyl sulfide, DTT and possibly solvent molecules. The intermediate already has a tertiary structure close to the
WT protein (Natarajan and Cowan, 1997). For a mature 4Fe-4S(Se) cluster to form, the $S^{2-}/Se^{2-}$ must go into the partially formed cluster pocket, and replace the DTT solvent coordination sites. Here the difference in ionic radii may play an important role. The ionic radius of $S^{2-}$ is 1.84 Å, and that of $Se^{2-}$ is 1.98 Å. It is more difficult for $Se^{2-}$ to pass through the structural barriers to reach the right place, since it is larger than $S^{2-}$. This may explain the difference in adding them to a pre-incubated solution of apo/DTT/Fe$^{2+}$. X formed very rapidly in the case of $S^{2-}$ (Figure 5.4), but the formation of X-like species with $Se^{2-}$ is much slower (Figure 5.5). In the case where Fe$^{2+}$ was added to a solution of apo-HiPIP/DTT/$S^{2-}/Se^{2-}$, since the sulfide (selenide) is already there to coordinate iron, the difference in ionic radii does not add much significance any more. Our experimental result is consistent with this explanation.

The slow development of the mature 4Fe-4S(Se) cluster comes after Y species. Our kinetic data, both from freeze-quench EPR (Table 5.3) and from fluorescence (Table 5.4), suggest that there is basically no difference between $S^{2-}$ and $Se^{2-}$ in the formation rates of the mature cluster. It is noticed that selenide gives slightly higher rate constants than sulfide, but difference is not significantly large (other rate constants differ in an order of magnitude). This implies that Y probably has 4Fe and 4S(Se), arranged in an immature manner. Therefore the later rearrangement and any other slow processes in forming the mature cubane 4Fe-4S(Se) cluster do not need exogenous addition of sulfide (selenide).
Scheme 5.1: A Working Model for 4Fe-4S Cluster Assembly in HiPIP
Scheme 5.1

ApoHiPIP
+ DTT
+ S^2-
+ Fe^{2+}

>100 s \(^{-1}\)

= 2.1 ± 0.3 s \(^{-1}\)

\[ V \]
5.4.4 A Working Model for 4Fe-4S Cluster Assembly

From the above discussion, a working model illustrating the mechanism of 4Fe-4S cluster assembly is proposed in Scheme 5.1. In the presence of over 50 fold excess of DTT, single iron centers first bind to cysteiny1 sulfide with extra coordination provided by DTT and possibly, solvent H₂O molecules. This step is very rapid, and probably occurs in a diffusion-limited manner. If there is no sulfide around, these iron centers will eventually be bridged by cysteiny1 thiolate and possibly DTT or solvent molecules to form intermediate I, as discussed in Chapter 4. In the presence of sulfide, however, two of the iron centers, probably the adjacent ones with Cys43 and Cys46, will be bridged by sulfide, with DTT and solvent providing other coordinating ligands, thus forming intermediate species X. This would cause conformational changes on the protein backbone, and facilitate another similar di-iron center to form with coordination to two other cysteines, Cys63 and Cys77. The formation of the di-iron center in X is very fast with estimated rate constant $k_0$ around 100 s⁻¹ in the case of sulfide. Formation of the two diiron centers is accompanied by the rapid folding of the protein backbone. Further folding of the backbone brings these two di-iron centers closer, forming an intermediate species Y. In oxidized Y, both of these di-iron centers have Fe³⁺/Fe²⁺ configuration (S=1/2). Weak anti-ferromagnetic coupling interaction leads to a small (or even silent) EPR signal. The formation of Y (or the disappearance of X) is fast ($k_f$~25 s⁻¹ with sulfide) and transient. With Se²⁻, the exact moment was not caught in the freeze-quench EPR shots. The conformational changes and structural rearrangements do not stop here, although the global tertiary structure would be close to the WT at this stage. Further
rearrangements of these two di-iron centers include displacement of DTT and any solvent coordination by shared sulfide ligation, with possible simultaneous oxidation of one iron on each di-iron center, giving rise to the mature [4Fe-4S]$^{2+}$ cluster. These final rearrangements are slow steps. The source of oxidant for this transition is not clear now, possibly trace amount of oxygen in the solution. It may be some other biological oxidant in vivo.

We have noticed that this 4Fe-4S cluster assembly pathway has an analogue in synthetic studies. Hagen and coworkers defined reaction sequences resulting in self-assembly of [Fe$_4$S$_4$(SR)$_4$] clusters from simple reactants, in which they demonstrated elaboration of a tetranuclear cluster through mononuclear and then binuclear intermediates (Hagen, K. S., et al, 1981). 2Fe-2S clusters to 4Fe-4S cluster conversion have been reported in other biological systems, such as biotin synthase (Duin et al., 1997) and FNR (fumarate and nitrate reduction) (Khoroshilova et al., 1997). The cluster assembly mechanism for in vivo reconstitution of cytosolicaconitase from IRPI is not known, but it is likely that the mechanism we proposed here is relevant to other biological Fe-S cluster processes.

5.5 SUMMARY

In vitro 4Fe-4S cluster assembly was studied by freeze-quench EPR techniques and time-resolved fluorescence method. Fluorescence experiments have detected at least three kinetic intermediates. Freeze-quench EPR experiments identified a series of iron cluster intermediates, in which two important ones are well characterized as X and Y
species. Based on a set of control and reference experiments, including seleno-substituted reconstitution, a working model for the mechanism of in vitro 4Fe-4S cluster assembly in HiPIP has been proposed and presented in Scheme 5.1. Iron binding to cysteine residues is most likely the first event in cluster assembly. This step is probably diffusion-limited. DTT thiol groups (and possibly solvents) provide necessary coordination for these single iron centers. In the presence of sulfide, two of the adjacent single iron centers were rapidly (estimated rate constant > 100 s\(^{-1}\)) converted to a diiron center bridged by inorganic sulfide(s), giving rise to an intermediate species X. X has a distinct EPR signal when oxidized: \(g_1 = 1.96, g_2 = 2.00, g_3 = 2.08\). Formation of short bridges in X promote protein folding, and the formation of another di-iron center with a rate constant ~ 25 s\(^{-1}\).

The Y species then forms when these two di-iron centers are brought close enough to show anti-ferromagnetic coupling interaction. The formation of Y marked the beginning of a series of slow structural rearrangements, a kinetically biphasic process, with rate constants around 0.3s\(^{-1}\) and 0.002 s\(^{-1}\). These rearrangements must include the final replacement of any thiol coordination, formation of sulfide bridges with three irons (two irons in X and Y), and oxidation of each of the di-iron centers. These are all required for final [4Fe-4S]\(^{2+}\) cluster formation, some of which are rate-limiting steps.

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IMAGE EVALUATION
TEST TARGET (QA-3)

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