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Characterization of heme and heme moiety in native and cobalt-substituted heme respiratory proteins by low temperature Fourier transform infrared spectroscopy

Park, Sungjo, Ph.D.

The Ohio State University, 1994
CHARACTERIZATION OF HEME AND HEME MOIETY IN NATIVE AND COBALT SUBSTITUTED HEME RESPIRATORY PROTEINS BY LOW TEMPERATURE FOURIER TRANSFORM INFRARED SPECTROSCOPY

DISSERTATION

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

By

Sungjo Park, B.S., M.S.

************

The Ohio State University

1994

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Adviser
Biophysics Graduate Program
ACKNOWLEDGEMENTS

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# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS** ................................................................................... ii

**VITA** ................................................................................................................ iii

**LIST OF TABLES** ........................................................................................... viii

**LIST OF FIGURES** .......................................................................................... x

**GLOSSARY** ........................................................................................................ xvi

## CHAPTER 

<table>
<thead>
<tr>
<th>I. INTRODUCTION</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Porphyrin Skeletal Modes</td>
<td>2</td>
</tr>
<tr>
<td>1.2 Assignment of in-plane Porphyrin Fundamental Modes</td>
<td>5</td>
</tr>
<tr>
<td>1.3 Out-of-plane Porphyrin Skeletal Modes</td>
<td>15</td>
</tr>
<tr>
<td>1.4 Vinyl Vibrational Modes</td>
<td>15</td>
</tr>
<tr>
<td>1.5 List of References</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>II. SPECTROSCOPIC CHARACTERIZATION OF OPTICAL PUMPING IN PHOTOLYZED HbCO</th>
<th>21</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Abstract</td>
<td>21</td>
</tr>
<tr>
<td>2.2 Introduction</td>
<td>21</td>
</tr>
<tr>
<td>2.3 Methods</td>
<td>24</td>
</tr>
<tr>
<td>2.4 Results</td>
<td>26</td>
</tr>
<tr>
<td>2.5 Discussion</td>
<td>34</td>
</tr>
<tr>
<td>2.6 Conclusions</td>
<td>39</td>
</tr>
<tr>
<td>2.7 List of References</td>
<td>41</td>
</tr>
</tbody>
</table>
III. SPECTROSCOPIC CHARACTERIZATION OF HEME MOIETY IN NATIVE AND COBALT SUBSTITUTED HEME PROTEINS BY FOURIER TRANSFORM INFRARED SPECTROSCOPY: IMPLICATION OF VINYL GROUPS AND DIOXYGEN LIGAND ....................................................... 44

3.1 Abstract ..................................................................................... 44
3.2 Introduction .............................................................................. 46
3.3 Methods ..................................................................................... 50
3.4 Results ....................................................................................... 52
3.5 Discussion .................................................................................. 71
3.6 Conclusions ............................................................................... 82
3.7 List of References ................................................................... 83

IV. INFRARED ASSIGNMENT OF PORPHYRIN SKELETAL AND SUBSTITUENT VIBRATIONAL MODES IN HEME RESPIRATORY PROTEINS AT LOW TEMPERATURE ............ 90

4.1 Abstract ..................................................................................... 90
4.2 Introduction .............................................................................. 91
4.3 Methods ..................................................................................... 93
4.4 Results ........................................................................................ 97
4.5 Discussion ........................................................... 119
  4.5.1 Photoperturbation absorbance difference spectroscopy .............................................................. 119
  4.5.2 In-plane $E_u$ and out-of-plane $A_{2u}$ porphyrin modes ........................................ 120
    4.5.2.1 In-plane $E_u$ modes ................................................. 120
    4.5.2.2 Out-of-plane $A_{2u}$ modes ........................................... 128
  4.5.3 Vinyl modes ........................................................................ 130
  4.5.4 Formyl modes ..................................................................... 134
  4.5.5 Peripheral substituent modes .............................................. 139
  4.5.6 Deuteration and pH Effects ................................................ 141
  4.5.7 Difficulties of band assignment in biological proteins .................................................. 143
4.6 Conclusions ............................................................................... 145
4.7 List of References ................................................................... 146
V. PHOTOPERTURBATION OF HEME A₃-FORMYL AND VINYL GROUPS OF CYTOCHROME C OXIDASE CO COMPLEX OBSERVED BY FOURIER TRANSFORM INFRARED SPECTROSCOPY .............................................................. 154

5.1 Abstract ..................................................................................... 154
5.2 Introduction .............................................................................. 154
5.3 Methods ..................................................................................... 157
5.4 Results ...................................................................................... 158
5.5 Discussion .................................................................................. 170
5.6 List of References ................................................................. 175

VI. SUMMARY .................................................................................... 179

BIBLIOGRAPHY .................................................................................. 182

APPENDIX .......................................................................................... 198
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. (A) Reducible representation of metalloporphyrin in $D_{4h}$ symmetry. The substituent groups are treated as point masses. (B) Character table in $D_{4h}$ symmetry</td>
<td>4</td>
</tr>
</tbody>
</table>
10. Calculated out-of-plane vibrations for Ni\textsuperscript{II}(OMP) and assignments for Ni(OEP). (From Spiro and Li, Biological Application of Resonance Raman Spectroscopy, Vol III, 1988, Wiley-Interscience, pp. 1-37) ................................................................. 17

11. Absorptivities and negative peak frequencies of selected bands .......... 70

12. Infrared frequencies of HbCO, MbCO and CcO-CO assignable to porphyrin fundamental $E_u$ and $A_{2u}$ modes, and imidazole modes ........................................................................................................ 116

13. Infrared frequencies of HbCO, MbCO and CcO-CO assignable to vinyl modes ........................................................................................................ 117

14. Formyl modes of cytochrome c Oxidase ............................................ 118

15. Assignments of the carbonyl stretching mode of the formyl group in cytochrome c oxidase and model compounds ...................................................... 167

16. Analysis of heme a, 8-formyl group in cytochrome c oxidase .......... 169

17. Tentative assignment of farnesyl derivatives ..................................... 202

18. Assignment of benzaldehyde ............................................................. 204
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>3. Vinyl vibrations of Olefin. The left-hand column illustrates the in-plane vibrations and left-hand column illustrates the out-of-plane vibrations (+ and -) of vinyl group (From Colthup et al. Introduction to Infrared and Raman Spectroscopy, 1990, Academic Press, p.248)</td>
<td>18</td>
</tr>
<tr>
<td>4. FT-IR absorbance difference spectrum of HbCO at 10 K shows A and B substates. The 1905 cm⁻¹ band results from the natural abundance of heavy isotopes ¹³C¹⁶O and ¹²C¹⁸O</td>
<td>27</td>
</tr>
<tr>
<td>5. (A). Absorbance ratio of photodissociated B₂/B₁ in HbCO with continuous light photodissociation at 10, 20 and 30 K. (B) Fractional photolysis of carbon monoxide in HbCO obtained simultaneously with (A) (From S. Park., M.S. Thesis, The Ohio State University, Columbus, Ohio)</td>
<td>28</td>
</tr>
</tbody>
</table>
6. Absorbance changes of $B_2$ and $B_1$ substates in HbCO at different time (1018 & 11505 sec) during continuous illumination from tungsten project lamp at 10 K ........................................................... 31

7. The effect of temperatures of CO in photolyzed CO. Apparent integrated absorptivity and fractional photolysis of CO were collected from 10 to 50 K (From S. Park., M.S. Thesis, The Ohio State University, Columbus, Ohio) .......................................... 32

8. Absorbance changes of $B_2$ and $B_1$ substates in sperm whale MbCO at different time (1018 & 11505 sec) during continuous illumination from tungsten project lamp at 10 K ............................................................................................................... 35


10. (A). Light minus dark photodissociation spectra of dioxygen from oxycobalt substituted Mb and Hb at 11.7 Kelvin. Each protein was prepared in a pair of BaF$_2$ windows with 7 microns spacer: 
(a) proto-Co-MbO$_2$; (b) proto-Co-HbO$_2$; (c) meso-Co-MbO$_2$.
Each spectrum was normalized for the absorption of 1134 cm$^{-1}$ band. (B). The absorbance double-difference spectrum of [(L/D)proto-Co-MbO$_2$ - (L/D)meso-Co-MbO$_2$] ................. 54

11. (A). Light minus dark photodissociation spectra of HbO$_2$, Hb$^{18}$O$_2$, and HbCO at 10.5 K. Each protein was prepared in a pair of BaF$_2$ infrared windows and KCl window cryostat cube with 24.8 microns spacer. (B). The absorbance double-difference spectrum of 
[(L/D)Hb$^{18}$O$_2$ - (L/D)Hb$^{16}$O$_2$]. (C). The absorbance double-difference spectrum of 
[(L/D)Hb$^{18}$O$_2$ - (L/D)HbCO] ........................................................................ 57

12. (A). Light minus dark photodissociation spectra of sperm whale MbO$_2$, Mb$^{18}$O$_2$, and MbCO at 13.8 K. Each protein was prepared in a pair of BaF$_2$ infrared windows and KCl window cryostat cube with 24.8 microns spacer. (B). The absorbance double-difference spectrum of [(L/D)Mb$^{18}$O$_2$ - (L/D)Mb$^{16}$O$_2$]. (C). The absorbance double-difference spectrum of [(L/D)Mb$^{18}$O$_2$ - (L/D)MbCO] ............... 63
13. Light minus dark photodissociation spectra of bovine heart MbO₂ and MbCO at 11.5 K. Each protein was prepared in a pair of BaF₂ infrared windows and KCl wind cryostat cube with 7 microns spacer

14. Relaxation rate of two apparent dioxygen bands in meso-Co-MbO₂ at 12 K. The 1104 cm⁻¹ peak is denoted by a closed circle, and 1136 cm⁻¹ peak is denoted by a closed square. N(B) is expressed by ΔA(t)/ΔA(t=0) where A(t) refers to the absorbance difference between a spectrum taken at time, t, and A(t=0) is the maximum absorbance from the continuous light spectrum at 12 K

15. Absorbance difference spectra of Hb derivatives at above freezing temperature. (From S. Park., M.S. Thesis, The Ohio State University, Columbus, Ohio)

16. Photoperturbation (light/dark) FT-IR spectra of HbCO at 10 K was prepared on a single KRS-5 window and collected by Dr. Alben. The spectrum was collected with liquid helium cooled Zn:Ge detector and a CsI beamsplitter. The strong negative peak at 666 cm⁻¹, denoted by ⭐, is the bending mode of CO₂ gas which is present in the infrared radiation beam path

17. Photoperturbation (light/dark) FT-IR spectrum of HbCO was prepared in H₂O, and in D₂O and Hb¹³CO at 10 K. The KBr beamsplitter absorption band at 1267 cm⁻¹ is completely uncompensated. Spectrum was normalized for the absorption of 1236 cm⁻¹ peak

18. Photoperturbation (light/dark) FT-IR spectra of sperm whale MbCO prepared in H₂O, in D₂O, a site-directed mutant MBCO (HisE7>Phe) at 10 K. The KBr beamsplitter absorption band at 1267 cm⁻¹ is completely uncompensated. Spectrum was normalized for the absorption of 1239 cm⁻¹ peak
19. Photoperturbation (light/dark) FT-IR spectra of sperm whale MbCO at different pH at 10 K:
(A) pH 8.5, 50 mM Tris-HCl; (B) pH 7.0, 50 mM Bis-Tris; (C) pH 6.0, 30 mM MES. The KBr beamsplitter absorption band at 1267 cm$^{-1}$ is completely uncompensated. Spectrum was normalized for the absorption of 1239 cm$^{-1}$ peak ............................................................ 102

20. Photoperturbation (light/dark) FT-IR spectra of bovine heart, horse heart MbCO, and sperm whale MbCO at 10 K. The KBr beamsplitter absorption band at 1267 cm$^{-1}$ is completely uncompensated. Spectrum was normalized for the absorption of 1239 cm$^{-1}$ peak ........................................................................................................ 103

21. Photoperturbation (light/dark) FT-IR spectra of HbO$_2$, bovine heart MbO$_2$, and sperm whale MbO$_2$ at 10 K. The peaks at around 1390 cm$^{-1}$ with 0.5 cm$^{-1}$ bandwidth in HbO$_2$ are water vapor absorptions. Spectrum was normalized for the absorption of an apparent dioxygen band at 1106 cm$^{-1}$ (HbO$_2$) and 1104 cm$^{-1}$ (MbO$_2$) ........................................................................................................ 104

22. (A). Photoperturbation (light/dark) FT-IR spectra of heme a$_3$ of CcO-CO at 13 K, collected in the "forward" direction of moving mirror. (B). Photoperturbation (light/dark) FT-IR spectra of heme a$_3$ of CcO-CO at 13 K, measured in the "forward" and "reverse" directions of moving mirror, and their difference ..................... 105

23. Photoperturbation (light/dark) FT-IR spectra of protoporphyrin IX in HbCO and bovine heart MbCO, and heme A in CcO-CO at 10 K: (A) low frequency region (920 - 1350 cm$^{-1}$); (B) high frequency region (1300 - 1720 cm$^{-1}$) ................................................................. 107

24. Photoperturbation absorbance double-difference spectra of Hb and Mb in H$_2$O and D$_2$O solution used in Figures 17 and 18 at 10 K: (A) (L/D)HbCO in H$_2$O - (L/D)HbCO in D$_2$O; (B) (L/D)MbCO in H$_2$O - (L/D)MbCO in D$_2$O, Sperm Whale ......... 109

25. Photoperturbation absorbance double-difference spectra of Mb at different pH at 10 K, used in Figure 19: (A) (L/D)MbCO: pH 8.5 - pH 7.0; (B) (L/D)MbCO: pH 8.5 - pH 6.0 .................................................... 111
26. Photoperturbation absorbance double-difference spectra at 10 K used in Figures 17 and 18:
(A) (L/D)SW-MbCO - (L/D)MbCO (HisE7>Phe);
(B) (L/D)Hb^{12}CO - (L/D)Hb^{13}CO

27. Difference spectra for Lorentzian bands resulting from bandwidth and frequency changes. (A) Bandwidth change only: \( \Gamma_A = 12 \text{ cm}^{-1}, \Gamma_B = 8 \text{ cm}^{-1} \); \( \Delta \nu_0 = 0 \text{ cm}^{-1} \). (B) Frequency shift only: \( \Gamma_A = \Gamma_B = 10 \text{ cm}^{-1} \); \( \Delta \nu_0 = 0.5 \text{ cm}^{-1} \). (C) Both bandwidth and frequency changes: \( \Gamma_A = 12 \text{ cm}^{-1}, \Gamma_B = 8 \text{ cm}^{-1} \);
\( \Delta \nu_0 = 0.5 \text{ cm}^{-1} \) (From Laane, J. Chem. Phys. (1981) 75:2539-2545)

28. Room temperature visible spectrum of cytochrome c oxidase CO complex following low temperature photodissociation studies illustrated in Figures 29, 30, and 32

29. Fourier transform infrared absorbance difference spectrum of the CO complex of cytochrome c oxidase at 12 K, "after" minus "before" photodissociation (light minus dark)

30. The photoperturbation infrared spectrum (light minus dark) of heme \( a_3 \) of cytochrome c oxidase at 13 Kelvin. The beam splitter band is denoted by ★

31. Absorbance difference spectrum (----) of two Gaussian bands (—) with identical half-widths, \( \Gamma \) (FWHH), normalized to the same peak intensity \( A_p \), but separated in frequency by \( \Delta \nu_0 \). The peak-to-trough absorbance difference is \( \Delta A \), and frequency difference is \( \delta \). The frequency scale is normalized to the half-width. (From Alben and Fiamingo, Optical Techniques in Biological Research, 1984, Academic Press. pp.133-179)

32. Absorbance difference spectra from Figure 30 from 1600-1700 cm\(^{-1}\) plotted with an expanded abscissa to show the frequency-shifted formyl C=O and vinyl \( \text{C} = \text{C} \) bands at 1662.1 cm\(^{-1}\), and 1629.7 cm\(^{-1}\). Some of residual atmospheric water vapor absorptions are denoted by •

33. FT-IR absorbance spectra of farnesyl derivatives (neat solution) with a 16.5 microns spacer at above freezing temperature:
(A) Farnesyl Acetate; (B) Farnesol, scale expanded plot in the region of 1350 - 1400 cm\(^{-1}\) is inserted; (C) Farnesyl Bromide, scale expanded plot in the region of 1350 - 1400 cm\(^{-1}\) is inserted
34. FT-IR absorbance spectrum of 0.01 M Benzaldehyde in CCl₄ with 0.107 mm spacer at above freezing temperature 203

35. Photoperturbation (light/dark) FT-IR spectra of sperm whale MbCO at 10 K illustrated in Figures 18, 20, and 23 measured in the "forward" and "reverse" directions of moving mirror, and their difference. Instrumental contribution bands in the difference spectrum are denoted by ★ 205

36. Single beam spectra of HbCO, sperm whale MbCO, and cytochrome c oxidase illustrated in Figure 23 206

37. Photoperturbation (light/dark) FT-IR spectrum cytochrome c oxidase illustrated in Figure 22. After the spectral collection of Figure 22, protein sample was stored at -20°C for 7 days, and FT-IR spectrum was obtained 208
GLOSSARY

DP        Deuteroporphyrin IX dimethyl ester
(D)FDP    (di)Formyl deuteroporphyrin IX dimethyl ester
OE(M)P    Octaethyl(methyl)porphyrin
Ni(OEP)   Ni$^{ll}$ (octaethylporphyrin)
Ac        Acetyl
ImH       Imidazole
Py        Pyridine
Pyr       Pyrrole
TPP       Tetraphenylporphyrin
NiP       Ni$^{ll}$ porphine
PP        Protoporphyrin
Pa        Heme A porphyrin
CcO       Cytochrome c Oxidase
Hb        Hemoglobin
Mb        Myoglobin
Et        Ethyl
CHAPTER I
INTRODUCTION

The primary objective of this research is to characterize the photoperturbed bands of biological heme respiratory proteins with dioxygen and carbon monoxide ligands observed in low temperature photodissociation absorbance difference spectrum by use of FT-IR spectroscopy. This spectral region contains intense CO absorption bands (1900 - 2200 cm\(^{-1}\)) as well as many bands of the heme (500 - 1700 cm\(^{-1}\)) that are perturbed by photodissociation of ligand. The highly polarizable CO has a strong absorption, and is a useful probe in local molecular interactions. Of great interest are photoperturbed bands with weak intensity, which have not been observed by previous FT-IR studies. These are expected to contain infrared active modes of in-plane and out-of-plane porphyrin skeletal motions, a variety of vinyl and formyl vibrational modes, axial histidylimidazole modes, and dioxygen vibrational band(s) in case of heme dioxygen complexes. In this study, not only have native and cobalt substituted heme respiratory proteins been used, but also a site-directed mutant (HisE7>Phe) has been used to understand molecular interactions of axial ligand.
The first part of this research will deal with the background information of metalloporphyrin as \( \text{D}_{4h} \) symmetry and vinyl modes. After that, an optical pumping in photolyzed HbCO is presented in chapter 2 to understand molecular interactions when the additional photon is absorbed by the heme. Dioxygen vibrational bands and vinyl asymmetric deformation modes are identified and characterized in chapter 3. The complete infrared assignment of porphyrin fundamental modes and heme substituent modes observed in low temperature photodissociation absorbance difference spectrum is presented in chapter 4. These assignments may be useful as sensitive monitors of porphyrin conformation as well as interactions of the side chains with proteins residues. The detailed analysis of heme A3-formyl and vinyl groups of cytochrome c oxidase CO complex is presented in chapter 5.

1.1 Porphyrin Skeletal modes

Metalloporphyrins are important for a variety of functions in biological systems. They are found in the respiratory proteins of Hb and Mb, electron transfer protein of cytochrome c, and terminal respiratory enzyme of cytochrome c oxidase. Figure 1 gives the structure of metalloporphyrin. If the substituent groups of octaethylmetalloporphyrin (\( \text{M}^{2+}\text{OEP}, \text{M}^{2+}\text{porphin}^2^- \)) are treated as point masses, the 37-atom model has a reducible representation in \( \text{D}_{4h} \) symmetry presented in Table 1. In order to reduce the reducible representation to their irreducible representations in the \( \text{D}_{4h} \) point group, the following equation form was used (Colthup et al.1990).
Figure 1. Structural diagram of a $D_4h$ metalloporphyrin with atom labeling. For porphine, $X=Y=H$, while for TPP, $Y=H$, $X=\text{phenyl}$. For OEP, $X=H$, $Y=\text{ethyl}$. (From Spiro and Li Biological Application of Resonance Raman Spectroscopy, Vol III, 1988, Wiley-Interscience. 1-37).
Table 1. (A) Reducible representation of metalloporphyrin in $D_{4h}$ symmetry. The substituent groups are treated as point masses. (B) Character table in $D_{4h}$ symmetry

(A)

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<tr>
<td>$E_u$</td>
<td>2</td>
<td>0</td>
<td>-2</td>
<td>0</td>
<td>0</td>
<td>2</td>
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<td>0</td>
<td>0</td>
<td>-2</td>
</tr>
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</table>

$\alpha_{xx} + \alpha_{yy}, \alpha_{zz}$

$\alpha_{xx} - \alpha_{yy}$

$\alpha_{yy}$

$(R_x, R_y)$

$(T_x, T_y)$
number of irreducible representations of a given type

\[ = \frac{1}{\text{order}} \Sigma (\text{character of reducible representations}) \times (\text{character of irreducible representations}) \] ............... (1.1)

Therefore, for example, \( n_{A_{1g}} \)

\[ = \frac{1}{16} \{ 37x3x1 + 2x1x1 + (-1)x1x1 + 2x1x(-3) + 2x1x(-5) + 1x1x37 + 2x1x3 + 2x1x5 + 2x1x(-1) + 1x1x(-3) \} = \frac{144}{16} = 9A_{1g} \] ........................................ (1.2)

The reducible representations for all motions of the \( M^{2+} \text{OEP} \) is therefore reduced to the followings: \( 9A_{1g} + 3A_{1u} + 9A_{2g} + 7A_{2u} + 9B_{1g} + 5B_{1u} + 9B_{2g} + 4B_{2u} + 9E_{g} + 19E_{u} \). By removing translational (\( A_{2u} \) and \( E_{u} \)) and rotational (\( A_{2g} \) and \( E_{g} \)) motions, \( M^{2+} \text{OEP} \) will have remaining vibrational modes as followings:

\[ 9A_{1g} + 3A_{1u} + 8A_{2g} + 6A_{2u} + 9B_{1g} + 5B_{1u} + 9B_{2g} + 4B_{2u} + 8E_{g} + 18E_{u} \]

This can be divided into in-plane and out-of-plane porphyrin skeletal modes based on \( \sigma_{u} \) irreducible representations.

\[ \Gamma_{\text{in-plane}} = 9A_{1g} + 8A_{2g} + 9B_{1g} + 9B_{2g} + 18E_{u} \] ............................................. (1.3)

\[ \Gamma_{\text{out-of-plane}} = 3A_{1u} + 6A_{2u} + 5B_{1u} + 4B_{2u} + 8E_{g} \] ............................................. (1.4)

Among these, \( A_{1g}, A_{2g}, B_{1g}, B_{2g}, \) and \( E_{g} \) modes are Raman active, whereas only \( E_{u} \) and \( A_{2u} \) modes are infrared active.

1.2 Assignment of in-plane Porphyrin Fundamental modes

The initial normal coordinate analysis of \( \text{Ni(OEP)} \) by Abe and co-workers (Abe et al.1978) has been widely accepted in interpreting hemes and model compounds, and is summarized in Table 2. After this finding, Spiro and co-workers applied the assignment of porphyrin skeletal modes not only to find vinyl

<table>
<thead>
<tr>
<th>Ni(OEP)</th>
<th>assignment of E&lt;sub&gt;n&lt;/sub&gt;</th>
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<tbody>
<tr>
<td>..</td>
<td>v36 v(C&lt;sub&gt;α&lt;/sub&gt;H)</td>
</tr>
<tr>
<td>1604</td>
<td>v37 v(C&lt;sub&gt;α&lt;/sub&gt;C&lt;sub&gt;α&lt;/sub&gt;)</td>
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<tr>
<td>1557</td>
<td>v38 v(C&lt;sub&gt;α&lt;/sub&gt;C&lt;sub&gt;α&lt;/sub&gt;)</td>
</tr>
<tr>
<td>1487</td>
<td>v39 v'(C&lt;sub&gt;α&lt;/sub&gt;C&lt;sub&gt;α&lt;/sub&gt;)</td>
</tr>
<tr>
<td>1443</td>
<td>v40 v'(C&lt;sub&gt;α&lt;/sub&gt;C&lt;sub&gt;β&lt;/sub&gt;)</td>
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<tr>
<td>1389</td>
<td>v41 v(C&lt;sub&gt;α&lt;/sub&gt;N)</td>
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<tr>
<td>1268</td>
<td>v42 δ(C&lt;sub&gt;α&lt;/sub&gt;H)</td>
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<tr>
<td>1148</td>
<td>v43 v'(C&lt;sub&gt;α&lt;/sub&gt;Et)</td>
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<tr>
<td>1113</td>
<td>v44 v(C&lt;sub&gt;α&lt;/sub&gt;Et)</td>
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<tr>
<td>993</td>
<td>v45 v'(C&lt;sub&gt;α&lt;/sub&gt;N)</td>
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<td>924</td>
<td>v46 v'(C&lt;sub&gt;α&lt;/sub&gt;Et)</td>
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<td>726</td>
<td>v47 v(C&lt;sub&gt;α&lt;/sub&gt;Et)</td>
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<tr>
<td>605</td>
<td>v48 δ(C&lt;sub&gt;α&lt;/sub&gt;Et)</td>
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<tr>
<td>550</td>
<td>v49 δ(C&lt;sub&gt;α&lt;/sub&gt;C&lt;sub&gt;α&lt;/sub&gt;C&lt;sub&gt;α&lt;/sub&gt;)</td>
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<tr>
<td>..</td>
<td>v50 δ(C&lt;sub&gt;α&lt;/sub&gt;Et)</td>
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<td>..</td>
<td>v51 δ(C&lt;sub&gt;α&lt;/sub&gt;Et)</td>
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<td>287</td>
<td>v52 δ(C&lt;sub&gt;α&lt;/sub&gt;Et)</td>
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<tr>
<td>..</td>
<td>v53 δ(C&lt;sub&gt;α&lt;/sub&gt;Et)</td>
</tr>
</tbody>
</table>

v' and δ represent the symmetry coordinates of antisymmetric stretching and deformation vibrations about the C<sub>2</sub> axis of pyrrole ring, respectively.
vibrational modes (Choi et al.1982; Choi et al.1982), but also to characterize biological heme proteins of CcO, and model hemes like PP and Pa (Choi et al.1983). Their assignments are given in Table 3 and 4. More detailed studies by Willems and Bocian (Willems and Bocian, 1984; Willems and Bocian, 1985) pointed out that the $E_u$ porphyrin fundamental modes are sensitive to the effects of symmetry lowering by peripheral substituents not only in intensity but also in observed frequencies. Their assignment is given in Table 5.

An alternative assignment have been proposed for $E_u$ porphyrin skeletal modes of Ni(OEP) by Kincaid et al. (Kincaid et al.1983) in matrix-isolated metal complex studies. They suggest that certain bands attributed to $E_u$ ($v_{39, 40, 41, 44}$) core modes by Abe and co-workers (Abe et al.1978) actually correspond to internal modes of the $C_p$ ethyl side chains. Their assignment is shown in Table 6.

Recently, Spiro and coworkers (Li et al.1990) reexamined the vibrational spectra of Ni(OEP) and the normal coordinate analysis of Ni(OEP) with inclusion of methylene hydrogen atoms, pointing out that the original study of Abe et al. (1978) was limited to excitation at 488.0 nm and 514.5 nm, neither of which is fully resonant with any of the $\pi-\pi^*$ electronic transitions. There are little differences for Raman active modes, but much differences are present in infrared active $E_u$ modes between these two works. Their assignment is given in Table 7.

Biological applications of porphyrin modes are also summarized in Table 8 and 9.
Table 3. Porphyrin skeletal and vinyl modes of Ni(OEP) and Ni(PP) from Choi et al. (J. Am. Chem. Soc. (1982) 104:4337-4344)

<table>
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<tr>
<th>Ni(OEP)</th>
<th>Ni(PP)</th>
<th>Skeletal and Vinyl Mode</th>
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</thead>
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<tr>
<td></td>
<td>1634&lt;sup&gt;*&lt;/sup&gt;</td>
<td>v C=C</td>
</tr>
<tr>
<td></td>
<td>1620</td>
<td>v C=C</td>
</tr>
<tr>
<td>1604</td>
<td>1610&lt;sup&gt;*&lt;/sup&gt;</td>
<td>v37 (C&lt;sub&gt;a&lt;/sub&gt;-C&lt;sub&gt;b&lt;/sub&gt;)</td>
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<td>1557</td>
<td>1567</td>
<td>v38 (C&lt;sub&gt;a&lt;/sub&gt;-C&lt;sub&gt;b&lt;/sub&gt;)</td>
</tr>
<tr>
<td>1486</td>
<td>1456</td>
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</tr>
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<td>1442</td>
<td>1440</td>
<td>v40 (C&lt;sub&gt;a&lt;/sub&gt;-C&lt;sub&gt;b&lt;/sub&gt;)</td>
</tr>
<tr>
<td></td>
<td>1434&lt;sup&gt;*&lt;/sup&gt;</td>
<td>δ&lt;sub&gt;s&lt;/sub&gt;=CH&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>1386</td>
<td>1377</td>
<td>v41 (C&lt;sub&gt;a&lt;/sub&gt;-N)</td>
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<td>1345</td>
<td>δ&lt;sub&gt;s&lt;/sub&gt;=CH&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>1262</td>
<td>v42 (δC&lt;sub&gt;a&lt;/sub&gt;-H)</td>
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<td>1148</td>
<td>1165</td>
<td>v43 (C&lt;sub&gt;b&lt;/sub&gt;-S), or v(C&lt;sub&gt;b&lt;/sub&gt;-C&lt;sub&gt;b&lt;/sub&gt;)</td>
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<td>γ (CH=)</td>
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<td>984</td>
<td>v45 (C&lt;sub&gt;a&lt;/sub&gt;-N)</td>
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<td>γ&lt;sub&gt;s&lt;/sub&gt; (=CH&lt;sub&gt;2&lt;/sub&gt;)</td>
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* Observed in RR, Otherwise in IR
Table 4. IR and Raman frequencies assignable to porphyrin skeletal, vinyl and formyl modes from Choi et al. (J. Am. Chem. Soc. (1983) 105:3692-3707).

<table>
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<th>Pa*</th>
<th>COX&lt;sub&gt;red&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>COX&lt;sub&gt;cit&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Assignment</th>
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<td>1676</td>
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<td>1624</td>
<td>1626</td>
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<td>1547, 1574&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1547, 1574&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ν38</td>
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<td>ν39</td>
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ClFe<sup>m</sup>PP and ClFe<sup>m</sup>Pa
PP & Pa prepared in CsI
* IR observation. b Raman observation. c splitting. d formyl group.
e or γs=CH<sub>2</sub>
Table 5. Porphyrin in-plane skeletal modes and 2,4 substituents from Willems et al. (J. Am. Chem. Soc. (1984) 106:880-890)

<table>
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<th>NiOEP</th>
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<th>Ni(2Ac-DP)</th>
<th>Ni(2,4-DiAcDP)</th>
<th>Ni(4-FDP)*</th>
<th>Ni(2,4-DFDP)</th>
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Ni(porphyrin) prepared in KBr
b splitting
Table 6. Observed IR frequencies of metal(OEP) and assignments from Kincaid et al. (*J. Phys. Chem.* (1983) 87:3096-3101)

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<td>v37 v(C=C)</td>
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<td>1378 0</td>
<td>ethyl</td>
</tr>
<tr>
<td>1322</td>
<td>m</td>
<td>1322</td>
<td>1322</td>
<td>1321 1</td>
<td>ethyl</td>
</tr>
<tr>
<td>1275</td>
<td>s</td>
<td>1277</td>
<td>1276</td>
<td>1276 -1</td>
<td>v41 v(C=C),v(C=C)</td>
</tr>
<tr>
<td>1231</td>
<td>s</td>
<td>1232</td>
<td>1228</td>
<td>1227 4</td>
<td>v42 v(C=C),v(C=N)</td>
</tr>
<tr>
<td>1153</td>
<td>s</td>
<td>1154</td>
<td>1154</td>
<td>1154 -1</td>
<td>v43 v(C=N)δ(C=C,H)</td>
</tr>
<tr>
<td>1133</td>
<td>w</td>
<td>1133</td>
<td>1137</td>
<td>1136 -3</td>
<td>v44 v(C=N)δ(C=C,H)</td>
</tr>
<tr>
<td>1119</td>
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<td>1118</td>
<td>1117</td>
<td>1117 2</td>
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</tr>
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<tr>
<td>1061</td>
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<td>1061</td>
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<td>1021</td>
<td>s</td>
<td>1021</td>
<td>1020</td>
<td>1019 2</td>
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</tr>
<tr>
<td>996</td>
<td>s</td>
<td>994</td>
<td>988</td>
<td>991 5</td>
<td>v45 v(C=N),v(C=C)</td>
</tr>
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<td>s</td>
<td>960</td>
<td>960</td>
<td>959 0</td>
<td>ethyl</td>
</tr>
<tr>
<td>927</td>
<td>m</td>
<td>927</td>
<td>922</td>
<td>923 4</td>
<td>v46 v(C=C),v(C=C)</td>
</tr>
<tr>
<td>846</td>
<td>s</td>
<td>846</td>
<td>846</td>
<td>843 3</td>
<td>π(C=C)</td>
</tr>
<tr>
<td>834</td>
<td>m</td>
<td>833</td>
<td>831</td>
<td>830 4</td>
<td>π(C=C)</td>
</tr>
<tr>
<td>754</td>
<td>s</td>
<td>754</td>
<td>752</td>
<td>752 2</td>
<td>π(skeletal)</td>
</tr>
<tr>
<td>742</td>
<td>w</td>
<td>741</td>
<td>744</td>
<td>744 -2</td>
<td>π(skeletal)</td>
</tr>
<tr>
<td>726</td>
<td>m</td>
<td>729</td>
<td>729</td>
<td>722 4</td>
<td>v47 v(C=N)</td>
</tr>
<tr>
<td>703</td>
<td>s</td>
<td>700</td>
<td>701</td>
<td>703 0</td>
<td>π(skeletal)</td>
</tr>
</tbody>
</table>

Observed by IR. v38,39,42,45 are metal sensitive
* intensity: s strong, m medium, w weak. * Ni(OEP) - Fe(OEP)
Table 7. Porphyrin in-plane skeletal and vinyl mode frequencies and assignments from Li et al. (*J. Phys. Chem.* (1990) 94:47-61)

<table>
<thead>
<tr>
<th>Ni(OEP)</th>
<th>Ni(P)</th>
<th>Ni(TPP)</th>
<th>mode</th>
</tr>
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<tbody>
<tr>
<td>[1637]</td>
<td>1624</td>
<td>[1586]</td>
<td>v37 \ v(C_a-C_a)^{\text{sym}}</td>
</tr>
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<td>1604</td>
<td>1547</td>
<td>[1552]</td>
<td>v38 \ v(C_b-C_b)</td>
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<td>1501</td>
<td>1462</td>
<td>[1473]</td>
<td>v39 \ v(C_a-C_a)^{\text{sym}}</td>
</tr>
<tr>
<td>1456, 1440</td>
<td></td>
<td></td>
<td>CH_2 scissor</td>
</tr>
<tr>
<td>1396</td>
<td>1385</td>
<td>[1403]</td>
<td>v40 \ v(Pyr quater-ring)</td>
</tr>
<tr>
<td>1378</td>
<td></td>
<td></td>
<td>CH_2 wag</td>
</tr>
<tr>
<td>[1346]</td>
<td>1319</td>
<td>[1331]</td>
<td>v41 \ v(Pyr half-ring)^{\text{sym}}</td>
</tr>
<tr>
<td>1323</td>
<td></td>
<td></td>
<td>CH_2 wag</td>
</tr>
<tr>
<td>1275</td>
<td></td>
<td></td>
<td>CH_1 twist</td>
</tr>
<tr>
<td>1231</td>
<td>1150</td>
<td>[233]</td>
<td>v42 \ \delta(C_a-X)</td>
</tr>
<tr>
<td>1153</td>
<td>[3097]</td>
<td>[3097]</td>
<td>v43 \ v(C_b-Y)^{\text{sym}}</td>
</tr>
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<td>1033</td>
<td>[1003]</td>
<td>v44 \ v(Pyr half-ring)^{\text{sym}}</td>
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<td>1021</td>
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<td>\nu(C_1C_2)</td>
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<tr>
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<td>[3087]</td>
<td>[3100]</td>
<td>v45 \ v(C_b-Y)^{\text{sym}}</td>
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<td>927</td>
<td>806</td>
<td>[864]</td>
<td>v46 \ \delta(Pyr)^{\text{sym}}</td>
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<td>766</td>
<td>995</td>
<td>[1023]</td>
<td>v47 \ v(Pyr breathing)</td>
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<tr>
<td>754, 726</td>
<td></td>
<td></td>
<td>CH_2 rock</td>
</tr>
<tr>
<td>605</td>
<td>745</td>
<td>[895]</td>
<td>v48 \ \delta(pyr)^{\text{sym}}</td>
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</table>

Note: X,Y = H, H (NiPP); H, C\_5H\_5 (NiOEP); and C\_6H\_6, H (NiTPP)
[ ]: calculated not observed by IR

<table>
<thead>
<tr>
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<td>v38</td>
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<td>1367</td>
<td>v41</td>
</tr>
<tr>
<td>1329</td>
<td>δ=CH2</td>
</tr>
<tr>
<td>1310</td>
<td>δ=CH=</td>
</tr>
<tr>
<td>1173</td>
<td>v43</td>
</tr>
<tr>
<td>1115</td>
<td>v44</td>
</tr>
<tr>
<td>955</td>
<td>v46</td>
</tr>
<tr>
<td>735</td>
<td>v47</td>
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<table>
<thead>
<tr>
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<th>Cyta$_2^{2+}$</th>
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<td></td>
<td>v C=O</td>
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<td>1618</td>
<td>1622</td>
<td>1625</td>
<td>v C=C</td>
</tr>
<tr>
<td></td>
<td>1607</td>
<td>1609</td>
<td>v10 (R)</td>
</tr>
<tr>
<td>1585</td>
<td>1579</td>
<td>1586</td>
<td>v2 (R)</td>
</tr>
<tr>
<td>1567</td>
<td>1569</td>
<td>1568</td>
<td>v37 ?</td>
</tr>
<tr>
<td>1541</td>
<td></td>
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</tr>
<tr>
<td>1516</td>
<td>1517</td>
<td></td>
<td>v11 (R)</td>
</tr>
<tr>
<td>1469</td>
<td>1464</td>
<td></td>
<td>v28 (R)</td>
</tr>
<tr>
<td>1398</td>
<td>1399</td>
<td></td>
<td>δCH or v20,29</td>
</tr>
<tr>
<td>1368</td>
<td>1355</td>
<td>1354</td>
<td>v4 (R)</td>
</tr>
<tr>
<td>1328</td>
<td>1331</td>
<td></td>
<td>δs=CH$_2$</td>
</tr>
<tr>
<td>1306</td>
<td>1305</td>
<td></td>
<td>δCH=</td>
</tr>
<tr>
<td>1286</td>
<td>1289</td>
<td></td>
<td>v42</td>
</tr>
<tr>
<td>1255</td>
<td></td>
<td></td>
<td>v (5+9) ?</td>
</tr>
<tr>
<td>1244</td>
<td>1247</td>
<td>1246</td>
<td>v (5+9) ?</td>
</tr>
<tr>
<td>1227</td>
<td>1225</td>
<td>1226</td>
<td>v13 (R)</td>
</tr>
<tr>
<td>1182</td>
<td>1181</td>
<td></td>
<td>v30 (R)?</td>
</tr>
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<td>1159</td>
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<td>v30 (R)?</td>
</tr>
<tr>
<td>1133</td>
<td>1129</td>
<td>1131</td>
<td>v (6+8) (R)</td>
</tr>
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<td>1115</td>
<td>1113</td>
<td>v22 (R)</td>
</tr>
<tr>
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<td>745</td>
<td>746</td>
<td>v16 (R)</td>
</tr>
<tr>
<td>683</td>
<td>682</td>
<td>679</td>
<td>v7 (R)</td>
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</tbody>
</table>

(R) Raman active selection rule
1.3 Out-of-plane Porphyrin Skeletal Modes

The out-of-plane modes were categorized according to local motions of various structural elements of the porphyrin (Choi and Spiro, 1983; Li et al., 1989; Spiro and Li, 1988) in Figure 2: They are i) out-of-plane wagging modes of $\gamma C_6^\alpha H$, $\gamma C_6^\beta Y$, $\gamma C_6^\gamma C_6^\gamma$, and the central metal atom, ii) pyrrole tilting, translation, and swivel modes, iii) internal folding of the pyrrole rings, which are symmetric and antisymmetric with respect to the two-fold axis. Spiro and Li (Spiro and Li, 1988) calculated out-of-plane vibrations of Ni(OEP) and observed only $A_{2u}$ modes in their spectra (see Table 10). However, by vibronic mixing of in-plane ($E_u$) and out-of-plane ($A_{2u}$) electronic transitions ($E_u \times A_{2u} = E_g$), out-of-plane modes could be enhanced and observed in the resonance Raman spectrum (Choi and Spiro, 1983; Li et al., 1989).

1.4 Vinyl Vibrational Modes

The vinyl group at 2 and 4 positions of protoporphyrin IX has been suggested (Alben and Caughey, 1968; Warshel and Weiss, 1981; Rousseau et al., 1983; Sono and Asakura, 1975; Asakura and Sono, 1974) as a molecular modulator. It controls the ligand binding affinity by steric repulsion or electronic withdrawing effects of the vinyl group through the porphyrin $\pi$-electron density. Primarily, identification of vinyl modes was based on the assignment of Spiro and co-workers who have examined the nickel(II) protoporphyrin IX dimethyl ester and its derivatives deuterated the $\alpha$ and $\beta$ carbon atoms of the vinyl groups in the
RR studies (Choi et al.1982; Choi et al.1982), as summarized in table 3. Vinyl vibration of olefin is given in Figure 3


<table>
<thead>
<tr>
<th>Symmetry</th>
<th>Observed NiOEP ( \nu (\text{cm}^{-1}) )</th>
<th>Calculated Frequencies ( \nu (\text{cm}^{-1}) )</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A_{1u} )</td>
<td>591.0</td>
<td>366.4</td>
<td>Pyr. fold (as)</td>
</tr>
<tr>
<td></td>
<td>366.4</td>
<td></td>
<td>( \gamma_{C_4Y} )</td>
</tr>
<tr>
<td></td>
<td>79.7</td>
<td></td>
<td>Pyr. swivel</td>
</tr>
<tr>
<td>( B_{1u} )</td>
<td>826.2</td>
<td>610.4</td>
<td>Pyr. fold (as)</td>
</tr>
<tr>
<td></td>
<td>610.4</td>
<td></td>
<td>( \gamma_{C_3H} )</td>
</tr>
<tr>
<td></td>
<td>458.1</td>
<td>130.5</td>
<td>( \gamma_{C_6C_3} + \gamma_{C_5Y} )</td>
</tr>
<tr>
<td></td>
<td>458.1</td>
<td>41.4</td>
<td>Pyr. swivel</td>
</tr>
<tr>
<td>( A_{2u} )</td>
<td>835</td>
<td>834.6</td>
<td>( \gamma_{C_3H} )</td>
</tr>
<tr>
<td></td>
<td>605</td>
<td>606.5</td>
<td>Pyr. fold (s)</td>
</tr>
<tr>
<td></td>
<td>355</td>
<td>356.5</td>
<td>Pyr. tilt + ( \gamma_{C_6C_3} )</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>282.5</td>
<td>( \gamma_{C_6C_3} + \gamma_{C_5Y} )</td>
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<tr>
<td></td>
<td>120</td>
<td>116.9</td>
<td>( \gamma_{C_5Y} + \gamma_{C_6C_3} )</td>
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<td></td>
<td></td>
<td>30.0</td>
<td>( \gamma_{NM} )</td>
</tr>
<tr>
<td>( B_{2u} )</td>
<td>537.4</td>
<td>294.7</td>
<td>Pyr. fold (s)</td>
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<tr>
<td></td>
<td>294.7</td>
<td></td>
<td>Pyr. tilt + ( \gamma_{C_5Y} )</td>
</tr>
<tr>
<td></td>
<td>143.2</td>
<td>27.4</td>
<td>( \gamma_{C_5Y} + \text{Pyr. tilt} )</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>Pyr. translation</td>
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<tr>
<td>( E_g )</td>
<td>828.9</td>
<td></td>
<td>( \gamma_{C_3H} )</td>
</tr>
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<td>602.9</td>
<td></td>
<td>Pyr. fold (as)</td>
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<td>543.1</td>
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<td>Pyr. fold (s)</td>
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<td>421.1</td>
<td>283.4</td>
<td>( \gamma_{C_6C_3} + \gamma_{C_5Y} )</td>
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<td></td>
<td>283.4</td>
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<td>Pyr. tilt + ( \gamma_{C_6Y} )</td>
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<td>143.2</td>
<td>205.6</td>
<td>( \gamma_{C_6C_3} + \gamma_{C_5Y} )</td>
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<td></td>
<td></td>
<td>97.9</td>
<td>( \gamma_{C_5Y} + \text{Pyr. fold} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pyr. swivel</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pyr. swivel</td>
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</table>
Figure 3. Vinyl vibrations of Olefin. The left-hand column illustrates the in-plane vibrations and left-hand column illustrates the out-of-plane vibrations (+ and -) of vinyl group (From Colthup et al. Introduction to Infrared and Raman Spectroscopy, 1990, Academic Press, p.248).
LIST OF REFERENCES


CHAPTER II

Spectroscopic Characterization of Optical Pumping in Photolyzed HbCO

2.1 Abstract

Low temperature photodissociation Fourier transform infrared spectroscopy has been used to characterize optical pumping of photodissociated CO from HbCO with continuous illumination. A photodissociation absorbance difference spectrum of HbCO shows the infrared Fe-CO absorptions (A substates: $A_0$, $A_1$ and $A_2$) and the photodissociated CO (B substates: $B_0$, $B_1$ and $B_2$). The ratio of B substate intensities is dependent on both sample temperature and the illumination time of the photolyzing light. The $B_2$ band disappears at 35 K but represents the dominant B substate at 10 K. At 10 K, the intensity ratio of $B_2/B_1$ substates increases from 1 to 2 under the continuous photolyzing conditions. These results will provide additional information regarding molecular interactions between the heme of proteins and small ligand molecule when the additional photon is absorbed by the heme.
2.2 Introduction

Photolysis studies have been extensively used to understand the molecular interactions between small ligand molecules and macromolecules for nearly a century (Brunori and Giacommetti, 1981; Keilin and Hartree, 1955; Gibson and Ainsworth, 1957). In these experiments, CO is a very useful probe of local molecular interactions because of a strong transition dipole and intense infrared absorptions. FT-IR absorbance difference spectra have demonstrated the ability to examine the structure of photolyzed MbCO (Fiamingo and Alben, 1985), to obtain structural information of the iron-copper binuclear center in mammalian cytochrome oxidase (Fiamingo et al.1982; Fiamingo et al.1986; Fiamingo et al.1990), and to define the coordination structure of the cytochrome a$_3$Fe:Cu$_b$ centers with a site-directed mutagenesis technique in bacterial cytochrome oxidase (Calhoun et al.1993; Thomas et al.1993; Thomas et al.1993).

Basically, the frequency deviation between free CO gas (center frequency 2144 cm$^{-1}$) and CO complexed to metal proteins provides a yard stick to reveal the strength of interactions. In the simplest approximation, lower frequency indicates a greater perturbation from the free gas, an increased binding enthalpy, a lower CO force constant, a longer equilibrium CO distance, and a larger change in dipole moment with the vibrational coordinate. In the case of MbCO, three photolyzed CO vibrational frequencies are observed at 2144, 2131, 2119 cm$^{-1}$, and labeled as $B_0$, $B_1$, and $B_2$ substates respectively (Alben et al.1982). $B_0$ substate is a free CO gas in the distal heme pocket, and $B_1$ and $B_2$ represent the CO that is
weakly bound to heme or to the wall of heme pocket. The $B_2$ substate is
 displaced twice as much as the $B_1$ substate from free CO gas, suggesting that it
is a stronger perturbation than $B_1$ substate. The $B$ substates also provide the
possible configurations of photodissociated CO molecules in distal heme pocket
(Fiamingo and Alben, 1985). In the bound state of MbCO, three vibrational
frequencies are observed at 1969, 1945, and 1927 cm$^{-1}$, which represent the
coordination of CO to iron with different conformations (Alben et al.1982).

When a visible photon which is used in flash photolysis experiments is
absorbed by the heme, the CO is dissociated from iron and the spin state of iron
is changed. When the level of fractional photolysis approaches 100%, the
absorption of additional photon energy will be dissipated as kinetic energy by
translational and rotational motions with protein surroundings or an increase of
local molecular temperature up to several hundreds of Kelvin (Henry et al.1986;
Anfinrud et al.1989). The distribution of this energy may result in configurational
and conformational substates of protein which are different from the initial
conformation of the protein prior to absorption of light. Recently, Alben (Alben,
1993) has raised the question about the distinction between a distribution of
conformational substates induced by the absorption of secondary photons by the
heme and a similar ground state distribution by cooling from room temperature.
This is important to define initial conditions regarding molecular interactions
between the heme and small ligand molecule in flash photolysis experiments when
the additional photon energy is introduced.
In an attempt to address the distribution of conformational substates by additional absorption of light, we have used continuous illumination to photolyze HbCO at low temperatures. The low temperature photodissociation spectrum of HbCO shows three Fe-CO bands ($A_0: 1967 \text{ cm}^{-1}$, $A_1: 1950 \text{ cm}^{-1}$, $A_2: 1926 \text{ cm}^{-1}$) and three photolyzed CO bands ($B_0: 2144 \text{ cm}^{-1}$, $B_1: 2130 \text{ cm}^{-1}$, $B_2: 2118 \text{ cm}^{-1}$). The observation of intensity changes of B substates at different time during the continuous illumination in photodissociated HbCO spectra suggests an optical pumping mechanism from $B_1$ to $B_2$. The optical pumping may be a possible device to dissipate the kinetic energy and shows the distribution of conformational substates induced by additional photolyzing light.

2.3 Methods

The main component of human Hb $A_0$ was separated from the hemolysate by the method of Robley et al. (Williams and Tsay, 1973). This purified hemoglobin solution was applied to a Sephadex G-25 column, eluted with 0.1 M NaCl pH 7.5, concentrated to 20 mM with Amicon ultrafiltration, and then stored in liquid nitrogen until use. HbCO was obtained by exposing the purified HbO$_2$ to CO gas (Matheson C. P. 99.5% pure) anaerobically. Sperm whale Mb purchased from Sigma (St. Louis, MO) was reduced and converted to MbCO as described in Alben et al. (Fiamingo and Alben, 1985)

The visible absorption spectra of HbCO and MbCO at 4°C was obtained with a Cary model 17DX spectrometer. All infrared spectra were recorded at 0.5 cm$^{-1}$ resolution with a Mattson Sirius 100 Fourier transform infrared (FT-IR)
interferometer which was fitted with a liquid nitrogen cooled photovoltaic (HgCd)Te detector or InSb detector and germanium-coated KBr beamsplitter. Visible light was excluded from the cryostat by use of an As$_2$Se$_3$ window which transmits infrared radiation. Low temperatures were obtained by use of a Lake Shore Cryotronics closed cycle helium refrigerator, Model LTS-21-D70C. Cryostat cell temperature was measured by use of a Lake Shore Cryotronics digital thermometer, model DRC-70, with a calibrated silicon diode probe.

HbCO samples were pressed between a pair of CaF$_2$ windows, using either 27.6 or 7 micrometer spacers. The exact cell path lengths were measured from the interference fringes. These channel spectra are caused by the reflection of a portion of the light from the empty cell as described in the Alben et al. (Alben and Fiamingo, 1984).

Photolysis made use of a 500 W tungsten lamp focused through a slide projector and passed through a water filter (2 cm) to remove infrared radiation beyond 1.2 micrometers. The intensity of the light was measured with a Scientech Laser Power Meter Model 360001 at the sample position of the cryostat. The average light intensity measured was $110 \pm 3$ mW/cm$^2$. This value varied depending on the light path and focus on the sample.

Single beam spectra in the dark were obtained from the Fourier transform of 8192 scans of full double-sided interferograms with triangular apodization. Another set of single beam spectra was also obtained by successive logarithmic increases in the number of signal-averaged scans from 32 to 8192 scans in the
presence of continuous light. Photodissociation absorbance spectra (light - dark) were obtained from the ratio of single beam spectra in the presence (light) and in the absence (dark) of continuous illumination. Between each set of data collection, the sample was relaxed in the dark at 180 K for at least 30 minutes. At this temperature the photodissociated CO recombined to the heme iron, and the photolyzed heme groups fully relaxed (Alben et al. 1982; Fiamingo and Alben, 1985). The degree of photodissociation of iron bound CO was measured from the Fe-CO vibrational frequency at 1950 cm \(^{-1}\) (Alben and Caughey, 1968) in the single beam spectrum. Low temperature FT-IR spectra of photodissociated MbCO were obtained in a similar manner as used in HbCO.

2.4 Results

The low temperature photodissociation spectrum of HbCO in Figure 4 reveals the infrared Fe-CO absorptions (A substates: A\(_0\) at 1967, A\(_1\) at 1950, A\(_2\) at 1926 cm \(^{-1}\)) and the photodissociated CO absorptions (B substates: B\(_0\) at 2144 cm \(^{-1}\), B\(_1\) at 2130 cm \(^{-1}\), B\(_2\) at 2118 cm \(^{-1}\)) similar to the MbCO photodissociation spectrum (Alben et al. 1982; Fiamingo and Alben, 1985). The B\(_0\) substate has been assigned to free CO gas in the heme pocket (Alben et al. 1982), and is so weak that we exclude it from the analysis in this study. Figure 5 shows the intensity ratio of B\(_2\)/B\(_1\) and total fractional photolysis of CO at three different temperatures with continuous illumination. With continuous photodissociation of HbCO at 10 K, the intensity ratio of B\(_2\)/B\(_1\) surprisingly increases from 1 to 2
Figure 4. FT-IR absorbance difference spectrum of HbCO at 10 K shows A and B substates. The 1905 cm\(^{-1}\) band results from the natural abundance of heavy isotopes \(^{13}\)C\(^{16}\)O and \(^{12}\)C\(^{18}\)O.
Figure 5. (A). Absorbance ratio of photodissociated B₂/B₁ in HbCO with continuous light photodissociation at 10, 20 and 30 K designated by •, ▲, and ▼ respectively. (B) Fractional photolysis of carbon monoxide in HbCO obtained simultaneously with (A). (From, S. Park., M.S., Thesis, The Ohio State University, Columbus, Ohio).
Figure 5 (continued)

![Graph showing the photolysis of CO over time. The graph has a logarithmic scale for time and shows data points for different conditions.](image)

(B)
during this experimental period, while the total fractional photodissociation of CO remains close to 100%. More than 4 hours illumination was not enough to reach a photostationary equilibrium at 10 K under our photolyzing conditions. The absorbance ratio of $B_2/B_1$ is constant at 20 K, indicating that B substates are able to reach a photostationary equilibrium at this temperature. The fractional photolysis of CO remains about 75% at this temperature. At 30 K, the absorbance ratio is close to zero during the continuous illumination, and fractional photolysis of CO is about 48%. The intensity increment of the $B_2/B_1$ substate ratio at 10 K, therefore, must be due to a photoactivated pumping of CO molecules from $B_1$ to $B_2$ by an additional absorption of photon energy.

Figure 6 is a spectral representation of optical pumping. It shows the absorbance difference spectra of B substates at different time ($t = 1018$ and 11505 sec) during continuous illumination with a project lamp light. The total integrated absorptivity of ($B_2 + B_1$) substates in these two spectra is identical, such that only the relative amount of each substate is changed within the limits of signal/noise ratio. The more light is absorbed by the heme, the more the $B_1$ substate loses its intensity so that $B_2$ substate gains its intensity. This result indicates not only that there is no transition between A and B substates in HbCO under continuous illumination, but also that the change of intensity ratio from 1 to 2 at 10 K represents photoactivated pumping of photolyzed CO from $B_1$ to $B_2$. This optical pumping is independent of additional influx of CO into B substate from Fe-CO.
Figure 6. Absorbance changes of $B_2$ and $B_1$ substates in HbCO at different time (1018 & 11505 sec) during continuous illumination from tungsten project lamp at 10 K.
Figure 7. The effect of temperatures of CO in photolyzed CO. Apparent integrated absorptivity and fractional photolysis of CO were collected from 10 to 50 K. (From S. Park., M.S. Thesis, The Ohio State University, Columbus, Ohio).
Figure 7 shows the relationships among the B substates, temperatures (10 - 50 K), and fractional photolysis of CO after approximately 2 hours flash photodissociation. The apparent integrated absorptivity \( f_i \) was calculated from the following equations:

\[
A = \epsilon \times l \times c 
\]

(2.1)

band area \( (F) \):

\[
F = \int A(v)dv = c \times l \int \epsilon(v)dv
\]

(2.2)

apparent integrated absorptivity \( (f_i) \):

\[
f_i = F / (c \times l)
\]

(2.3)

where \( \epsilon \): extinction coefficient

\( c \): concentration of HbCO

\( l \): path length

The \( B_2 \) band disappears at around 30 to 35 K, but represents the dominant B substate at 10 K. At 15 K, \( B_2 \) and \( B_1 \) band have similar absorptivities. The absorptivity of \( B_1 \) band increases gradually from 10 to 30 K, and decreases beyond that temperature. The fractional photolysis of CO and apparent integrated absorptivity of \( (B_2 + B_1) \) both decreases with increasing the temperature. From Figure 7, we calculate the total integrated absorptivity of \( (B_2 + B_1) \) substate at each temperature by following equation:

Total integrated absorptivity = \( f_i / \) (fractional photolysis of CO/100) ......... (2.4)

The total integrated absorptivity appears to be invariant with temperature at 1.2 ± 0.1 mM\(^{-1}\)cm\(^2\), which is slightly lower than that of MbCO (Alben et al.1982).
This observation also supports an optical pumping mechanism between \( B_2 \) and \( B_1 \) substates rather than an additional photolysis of CO because the sum of the area of \((B_2 + B_1)\) is constant.

Unlike HbCO, the continuous illumination of sperm whale MbCO does not show the optical pumping between \( B_1 \) and \( B_2 \) at 10 K under our experimental conditions. Figure 8 represents the absorbance difference spectra of the B substate in MbCO at different time (\( t = 1018 \) and 11505 sec). Within our experimental uncertainties, the intensities of the \( B_2 \) and \( B_1 \) substates in both spectra are identical. The relative weak intensity of \( B_2 \) substate in MbCO than in HbCO is probably due to the fast relaxation of photolyzed CO.

2.5 Discussion

The data reported here help to define the condition of the photolyzed CO molecules upon photodissociation of HbCO at low temperatures. Photodissociation of CO from HbCO leads to the photolyzed B substates in which the CO is associated with the distal heme pocket wall or the heme group itself along its pathway of escape. We have monitored two populations of photolyzed CO (\( B_1 \) and \( B_2 \)) which can be distinguished by the CO vibrational stretching frequency. The continuous illumination of HbCO by visible light leads to essentially 100% photodissociation of CO with photoactivated optical pumping from \( B_1 \) substate to \( B_2 \) substate by the absorption of additional photons. This photoactivation is independent of the additional photodissociation of CO from
Figure 8. Absorbance changes of $B_2$ and $B_1$ substates in sperm whale MbCO at different time (1018 & 11505 sec) during continuous illumination from tungsten project lamp at 10 K.
Fe-CO because the total population of B$_2$ and B$_1$ substates is invariant. The photon energy in this optical pumping must be sufficient to overcome the energy barrier of approximately 4 KJ/mol between B$_2$ and B$_1$ substates (Alben et al. 1982), and the barrier of rapid cooling protein matrix to equilibrate the sample holder temperature of the cryostat to 10 K. By increasing the temperature the B$_2$ and B$_1$ substates reach their equilibrium because the rate of relaxation eventually becomes comparable to the photon absorption from the photolyzing light. These observations suggest that B$_2$ substate is entropically stabilized.

A possible model for the optical pumping mechanism of HbCO can be presented by the following scheme (a modification of that proposed by Alben et al. (1982) in MbCO photodissociation studies).

\[
\begin{array}{c}
\text{hv} \\
\text{A (A$_0$, A$_1$, A$_2$)} \quad TR \quad \text{TR} \\
\text{hv} \quad \text{hv}
\end{array}
\]

\[
\begin{array}{c}
\rightarrow B_2 \\
\rightarrow B_1
\end{array}
\]

where TR: Thermal Relaxation

Thermal relaxation leads to the rebinding of B substates to A substates as suggested by Frauenfelder and co-workers (Alben et al. 1982; Mourant et al. 1993).

The nature of the B$_2$ and B$_1$ substates in the heme pocket is unknown. The B$_2$ substate has a lower vibrational frequency at 2118 cm$^{-1}$ and is perturbed from free CO more than is B$_1$ substate (2130 cm$^{-1}$). This means that the B$_2$ substate has
stronger interactions and a lower ground state than the $B_1$ substate. However, the $B_2$ substate is dissociated at lower temperature than is the $B_1$ substate, indicating that the $B_2$ substate involves a weaker interaction. This paradox can be resolved if the $B_2$ substate involves a weak association in addition that found in the $B_1$ substate so that the $B_2$ substate is entropically stabilized by two non-covalent bands. An increase in kinetic energy (temperature) will break one of these bands and go to $B_1$ substate.

A visible photon that initiates photodissociation contains a significant amount of excess energy above that required for photolysis. Molecular dynamics simulation of the photodissociation event in $\alpha$-HbCO indicate that the energy difference between high spin ferrous in-plane and out-of-plane conformations is 2.5-5.0 Kcal/mol (Henry et al.1985). This is far less than the energy of a single dissociating photon, which is typically about 57 Kcal/mol (for 500 nm light). It can then be expected that the additional photon energy will be dissipated through kinetic energy imparted to the photolyzed CO, thermal motion of the amino acid residues in Van der Waals contact with the heme, and raising of the temperature of chromophore by several hundreds K (Henry et al.1986; Anfinrud et al.1989). The hot heme is cooled down by interacting with the surrounding matrix on a picosecond time scale. An optical pumping mechanism for the $B_2/B_1$ interconversion appears to support one of the several possible pathways to dissipate kinetic energy. The additional absorption of photon energy, after 100%
fractional photodissociation of CO from Fe-CO is achieved, is dissipated through the photolyzed CO as an optical pumping at 10 K.

Flash photolysis experiments by Frauenfelder and co-workers have demonstrated a nonexponential nature of the several processes involved in geminate recombination at low temperature and suggested that this arises from a distribution of micro- and macro- conformational substates, each of which is separated by an activation energy in each substate (Mourant et al.1993; Nienhaus et al.1992; Frauenfelder et al.1991; Steinbach et al.1991; Austin et al.1975). Their recent application of temperature-derivative spectroscopy to MbCO has revealed the transition between the B substates during the relaxation process (Mourant et al.1993). Below 25 K, substate B₂ (2119 cm⁻¹) goes to substate B₁ (2131 cm⁻¹) and then recombines to form Fe-CO complex. Our data, presented here, suggest the opposite of this transition as an optical pumping mechanism under continuous illumination conditions in HbCO. If the rebinding process from B₂ is simply the reversal of photodissociation at low temperatures, our observations indicate the photoactivation process between B substates is in the opposite direction in the relaxation process by Frauenfelder and co-workers (Mourant et al.1993; Alben et al.1982). Even though the perturbations of B substates in HbCO are almost identical to that of MbCO as reflected in the less than 1 cm⁻¹ deviations in the observed frequencies of B substates in MbCO and HbCO (Park, 1988; Song et al.1993), the optical pumping between B substates in sperm whale MbCO was not
detected at 10 K under our identical experimental conditions in Figure 8. Therefore the optical pumping mechanism from B₁ to B₂ at 10 K may be a unique process in HbCO, presumably because of B₂ substate in HbCO is energetically favorable than in MbCO. However, the remaining question is whether MbCO at liquid helium temperature behaves similarly to HbCO. Further experiments are required to solve this problem.

Optical pumping may be important to define the distribution of initial conformational substates of protein in flash photolysis experiments at low temperatures. As presented here, the distribution among substates generated by the absorption of light is dependent on the illumination conditions. This result indicates that photolysis measurements should be made while being cautious of excess kinetic energy that is absorbed by the heme, because this kinetic energy may affect the observations of initial photodissociation state. In the case of relaxation experiments after photolysis, the illumination conditions can change the initial photolyzed CO conditions after obtaining 100% photodissociation of CO. The optical pumping of photolyzed CO clearly distinguishes the distribution of conformational substates followed by the absorption of additional photon from the ground photodissociation state distribution.
2.6 Conclusions

Absorption of a visible photon by the heme causes photodissociation CO from Fe-CO, and changes spin state of iron. Absorption of an additional photon introduces energy that must be dissipated in the form of translational and rotational interactions with protein surroundings, and this will alter the distribution of conformation substates of protein. We have observed an optical pumping mechanism in photolyzed CO as a means of kinetic energy dissipation, and a distribution of conformation substates of protein which is different from the initial distribution of ground state induced by the initial absorption of light. This observation provides an additional view of molecular interactions between the heme and the small ligand when the excess kinetic energy is absorbed by the heme.
LIST OF REFERENCES


CHAPTER III

Spectroscopic Characterization of Heme Moiety in Native and Cobalt Substituted Heme Proteins by Fourier Transform Infrared Spectroscopy: Implication of Vinyl Groups and Dioxygen Ligand

3.1 Abstract

Native and cobalt substituted heme proteins have been used to obtain structural information regarding the heme moiety by use of Fourier transform infrared (FT-IR) spectroscopy at cryogenic temperatures. The structural difference between meso- and protoporphyrin cobalt complexes enables us to identify the vinyl asymmetric deformation mode (CH$_2$ rock) in low temperature photodissociation absorbance spectra. This CH$_2$ rock mode has also been observed in native hemoglobin (Hb) and myoglobin (Mb). The solid assignment of the vinyl asymmetric deformation mode is essential to evaluate vibrational bands of dioxygen complex because of the complications in the assignment of vibrational dioxygen bands that may result from this vinyl mode. The photodissociation spectra of cobalt dioxygen complexes also show the apparent dioxygen vibrational bands, which is in good agreement with resonance Raman
observations of Tsubaki and Yu (Proc. Natl. Acad. Sci. (1981) 78, 3581) and Proniewicz and Kincaid (J. Am. Chem. Soc. (1990) 112, 675), but no third dioxygen infrared vibrational band was observed as suggested by RR spectroscopy. Relaxation studies of two dioxygen vibrational bands in the Co complex at 10 K do not substantiate the vibrational coupling or perturbations of dioxygen bands proposed by RR studies at above freezing temperature (Tsubaki and Yu, Proc. Natl. Acad. Sci. (1981) 78, 3581; Proniewicz and Kincaid, J. Am. Chem. Soc. (1990) 112, 675). The low temperature photodissociation spectrum of native HbO₂ and MbO₂ is nearly identical to that of HbCO and MbCO except that some bands show stronger intensities than others. These findings indicate that the apparent dioxygen band observed at 1106 cm⁻¹ in HbO₂ (1104 cm⁻¹, MbO₂) may be resonance enhanced or overlapped with either an axial imidazole mode or a porphyrin ring mode. Unlike infrared spectrum at above freezing temperature, low temperature photodissociation spectrum of Hb¹⁸O₂ and Mb¹⁸O₂ shows directly the appearance of new bands due to isotopic substitution at 1066 cm⁻¹ and 1065 cm⁻¹ respectively. But, the absorbance double-difference spectrum of [(L/D)Hb¹⁸O₂ - (L/D)Hb¹⁶O₂] exhibits multiple dioxygen (¹⁶O₂) sensitive bands and a single heavy isotope dioxygen (¹⁸O₂) band. Multiple bands, except the putative 1106 cm⁻¹ (1104 cm⁻¹), seem to arise from the conformational changes associated with porphyrin bands.
3.2 Introduction

The vinyl groups at the 2 and 4 positions of protoporphyrin (IX) have been suggested (Alben and Caughey, 1968; Warshel and Weiss, 1981; Rousseau et al. 1983; Sono and Asakura, 1975; Asakura and Sono, 1974) as a molecular modulator. These control the ligand binding affinity by a steric mechanism or electronic withdrawing effects of the vinyl group through the porphyrin \( \pi \)-electron density. The vinyl stretching frequency, \( v(C=C) \), was observed in RR spectra of biological samples of insect and human Hb(Gersonde et al. 1989; Rousseau et al. 1983), and cytochrome oxidase (de Paula et al. 1990) to probe the conformation of vinyl group. Primarily, the identification of vinyl was based on the assignment by Spiro and co-workers who have examined the nickel(II) protoporphyrin IX dimethyl ester and its derivatives deuterated the \( \alpha \) and \( \beta \) carbon atoms of the vinyl groups in the RR studies (Choi et al. 1982; Choi et al. 1982).

The proto, meso and deuterocobalt substituted heme proteins (Figure 9) were used to obtain information of thermodynamic characteristics of reversible oxygenation (Yamamoto et al. 1974; Yonetani et al. 1974), and spectroscopic properties of ligand binding (Yonetani et al. 1974; Yonetani et al. 1974; Woodruff et al. 1974). These proteins are also the ideal porphyrin substituents to identify the various vinyl modes of biological heme proteins instead of looking at the model porphyrins because they have been characterized in detail to compare the steric and electronic factors which control oxygen binding in the native proteins.

Initially, the infrared frequency of the bound dioxygen in Co-HbO$_2$ was observed at 1105 cm$^{-1}$ and a possible charge-transfer formulation of Co$^{III}$ - O$_2$ was suggested (Maxwell and Caughey, 1974). However, multiple bands were reported in Co-HbO$_2$ and Co-MbO$_2$ by Tsubaki and Yu (Tsubaki and Yu, 1981) who detected three isotope sensitive bands in the resonance Raman spectra at 1107, 1137, and 1153 cm$^{-1}$. They proposed that the dioxygen stretching mode is perturbed by accidentally degenerate porphyrin ring mode, and that there are two independent dioxygen vibrational stretching frequencies representing two different species: hydrogen bonded and non hydrogen bonded.

A more recent study of dioxygen adducts of cobalt porphyrins by Proniewicz and Kincaid (Proniewicz and Kincaid, 1990) has also found multiple bands due to vibrational coupling between dioxygen and internal modes of the associated axial ligands. According to this work, there is only one O$_2$ adduct present whose inherent $\nu$(O-O) frequency is 1139 cm$^{-1}$, and histidylimidazole fragment possesses internal modes at 1148, 1109, and 1085 cm$^{-1}$. Thus, coupling of $\nu$(O-O) with the nearby 1148 cm$^{-1}$ mode results in a decrease of dioxygen
stretching modes to 1136 cm⁻¹ and moderate intensity in the 1151 cm⁻¹. The 1109 cm⁻¹ mode is also weakly coupled to ν(O-O) as is evident by its weak activation and small shift (2 cm⁻¹) to 1107 cm⁻¹. This interpretation does not require the existence of two structures as proposed by Tsubaki and Yu (Tsubaki and Yu, 1981).

The identification of dioxygen stretching modes in native Hb and Mb have not been well resolved either. After initial observation of dioxygen stretching band at 1107 cm⁻¹ (Barlow et al.1973), Alben et al., (Alben et al.1978) reported an additional band at 1156 cm⁻¹ in the infrared spectrum of oxyhemoglobin, and proposed that two bands at 1107 cm⁻¹ and 1156 cm⁻¹ could be interpreted as due to Fermi resonance with the first overtone of the Fe-O stretching mode at 567 cm⁻¹ (Brunner, 1974). Recently, Caughey and co-workers (Potter et al.1987) reported two genuine dioxygen stretching bands of HbO₂, near those of cobalt substituted Hb at 1125 and 1155 cm⁻¹ (Tsubaki and Yu, 1981; Proniewicz and Kincaid, 1990). They attributed the observed 30 cm⁻¹ difference in the two dioxygen stretching bands to a conformational difference in oxygen binding site, rather than a hydrogen bonding as suggested in cobalt substituted heme proteins (Tsubaki and Yu, 1981).

In this report, we obtained (light minus dark) absorbance difference FT-IR spectra with 0.5 cm⁻¹ spectral resolution at low temperatures to characterize the heme moiety because absorbance difference spectrum is very sensitive to a small
frequency change which may contain a biological significance. For example, the application of FT-IR difference spectroscopy has demonstrated that the protonation of a single amino acid residue can be identified in the proton transfer mechanism of bacteriorhodopsin (Rothschild et al. 1988; Bousche et al. 1991). It has also been used to study light-induced electron transfer photosystem II (MacDonald and Barry, 1992), and redox-induced conformational changes in heme proteins (Schlereth and Mantele, 1992; Schlereth and Mantele, 1993). Here, we have examined the vinyl modes in cobalt substituted and native Hb and Mb complexes at low temperature. In addition to vinyl modes, these FT-IR absorbance difference spectra also contain unique information regarding the dioxygen vibrational bands both in cobalt and native heme protein dioxygen complexes because the dioxygen ligand will be photodissociated from the cobalt or iron in the presence of light. These spectra might clearly identify the dioxygen vibrational bands unambiguously.

3.3 Methods

Proto and meso Co-MbO₂, and proto Co-HbO₂ were prepared by the methods of Yonetani et al. (Yamamoto et al. 1974; Yonetani et al. 1974) and oxygenated. Human HbO₂ and HbCO were prepared as described by Moh et al. (Moh et al. 1987). Sperm whale ferric Mb (Type II) obtained from Sigma was dissolved in 50 mM Tris-HCl buffer pH 8.5. This protein solution was reduced with a small amount of solid dithionite, and subsequently passed through the
Sephadex G-25 column equilibrated with Tris buffer. The resultant sperm whale MbO$_2$ sample was concentrated by pressure ultrafiltration (Amicon: YM-10 membrane). Sperm whale MbCO was obtained from MbO$_2$ by passing CO gas (Matheson, 99.5%) over the solution with gentle stirring for 1 h. Preparations were stored in liquid nitrogen until use. Dioxygen isotope substitution of Hb was obtained by at least 6 cycles of alternate repeated evacuation and flushing with $^{18}$O$_2$ gas (95 atom %; Cambridge Isotope Laboratories), and used immediately to collect FT-IR spectrum. Visible spectra were obtained with a Cary model 17DX spectrophotometer. All infrared spectra were recorded at 0.5 cm$^{-1}$ resolution with a Mattson Sirius 100 Fourier transform infrared interferometer which was fitted with a liquid nitrogen cooled photovoltaic (HgCd)Te detector. Low temperature were obtained by use of a Lake Shore Cryotronics closed cycle helium refrigerator, Model LTS-21-D70C. Cryostat cell temperature was measured by use of a Lake Shore Cryotronics digital thermometer, model DRC-70, with a calibrated silicon diode probe. Photolysis made use of a 500 W tungsten lamp focused through a slide projector and passed through a water filter (2 cm) to remove infrared radiation beyond 1.2 micrometers. Photodissociation absorbance spectra of (light - dark), were obtained from the ratio of single beam spectra in the presence (light) and in the absence (dark) of visible radiation.
3.4 Results

Absorbance difference spectroscopy has been widely used in many biological systems. This method is particularly sensitive to changes in bandwidth and frequency shifts in the absorbance difference spectrum (Alben and Fiamingo, 1984; Alben and Bare, 1978). The photodissociation of FT-IR absorbance difference spectrum of MbCO (Fiamingo and Alben, 1985; Alben et al. 1982) is one example exhibiting a frequency shift. It shows the major Fe-CO band (1945 cm\(^{-1}\)) in the dark and photolyzed CO bands (2131 and 2119 cm\(^{-1}\)) in the light pointing down or up at low temperatures because the frequency shift is much larger than the bandwidth. Alternatively, if the frequency shift is less than the bandwidth, the appearance of frequency shifted bands in the absorbance difference spectrum is roughly similar to the first derivative of an absorption band. A change in the bandwidth also results in an appearance similar to second derivative or to a \(\sin x/x\) function in the absorbance difference spectrum. All of these possibilities are expected to occur in our FT-IR absorbance difference spectra. In this paper, we report negative peak frequencies instead of reporting zero-crossing frequencies in the (light/dark) photodissociation absorbance difference spectrum, because the negative peak is the vibrational mode present in the dark, and also because the precise knowledge of band shapes is also unknown to evaluate frequency shift (Alben and Fiamingo, 1984; Laane and Kiefer, 1980; Laane, 1981).
Low temperature (light/dark) photodissociation absorbance difference spectra of cobalt substituted heme protein complexes are shown in Figure 10. The vinyl deformation modes (CH$_2$ rock) of heme substituents, expected to occur at around 1080 cm$^{-1}$ from olefin and nickel porphyrin studies (Colthup et al.1990; Choi et al.1982; Choi et al.1982), is unambiguously identified by a structural comparison between meso- and proto Co protein complexes in the absorbance double-difference spectrum of [(L/D)proto-CO-MbO$_2$ - (L/D)meso-Co-MbO$_2$] in Figure 10-B. The large amplitude first-derivative frequency shifted bands at 1079 and 1095 cm$^{-1}$ (zero-crossing frequency) are due to vinyl asymmetric deformation mode and the intense negative peak at 1132 cm$^{-1}$ is due to half-bandwidth and frequency changes of dioxygen vibrational band. The vinyl CH$_2$ rock modes are observed at 1082 and 1098 cm$^{-1}$ in proto-Co-MbO$_2$, and at 1085 and 1095 cm$^{-1}$ in proto-Co-HbO$_2$. These two bands are new features in the assignment of vinyl asymmetric deformation because previous studies (Choi et al.1982; Choi et al.1982; Lee et al.1986; Desbois et al.1984) observed only one of two bands at 1085 cm$^{-1}$. The splitting of the vinyl rocking mode in Co-Hb and Co-Mb may be due to different positions of vinyl groups in the porphyrin ring or in-phase and out-of-phase deformation. The observed two intense negative peaks at 1104 and 1135 cm$^{-1}$ in Co complexes at 10 K are assigned the dioxygen vibrational band of the Co-O$_2$ protein complex, as these frequencies are in good agreement with the assignment of RR studies at above freezing temperature (Tsubaki and Yu, 1981; Proniewicz and Kincaid, 1990). However, the observed distinctive third dioxygen
Figure 10. (A). Light minus dark photodissociation spectra of dioxygen from oxycobalt substituted Mb and Hb at 11.7 Kelvin. Each protein was prepared in a pair of BaF$_2$ windows with 7 microns spacer: (a) proto-Co-MbO$_2$; (b) proto-Co-HbO$_2$; (c) meso-Co-MbO$_2$. Each spectrum was normalized for the absorption of 1134 cm$^{-1}$ band. (B). The absorbance double-difference spectrum of [(L/D)proto-Co-MbO$_2$ - (L/D)meso-Co-MbO$_2$].
Figure 10 (continued)

(B)
vibrational band at around 1150 cm\(^{-1}\) by RR studies was not detected in FT-IR spectra of proto-Co-Mb and Hb within our instrumental limitations. The negative 1075 cm\(^{-1}\) peak in proto-Co-Hb\(_{O_2}\) seems to be a characteristic band of Co-Hb and also observed in native Hb in Figure 11.

To compare the vinyl deformation and dioxygen vibrational bands of native heme proteins with cobalt substituted heme protein complexes, low temperature (light/dark) photodissociation FT-IR absorbance difference spectra of Hb (Figure 11) and Mb (Figure 12) were obtained. These native heme proteins showed different photoperturbations from the cobalt complexes, probably due to the different electron occupation of d orbitals (Kincaid et al.1983) or a different apparent quantum yield (Hoffman and Gibson, 1978). There are no major differences between Hb\(_{O_2}\) and HbCO in the photodissociation absorbance difference spectra in Figure 11. Every single photoperturbed peak observed in Hb\(_{O_2}\) is also present in HbCO, although some bands show stronger intensities than others. Two intense negative peaks at 1082 and 1093 cm\(^{-1}\) are ascribed to vinyl rocking modes which were identified in this study. These vinyl band shift to lower frequencies upon photodissociation of O\(_2\). The low temperature photodissociation spectrum of Hb\(^{18}\)O\(_2\) shows the appearance and disappearance of band directly: \(i\) A new negative peak at 1066 cm\(^{-1}\) is appeared. \(ii\) The 1106 cm\(^{-1}\) peak loses its intensity, such that the shape of peak is similar to that of HbCO, but not completely disappeared. \(iii\) There are no significant spectral
Figure 11. (A). Light minus dark photodissociation spectra of HbO₂, Hb¹⁸O₂, and HbCO at 10.5 K. Each protein was prepared in a pair of BaF₂ infrared windows and KCl window cryostat cube with 27.6 microns spacer. (B). The absorbance double-difference spectrum of [(L/D)Hb¹⁸O₂ - (L/D)Hb¹⁸O₂]. (C) The absorbance double-difference spectrum of [(L/D)Hb¹⁸O₂ - (L/D)HbCO].
Figure 11 (continued)
Figure 11 (continued)
differences in 1132 cm\(^{-1}\) and 1150 cm\(^{-1}\) region in both Hb\(^{16}\)O\(_2\) and Hb\(^{18}\)O\(_2\). The HbCO photodissociation difference spectrum has well resolved peaks at around 1133 cm\(^{-1}\) and 1170 cm\(^{-1}\) contrary to HbO\(_2\). The intense 1236 cm\(^{-1}\) negative peak in HbCO photodissociation spectrum is the characteristic of porphyrin-Fe-CO complexes which has about 1/10 times intensity compared to the vibrational stretching intensity of Fe-CO observed at 1950 cm\(^{-1}\).

To evaluate the dioxygen vibrational bands, the absorbance double-difference spectrum of \([(L/D)\text{Hb}^{18}\text{O}_2 - (L/D)\text{Hb}^{16}\text{O}_2]\) was obtained because the photoperturbed bands of these two spectra are identical except in the expected dioxygen vibrational band region. The proper choice of scaling factor is critical in the absorbance double-difference spectrum, and our choice of scaling factor is reasonable because the photoperturbed bands above 1200 cm\(^{-1}\) region are quite well canceled out. The absorbance double-difference spectrum presented in this study is significantly different from the previous studies (Potter et al. 1987; Barlow et al. 1973) not only for improved signal/noise ratio and 0.5 cm\(^{-1}\) spectral resolution, but also for the direct comparison of isotope sensitive bands in the low temperature photodissociation spectrum. One negative peak (1066 cm\(^{-1}\)) with a shoulder (1058 cm\(^{-1}\)) and four positive peaks (1092, 1106, 1126 and 1165 cm\(^{-1}\)) are observed in \([(L/D)\text{Hb}^{18}\text{O}_2 - (L/D)\text{Hb}^{16}\text{O}_2]\) absorbance double-difference spectrum in Figure 11-B. The negative peak is due to the vibrational modes of \(^{18}\text{O}_2\), and the positive peaks are due to the vibrational modes of \(^{16}\text{O}_2\) assuming that there are
no conformational and structural differences on the isotope substitution except dioxygen vibrational modes.

The observed negative peak at 1066.2 cm\(^{-1}\) in Figure 11-B must be attributed to \(^{18}\)O\(_2\) vibrational band because this peak appeared exclusively and directly in the low temperature photodissociation spectrum of Hb\(^{18}\)O\(_2\) (Figure 11-A). The frequency shift calculation based on simple harmonic oscillator also confirms our assignment \((\nu^{18}/\nu^{16} = 0.9629 \& (\mu^{16}/\mu^{18})^{1/2} = 0.9428)\). The shoulder of this negative peak was observed in all Hb derivatives, so it may be due to an unidentified porphyrin ring or porphyrin substituent vibrational band. As the 1093 cm\(^{-1}\) peak is unambiguously confirmed in this study, the positive 1092 cm\(^{-1}\) peak in the absorbance double-difference spectrum must be ascribed to a slight conformational difference of vinyl deformation rather than a dioxygen vibrational band. The almost identical appearance of vinyl asymmetric deformation in low temperature photodissociation spectra of Hb\(^{16}\)O\(_2\) and Hb\(^{18}\)O\(_2\) (Figure 11-A) also supports the conformational difference of vinyl. The intense 1106 cm\(^{-1}\) positive peak must be due to a dioxygen (\(^{16}\)O\(_2\)) stretching band because this peak shows the strongest absorption in low temperature photodissociation spectrum of Hb\(^{16}\)O\(_2\) among Hb\(^{18}\)O\(_2\) and HbCO in addition to the decreased intensity which is observed directly in low temperature photodissociation spectrum of Hb\(^{18}\)O\(_2\). However, the shape of 1106 cm\(^{-1}\) band in the absorbance double-difference spectrum in Figure 11-B suggests multiple bands rather than a single band. The composition of the
1106 cm\(^{-1}\) peak may result in resonance enhancement or overlap with either an axial imidazole, or a porphyrin mode. This peak has a good frequency correlation with that of histidylimidazole (Salama and Spiro, 1978; Walters and Spiro, 1983; Hodgson et al.1980; Hodgson et al.1980). Unlikely direct evidence of 1106 cm\(^{-1}\) band as a dioxygen (\(^{16}\text{O}_2\)) vibrational band, the other two positive peaks with absorbance maxima at 1126 and 1165 cm\(^{-1}\) are not definitively assigned to dioxygen (\(^{16}\text{O}_2\)) vibrational bands because the distinctive features were not observed in the low temperature photodissociation spectrum of Hb\(^{18}\text{O}_2\) in this spectral region.

The absorbance double-difference spectrum of \([(L/D)\text{Hb}^{18}\text{O}_2 - (L/D)\text{HbCO}]\) in Figure 11-C appears to present more information about dioxygen vibrational bands. This spectrum shows \(^{18}\text{O}_2\) vibrational band at 1066 cm\(^{-1}\) in addition to several unidentified bands. As expected, the 1106 cm\(^{-1}\) dioxygen (\(^{16}\text{O}_2\)) peak is completely absent, however, the positive peaks at 1127, 1133, and 1167 cm\(^{-1}\) have almost identical frequencies to those observed in absorbance double-difference spectrum of \([(L/D)\text{Hb}^{18}\text{O}_2 - (L/D)\text{Hb}^{16}\text{O}_2]\) in Figure 11-B. Therefore, only putative 1106 cm\(^{-1}\) peak seems to be a dioxygen vibrational band.

Photodissociation absorbance difference spectra of sperm whale Mb derivatives were collected and presented in Figure 12. The general photodissociation patterns between Mb\(^{18}\text{O}_2\) and MbCO are closely related to the
Figure 12. Light minus dark photodissociation spectra of sperm whale MbO₂, Mb⁻¹⁸O₂, and MbCO at 13.8 K. Each protein was prepared in a pair of BaF₂ windows and KCl window cryostat cube with 27.6 microns spacer. (B). The absorbance double-difference spectrum of [(L/D)Mb⁻¹⁸O₂ - (L/D)Mb⁻¹⁸O₂]. (C) The absorbance double-difference spectrum of [(L/D)Mb⁻¹⁸O₂ - (L/D)MbCO].
Figure 12 (continued)

![Graph of Figure 12](B)

- Wavenumber range: 1000 to 1300
- Absorbance range: -0.002 to 0.0025
- Peaks at 1065, 1087, 1119, 1124, and 1141
- Mbd2-1 abs
Figure 13. Light minus dark photodissociation spectra of bovine heart MbO₂ and MbCO at 11.5 K. Each protein was prepared in a pair of BaF₂ windows and KCl wind cryostat cube with 7 microns spacer.
corresponding derivatives of Hb. The vinyl rocking mode at around 1081 and 1097 cm\(^{-1}\) in Mb derivatives is identified. The frequency shifted pattern also shows that the vinyl deformation mode is shifted from higher (in the dark spectrum) to lower frequency (in the light spectrum) by the photodissociation of ligand molecules. The pattern of this shift is identical to that of Hb. Unlike Hb\(_2\), a peak with medium intensity at 1125 cm\(^{-1}\) is present in the low temperature photodissociation spectrum of Mb\(_2\), suggesting the involvement of dioxygen vibrational band. The existence of 1125 cm\(^{-1}\) and very weak 1120 cm\(^{-1}\) negative peaks in the low temperature photodissociation spectrum of Mb\(_2\) are due neither to water vapor peaks nor an instrumental artifact, and are also observed in the low temperature photodissociation spectrum of beef heart Mb\(_2\) (Figure 13). Thus, these bands are primarily due to photoperturbations of Mb\(_2\). The low temperature photodissociation spectrum of Mb\(^{18}\)O\(_2\) shows the appearance of new peak at 1065 cm\(^{-1}\), and the reduced-intensity peak at 1105 and 1125 cm\(^{-1}\) peaks. The decreased intensity of the 1125 cm\(^{-1}\) band seems to be the characteristic of Mb\(_2\), and is not observed in low temperature photodissociation spectrum of Hb\(_2\) (Figure 11-A). The low temperature photodissociation spectrum of MbCO shows the intense enhancement of the vinyl rocking modes as well as two negative peaks at 1120 and 1238 cm\(^{-1}\). The enhancement of the vinyl mode in MbCO may be due to different polarization of the vinyl group in MbCO than Mb\(_2\). As observed in HbCO, the 1238 cm\(^{-1}\) peak is characteristic of porphyrin-Fe-CO complex, but has not yet been definitively assigned.
The absorbance double-difference spectrum of \((L/D)\text{Mb}^{18}\text{O}_2 - (L/D)\text{Mb}^{16}\text{O}_2\) in Figure 12-B shows two negative peaks (1065 and 1088 cm\(^{-1}\)) and four positive peaks (1105, 1120, 1125 and 1157 cm\(^{-1}\)). The intense negative peak at 1065 cm\(^{-1}\) originates from the vibrational mode of dioxygen \((^{18}\text{O}_2)\) band in \text{Mb}^{18}\text{O}_2, because it is obvious in the low temperature photodissociation spectrum of \text{Mb}^{18}\text{O}_2 in Figure 12-A. As no definitive spectral changes are observed in the photodissociation spectrum of \text{Mb}^{18}\text{O}_2 compared to \text{Mb}^{16}\text{O}_2 in vinyl deformation spectral region, the second negative 1088 cm\(^{-1}\) peak seems to be due to a difference in the vinyl deformation modes comparable to 1092 cm\(^{-1}\) band in the \((L/D)\text{Hb}^{18}\text{O}_2 - (L/D)\text{Hb}^{16}\text{O}_2\) absorbance double-difference spectrum. The intense 1105 cm\(^{-1}\) peak in the absorbance double-difference spectrum must be ascribed to the dioxygen \((^{16}\text{O}_2)\) vibrational band of \text{Mb}^{16}\text{O}_2 because of reduced intensity observed in low temperature photodissociation spectrum of \text{Mb}^{18}\text{O}_2 directly. The rest of the observed positive peaks are for the most part comparable to bands observed in the \((L/D)\text{Hb}^{18}\text{O}_2 - (L/D)\text{Hb}^{16}\text{O}_2\) absorbance double-difference spectrum, except that a new band is present at 1120 cm\(^{-1}\). This band is present as a weak negative peak in \text{Mb}^{16}\text{O}_2, absent in \text{Mb}^{18}\text{O}_2, and present in \text{MbCO} with a strong intensity in Figure 12-A. The absorbance difference spectrum of \((L/D)\text{Mb}^{18}\text{O}_2 - (L/D)\text{MbCO}\) in Figure 12-C shows not only \(^{16}\text{O}_2\) vibrational bands but also vinyl asymmetric deformation modes and several unidentified bands.
Table 11 is a summary of absorptivities and negative peak frequencies of cobalt and native heme proteins observed in low temperature photodissociation spectra. The frequencies reported for vinyl asymmetric deformation frequencies are those for zero-crossing in this table. The concentration of each protein was obtained from the visible spectra. As the exact fractional photolysis is unknown for the dioxygen complexes, fractional photolysis was not considered to calculate the absorptivity. Among cobalt and native heme proteins, meso-Co-MbO$_2$ has the highest absorptivity in the observed dioxygen vibrational bands.

Several independent samples were collected in replicate, and the absorbance difference bands were well reproduced. We have tested against instrumental artifacts by the comparison of light/dark photodissociation spectra of metHb and HbCN, which are unphotolizable, and the dark/dark spectrum of HbCO under identical experimental conditions. The photodissociation spectra of these studies show no photodissociated bands above 100 microabsorbance peak to peak noises. Therefore, the absorbance difference spectra presented here are attributed to molecular changes associated with photodissociation of ligands such as O$_2$ and CO, and are independent of thermal effects associated with light absorption.
### Table 11. Absorptivities and Negative Peak Frequencies of Selected Bands.

Unit: mM·cm⁻¹

<table>
<thead>
<tr>
<th>Peak-to-peak frequencies &amp; assignment</th>
<th>meso-CoMbO₂</th>
<th>proto-CoMbO₂</th>
<th>proto-CoHbO₂</th>
<th>¹native HbO₂</th>
<th>²native HbCO</th>
<th>native MbO₂</th>
<th>native MbCO</th>
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<tr>
<td>1074₄⁻1077ᵀ</td>
<td>0.04 (0.01)</td>
<td>0.01 (0.005)</td>
<td>0.03 (0.005)</td>
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<td>0.06 (0.005)</td>
<td>0.14 (0.005)</td>
<td>0.56 (0.005)</td>
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<td>0.32 (0.01)</td>
<td>0.14</td>
<td>1081.7 cm⁻¹</td>
<td>1079.2 cm⁻¹</td>
<td>1079.0 cm⁻¹</td>
<td>1078.2 cm⁻¹</td>
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<tr>
<td>w(vinyl)</td>
<td>1079.6 cm⁻¹</td>
<td>1091.9 cm⁻¹</td>
<td>1089.8 cm⁻¹</td>
<td>1089.4 cm⁻¹</td>
<td>1094.1 cm⁻¹</td>
<td>1093.1 cm⁻¹</td>
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<tr>
<td>1087ᵀ⁻1093Ⱶ</td>
<td>0.44</td>
<td>0.28</td>
<td>1095.0 cm⁻¹</td>
<td>1091.9 cm⁻¹</td>
<td>1089.8 cm⁻¹</td>
<td>1089.4 cm⁻¹</td>
<td>1094.1 cm⁻¹</td>
</tr>
<tr>
<td>w(vinyl)</td>
<td>1093.6 cm⁻¹</td>
<td>1105.0 cm⁻¹</td>
<td>1106.7 cm⁻¹</td>
<td>1106.7 cm⁻¹</td>
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<tr>
<td>1107ᵀ⁻1107Ⱶ wO₃ + Im</td>
<td>0.87 (0.01)</td>
<td>0.31</td>
<td>1104.0 cm⁻¹</td>
<td>1105.0 cm⁻¹</td>
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<td></td>
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<tr>
<td>1117ᵀ⁻1122Ⱶ</td>
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<tr>
<td>wO₃ + wO₃</td>
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<td>1134.2 cm⁻¹</td>
<td>1134.0 cm⁻¹</td>
<td>1131.8 cm⁻¹</td>
<td>1132.8 cm⁻¹</td>
<td>1124.6 cm⁻¹</td>
<td>-</td>
</tr>
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<td>0.08</td>
<td>0.12</td>
<td>1235.4 cm⁻¹</td>
<td>1235.7 cm⁻¹</td>
<td>1237.6 cm⁻¹</td>
</tr>
</tbody>
</table>

* Absorptivities were not normalized for fractional photolysis. Hb: Human Hb, Mb: Sperm Whale. Im: imidazole
¹ Average of three independent data set.
² Average of four independent data set.
³ †: positive peak, ‡: negative peak
⁴ zero-crossing frequencies in this row
⁵ negative peak frequencies in this row
⁶ uncertainties
3.5 Discussion

*Low temperature photodissociation absorbance difference spectroscopy:* Low temperature photodissociation absorbance difference spectroscopy has a great advantage over above freezing absorbance difference spectroscopy in the sense that no reference sample preparation is necessary because of its own self-reference. Absorbance difference spectrum is computed from a logarithmic ratio of the single beam spectrum obtained in the absence of light (dark) and in the presence of light (light) at the indicated cryogenic temperatures. Therefore we can easily detect not only the absorbance difference between the metal coordinated and unligated ligand states but also the small frequency differences.

As absorbance difference spectroscopy is very sensitive to small difference in band center frequency, a shift of one hundredth of the half-bandwidth is readily measured (Alben and Fiamingo, 1984; Alben and Bare, 1978; Laane and Kiefer, 1980; Laane, 1981). A similar error of measurement of center frequency due to instrumental non-reproducibility would also be measured and might be confused with a real frequency shift of an absorption band (Park and Alben, 1993). Thus frequency reproducibility and calibration are essential. We measured the frequency accuracy corrected to vacuum that the instrumental inaccuracy was to be $0.024 \pm 0.02 \text{ cm}^{-1}$ ($1:100,000$), by comparison of spectra of carbon monoxide gas at low partial pressure with literature values (Cole, 1977).
**Vinyl deformation mode**: The direct comparison between the meso and proto species (Figure 9) has allowed us to assign two strongly enhanced vinyl asymmetric deformation (CH$_2$ rock) bands in biological heme proteins. The observation of two vinyl deformation bands is a new feature in the vibrational assignment of vinyl mode because the previous studies (Choi et al.1982; Choi et al.1982; Lee et al.1986) based on Ni(II) protoporphyrin dimethyl ester and its derivatives deuterated at $\alpha$ and $\beta$ atoms of the 2,4-vinyl groups showed one CH$_2$ rock band instead of two band in RR spectra. The difference in the assignment of vinyl deformation between IR and RR studies may originate from the different selection rules or the difference between model porphyrins or biological heme proteins which have a certain orientation of vinyl-protein interaction (La Mar et al.1978). The complete assignment and characterization of vinyl mode seem to be critical to investigate the dioxygen vibrational mode of heme proteins, not only because the frequency of dioxygen heavy isotope (Tsubaki and Yu, 1981; Proniewicz and Kincaid, 1990) but also because an unidentified absorption in the absorbance double-difference spectra with different heme ligands (Potter et al.1987) appears to occur in this spectral region. The vinyl deformation bands were observed, but not identified in the absorbance double-difference spectra of Potter et al. (Potter et al.1987) (Figure 3 of that work), which showed the first derivative shape of an absorption band of CH$_2$ rock mode. RR spectra of Tsubaki et al. (Tsubaki and Yu, 1981) (Figure 4 of that work) and Proniewicz et al. (Proniewicz and Kincaid, 1990) (Figure 3 of that work) also seem to implicate
this complications of the vinyl deformation mode.

The zero-crossing frequencies of vinyl rock mode from cobalt-substituted and native heme proteins are summarized in Table 11. Studies of vinyl substituted porphyrin model compounds show the variation of C=C vibrational frequency depending on the angles of vinyl and porphyrin ring (Bocian et al.1986; Gersonde et al.1989). Assuming that the vinyl CH$_2$ rock mode also reflects the orientation of the vinyl and porphyrin ring, the vinyl orientations of proto-Co-HbO$_2$ and native MbCO are quite different from other protein complexes. The almost identical frequency of CH$_2$ rocking in native HbO$_2$ and HbCO may result from an identical localization of vinyl groups or an almost identical $\pi$ electron delocalization in porphyrin. However, the difference of CH$_2$ rocking frequency between Hb and Mb may be attributed to the amino acid side chains of the globin. This seems to be supported by RR studies indicating that the stretching and deformation of vinyl groups are conformation dependent, reflecting protein-induced differences of vinyl side chains (Desbois et al.1984; DeVito and Asher, 1989). The vinyl asymmetric deformation can be ascribed to a composition of 43% CH$_2$ rocking mode, 13% CN stretching, and the reminder heme ring stretching from normal mode calculations by Lee et al.(Lee et al.1986).

The CH$_2$ asymmetric deformation mode in heme proteins is a very strong infrared active mode and can be used as a local molecular probe to understand the functional and structural role of porphyrin vinyl substituents that have not been understood in great detail.
Cobalt substituted proteins: Contrary to the different intensities of multiple dioxygen peaks with RR data (Tsubaki and Yu, 1981; Proniewicz and Kincaid, 1990), low temperature photodissociation FT-IR spectra of cobalt substituted heme proteins showed two dioxygen stretching bands with nearly equal intensities both in meso and proto Co-Mb. The half-bandwidth of two dioxygen stretching bands in meso-Co-MbO₂ is similar, but is not similar in proto-Co-MbO₂. Within the limitations of signal/noise, no distinctive third dioxygen band was observed, as suggested by RR spectroscopy at around 1150 cm⁻¹ at room temperatures. Possible reasons may be: i) The configuration of dioxygen allows to have a very little change in the net transition dipole, possibly having the side-on geometry. ii) The 1150 cm⁻¹ is not an infrared active mode, it is a Raman active. iii) The 1150 cm⁻¹ dioxygen vibrational band is an unphotolizable species, or once it is photolyzed, the gemination rate is too fast to allow us for its detection under our experimental conditions. iv) At 10 K, the configuration of dioxygen does not allow for the existence of this frequency band relative to the room temperature configuration which is associated with the ligand dependent difference in protein conformation. Even though the side-on geometry is stereochemically possible and was interpreted in infrared spectra of model hemes without a back axial ligand (Watanabe et al.1984), it may be too unstable in biological systems (Duff et al.1979). The common observation between RR and IR is the complete absence of the high frequency band (1150 cm⁻¹) in meso-Co-MbO₂ (Tsubaki and Yu, 1981; Mackin et al.1983; Yu and Kerr, 1988).
Relaxation studies of the two apparent dioxygen bands may differentiate the perturbation or vibrational coupling from two different conformations. If observed two apparent dioxygen bands are primarily due to perturbation or coupling, the expected relaxation rate might be similar. Otherwise the observed dioxygen bands should be ascribed to different conformations. Our preliminary studies of relaxation rate of dioxygen bands (about 1105 and 1135 cm\(^{-1}\)) in meso-Co-MbO\(_2\) suggest the existence of different conformations rather than vibronically coupled or perturbed bands (Tsubaki and Yu, 1981; Proniewicz and Kincaid, 1990) because of the different relaxation rates of the two apparent dioxygen bands (Figure 14). Also these dioxygen bands can not be considered as a Fermi resonance as suggested Alben et al. (Alben et al. 1978) in native HbO\(_2\) because the first overtone of v(Co-O) is 1078 cm\(^{-1}\) (539 cm\(^{-1}\) x 2) is too low to interact the dioxygen vibrational bands (Tsubaki and Yu, 1981).

The absorptivity in Table 11 suggests that meso-Co-MbO\(_2\) has a higher fractional photodissociation yield among the cobalt substituted and native heme proteins, assuming that all proteins are 100% saturated with dioxygen based on visible spectra collected at room temperature. The higher fractional photolysis in meso-Co-MbO\(_2\) reasonably agrees with the optical photodissociation spectra, indicating that the percentage of the photodissociated form decreased in the order meso>proto>deutero (Iizuka et al. 1974). The higher apparent photodissociation may be due to the possible electronic interactions of porphyrin
Figure 14. Relaxation rate of two apparent dioxygen bands in meso-Co-MbO₂ at 12 K. The 1104 cm⁻¹ peak is denoted by a closed circle, and 1136 cm⁻¹ peak is denoted by a closed square. $N(B)$ is expressed by $\Delta A(t)/\Delta A(t=0)$ where $A(t)$ refers to the absorbance difference between a spectrum taken at time, $t$, and $A(t=0)$ is the maximum absorbance from the continuous light spectrum at 12 K.
substituents group through the entire porphyrin ring as suggested by Lizuka et al. (Tucker et al. 1978).

**Interpretation of apparent dioxygen bands of native heme proteins:** Previous infrared studies of native Hb and Mb with 2.8 cm⁻¹ spectral resolution (Potter et al. 1987) collected at above freezing temperature showed three apparent dioxygen (¹⁶O₂) vibrational bands at around 1155 cm⁻¹ (Δυ/2 = 15 cm⁻¹) with a medium intensity, at around 1130 cm⁻¹ (Δυ/2 = unreported) with a low intensity, and at 1107 cm⁻¹ (Δυ/2 = 8 cm⁻¹) with a strong intensity. These were interpreted as two conformers similar to the interpretation of Tsubaki and Yu (Tsubaki and Yu, 1981). In case of the dioxygen heavy isotope (¹⁸O₂), two dioxygen (¹⁸O₂) vibrational bands were observed (Potter et al. 1987). However, as discussed previously (Nakamoto and Czernuszewicz, 1993; Proniewicz and Kincaid, 1990), the detection and interpretation of dioxygen stretching bands with RR and IR spectroscopic method are still open questions because of instability of dioxygen complex and an instrument, and lack of complete understanding of perturbation mechanism. Furthermore, as discussed here, the highly overlapping frequency of vinyl deformation with ¹⁸O₂ vibrational band seems to complicate the analysis.

Low temperature photodissociation spectra of native heme proteins presented here may give an unique solution for these uncertainties because we can isolate and identify the vinyl deformation mode, and trap the dioxygen bands
generated by photodissociation, in addition to collecting spectra with highly improved signal/noise ratio. It also provides us a great advantage against above freezing infrared spectrum because the isotope sensitive bands can be detected directly with an appearance or disappearance in the low temperature photodissociation spectrum. It may also provide a crucial information about the molecular perturbations indirectly because the proposed unidentified perturbations (Potter et al. 1987; Tsubaki and Yu, 1981) due to dioxygen ($^{16}\text{O}_2$) stretching band with porphyrin or an axial ligand are completely absent in the low temperature photodissociation spectra of Hb$^{18}\text{O}_2$ (Mb$^{18}\text{O}_2$) and HbCO (MbCO). All of the observed peaks in this study are primarily due to the photoperturbation of heme, heme substituents, dissociation of $^{18}\text{O}_2$ (or CO) and heme surroundings possibly including axial imidazole.

All of our photodissociation spectra of Hb$\text{O}_2$, HbCO and Hb$^{18}\text{O}_2$ in Figure 10 exhibit every apparent dioxygen ($^{16}\text{O}_2$) vibrational frequencies reported by Potter et al. (Potter et al. 1987). This may be due to fact that apparent dioxygen stretching bands seem to be resonance enhanced band or overlapped. Especially it is quite obvious that the putative 1107 cm$^{-1}$ dioxygen vibrational band has resonance enhancement. In general, perturbation studies suggest that perturbations between one band and an accidentally degenerate band splits a single band into several bands (Tsubaki and Yu, 1981; Yu and Kerr, 1988; Bajdor et al. 1984; Kincaid et al. 1985). Therefore, we expected to observe a different band
shape of 1093 cm\(^{-1}\) peak in our low temperature photodissociation spectrum of Hb\(^{18}\)O\(_2\) compared to Hb\(^{16}\)O\(_2\) and HbCO in Figure 11, because above freezing infrared studies by Potter et al. (Potter et al. 1987) showed two dioxygen (\(^{18}\)O\(_2\)) isotope sensitive bands at 1064 and 1094 cm\(^{-1}\). In our photodissociation spectrum of Hb\(^{18}\)O\(_2\) in Figure 11, there is only one isotope sensitive band at 1066 cm\(^{-1}\) directly. However no distinctive spectral changes of band shape at 1093 cm\(^{-1}\) was observed. This observation also applies to the dioxygen (\(^{18}\)O\(_2\)) vibrational bands in low temperature photodissociation spectrum of MbO\(_2\) in Figure 12. Therefore, these results strongly suggest that the 1094 cm\(^{-1}\) peak observed at above freezing infrared studies (Potter et al. 1987) seems to be unlike a dioxygen band in Hb\(^{18}\)O\(_2\).

The assignment of vinyl deformation mode has helped us to obtain a single dioxygen (\(^{18}\)O\(_2\)) heavy isotope vibrational band representing a single conformer. However, multiple dioxygen (\(^{16}\)O\(_2\)) vibrational bands in the absorbance double-difference spectra of heme protein (\(^{18}\)O\(_2\) - \(^{16}\)O\(_2\)) may indicate either perturbations of the dioxygen band which generate several dioxygen bands, or a single dioxygen band and several unidentified bands due to conformational difference or porphyrin skeletal modes. The intense resonance enhancement of the 1106 cm\(^{-1}\) in HbO\(_2\) (1104 cm\(^{-1}\) MbO\(_2\)) compared to Hb\(^{18}\)O\(_2\) and HbCO suggest a definitive dioxygen vibrational band. However, this band seems to be a composite band with an axial imidazole or porphyrin band. The rest of the apparent dioxygen bands
remain to be identified by further experiments.

The uncertainties in dioxygen vibrational bands may be due to the lack of the complete assignment of porphyrin ring vibrational modes. According to the normal mode analysis of Ni(OEP) as a $D_{4h}$ symmetry (Abe et al. 1978; Li et al. 1990; Willems and Bocian, 1984; Willems and Bocian, 1985), porphyrin fundamental in-plane ($E_u$) and out-of-plane ($A_{2g}$) modes are infrared active. We are expected to observe $v_4$ (≈1230 cm$^{-1}$), $v_{43}$ (≈1155 cm$^{-1}$), and $v_{44}$ (≈1133 or 1110 cm$^{-1}$) in-plane ($E_u$) modes (Abe et al. 1978; Li et al. 1990; Kincaid et al. 1983; Choi et al. 1982) in our spectral region. The $v_{43}$ and $v_{44}$ modes may accidently occur the dioxygen stretching frequency, or the observed dioxygen vibrational bands may be porphyrin ring mode rather than genuine dioxygen bands. The above freezing temperature absorbance double-difference spectra reveals the presence of 1155 cm$^{-1}$ band in (metHb-HBCO) and (HbCN-HbCO) (Figure 15).

The isotope shift in Figures 11 and 12 shows a reasonable agreement with the corresponding shifts from the expected isotope shift calculation of a simple harmonic oscillator (expected position: 1044 cm$^{-1}$; $v^{16}/v^{18} = 0.9629 \& (\mu^{16}/\mu^{18})^{1/2} = 0.9428$). However, the small deviation (0.02) between frequency ratio ($v^{18}/v^{16}$) and reduced mass ratio ($(\mu^{16}/\mu^{18})^{1/2}$), even though its value is small, must be explained. In general, coupling of the diatomic stretching mode with another mode of vibration is responsible for most of the unexpected deviation (Arakawa
Figure 15. Absorbance difference spectra of Hb derivatives at above freezing temperature. (From S. Park., M.S. Thesis, The Ohio State University, Columbus, Ohio).
et al. 1994). In this case, a considerable vibrational energy is localized in other parts of polyatomic molecule, so that the diatomic molecule can no longer provide the correct isotope frequency shift. Therefore, the small deviation observed in isotope shift suggests that dioxygen stretching mode at 1106 cm$^{-1}$ ($HbO_2$) is coupled with an axial imidazole mode or unidentified porphyrin ring mode.

3.6 Conclusions

We have used the novel approach of FT-IR photodissociation at cryogenic temperatures to obtain an exclusive spectral information in the heme moiety of cobalt substituted and native heme proteins. This approach provides an excellent way to isolate and identify the vinyl asymmetric deformation and dioxygen vibration because all of these bands are observed directly in low temperature photodissociation spectrum, and are heavily overlapped with protein mode at above freezing infrared spectrum so that the isolation is complicated. Our interpretations in this study are limited only to vinyl deformation and dioxygen vibrational mode. Thus, uncertainties of photoperturbed bands remain because of a lack of information on the assignment of porphyrin skeletal mode and axial imidazole modes. Further studies, involving the complete assignment of infrared active porphyrin mode and axial histidylimidazole mode will provide a more definitive information of the dioxygen stretching bands in the biological heme proteins.
LIST OF REFERENCES


CHAPTER IV
Infrared Assignment of Porphyrin Skeletal and Substituents
Vibrational Modes in Respiratory Heme Proteins
at Low Temperature

4.1 Abstract

We have observed infrared active in-plane and out-of-plane porphyrin fundamental modes ($E_u$ and $A_{2u}$) as well as porphyrin side chain modes in respiratory heme proteins by low temperature FT-IR spectroscopy. The high frequency (>1000 cm$^{-1}$) spectra of HbCO and MbCO show similar spectral perturbations of infrared active $E_u$ modes and vinyl modes ($CH_2$ scissor and $CH_2$ rock), indicating that Hb and Mb have nearly identical polarization over the entire porphyrin $\pi$ electron system including vinyl peripheral substituent attached at 2,4-positions of porphyrin ring. Myoglobin CO complexes from different species show almost identical spectral perturbations. This result suggests that photoperturbation occurs only in active heme site of Mb not in the protein matrix. Unlike infrared spectra of HbCO and MbCO, CcO-CO shows different vinyl frequencies, most likely due to either the trans disposition of the vinyl group.
with respect to the formyl at the 8 position or the farnesyl side chain at 2 position in heme A. The less overlapped low frequency (<1000 cm\(^{-1}\)) spectrum of partially dehydrated Hb clearly indicates the methine hydrogen out-of-plane vibrational mode. We also observe the strong enhanced at around ~1106 cm\(^{-1}\), which is tentatively assigned as an axial imidazole band. Although the entire band assignment is far from the complete, the results obtained by the novel approach of low temperature photoperturbation FT-IR spectroscopy can be used as a local molecular probe for dynamic and structural studies of the heme core, including side chains, within the biological protein matrix.

4.2 Introduction

Hemoglobin (Hb) and myoglobin (Mb) that have heme \(b\) as a heme core are simple respiratory proteins. Hb binds oxygen in a lung and carries it to tissues where the oxygen is stored as \(\text{MbO}_2\) and released for respiration (Antonini and Bronori, 1971). However, the terminal respiratory heme protein of cytochrome c oxidase (CcO) has two iron porphyrin (heme a and a\(_3\)) and two copper sites (Cu\(_A\) and Cu\(_B\)) (Babcock and Wikstrom, 1992; Chan and Li, 1990; Blair et al. 1983; Wikstrom et al. 1981). The heme a and Cu\(_A\) participate in electron transfer from cytochrome c to the binuclear site, which consists of heme a\(_3\) and Cu\(_B\). Dioxygen is bound and reduced to water at the binuclear site.
Many physicochemical techniques have proven the protein quaternary and tertiary structural changes in the simple respiratory heme proteins of Hb and Mb upon the oxygen binding (Bare et al.1975; Fiamingo and Alben, 1985; Moh et al.1987; Norvell et al.1975; Baldwin, 1980; Phillips, 1980; Shaanan, 1983; Choi et al.1982) in order to apply these understandings to more complex biological molecules like cytochrome c oxidase. But the understanding of the dynamics and structural studies of the active heme site upon oxygen binding is far from the complete because of the incomplete understanding of porphyrin vibrational modes of respiratory heme proteins.

The relationships of vibrational modes to porphyrin structure have been made indirectly by detailed comparison of the vibrational spectra of hemes with those of simple and highly symmetric compounds. Abe and co-workers (Abe et al.1978) proposed the assignment of all in-plane vibrations based on isotope substitution and an analysis of combination modes of Ni(OEP). These are in good agreement with their normal coordinate analysis. Alternative assignments have been proposed by several workers based on RR spectra with a more close resonant excitation of Ni(OEP) (Li et al.1990) and IR spectra of matrix-isolated complexes of divalent OEP (Kincaid et al.1983).

An application of porphyrin and side chain modes observed in Ni(OEP) to biological heme proteins has been done by RR spectroscopy (Abe et al.1978; Li et al.1990) where $A_{1g}$, $A_{2g}$, $B_{1g}$ and $B_{2g}$ are enhanced in $D_{4h}$ symmetry.
However, the remaining IR active degenerated $E_u$ modes in $D_{4h}$ symmetry system have never been characterized in biological heme proteins by infrared spectroscopy. The assignment of infrared active porphyrin and side chain modes in the heme proteins may be useful as sensitive monitors of porphyrin conformation as well as the interactions of the side chains with proteins residues.

In the present work, we have undertaken to assign the in-plane and out-of-plane porphyrin skeletal modes and porphyrin peripheral modes of heme $b$ (Hb and Mb) and heme $a$ (CcO) with the aid of frequency correlations to the recently assigned modes of Ni(OEP) by Spiro and co-workers (Li et al.1990) using low temperature infrared spectroscopy. Deuterium and pH effects on the porphyrin skeletal and porphyrin side chains have been investigated. We also have investigated the distal histidine effects on the porphyrin skeletal and peripheral modes by use of a site-directed mutant Mb(HisE7>Phe). We show that the frequencies of porphyrin skeletal and peripheral modes in heme $a$ (CcO) are different from in heme $b$ (Mb and Hb), and that deuterium, pH, and the mutant cause the global conformational effects.

4.3 Methods

*Preparation of human Hb:* Fresh human blood sample was collected in clinical Vacutainer® containing sodium heparin. Red cells were washed three times with 0.9% saline and hemolyzed with 1 volume of $H_2O$ and 0.5 volume of toluene.
After centrifugation (20,000 rpm), hemolysate were passed through Sephadex G-25 column equilibrated with 0.1 M NaCl to remove the strong infrared absorption of diphosphoglycerate. The resulting solution was concentrated by pressure ultrafiltration (Amicon:X-50 membrane) to a heme concentration of about 20 mM and then exposed to $^{12}$CO gas (Matheson C. P. 99.5 %), or $^{13}$CO (enriched to 90% $^{13}$C) purchased from Monsanto Research Corp., Mound Facility (Miamisburg OH), or O$_2$ gas (Bare et al.1975; Moh et al.1987).

HbCO solution prepared above was diluted with 25 mM deuterated Tris-HCl buffer, pH 7.1 (pH meter reading) and concentrated by pressure ultrafiltration cycles of a 10-fold volume excess for 4 times over 72 hr. The resulting HbCO was equilibrated with CO gas. The replacement of H$_2$O by D$_2$O was estimated to be greater than 90% since the near-infrared absorption of water at 1430 nm was not observed in this preparation (Bare et al.1975; Moh et al.1987).

**Preparation of sperm whale Mb:** About 15 mM of oxidized sperm whale Mb (Sigma) was prepared in 0.1 M Tris-HCl, pH 7.3. Mb solution was placed on ice for 30 min and spun for 30 min at 35000 g with SS-34 rotor. The supernatant was collected. The Mb was made MbCO by passing CO gas over the solution with gentle stirring for 1 hr, and then deaerated 1 M Na$_2$S$_2$O$_4$, prepared in 1 M Tris-HCl, pH 8.5, was added to a 3-fold molar excess. The pH of MbCO (pH = 6.95) was measured by inserting electrode into the solution that was exposed to air.
To maintain the constant pH even after the addition of Na₂S₂O₄, MbCO was diluted with desired pH buffers saturated with CO (Tris-HCl pH 8.5, Bis-Tris pH 7.0, MES pH 6.0) and concentrated for at least 3 times, and finally resaturated with CO gas.

Deuterium-exchanged sperm whale metMb was prepared by 5 times dilution and reconcentration cycles of a 10-fold volume excess with 50 mM deuterated Tris-HCl, pH 8.1 (pH meter reading) over 70 hr. The protein solution was reduced with a small amount of solid Na₂S₂O₄ under CO gas, and stirred for 1 hr. The reduced MbCO was washed with deuterated Tris-HCl buffer and reconcentrated for at least 3 times to maintain the constant pD, and finally resaturated with CO gas.

Preparation of bovine heart Mb: Bovine heart Mb was prepared as oxy form and equilibrated with CO gas (Wittenberg and Wittenberg, 1981).

Preparation of horse heart Mb: About 8 mM of oxidized horse heart myoglobin (Sigma) was prepared in deaerated H₂O, placed on ice for 30 min, and spun for 30 min at 35000 g with SS-34 rotor. The final volume of Mb was 1.5 mL. The Mb was made MbCO by passing CO gas over the Mb solution (1.5 mL) with gentle stirring for 1 hr, and then 800 µM cytochrome c and 200 mM ascorbate (both are prepared in 50 mM Tris-HCl, pH 7.4) were added about 1.5 mL respectively (Fiamingo and Alben, 1985).
Preparation of a site-directed mutant MbCO (HisE7>Phe): Sperm whale mutant Mb (HisE7>Phe) was kindly donated by Dr. Takashi Yonetani from University of Pennsylvania, and converted to MbCO in 50 mM Tris-HCl, pH 8.5 by the methods as described above.

Preparation of Cytochrome c Oxidase: Beef heart cytochrome c oxidase was isolated by the method of Hartzell and Beinert (Hartzell and Beinert, 1974). Following addition of 1 equivalent of ascorbate and 0.05 equivalent of cytochrome c, the enzyme samples were thoroughly degassed on a vacuum apparatus and subsequently exposed to 1 atmosphere of CO gas at 4°C overnight. This preparation was kindly donated by Drs. Lian-Ping Pan and Sunney I. Chan from California Institute of Technology.

Spectroscopy: The enzyme preparations were stored in liquid nitrogen until used. Visible spectra of respiratory proteins at 4°C were obtained with a Cary model 17DX spectrometer. The protein solution was prepared between a pair of BaF$_2$ windows or CaF$_2$ windows with 24.8 or 7 micrometers spacer or HbCO sample prepared on a single KRS-5 window. All infrared spectra were recorded at 0.5 cm$^{-1}$ resolution with a Mattson Sirius 100 Fourier transform infrared interferometer which was fitted with a liquid nitrogen cooled photovoltaic (HgCd)Te detector for measurements below 1800 cm$^{-1}$ or InSb detector for measurements above 1800 cm$^{-1}$, or liquid helium cooled Zn:Ge detector for measurements below 1000 cm$^{-1}$ down to 500 cm$^{-1}$ (collected by Dr. J. O. Alben).
The detectors were fitted with appropriate low-pass filters. The spectrophotometer was calibrated with low partial pressure of carbon monoxide gas. Low temperature were obtained by use of a Lake shore Cryotronics closed cycle helium refrigerator, Model LTS-21-D70C. Cryostat cell temperature was measured by use of a Lake Shore Cryotronics digital thermometer, model DRC-70, with a calibrated silicon diode probe. Cryostat cube windows were fitted with appropriated IR windows of CaF$_2$, KCl or KBr. Photolysis made use of a 500 W tungsten lamp focused through a slide projector and passed through water filter (2 cm) to remove infrared radiation beyond 1.2 micrometers.

4.4 Results

Figure 16 shows the photoperturbation FT-IR spectra of a partially dehydrated film of human HbCO collected by Dr. J. O. Alben at 10 K. This spectrum with a less overlapped region below 1000 cm$^{-1}$ clearly shows IR bands, which are assignable to porphyrin skeletal in-plane $E_u$ and out-of-plane $A_{2u}$ modes. Figure 17 shows photoperturbation FT-IR spectra of HbCO prepared in H$_2$O and in D$_2$O and Hb$^{13}$CO. Sperm whale MbCO prepared in H$_2$O and in D$_2$O, and a site-directed mutant MbCO (HisE7>Phe) are presented in the photoperturbation spectra in Figure 18. The photoperturbation spectra of MbCO prepared at a different pH and prepared from bovine heart, horse heart, and sperm whale MbCO are shown in Figures 19 and 20, respectively. The structural photoperturbations of MbCO from different species are nearly identical indicating
that photoperturbation we observed in mid-ir region occurs only in active heme site of Mb, not in the protein matrix. Figure 21 shows photoperturbation spectra of HbO₂, and bovine heart and sperm whale MbO₂. Photoperturbation spectrum of CcO-CO is shown in Figure 22. Especially, the measurements of "forward" and "reverse" directions of moving mirror, and their difference are shown in Figure 22, so that the instrumental artifacts are clearly identified in the difference spectrum. The different photoperturbation patterns among HbCO, MbCO and CcO-CO are shown in Figure 23. Photoperturbation absorbance double-difference spectra in various conditions are shown in Figures 24, 25, and 26.

The observed IR bands assignable to porphyrin skeletal in-plane and out-of-plane modes in addition to tentatively assigned imidazole mode are given in Table 12. The numbering of the modes are given according to the scheme used by Abe et al. (Abe et al.1978) for Ni(OEP), however the assignment of porphyrin fundamental modes are given by retaining the recent scheme proposed Li et al. (Li et al.1990). Assignments for IR vinyl modes in Hb, Mb and CcO are given in Table 13 following the scheme used by Choi et al. (Choi et al.1982) for Ni(PP). The observed IR bands related to formyl modes of CcO-CO are given in Table 14. There are several bands which have not yet been assigned in our photoperturbation spectra.
Figure 16. Photoperturbation (light/dark) FT-IR spectrum of HbCO at 10 K was prepared on a single KRS-5 window, and collected by Dr. Alben. The spectrum was collected with liquid helium cooled Zn:Ge detector and a CsI beamsplitter. The strong negative peak at 666 cm⁻¹, denoted by ★, is the bending mode of CO₂ gas which is present in the infrared radiation beam path.
Figure 17. Photoperturbation (light/dark) FT-IR spectra of HbCO prepared in H$_2$O, and in D$_2$O and Hb$^{13}$CO at 10 K. The KBr beamsplitter absorption band at 1267 cm$^{-1}$ is completely uncompensated. Spectrum was normalized for the absorption of 1236 cm$^{-1}$ peak.
Figure 18. Photoperturbation (light/dark) FT-IR spectra of sperm whale MbCO prepared in H₂O, in D₂O, a site-directed mutant MBABO (HisE7>Phe). The KBr beamsplitter absorption band at 1267 cm⁻¹ is completely uncompensated. Spectrum was normalized for the absorption of 1239 cm⁻¹ peak.
Figure 19. Photoperurbation (light/dark) FT-IR spectra of sperm whale MbCO at different pH: (A) pH 8.5, 50 mM Tris-HCl; (B) pH 7.0, 50 mM Bis-Tris; (C) pH 6.0, 30 mM MES. The KBr beamsplitter absorption band at 1267 cm⁻¹ is completely uncompensated. Spectrum was normalized for the absorption of 1239 cm⁻¹ peak.
Figure 20. Photoperturbation (light/dark) FT-IR spectra of bovine heart MbCO, horse heart MbCO, and sperm whale MbCO at 10 K. The KBr beamsplitter absorption band at 1267 cm\(^{-1}\) is completely uncompensated. Spectrum was normalized for the absorption of 1239 cm\(^{-1}\) peak.
Figure 21. Photoperturbation (light/dark) FT-IR spectra of HbO$_2$, bovine heart MbO$_2$, and sperm whale MbO$_2$. The peaks at around 1390 cm$^{-1}$ with 0.5 cm$^{-1}$ bandwidth in HbO$_2$ are water vapor absorptions. Spectrum was normalized for the absorption of an apparent dioxygen band at 1106 cm$^{-1}$ (HbO$_2$) and 1104 cm$^{-1}$ (MbO$_2$).
Figure 22. (A). Photoperturbation (light/dark) FT-IR spectra of heme $a_1$ of CcO-CO at 13 K, collected in the forward direction of moving mirror. (B). Photoperturbation (light/dark) FT-IR spectra of heme $a_1$ of CcO-CO at 13 K, measured in the "forward" and "reverse" directions of moving mirror, and their difference.
Figure 22 (continued)

![Absorbance vs. Wavenumber Graph](B)

- Forward
- Reverse
- Forward - Reverse
Figure 23. Photoperturbation (light/dark) FT-IR spectra of protoporphyrin IX in HbCO and bovine heart MbCO, and heme A in CcO-CO: (A) low frequency region (920 - 1350 cm⁻¹); (B) high frequency region (1300 - 1720 cm⁻¹)
Figure 23 (continued)
Figure 24. Photoperturbation absorbance double-difference spectra of Hb and Mb in H$_2$O and D$_2$O solution used in Figures 17 and 18 at 10 K: (A) (L/D)HbCO in H$_2$O - (L/D)HbCO in D$_2$O; (B) (L/D)MbCO in H$_2$O - (L/D)MbCO in D$_2$O, Sperm Whale.
Figure 25. Photoperturbation absorbance double-difference spectra of Mb at different pH at 10 K used in Figure 19: (A) (L/D)MbCO: pH 8.5 - pH 7.0; (B) (L/D)MbCO: pH 8.5 - pH 6.0.
Figure 25 (continued)
Figure 26. Photoperturbation absorbance double-difference spectra at 10 K used in Figures 17 and 18: (A) (L/D)SW-MbCO - (L/D)MbCO (HisE7>Phe); (B) (L/D)Hb^{12}CO - (L/D)Hb^{13}CO.
Figure 26 (continued)
Figure 27. Difference spectra for Lorentzian bands resulting from bandwidth and frequency changes. (a) Bandwidth change only: $\Gamma_A = 12 \text{ cm}^{-1}$, $\Gamma_B = 8 \text{ cm}^{-1}$; $\Delta v_0 = 0$. (b) Frequency shift only: $\Gamma_A = \Gamma_B = 10 \text{ cm}^{-1}$; $\Delta v_0 = 0.5 \text{ cm}^{-1}$. (c) Both bandwidth and frequency changes: $\Gamma_A = 12 \text{ cm}^{-1}$, $\Gamma_B = 8 \text{ cm}^{-1}$; $\Delta v_0 = 0.5 \text{ cm}^{-1}$ (From Laane, *J. Chem. Phys.* (1981) 75:2539-2545)
Table 12. Infrared frequencies of HbCO, MbCO and CcO-CO assignable to porphyrin fundamental $E_u$ and $A_{2u}$ modes, and imidazole modes.

<table>
<thead>
<tr>
<th>Modes*</th>
<th>HbCO</th>
<th>MbCO</th>
<th>CcO-CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_u(v_{so})$</td>
<td>1399↓</td>
<td>1397↓</td>
<td>1402↓ ?</td>
</tr>
<tr>
<td>$E_u(v_{se})$</td>
<td>1378↓</td>
<td>1373↓</td>
<td>1368*</td>
</tr>
<tr>
<td>$E_u(v_{so})$</td>
<td>1236↓</td>
<td>1239↓</td>
<td>1236↓</td>
</tr>
<tr>
<td>$E_u(v_{se})$</td>
<td>1170↓ (or 1150↓)</td>
<td>1163↓ (or 1148↑)</td>
<td>1167* (or $v(C_\equiv CH)$)</td>
</tr>
<tr>
<td>$E_u(v_{so})$</td>
<td>1115↑ (or 1133↓)</td>
<td>1110,1115↑ (or 1121↓)</td>
<td>1119↑ (or 1138↓)</td>
</tr>
<tr>
<td>ImH</td>
<td>1107↓</td>
<td>1106↓</td>
<td>-</td>
</tr>
<tr>
<td>$E_u(v_{se})$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$E_u(v_{so})$</td>
<td>933↑ (or 956↓)</td>
<td>933↑ (or 955↓)</td>
<td>963↓</td>
</tr>
<tr>
<td>$E_u(v_{se})$</td>
<td>715*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$E_u(v_{so})$</td>
<td>598*</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$A_{2u}(\gamma_{C\equiv H})$</td>
<td>830*</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>


* or CH$_3$ in-phase deformation

* observed in KRS-5 single window photoperturbation spectrum of partially dehydrated HbCO.

* splitting. * zero-crossing frequency

↑: positive peak, ↓: negative peak

?: uncertain, -: not observed
Table 13. Infrared frequencies of HbCO, MbCO and CcO-CO assignable to vinyl modes.

<table>
<thead>
<tr>
<th>Modes</th>
<th>HbCO</th>
<th>MbCO</th>
<th>CcO-CO</th>
</tr>
</thead>
<tbody>
<tr>
<td>vC=O</td>
<td>-</td>
<td>-</td>
<td>1630</td>
</tr>
<tr>
<td>δ_2=CH₂ (CH₂ scissor)</td>
<td>IR inactive</td>
<td>IR inactive</td>
<td>IR inactive</td>
</tr>
<tr>
<td>δ_2=CH½ (CH½ scissor)</td>
<td>1340</td>
<td>1337</td>
<td>1337 (or 1368)?</td>
</tr>
<tr>
<td>δCH= (CH rock)</td>
<td>-</td>
<td>-</td>
<td>1304</td>
</tr>
<tr>
<td>δ_2=CH₂ (CH₂ rock)</td>
<td>1079 &amp; 1089</td>
<td>1077 &amp; 1093</td>
<td>1082</td>
</tr>
<tr>
<td>γCH= (CH wag)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>γ₂CH= (CH₂ wag)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* zero-crossing frequencies in our photoperturbation spectra


* in-phase and out-of-phase combination

? uncertain

- not observed
Table 14. Formyl Modes of Cytochrome c Oxidase

<table>
<thead>
<tr>
<th>Modes</th>
<th>Benzaldehyde*</th>
<th>(ImH)₃Heme a²⁺</th>
<th>Ni(2-FDP)⁺⁺</th>
<th>Ni(4-FDP)⁺⁺</th>
<th>Ni(2,4-FDP)⁺⁺</th>
<th>CcO-CO*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\nu$(C=O)</td>
<td>1709/1694¹</td>
<td>1656</td>
<td>1661/1667</td>
<td>1666/1666</td>
<td>1674,1665⁶/1663</td>
<td>1662/</td>
</tr>
<tr>
<td>$\nu$(Cb-CHO)</td>
<td>1203/1206</td>
<td>1220</td>
<td>/1174 (v43)</td>
<td>/1174 (v43)</td>
<td>1226 /1161 (v427) (v437)</td>
<td>1167/ (v437)</td>
</tr>
<tr>
<td>$\delta$(Cb-CO-H)</td>
<td>1388/1395</td>
<td>/1390</td>
<td>1283/</td>
<td>1265/</td>
<td>1395,1282⁴/</td>
<td>1285/</td>
</tr>
</tbody>
</table>

* assignment of benzaldehyde modes from Zwarich et al. (J. Mol. Spectrosc. (1971) 38:336-357)
IR frequency collected in this study

¹ RR frequency from Choi et al. (J. Am. Chem. Soc. (1983) 105:3692-3707)

² from Choi et al. (J. Am. Chem. Soc. (1983) 105:3692-3707)

³ from Willems et al. (J. Phys. chem. (1985) 89:234-239)

⁴ out-of-phase and in-phase modes

⁶ in this study, zero-crossing frequency in the frequency shifted band

¹¹ IR frequency/RR frequency
The particular difficulty encountered in high frequency region of photoperturbation spectra concerns the water vapor absorptions that occur in infrared radiation beam path. The optical bench was flushed with highly dried compressed air, and half-wavenumber spectral resolution is used so that perturbations of the sample is clearly distinguished from the atmosphere water absorptions. The instrumental non-reproduciblity of beamsplitter absorption at 1267 cm$^{-1}$ was also measured and characterized (Park and Alben, 1993).

4.5 Discussion

4.5.1 Photoperturbation absorbance difference spectroscopy

In comparison between two closely related biological molecules small structural changes that may have biological significance can give rise to small frequency differences in the difference spectrum. Inversely small frequency differences observed in difference spectrum would be a reliable indicator of structural differences between the biological molecules. The limitation for the detection of frequency differences between two biological samples has primarily been due to experimental irreproducibility and low spectral resolution. FT-IR spectroscopy used in this study has an ability to collect full-double sided interferogram with 0.5 cm$^{-1}$ spectral resolution. Especially, the mean deviation of observed frequency of this instrument is 0.024 cm$^{-1}$ compared to literature values (Cole, 1977).
Difference spectroscopy has been discussed in detail by Laane et al. (Laane and Kiefer, 1980; Laane, 1981). Three possible band shapes can be observed in the difference spectrum based on frequency shift only, bandwidth change only, and both frequency and bandwidth changes (Figure 27). All of these complicated band shapes are expected to be observed in the mid-IR photoperturbation spectrum. In this study, we would like to report negative or positive peak frequencies instead of zero-crossing frequencies in the (light/dark) photodissociation absorbance difference spectrum, because the precise knowledge of band shapes is unknown in order to evaluate frequency shift (Alben and Fiamingo, 1984; Laane and Kiefer, 1980; Laane, 1981). However, zero-crossing frequencies are reported here in the obvious frequency-shifted bands as observed in vinyl and formyl modes.

4.5.2 In-plane \( E_u \) modes and out-of-plane \( A_{2u} \) porphyrin modes

4.5.2.1 In-plane \( E_u \) modes

Since Abe and co-workers (Abe et al. 1978) observed the normal mode analysis of Ni(OEP) as a \( D_{4h} \) symmetry, the assignment of in-plane modes of porphyrin complexes has been studied extensively (Kitagawa and Ozaki, 1987; Kitagawa et al. 1978; Choi et al. 1983; Li et al. 1990; Li et al. 1990; Willems and Bocian, 1984; Willems and Bocian, 1985). If the substituent groups of metalloporphyrins are treated as point masses, the 37-atom model has 71 in-plane modes (2N-3), which are classified as follows (Abe et al. 1978; Li et al. 1990):
Among the in-plane modes, the totally symmetric vibrations, $A_{1u}$, and the totally non symmetric $A_{2g}$, $B_{1g}$, and $B_{2g}$ modes are enhanced by the intense $B$, and $Q_0$ and $Q_1$ (at about 550 and 500 nm) Raman excitation (Li et al.1990). The remaining degenerate $E_u$ modes are infrared active. However, the splitting of in-plane vibrations into two different frequencies with the lifting of $x,y$ degeneracy has been suggested by Woodruff et al. (Woodruff et al.1982). Spiro and co-workers (Choi et al.1983) presented the evidence of splitting that the $v_{38}$ ($E_u$) mode in $D_{4h}$ symmetry of heme A porphyrin (Pa) was split into two modes observed at 1544 and 1512 cm$^{-1}$ by IR spectroscopy, possibly by lowering the symmetry to $D_{2h}$ or to $C_2$.

By virtue of peripheral asymmetric disposition, the presence of the vinyl substitutions of 2 and 4 positions in a protoporphyrin IX lowered the symmetry of the chromophore so that some of infrared active in-plane $E_u$ modes ($v_{37}$, $v_{38}$, $v_{44}$, $v_{45}$) became Raman active (Choi et al.1983; Choi et al.1982; Babcock, 1988). More detailed studies by Willems and Bocian (Willems and Bocian, 1984; Willems and Bocian, 1985) pointed out that these $E_u$ modes are sensitive to the effects of symmetry lowering by peripheral substituents not only in intensity but also in observed frequencies. In contrast to the presence of infrared modes in the RR spectrum, no Raman active modes have been reported in the infrared spectrum (Choi et al.1983; Choi et al.1982; Willems and Bocian, 1984; Willems
and Bocian, 1985).

Historically, the initial normal coordinate analysis of Ni(OEP) by Abe et al. (Abe et al.1978) has been widely accepted in interpreting hemes and model compounds such as nickel(II) acetyl and formyl deuteroporphyrins (Choi et al.1982; Choi et al.1983; Willems and Bocian, 1984; Willems and Bocian, 1985), and RR studies of biological heme proteins (Choi et al.1983; Centeno and Babcock, 1991; Babcock, 1988; Argade et al.1986). Alternative assignments have been proposed for Eu porphyrin macrocycle modes of Ni(OEP) by Kincaid et al. (Kincaid et al.1983). They demonstrated the metal sensitive Eu modes in matrix-isolated metal complexes, suggesting that certain bands attributed to Eu ($v_{30}, v_{40}, v_{41}, v_{44}$) core modes by Abe and co-workers actually correspond to internal modes of the $C_\beta$ ethyl side chains.

Recently, Spiro and co-workers (Li et al.1990) examined the vibrational spectra of Ni(OEP) including the ethyl substituent involvement, and found the ethyl C-C stretching and C-H bending modes in their spectrum. They also reexamined the normal coordinate analysis of Ni(OEP) with inclusion of methylene hydrogen atoms, pointing out that the original Abe's study of Ni(OEP) was limited to excitation at 488.0 nm and 514.5 nm, neither of which is fully resonant with any of the $\pi-\pi^*$ electronic transition. There are little differences for Raman active modes, but there are many differences present in infrared active
modes between these two works. Their $E_u$ mode assignments were taken from the studies of Kincaid et al. (Kincaid et al. 1983) except that $v_{41}$ and $v_{37}$ porphyrin skeletal mode assigned by Kincaid and co-workers correspond to the deformation of CH$_2$ and $v_{38}$ respectively. Under the same equivocal $E_u$ mode assignments, we have chosen to retain the recent scheme of Spiro and co-workers obtained from Ni(OEP) studies (Li et al. 1990) in order to assign the infrared active heme skeletal modes in biological heme proteins (Table 12).

$v_{46}$ [$\delta$(Pyr def)$_{pyr}$] and $v_{47}$ [v(Pyr breathing)]: The less overlapped low frequency region of HbCO collected on a single KRS-5 window clearly allows us to assign $v_{46}$ and $v_{47}$ modes at 598 and 715 cm$^{-1}$ respectively based on the previous assignment of Ni(OEP) at 605 and 726 cm$^{-1}$ (Li et al. 1990; Kincaid et al. 1983; Choi et al. 1982).

$v_{46}$ [$\delta$(Pyr def)$_{sym}$]: The consistent assignment of $v_{46}$ has been made in Ni(OEP) and Ni(PP) at 927 and 963 cm$^{-1}$ respectively (Abe et al. 1978; Li et al. 1990; Kincaid et al. 1983; Choi et al. 1982). However, a clear evidence of doubling of Ni(2-AcDP) and Ni(2-FDP) has been observed by Willems and Bocian (Willems and Bocian, 1984), where the frequency separations between the splitted bands were about 32 cm$^{-1}$ and 8 cm$^{-1}$ respectively. They also noticed that $v_{46}$ is quite sensitive to 2,4 substituent groups, so that $v_{46}$ of Ni(2-FDP) and Ni(4-DFP) was observed near 905 cm$^{-1}$ (Willems and Bocian, 1985). Matrix isolation studies by
Kincaid et al. (Kincaid et al.1983) suggested that $v_{46}$ is sensitive not only to substituent but also to metal.

The $v_{46}$ can be assigned either at 933 cm$^{-1}$ or at 956 cm$^{-1}$ in this study. However, our photoperturbation spectrum does not seem to have the definitive splitting of the $v_{46}$ porphyrin macrocycle mode because of photopertrubed band shape: positive peak (933 cm$^{-1}$), negative peak (956 cm$^{-1}$).

$v_{45}$ [$v(C_3-Y)_{asym}$]: The reported frequency of $v_{45}$ is the region of vinyl CH wag mode (Choi et al.1983) so that the degeneracy is expected. Like $v_{46}$ mode, the vibration of $v_{45}$ is susceptible to 2,4 substituent groups as well as metal (Kincaid et al.1983; Willems and Bocian, 1984), and Raman activation of this mode has been observed in MbF (991 cm$^{-1}$) and HbF (999 cm$^{-1}$) (Choi et al.1982), possibly due to the symmetry lowering. No distinctive photoperturbation was observed in our spectra so that the assignment of this mode is postponed.

$v_{44}$ [$v(Pyr\text{ half-ring})_{asym}$]: The original work of $v_{44}$ porphyrin skeletal mode by Abe et al. (Abe et al.1978) was identified at 1113 cm$^{-1}$ in Ni(OEP) and confirmed in model porphyrin and biological heme proteins studies (Choi et al.1982; Choi et al.1983; Choi et al.1982; Willems and Bocian, 1984; Willems and Bocian, 1985). But recent observation by Kincaid et al. (Kincaid et al.1983) and Li et al. (Li et al.1990) showed different assignments. They suggested the 1133 cm$^{-1}$ band as a $v_{44}$ mode, but did not rule out the possibility of the assignment at 1113 cm$^{-1}$ as
a core mode proposed by Abe et al. (Abe et al. 1978).

We wish to assign 1115 cm$^{-1}$ (HbCO), 1110 and 1115 cm$^{-1}$ (MbCO), and 1119 cm$^{-1}$ (CcO-CO) peaks as a $\nu_{44}$ mode, or an alternative assignment will be 1133 cm$^{-1}$ (HbCO), 1121 cm$^{-1}$ (MbCO), and 1138 cm$^{-1}$ (CcO-CO) even though there is a frequency difference between HbCO and MbCO. However, the uncertainties are still remains in the assignment of $\nu_{44}$ in Mb because of splitting and deuterium sensitivity of $\nu_{44}$ core mode in this study (Figure 18). Also this region seems to contain the vibrational modes of axial imidazole (Salama and Spiro, 1978; Walters and Spiro, 1983; Hodgson et al. 1980; Hodgson et al. 1980).

$\nu_{43} [\nu(C_{v}-Y)]_{nm}$: There are general agreements in the assignment of $\nu_{43}$ porphyrin core mode in Ni(OEP) (Abe et al. 1978; Li et al. 1990; Kincaid et al. 1983; Choi et al. 1982; Willems and Bocian, 1984) at around 1150 cm$^{-1}$. This mode is very sensitive to the effects of degeneracy lifting substituents, which was demonstrated by Willems and Bocian (Willems and Bocian, 1984; Willems and Bocian, 1985). In our photoperturbation spectra of Hb and Mb, 1170 cm$^{-1}$ (HbCO) and 1168 cm$^{-1}$ (MbCO) bands appear to be the $\nu_{43}$ porphyrin core mode, but the vibrational bands at 1150 (HbCO) and 1148 (MbCO) cm$^{-1}$ may be an alternative assignment.

Unlike to Hb and Mb, the $\nu_{43}$ mode in CcO-CO has a significant contribution from $\nu(C_{v}-CHO)$ mode of formyl group (Willems and Bocian, 1985; Willems and Bocian, 1984). Therefore, the 1167 cm$^{-1}$ in CcO-CO may be ascribed to the degenerated band with $\nu_{43}$ and $\nu(C_{v}-CHO)$ of formyl, or the assignment
of \nu(C=CHO).

\nu_{42} [\delta(C=\text{X})]: The assignment of \nu_{42} is ambiguous. Several workers (Abe et al.1978; Choi et al.1982; Willems and Bocian, 1984) reported this mode at 1268 cm\(^{-1}\). But Kincaid and co-workers (Kincaid et al.1983) reported other frequency (1231 cm\(^{-1}\)) for this mode based on disappearance upon deuteration, nitrogen isotope shift, and its metal sensitivity. The intensity of \nu_{42} porphyrin skeletal mode was pretty strong (Kincaid et al.1983; Abe et al.1978; Choi et al.1982; Willems and Bocian, 1984). We like to assign this porphyrin core mode at 1236 cm\(^{-1}\) (HbCO), at 1239 cm\(^{-1}\) (MbCO), and 1236 cm\(^{-1}\) (CcO-CO) based on strong intensification.

Low temperature photoperturbation spectra of HbO\(_2\) and MbO\(_2\) (Figure 21), however, shows a significant decrease of intensity in \nu_{42} band, suggesting this mode as a characteristic peak of porphyrin-Fe-CO complex instead of \nu_{42} porphyrin skeletal mode.

\nu_{41} [\nu(\text{Pyr half-ring})_{\text{sym}}]: There are several uncertainties in the assignment of \nu_{41} core porphyrin core mode. Abe et al. (Abe et al.1978) observed this mode at 1389 and 1377 cm\(^{-1}\) in Ni(OEP) and Ni(PP), but matrix-isolated metal studies by Kincaid et al. (Kincaid et al.1983) assigned 1275 cm\(^{-1}\) band to a \nu_{41} in Ni(OEP). Furthermore, recent studies by Spiro and co-workers (Li et al.1990) presented that the 1275 cm\(^{-1}\) band was a CH\(_2\) twist mode instead of core mode, and \nu_{41}
mode could not be observed in Ni(OEP) analysis. According to the normal coordinate analysis by Spiro and co-workers (Li et al. 1990), the expected value was 1346 cm\(^{-1}\), which was about 12 cm\(^{-1}\) higher than that of Abe's calculation (Abe et al. 1978). Analysis of Ni(FDP) also showed (Willems and Bocian, 1984; Willems and Bocian, 1985) that there were doubling effects on this mode, at 1379 and 1360 cm\(^{-1}\).

Our present data do not appear to show the obvious photoperturbation in the expected spectral region, but the small perturbation at 1378 cm\(^{-1}\) (HbCO), 1373 cm\(^{-1}\) (MbCO), and 1368 cm\(^{-1}\) (CcO-CO) may be assigned to \(v_{41}\) mode. However, the assignment of \(v_{41}\) seems to be mixed with a \(CH_3\) in-phase deformation, which will be explained in detail in another section.

\(v_{40}\) [\(v_{\text{Pyr quater-ring}}\)]: There are differences in the assignment of this band between Kincaid and Abe (Kincaid et al. 1983; Abe et al. 1978). Kincaid and co-workers (Kincaid et al. 1983) described the 1396 cm\(^{-1}\) peak as a \(v_{40}\) and the 1443 cm\(^{-1}\) previously assigned by Abe et al., was explained as ethyl \(CH_2\) scissor mode in Ni(OEP). Both of their interpretations were based on deuterium insensitivity. We wish to assign \(v_{40}\) at 1399 cm\(^{-1}\) (HbCO), at 1397 cm\(^{-1}\) (MbCO), and 1402 cm\(^{-1}\) (CcO-CO), because there are no perturbation bands observed at 1440 cm\(^{-1}\) region in HbCO and MbCO.
$v_{39}, v_{38}$ and $v_{37}$: The $v_{39}$ core mode has a very weak intensity, and the assignment of $v_{38}$ and $v_{37}$ seems to be complicated because of disagreement in the assignment (Kincaid et al. 1983; Li et al. 1990; Choi et al. 1982; Abe et al. 1978). Therefore, we like to retain the assignment of these modes.

4.5.2.2 Out-of-plane $A_{2u}$ modes

We have extended our spectral region down to 500 cm$^{-1}$ using liquid helium cooled Zn:Ge detector to observe the out-of-plane modes of HbCO (Figure 16). These modes are expected to observe at low frequencies below 1000 cm$^{-1}$. There are 34 out-of-plane vibrations (N-3) are possible, if the substituents of metalloporphyrin are treated as point masses in $D_{4h}$ point group (Choi and Spiro, 1983; Li et al. 1989):

$$
\Gamma_{\text{out-of-plane}} = 3A_{1u} + 6A_{2u} + 5B_{1u} + 4B_{2u} + 8E_g \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots 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central metal atom, ii) pyrrole tilting, translation, and swivel modes, iii) internal folding of the pyrrole rings, which are symmetric and antisymmetric with respect to the two-fold axis.

\(\gamma_{C_m}H\): The infrared active \(C_mH\) wags are expected to observe at 700-900 cm\(^{-1}\) in model porphyrin studies (Alben, 1978; Colthup et al. 1990). This mode involves hydrogen motions, and is far above the other out-of-plane modes. Kitagawa et al. (Kitagawa et al. 1978) have assigned a 843 cm\(^{-1}\) infrared band of Ni(OEP) to \(\gamma_{C_m}H\) of porphyrin \(A_{2u}\) skeletal mode, which shifts to 680 cm\(^{-1}\) upon meso deuteration. However, the 841 cm\(^{-1}\) RR band of \((\text{Im}H)_{2}\)PP was assigned to \(E_g\) \(\gamma_{C_m}H\) by Choi et al. (Choi and Spiro, 1983) and this out-of-plane transition could be provided by a porphyrin \(\pi (a_{2u}) \rightarrow Fe d_{2g} (a_{1g})\) charge transfer. They suggest (Choi and Spiro, 1983) that the \(A_{2u}\) and \(E_g\) of \(\gamma_{C_m}H\) are essentially degenerate. Spiro and Li (Spiro and Li, 1988) calculated out-of-plane vibrations for Ni(OMP) at 834.6 cm\(^{-1}\) and found the \(\gamma_{C_m}H\) of porphyrin \(A_{2u}\) mode in Ni(OEP) at 835 cm\(^{-1}\).

In our photoperturbation spectrum of HbCO collected in the single KRS-5 (Figure 16), the \(\gamma_{C_m}H\) mode is assigned at 830 cm\(^{-1}\) with a strong perturbation.

Other out-of-plane modes: The remaining modes, involving wagging of the methine bridges, of the metal atom, and translation and rotation of the pyrrole rings are expected to occur below 400 cm\(^{-1}\), so that the analysis was excluded in this study.
4.5.3 Vinyl modes \([CH=CH_2]\)

The vinyl modes as porphyrin peripheral substituents were studied extensively by Choi et al. (Choi et al.1982) via the frequency shifts observed upon vinyl deuteration at \(\alpha\) and \(\beta\) positions in nickel protoporphyrin IX dimethyl esters and its derivatives. The application of this assignment into RR spectra of biological heme proteins has been obtained unambiguously (Choi et al.1982; Choi et al.1983; Choi et al.1982; Argade et al.1986). However, contrary to RR studies, the application of vinyl assignment by Choi et al. (Choi et al.1982) into biological respiratory heme proteins has never been obtained by the infrared studies, which has a different selection rule. The vinyl group has 10 internal vibrational modes (Choi et al.1982). Among these, the CH and CH\(_2\) in- and out-of-phase stretching modes observed at ~3000 cm\(^{-1}\) and asymmetry CH wag at ~630 cm\(^{-1}\) will be excluded because of spectral limitation. The remaining six vinyl vibrational modes will be examined in this photoperturbation infrared studies (Table 13).

We do not observe the splitting of vinyl CH\(_2\) symmetric deformation mode (scissor) in biological heme proteins as suggested by Choi et al. (Choi et al.1982) in Ni(PP) studies, but we do observed the splitting of vinyl CH\(_2\) asymmetric deformation mode (rock) in Hb and Mb, which seems to be the uniqueness of infrared studies.

\(\nu(\text{C}=\text{C})\): We have observed vinyl stretching only in the low temperature photodissociation spectrum of CcO-CO, but not in that of HbCO and MbCO. The C=\(\text{C}\) stretching band is suggested to be infrared inactive or quite weak band
but Raman active when it has center of symmetry (Colthup et al.1990). This supports that unlike the absence of vinyl stretching mode in our spectrum of HbCO and MbCO, RR studies (Choi et al.1982) showed $\nu(\text{C}=\text{C})$ at 1618 cm$^{-1}$ for deoxyMb and 1622 cm$^{-1}$ for HbF. The observation of $\nu(\text{C}=\text{C})$ in CcO-CO, however, suggests the possible involvement of photoperturbation in farnesyl side chain which has three C=C bonds, or asymmetry in the trans disposition of vinyl substituent at 4 position with respect to the formyl group at 8 position or the farnesyl side chain at 2 position in heme A.

The infrared absorption spectra of farnesyl derivatives show the weak intensity of $\nu(\text{C}=\text{C})$ at around 1670-1650 cm$^{-1}$ depending on substituent group. However, the conjugation of double bond reduces the force constant of C=C, and subsequently lowers the frequency about 10-50 cm$^{-1}$ (Colthup et al.1990). Therefore the our assignment of $\nu(\text{C}=\text{C})$ at 1630 cm$^{-1}$ (zero-crossing frequency) seems to be acceptable (Figure 22). The observed $\nu(\text{C}=\text{C})$ of Pa at 1628 cm$^{-1}$ by IR, and of CcC at 1626 by RR (Choi et al.1983) also support our assignment.

$\delta_4(=\text{CH}_2)$: The initial assignment of CH$_2$ symmetric deformation (scissor) was reported by Spiro and co-workers in Ni(PP) at 1434 cm$^{-1}$ by RR and at 1345 cm$^{-1}$ by IR (Choi et al.1982). The large splitting of $\delta_4(=\text{CH}_2)$ as much as 100 cm$^{-1}$ was ascribed to the differential coupling with porphyrin skeletal modes. Infrared studies of PP and Pa (Choi et al.1983) showed only one $\delta_4(=\text{CH}_2)$ band at 1338 cm$^{-1}$. Therefore, the higher frequency $\delta_4(=\text{CH}_2)$ mode seems to be infrared
inactive. In our photoperturbation spectra, we observed the vinyl scissor mode of HbCO and MbCO at 1340 and 1337 cm\(^{-1}\) (zero-crossing frequencies) with a strong amplitude. However, the assignment of this mode in CcO-CO seems to be either 1337 cm\(^{-1}\) or 1368 cm\(^{-1}\) (zero-crossing frequency). In general, scissor mode is a quite strong in protoheme proteins (Spiro and Strekas, 1974; Adar and Erecinska, 1974). The assignment of 1337 cm\(^{-1}\) was based on frequency correlation, and that of 1368 cm\(^{-1}\) was based on intensity correlation. Alternatively, assignment of latter 1368 cm\(^{-1}\) band may be CH\(_3\) in-plane deformation.

\(\delta(CH=)\): No infrared active band of CH rock has been reported in Ni(PP), and Hb and Mb (Choi et al.1982; Choi et al.1982). However, the model porphyrin of PP and Pa showed the \(\delta(CH=)\) mode at 1301 cm\(^{-1}\) by IR studies (Choi et al.1983). In this study, we observed strongly enhanced CH rock mode at 1304 cm\(^{-1}\) (zero-crossing frequency) in CcO-CO but not in HbCO and MbCO (Figures 16, 17 and 18). Alternative assignment of \(\delta(CH=)\) comes from RR studies (Argade et al.1986), which observed 1306 cm\(^{-1}\) peak and described as a \(v_{21}\) Raman active mode or a \(\delta(CH=)\). If \(D_{4h}\) symmetry is still worth in heme A of CcO-CO, our assignment of \(\delta(CH=)\) at 1301 appears to be correct.

\(\delta_{=CH_2}\): Low temperature photoperturbation studies of cobalt substituted meso and proto MbO\(_2\) has allowed us to assign two CH\(_2\) asymmetric deformation mode (Park, S., Yonetani, T., Alben, J.O., manuscript in preparation) instead of
one band observed in IR studies of Ni(PP) at 1080 cm\(^{-1}\) (Choi et al.1982) and RR studies of biological heme proteins (Argade et al.1986; Ching et al.1985; Desbois et al.1984; Lee et al.1986). The spectral region of CH\(_2\) rock seems to be complicated because of vibronic coupling with the possible axial ligand (Proniewicz and Kincaid, 1990), or the involvement of histidylimidazole modes (Hodgson et al.1980; Hodgson et al.1980). The CH\(_2\) asymmetric deformation mode is assigned in our photodissociation spectra of HbCO and MbCO at 1089 and 1079 cm\(^{-1}\), and 1093 and 1077 cm\(^{-1}\) (zero-crossing frequencies), respectively (Figures 16, 17 and 18). The splitting of this mode in the infrared spectrum may be due to a different position of vinyl groups in porphyrin ring or in-phase and out-of-phase combination. However, the photoperturbations spectrum of CcO-CO shows only one assignable vinyl asymmetric deformation at 1082 cm\(^{-1}\) (zero-crossing frequency), which is comparable to model porphyrin studies of PP observed at 1087 cm\(^{-1}\) by IR studies (Choi et al.1983). Therefore, the splitting of \(\delta_{\ell}(=\text{CH}_2)\) seems to be the characteristic of porphyrin IX in biological proteins.

The strong intensification of CH\(_2\) rock mode was ascribed to the specific interactions of with the \(\gamma(\text{CH}=)\), which appeared to be enhanced by \(\nu_{45}\) (Choi et al.1982).

\(\gamma(\text{CH}=)\) & \(\gamma_{\ast}(=\text{CH}_2)\): The out-of-plane trans CH wag and CH\(_2\) wag is expected at around 994 and 903 cm\(^{-1}\) from the infrared studies of Ni(PP) (Choi et al.1982), and \(\gamma(\text{CH}=)\) is accidently degenerate with \(\nu_{45}\) porphyrin skeletal mode (Choi et al.1983). These modes seem to be so weak in our photoperturbation spectra, so
that we exclude the assignment.

**General feature of vinyl modes:** Except $v(C=C)$, all the observed deformation modes go to lower frequency (in the dark) from higher frequency (in the light) upon the photodissociation of CO.

### 4.5.4 Formyl modes

The formyl group in heme A of CcO is a strong electron withdrawing group, and has been used as a local molecular probe (Babcock, 1988; Babcock and Wikstrom, 1992; Chan and Li, 1990). The frequency assignments have been made from RR spectra of heme A and purified cytochrome c oxidase (de Paula et al.1990; Han et al.1991; Argade et al.1986; Babcock et al.1981; Ching et al.1985; Sassaroli et al.1988; Han et al.1990). Three formyl modes are expected to observe in our infrared spectral region (Zwarich et al.1971; Choi et al.1983; Willems and Bocian, 1985; Willems and Bocian, 1984): $v(C=O)$, $v(C=CHO)$, and $\delta(C=CO\cdot H)$. These are shown in Figure 22 and summarized in Table 14. In this study, the vibrational photoperturbations of formyl appears to result from cytochrome a$_1$-heme instead of cytochrome a-heme, since the relaxation of photoexcited cytochrome a-heme is expected to complete in the sub-microsecond (or picosecond) time range, long before the present observations were begun.
\(\delta(C_\alpha-\text{CO-H})\): The formyl hydrogen deformation was assigned initially in \([(\text{ImH})_2\text{heme}]^{2+}\) at 1390 cm\(^{-1}\), based on deuteration shift of formyl to 1077 cm\(^{-1}\) by RR studies (Choi et al. 1983). Extended infrared studies of Ni(FDP) species showed that \(\delta(C_\alpha-\text{CO-H})\) occurs at 1283 and 1265 cm\(^{-1}\) for Ni(2-FDP) and Ni(4-FDP) respectively (Willems and Bocian, 1985; Willems and Bocian, 1984). In case of Ni(2,4-FDP), two frequencies were observed at 1395 and 1282 cm\(^{-1}\) possibly due to in-plane and out-of-plane deformations. The former 1395 cm\(^{-1}\) was severely obscured by the \(\nu_{40}\) porphyrin skeletal mode in the spectrum. By retaining the infrared assignment (Willems and Bocian, 1985; Willems and Bocian, 1984), we observed formyl hydrogen deformation mode at 1285 cm\(^{-1}\) (zero-crossing frequency).

\(\nu(C_\beta-\text{CHO})\): The \(C_\beta\) and formyl stretching is expected to be observed in the range of 1150-1250 cm\(^{-1}\) from benzaldehyde and model heme A studies (Choi et al. 1983; Gambi et al. 1980; Zwarich et al. 1971), but no direct assignment has been made by infrared studies (Choi et al. 1983; Willems and Bocian, 1984; Willems and Bocian, 1985; Babcock, 1988). The \(\nu(C_\beta-\text{CHO})\) of heme A was observed at 1220 cm\(^{-1}\) by RR studies (Choi et al. 1983), and interpreted as an isolated motion. However, model studies of Ni(FDP) species by IR and RR (Willems and Bocian, 1985; Willems and Bocian, 1984) suggests that \(\nu(C_\beta-\text{CHO})\) mode significantly contributes to \(\nu_{42}\) and \(\nu_{43}\) porphyrin \(E_u\) skeletal modes at least in case of Ni(2,4-FDP). It was also observed from Ni(FDP) studies (Willems and Bocian, 1985;
Willems and Bocian, 1984) that no infrared frequency of $\nu$(C_b-CHO) was reported in both Ni(2-FDP) and Ni(4-FDP), but the 1226 cm$^{-1}$ by IR and 1161 cm$^{-1}$ by RR were assigned to $\nu$(C_b-CHO) in Ni(2,4-FDP) possibly due to in-phase and out-of-phase combinations. Recently, Babcock (Pyle, 1993) suggests that the 1163 and 1167 cm$^{-1}$ bands in Ni(2-FDP) and Ni(4-FDP), assigned as $\nu_{43}$ porphyrin skeletal mode by Willems and Bocian (Willems and Bocian, 1985), are $\nu$(C_b-CHO) modes based on the observation of nondegenerate components of $\nu_{43}$ porphyrin skeletal mode in higher symmetry metalloporphyrins.

With the consideration of nondegeneration with porphyrin skeletal mode, a strong perturbation at 1167 cm$^{-1}$ (zero-crossing frequency) in our CcO-CO appears to be the $\nu$(C_b-CHO). However, we can not rule out the degeneration with $\nu_{43}$.

$\nu$(C=O): Unlike the vinyl modes in heme A which are essentially insensitive to changes of iron spin or valence states, the $\nu$(C=O) of formyl group is very sensitive to changes at the metal and ligand (Babcock, 1988; Choi et al.1983). The $\nu$(C=O) of formyl has been observed not only model compounds (Willems and Bocian, 1984; Willems and Bocian, 1985; Choi et al.1983; Steelandt-Frentrup et al.1981; Tsubaki et al.1980) by IR and RR, but also plant and animal cytochrome c oxidase (de Paula et al.1990; Han et al.1991; Gregory and Ferguson-Miller, 1989; Salmeen et al.1973; Babcock et al.1981; Argade et al.1986; Ching et al.1985; Sassaroli et al.1988; Han et al.1990; Choi et al.1983) by RR studies. However, no
infrared observation has been made in biological respiratory heme proteins.

The largest amplitude first derivative-shaped band in our photoperturbation spectrum of CcO-CO is centered at 1662 cm\(^{-1}\) (zero-crossing frequency), and is assigned to a \(\nu(C=O)\) of the formyl. This spectral region also has a significant contribution from instrument because of rapid decrease of infrared transmission due to protein amide I mode, so that it seems to be important to evaluate formyl stretching mode assignment (Park and Alben, 1993).

The spectral measurements of "forward" and "reverse" directions of moving mirror is an excellent way to distinguish sample perturbations from instrumental artifacts, since the sampling points in the two directions are different, and yield independent data collections and phase corrections. Figure 22-B shows the nearly identical photoperturbations in the forward and reverse directions, and the their difference spectrum does not indicate any instrumental contributions. Single beam spectrum of CcO-CO (see Appendix Figure 36) also shows good infrared transmission in amide I region.

However, zero-contributions of instrumental artifact always do not happen. Photoperturbation (light/dark) FT-IR spectra of sperm whale MbCO, measured forward and reverse directions of moving mirror movement, show small instrumental contributions at 1007, 1260, 1384, 1474, 1555, and 1652 cm\(^{-1}\) (see Appendix Figure 35). The beamsplitter absorption at 1260 cm\(^{-1}\) is significant relative to forward and reverse perturbations spectrum, and must be considered
during vibrational analysis. The presence of amide I and II absorption bands (≈ 1652 and 1555 cm⁻¹) in the difference spectrum suggests the instrumental fluctuations, which are accentuated in regions of strong absorption bands. Single beam spectrum of sperm whale MbCO (see Appendix Figure 36) also shows a weak infrared transmission in this spectral region.

Therefore, under these evaluations, at least, the formyl v(C=O) does not result from a sample perturbation, rather than from instrumental contribution, and our assignment seems to be reasonable.

Polarization of formyl and vinyl: The present data of formyl and vinyl provide new insight into the heme polarization. Our observation of formyl and vinyl modes is consistent with excitation of a low spin (S=0) to a high spin (S=2) iron, and conversion of a tetragonal to a square-pyramidal heme complex, when heme is perturbed by the photodissociation of CO. The observed frequency increase of v(C=C) and v(C=O) from lower (in the dark) to higher frequency (in the light) upon photodissociation are consistent with decreased overlap of the pyrrole nitrogen pₓ and iron dₓ and dᵧ orbitals. This may result in a decreased peripheral polarization of the heme and a decreased negative charge on the peripheral substituents, with the additional increase in C=O and C=C stretching frequencies.
4.5.5 Peripheral substituent modes

Axial imidazole perturbation: Imidazole has its characteristic role in most of heme proteins especially in active-site. In case of Hb and Mb, iron is bonded to 5th-ligand imidazole of proximal histidine. The 6th-exogenous ligand, dioxygen in the case of HbO₂ or MbO₂, which is coordinated to heme iron has been suggested as a possible hydrogen bonding to the imidazole of distal histidine (Norvell et al.1975; Phillips and Schoenborn, 1981; Petsko et al.1978; Shaanan, 1982; Yonetani et al.1974; Ikeda-Saito et al.1977).

From the photoperturbation studies of dioxygen (¹⁶O₂), dioxygen isotope (¹⁸O₂) and CO complexes in the heme proteins (S. Park, T. Yonetani, J. O. Alben, manuscript in preparation), the putative dioxygen band at 1106 cm⁻¹ in HbO₂ appears to be a composite band with a imidazole of histidine either axial or distal, because not only the intensity change observed at this band upon isotope exchange, but also the 1106 cm⁻¹ peak has a good frequency correlation of histidylimidazole (Salama and Spiro, 1978; Walters and Spiro, 1983; Hodgson et al.1980; Hodgson et al.1980). Thus, the observed 1106 cm⁻¹ (HbCO) and 1104 cm⁻¹ (MbCO) bands in our photoperturbation spectra seem to have a contribution from an imidazole mode (Table 12)

The photoperturbation spectrum of a mutant MbCO (HisE7>Phe) may provide the information about the involvement of imidazole, because we would expect to have a massive change of spectral photoperturbations if distal imidazole
is involved (Figure 18). The subtle alterations in our absorbance double-difference spectrum (Figure 26-A) were observed in the region of vinyl and imidazole modes. An interesting feature in the absorbance double-difference spectrum is the appearance of frequency shifted band at 1225.2 cm$^{-1}$ (zero-crossing frequency), which has not been yet assigned definitively. Thus, all these changes must be attributed to polarization effects of the porphyrin through global conformation effects rather than a distal histidine substitution. Therefore, we can rule out the possible involvement of distal imidazole of histidine in our tentatively assigned imidazole mode at least.

CH$_3$ deformation: The CH$_3$ in-phase and out-of-phase deformation is expected at around 1380 and 1460 cm$^{-1}$ respectively (Colthup et al. 1990). When two methyls are bonded to one saturated carbon, two bands of CH$_3$ in-phase deformation appear with nearly equal intensity near 1385 and 1368 cm$^{-1}$ (Colthup, 1981; Colthup et al. 1990). This splitting was interpreted by the differences in H···H steric repulsion during the vibration (Benko and Yu, 1983). It is observed the splitting of CH$_3$ in-phase deformation in the infrared absorption spectra of farnesyl derivatives, which show the slitting of CH$_3$ in the in-phase deformation mode (Park et al. unpublished work)

By retaining the photoperturbation of farnesyl group as discussed in $\nu$(C=C), the strong amplitude band at 1368 cm$^{-1}$ (zero-crossing frequency) in our spectrum of CcO-CO is likely to be assigned as an in-phase deformation of CH$_3$,
of farnesyl side chain (Figure 2). Unlike the CcO-CO, the CH₃ in-phase deformation in our photoperturbation spectra of HbCO and MbCO (Figures 16, 17, 18 and 20) was observed with a less intense amplitude. As discussed previously, porphyrin skeletal mode ν₄₁ is expected to be mixed with CH₃ in-phase deformation.

The frequency assignment of CH₃ out-of-plane deformation seems to be more complicated with the combination of CH₂ deformation, therefore the assignment is excluded in this study.

4.5.6 Deuteration and pH Effects

Two deuterium sensitive bands were observed directly in the photoperturbation spectrum of MbCO at 1036 cm⁻¹ and 1110 cm⁻¹ (Figure 18). The former 1036 cm⁻¹ peak has a frequency shift to 1038 cm⁻¹ upon deuteration, but the latter 1110 cm⁻¹ loses its intensity upon deuteration. The 1036 cm⁻¹ peak seems to be the characteristic peak of native Mb, and has not been yet assigned definitively. In general deuterium exchange lowers the stretching or bending frequency of the deuterated chemical group of the molecule. Therefore, the 1036 cm⁻¹ peak in MbCO seems to be a certain vibrational mode due to the interactions with neighboring molecules rather than an isolated vibrational mode, so that the deuterium substitution will strengthen the interaction and increase the vibrational frequency. Unlike Mb, no apparent changes were observed in the photoperturbation spectrum of deuterated HbCO (Figure 17). However, the
absorbance double-difference spectra of (HbCO in H₂O - HbCO in D₂O; MbCO in H₂O - MbCO in D₂O) in Figure 24 show the minor alterations in the vinyl deformation and tentatively assigned imidazole modes in addition to yet unidentified bands.

Also no apparent changes were observed in the photoperturbation spectra of MbCO at different pH (Figure 19). The absorbance double-difference spectra of MbCO at pH 8.5, 7.0 and 6.0, however, show that vinyl modes and tentatively assigned imidazole modes appear to be very susceptible to pH changes of MbCO in Figure 25. Especially, these changes are intensified in the absorbance double-difference spectrum between pH 8.5 and 6.0. There are 12 histidine residues in the sperm whale Mb, which may be responsible the change in the tentatively assigned imidazole mode.

Many physicochemical techniques (Shimada and Caughey, 1982; Bothelo and Gurd, 1978; Bothelo et al. 1978; Wilbur and Allerhand, 1977; Ramsden and Spiro, 1989) has been applied to identify the pKₐ value of histidine imidazole of Mb, which is responsible for the influence of ligand binding affinity and the spectral properties. Shimada and Caughey (Shimada and Caughey, 1982) observed that the protein residue with a pKₐ = 6.0, neither proximal nor distal histidine imidazole, is responsible for the spectral changes. The increase of CO rebinding to Mb is observed when the pH is lowered from neutrality (Doster et al. 1982), suggesting the involvement of His-64(E7) protonation with a pH 5.7. However,
Wilbur and Allerhand (Wilbur and Allerhand, 1977) assigned His-64 to a pK$_a$ 4.4 with $^{13}$C NMR studies. Proton NMR studies (Bothelo and Gurd, 1978; Bothelo et al.1978) suggested that His-113(G14) and His-119(GH1) with pK$_a$ values of 5.5, located away from the heme, are responsible for the modulation of CO rebinding and for the change of the optical spectrum. Ramsden and Spiro (Ramsden and Spiro, 1989) also suggested that the protonation of remote histidine residues from the heme induces a global change in the protein structure which alters the heme core environments. Recently, a site-directed mutagenesis technique (Li et al.1994) has been used to pinpoint the relative importance of polar versus steric interactions in the CO binding properties and conformation changes.

Under these considerations, the isolation of specific histidine imidazole residues which are responsible for the change in our photoperturbation spectrum seems to be infeasible in this study, but the subtle alterations in deuterated Hb and Mb might be attributed to the polarization effects of the porphyrin ring or global conformational effects, rather than the changes in a single amino acid residue which is solely responsible for.

4.5.7 Difficulties of band assignment in biological proteins

The assignment of porphyrin skeletal modes in biological heme proteins has been suggested (Kitagawa et al.1978; Argade et al.1986; Li et al.1990; Li et al.1990; Willems and Bocian, 1984; Willems and Bocian, 1985), since Abe and co-
workers obtained normal coordinated analysis of Ni(OEP) (Abe et al.1978), which was a bench mark in the $D_{4h}$ symmetry heme proteins. After their initial assignment of porphyrin fundamental modes, many spectroscopic approaches have been applied to correct and modify the initial assignment in Ni(OEP), and finally came to common observations in the assignment of Raman active modes. Contrary to highly symmetric porphyrin complex, however, most of biological respiratory heme proteins have either heme $a$ or heme $b$ (protoporphyrin IX) in their core center, which probably has a significantly decreased symmetry down to $D_{2h}$ or $C_2$. For example, Raman active modes of $v_2(A_{1g})$ and $v_{11}(B_{1g})$ were observed at 1602 and 1576 cm$^{-1}$ in Ni(OEP) (Kitagawa and Ozaki, 1987; Li et al.1990), while they were assigned at 1585 and 1516 cm$^{-1}$ in cytochrome c oxidase (Argade et al.1986), respectively. Symmetry lowering also affects the involvement of some of infrared active modes in the resonance Raman spectrum (Choi et al.1983; Choi et al.1982; Willems and Bocian, 1984; Willems and Bocian, 1985).

Contrary to the assignment of Raman active modes by RR spectroscopy, the assignment of infrared active modes seems to be more difficult even in the highly symmetric Ni(OEP) complex because of the involvement of side chains. Several major changes in the assignment of infrared active porphyrin skeletal modes in Ni(OEP) (Kincaid et al.1983; Li et al.1990) have been made, after the initial assignment by Abe and co-workers (Abe et al.1978) as discussed in in-plane $E_a$ modes section. Our infrared active assignments of porphyrin skeletal and side chain modes with the aid of frequency correlations to highly symmetric Ni(OEP)
are the first approach in biological heme proteins. These assignments may provide a means of monitoring porphyrin conformation and the interactions of porphyrin peripheral groups with protein residues.

4.6 Conclusions

We have demonstrated the use of low temperature photoperturbation FT-IR spectroscopy to probe the dynamics and structural studies of heme proteins. These include the involvement of in-plane and out-of-plane porphyrin macrocycle modes, vinyl side chains, formyl modes, and in addition to tentatively assigned axial imidazole modes within the biological protein matrix. This technique also demonstrate the identification of infrared active modes that was rarely detected by RR spectroscopy. These findings will provide an additional information in detailed understanding of protein conformational changes. Not only porphyrin modes but also vinyl and formyl modes will be a useful probe of local molecular interactions and protein dynamics in future studies.
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5.1 Abstract

Purified cytochrome $c$ oxidase from beef heart has been studied by Fourier transform infrared absorbance difference spectroscopy. Photolysis at 10-20 Kelvin results in dissociation of $a\beta$FeCO, formation of Cu$\beta$CO, and perturbation of the $a\beta$-heme. The perturbation spectrum between 900 and 1700 cm$^{-1}$ contains information about the binuclear center. Frequency shifted bands due to the 8-formyl and 4-vinyl groups of the $a\beta$-heme have been identified. The frequency shifts have been interpreted as due to a change in porphyrin polarization with change in spin state of the iron by photodissociation of CO.

5.2 Introduction

Cytochrome $c$ oxidase is the terminal respiratory enzyme in eucaryotic plants and animals. It catalyzes the reduction of molecular oxygen to water, and conserves the chemical energy for use in cellular processes. It consists of two iron
porphyrin (heme $a$ and $a_j$) and two copper sites ($\text{Cu}_A$ and $\text{Cu}_B$) (Wikstrom et al. 1981; Babcock and Wikstrom, 1992; Chan and Li, 1990). The heme $a$ and $\text{Cu}_A$ participate in electron transfer from cytochrome $c$ to the binuclear site, which consists of heme $a_j$ and $\text{Cu}_B$. Dioxygen is bound and reduced to water at the binuclear site. The molecular interactions between metal-bound axial ligand and its molecular surroundings provides the chemical basis for biological control of respiratory processes (Woodruff et al. 1991; Chan and Li, 1990).

The formyl group at the 8-position on the porphyrin ring of heme $a_j$ provides a molecular probe of such interactions. Although the formyl group is a weak scatterer, it has been studied by resonance Raman spectroscopy, and frequency assignments have been made from heme A and from purified cytochrome $c$ oxidase (Salmeen et al. 1973; Callahan and Babcock, 1983; Babcock and Callahan, 1983; de Paula et al. 1990; Ching et al. 1985; Argade et al. 1986; Sassaroli et al. 1988; Choi et al. 1983). Suggestions have been presented that the formyl group of heme A in cytochrome oxidase may be hydrogen bonded to a protein group and/or the formyl group may be sensitive to conformational changes in the protein (Sassaroli et al. 1988). Such interactions may affect the chemical reactivity of ligands coordinated to the iron.

Fourier transform infrared spectroscopy (Alben and Fiamingo, 1984) has been used to monitor and characterize the heme $a_j$ and $\text{Cu}_B$ binuclear site following photodissociation of the carbon monoxide from the $a_j$ heme-CO
The photodissociated CO forms a complex with Cu$_B$ that is stable at temperatures below 140 Kelvin (Alben et al. 1982; Alben et al. 1982; Fiamingo et al. 1982; Alben et al. 1981; Fiamingo et al. 1986). Photodissociation of the $a_2$FeCO complex of cytochrome c oxidase has provided essential information about structural interactions at the $a_2$Fe:Cu$_B$ site. Measurements at low temperature (10-180 Kelvin) by Fourier transform infrared spectroscopy provided the first observation of Cu$_B$CO complex (Alben et al. 1982; Alben et al. 1982), and demonstrated that an unhindered path exists between $a_2$Fc and Cu$_B$. Photodissociation of $a_2$FeCO results in very rapid formation of Cu$_B$CO even at 10 Kelvin, and thermal dissociation of the Cu$_B$ complex proceeds at measurable rates above 140 Kelvin with reformation of the $a_2$FeCO complex. In the reduced CO complex, $a_2$Fe and Cu$_B$ must be close enough to provide a clear path between them, but far enough apart so that the 2.5 cm$^{-1}$ bandwidth of $a_2$FeCO at 1963 cm$^{-1}$ is not broadened by interaction with the adjacent Cu$_B$ complex. We therefore estimated the distance between these metal centers to be 4-6 Angstroms in the reduced CO complex (Fiamingo et al. 1982; Alben et al. 1981).

In this study, we optimized sampling conditions and instrumentation to permit observation of infrared perturbation spectra of cytochrome c oxidase in aqueous solution between 950 cm$^{-1}$ to 1700 cm$^{-1}$ at high signal/noise ratio. Photodissociation of the cytochrome $a_2$FeCO perturbs the porphyrin ring and substituents such as 8-formyl and 4-vinyl groups, resulting in frequency shifts that
are observed by absorbance difference spectroscopy (light - dark).

5.3 Methods

Beef heart cytochrome c oxidase was isolated by the method of Hartzell and Beinert (Hartzell and Beinert, 1974; Pan et al. 1991). Following addition of 1 equivalent of ascorbate and 0.05 equivalents of cytochrome c, the enzyme samples were thoroughly degassed on a high-vacuum apparatus and subsequently exposed to 1 atmosphere of CO gas (Matheson, 99.5%) at 4°C overnight. The enzyme preparation was stored in liquid nitrogen until used. Visible spectra of cytochrome c oxidase at 4°C were obtained with a Cary model 17DX spectrometer. All infrared spectra were recorded at 0.5 cm\(^{-1}\) resolution with a Mattson Sirius 100 FT-IR interferometer which was fitted with a liquid nitrogen cooled photovoltaic (HgCd)Te detector for measurements below 1800 cm\(^{-1}\), or with a liquid nitrogen cooled InSb detector for measurements above 1800 cm\(^{-1}\). The detectors were fitted with appropriate low-pass optical filters. Low temperature was obtained by use of a Lake Shore Cryotronics closed cycle helium refrigerator, Model LTS-21-D70C. Cryostat cell temperature was measured by use of a Lake Shore Cryotronics digital thermometer, model DRC-70, with a calibrated silicon diode probe. Photolysis made use of a 500 W tungsten lamp focused through a slide projector and passed through a water filter (2 cm) to remove infrared radiation beyond 1.2 micrometers. Photodissociation absorbance difference spectra (light - dark), were obtained from the ratio of single beam
spectra in the absence (dark) and in the presence (light) of visible radiation.

The optical bench was flushed with highly dried compressed air to remove water vapor absorption that occurs in the infrared beam path. The residual atmospheric water vapor absorptions were identified by 0.5 cm\(^{-1}\) half-bandwidth, and removed by absorbance subtraction with water vapor spectrum collected independently.

5.4 Results

Purified cytochrome \(c\) oxidase was characterized by visible and infrared spectroscopy of the carbon monoxide complex. The visible spectrum (Figure 28) is characteristic of the reduced CO complex. The bands at 430.8 and 594.3 nm are characteristic of the \(a_{2}\)FeCO complex, while the absorptions at 443.5 and 603.3 nm are characteristic of the reduced cytochrome \(a\). The visible spectrum in Figure 26 was recorded after the infrared measurements in Figures 29, 30, and 32, and thus represents the final state of the enzyme.

Photodissociation of the purified cytochrome oxidase CO complex at 12 Kelvin yielded infrared spectra of the CO complexes (Figure 29) similar to those previously reported for beef heart mitochondria, rat heart myocytes, and purified cytochrome oxidase (Fiamingo et al.1982; Fiamingo et al.1986; Dyer et al.1989). \(Alpha\)-form conformers at 12 Kelvin exhibit an absorption due to \(a_{2}\)FeCO at 1962.5 cm\(^{-1}\) and a split CuBCO band at 2054.8 and 2064.8 cm\(^{-1}\), and \(beta\)-forms
Figure 28. Room temperature visible spectrum of cytochrome c oxidase CO complex following low temperature photodissociation studies illustrated in Figures 29, 30, and 32.
Figure 29. Fourier transform infrared absorbance difference spectrum of the CO complex of cytochrome c oxidase at 12 K, "after" minus "before" photodissociation (light minus dark).
exhibit $a_3\text{FeCO}$ bands at 1944.1, 1948.2 and 1957 cm$^{-1}$, and a single $\text{Cu}_b\text{CO}$ band at 2041.3 cm$^{-1}$. The ratio of $\text{beta/alpha}$-form (0.079) was less than that observed with beef heart tissue or myocytes isolated from rat heart (Fiamingo et al.1986), but similar to that observed in mitochondria (Fiamingo et al.1982) isolated from beef heart. Similar spectra were previously measured with dithionite-reduced and auto-reduced (mixed valence) CO complexes of purified cytochrome $c$ oxidase (D. Bickar, S. Park, C. Bonaventura, and J. O. Alben, unpublished data). The frequency accuracy is limited only by noise and baseline uncertainty, since the instrumental inaccuracy was measured to be $+0.024 \pm 0.02$ cm$^{-1}$ (1:100,000) corrected to vacuum, by comparison of spectra of carbon monoxide gas at low partial pressure with literature values (Cole, 1977). Sample composition and concentration (3.3mM, 7.0 micrometer optical path) were determined from the 12 Kelvin photodissociation difference spectrum of the iron carbonyl at 1962.5 cm$^{-1}$ (Figure 29), assuming an integrated absorptivity of 28 mM$^{-1}$cm$^{-2}$ (Yoshikawa et al.1977).

Following collection of the cytochrome oxidase CO spectra in Figure 29, the cryostat was moved to a second Mattson interferometer fitted with a photovoltaic (HgCd)Te detector for measurements through the amide and fingerprint spectral regions (900-1800 cm$^{-1}$). The sample temperature was raised to 200 Kelvin for 20 minutes to thermally dissociate the $\text{Cu}_b\text{CO}$ with reformation of $a_3\text{FeCO}$, and then recooled to 13 Kelvin. Infrared data collected before and
after a 10 minute photodissociation show perturbations of many bands in the absorbance difference spectrum (Figure 30). The spectral observations were replicated an additional four times by relaxation at 200 Kelvin, cooling to 10-20 Kelvin, and signal averaging between 2048 and 16384 interferograms each before and after a 10 minute photolysis. The absorbance difference bands were well reproduced. A second sample yielded a similar difference spectrum. Especially, the presence of residual atmospheric water vapor absorptions in amide region is clearly distinguished from spectral perturbation of cytochrome oxidase CO by use of 0.5 cm⁻¹ resolution.

Frequency shifted bands (Alben and Fiamingo, 1984; Alben and Bare, 1978) have an appearance roughly similar to the first derivative of an absorption band, and are identified by the frequency of the zero-crossing and the amplitude of the absorption difference (Figure 31). Other perturbations may result in a change in absorptivity, so the difference band is only pointed up or down, or in a change in bandwidth which results in an appearance roughly similar to a second derivative or to a \( \sin x/x \) function. The largest amplitude first derivative-shaped band is centered at \( 1662.1 \pm 0.3 \) cm⁻¹ (\( \Delta \varepsilon = 1.16 \pm 0.07 \) mM⁻¹cm⁻¹), and is assigned to a frequency shifted \( \nu(CO) \) of the 8-formyl group of the cytochrome \( a_3 \) heme because of an excellent frequency correlation with RR studies of model heme A and plant and animal cytochrome c oxidase (de Paula et al.1990; Han et al.1991; Gregory and Ferguson-Miller, 1989; Salmeen et al.1973; Babcock et al.1981;
Figure 30. The photoperturbation infrared spectrum (light minus dark) of heme $a_3$ of cytochrome $c$ oxidase at 13 Kelvin. The beam splitter band is denoted by ★.
Figure 31. Absorbance difference spectrum (----) of two Gaussian bands (—) with identical half-widths, $\Gamma$ (FWHH), normalized to the same peak intensity $A_o$, but separated in frequency by $\Delta v_0$. The peak-to-trough absorbance difference is $\Delta A$, and frequency difference is $\delta$. The frequency scale is normalized to the half-width. (From Alben and Fiamingo, Optical Techniques in Biological Research, 1984, Academic Press. pp.133-179).
Figure 32. Absorbance difference spectra from Figure 30 from 1600-1700 cm\(^{-1}\) plotted with an expanded abscissa to show the frequency-shifted formyl C=O and vinyl C=C bands at 1662.1 cm\(^{-1}\), and 1629.7 cm\(^{-1}\). Some of residual atmospheric water vapor absorptions are denoted by •.
Argade et al. 1986; Ching et al. 1985; Sassaroli et al. 1988; Han et al. 1990; Choi et al. 1983; Dong et al. 1990; Willems and Bocian, 1984; Steelandt-Frentrup et al. 1981) summarized in Table 15. Additional bands appear at 1081.7 ± 0.4 cm⁻¹ (Δε = 0.50 ± 0.03 mM⁻¹ cm⁻¹), 1304.2 ± 0.4 cm⁻¹ (Δε = 0.39 ± 0.04 mM⁻¹ cm⁻¹), 1368.3 ± 0.1 cm⁻¹ (Δε = 0.46 ± 0.04 mM⁻¹ cm⁻¹), and 1629.7 ± 0.3 cm⁻¹ (Δε = 0.35 ± 0.03 mM⁻¹ cm⁻¹). We assign the band at 1081.7 cm⁻¹ to a =CH₂ rocking mode and the 1629.7 cm⁻¹ band to a C=C stretching mode of the 4-vinyl group of the cytochrome a₂-heme, in agreement with Choi, et al. (Choi et al. 1982; Choi et al. 1982). The observation of a single =CH₂ rock mode and ν(C=C) mode in cytochrome oxidase appears to be different from that of hemoglobin and myoglobin, which exhibits two frequencies of =CH₂ rock mode and no perturbation of vinyl C=C stretching mode (S. Park, T. Yonetani, and J. O. Alben, in preparation). This difference seems to be ascribed to the trans disposition of vinyl group with respect to the 8-formyl group, or the 2-farnesyl side chain of the cytochrome a₂-heme. The bands at 1304.2 and 1368.3 cm⁻¹ may be due to vinyl CH bending modes. The strong downward-pointing difference band at 1235 cm⁻¹ is characteristic of porphyrin-Fe-CO complexes, but has not yet been definitively assigned. These vibrations must be associated with the cytochrome a₂-heme since the low temperature is expected to restrict the observed spectral perturbations to the photodissociated heme and its immediate vicinity. The smaller amplitude bands will be discussed in a later work (S. Park and J. O. Alben, in preparation).
Table 15. Assignments of the Carbonyl Stretching mode of the Formyl Group in Cytochrome Oxidase and Model Compounds.

<table>
<thead>
<tr>
<th>heme</th>
<th>coordination no. and spin state*</th>
<th>frequency cm(^{-1})</th>
<th>Resolution cm(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model Compounds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heme a(^{2+})(2-MeIm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in CH(_2)Cl(_2)</td>
<td>5, hs, RR</td>
<td>1660(^b)</td>
<td>6</td>
</tr>
<tr>
<td>in H(_2)O</td>
<td>5, hs, RR</td>
<td>1640(^b)</td>
<td>6</td>
</tr>
<tr>
<td>heme a(^{2+})(N-MeIm)(_2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in CH(_2)Cl(_2)</td>
<td>6, ls, RR</td>
<td>1645(^c)</td>
<td>6</td>
</tr>
<tr>
<td>in H(_2)O</td>
<td>6, ls, RR</td>
<td>1633(^c)</td>
<td>6</td>
</tr>
<tr>
<td>Ni(2-FDP)</td>
<td>IR</td>
<td>1661(^d)</td>
<td></td>
</tr>
<tr>
<td>Ni(4-FDP)</td>
<td>IR</td>
<td>1666(^d)</td>
<td></td>
</tr>
<tr>
<td>Ni(2,4-FDP)</td>
<td>IR</td>
<td>1674, 1665(^d)</td>
<td></td>
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<tr>
<td>Cytochrome a(_3),</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(^{2+})</td>
<td>5, hs, RR</td>
<td>1665(^*)</td>
<td>6</td>
</tr>
<tr>
<td>Fe(^{3+})(CO)</td>
<td>6, ls, RR</td>
<td>1666(^f)</td>
<td>5</td>
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<tr>
<td>Fe(^{3+})(CN)</td>
<td>6, ls, RR</td>
<td>1644(^*)</td>
<td>4</td>
</tr>
<tr>
<td>Fe(^{2+})</td>
<td>6, hs, RR</td>
<td>1671(^*)</td>
<td>4</td>
</tr>
<tr>
<td>Fe(^{3+})(CO)</td>
<td>5, hs, RR</td>
<td>1674(^b)</td>
<td></td>
</tr>
<tr>
<td>Mixed Valence</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cytochrome a(_3),</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe(^{3+}*)(CO)</td>
<td>5, hs, RR</td>
<td>1668(^*)</td>
<td></td>
</tr>
<tr>
<td>Fe(^{3+}*)(CO/O(_2))</td>
<td>6, ls, RR</td>
<td>1671(^*)</td>
<td></td>
</tr>
</tbody>
</table>

* hs, high spin; ls, low spin; RR, resonance Raman; IR, infrared.


\(^c\) Baboock, G. T., and Callahan, P. M., Biochemistry (1983) 22:2314-2319.


\(^i\) Han, S., Ching, Y.-c, and Rousseau, D. L., Biochemistry (1990) 29:1380-1384.

\(^j\) Photoproduct of CO in the presence of O\(_2\).

\(^k\) Photoproduct
The absorbance difference spectrum was analyzed (Alben and Fiamingo, 1984; Alben and Bare, 1978; Laane and Kiefer, 1980; Laane, 1981; Rousseau, 1981) to measure the frequency shift of the formyl band upon photodissociation. This is possible since the symmetry of the frequency shifted band suggests negligible overlap with adjacent bands. The frequency of the zero-crossing can be precisely measured, and represents the mean of the band center frequencies before and after photodissociation. Analysis of the frequency shift (Figure 31) observed by absorbance difference spectroscopy (Figure 32) depends upon knowledge of the absorbance maximum ($A_o$) of the frequency-shifted band, the half-bandwidth ($\Gamma = \text{FWHH}$), and the shape of the band (e.g. gaussian and lorentzian), in addition to the observed peak to trough absorbance difference ($\Delta A = 0.0026$). We have not directly observed the total infrared absorption band due to the 8-formyl group, but can approximate the required information by comparison with benzaldehyde in the following manner. We assume the integrated absorptivity ($B$), and type of band shape (gaussian, lorentzian, etc.), but not $A_o$ or $\Gamma$, are the same for the formyl groups of heme A and benzaldehyde. Then from the Beer-Lambert relation and letting band area $\alpha \equiv (1/2) \cdot A_o \cdot \Gamma$, it follows that $A_o \Gamma / A_i \Gamma_i = c \lambda_c / c \lambda_i$, and $A_o = (A_i \Gamma / A_o \Gamma_o)(c \lambda_c / c \lambda_i)$, where subscripts 1 and o refer to benzaldehyde and cytochrome c oxidase, and $c$ and $l$ refer to concentration and pathlength, respectively. The parameters for the analysis of benzaldehyde and cytochrome c oxidase are given in Table 16. For this sample of cytochrome a$_3$ (Figures 29, 30, and 32), $A_o = 0.055 / \Gamma_o$. Limiting values
Table 16. Analysis of Heme α₃ 8-formyl Group in Cytochrome c Oxidase

(A) Benzaldehyde

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Total integrated absorptivity of aldehyde group in benzaldehyde at 1709.0 cm⁻¹</td>
<td>27 mM⁻¹ cm⁻²</td>
</tr>
<tr>
<td>Absorptivity of aldehyde group in benzaldehyde at 1709.0 cm⁻¹</td>
<td>0.356 Absorbance</td>
</tr>
<tr>
<td>Path length</td>
<td>0.107 mm</td>
</tr>
<tr>
<td>Concentration of benzaldehyde</td>
<td>10 mM</td>
</tr>
<tr>
<td>Half-bandwidth (Γ)</td>
<td>7.2 cm⁻¹</td>
</tr>
</tbody>
</table>

(B) Cytochrome c Oxidase

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of cytochrome c oxidase (From FeCO absorption spectrum)</td>
<td>3.3 mM (cₒ)</td>
</tr>
<tr>
<td>Path length</td>
<td>7.0 x 10⁻⁴ cm</td>
</tr>
<tr>
<td>δ</td>
<td>5.2 cm⁻¹</td>
</tr>
<tr>
<td>∆A</td>
<td>0.0026 Absorbance</td>
</tr>
<tr>
<td>∆v₁/₄</td>
<td>4.0 cm⁻¹</td>
</tr>
<tr>
<td>Heme α₃ formyl zero-crossing frequency</td>
<td>1662.1 cm⁻¹</td>
</tr>
</tbody>
</table>

(C) Analysis of frequency shift

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Limiting Conditions¹</td>
<td>Γ ≥ ∆v₁/₄, ∆vₒ ≤ δ, Γ ≤ (δ/0.849, or δ/0.577, )</td>
</tr>
<tr>
<td>Equations for small frequency shift¹</td>
<td>∆vₒ ≈ 0.350 (∆A/Aₒ) (Γ)ᵣ</td>
</tr>
<tr>
<td></td>
<td>∆vₒ ≈ 0.385 (∆A/Aₒ) (Γ)ᵣ</td>
</tr>
<tr>
<td>Gaussian Band Shape ∆vₒ</td>
<td>0.3 - 0.6 cm⁻¹</td>
</tr>
<tr>
<td>Lorentzian Band Shape ∆vₒ</td>
<td>0.3 - 1.5 cm⁻¹</td>
</tr>
</tbody>
</table>

of the $a_j$-heme formyl half-bandwidth ($\Gamma = \text{FWHH}$) are given by the following inequalities (Alben and Fiamingo, 1984; Rousseau, 1981; Laane and Kiefer, 1980; Laane, 1981): $\Gamma \geq \Delta v_{1/4}$; $\Gamma_1 \leq \delta/0.849$ (gaussian), or $\Gamma_1 \leq \delta/0.577$ (lorentzian); and $\Delta v_o \leq \delta$, where these parameters are defined in Figure 29. For cytochrome $c$ oxidase, $\Delta v_{1/4} = 4.0 \text{ cm}^{-1}$, and $\delta = 5.2 \text{ cm}^{-1}$. These relations lead to limiting values of $\Gamma = 4$ to $6 \text{ cm}^{-1}$ (gaussian) and $\Gamma = 4$ to $9 \text{ cm}^{-1}$ (lorentzian) for the $a_j$-formyl group, and to center frequency shifts $\Delta v_o = 0.3$ to $0.6 \text{ cm}^{-1}$ (gaussian), or $\Delta v_o = 0.3$ to $1.5 \text{ cm}^{-1}$ (lorentzian) from the calculation of small frequency shifts equations given in Table 16. This approximation is exclusively accurate for $\Delta v_o \leq (\Gamma_{1/4})$ (Alben and Fiamingo, 1984; Laane and Kiefer, 1980; Laane, 1981).

5.5 Discussion

The data reported here in sequential experiments on the identical sample of purified cytochrome $c$ oxidase help to define the molecular changes that occur with photodissociation of the CO complex at 10-20 Kelvin. The purified oxidase appears to be identical to that previously reported for bovine heart mitochondrial preparations, and differs from the oxidase in rat myocyte or beef heart tissue preparations only in the ratio of beta- to alpha-forms (Fiamingo et al.1982; Fiamingo et al.1986). The observations of mid-infrared absorbance difference spectra, newly presented here, confirm that major perturbations occur at the cytochrome $a_j$-heme upon photodissociation even at 10-20 Kelvin. Frequency-
shifted bands have been assigned to the 8-formyl and 4-vinyl groups. As yet unassigned bands may include a proximal histidine coordinated to the $a_1$Fe, histidines coordinated to Cu$_B$, and other vibrational modes of the $a_1$-heme. It appears unlikely that any of the vibrational perturbations may be assigned to heme $a$, since relaxation of its photoexcited heme is expected to be complete in the sub-microsecond (or picosecond) time frame, long before the present observations were begun. Geminate recombination of the photolyzed iron-histidine bond was observed to occur in less than 20 psec in both cytochrome b$_3$ and cytochrome c by Jongeward, et al. (Jongeward et al.1988). No groups in the protein other than heme A absorb visible light, and thus would not be perturbed. The low temperature precludes motions of the protein except as required for dissipation of the kinetic energy that results from absorption of a photon by the heme.

The present data provide new insight into the photodissociation process. Not only does the $a_1$Fe-coordinated CO receive enough kinetic energy to reach the adjacent Cu$_B$, but the $a_1$-heme is perturbed in a manner consistent with excitation of a low spin ($S = 0$) to a high spin ($S = 2$) iron, and conversion of a tetragonal to a square-pyramidal heme complex. The observed increase in frequencies of the 8-formyl $v$(C=O) and 4-vinyl $v$(C=C) bands with photodissociation are consistent with decreased overlap of the pyrrole nitrogen $p_z$ and iron $d_\alpha$ and $d_\pi$ orbitals. This results in a decreased peripheral polarization
of the heme and a decreased negative charge on the peripheral substituents, with
the concomitant increase in C=O and C=C stretching frequencies. The foregoing
changes in heme polarization with photodissociation of CO are analogous to
effects on v(CO) of axially coordinated carbon monoxide that result from
differences in polarization by porphyrin or axial ligand substituent groups
transmitted through the iron in either the cis- or trans- directions (Alben and
Caughey, 1968). They are also consistent with a high spin iron in the
photodissociated state observed by time-resolved magnetic circular dichroism of
beef cytochrome c oxidase and by magnetic circular dichroism in cytochrome ba,
from *Thermus thermophilus* (Goldbeck et al.1992). These data rule out the
formation with CO photodissociation of a low spin heme a, complex by a strong
field ligand such as imidazole or methionine sulfur, but do not rule out a
photodissociable weak sigma-donor ligand such as a farnesyl ethylene group, as

Absorbance difference spectroscopy is very sensitive to small differences
in band center frequency, such that a shift of one tenth of the half-bandwidth is
readily measured. A similar error of measurement of center frequency due to
instrumental non-reproducibility also would be measured (Park and Alben, 1993)
and might be confused with a real frequency shift of an absorption band. Thus
frequency reproducibility and calibration are essential. Careful measurements of
CO rotational transitions between 2100-2200 cm⁻¹ indicated a reproducibility of
±0.02 cm⁻¹, which is limited by measurement noise, phase shift (drift), and laser
mode-hopping. Another particular concerns in the absorbance difference spectroscopy are the atmospheric water vapor absorptions in the \( \nu(C=O) \) and \( \nu(C=C) \) region, and noise problem. Flushing the optical bench with highly dried air was enough to remove the major atmospheric water vapor absorptions that occur in the infrared beam path, and the minor residual water vapor absorptions are clearly identified by 0.5 cm\(^{-1}\) half-bandwidth absorption band and the comparison of water vapor absorption spectrum collected at 0.5 cm\(^{-1}\) spectral resolution independently. Therefore the high spectral resolution interferometer we used in this study appears to be essential to evaluate a water vapor absorption. Noise is also carefully evaluated by taking an absorbance (dark/dark) difference spectrum under identical conditions, which showed non of perturbation bands except 80 microabsorbance peak to peak noises. These effects are all small, and do not contribute significantly to the formyl-group frequency shifts reported here. The greater concern is the lack of precise knowledge of the absorption band shape or intensity. Thus while measurements of absorbance difference zero-crossing as in Figure 31 yield very accurate estimates of the mean of band center frequencies due to the perturbation, the frequency shift \( (\Delta \nu_o) \) is model dependent and therefore less well defined. The small observed formyl frequency shift makes it unlikely that CO photodissociation is accompanied by a change in H-bonding to the formyl group, but is consistent with formation of a penta-coordinate high spin iron.
Similar formyl frequencies have been reported from resonance Raman data. The frequency shift we observe is, however, much smaller than reported from Raman spectroscopy. This appears to be a function of the conditions of measurement. Sassaroli, et al. (Sassaroli et al. 1988), reported a formyl frequency of the photoprodut at 1666 cm⁻¹ when a low laser flux density was used, and at 1674 cm⁻¹ with high flux density. The latter frequency may be affected by polarization effects due to local conformational changes associated with heat absorption by the porphyrin.
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and functional implications of the cytochrome a3 transients after
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Yoshikawa, S., Choc, M. G., O'Toole, M. C., and Caughey, W. S. 1977. An
Infrared Study of CO Binding to Heart Cytochrome c Oxidase and Hemoglobin
Low temperature Fourier transform infrared photodissociation spectroscopy is a powerful tool to obtain a structural information about heme and heme moiety vibrational modes, and photodissociated ligand molecules. Absorption of a visible photon by the heme causes photodissociation of CO or $O_2$ from the heme, changes spin state of iron, and perturbs the heme by changing the localization of porphyrin $\pi$ electron density. Absorption of an additional photon introduces energy that must be dissipated as a kinetic energy by translational and rotational interactions with protein surroundings, and this will alter the distribution of conformational substates of protein. Optical pumping mechanism in photolyzed CO molecules of hemoglobin was observed as a means of kinetic energy dissipation, and a distribution of conformational substates of protein which is different from the initial distribution of ground state induced by the initial absorption of light.
The structural difference between meso- and protoporphyrin cobalt complexes provides a solid information about vinyl asymmetric deformation mode observed as two bands instead of one band suggested by resonance Raman studies. The splitting of the vinyl modes is probably due to in-phase and out-of-phase deformation or two different positions in porphyrin ring. This assignment contributes to isolate apparent dioxygen vibrational bands in native and cobalt substituted heme respiratory proteins. Unlike the infrared spectrum at above freezing temperature, low temperature FT-IR photodissociation spectrum of Hb$^{18}$O$_2$ and Mb$^{18}$O$_2$ shows directly the appearance of new bands due to isotope substitution.

The detailed analysis of low temperature FT-IR photodissociation spectra in Mb, Hb and CcO with the aid of frequency correlations to the recently assigned modes of Ni(OEP) provides an assignment of infrared active in-plane and out-of-plane porphyrin skeletal modes and vinyl stretching and deformation modes, in addition to tentatively assigned histidylimidazole mode. Formyl vibrational modes of cytochrome a$_3$ in CcO was isolated and assigned. The infrared active frequencies of porphyrin skeletal and vinyl modes in heme a (CcO) are different from heme b (Mb & Hb), and the deuterium, pH, and the mutant cause the global conformational effects.

Although the entire band assignment in low temperature FT-IR photodissociation spectrum is far from the complete, the results obtained by novel approach may be useful as local molecular probes of porphyrin conformation as
well as the interactions of the side chains with protein residues.
BIBLIOGRAPHY


APPENDIX

FT-IR Absorbance Spectra and Their Corresponding Tables
Figure 33. FT-IR absorbance spectra of farnesyl derivatives (neat solution) with a 16.5 microns spacer at above freezing temperature: (A) Farnesyl Acetate; (B) Farnesol, scale expanded plot in the region of 1350 -1400 cm$^{-1}$ is inserted; (C) Farnesyl Bromide, scale expanded plot in the region of 1350 -1400 cm$^{-1}$ is inserted.
Figure 33 (continued)

![Graph showing the absorbance of Farnesol over a range of wavenumbers](B)
Figure 33 (continued)

Farnesyl Bromide

Absorbance

Wavenumber

(C)
Table 17. Tentative assignment of farnesyl derivatives (in this work).

<table>
<thead>
<tr>
<th>Farnesyl Acetate</th>
<th>Farnesol</th>
<th>Farnesyl Bromide</th>
<th>Tentative Assignment</th>
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<tbody>
<tr>
<td>3334</td>
<td></td>
<td></td>
<td>vOH</td>
</tr>
<tr>
<td>2966.6</td>
<td>2967.4</td>
<td>2967.5</td>
<td>asy CH₃</td>
</tr>
<tr>
<td>2920.9</td>
<td>2921</td>
<td>2922.7</td>
<td>asy CH₂</td>
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<td>2855.9</td>
<td>2855.8</td>
<td>2854.9</td>
<td>sy CH₃, CH₃</td>
</tr>
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<td>1742.6</td>
<td></td>
<td>1741.8 (?)</td>
<td>v C=O</td>
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<tr>
<td></td>
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<td>Contamination</td>
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</tr>
<tr>
<td>1670.5</td>
<td>1669.9</td>
<td>1657</td>
<td>v C=C</td>
</tr>
<tr>
<td>1445.8</td>
<td>1446.6</td>
<td>1447.5</td>
<td>Bending (?)</td>
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<td>1381.4</td>
<td>1382.2</td>
<td>1383</td>
<td>CH₃ in-plane deformation</td>
</tr>
<tr>
<td>1365.2</td>
<td>1376.4</td>
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<td>CH₃ in-plane deformation</td>
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<tr>
<td></td>
<td>1236.6</td>
<td></td>
<td>-C=O</td>
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<td>1232.1 (s)</td>
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<td>-C-O</td>
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<td></td>
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<td>1200 (s)</td>
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<td>1108.8</td>
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<td>1000.9 (s)</td>
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<td>-C-O stretching</td>
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<tr>
<td></td>
<td></td>
<td>922.6</td>
<td></td>
</tr>
</tbody>
</table>

s: strong
Figure 34. FT-IR absorbance spectrum of 0.01 M Benzaldehyde in CCl₄ with 0.107 mm spacer at above freezing temperature.
Table 18. Assignment of benzaldehyde.

<table>
<thead>
<tr>
<th>( \text{C}_6\text{H}_5\text{CHO} )</th>
<th>Assignment*</th>
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<tbody>
<tr>
<td>1730.7</td>
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<tr>
<td>1708.97</td>
<td>( v(\text{C}=\text{O}) )</td>
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<tr>
<td>1653.7</td>
<td>combination</td>
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<tr>
<td>1607.4</td>
<td></td>
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<tr>
<td>1597.9</td>
<td>( v_{8a} )</td>
</tr>
<tr>
<td>1586.0</td>
<td>( v_{5b} )</td>
</tr>
<tr>
<td>1456.0</td>
<td>( v_{18b} )</td>
</tr>
<tr>
<td>1430</td>
<td></td>
</tr>
<tr>
<td>1387.7</td>
<td>( \delta(\text{H-CO}), (\text{C-H aldehyde bend}) )</td>
</tr>
<tr>
<td>1337.4</td>
<td></td>
</tr>
<tr>
<td>1309.9</td>
<td>( v_3 )</td>
</tr>
<tr>
<td>1285.6</td>
<td>( v_{14} )</td>
</tr>
<tr>
<td>1268.6</td>
<td></td>
</tr>
<tr>
<td>1202.7</td>
<td>( v(\phi\text{-CHO}) )</td>
</tr>
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<td>1166.6</td>
<td>( v_{9b} )</td>
</tr>
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<td>1085.5</td>
<td></td>
</tr>
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<td></td>
</tr>
<tr>
<td>1066.5</td>
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Figure 35. Photoperturbation (light/dark) FT-IR spectra of sperm whale MbCO at 10 K illustrated in Figures 18, 20, and 23 measured in the "forward" and "reverse" directions of moving mirror, and their difference. Instrumental contribution bands in the difference spectrum are denoted by *. 
Figure 36. Single beam spectrum of sperm whale MbCO, HbCO, and cytochrome c oxidase illustrated in Figure 23.
Figure 36 (continued)

Cytochrome c Oxidase
Figure 37. Photoperturbation (light/dark) FT-IR spectrum of cytochrome c oxidase illustrated in Figure 22, protein sample was stored at -20°C for 7 days and FT-IR spectrum was obtained.