Spectroscopic Probes of Protein Dynamics: Hemoglobin and Myoglobin

NC State University
The iron in heme is the binding site for oxygen and peroxide.

Heme is iron protoporphyrin IX.

Functional aspects in Mb.
The iron in heme is the binding site for oxygen and peroxide.

Heme is iron protoporphyrin IX.

Functional aspects in Mb

1. Discrimination against CO binding.
The iron in heme is the binding site for oxygen and peroxide.

Heme is iron protoporphyrin IX.

Functional aspects in Mb

1. Discrimination against CO binding.

2. O$_2$ is the physiologically relevant ligand, but it can oxidize iron (autooxidation).
Myoglobin

PDB: 1A6G

SCOP
Class: All α proteins
Superfamily: Globin-like
Family: Globins

Vojtechovsky, Berendzen, Schlicting
The substrate of DHP binding site

4-iodophenol

Lebioda et al., J.Biol.Chem. (2000) 275, 18712
Catalytic activity in a hemoglobin

DHP + DBQ + H₂O
Dibromo Quinone

DHP + TPB + H₂O₂
Tribromo phenol

Absorbance (a.u.)

Wavelength (nm)
Conformational Substates

Proteins can assume a huge number of slightly different structures (conformational substates), which can be represented by nearly isoenergetic minima in an energy landscape with a typical barrier height of 10 kJ/mol.

What is the functional role of these substates?
Enzymatic catalysis involves lowering of the energy barrier

Protein fluctuations are certainly required for catalysis. However, the important question is: How is specificity built into the catalytic mechanism?
Non-equilibrium relaxation

Protein-substrate, protein-protein and protein-DNA interactions usually do not occur under equilibrium conditions in living systems.

Non-equilibrium relaxations from one state to another occur in many situations in biology:

Ligand binding and transport
Signaling
Electron Transfer
Enzymatic Catalysis
Protein Folding
MbCO

The peptide backbone is shown as a ribbon that follows the a-helical structure of myoglobin.

The structure shown is at equilibrium.

Conformational substates are called A states.

Teng, Srajer, Moffat
Mb:CO
The photoproducrt.

Iron moves out of the heme plane when CO is photolyzed.

CO moves to a docking site and is parallel to the heme plane.

Conformational substates are called B states.
Deoxy Mb

The deoxy structure has no CO ligand.

The protein backbone has shifted to permit a water to enter the distal pocket.

This form is often referred to an S state.
A comparison of three structures shows the changes in both proximal and distal histidines.

Protein Data Bank 1A6N 1AJG 1AJH
Deoxy Mb

\[ \text{Mb:CO} \]

\[ \text{MbCO} \]

These structures represent the three states of a kinetic scheme.

Teng, Srajer, Moffat
Absorption Spectroscopy: Introduction to Vibronic Coupling
Porphine orbitals

e\_g

\(a\_2u\)

\(e\_g\)

\(a\_1u\)
The four orbital model is used to represent the highest occupied and lowest unoccupied MOs of porphyrins.

The two highest occupied orbitals \((a_{1u}, a_{2u})\) are nearly equal in energy. The \(e_g\) orbitals are equal in energy. Transitions occur from:

\[ a_{1u} \rightarrow e_g \text{ and } a_{2u} \rightarrow e_g. \]
The transitions from ground state $\pi$ orbitals $a_{1u}$ and $a_{2u}$ to excited state $\pi^*$ orbitals $e_g$ can mix by configuration interaction.

Two electronic transitions are observed. One is very strong (B or Soret) and the other is weak (Q). The transition moments are:

$M_B = M_1 + M_2$

$M_Q = M_1 - M_2 \approx 0$
The porphine ring is an aromatic ring that has a fourfold symmetry axis.

The ring and metal can be considered separately. The ring has been successfully modeled using the Gouterman four orbital model. In globins the iron is Fe(II) and can be either high spin or low spin. MbCO ------ low spin. Deoxy Mb - high spin.
There are four excited state configurations possible in $D_{4h}$ symmetry. These are denoted B (strong) and Q(weak).

$$|B^0_y\rangle = \frac{1}{\sqrt{2}} \left( a_{2u} e_{gy} + a_{1u} e_{gx} \right)$$

$$|Q^0_y\rangle = \frac{1}{\sqrt{2}} \left( a_{2u} e_{gy} - a_{1u} e_{gx} \right)$$

$$|B^0_x\rangle = \frac{1}{\sqrt{2}} \left( a_{2u} e_{gx} + a_{1u} e_{gy} \right)$$

$$|Q^0_x\rangle = \frac{1}{\sqrt{2}} \left( a_{2u} e_{gx} - a_{1u} e_{gy} \right)$$
The absorption cross section

The absorption cross section for a Franck-Condon active transition is proportional to:

\[
\frac{\langle i | e \sigma | f \rangle^2}{E_f - E_i - \hbar \omega - i \Gamma_f}
\]

Here i and f represent individual vibrational levels in each electronic manifold. The polarization can be \( \sigma = x, y, \) or z.
The Herzberg-Teller expansion

The Herzberg-Teller expansion of a particular vibronic level is given by:

\[ |n_v\rangle = |n\rangle^0|v\rangle + \sum_r \sum_K \frac{\langle r| \left( \frac{\partial H}{\partial Q_K} \right) |n\rangle \langle u|Q_K|v\rangle}{E_{n,v} - E_{r,u}} |r\rangle|u\rangle \]

This expansion describes how electronic states \( n \) and \( r \) can mix by virtue of distortions of the molecular geometry.
Vibronic matrix elements

Interstate Herzberg-Teller coupling:
\[
\left\langle B^0_y \left| \frac{\partial H}{\partial Q} \right| Q^0_y \right\rangle = \left\langle a_{2u} \left| \frac{\partial H}{\partial Q} \right| a_{2u} \right\rangle
\]
\[
- \left\langle a_{1u} \left| \frac{\partial H}{\partial Q} \right| a_{1u} \right\rangle + \left\langle e_{gy} \left| \frac{\partial H}{\partial Q} \right| e_{gy} \right\rangle - \left\langle e_{gx} \left| \frac{\partial H}{\partial Q} \right| e_{gx} \right\rangle
\]

Intrastate Jahn-Teller coupling:
\[
\left\langle Q^0_y \left| \frac{\partial H}{\partial Q} \right| Q^0_y \right\rangle = \left\langle a_{1u} \left| \frac{\partial H}{\partial Q} \right| a_{1u} \right\rangle
\]
\[
+ \left\langle a_{2u} \left| \frac{\partial H}{\partial Q} \right| a_{2u} \right\rangle + \left\langle e_{gy} \left| \frac{\partial H}{\partial Q} \right| e_{gy} \right\rangle + \left\langle e_{gx} \left| \frac{\partial H}{\partial Q} \right| e_{gx} \right\rangle
\]
Vibronic theory of absorption

The extinction coefficient is proportional to the square of the transition moment:

\[ \varepsilon = \sum_v \left( \frac{\langle i | e\sigma | f \rangle^2 \langle 0 | \nu \rangle^2}{E_{f'} - E_{i0} - \hbar \omega - i\Gamma_f} + \left( \sum_r \sum_K \frac{\langle r | \left( \frac{\partial H}{\partial Q_K} \right) | f \rangle \langle u | Q_K | \nu \rangle \langle i | e\sigma | r \rangle}{E_{f',v} - E_{r,u}} \right)^2 \langle 0 | u \rangle^2 \right) \]

FC part

Vibronic part
Transition moments in the four orbital model with configuration interaction

\[ r^0_\sigma \equiv \langle G|e\sigma|Q^0_\sigma \rangle \]
\[ R^0_\sigma \equiv \langle G|e\sigma|B^0_\sigma \rangle \]

The mixing of the B and Q states is represented by the angle \( \alpha \):

\[ r_\sigma = \langle G|e\sigma|Q_\sigma \rangle = \cos(\alpha)\langle G|e\sigma|Q^0_\sigma \rangle + \sin(\alpha)\langle G|e\sigma|B^0_\sigma \rangle \]
\[ = \cos(\alpha)r^0_\sigma + \sin(\alpha)R^0_\sigma \]
\[ R_\sigma = \langle G|e\sigma|B_\sigma \rangle = \cos(\alpha)\langle G|e\sigma|B^0_\sigma \rangle + \sin(\alpha)\langle G|e\sigma|Q^0_\sigma \rangle \]
\[ = \cos(\alpha)R^0_\sigma + \sin(\alpha)r^0_\sigma \]
The B and Q bands for B$_{1g}$ and B$_{2g}$ symmetry vibronic matrix elements

\[ \varepsilon = R_{\sigma}^{02} \left( \frac{\sin^2(\alpha)}{(E_{Q0} - \hbar \omega)^2 + \Gamma_Q^2} + \frac{\cos^2(\alpha)}{(E_{B0} - \hbar \omega)^2 + \Gamma_B^2} \right) \]

\[ + R_{\sigma}^{02} b_g^2 \left( \frac{\sin(\alpha)\sin(2\alpha)}{\hbar \omega_K} + \frac{\cos(\alpha)\cos(2\alpha)}{E_Q^0 - E_B^0 + \hbar \omega} \right) \left( \frac{\cos(\alpha)\sin(2\alpha)}{\hbar \omega_K} + \frac{\sin(\alpha)\cos(2\alpha)}{E_Q^0 - E_B^0 - \hbar \omega} \right) \]

Franck-Condon part

Herzberg-Teller part
Vibronic lineshapes as a function of angle $\alpha$ for $B_{1g}$ vibronic modes
Vibronic lineshapes for $B_{1g}$ modes as a function of vibronic strength ($\alpha = 1^\circ$)
Vibronic lineshapes for $B_{1g}$ modes as a function of vibronic strength ($\alpha = 15^\circ$)
The B and Q bands for $A_{2g}$ symmetry vibronic matrix elements

\[ \epsilon = R_{\sigma}^0 \left( \frac{\sin^2(\alpha)}{(E_{Q0} - \hbar \omega)^2 + \Gamma_Q^2} \right) + \frac{\cos^2(\alpha)}{(E_{B0} - \hbar \omega)^2 + \Gamma_B^2} \]

\[ + R_{\sigma}^0 a_{2g}^2 \left( \frac{\cos^2(\alpha)}{(E_Q^0 - E_B^0 + \hbar \omega)^2 + \Gamma_Q^2} \right) + \frac{\sin^2(\alpha)}{(E_{Q1} - \hbar \omega)^2 + \Gamma_Q^2} \]

Franck-Condon part

Herzberg-Teller part
Vibronic lineshapes for $A_{2g}$ modes as a function of vibronic strength ($\alpha = 15^\circ$)
Ligand recombination is a sum of single exponential processes at room temperature.

\[ S(t) = \Phi_{ge}e^{-(k_{gem} + k_{esc})t} + \Phi_{bi}e^{-k_{bi}t} \]

Difference spectrum from nanosecond transient absorption spectroscopy.
The heme iron center moves out of the heme plane and the porphyrin macrocycle domes upon deligation of CO.
The ligation of CO changes the spin state of the heme iron

Low spin Fe(II)  

S = 0

High spin Fe(II)  

S = 2

$S = 0$

$S = 2$

$d_{z^2}$

$d_{x^2-y^2}$

$d_{xz}, d_{yz}$

$d_{xy}$

$d_{x^2-y^2}$

$d_{z^2}$

$d_{yz}$

$d_{xy}$

$d_{xz}$
Absorption spectra for MbCO and deoxy Mb
Infrared Spectroscopy
Structural heterogeneity in myoglobin has been proposed based on the observation of multiple CO stretching bands

- The bands are observed at 1966 cm\(^{-1}\), 1945 cm\(^{-1}\), and 1927 cm\(^{-1}\).
- The heterogeneity has been attributed to conformational substates.
- Rebinding to each substate is also observed to be non-exponential.
- Are there many relevant tiers of substates?
The origin of the A states is the hydrogen bonding conformations to CO.
Infrared spectra of myoglobin

Protein Data Bank 2MGK
Wild type Mb IR spectra show multiple bands

Quillen, Phillips et al.
JMB 1993, 234, 140-155
Mutants at the V68 position also show multiple bands
The H64V mutant shows a single IR band.

![Graph and molecular structure showing absorbance peaks and frequency analysis.](image)
DFT calculation of $\nu_{\text{CO}}$ frequencies

Multiple hydrogen bonding interactions
DFT calculation of $\nu_{\text{CO}}$ frequencies

Single hydrogen bonding interaction
DFT calculation of $\nu_{\text{CO}}$ frequencies

No hydrogen bonding interaction
DFT calculation in an applied electric field

Franzen JACS (2002) 124, 13271
DFT calculation of $\nu_{\text{CO}}$ frequencies

Stark tuning rate is 2.4 cm$^{-1}$/MV/cm. This is value predicted from correlations shown on right. Park and Boxer JPC 1999, 103, 9013
Cryogenic Spectroscopy
At less than 10K photolyzed Mb*CO recombines by nuclear tunneling

\[ k = A e^{-\frac{H_{BA}}{RT}} \]
Current hypothesis

The iron coordinate controls the barrier to CO rebinding and explains the distribution of energy barriers.

Austin et al. Biochemistry 1975, 14, 5355
Iron relaxation hypothesis rests on the assignment of charge transfer band III

Experimental observation:

Mb*CO at $t_1$

Steinbach et al. Biochemistry 1991, 30, 3988
Iron relaxation hypothesis rests on the assignment of charge transfer band III

Experimental observation:

$\text{Mb}^*\text{CO at } t_2$

Steinbach et al. Biochemistry 1991, 30, 3988
Iron relaxation hypothesis rests on the assignment of charge transfer band III

Experimental observation: Kinetic holeburning

\[ \text{Mb}^*\text{CO} \ t_2 - t_1 \]

Steinbach et al. Biochemistry 1991, 30, 3988
Assignment of band III

The interpretation of the data depends critically on the assignment of band III.

Band III has been assigned based on single crystal absorption data.

However, there have been problems with the interpretation from the very earliest experiments.
Interpretation of kinetic hole burning

Conformational substates are correlated with the band III maximum and the enthalpy of rebinding and the rate constant distribution.

Steinbach et al. Biochemistry 1991, 30, 3988
Ultrafast Spectroscopy
Ligand dynamics, protein dynamics, and fast recombination can be monitored by ultrafast spectroscopy.

- **Nd:YLF Laser**
- **Mode locked Ti:sapphire Laser**
- **Regenerative amplifier**
- **BBO**
- **Continuum**
- **Delay line**
- Frequency doubled 100 fs pulses 1000 Hz

Time resolution from 100 fs to nanoseconds.

MbCO Sample
Ultrafast near-infrared spectroscopy shows that a protein relaxation follows photolysis.

Band III at 10 ps, 10 ns, 100 ns, and 10 µs

Non-exponential band III decay in buffer solution $\eta = 1\text{cp}$

Jackson, Lim, Anfinrud
Chem. Phys. 1994, 180, 131-140
Ultrafast mid-infrared shows CO trapping in the distal pocket

Two CO orientations in infrared bands $B_1$ and $B_2$

Spectral bands are rotamers of CO in Mb

Nature structural Biology
Lim, Jackson, Anfinrud, 1997, 4, 209
What is the structure origin of the viscosity dependent protein relaxation?

The heme iron out-of-plane motion could change heme spectra

- band III $a_{1u}, a_{2u} \rightarrow d\pi$ charge transfer band and therefore its frequency maximum is thought to depend on the heme iron position.

- re-binding enthalpy $H$ is thought to depend on the heme iron position and there is a connection between $H$ and the position of band III (kinetic hole burning).
The iron hypothesis

“The red shift of the Soret band and band III by the same amount $\Delta \nu = 140 \text{ cm}^{-1}$ suggests that changes in a single coordinate affect both the Soret band and band III. We expect that the primary coordinate associated with the heme relaxation from Mb* to Mb involves heme doming, characterized by the iron out-of-plane displacement.”

Srajer and Champion *Biochemistry* (1991), 30, 7390

“The similarity of the deoxyheme (Soret) spectral changes to those observed for hemoglobin suggests that they correspond to a displacement of the iron relative to the heme plane that is coupled to a protein conformational change on the proximal side of the heme.”

The iron hypothesis

“As the effective coordinate involved in the time-dependent barrier (responsible for non-exponential NO rebinding kinetics), we focus on the Fe-heme distance. It is, in turn, modulated by the protein relaxation. The latter is the major factor that regulates the distribution of geminate rebinding rates over a picosecond time scale at room temperature.”

Petrich, Karplus, and Martin Biochemistry (1991), 30, 3986
What structural feature is responsible for the relaxation?
An alternative view of relaxation

Protein relaxation involves CO docking \textit{not} the iron out-of-plane motion.

1. Iron out-of-plane motion occurs in < 2 ps.
2. NO recombination is biexponential. The two components are due to the rotamers of NO.
3. The Soret band and band III shift couple to distal mutations, and are not coupled to proximal mutations.
4. Band III is a vibronically coupled charge transfer band! Band III is coupled exclusively to non-totally symmetric modes.
5. MCD spectra are consistent with vibronic coupling model.
6. Temperature dependent $\nu_{Fe-L}$ mode is consistent with anharmonic coupling and not conformational substates in deoxy Mb.
Ultrafast NO recombination kinetics show an acceleration in rate with increasing $\eta$

Maximum entropy method
1. Two populations
2. Tends towards a single population at high $\eta$

Picosecond kinetics of photolyzed MbNO

Shreve, Franzen, and Dyer
JPC 1999, 103,7969
FTIR data show that there are two populations of NO.

Photoprocess states can interconvert even at 10 K.

The lower wavenumber band corresponds to a lower barrier.

Miller, Chance et al. Biochemistry 1997, 40, 12199
Protein relaxation can be monitored by Soret band and Band III frequency shifts

Comparison of time-dependent frequency shifts

Band III shift
Observed in 75% glycerol/buffer

Soret band shift
\( \eta = 30 \text{ cP at } 290 \text{ K} \)
\( \eta = 300 \text{ cP at } 250 \text{ K} \)

Franzen and Boxer
JBC 1997, 272, 9655
Time-resolved absorption spectroscopy shows protein relaxation following photolysis does not depend on the proximal ligand.

MbCO in 75% glycerol-buffer solution.

Franzen and Boxer JBC 1997, 272, 9655
Near-infrared bands of heme in deoxy myoglobin

Band III

Magnetic Circular Dichroism

Absorption

Circular Dichroism

Single crystal polarized absorption spectra

Eaton et al. JACS 1978, 100, 4991
Near-infrared charge transfer bands of the heme in deoxy myoglobin

Eaton et al. JACS 1978, 100, 4991

<table>
<thead>
<tr>
<th>band</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
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<td>10 900</td>
<td>12 250</td>
<td>13 200</td>
<td>15 150</td>
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<td>290</td>
<td>310</td>
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<td>150</td>
<td>160</td>
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<td>160</td>
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<td>130</td>
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<td>polarization</td>
<td>$z$</td>
<td>mostly $z$</td>
<td>in-plane</td>
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</table>
Raman Spectroscopy
A resonance Raman spectrum is obtained by laser light scattering. Inelastic light scattering produces a frequency shift. There is exchange of energy between the vibrations of the molecule and the incident photon.
Resonance Raman spectrum for excitation of heme Soret band

![Graph showing Resonance Raman spectrum for excitation of heme Soret band](image-url)
Soret (B) band Resonance Raman spectra of MbCO and Deoxy Mb

![Figure showing Raman spectra and molecular structures](image-url)

- **Raman Intensity**
- **Raman Shift (cm⁻¹)**
- **ν₈**, **ν₁₈**, **Fe-His**, **Fe-C**, **ν₇**

- **Deoxy Mb** (blue)
- **MbCO** (red)
B band Resonance Raman spectra of MbCO and Deoxy Mb
The cooperative R - T switch relies on iron displacement to communicate between $\alpha$ and $\beta$ subunits.

Hemoglobin is composed of two $\alpha$ and two $\beta$ subunits whose structures resemble myoglobin.

Ultrafast resonance Raman spectroscopy shows that heme doming occurs in \( \approx 1 \) ps.

Equilibrium HbCO

Difference spectra obtained by subtraction of the red spectrum from spectra obtained at the time delays shown.

The frequency of the iron-histidine vibration shows strain in T state

The comparison of photolyzed HbCO in the R state and the equilibrium T state.
Hb*CO at 10 ns
Fe-His = 230 cm⁻¹
Deoxy Hb
Fe-His = 216 cm⁻¹
The lower frequency indicates weaker bonding interaction and coupling to bending modes.
The motion of the F-helix tugs on the proximal histidine and introduces strain.

The frequency lowering in the T state arises from weaker Fe-His ligation and from anharmonic coupling introduced by the bent conformation of the proximal histidine.

R state
The frequency lowering in the T state arises from weaker Fe-His ligation and from anharmonic coupling introduced by the bent conformation of the proximal histidine.
Time-resolved resonance Raman can follow the R - T structure change

Strain is introduced in stages as intersubunit contacts are made. Based on the x-ray data it was proposed that the iron displacement from the heme plane is a trigger for the conformational changes.

Scott and Friedman JACS 1984, 106, 5877
Many Peroxidases belong to the Cytochrome c Peroxidase family

PDB: 1A2F
Cytochrome c Peroxidase (CCP)
Class: All $\alpha$ proteins
Superfamily: Heme peroxidases
Family: CCP-like

PDB: 2ATJ
Horseradish Peroxidase (HRP)
Class: All $\alpha$ proteins
Superfamily: Heme peroxidases
Family: CCP-like

Goodin and McCree Scripps Institute
Dehaloperoxidase is a peroxidase that belongs to the globin family

**PDB: 1A6G**
Myoglobin (Mb)
Class: All α proteins
Superfamily: Globin-like
Family: Globins

Vojtechovsky, Berendzen, Schlichting

**PDB: 1EW6**
Dehaloperoxidase (DHP)
Class: All α proteins
Superfamily: Globin-like
Family: Globins

Comparison of DHP and Mb Structures

比起DHP和Mb结构
Resonance Raman spectroscopy of iron-histidine stretching mode of deoxy dehaloperoxidase

The frequency of the Fe-His mode is intermediate between that of myoglobin (HHMb) and horseradish peroxidase (HRP).

Franzen et al., JACS (1998), 120, 4658-4661
Model for proximal hydrogen bonding

Peroxidase Catalytic Triad
Asp-His-Fe

This is the “push”
In peroxidase mechanism

Franzen JACS 2001,123, 12578
Model for proximal hydrogen bonding

Dehaloperoxidase Catalytic Triad
C=O-His-Fe?

Hydrogen bond strength is intermediate between Mb and HRP/CcP.

Franzen JACS 2001, 123, 12578
General expression for Raman scattering transition polarizability

The transition polarizability involves an electric field interaction for the incident wave $\sigma$ and the scattered wave $\rho$:

$$\left( \alpha_{\rho\sigma} \right)_{if} = \sum_n \frac{\langle i|e\sigma|n \rangle \langle n|e\rho|f \rangle}{E_n - E_f - \hbar\omega - i\Gamma_n}$$
Raman scattering transition polarizability for vibronic bands

Substituting in the H-T expansion we have for $\sigma$:

$$
\left(\alpha_{\rho \sigma}\right)_{01} = \sum \frac{\langle i0|e\sigma|r0 \rangle \langle r0|(\partial H/\partial Q)|n1 \rangle}{E_{r0} - E_{n1}} \frac{\langle 1|Q|0 \rangle \langle n1|e\rho|i1 \rangle}{E_{n1} - E_{i0} - \hbar\omega - i\Gamma_n}
$$

and for $\rho$:

$$
\left(\alpha_{\rho \sigma}\right)''_{01} = \sum \frac{\langle i0|e\sigma|n0 \rangle \langle 0|Q|1 \rangle \langle r1|(\partial H/\partial Q)|n0 \rangle}{E_{r1} - E_{n0}} \frac{\langle r1|e\rho|i1 \rangle}{E_{n0} - E_{i0} - \hbar\omega - i\Gamma_n}
$$
Vibronic matrix elements for B\textsubscript{1g} and B\textsubscript{2g} vibronic modes

\[
\begin{align*}
\langle \alpha_{xy}^{bg} \rangle &= - b_g R_\sigma^{02} \\
&= \left( \frac{\sin^2(\alpha)\sin(2\alpha)}{\hbar \omega_K} + \frac{\sin(\alpha)\cos(\alpha)\cos(2\alpha)}{E_{B_0} - E_{Q_0} + \hbar \omega_K} \right) - \frac{\cos^2(\alpha)\sin(2\alpha)}{\hbar \omega_K} + \frac{\sin(\alpha)\cos(\alpha)\sin(2\alpha)}{E_{B_0} - E_{Q_0} - \hbar \omega_K} \\
&= \left( \frac{\sin(\alpha)\cos(\alpha)\sin(2\alpha)}{E_{B_0} - E_{Q_0} - \hbar \omega_K} - \frac{\sin^2(\alpha)\sin(2\alpha)}{\hbar \omega_K} \right) \left( \frac{\cos^2(\alpha)\sin(2\alpha)}{\hbar \omega_K} - \frac{\sin(\alpha)\cos(\alpha)\sin(2\alpha)}{E_{B_0} - E_{Q_0} + \hbar \omega_K} \right) \\
&= \left( \frac{\sin(\alpha)\cos(\alpha)\sin(2\alpha)}{E_{B_1} - \hbar \omega} + \frac{\cos^2(\alpha)\sin(2\alpha)}{\hbar \omega_K} + \frac{\sin(\alpha)\cos(\alpha)\sin(2\alpha)}{\hbar \omega_K} \right) - \frac{\cos^2(\alpha)\sin(2\alpha)}{\hbar \omega_K} \left( \frac{\sin(\alpha)\cos(\alpha)\sin(2\alpha)}{E_{B_0} - E_{Q_0} - \hbar \omega_K} \right)
\end{align*}
\]
Vibronic Raman excitation profile as a function of angle \( \alpha \) for \( B_{1g} \) modes.

Predicted Jahn-Teller enhancement.
Vibronic Raman excitation profile for the Q band for $B_{1g}$ modes
Vibronic Raman excitation profile as a function of vibronic strength of $B_{1g}$ modes for $\alpha = 1^\circ$
Vibronic Raman excitation profile as a function of vibronic strength of $B_{1g}$ modes for $\alpha = 15^\circ$
Vibronic matrix element for $A_{2g}$ vibronic modes

$$
(\alpha_{xy}^{a_{2g}}) = - a_{2g} R_\sigma^{02} \cos(\alpha) \sin(\alpha)
$$

$$
\left( \frac{1}{E_{B0} - E_{Q0} + \hbar \omega_K} + \frac{1}{E_{B0} - E_{Q0} - \hbar \omega_K} \right) + \frac{1}{E_{B0} - \hbar \omega - i \Gamma_B} - \frac{1}{E_{Q1} - \hbar \omega - i \Gamma_Q} - \frac{1}{E_{B1} - \hbar \omega - i \Gamma_B}
$$

The $A_{2g}$ modes can be easily assigned from the depolarization ratio. These modes are predicted to have $\rho = \infty$. Experimentally, large $\rho >> 0.75$ are observed.
Vibronic Raman excitation profile as a function of vibronic strength of $A_{2g}$ modes for $\alpha = 15^\circ$
Raman spectra for the Soret, Q, and III

Franzen et al. JACS (2002) 124, 7146
Q band Resonance Raman spectra

\[ \nu_4 = 1357 \text{ cm}^{-1} \]
\[ \nu_{19} = 1561 \text{ cm}^{-1} \]
\[ \nu_{11} = 1546 \text{ cm}^{-1} \]
\[ \nu_{10} = 1610 \text{ cm}^{-1} \]

\[ \nu_{13} = 1211 \text{ cm}^{-1} \]
\[ \nu_3 = 1472 \text{ cm}^{-1} \]
\[ \nu_{28} = 1523 \text{ cm}^{-1} \]

514.5 nm
565 nm
575 nm
585 nm
595 nm

Raman Shift (cm\(^{-1}\))
REPs of $B_{1g}$ modes

![Graph showing absorbance vs wavenumber for $B_{1g}$ modes with peaks at 1546 cm$^{-1}$, 1610 cm$^{-1}$, and 1211 cm$^{-1}$]

- $v_{11}$
- $v_{10}$
REPs of $A_{2g}$ modes

$\nu_{19}$

$\nu_{20}$

$\nu_{21}$

Absorbance / REP

Wavenumber (cm$^{-1} \times 10^3$)
Band III resonance Raman spectra

Franzen et al. JACS (2002) 124, 7146
REP of high frequency band III modes

Franzen et al. JACS (2002) 124, 7146
REP of high frequency band III modes

Franzen et al. JACS (2002) 124, 7146
Significance of Mb substates

Conformational substates must include functional tests for specific functional states.

The biological role of myoglobin is consistent with relaxation and conformational states of the distal pocket.

There are specific conformational states that are important
1. The docking site (transient)
2. The distal histidine interaction (closed form)
3. The open form

Inhomogeneous broadening is a background that is always present.
The 150 cm$^{-1}$ $B_{1g}$ mode is $\nu_{18}$ in MbCO and a mixed $\nu_{18}, \gamma_{16}$ mode in deoxy Mb
The side view reveals significant out-of-plane character for $\nu_{18}$ in deoxy Mb.
Magnetic Circular Dichroism
The Perimeter Model

The porphine ring has D_{4h} symmetry. The aromatic ring has 18 electrons. The p system approximates circular electron path.

\[ \Phi = \frac{1}{\sqrt{2\pi}} e^{im\phi} \]
MCD spectra

\[ \Delta \varepsilon(\nu) = A_1 \left( - \frac{\partial f(\nu)}{\partial \nu} \right) + \left( B_0 + \frac{C_0}{k_BT} \right)f(\nu) \]

\[ \frac{\Delta \varepsilon_m(\nu)}{\varepsilon(\nu)} = \frac{A_1 \mu_B}{D_0} \frac{\left( - \frac{\partial f(\nu)}{\partial \nu} \right)}{f^*} \]

\[ \Delta L_z = 2 \frac{A_1}{D_0} \]

Franzen, JPC Accepted
MbCO MCD spectra follow the PM

The spectra are A-term MCD as shown by the derivatives of the absorption spectrum (red). The \((Q \text{ MCD}) = 9 \times (B \text{ MCD})\). Franzen, JPC Accepted
Deoxy MCD spectra are anomalous

C-term 4 time larger than MbCO!  A-term but with vibronic structure
MCD spectra: Vibronic coupling in the Perimeter Model

Franzen, JPC Accepted
MCD spectra: Vibronic coupling in the Perimeter Model

Metal

| 1 = ±2 |
| 1 = ±1 |
| 1 = 0 |

Porphyrin

| CT |
| \( \pi - \pi^* \) |

Vibronic Distortions

- \( N = 0 \)
- \( N = 1 \)
- \( N = 2 \)

B: \( 4N + 2 \)

A: \( 4N \)
Conclusions for band III

Although band III behaves as a ring-to-metal charge transfer band it is vibronically coupled to the Soret band. It is not the iron-histidine mode that is coupled to the transition, but rather in-plane non-totally symmetric modes.

1. The polarization arises from vibronic coupling to in-plane non-totally symmetric modes.

2. The small charge displacement results from strong mixing of III with the Soret band.

3. The temperature dependence arises from thermal population of the $B_{1g}$ mode $\nu_{18}$ at 150 cm$^{-1}$. 
Anharmonic Coupling
Temperature dependence of the H93G(Im) axial mode

Franzen, Fritsch, Brewer JPC Accepted

![Graph showing temperature dependence of the H93G(Im) axial mode.](image)

Franzen, Fritsch, Brewer JPC Accepted
Temperature dependence of the H93G(2-Me Im) axial mode
Temperature dependence of the H93G(H₂O) axial mode
Differences in anharmonicity for axial ligands

Experimental approach shows that axial ligands differ.

Horse heart is closest to H93G(4-Me Im).

H93G(2-Me Im) is the most anharmonic.
DFT calculated mode-mode anharmonic coupling

Comparison with experiment

Frequency shift of axial-ligand mode calculated for displacement of iron doming mode.

Only H93G(1-Me Im) excluded because of Fermi resonance.
Iron-ligand stretching and doming modes

$\nu_{\text{Fe-L}}$

4-Me Im

143 cm$^{-1}$

1-Me Im

161 cm$^{-1}$

$\nu_{\text{Fe-doming}}$

77 cm$^{-1}$

81 cm$^{-1}$
Iron-ligand stretching and doming modes

$\nu_{\text{Fe-L}}$

Im

$170 \text{ cm}^{-1}$

$143 \text{ cm}^{-1}$

$\nu_{\text{Fe-doming}}$

4-Br Im

$87 \text{ cm}^{-1}$

$70 \text{ cm}^{-1}$
Significance of substates

Functional test is needed.

In spectroscopy solvation states give rise to inhomogeneous broadening.

Propose the same distinction for kinetics. Non-exponential kinetics in myoglobin is due to an inhomogeneous distribution.

The inhomogeneous linewidth depends on the environment and hence is not the same for all proteins.
Myoglobin function depends on specific distal dynamics

Prevent autooxidation: docking site

Discriminate against CO binding: histidine 64

Trigger for $O_2$ release: low pH form?

These functional criteria are all consistent with the alternative view. They all involve dynamics on the distal side.
A picture of the open and closed states of myoglobin

H64

pH 7
ν_{CO} = 1943 cm^{-1}
pH 5
ν_{CO} = 1967 cm^{-1}

Protein Data Bank 1VXC, 2MGK
Yang and Phillips JMB 1996, 256, 762