Investigations of Protein Induced Heme Distortion Using Raman and Vibrational Coherence Spectroscopy

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A dissertation submitted to

The Faculty of
the College of Science of
Northeastern University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

December 5, 2013

Dissertation directed by

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Acknowledgements

This thesis is a product of many years’ work. It would have been impossible to accomplish without the help and support from my committee members, my colleagues, my friends, and my family.

First of all, I would like to express my deepest gratitude to my advisor, Professor Paul Champion for his excellent guidance, patience, and support. I am very thankful to Professor Tim Sage and Professor Armen Stepanyants for serving as my thesis committee member and providing their kind help and useful comments regarding my dissertation.

I would like to thank my colleagues and my friends for their generous help and support: Dr. Abdelkrim Benabbas, Dr. Weiqiao Zeng, Dr. Zhengyu Zhang, Dr. Venugopal Karunakaran, Dr. Minoru Kubo, Yunbin Zhang, Dr. Fei Wang, and Dr. Jingyun Zhang.

Most of all, I would like to thank my wife, Chen Lu, and our parents for their love and enormous support through the years of my PhD study.
Abstract of Dissertation

This thesis focuses on studying the protein induced heme out-of-plane distortion and its impact on heme reactivity. Using optical absorption spectroscopy, resonance Raman spectroscopy, and femtosecond vibrational coherence spectroscopy (VCS), we studied low frequency spectral density changes upon equilibrium unfolding of ferric cytochrome c, the effect of heme out-of-plane deformation on its electron transfer rate in cytochrome c, ligand switching associated with the inactive form of cytochrome p450, and oxygen binding kinetics in H-NOX.

General background knowledge regarding heme proteins’ structures and biological functions is provided in chapter 1. Chapter 2 describes the femtosecond pump-probe vibrational coherence spectroscopy, and the data analysis method to process the VCS signal.

In chapter 3, we studied the equilibrium unfolding process of ferric horse heart cytochrome c (cyt c), induced by guanidinium hydrochloride (GdHCl). This unfolding was systematically studied using UV-vis absorption spectroscopy, resonance Raman spectroscopy and vibrational coherence spectroscopy (VCS). The unfolding process was successfully fit using a three-state model, which included the fully folded (N) and unfolded (U) states, along with an intermediate (I) assigned to a Lys bound heme. The VCS spectra revealed for the first time several low frequency heme modes that are sensitive to cytochrome c unfolding: $\gamma_a$ (~50 cm$^{-1}$), $\gamma_b$ (~80 cm$^{-1}$), $\gamma_c$ (~100 cm$^{-1}$), and $\nu_s$(His-Fe-His) at 205 cm$^{-1}$. These out-of-plane modes have potential functional relevance and are activated by protein-induced heme distortions. The free energies for the N-I and the I-U transitions at pH 7.0 and 20°C were found to be 4.6 kcal/M and 11.6 kcal/M, respectively. Imidazole was also introduced to replace the methionine ligand so the unfolding can be modeled as a two-state system. The intensity of the mode $\gamma_b$~80 cm$^{-1}$ remains nearly constant during the unfolding process, while the amplitudes of the other low frequency
modes track with spectral changes observed at higher frequency. This confirms that the heme deformation changes are coupled to the protein tertiary structural changes that take place upon unfolding. These studies also reveal that damping of the coherent oscillations depends sensitively on the coupling between heme and the surrounding water solvent.

In chapter 4, we studied the effect of heme out-of-plane deformation on its electron transfer rate in cytochrome c. The ruffling deformation of its heme cofactor has been suggested to relate to its redox properties and electron transfer rate. However, there is no direct experimental evidence demonstrating this correlation. In this work, we studied *Pseudomonas aeruginosa* (Pa) cytochrome c$_{551}$ and its F7A mutant. These two proteins, though similar in their x-ray crystal structure, display a significant difference in their heme out-of-plane (OOP) deformations, mainly along the ruffling coordinate. Resonance Raman and vibrational coherence measurements also indicate significant differences in ruffling-sensitive modes, particularly the low-frequency $\gamma_a$ mode found between $\sim$50-60 cm$^{-1}$. This supports previous assignments of $\gamma_a$ as having a large ruffling content. Measurement of the photoreduction kinetics finds an order of magnitude decrease of the photoreduction cross-section in the F7A mutant, which has nearly twice the ruffling deformation as the wild type. When the results of photoreduction in cytochrome c are also considered, it appears that heme ruffling has an exponential effect on the electron transfer rates that is consistent with a modulation of the transfer distance on the order of the heme dimension. Since other factors that could influence electron transfer rate appear to have a minimal influence in these proteins, this result provides direct evidence showing that the protein induced ruffling distortion can play a major role in controlling the electron transfer rate.

In chapter 5, we studied the inactive form of cytochrome p450. It is generally accepted that the inactive P420 form of cytochrome P450 (CYP) involves the protonation of the native
cysteine thiolate to form a neutral thiol heme ligand. On the other hand, it has also been suggested that recruitment of a histidine to replace the native cysteine thiolate ligand might underlie the P450→P420 transition. Here we discuss resonance Raman investigations of the H93G myoglobin (Mb) mutant in the presence of tetrahydrothiophene (THT) or cyclopentathiol (CPSH), and on pressure-induced cytochrome P420\textsubscript{cam} (CYP101), that show a histidine becomes the heme ligand upon CO binding. The Raman mode near 220 cm\(^{-1}\), normally associated with the Fe-histidine vibration in heme proteins, is not observed in either reduced P420\textsubscript{cam} or the reduced H93G Mb samples, indicating that histidine is not the ligand in the reduced state. The absence of a mode near 220 cm\(^{-1}\) is also inconsistent with a generalization of the suggestion that the 221 cm\(^{-1}\) Raman mode, observed in the P420-CO photoprodut of inducible nitric oxide synthase (iNOS), arises from a thiol-bound ferrous heme. This leads us to assign the 218 cm\(^{-1}\) mode observed in the 10 ns P420\textsubscript{cam}-CO photoprodut Raman spectrum to a Fe-histidine vibration, in analogy to many other histidine bound heme systems. Additionally, the inverse correlation plots of the \(\nu_{\text{Fe-His}}\) and \(\nu_{\text{CO}}\) frequencies for the CO adducts of P420\textsubscript{cam} and the H93G analogs provide supporting evidence that histidine is the heme ligand in the P420-CO bound state. We conclude that, when CO binds to the ferrous P420 state, a histidine ligand is recruited replacing the thiol (or water) heme ligand. The common existence of a HXC-Fe motif in many CYP systems allows the C→H ligand switch to occur with only minor conformational changes. One suggested scenario involves the addition of another turn in the proximal L helix so that the Cys ligand becomes part of the helix and the His residue from the adjoining loop region becomes the heme ligand in P420-CO. In other systems, such as iNOS and CYP3A4 (where the HXC-Fe motif is not found) a somewhat larger conformational change would be necessary to recruit a nearby histidine.
In chapter 6, we studied heme nitro oxide and/or oxygen binding domain (H-NOX). The heme cofactor in wild-type H-NOX shows the largest out-of-plane distortions observed to date, majorly due to the nonbonded contact of Pro115 with the heme. Mutation of Pro115 to Ala drastically reduces these heme distortions. Resonance Raman, optical absorption and vibrational coherence spectroscopies are used to investigate the mechanisms of NO and O2 binding to Tt-HNOX and its P115A mutant. Vibrational Coherence spectra of the oxy-complexes provide a clear evidence for the activation of an iron-histidine mode around 217 cm\(^{-1}\) following photoexcitation, indicating that O2 dissociates in both proteins. The quantum yield of O2 photolysis is very low, particularly in the wild type (<2%). Geminate recombination of O2 and NO in both proteins is very fast and highly efficient. This indicates that the distal heme pocket in these proteins is tightly packed, and forms an efficient trap, preventing the bound ligand from escaping into the solvent upon thermal dissociation. This, along with the low photolysis quantum yield, explains the unusually high O2 affinity in Tt H-NOX and its P115A mutant as compared to that in myoglobin.

Appendix A reports the hydration study on cytochrome c thin film. Appendix B provides a useful control experimental result for chapter 3. Appendix C reports VCS experimental result for green fluorescent protein.
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Chapter 1

General Introduction

Proteins are biological macromolecules that consist of a sequence of amino acids. They play a broad range of roles in most biological processes. Among them, heme proteins, which contain heme prosthetic groups, form a particularly important class of proteins that attract an enormous amount of research interests. Heme proteins perform a wide range of biological functions including oxygen transportation and storage\(^1, 2\), NO scavenger\(^3\), intracellular oxygen and iron\(^4, 5\), oxygen sensing\(^6\), activation of oxygen-containing molecules\(^7\), enzymatic transformations of organic molecules\(^8, 9\), drug detoxification\(^10\), NO synthesis and regulation\(^8, 9\), oxidative metabolism\(^7\), electron transfer, cellular signaling, apoptosis, and regulation of DNA expression\(^11\).

Heme consists of four pyrrole groups, which are interconnected by methine bridges to form a tetrapyrrole ring. The four nitrogen atoms in the center of the porphyrin ring covalently bind an iron atom, which can form two additional bonds, one on either side of the heme plane. The tetrapyrrole ring has attached to eight side groups: four methyl, two vinyl, and two propionate, which can be arranged in many ways. However, the most common configuration that is found in biological systems is protoporphyrin IX (figure 1.1 (heme b)).

There are several types of heme found in nature. The most common types of heme are heme b and heme c. Heme b is iron–protoporphyrin IX and binds noncovalently to protein, whereas heme c is characterized by the presence of two (rarely, one) covalent thioether bonds, formed between Cys side chains and the heme vinyl groups at positions 2 and 4. The stereochemistry of heme attachment is the same in all known examples of heme c, and the vinyl groups at positions 2 and 4 are attached to the N- and C-terminal Cys, forming a CXXCH motif,
respectively. This motif is a unique feature shared with nearly all c-type hemes(1). Other less common derivatives of heme include heme d1, presented in cytochrome cd1 nitrite reductase, heme a, found in cytochrome c oxidase, and the related heme o, found in some bacterial oxidases.(12)

Figure 1.1 Chemical structures of heme b (left) and heme c(right). The Fisher number system for heme substituents is shown for heme b. The curve in heme c represents a peptide segments. Usually 2 residues separate the two Cys, and His axil ligand follows the Cys attached to position 4.(12)

The diverse range of biological functions found for heme proteins is defined by the protein matrix surrounding the heme group. In this thesis, we focus on studying the protein induced heme out-of-plane distortion, and its impact on heme structure and reactivity. Three classes of heme proteins are investigated: cytochrome c (cyt c), cytochrome p450, and heme-NO and oxygen binding (H-NOX) domain.

Cytochrome c is an important charge transfer protein. Cyt c normally resides in the spaces within the cristae of the inner mitochondrial membrane (IMM) and is effectively sequestered by narrow cristae junctions. Within the IMM, cyt c participates in the mitochondrial
electron-transport chain, using its heme group as a redox intermediate to shuttle electrons between cyt c reductase and cyt c oxidase in the mitochondrial membrane, in order to facilitate respiration.\(^{(2, 3)}\) However, when the cell detects an apoptotic stimulus, such as DNA damage, metabolic stress, or the presence of unfolded proteins, the intrinsic apoptotic pathway is triggered, and mitochondrial cyt c is released into the cytoplasmic matrix \(^{(4-6)}\). In the early stages of apoptosis, cyt c acts as a catalyst for peroxidation of cardiolipin, which accounts for \(\sim 10\%\) of total mitochondria membrane. Cardiolipin peroxidation leads to permeabilization of the outer mitochondrial membrane, and releases cyt c and other proapoptotic proteins into the cytoplasm, which initiates the formation of the apoptosome and the caspase cascade that leads to cell death.\(^{(7, 8)}\)

Another important class of heme proteins investigated in this thesis is cytochrome p450 (CYP). The cytochrome P450s is a large family of enzymes, which are involved in drug metabolism, toxicity, xenobiotic degradation, and biosynthesis\(^{(9)}\). There are almost 4000 identified P450 genes discovered so far. The cytochrome P450s have been found in all branches of the “tree of life” that catalogs the diversity of life forms. The important metabolic role, together with the unique chemistry and physical properties of the cytochrome P450s, provides a strong attraction for scientists in many disciplines.

P450 enzymes share a common overall fold and topology (Figure 1.2), despite less than 20% sequence identity across the gene superfamily.\(^{(13)}\) The conserved P450 structural core is formed by a four-helix bundle, composed of three parallel helices labeled D, L, and I and one antiparallel helix E.\(^{(14)}\) The prosthetic heme group is confined between the distal I helix and proximal L helix, and bound to the adjacent Cys-heme-ligand loop, which contains the P450 signature amino acid sequence FXXGX(H or R)XCXG. The absolutely conserved cysteine is the
proximal or “fifth” ligand to the heme iron. This sulfur ligand is a thiolate and is the origin of the characteristic namegiving 450-nm Soret absorbance observed for the ferrous-CO complex. Typically, the proximal Cys forms two hydrogen bonds with neighboring backbone amides.

Figure 1.2 The fold of cytochrome P450s is highly conserved and shown in a ribbon representation (distal face). Substrate recognition sequence (SRS) regions are shown in black and labeled. R-Helixes mentioned in the text are labeled with capital letters.(15)

Heme nitro oxide and/or oxgen binding domain (H-NOX), is a newly discovered family of heme-based sensor proteins; widely existing in both aerobic and anaerobic bacteria. In eukaryotes, H-NOX domains form the heme domain of soluble guanylate cyclase (sGC), which is the prototype of mammalian NO sensors.(7) In prokaryotes, H-NOX proteins appear to fall into one of two classes. One type is a stand-alone protein most often found in a predicted operon with a histidine kinase and less frequently with diguanylate cyclase domain. The other class is fused to methyl-accepting chemotaxis domains in the same open reading frame(16-18). H-NOX domains from the majority of eukaryotes and facultative aerobic prokaryotes do not bind O2, but
they bind NO and form 5C complexes that are similar to SGC-NO, whereas H-NOX proteins from obligate aerobic prokaryotes, including Tr-HNOX, bind both NO and O₂ forming stable 6C complexes.(19, 20) Homology to sGC as well as genomic placement suggests that H-NOX domains in prokaryotes are likely to serve as sensors for gases such as O₂ and NO.

Optical spectroscopies techniques, including absorption, Raman and ultrafast pump-probe spectroscopies, have been served as very powerful tools to study structure and dynamics of heme proteins for decades. The optical properties of the heme proteins are mainly determined by the prosthetic heme cofactor. The optical absorption band of heme is defined by a strong Soret band, or B band, ranging from 360 nm to 460 nm, which peaks ~ 410 nm, and a weaker Q band located in the visible region (500–600 nm). Both B and Q bands are due to π→π* electronic transition of the porphyrin ring of the heme group, and are sensitive to the heme iron oxidation, spin, and ligation state.

Resonance Raman spectra has played an important role in investigation of heme proteins.(9) It can selectively enhance the vibrational modes of an absorbing chromophore without interference from the vibrational modes of the protein. This ability allows us to investigate the structure, the surrounding protein matrix, and electronic properties of the heme in functional states of the protein. The acquired spectra are rich with structural information. The frequencies and intensities of the spectral are sensitive to heme ligation, and the interactions with the associated protein fragments in the active site. But due to the strong absorbance, Rayleigh and quasi-elastic scattering of solvent, resonance Raman spectroscopy cannot reliably detect heme modes below ~150 cm⁻¹ in the aqueous phase.(21)

Development of ultrafast lasers, with pulse duration in the 5-100 fs range, has made it possible to induce and monitor coherent nuclear motions in the heme on femtosecond time scale.
This thesis is based on femtosecond vibrational coherence spectroscopy (VCS) that has a unique capability in observing the low frequency dynamics of molecular. Along with absorption and Raman spectroscopy, this work attempt to provide better understanding to interaction between heme and its surrounding protein matrix. The low frequency modes we observe below 200 cm\(^{-1}\) using VCS can have significant thermal populations, and their interaction with the thermal bath and the surrounding amino acids can be important to the reactivity of heme. Studies of these low-frequency modes can improve our understanding of how dynamic structures relate to protein function. Generally, functionally important heme modes, such as “doming” and “ruffling”, which lie in the low frequency region below 200 cm\(^{-1}\), are delocalized and involve many nuclei, possibly even mixing with other modes in the surrounding protein material. Resonance Raman and infrared spectroscopies cannot reliably detect heme modes below ~150 cm\(^{-1}\) in the aqueous phase, due to the strong absorbance, Rayleigh and quasi-elastic scattering\(^{(21)}\) of water. On the other hand, the self-heterodyne nature of the VCS experiment allows it to extract the low frequency vibrational modulations of the third order polarization of the heme. We have previously investigated the low-frequency modes of a variety of heme proteins, using Soret band excitation.\(^{(22-31)}\) Unlike the higher frequency modes (> 200cm\(^{-1}\)), the low frequency modes (which have weaker force constants) are more easily distorted from equilibrium by the protein surroundings. These modes are activated in VCS when the protein induces symmetry breaking nonplanar heme distortions.\(^{(29)}\) In addition, these modes take on a special functional significance because of their thermal accessibility. The low-frequency coherence spectra offer a unique window into how the surrounding protein environment can alter these important thermally active heme modes.

**References**

2. Operator's manual the coherent mira model 900f laser (1997)


Chapter 2

Experimental Methods

2.1 The ultrafast pump-probe laser system

Ultrafast pulse lasers have a wide range of usage in scientific research and industrial applications. Ultra short laser pulses make it possible to probe nature on the femtosecond time scales, allowing the time-resolved study of a number of ultrafast chemical, biological, and physical processes. The generation of ultrafast laser pulses relies on mode locking techniques, where the phases of oscillating longitudinal modes in laser cavity are locked together.

The Ti:Sapphire lasers are among the most widely used sources of ultrashort pulse lasers owing to their advantages over previous dye-based lasers: good thermal conductivity and large gain bandwidth.(1-3) By employing Ti:Sapphire gain medium, the pulse is generated with a passive mode-locking through a mechanism called optical Kerr effect(4, 5). This nonlinear effect describes that the refractive index of a dielectric medium is a function of the photon intensity, which can be written as \( n(I) = n_{\text{linear}} + n_{\text{non-linear}}(I) \), where \( n \) is the refractive index of the medium and \( I \) is the intensity of laser beam. Because of the non-uniform power density distribution of a Gaussian beam, the refraction index in the center is greater than at the edge. A gradient index lens is formed, which is known as optical Kerr effect. The Kerr lens changes the spatial profile of the laser beam through self-focusing. The self-focusing results in a higher round trip gain in the mode-locked pulses with high peak power than in the continuous wave (cw) with low peak power, due to an increased overlap between the pumped gain profile and the circulating cavity mode. An aperture is placed at a position in the laser cavity to increase the round trip loss of the cw mode. This results in lasing from only the mode-locked pulses. More complete and detailed descriptions of Ti:Sapphire laser are available elsewhere.(4-7)
**Figure 2.1** High signal to noise femtosecond vibrational coherence spectroscopy setup.

The femtosecond coherence vibrational spectroscopy (VCS) used in this thesis is shown in Fig. 2.1. Full description of this setup is available in ref (7). Briefly, the laser system consists of Ti:Sapphire self-mode-lock oscillator (Mira-900F, Coherent, Inc.) which is capable of generating femtosecond pulses (50-100 fs) with a variable carrier wavelength between 800 and 910 nm. Each pulse contains approximately 10 nJ of energy and the pulse repetition rate is 76 MHz. The degenerated pump and probe pulses used in the sample measurement need to be in resonance with the heme Soret absorption band (400-440 nm). These pulses are obtained by doubling the horizontal polarized IR pulse from MIRA using a 0.25 mm BBO crystal. A pair of SF10 prisms, which is placed after BBO crystal, is used to control the chirp induced by the optics in the optical path and minimize the total chirp before the pulse reaches the sample. With a beam splitter, the second harmonic vertical polarized light is split into pump and probe beams with a power ratio of 2:1. The delay between pump and probe is controlled by a motorized translation...
stage on the probe beam. The step size is 1 μM, and each step results in a 6.67 fs change in delay time.

For the measurement of heme samples, the absorption changes is small \((ΔI/I_0 \approx 10^{-4} - 10^{-5})\) upon the pump excitation. A lock-in amplifier technique\(^{15-17}\) is used in order to obtain a high signal to noise ratio (SNR) detection. The technique consists of modulating the pump beam using an acousto-optic modulator (AOM) (Neos Technologies) at approximately 1.5 MHz which is driven by the lock-in amplifier. The modulating frequency is chosen in order to move detection away from the 1/f laser noise structure. The power of pump beam is reduced by half after AOM, which result in a 1:1 ratio for the power of pump and probe beams. The pump induced change in transmission signal is then monitored by the probe beam that is detected by either of two methods: “open band” or “detuned”.

The polarizations of the pump and probe pulses are set to be perpendicular to each other, in order to improve the rejection of pump leakage into the detector. The pump leakage results in a positive background signal, and is the main source of noise in the experiment. We use a quarter-wave-plate \((\lambda/4)\) on each beam in order to minimize the polarization ellipticity, to \(~0.1\%\), in conjunction with a half-wave-plate \((\lambda/2)\) that ensures that the linear polarization orientation is 90° relative to the other beam polarization.

The zero delay time and the autocorrelation techniques used to optimize the experimental setup have been described in detail elsewhere\(^8\). Briefly, the zero-delay time is determined interferometrically by inserting a mirror in front of the sample focusing lens. By performing small mirror adjustments, the pump and probe beams are overlapped on the beam splitter and the transmitted light is captured by a photodiode that is AC coupled to an oscilloscope. The zero
delay time is found by moving the optical delay line so that an interferogram is observed when the two beams pathlength are equal. Following the zero delay time, an autocorrelation is performed to ensure that the beam overlap is optimal, and to minimize the pulse chirp by changing the distance between the two prisms. The autocorrelation measurement is done by focusing (using a 3 or 4 inch focal length lens) the pump and probe beams on a BBO crystal (for $\lambda > 413$ nm) in a near parallel geometry in which the second harmonic pulse (UV) is detected between pump and probe beams (due to symmetrical k vectors). The autocorrelation geometry should be preserved for the sample measurement so that the beam overlap is optimal and the zero-time is precisely determined. The same geometry of the autocorrelation measurement is used in a near parallel geometry to focus the pump and probe beams unto a rotating sample cell (1 mm pathlength).

There are two detection schemes used in the VCS measurement: “open band” and “detune”. In the open band scheme (9-12) we employ a Si photodiode to collect the entire spectral bandwidth of the probe pulse. Whereas, in the detuned scheme, a monochromator is used to select a specific spectral range within the probe bandwidth (a pass band of 0.5 nm that is typically shifted 5 nm from the carrier frequency) and this is detected with a photomultiplier. In open band scheme, the detected signal is differential probe light transmitted through the sample, $S(\tau)$, whereas in detune scheme, its spectral component $S(\omega, \tau)$ is measured. The pump and probe pulses can be described as $E_{\text{pump}}(t)$ and $E_{\text{probe}}(t+\tau)$, where $\tau$ is the delay between them. $S(\omega, \tau)$ and $S(\omega, \tau)$ can be described as:

$$S(\tau) = -\int_{-\infty}^{\infty} dt E_{\text{probe}}(t - \tau) \left( \frac{\partial P(t, \tau)}{\partial t} \right)$$
\[ S(\tau) = \int_0^\infty S(\omega, \tau) d\omega \]

where \( P(t, \tau) \) is the polarization induced by the pump and probe fields in the sample.

The open band measurements are more suitable for identification of very low frequency modes and are free of solvent signal(13), but are affected by the presence of elastic interactions which make the data analysis a bit more challenging. The higher frequencies are also poorly represented in the detected signal. On the other hand in a detuned scheme, we can get rid of most of the elastic components of the measured signal, and the detection of higher frequencies is selectively enhanced. But in return, the lower frequencies are more difficult to detect correctly, and signal may be contaminated with solvent signal.(13, 14) So two detection schemes are complementary and help resolve or clarify different ranges of frequencies. They can be correlated with Raman data and in general, there is a very good correlation between the results of frequency domain measurement and the time domain measurements.

A different signal collecting geometry using Galvo scanning mirror are used in order to measure sample that can’t fitting in the rotating sample cell mount, e.g. low temperature measurement that requires the sample to be kept in a cryogenic chamber. As shown in figure 2.2, the Galvo scanning mirror system consists of two scanning mirrors. Each mirror is responsible for scanning the beam along one direction. So with controlling computer program, the collective motion of two mirrors can scan the sample in a pattern for desired purpose (e.g. avoid damaging the sample), at 10~100 times the speed of rotating cell.
2.2 Data Analysis

Femtosecond vibrational coherence spectroscopy (VCS) typically measures signals that consist of damped oscillations imposed on a monotonically decaying background. The power spectrum is generated from the time resolved data by using a linear predictive singular value decomposition (LPSVD) algorithm\textsuperscript{27,28} that simultaneously fits both the oscillations and the monotonic exponential decay, which can be written as:

\[ S = \Sigma_i S_i \cos(\omega_i t + \phi_i) \exp(-t/\tau_i) \]

The LPSVD algorithm finds the parameters of the power spectrum: (frequency, $\omega_i$, phase, $\phi_i$, amplitude, $S_i$ and damping time constant, $\tau_i$), that compose the oscillatory signal and uses them to display the power spectrum of the sample. Maximum entropy method (MEM) method\textsuperscript{(15)}, which is frequently used in the analysis of the rebinding kinetics data, is not applicable to fit the data that has oscillatory components. MEM treats the vibrational coherence
as noise and, as a result, the fitting procedure minimizes the sum of all residuals. But when we consider a damped oscillation, the integral of the displacement over time is not generally zero. This can lead to an unwanted distortion of the actual vibrational coherence signal amplitudes and frequencies when further frequency analysis tools such as LPSVD are applied.

References


Chapter 3

Low Frequency Spectral Density of Cytochrome c upon Equilibrium Unfolding

3.1 Introduction

Cytochrome c (cyt c) is a small, but very important heme protein, that shuttles electrons between cytochrome c reductase and cytochrome c oxidase in the mitochondrial membrane in order to facilitate respiration.(1, 2) It has also recently been discovered that cyt c plays an important role in the cellular apoptosis process. In the early stages of apoptosis, cyt c acts as a catalyst for peroxidation of cardiolipin. Cardiolipin peroxidation leads to permeabilization of the outer mitochondrial membrane and releases cyt c and other proapoptotic proteins into the cytoplasm, which initiates the formation of the apoptosome and the caspase cascade that leads to cell death.(3, 4)

The heme group (Fe-protoporphyrin IX) is the functional center of cyt c. The heme iron is axially coordinated to His18 (proximal ligand) and Met80 (distal ligand) in its native solution state. The porphyrin ring is also covalently anchored to the protein by two thioether linkages with Cys 14 and Cys 17 forming a CXXCH motif that is a unique feature of all c-type hemes(5). In cyt c, the residues between the two Cys are alanine and glutamine at positions 15 and 16, and H represents the histidine ligand at position 18.

Due to its stable protein structure, cyt c is an ideal model and is of great interest for protein folding/unfolding studies where various dynamic intermediates have been characterized.(6-10) The Soret band excited resonance Raman (RR) spectra are known to be highly sensitive to the heme configuration(11, 12) and the high frequency “marker bands” ($\nu_4$, $\nu_3$, $\nu_2$, and $\nu_{10}$) have been used to characterize the heme ligation status of various unfolding intermediates.(13-17) The low frequency RR spectra (200cm$^{-1}$ - 800cm$^{-1}$) contain many out-of-
plane (OOP) modes, which would not be Raman active in the ideal $D_{4h}$ symmetry point group of the heme core. Many of the low-frequency modes observed in the RR spectrum of native cyt c arise from the highly ruffled and distorted structure of the heme that is imposed by the architecture of the folded protein.(18) Despite numerous RR studies of cyt c unfolding, there appears to be no systematic study that has focused on how the low frequency heme vibrational modes respond to unfolding near the physiologically relevant neutral pH. In this sense, the low-frequency heme Raman modes are useful “reporters” of the protein fold and the heme distortions induced by the folding process.

In this work, we use vibrational coherence spectroscopy (VCS) and RR spectroscopy to systematically study changes in the low frequency vibrations of cyt c arising from guanidinium hydrochloride (GdHCl) induced unfolding at pH 7.0. The low frequency modes we observe below 200 cm$^{-1}$ using VCS can have significant thermal populations and their interaction with the thermal bath and the surrounding amino acids can be important to the electron transport reaction mechanism. Studies of these low-frequency modes can improve our understanding of how dynamic structures relate to protein function. Generally, functionally important heme modes, such as “doming” and “ruffling”, which lie in the low frequency region below 200 cm$^{-1}$, are delocalized and involve many nuclei, possibly even mixing with other modes in the surrounding protein material. Resonance Raman and infrared spectroscopies cannot reliably detect heme modes below $\sim$150 cm$^{-1}$ in the aqueous phase, due to the strong absorbance, Rayleigh and quasi-elastic scattering(19) of water. On the other hand, the self-heterodyne nature of the VCS experiment allows it to extract the low frequency vibrational modulations of the third order polarization of the heme. We have previously investigated the low-frequency modes of a variety of heme proteins, using Soret band excitation.(20-29) Unlike the higher frequency modes (>
200cm$^{-1}$), the low frequency modes (which have weaker force constants) are more easily distorted from equilibrium by the protein surroundings. These modes are activated in VCS when the protein induces symmetry breaking nonplanar heme distortions(27). In addition, these modes take on a special functional significance because of their thermal accessibility. The low-frequency coherence spectra offer a unique window into how the surrounding protein environment can alter these important thermally active heme modes.

In this paper we use UV-vis spectroscopy and a previously proposed procedure(30) to observe an intermediate state of unfolded cyt c in the range of 1.5 - 3 M GdHCl. Further investigations reveal that the intermediate state has little effect on the higher frequency RR spectra. However, we observe that the low frequency VCS spectra changes dramatically upon GdHCl induced equilibrium unfolding. We also investigate unfolding in the imidazole-cyt c complex, which bypasses the intermediate ligation states found in the native system. Both the axial ligands and the Soret band peak position of this complex remain fixed upon unfolding by GdHCl. In this well-controlled two-state system, the RR and VCS spectra are not affected by changing resonance conditions due to shifts in the Soret band that arise from intermediate ligation states. This allows normalization of the time domain data and helps to quantify the relative amplitudes of the coherent oscillations between the folded and unfolded states. The VCS spectra reflect a significant structural change of the heme chromophore as the protein is unfolded. This demonstrates that the protein architecture can apply forces that are large enough to modify the heme structure, at least along these lower frequency “soft” modes. Insofar as these low frequency modes are thermally accessible, and potentially involved in the activation of transition or tunneling states, it suggests how the ubiquitous heme group can have its reactivity
“tuned” by different protein structures to perform such a wide ranging set of functions.(5, 27, 31-38)

3.2 Experimental Section

Sample Preparation. Horse heart cytochrome c (cyt c) and GdHCl (molecular biology grade) were purchased from Sigma-Aldrich Co. and were used without further purification. The buffer containing GdHCl was prepared using 0.05M potassium phosphate at pH 7.0±0.05. The concentration of GdHCl was determined by its refractive index.(39) The imidazole-cyt c complex was prepared by adding small volume aliquots of 10 M imidazole stock solution to the total solution volume to obtain the desired concentration. Samples were freshly prepared before the spectroscopy experiments and were equilibrated for at least 30 minutes before any measurement. The absorption spectra were recorded (U-4100, Hitachi) after the preparation procedure to ensure that all chemical modifications were achieved. For VCS experiments the final concentration of protein samples was adjusted to O.D. = 1±0.05 in a 1 mm path length quartz sample cell at the selected excitation wavelength. The absorption spectra were also taken following the vibrational spectroscopy experiments to confirm the integrity of the samples during the laser exposure. All experiments were performed at room temperature.

Optical Systems. The femtosecond vibrational coherence spectroscopy system has been described in details elsewhere.(21, 40) Briefly, a laser pulse at 412 nm was generated and split into two arms: pump and probe. The pump arm was modulated by an acoustic-optical modulator controlled by a lock-in amplifier at 1.5 MHz. A translation stage on the probe arm controlled the delay between the pump and probe pulses. The polarization of the pump and probe beams were adjusted to be perpendicular to each other, allowing for both polarization and spatial filtering. The full width at half maximum of the pump and probe pulses was ~70 fs at the sample position.
Both beams were focused into a spinning sample cell using a three inch lens in a near parallel geometry. After the sample, the beams were re-collimated and the pump light was spatially blocked and further extinguished by a polarization analyzer, so that only the probe beam, modulated by the pump, was detected.

Two detection schemes, “open band” and “detuned”, are used to obtain the VCS spectra. In the open band scheme (21, 25, 40, 41) we employ a Si photodiode to collect the entire spectral bandwidth of the probe pulse, whereas in the detuned scheme, a monochromator is used to select a specific spectral range within the probe bandwidth (a pass band of 0.5 nm that is typically shifted 5 nm from the carrier frequency) and this is detected with a photomultiplier. The detuned scheme selectively enhances the higher frequency oscillations (25, 42, 43) within the full probe bandwidth and this gives improved reliability in the 200 cm$^{-1}$ to 400 cm$^{-1}$ region.

Resonance Raman spectra were obtained using a standard 90$^\circ$ light collecting geometry and a single grating monochromator (Acton SP2500i with 1800g/mm UV holographic grating, Princeton Instruments). Details of the setup have been described elsewhere.(27) Samples were placed in a standard quartz cuvette (Precision Cells, Inc.) and excited with ~5mW of the 413.1 nm line from a krypton laser (Innova 300, Coherent).

**Data Analysis.** The experimental VCS data contain two basic components that arise from electronic and vibrational population transfer and from vibrational coherence. The population transfer component is usually dominant under resonance conditions. Important information such as ligand rebinding and vibrational cooling is carried in this component. This component must be separated to reveal the underlying vibrational coherence signal, particularly under open band conditions. The digitization of the experimental signal is done by the lock in amplifier on a 24-bit scale, which offers a sufficient dynamic range to observe the low amplitude coherence signal.
The fractional change of the transmittance \( \Delta T/T \) for the open band oscillatory signals was estimated to be on the order of \( \sim 10^{-4} - 10^{-5} \).

To generate the power spectrum amplitudes from the time-domain oscillatory signal, we used a linear predictive singular value decomposition (LPSVD) algorithm that can fit both the monotonic background and the damped oscillations simultaneously, as described by
\[
\sum_i a_i \exp(-t/\tau_i) \cos(\omega_i t + \phi_i)
\]
A few parameters can be controlled during the fitting procedure, such as the number of oscillations. The coherence coupling signal around time zero was truncated before the fitting analysis as illustrated in Fig. 6a. Data points within the truncated region (< 200 fs) were not included in the data analysis in order to eliminate the influence of the coherence coupling signal near time zero. The maximum entropy method (MEM) is not used to fit the population decay background when the coherence data is extracted. The MEM treats the vibrational coherence as noise and, as a result, the fitting procedure minimizes the sum of all residuals. But when we consider a damped oscillation, the integral of the displacement over time is not generally zero. This can lead to an unwanted distortion of the actual vibrational coherence signal amplitudes and frequencies when further frequency analysis tools such as LPSVD are applied.

3.3 Results

Equilibrium unfolding of cyt c. The Soret band region of cyt c is displayed in Fig. S1 of the Supporting Information (SI) as a function of GdHCl concentration at pH 7.0 showing the absorption spectra as unfolding takes place under equilibrium conditions. The lower part of the figure shows the difference spectra of the unfolded cyt c with respect to the absorption spectrum of native cyt c. The peak of the Soret band blue-shifts from 409 nm to 406.5 nm as the GdHCl concentration is increased, reflecting the heme ligation switch from Met80 in native cyt c to
either a His33 or His26 ligand as the protein is unfolded by GdHCl.\(^\text{6, 7, 44}\) The equilibrium unfolding of cyt c at pH 7.0 is not a simple two-state process\(^\text{45}\), as shown in the difference spectra of Fig. S1, where no clear isosbestic point is observed as the concentration of GdHCl is increased. Using \(^1\text{H}\) NMR spectra, Russell and Bren reported\(^\text{46}\) an intermediate state in which Lys (at position 79, 73 or 72) replaces native Met80 ligand under the conditions of 1.5-2.3 M GdHCl at pH 7.0 and 30 °C. A similar misligated intermediate state with Lys ligation is also observed under alkaline conditions.\(^\text{15}\)

We adopt here the three-state model proposed by Latypov et al.\(^\text{45}\), which includes the equilibrium native state (N), an intermediate state (I), and an unfolded state (U). This allows all absorption spectra to be fitted consistently. The details of the fitting procedure and the results can be found in the SI. The Soret band maximum of the I-state is found at 407 nm and its population reaches a maximum (~80%) at 2.5 M GdHCl. Latypov et al.\(^\text{45}\) observed a similar unfolding intermediate state with a Soret band peak at the same position for cyt c at pH 5.0. The I-state is suggested to be similar to the “A” state\(^\text{47}\), a compact yet nonnative state, in which the iron-Met80 bond is broken and replaced by an unknown ligand with concomitant loss of the 695nm absorption band, which is attributed to a Met80 sulfur \(\rightarrow\) Fe (III) charge transfer. There is also a low Trp59 fluorescence yield, indicating quenching as a result of a short Trp to heme distance.\(^\text{45, 47}\).

The resonance Raman spectra of native cyt c has been assigned by Hu et al.\(^\text{18}\). At pH 7.0, the heme iron in native cyt c is 6-coordinate low spin (6cLS) and is characterized by its high frequency resonance Raman marker bands \(\nu_4\), \(\nu_3\), \(\nu_2\) and \(\nu_{10}\). The heme remains 6cLS when cyt c is fully unfolded by GdHCl at pH 7 with His33 (~80%) or His26 (~20%) replacing the distal Met80 heme ligand.\(^\text{44}\) Upon unfolding the heme marker bands downshift by \(~2\)-4 cm\(^{-1}\), due to
changes in the heme core size. This is the result of a decreased heme deformation associated with the Met-His ligand-switch. (11, 17)

Figure 3.1  a) Low frequency resonance Raman spectra (100 – 800 cm\(^{-1}\)) of ferric hh cyt c as it undergoes equilibrium unfolding at pH 7.0 induced by GdHCl, which is varied from 0 M (folded) to 4 M (unfolded). The spectra are normalized with respect to the 1008 cm\(^{-1}\) GdHCl peak (not shown). b) Relative amplitude changes of selected unfolding sensitive modes as a function of GdHCl concentration. The relative changes are normalized to unity at the maximum value for each mode.

The low frequency RR spectral region (~200-800 cm\(^{-1}\)) of native cyt c is more complicated. In Fig. 1a, we show the low frequency spectra of cyt c at pH 7.0 as unfolding takes place. There is no axial ligand mode present in the spectra. (18) Most of the in-plane modes are

25
relatively stable as the protein is unfolded, however, some in-plane modes lose their intensities such as $\nu_{51}$ (304 cm$^{-1}$) and $\nu_{7}$ (701 cm$^{-1}$). On the other hand, all of the out-of-plane (OOP) modes display a drastic loss of intensity (the 522 cm$^{-1}$ mode ($\gamma_{12}$) overlaps the broad GdHCl peak at 524 cm$^{-1}$, so the change of its amplitude is not readily observable). The other OOP modes include: 750 cm$^{-1}$ ($\gamma_{15}$), 730 cm$^{-1}$ ($\gamma_{5}$), 569 cm$^{-1}$ ($\gamma_{21}$), 442 cm$^{-1}$ ($\gamma_{22}$), 398 cm$^{-1}$ ($\delta(C_{\beta}C_{a}S)$), and 230 cm$^{-1}$ ($\gamma_{24}$). Table 1 presents a summary of some of the key low frequency Raman active modes. The $\gamma_{15}$ mode has $B_{2u}$ symmetry, $\gamma_{5}$ has $A_{2u}$ symmetry, and the $\gamma_{21}$, $\gamma_{22}$, $\gamma_{24}$ modes are of $E_{g}$ symmetry in the $D_{4h}$ point group. The Raman activity of these OOP modes is enhanced in native cyt c, due to symmetry lowering induced by nonplanar heme distortions along the low frequency OOP mode coordinates. An interesting anomaly in the spectra is the emergence of a mode at 205 cm$^{-1}$ that gains intensity upon unfolding.

We summarize in Fig. 1b the relative amplitude changes of $\gamma_{21}$, $\gamma_{22}$, $\nu_{51}$ and the 205 cm$^{-1}$ mode, where the Raman signal is normalized using the GdHCl peak at 1008 cm$^{-1}$. There are some small intensity changes (20% - 30% change) for $\gamma_{21}$, $\gamma_{22}$, and $\nu_{51}$ at [GdHCl] $\leq$ 2 M that indicate local environment changes associated with the formation of the I-state. However, the largest intensity change, especially the appearance of the 205 cm$^{-1}$ mode, happens between 2-3 M GdHCl. This transition is consistent with a growing population of the U-state as shown in Figs. S2 and S3 of the SI. When the protein is unfolded, the altered tertiary structure relaxes its constraint on the heme and it adopts a more planar structure. As a result, the OOP Raman modes, which are enhanced in native cyt c due to the significantly ruffled geometry, are weakened(27). The 398 cm$^{-1}$ mode $\delta(C_{\beta}C_{a}S)$ is the bending involving the $\beta$-carbon, the sulfur atom at Cys14(17), and the carbon atom bridging them. It can be taken as a characteristic peak for the formation of the native tertiary structure of the heme pocket.(14) The weakening of the $\gamma_{21}$ peak also
characterizes a less ruffled heme distortion and is also a good marker for unfolding.(14, 47) However, it is worth noting that the $\gamma_{21}$ mode as a distortion marker appears to apply only to c-type hemes. In other heme systems, such as NP4-CN(27), NP-H2O(27), and LPO(48), this mode is not observed even though strong ruffling distortions are present.

<table>
<thead>
<tr>
<th>Mode Assignment</th>
<th>VCS</th>
<th>Raman</th>
<th>Intensity change upon unfolding</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma_a$, significant ruffling content</td>
<td>45-50 cm$^{-1}$</td>
<td>205 cm$^{-1}$</td>
<td>+</td>
</tr>
<tr>
<td>$\gamma_b$, unassigned</td>
<td>73-78 cm$^{-1}$</td>
<td>267 cm$^{-1}$</td>
<td>0</td>
</tr>
<tr>
<td>$\gamma_c$, mixed with ruffling</td>
<td>97-114 cm$^{-1}$</td>
<td>341 cm$^{-1}$</td>
<td>0</td>
</tr>
<tr>
<td>$v_s$, symmetric bis-His stretch</td>
<td>203-205 cm$^{-1}$</td>
<td>341 cm$^{-1}$</td>
<td>0</td>
</tr>
<tr>
<td>$\nu_9$, $\delta(C_\delta C_1)_{sym}$</td>
<td>264-269 cm$^{-1}$</td>
<td>304 cm$^{-1}$</td>
<td>0</td>
</tr>
<tr>
<td>$\nu_{51}$, $\delta(C_\beta C_1)_{asym}$</td>
<td>341-351 cm$^{-1}$</td>
<td>398 cm$^{-1}$</td>
<td>0</td>
</tr>
<tr>
<td>$\nu_8$, $\nu(FeN)$</td>
<td>341 cm$^{-1}$</td>
<td>341 cm$^{-1}$</td>
<td>0</td>
</tr>
<tr>
<td>$\delta(C_\beta C_\alpha S)$</td>
<td>398 cm$^{-1}$</td>
<td>398 cm$^{-1}$</td>
<td>0</td>
</tr>
<tr>
<td>$\gamma_{22}$, pyrrole swivel</td>
<td>442 cm$^{-1}$</td>
<td>442 cm$^{-1}$</td>
<td>0</td>
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<tr>
<td>$\gamma_{21}$, pyrrole fold$_{sym}$</td>
<td>569 cm$^{-1}$</td>
<td>569 cm$^{-1}$</td>
<td>0</td>
</tr>
<tr>
<td>$v_7$, $\delta(\text{pyrrole deform})_{sym}$</td>
<td>701 cm$^{-1}$</td>
<td>701 cm$^{-1}$</td>
<td>0</td>
</tr>
<tr>
<td>$\gamma_{15}$, pyrrole fold$_{sym}$</td>
<td>730 cm$^{-1}$</td>
<td>730 cm$^{-1}$</td>
<td>0</td>
</tr>
<tr>
<td>$\gamma_{15}$, pyrrole fold$_{sym}$</td>
<td>750 cm$^{-1}$</td>
<td>750 cm$^{-1}$</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.1 Low-frequency modes sensitive to cyt c unfolding. Assignment of resonance Raman modes is based on Ref. 18. The intensity changes are denoted as “+” for increase and “−” for decrease, while “0” indicates little change.
Figure 3.2  Correlations between Raman and coherence spectra for unfolded ferric cyt c with 4M GdHCl at pH 7.0. The Raman spectrum (blue) was measured with excitation at 413.1 nm, whereas the open band (black) and detuned (red) coherence spectra (displaced below the Raman spectrum) were measured at a carrier wavelength of 412 nm. The detuned coherence data were collected with a 0.5 nm spectral window, detuned 5 nm to the blue of the carrier wavelength. The insets show the time domain VCS data (circles) for the detuned (upper) and open band (lower) conditions. The LPSVD fits are shown as red lines in the inserts. The Raman peak marked with “∗” corresponds to a GdHCl peak.

Vibrational coherence spectra of the fully unfolded cyt c (4M GdHCl at pH 7.0) are displayed in Fig. 2. The top trace shows resonance Raman spectra of unfolded cyt c, while the lower curves display the detuned (middle) and open band (lower) coherence spectra. The time domain oscillatory signals are shown in the insert. There are good correlations between the resonance Raman, detuned coherence, and open band coherence spectra. The modes at 205 cm\(^{-1}\), 267 cm\(^{-1}\) (\(\nu_9\)), and 341 cm\(^{-1}\) (\(\nu_8\)) are present in all three spectra and display a very good correlation. The shoulder near 170 cm\(^{-1}\) in the detuned spectrum is not observed in the open band spectra. This might be due to its weak intensity, which allows detection only when using the enhancement obtained from the selective wavelength detection scheme. This figure demonstrates clearly that the VCS and Raman spectrum share the same mode selection rules(42).
Figure 3.3  Open band VCS spectra of ferric cyt c measured at 412 nm with a 70 fs laser pulses as GdHCl is added to induce equilibrium unfolding. The concentration of GdHCl varies between 0-5 M. The left panel displays the oscillatory components (black circles) and the LPSVD fits (red lines). The blue and magenta oscillations represent the individual ~45-50 cm$^{-1}$ ($\gamma_a$) and ~75-80 cm$^{-1}$ ($\gamma_b$) oscillations, which are offset from the raw data for display. The right panel shows the amplitudes of the corresponding power spectrum. The amplitudes of all spectra and oscillations are given in arbitrary units.

Figure 3 displays the open band VCS oscillations and the LPSVD-derived spectra as cyt c undergoes equilibrium unfolding with increasing GdHCl concentration from 0-5.0 M at pH 7.0. The amplitude of the oscillations and the frequency domain spectra have been placed on a common scale. For native cyt c, the mode at 45-50 cm$^{-1}$ ($\gamma_a$) dominates the oscillatory signal as shown in the top panel. In addition, there are two smaller peaks present at 76-80 cm$^{-1}$ ($\gamma_b$) and at 102 cm$^{-1}$ ($\gamma_c$). On the other hand, when the protein is unfolded, there is a clear change in the oscillatory pattern showing that the dominating low frequency oscillation, $\gamma_a$, fades away and
higher frequency oscillations gain relative intensity. For example, the mode at \( \sim 80 \text{ cm}^{-1} (\gamma_b) \), the \( 205 \text{ cm}^{-1} \) mode, and the \( 269 \text{ cm}^{-1} \) mode dramatically gain intensity relative to the \( \sim 50 \text{ cm}^{-1} (\gamma_a) \) and \( 102 \text{ cm}^{-1} (\gamma_c) \) modes. Additional spectra that help to reveal changes in absolute intensity are presented below for the two-state unfolding of imidazole bound cyt c.

![Figure 3.4](image)

**Figure 3.4** A plot of the damping time constants of ferric cyt c as a function of GdHCl concentration for the 5 principal components of the VCS spectra shown in Fig. 3. The time constants are obtained by LPSVD fitting. Because \( \gamma_c \) is not present in the unfolded state, and the \( 205 \text{ cm}^{-1} \) mode is not present in the folded state, their damping times at the corresponding concentrations are not shown.

In Fig. 4 we present the damping constants of the coherent modes as a function of GdHCl concentration. For \([\text{GdHCl}] \leq 2 \text{ M}\), \( \gamma_a \) and \( \gamma_c \) show nearly identical damping times \( \tau \sim 500 \text{ fs} \), while the damping time for \( \gamma_b \) is near \( \tau \sim 1000 \text{ fs} \). Interestingly, when the protein is unfolded \(( [\text{GdHCl}] \geq 3 \text{ M} )\), the damping times of all OOP modes converge to \( \sim 350 \text{ fs} \) while the in-plane mode \( \nu_9 \) \((267\text{cm}^{-1})\) maintains a longer damping time constant of \( \sim 600 \text{ fs} \). The minimum value of the damping constants appears at 2.8-3 M GdHCl, where the protein may be undergoing a fast exchange between N/I and U states. The faster damping time is what accounts for the broadened low frequency spectral lineshapes observed in Fig. 3 with 2.8 M and 3M GdHCl.
Figure 3.5  Open band VCS spectra of unfolded ferric cyt c (at 4 M GdHCl) measured with 70 fs laser pulses at 412 nm, 418 nm, 425 nm and 430 nm. The left panel displays the oscillatory components (black circles) and LPSVD fits (red lines). The blue and magenta oscillations represent the fits to the individual ~50 cm$^{-1}$ ($\gamma_a$) and ~80 cm$^{-1}$ ($\gamma_b$) modes and are shifted for clarity. The right panel shows the amplitudes of the corresponding power spectra. The phases and damping times of these modes are given in Table 2.

Figure 5 shows a VCS “excitation profile” of unfolded cyt c. The excitation wavelength was tuned from 412 nm to 430 nm to systematically examine the low frequency mode dependence on the changing resonance conditions. The phase and damping time constants for each mode are summarized in Table 2. While the phases remain in the same quadrant and the damping times stay relatively fixed to within ~ 0.1 ps, the relative amplitudes of $\gamma_a$ and $\gamma_b$ compared to the 205 cm$^{-1}$ and 269 cm$^{-1}$ modes are much weaker when tuning away from the 406 nm Soret band peak of unfolded cyt c. This suggests that the lower frequency components of the signal, $\gamma_a$ and $\gamma_b$, are more strongly peaked closer to the Soret maximum than are the higher frequency modes as is predicted theoretically\cite{41, 49}. Excitation profile studies of low frequency modes in native cyt c (work in preparation) reveal a mode at 60 cm$^{-1}$ that is greatly enhanced when tuning the excitation wavelength from 412 nm to 435 nm. This demonstrates that the excitation profile of ferric cyt c with its native Met80 ligand is fundamentally different from the
unfolded (bis-His ligated) protein and supports the idea that the 60 cm\(^{-1}\) mode is related to underlying charge transfer states that are present when Met is a ligand. (50)

<table>
<thead>
<tr>
<th>(\lambda_{\text{pump}})</th>
<th>(\gamma_a) (degree)</th>
<th>Damping (fs)</th>
<th>(\gamma_b) (degree)</th>
<th>Damping (fs)</th>
<th>(v_s) (degree)</th>
<th>Damping (fs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>412 nm</td>
<td>−38</td>
<td>395</td>
<td>−66</td>
<td>341</td>
<td>54</td>
<td>387</td>
</tr>
<tr>
<td>418 nm</td>
<td>−76</td>
<td>403</td>
<td>−69</td>
<td>488</td>
<td>29</td>
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<td>−4</td>
<td>574</td>
<td>−99</td>
<td>456</td>
<td>5</td>
<td>284</td>
</tr>
</tbody>
</table>

Table 3.2 The phase and damping time of low frequency modes of unfolded ferric cyt c.

**Equilibrium unfolding of the cyt c-imidazole complex.** In order to better understand the changes in the VCS spectra of native cyt c as it is unfolded, we performed a comparative study of the cyt c-imidazole complex. In this complex, which behaves like a two-state system, an exogenous imidazole ligand replaces Met80 leading to conformational changes of the backbone near residue Met80 as well as the repositioning of some residues in the distal cavity (51). The large displacement of these residues accounts for imidazole’s low binding affinity: 29 M\(^{-1}\) at 18°C (52). On the other hand, when cyt c is unfolded, the heme is exposed to the solution and the barriers for exogenous ligand binding are greatly reduced. As a result, the binding affinity is much higher in the unfolded state and imidazole binds at 0.01 M concentration. As with imidazole-bound microperoxidase 8 (MP8), when the heme is exposed to solvent, even the proximal His18 can be replaced by imidazole if the concentration is high enough (4 M) (53).

Fig. S4a shows the Soret absorption band of the folded and unfolded cyt c-imidazole complex at different imidazole concentrations. The peak of Soret absorption band does not change when the complex is unfolded in 4M GdHCl at 0.01 M imidazole concentration, but the peak shifts about 1.5 nm to the red at higher imidazole concentration (4M), indicating
replacement of the proximal His. As a result, we compare the sample for folded cyt c in 1M imidazole (no GdHCl) with the unfolded sample (4M GdHCl) in 0.01 M imidazole. These conditions generate the same ligation status (His18 and imidazole) in the two samples and there is no obvious shift of Soret absorption maximum. Interestingly, the full-width-at-half-maximum (FWHM) of the Soret absorption band of the unfolded imidazole complex is slightly narrower (less than 5%) compared to the folded form.

Using the normal coordinate structural decomposition (NSD) method,(37, 38, 54) we can quantitatively characterize the nonplanar heme distortions. NSD describes out-of-plane distortions by using the lowest frequency OOP mode of each symmetry type. In our analysis, we use the optimized planar ferrous porphine (including the iron atom) with D$_{4h}$ symmetry as the reference structure. The displacement along each normal coordinate was calculated in the mass-weighted coordinate space using the scalar product $(X - X_0) \cdot Q_\alpha$, where $X$ and $X_0$ are the mass-weighted atomic coordinates of the input and reference structures, and the unit vectors, $Q_\alpha$, are taken from the mass-weighted normal modes, $\alpha$, of the reference structure. Fig. S4b in the SI displays the NSD analysis of the out-of-plane heme core distortions for native cyt c (PDB# 1HRC) and its imidazole complex based on the NMR structure (PDB #1FI7).(51) Here it must be pointed out that the NMR structure lacks resolution and there are ambiguities in the heme conformation due to lack of protons in the carbon core. However, the NOE data(51) does include the protons on the meso carbons, which can be used to estimate the distortion along the ruffling coordinate. The imidazole and His protons, along with knowledge of the mean porphyrin plane position, should also allow a reasonable estimate of the doming distortions. With these caveats, we note that the NSD of the imidazole complex also suggests a strong ruffling distortion of $\sim$ 2.5 amu$^{1/2}$Å, which is somewhat less than the $\sim$ 3.5 amu$^{1/2}$Å found in native cyt c. It also displays
some saddling ($\sim 1.5$ amu$^{1/2}$Å) and doming ($\sim 1$ amu$^{1/2}$Å) distortions. The negative sign for doming suggests that the iron is displaced toward the distal (imidazole bound) side.

Given the potential ambiguity of the NMR structure for imidazole bound cyt c, we compare the RR spectra of the folded and unfolded imidazole complex in Fig. S4c. The RR spectrum of the folded complex is very similar to native cyt c. Especially noteworthy are the strong unfolding sensitive modes at $\sim 400$ cm$^{-1}$ ($\delta(C_{\beta}C_{\alpha}S)$) and $569$ cm$^{-1}$ ($\gamma_{21}$). When we compare to the RR spectrum of native cyt c in the top panel of Fig. 1a, we see that the native cyt c, which has larger ruffling distortion, shows stronger $569$ cm$^{-1}$ mode. This supports the argument that $\gamma_{21}$ is enhanced by the ruffling distortion of c-type heme.(47) When the complex is unfolded, these two modes lose their intensities, and the $205$ cm$^{-1}$ band emerges, indicating that the more planar heme structure associated with the unfolding helps to enhance the $205$ cm$^{-1}$ mode. Such an effect could arise if the $205$ cm$^{-1}$ mode undergoes significant mode mixing and becomes “diluted” when the heme is in a more highly distorted state inside the folded protein. Generally, the changes associated with unfolding the imidazole-cyt c complex are very consistent with the observations for equilibrium unfolding of native cyt c.

However, in order to gain a better perspective on how the low frequency modes of cyt c and its imidazole complex change during unfolding, we would like to be able to measure the oscillation intensities in an absolute sense. Unfortunately, under different experimental conditions, the absolute strength of the signal is not easily comparable and an internal standard would be useful to better normalize the amplitudes of the oscillations. The VCS experiment uses a near parallel, but still a crossed beam geometry, i.e. there is a small angle between the direction of the pump and probe beams focused on the sample.(21) This means that the detection of the self-heterodyned signal is very sensitive to absorbance of the sample and alignment of the optics.
(beam overlap within the sample). The intensity at time zero provides an inherently strong signal that is detected when the pump and probe pulses overlap in time. This signal has been studied in detail in prior work (55, 56) so that the detected signal (57) at \( t > 0 \) can, in principle, be normalized by the signal intensity at time zero, which can act as an internal standard. The signal at time zero will be approximately invariant so long as key experimental variables, including the sample OD, are kept constant. Under these conditions, the amplitude of the coherent oscillations can be normalized to the height of the signal at time zero so that the VCS spectra of different samples can be compared.

Figure 3.6  (a) Open band optical responses for the folded and unfolded ferric cyt c-imidazole complex. Both traces were normalized at the peak of the coherence spike and the data within shaded window were truncated and were not included in the LPSVD fitting procedure. (b) VCS signals and power spectrum amplitudes for the same samples as shown in panel (a). The left panel displays the oscillatory components (black circles) and the LPSVD fits (red lines). The right panel shows the corresponding power spectrum amplitudes. The amplitudes of the power spectra are normalized using the kinetic traces shown in panel (a).
Fig. 6a shows the optical response of the folded and unfolded cyt c-imidazole complex. For this pair of samples, the ligand coordination remains unaltered, the peak of the Soret band is fixed and the change in its width is less than 5%. Assuming the intensities of the signals at time zero are the same in the two cases, we normalize the traces to unity at t=0. In Fig. 6b, the amplitude of the oscillations and the VCS spectra are scaled with the same normalization. We see that when the complex is unfolded, the amplitude of the 80 cm\(^{-1}\) mode (\(\gamma_b\)) is roughly unchanged, while the 50 cm\(^{-1}\) mode (\(\gamma_a\)) loses more than half of its intensity. The mode at 100 cm\(^{-1}\) (\(\gamma_c\)) totally disappears upon unfolding, while the 205 cm\(^{-1}\) mode emerges, and the modes at 267 cm\(^{-1}\) (\(\nu_9\)) and 341 cm\(^{-1}\) (\(\nu_8\)) stay relatively constant in agreement with the RR spectra. Note that the LPSVD spectra extracted for the folded and unfolded cyt c have mode frequencies for \(\gamma_a\), \(\gamma_b\), \(\gamma_c\), 205 cm\(^{-1}\), \(\nu_8\) and \(\nu_9\) that agree to within 2 cm\(^{-1}\).

![Figure 3.7](image)

Figure 3.7. Plot of the relative amplitude changes for the unfolding-sensitive low-frequency modes of native cyt c as a function of GdHCl concentration. The amplitude ratios with respect to \(\gamma_b\), which is relatively insensitive to unfolding, are plotted. The relative change is normalized to unity at the maximum value for each mode.

Application of this normalization method to the equilibrium unfolding of native cyt c (without added imidazole) is difficult, because the unfolding process involves denaturant concentration dependent populations of three states: N, I and U. Both the I- and U-states have Soret absorption peaks near 406 nm, however, the N-state has the Soret peak at 409 nm. However, because the \(\gamma_b\) mode amplitude stays approximately the same as the imidazole
complex unfolds, we have elected to use it as a reference to characterize the amplitude changes of the other modes in native cyt c. In Fig. 7 we plot the ratio of $\gamma_a/\gamma_b$, $\gamma_c/\gamma_b$, and 205 cm$^{-1}/\gamma_b$ as a function of GdHCl concentration. The amplitude ratios are normalized to unity at the maximum value of each ratio. Figure 7 shows that the low frequency mode intensities reflect major changes in heme structure taking place upon unfolding of native cyt c. It can also be seen that the VCS data (Fig. 7) are very consistent with the RR results (Fig. 1b).

3.4 Discussion

Mode Assignments. The combination of the low frequency RR spectra and VCS allows the full low frequency Raman spectral density of cyt c to be observed following GdHCl induced unfolding. The main unfolding sensitive modes below 800 cm$^{-1}$ include: $\gamma_a$, $\gamma_c$, $\gamma_{24}$, $\nu_{51}$, $\delta(C_{\beta}C_{\alpha}S)$, $\gamma_{22}$, $\gamma_{21}$, $\nu_7$, $\gamma_5$, $\gamma_{15}$ along with the $\nu_s$(His-Fe-His) mode at 205 cm$^{-1}$ (see Table 1). Because heme structural information for the unfolded protein is lacking (no crystal structure), specific mode assignments in the unfolded state are difficult. However, the simultaneous change of both the Raman and VCS modes implies systematic changes in the heme deformation take place. As discussed by Kubo et al., the amplitude of the oscillation of a given low-frequency OOP normal mode of the heme is proportional to the square of its distortion from the planar state, which helps us to understand the Raman activity of “soft” low frequency modes.(27)

In unfolded cyt c, the heme adopts a more planar structure as indicated by the reduced intensity of the OOP modes (Figs. 1b, 7); i.e., the strong ruffling distortion of the heme in native cyt c is significantly relaxed. From the NSD analysis of native cyt c shown in Fig. S4b, the heme deformation is mainly along the ruffling coordinate with only very small contributions from other normal coordinates, such as saddling. Thus, the enhanced amplitude of $\gamma_a$ in native cyt c should be due to the lowering of porphyrin symmetry mainly along the ruffling normal
coordinate. Considering \( \gamma_a \) is the dominant mode in the VCS spectra, we assign it to have a large contribution from ruffling motion. The assignment of the \( \gamma_a \) mode at \( \sim 45-50 \text{ cm}^{-1} \) to ruffling suggests that the c-type heme ruffles at a slightly lower frequency than found for the b-type heme in myoglobin and nitrophorin (NP4) where it was assigned to a mode near \( 60 \text{ cm}^{-1} \). However, in the NP4 study(27), it was noted that the ruffling mode “softens” from 72 to 53 \( \text{cm}^{-1} \) as the ruffling distortion was increased systematically using several different heme complexes. Because the heme in cyt c involves a ruffling distortion that is slightly larger than found for NP4-CN, where the ruffling frequency was assigned at 53 \( \text{cm}^{-1} \), the value for the frequency near 45-50 \( \text{cm}^{-1} \) in cyt c appears to be very consistent with what was found in the nitrophorin system(27).

Based on the NSD analysis in the SI, we assign \( \gamma_c \) to be a mode with a lesser amount of ruffling admixture, possibly with some saddling or waving content. It is not fully understood why the amplitude of \( \gamma_b \) remains roughly constant during the unfolding process. The data suggest that \( \gamma_b \) involves a mode with an inherent deformation that is relatively unaffected by the protein folding process. It is noteworthy that Shelnutt et al.(58) used the four-coordinate Ni-substituted heme in microperoxidase11 and cyt c to show that a significant level of porphyrin OOP distortion, due to forces associated with the CXXCH pentapeptide, is retained even when cyt c is unfolded. The intensity invariance of \( \gamma_b \) is likely related to such a distortion, which is relatively isolated from the protein folding process. Because the intensity of the coherent signal depends quadratically on the magnitude of the distortion(27), the observed reduction in the \( \gamma_a \) and \( \gamma_c \) intensities by \( \sim 90\% \) is consistent with residual distortions in the unfolded state that are \( \sim 32\% \) of the native amount. This amount of residual distortion is less than predicted using an optimized microperoxidase vacuum structure(58). On the other hand, it seems possible that the interaction between the guanidinium and the carbonyl groups may weaken the H-bonds that constrain the
CXXCH pentapeptide(58), resulting in additional relaxation of the heme distortions in the GdHCl unfolded structure.

The 205 cm\(^{-1}\) mode is somewhat anomalous because it gains intensity upon unfolding. Its appearance as well as its increasing amplitude with increasing [GdHCl] is very consistent in both the VCS and RR spectra. Based on the work of Mitchell et al. (59), this mode can be assigned to the symmetric stretch, \(\nu_s(\text{His-Fe-His})\), of the bis-histidine complex that is formed in the unfolded state. This is also consistent with conclusions based on RR studies of MP8 with added imidazole. (53) Additional measurements (data not shown) indicate that the depolarization ratio for this mode is \(~1/3\), which could arise from coupling to only a single (x or y) component of the transition dipole. This might be associated with asymmetry induced by the relative histidine-heme orientations.

**Damping of Coherent Motion.** The damping times of the coherent oscillations are summarized in Fig. 4, which provides a direct probe of the effects of protein folding and the heme exposure to solvent fluctuations. The damping of the coherence signal has three main sources: spectral inhomogeneity, vibrational lifetime, and “pure” dephasing; the latter being due to a disparity in the coupling of the surrounding fluctuations to the individual vibrational eigenstates involved in the coherent superposition. Previous studies have demonstrated that the low frequency coherences of the heme within a protein matrix have a much longer damping time than when exposed to solvent (22). Further temperature dependent studies of the damping time of low frequency modes in horseradish peroxidase (HRP) suggested that pure dephasing is an important source of the coherence decay within the protein environment. (22) Figure 4 demonstrates that the low frequency heme coherences can have significantly different damping times, presumably related to the specifics of how they are coupled to the environment. The nearly identical
damping for $\gamma_a$ and $\gamma_c$ between 0 - 2M GdHCl suggests that they may couple to the protein with a very similar mechanism in the folded state. Low frequency oscillations involve the cooperative motion of many atoms in the heme and even extend into the protein. It is likely that the low frequency oscillations ($\gamma_a$ and $\gamma_c$) in cyt c involve ruffling and/or saddling motions that involve the linkage between the pyrrole rings of the heme and the two thioether bridges linked to the protein. Thus, there exists strong coupling between these modes and the protein matrix leading to shorter decay times. In addition, it has been shown that a major heme energy relaxation “doorway” involves the heme propionate groups, which facilitate heme-solvent interaction in the folded state of myoglobin.(60, 61) Similar interactions could be involved in the dephasing of low-frequency heme modes.

In contrast to $\gamma_a$ and $\gamma_c$, the mode assigned as $\gamma_b$ has an intensity that is roughly independent of the cyt c folding process and it shows a ~ 1 ps damping time (Fig. 4), which is typical of most low frequency oscillations in a ferric heme.(22, 25, 26, 40) Unfolding has a dramatic impact on the damping time of this mode, reducing it to ~300fs and suggesting a more solvent-exposed heme environment. Generally, the low frequency OOP modes have a consistent damping constant of ~350 fs when unfolded. This can be contrasted with the ~600 fs damping time of the $\nu_9$ in-plane mode at 267 cm$^{-1}$. The damping times in the fully unfolded state are comparable with previous measurements on solvent-exposed ferric heme models, where the measured damping times were about ~ 500 fs.(40)

**Folding.** The protein folding process is often interpreted by energy landscape theory where the protein first collapses and then, through the search of native contacts gradually, forms a native folded structure within a funnel-like energy landscape.(62-64) The presence of misligated heme traps in cyt c slows the folding kinetics; for example, when His 33 (or His 26) binds to the heme
iron(44). This bis-histidine trap has been proposed by Bren et al., to be on the folding pathway, helping to guide the folding process toward the native structure.(46) The folding process of cyt c involves the sequential folding of five pseudocooperative units (foldons) in order of descending free energy: the N- and C- terminal helices, the 60s helix, the β sheets (residues 37-39 and 58-61), the 71-85 loop (Met80 loop) and the 40-57 loop, along with the omega loop containing His 26 and 33, which does not display foldon properties(62, 64-68).

The Met80 loop unfolding represents an early step on the major cyt c unfolding pathway with a free energy of ~6 kcal mol\(^{-1}\).(62, 69) The presence of the I-state (heme coordination with Lys 73 or Lys 79 in the Met80 loop) under various very mild denaturation conditions is associated with the destabilization of Met80 loop. From the global fitting of the equilibrium unfolding absorption spectra at pH 7 and 20°C (see SI), we determine the free energy (\(\Delta G_{NI}\)) for the transition from N to I to be 4.6 ± 0.3 kcal/mol and the \(\Delta G_{IU}\) from I to U to be 11.6 ± 0.4 kcal/mol, with a total change of free energy, \(\Delta G_{total}=16.2 \pm 0.5\) kcal/mol. The value of \(\Delta G_{total}\) at pH 7.0 and 20°C is higher than \(\Delta G_{total}\) under other experimental conditions. Bai et al.(65, 70) reported \(\Delta G_{total}=12.8\) kcal/mol at pH 7.0 and 30°C. However, since 1-3 kcal/mol decrease of \(\Delta G_{total}\) is expected for a temperature rise from 20°C to 30°C at pH 7.0, this is consistent with our observations.(70) Latypov et al.(45) reported \(\Delta G_{total}=9.8\) kcal/mol at pH 5.0, but the ~6 kcal/mol drop in \(\Delta G_{total}\) can be accounted for by the expected reduction of unfolding free energy in going from pH 7 to pH 5.(71) The value for \(\Delta G_{total}\) found here is also larger than the 11.2 kcal/mol found for urea-induced unfolding at pH 7.0 and 30°C(72). In addition to the lowering of the unfolding free energy due to the temperature increase, a difference between urea and GdHCl is also expected due to the fact that GdHCl is an ionic denaturant.(45, 73) On the other hand, the value of \(\Delta G_{NI}=4.62\) kcal/mol obtained here is close to the 4.0 kcal/mol found using
urea to induce the partial unfolding to form the intermediate state (72). It is also near to the ~6 kcal/mol unfolding free energy of the Met80 loop deduced from previous hydrogen exchange experiments (65, 70, 71) using a limited concentration range for GdHCl (0-2.0 M). These results strongly suggest that the N to I transition is correlated with the Met80 loop unfolding.

Despite the change of axial ligand in going from the N to I state as the Met80 loop unfolds, there are only very small amplitude changes in the low frequency RR and VCS modes for $[\text{GdHCl}] \leq 2\text{M}$. This suggests that the destabilized Met80 loop has only limited “responsibility” for the highly ruffled heme structure. Comparing Fig. 1b and Fig.7 with Fig.S3, we see that the changes of the low frequency Raman and VCS modes follow the population profile of the U-state, displaying a midpoint of 2.7 M (Fig. 1b and 7) and saturating at higher GdHCl concentrations. The midpoint concentration is nearly the same as $C_{\text{NU}}$ (2.65 kcal/mol, see SI). This indicates that heme structural changes are strongly correlated with cyt c’s global unfolding. Among the 5 foldon units in cyt c, the terminal helices have the highest unfolding free energy and this corresponds to the last step in unfolding (first step in refolding), i.e. its unfolding free energy and midpoint concentration define the free energy and midpoint concentration of global unfolding.(62, 71) The CXXCH motif, which is covalently connected to the porphyrin, is found on the N-terminal. The unfolding of this unit leads to a structural change of the CXXCH motif and this affects the deformation of porphyrin ring. Thus, our results suggest that cyt c controls the distortion of the heme through its CXXCH motif, which is consistent with prior studies(58, 74).

**Lipid Binding.** The structure changes associated with cyt c binding to model membrane systems (polyanions(75), phospholipid vesicles(76) and electrodes(77)) as well as intact mitochondria(78) has also been investigated. The studies of model membrane systems
demonstrated that cyt c undergoes a tertiary structural change upon binding. Such a change, either due to electrostatic binding or hydrophobic binding, again results in the loss of the Met80 ligand(76), which is replaced by either a His residue or water. The $\gamma_{21}$ peak intensity near 569 cm$^{-1}$ is generally reduced, which indicates partial relaxation of the ruffled heme structure(79). Studies using intact mitochondria(78) also reported that $\gamma_{21}$ has completely disappeared when cyt c is bound in the living mitochondrial membrane. This suggests that, when cyt c binds to the membrane, it might both unfold and disrupt the H-bonds constraining CXXCH pentapeptide(58), thus inducing more heme relaxation than is achieved using chemical denaturants alone. It appears that the relaxed heme structure may, in fact, be very important for the in vivo function of cyt c. This conclusion is also supported by recent studies of cardiolipin bound cyt c(80).

3.5 Summary

In summary, we find that low-frequency modes at $\sim$50 cm$^{-1}$, $\sim$80 cm$^{-1}$, and $\sim$100 cm$^{-1}$, as well as certain higher frequency Raman modes, especially the $\nu_4$( His-Fe-His) mode at 205 cm$^{-1}$, and the $\gamma_{21}$ mode (569 cm$^{-1}$) directly track the unfolding of cyt c. We infer that the primary heme structural change associated with the fully unfolded protein involves the ruffling distortion and we assign the mode near 45-50 cm$^{-1}$ ($\gamma_a$) as having significant ruffling content. We also observe that the unfolded state generally leads to more rapid decoherence and shorter damping times, presumably due to increased coupling between the heme and the solvent. Finally, we use a simple 3-state thermodynamic model to generate the free energy for unfolding in the steps N-I and I-U. The relative intensity changes of the low-frequency heme modes indicate that the protein-induced heme distortions, specifically involving the ruffling coordinate, are significantly (but not completely) relaxed during the final I to U unfolding transition. More importantly, the binding of cyt c to natural lipid membranes appears to relax the heme distortions even further.
3.6 Support Information

Soret band absorption spectra and fits for equilibrium unfolding of hh Cyt c.

a) Serial model

The fitting of the Soret band during the equilibrium unfolding of Cyt c is performed using a three-state model proposed by Latypov et al. In addition to the native (N) and unfolded (U) states, an intermediate (I) state was introduced by Latypov and coworkers in order to account for the unfolding equilibrium spectral changes. The following scheme is assumed:

\[
N \rightleftharpoons I \rightleftharpoons U
\]

where \( K_{NI} \) and \( K_{IU} \) are equilibrium constants that can be expressed as \( K = e^{-\frac{\Delta G}{RT}} \). We assume that the free energy for each transition varies linearly with the denaturant concentration, \( c \):

\[
\Delta G_i(c) = m_i(C_{m_i} - c),
\]

where \( C_{m_i} \) and \( m_i \) are the midpoint concentration and slope, respectively, and the index \( i \) stands for either NI or IU.

The extinction coefficient of the superposition of unfolding states, \( \varepsilon(v, c) \) is given by:

\[
\varepsilon(v, c) = \varepsilon_N(v)f_N + \varepsilon_I(v)f_I + \varepsilon_U(v)f_U
\]

This is a superposition of the absorption from the native (N), unfolded (U), and intermediate (I) states with fractional populations \( (f_N, f_I, f_U) \):

\[
f_N = \frac{1}{1 + K_{NI}K_{IU}}, \quad f_I = K_{NI}f_N, \quad f_U = K_{NI}K_{IU}f_I
\]

The global fitting procedure is performed using IGOR Pro (WaveMetrics, Inc.). The program simultaneously optimizes the fit to the absorption spectrum at each wavelength and GdHCl concentration using 7 common fitting parameters: \( C_{m_{NI}}, C_{m_{IU}}, m_{NI}, m_{IU} \), and three coefficients to construct the I-state absorption by a linear combination of the spectra at 0M, 2M and 5M GdHCl.

The fitting results are summarized in Table S1. The fitted absorption spectra are shown in Fig. S1, the spectra of the three states are summarized in Fig. S2, and the population of each state as a function of GdHCl concentration is depicted in Fig S3.

**Table 3.S1**  Thermodynamic parameters obtained by global fitting to the absorption spectra of hh Cyt c at pH 7.
<table>
<thead>
<tr>
<th>$C_{mNI}$ (M)</th>
<th>$m_{NI}$ (kcal mol$^{-1}$ M$^{-1}$)</th>
<th>$\Delta G_{NI}(0)$ (kcal mol$^{-1}$)</th>
<th>$C_{mIU}$ (M)</th>
<th>$m_{IU}$ (kcal mol$^{-1}$ M$^{-1}$)</th>
<th>$\Delta G_{IU}(0)$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.13±0.02</td>
<td>2.17±0.12</td>
<td>4.62±0.26</td>
<td>2.94±0.01</td>
<td>3.95±0.12</td>
<td>11.61±0.35</td>
</tr>
</tbody>
</table>

**Figure 3.S1**  Absorption spectra of hh cyt c (dotted line) and their fitted spectra (solid line) at pH 7 and [GdHCl] = 0 M (dark yellow), 1 M (yellow), 2 M (magenta), 2.5 M (cyan), 2.8 M (blue), 3 M (green), 4 M (red), and 5 M (black), by three-state global fits. The lower part shows the spectral difference with respect to the native cyt c absorption spectrum at 0 M GdHCl.
Figure 3.S2  Absorption spectra obtained from three-state global fits, native (black), intermediate (blue) and unfolded (red).

Figure 3.S3. Population of each state as function of GdHCl concentration obtained from three-state global fits. Native (black), intermediate (blue) and unfolded (red).
b) Parallel model  

The three-state unfolding process can also be explained in the context of a “parallel” scheme, which includes a direct N to U transition (global unfolding) in parallel to the N to I transition. This, in effect, closes the “thermodynamic loop” so the direct N to U transition is equivalent to N to I followed by I to U and the thermodynamic cycle links the equilibrium states (i.e., $K_{NU} = K_{NI}K_{IU}$). The “parallel” scheme is often used in NMR studies for the unfolding of cyt c. (65, 66, 70-72) The populations can be written in terms of the free energies connecting N-I and N-U states, denoted by $\Delta G_{NI}$ and $\Delta G_{NU}(=\Delta G_{total})$, respectively. $K_{NU}$, can be written as $K_{NU} = \exp(-\Delta G_{NU}/RT) = \exp(-\Delta G_{NI}/RT)\exp(-\Delta G_{IU}/RT) = K_{NI}K_{IU}$, so Eq. 2 still holds to describe the population of each state. The m-value, $m_{NU}$, and midpoint concentration, $C_{NU}$, for the N to U transition can be derived using:

$$\Delta G_{total} = \Delta G_{NU} = m_{NI}(C_{NI} - c) + m_{IU}(C_{IU} - c) = (m_{NI} + m_{IU}) ((m_{NI}C_{NI} + m_{IU}C_{IU})/(m_{NI} + m_{IU}) - c),$$

So that, when written in terms of the parameters for 3-state scheme, we have:

$$m_{NU} = m_{NI} + m_{IU}$$

$$C_{NU} = (m_{NI}C_{NI} + m_{IU}C_{IU})/m_{NU}$$

Thus for this study, we have $m_{NU} = 6.12\pm 0.17$ kcal mol$^{-1}$ M$^{-1}$, $\Delta G_{NU}(0)=16.23\pm 0.44$ kcal mol$^{-1}$, and $C_{NU}=2.65\pm 0.15$ M.

Table S2 summarizes the $\Delta G_{NI}$, $\Delta G_{NU}$, $m_{NI}$, and $m_{NU}$ values obtained in this work with other published data. The unfolding free energy and m-value obtained at 20 °C and pH 7 is higher than those obtained under less thermodynamically stable conditions.

**Table 3.S2.** Comparison of unfolding free energies and m-values. Eq.3 is used to derive the N-U parameters from ref 45 and this work. The unfolding of the Met80 loop in ref. 64 is taken to correspond to N-I. The reference numbers refer to the main text.

<table>
<thead>
<tr>
<th>Solution condition</th>
<th>$m_{NI}$ (kcal mol$^{-1}$ M$^{-1}$)</th>
<th>$\Delta G_{NI}$ (0) (kcal mol$^{-1}$)</th>
<th>$m_{NU}$ (kcal mol$^{-1}$ M$^{-1}$)</th>
<th>$\Delta G_{NU}$ (0) (kcal mol$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea, pH 7.0, 30 °C</td>
<td>0.5</td>
<td>4.0</td>
<td>1.5</td>
<td>11.2</td>
<td>Ref. 71</td>
</tr>
</tbody>
</table>
Imidazole-Cytochrome c complex and NSD analysis

Figure 3.S4. (a) The Soret band absorption spectra of folded cyt c and the GdHCl unfolded ferric cyt c-imidazole complex at different concentrations of GdHCl and imidazole. All spectra are normalized to O.D. = 1 at the peak of Soret band. (b) NSD analysis of the heme core distortions for hh cyt c (crystal structure) and the hh cyt c-imidazole complex (NMR structure). A displacement of 1 amu^{1/2}Å represents the square root of the sum of squares of the mass weighted displacements of the Fe and the 24 (4N, 20C) porphyrin atoms. The color coding for the modes is propelling (blue), ruffling (green), saddling (red), waving (light blue), waving (yellow), doming (purple), inverse doming (gray). The minus sign of displacement is defined only for doming and inverse doming to indicate the direction of Fe displacement (positive is proximal; negative is distal). The PDB IDs are 1AKK and 1FI7 for hh cyt c and cyt c-imidazole. (c) The Raman spectra in the low frequency region (100 – 800 cm^{-1}) of the folded (upper panel) and GdHCl-induced unfolded (lower panel) ferric cyt c imidazole complex.
References


Chapter 4

Heme Distortion, Low-Frequency Vibrational Excitations, and Electron Transfer in Cytochrome c

4.1 Introduction

Cytochrome (cyt) c is an important electron transfer protein that is involved in a variety of biological functions like photosynthesis, respiration, and apoptosis. (1) The heme group (Fe-protoporphyrin IX) is the functional center of cyt c. The heme iron is axially coordinated to His18 (proximal ligand) and Met80 (distal ligand) in its native solution state. The porphyrin ring is also covalently anchored to the protein by two thioether linkages with Cys 14 and Cys 17, which form a Cys-X-X-Cys-His (CXXCH) pentapeptide unit that is a unique feature shared by nearly all c-type hemes (1) (“XX” refers to other amino acids, e.g. Val and Ala, as in Pa cyt c551).

The heme in cyt c has a geometry that is dominated by a large ruffling distortion, induced by both the protein fold and by the CXXCH motif. (2, 3) Systematic analysis of x-ray crystal structures of heme proteins has shown that the proteins belonging to the same functional class share similar out-of-plane (OOP) heme distortions. (4-6) These protein induced OOP distortions are energetically unfavorable for the heme, and their evolutionary conservation implies that they have biological significance. Among them, doming and ruffling have been reasonably well characterized and correlated with protein functions. Doming is typically observed in oxygen storage or transport proteins like hemoglobin (7, 8) and myoglobin (9). Moreover, the coupling of heme doming to the protein conformational substates has been shown to be functionally significant in a variety of heme protein systems (10-12). On the other hand, heme ruffling, which is the primary topic of this work, is the dominant OOP deformation found in c-type
cytochromes(4-6, 13) and nitrophorins(14-16), which are involved in electron and NO transport, respectively.

![Figure 4.1](image)

**Figure 4.1.** Crystal structure and NSD analysis of hemes in ferric cyt c551 wild type and its F7A mutant. A displacement of 1 amu$^{1/2}$Å represents that the square root of the sum of squares of the displacements of Fe and 24 porphyrin (4N, 20C) atoms is 1 amu$^{1/2}$Å. The color coding for the modes is pro: propellering (blue), ruf: ruffling (green), sad: saddling (red), wav(x): waving$_x$ (light blue), wav(y): waving$_y$ (yellow), dom: doming (purple), invdom: inverse doming (gray). The minus sign of displacement is defined only for doming and inverse doming to indicate the direction of Fe displacement (+: proximal; −: distal). PDB IDs are 351C, and 2EXV for *Pa* cyt c$_{551}$ wt, and F7A mutant, respectively. The ruffling mode is shown at the lower left part of the figure and the arrows indicate the rotation of pyrrole rings with respect to Fe-N axis (dotted black lines).

As seen in Fig. 1, ruffling involves a pyrrole-ring twisting about the Fe-N bond. The ruffling distortion tilts the $p_z$ orbitals of the porphyrin nitrogens away from the heme normal and increases overlap of the porphyrin $\pi$ and iron $d_{xy}$ orbitals. It has been shown via NMR experiments(17) and DFT computation(17) that, in the absence of a strong $\pi$-acceptor axial ligand(18), a ruffling deformation increases the Fe 3$d_x$-based electron density on the iron center, which makes the heme meso-carbon electron donation to the iron 3$d_{xy}$ orbital less energetically favorable.(17) Ruffling destabilizes all three occupied Fe 3$d$-based molecular orbitals and decreases the positive and negative spin density on the $\beta$-pyrrole and meso-carbon,
respectively. Consequently, the electron transfer rate to the ferric heme is expected to decrease as a function of the ruffling deformation. In addition, when ruffling is considered in isolation, it decreases the reduction potential of ferric cyt c.

The CXXCH pentapeptide in cyt c may be critical to the ruffled structure and the function of cyt c. The CXXCH unit is thought to affect the heme reduction potential and it can influence heme deformation through the covalent bonding of the thioether groups and by hydrogen bonding within the pentapeptide. Furthermore, the CXXCH pentapeptide may have a biologically important role related to its proximity to the electron transfer partner binding site, as in the yeast cyt c peroxidase/cyt c complex. The local vibrational modes of heme in the 250 - 400 cm\(^{-1}\) region have been shown to strongly mix with the vibrational modes of the CXXCH motif. This suggests that the heme-CXXCH vibrational dynamic couplings can play a role in electron transfer by coupling the vibrations of the heme directly to vibrations of the CXXCH unit at the protein–protein interface. This coupling could help to transduce thermal energy or alter the reorganization energy and the barrier for electron tunneling.

Despite the great deal of work that has been done to investigate electron transfer and heme deformation in cyt c, no experiment has directly demonstrated a quantitative correlation between heme deformation and the electron transfer rate. Generally, the functionally important heme modes, such as doming and ruffling, are delocalized and involve many nuclei and lie in the low-frequency region below 200 cm\(^{-1}\). Infrared and resonance Raman spectroscopy cannot reliably detect heme modes below ~150 cm\(^{-1}\) in the aqueous phase, due to the strong absorbance, Rayleigh scattering, and quasi-elastic scattering of water. In contrast, vibrational coherence spectroscopy (VCS), makes it possible to extract vibrational modulations of the third-order
polarization of the heme at very low frequency, which provides access to this relatively unexplored region.

We have used this technique previously to investigate the low-frequency modes of a variety of heme proteins, using Soret band excitation. (26-35) Unlike the higher frequency modes (>200 cm\(^{-1}\)), the low-frequency modes (which have weaker force constants) are more easily distorted from equilibrium by the protein surroundings. These modes are activated in VCS when the protein induces symmetry-breaking nonplanar heme distortions. In addition, these modes take on a special functional significance because of their thermal accessibility. The low-frequency coherence spectra offer a unique window into how the surrounding protein environment can alter these important thermally active heme modes.

In this work, we studied \(Pa\) cyt c\(_{551}\) and its F7A mutant using absorption spectroscopy, resonance Raman spectroscopy and VCS. \(Pa\) cyt c\(_{551}\) and its F7A mutant have very similar crystal structures, but the mutant has a more ruffled heme geometry than the wild type. The investigations of this very similar pair of proteins revealed a clear difference between their resonance Raman and VCS spectra, reflecting the different degree of heme ruffling deformation. These observations support the previous assignment that \(\gamma_a\) (45~55 cm\(^{-1}\)) is a mode with major ruffling content in the c-type heme of cyt c (in contrast, the b-type heme of Mb has a doming mode near 40 cm\(^{-1}\)). We also investigated the photoreduction kinetics of the two proteins. The photoreduction cross-section determined for wt is an order of magnitude larger than for the more ruffled F7A mutant. Although the details of photoreduction in heme proteins are not fully understood, these measurements provide direct quantitative evidence that correlate an order of magnitude increase in the photoinduced electron transfer rate with a factor of two decrease in the ruffling distortion.
4.2 Materials and Methods

Sample Preparation. *Pa* cyt c\textsubscript{551} wt and F7A mutant was prepared as previously reported\cite{41, 42}. Horse heart cytochrome c was purchased from Sigma-Aldrich and *Pseudomonas aeruginosa* (*Pa*) cyt c\textsubscript{551} was prepared according to previously described methods\cite{41, 42}. All samples were further oxidized with small amount of ferricyanide. Samples were freshly prepared in 0.05 M pH 7.0 potassium phosphate buffer before any spectroscopy measurements. The absorption spectra were recorded (U-4100, Hitachi) after the preparation procedure to ensure that all chemical modifications were achieved. For VCS experiments, the final concentration of protein samples was adjusted to O.D. = 1 ± 0.05 in a 1 mm optical path length quartz sample cell at the selected excitation wavelength. The final concentrations of the samples are ~100 \( \mu \)M. The absorption spectra were also taken following the vibrational spectroscopy experiments to confirm the integrity of the samples during the laser exposure. For the kinetic measurements, the sample was passed through a PD-10 Sephadex G-25 column (GE Healthcare Bio-Sciences) twice to remove all remaining ferricyanide. Following this, the sample was sealed in a quartz cell and flushed with pure dry argon gas to remove oxygen. The ferrous cyt c that was used to measure the oxidation rate in the dark was prepared in 0.05 M potassium phosphate buffer by addition of sodium dithionite followed by centrifugation to remove excess reductant and deoxygenation with argon gas. All experiments were performed at room temperature.

Resonance Raman. Resonance Raman spectra were obtained using a standard 90\(^{\circ}\) light collecting geometry and a single grating monochromator (Acton SP2500i with 1800 g/mm UV holographic grating, Princeton Instruments). Details of the setup have been described elsewhere\cite{33}. Samples were placed in a standard quartz cuvette (Precision Cells, Inc.) and excited with ~ 5 mW of the 413.1 nm line from a krypton laser (Innova 300, Coherent).
Vibrational Coherence Spectroscopy. The femtosecond vibrational coherence spectroscopy system has been described in detail elsewhere (27, 43). Briefly, a 76 MHz laser pulse train at 412 nm was generated and split into two arms: pump and probe. The pump arm was modulated by an acoustic-optical modulator controlled by a lock-in amplifier at 1.5 MHz. A translation stage on the probe arm controlled the delay between the pump and probe pulses. The polarization of the pump and probe beams was adjusted to be perpendicular, allowing for both polarization and spatial filtering. The full width at half maximum of the pump and probe pulses was ~70 fs at the sample position. Both beams were focused into a spinning sample cell using a three inch lens in a near parallel geometry. After the sample, the beams were re-collimated and the pump light was spatially blocked and further extinguished by a polarization analyzer, so that only the probe beam, modulated by the pump, was detected. We used the open band scheme (27, 31, 44, 45) to obtain the VCS spectra. A Si photodiode collected the entire spectral bandwidth of the probe pulse.

Data Analysis. The LPSVD data analysis method used to extract oscillatory components of the VCS signal has been described in detail elsewhere (3). To generate the power spectrum amplitudes from the time-domain oscillatory signal, we used a linear predictive singular value decomposition (LPSVD) algorithm that can fit both the monotonic background and the damped oscillations simultaneously, as described by $\sum a_i \exp(-t/\tau_i)\cos(\omega_i t + \phi_i)$. A few parameters can be controlled during the fitting procedure, such as the number of oscillations. The coherence coupling signal around time zero was truncated before the fitting analysis. Data points within the truncated region (< 200 fs) were not included in the data analysis in order to eliminate the influence of the coherence coupling signal near time zero.
Photoreduction Kinetics Measurement. A crossed-beam transient absorption detection scheme was used to measure the photoreduction kinetics of *Pa* cyt c$_{551}$ and horse heart (hh) cyt c. The laser beam (at 413.1nm, krypton laser, Innova 300, Coherent) was expanded by a Thor Labs ED1-C20-MD engineered diffuser that homogeneously illuminated the sample in a 1 mm pathlength quartz cell (cross-section 10 x 10 mm$^2$). The 10 mm radius of the diffused beam at the sample position is adjusted to overfill the sample to ensure homogeneous excitation of the entire sample. The power measured after expansion is 11 mw so that the photon flux is found to be $J = 7.3 \times 10^{15}$ photons/s-cm$^2$. The absorption change of the sample due to photoreduction is recorded by a portable absorption spectrometer (Spectral Instruments, Inc. Tucson AZ), which was aligned about $\sim 30^\circ$ to the laser beam in order to avoid collecting scattering light. The experiment was repeated twice for each sample.

For each ferric *Pa* cyt c$_{551}$ sample, two loaded quartz cells were prepared aerobically as described above for the oxidized species. One is kept in the dark compartment within the Hitachi spectrometer; the other was used for the photoreduction kinetic measurements. An additional control experiment using ferrous cyt c was also performed. No oxidation was detected over time intervals corresponding to the photoreduction kinetic measurements. All kinetic experiments were performed twice with freshly prepared sample to assure the reproducibility of the results.

4.3 Results and Discussion

NSD Analysis and Resonance Raman Spectra. The wt *Pa* cyt c$_{551}$ and its F7A mutant share a very similar crystal structure(42), with a $\sim 0.6$ Å deviation of the mean square position of the C$_\alpha$ atoms.(42) However, a significant structural difference is found in the porphyrin OOP deformation along the ruffling coordinate. Using the normal coordinate structural decomposition (NSD) method,(4-6) the nonplanar heme distortions can be quantitatively characterized. NSD
describes out-of-plane distortions by using the lowest frequency OOP mode of each symmetry type. In our NSD analysis(33), we use the optimized planar reduced porphine (including the iron atom) with D$_{4h}$ symmetry as the reference structure. The displacement along each normal coordinate is calculated in the mass-weighted coordinate space using the scalar product $(X - X_0) \cdot Q_\alpha$, where $X$ and $X_0$ are the mass-weighted atomic coordinates of the x-ray input and reference structures. The unit vectors, $Q_\alpha$, are taken from the mass-weighted normal modes, $\alpha$, of the reference structure. As shown in Fig. 1, wt cyt c$_{551}$ displays a prominent ruffling distortion of 1.6 amu$^{1/2}$Å, and its distortions along the other normal coordinates are less than 0.5 amu$^{1/2}$Å. The mutation at position 7 leads to a 1.4 amu$^{1/2}$Å increase in the ruffling distortion compared to the wt, while the distortions along the other normal coordinates remain less than 0.5 amu$^{1/2}$Å. The difference along ruffling coordinate accounts for ~ 90% of the overall difference between wt and the F7A mutant, making these samples an ideal model system in which to study the effect of ruffling on electron transfer in the c-type heme.

Despite the 1.4 Å change in ruffling distortion, the optical absorption spectra display only a very small difference between the two c$_{551}$ proteins and both spectra are very similar to that of hh cyt c. The peak of the Soret absorption band is at 408 nm for the cyt c$_{551}$ compared to 409 nm for native hh cyt c. The FWHM of the Soret band for the two c$_{551}$ proteins are the same as for hh cyt c as can be seen from the absorption spectra shown in Figs. S1 and S2 in the SI. Upon mutation, the removal of the Phe ring produces a larger cavity near Cys12, which hosts three water molecules, making the cavity more polar(42). This polarity may affect the hydrogen bonds that constrain the CXXCH pentapeptide, leading to changes in the ruffling of the heme.(2, 3)

The low frequency resonance Raman spectra of oxidized wt and the F7A mutant are compared with hh cyt c in Fig. S3 of the SI. The observed spectra are aligned with the spectrum
of hh cyt c. However, a few changes in the c551 Raman spectra are observed: reduced intensity of \( \nu_9 \) (267 cm\(^{-1}\)), \( \nu_{31} \) (shifted from 304 cm\(^{-1}\) to 310 cm\(^{-1}\) ), \( \nu_8 \) (decreased intensity at 350 cm\(^{-1}\) ); \( \nu_{50} \) shifts from 359 cm\(^{-1}\) to 366 cm\(^{-1}\); the \( \delta(C_pC_aC_b) \) peak shifts from 412 cm\(^{-1}\) to 419 cm\(^{-1}\); and \( \nu_7 \) shifts from 701 cm\(^{-1}\) to 706 cm\(^{-1}\). As discussed previously, the low frequency spectra reflect coupling between the heme vibrations, the axial ligands, and the CXXCH motif. Thus, the change of the axial Met ligand orientation from R-met in hh cyt c to S-met in Pa cyt c551 along with the slightly different CXXCH configuration, are likely responsible for the changes of low frequency modes. The difference between the spectra of c551 wt and the F7A mutant are very small. The only difference is the weaker 379 cm\(^{-1}\) and \( \gamma_{21} \) modes in the less ruffled wt. (The higher frequency resonance Raman spectra of the wt and F7A mutant cyt c551 in the ferric and ferrous states are shown in Figs. S4, and S5 of the SI).

The \( \gamma_{21} \) mode near 568cm\(^{-1}\) is usually thought to be a ruffling distortion marker band because it shows intensity changes roughly proportional to the amount of the ruffling deformation (Fig. S6 in the SI). This relationship is consistent with previous cyt c unfolding studies(3, 46, 47). However, it is worth noting that the \( \gamma_{21} \) mode functions as a ruffling distortion marker band only in c-type hemes. In other heme systems, such as NP4-CN(33), NP-H2O(33), and LPO(48), this mode is not observed even though strong ruffling distortions are present.

**Figure 4.2.** Open-band VCS spectra of ferric Pa cyt c551 wt and F7A mutant at 412 nm. LPSVD fits and LPSVD power spectra are shown in the left and right panels, respectively.
**Low frequency VCS spectra.** The low frequency open band VCS spectra of wt *Pa* cyt c$_{551}$ and its F7A mutant are compared with hh cyt c in Fig. 2. The degenerate pump and probe pulses are centered at 412 nm. Open band VCS in electronic resonance has the unique ability to probe the low frequency region between 20-200 cm$^{-1}$ without interference from Rayleigh scattering or solvent signals.(25, 49, 50) The LPSVD spectra derived from the time domain data agree very well with frequency domain resonance Raman spectra in the region where overlap is possible (200 ~ 400 cm$^{-1}$). Comparisons between resonance Raman spectra and the VCS spectra are given in Fig. S7. The modes $\gamma_b$, $\gamma_c$, 155 cm$^{-1}$, 183 cm$^{-1}$, $\gamma_{24}$ (240 cm$^{-1}$), and $\nu_8$ are in excellent agreement among the three proteins. On the other hand, the mode at 133 cm$^{-1}$ for wt and 128 cm$^{-1}$ for F7A are not seen in cyt c, which might be due to the axial Met orientation difference between *Pa* cyt c$_{551}$ and hh cyt c. However, the general similarity of the low frequency spectra of *Pa* cyt c$_{551}$ and cyt c is consistent with the higher frequency resonance Raman spectra and implies that their porphyrin configurations are similar.

In a previous study of cyt c unfolding, the mode labeled $\gamma_a$ (Fig. 2) was suggested to have significant ruffling content(3). Because the wt *Pa* cyt c$_{551}$ and its F7A mutant have very similar Soret bands, and all other conditions in the VCS experiment were fixed, we can normalize the LPSVD spectra and compare the relative amplitude of the low frequency modes by using the peak of coherence coupling signal(3). Using this approach, we find the amplitude ratios $\gamma_a$(wt)/ $\gamma_a$(F7A) = 0.2 and $\gamma_b$(wt)/ $\gamma_b$(F7A) = 0.9. The small amplitude change of $\gamma_b$ is consistent with the cyt c unfolding study where $\gamma_b$ was found to be insensitive to changes in the ruffling distortion. As a result, $\gamma_b$ was assigned to a mode with very little or no ruffling content. The ratio of $\gamma_a$(wt)/ $\gamma_a$(F7A) = 0.2 is roughly equal to the square of the ratio of the ruffling distortion in the
two proteins, (~0.25). This result supports the assignment of $\gamma_a$ as primarily a ruffling mode and is consistent with the explanation for the Raman activity of “soft” low frequency modes proposed by Kubo et al.\textsuperscript{(33)}, where the amplitude is proportional to the square of its protein-induced distortion from the planar reference state.

**Figure 4.3.** Frequency of $\gamma_a$ is plotted as a function of ruffling deformation. Data points are from this work and NP4 work\textsuperscript{(33)}. Cyt c and Pa cyt $c_{551}$ data are in black squares, while NP4 data are in black circles. The data are fit by the solid curve using Eq. S8.3, as discussed in the SI. The fit results in values of $a = 0.09$ (amu$^{1/2}$/Å)$^{-1}$, and $a^2D_0/\mu_r=3.15\times10^3$ (rad/s)$^2$.

Inspection of Fig. 2 reveals an inverse correlation between the frequency of $\gamma_a$ and the magnitude of the porphyrin ruffling distortion. In wt cyt $c_{551}$, with a moderately ruffled heme, $\gamma_a$ is located at 58 cm$^{-1}$, which downshifts to 52 cm$^{-1}$ in the more ruffled F7A mutant. In hh cyt c, with its strongly ruffled heme, this mode (44 cm$^{-1}$) is located at an even lower frequency. A similar inverse correlation was observed for the ruffling mode in nitrophorin (NP4) as different axial ligands were added\textsuperscript{(33)}. The data from both works are plotted in Fig. 3 and are fitted with a straight line.

This inverse correlation demonstrates an OOP distortion induced mode frequency downshift, which probably reflects the anharmonic nature of the potential energy surface along the low frequency ruffling coordinate.\textsuperscript{(30, 51)} Generally, under an external force from the
surroundings, the potential along a given coordinate, \( q \), can be described as: \( U(q) = V(q) - qf_{ex} \), where \( f_{ex} \) is the applied external force and \( V(q) \) is the ground state potential in its absence. The equilibrium position, \( q_0 \), is determined by setting \( V'(q_0) - f_{ex} = 0 \). For small oscillations near \( q_0 \), the frequency is \( \sqrt{U''(q_0)/\mu_r} = \sqrt{V''(q_0)/\mu_r} \), where \( \mu_r \) is the reduced mass of the ruffling coordinate. If \( V(q) \) is a harmonic potential, there will be no change of frequency, because \( V''(q_0) \) is constant. However, the large distortion along the ruffling coordinate in cyt c_{551} and hh cyt c may exceed the limit for the harmonic approximation so that the observed frequency probes the anharmonicity of the potential surface(30). We discuss such a possibility in section S8 of the SI. The fit in Fig. 3 is found by assuming a Morse potential \( V(q) = D_e(1 - e^{-aq})^2 \), which allows the ruffling distortions to be correlated with an external force and the associated frequency \( \sqrt{V''(q_0)/\mu_r} \).

The above discussion is meant to provide a simple example demonstrating how relatively small anharmonic corrections can lead to observable shifts in a low-frequency heme mode such as ruffling. More careful diagnosis of the heme potential energy surface, when embedded in a protein, is needed to fully understand the frequency shifts of the observed low-frequency modes.

**Photoreduction Kinetics.** The difference in the ruffling distortion shown in Fig. 1 makes wt \( Pa \) cyt c_{551} and its F7A mutant an ideal system to investigate the relationship between the ruffling distortion and the electron transfer rate. The structural similarity between the two proteins limits the factors that can alter the electron transfer rate. The aromatic Phe7 sidechain of the wt is replaced in the mutant by a more polarized cavity close to Cys12 containing water. This cavity is \( \sim 10 \) Å away from the iron and is still further away from the \( \beta \)-pyrrole carbon and the solvent-exposed edge of the heme.(17) It is important to note that the relative orientation of the heme plane to the plane of Phe7 is nearly perpendicular (see Fig. S10 in the SI). This means that Phe7
is an unlikely electron donor in the photoreduction process (52-54). On the other hand, the relative position of the heme and the other aromatic side chains like Trp56 and Tyr27 stays nearly unchanged upon mutation. These residues are closer to the heme plane and more likely candidates to be the electron donor. Because of this we suggest that the mutation of Phe7 to Ala influences the photoreduction of heme primarily by modulation of its ruffling distortion.

We carried out experiments that measure the photoreduction kinetics of Pa cyt c{sub 551} using 413.1 nm excitation. Some results are plotted in Fig. 4a. The intensity of the sharp α-band absorption of the ferrous form near 550 nm was recorded as a function of time. (A comparison of the ferrous and ferric Pa cyt c{sub 551} absorption spectra are shown in Figs. S1 and S2 of the SI). We also measured the hh cyt c photoreduction kinetics in order to compare with a previous result (36). Photoreduction of cyt c is a much slower process than observed for Pa cyt c{sub 551}. On the time scale used in Fig. 4a, cyt c shows very little population evolution into the reduced form.

The photoreduction of Pa cyt c{sub 551} can be described by a 4-state model (36) consisting of DA, (DA)*, (D{sup +}A{sup -})*, D{sup +}A{sup -}, where D and A represent the electron donor (unknown) and acceptor (heme), respectively. The state DA represents the ground oxidized state and (DA)* represents a photoexcited or vibrationally hot oxidized state. The state (D{sup +}A{sup -})* represents the photoexcited/vibrationally hot reduced intermediate, while and D{sup +}A{sup -} is the reduced ground state. We can determine the photoreduction cross-section for cyt c{sub 551} using the photoreduction and photooxidation kinetic analysis provided previously (36):

\[ N_r = \sigma_r / (\sigma_o + \sigma_r) \]  
\[ k_{obs} = J(\sigma_o + \sigma_r) \]  

(1)

where \( J \) is the average photon flux, and \( k_{obs} \) is the observed rate of population change. The quantities \( \sigma_o \) and \( \sigma_r \) are the oxidation and reduction cross-sections, respectively, and \( N_r \) is the normalized photoreduced population in the photostationary state.
Figure 4.4. (a) Kinetic measurements of photoreduction. \( N_r(t) \) increases with irradiation time and is fitted with \( N_r = N_r(\infty) \, (\text{1} - \exp(-\sigma Jt)) \) and \( N_r(\infty) = \sigma_r/\sigma \), with \( \sigma = \sigma_r + \sigma_o \). The data for \( Pa \) cyt c\(_{551}\) wt, and F7A, and cyt c are shown as circles, triangles, and squares, respectively. The fitted curves are shown in red. Parameters used in the fits are summarized in Table 1. (b) The photoreduction cross-sections are plotted as a function of ruffling distortion and fit using an electronic overlap matrix element that goes as \( V_0 e^{-\beta(r_d + aq_0)} = V'_0 e^{-\beta aq_0} \). The value of \( \beta \) is taken as 2.0 \( \text{Å}^{-1} \), (55, 56) and the fit finds \( a = 1.2 \text{ amu}^{-1/2} \), which yields an increase in the “effective” tunnel distance, \( aq_0 \), of 1.9, 3.6, and 4.2 for the wt cyt c\(_{551}\), its F7A mutant, and hh cyt c, respectively.

<table>
<thead>
<tr>
<th></th>
<th>( Pa ) cyt c(_{551}) wt</th>
<th>( Pa ) cyt c(_{551}) F7A</th>
<th>hh cyt c</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \sigma_r )</td>
<td>( 6.4 \times 10^{-20} \text{ cm}^{-2} )</td>
<td>( 0.73 \times 10^{-20} \text{ cm}^{-2} )</td>
<td>( 4.3 \times 10^{-22} \text{ cm}^{-2} )</td>
</tr>
<tr>
<td>( \sigma_o )</td>
<td>( 3.6 \times 10^{-20} \text{ cm}^{-2} )</td>
<td>( 5.8 \times 10^{-20} \text{ cm}^{-2} )</td>
<td>( 0.88 \times 10^{-22} \text{ cm}^{-2} )</td>
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Table 4.1. Summary of photoreduction (\( \sigma_r \)) and photooxidation (\( \sigma_o \)) cross-section determined from Fig. 4.4a.

From the fits to the data, we can determine that the cross-sections for photoreduction and photooxidation, which are listed in Table 1. In addition to cyt c\(_{551}\), the photoreduction
experiments were carried out on cyt c as a control and the rates found agree within error with the values found by Gu et al. [36] who used a somewhat different methodology.

As mentioned in the experimental section, a control experiment with Pa cyt c551 samples was also performed to determine the rate of reduction in the absence of photons. The “dark” sample contained no observable ferrous population over an experimental time longer than the photoreduction kinetic measurements. Another control experiment using the ferrous sample also shows no observable oxidized population on the same time scale. This indicates that the “dark” reduction and oxidation rates are much smaller than the photon-induced electron transfer rates so that the “dark” ambient temperature oxidation and reduction reactions can be ignored as in previous work [36].

The results demonstrate that the photoreduction cross-section of Pa cyt c551 is nearly two orders of magnitude larger than that of hh cyt c, which is surprisingly large. Although the heme of cyt c exceeds the ruffling of wt cyt c551 by over a factor of two, it is only ~ 17% more ruffled than the F7A mutant (see Fig. S6). This is suggestive of a non-linear correspondence, where ruffling modulates the overlap of the donor orbital with the iron dπ acceptor orbital.

Although Pa cyt c551 has a similar tertiary structure to hh cyt c [1], we must consider the possibility that differences in the arrangement of the primary sequence of amino acids could also be responsible for its significantly enhanced photoreduction and photooxidation cross-sections compared to cyt c (see Table S11 in the SI). Although the aromatic amino acids are arranged differently in hh cyt c compared to c551, the closest aromatic amino acid in cyt c (Phe82) located in a near parallel geometry that is 7.3 Å away from the heme iron and 4.6 Å from the closest heme carbon atom. In Pa cyt c551 the closest amino acid is Tyr27, which also has a near parallel geometry and is 6.8 Å from the heme iron and 5.2 Å from the closest carbon atom on the heme.
Thus, the very similar heme-to-nearest aromatic residue distance for cyt c and Pa cyt c
suggests that the order of magnitude difference in the photoreduction cross-sections must depend
on something in addition to the donor-acceptor distance. Even when multiple donors are
considered, the effects of parallel channels are similar among the three samples and this does not
help to explain the order of magnitude differences in the photoreduction cross-section.

As mentioned earlier, the F7 residue of Pa cyt c_{551} is perpendicular to the heme and 9.9 Å
away from the heme iron and 7.6 Å from the closest heme carbon atom, making it highly
unlikely to be the primary e-donor in the wt protein. (Because the F7 distance from the heme in
Pa cyt c_{551} is larger than the heme-aromatic residue distances in cyt c, and because it has a
perpendicular orientation (52-54), we would expect the photoreduction cross-section to be smaller,
rather than an order of magnitude larger, if it was the primary electron donor). Thus, when we
eliminate the F7 residue as the primary electron donor in Pa cyt c_{551}, the different photoreduction
rates observed for wt and the F7A mutant must be reflecting the difference in the ruffling
distortion between these two nearly identical proteins. Given the similarity in donor acceptor
distances for cyt c and Pa cyt c_{551}, it also appears that the even more ruffled heme structure in cyt
c is implicated in the additional order of magnitude decrease of its photoreduction cross-section.
These results offer a direct experimental confirmation of the correlation between the heme
ruffling distortion and the electron transfer rate for heme reduction.

The photooxidation cross-sections listed in Table 1 are expected to be less sensitive to the
ruffling distortion because photooxidation probably involves electron loss from the ferrous π*
excited state. The ferrous iron is low-spin and already has fully filled iron t_{2g} orbitals, so that
there is no localized or delocalized hole state (associated with an unpaired d-electron) available
to modulate the tunnel distance. Moreover, the ruffling distortions are difficult to analyze in the
ferrous state because, although the ground state x-ray structure of ferrous Pa cyt c551 is known (21), the structure of its F7A mutant has not been reported.

**Nonlinear Ruffling Effect.** If the ruffling effect is assumed to be non-linear, as noted above, a simple exponential model can reasonably account for the photoreduction observations. In Fig 4b, we plot the rates as a function of the ruffling distortion and fit them using an exponential overlap model for the electronic matrix element, $V_{AB} = V_0 e^{-\beta(r_D + \alpha q_0)/2}$, which allows for an extra tunnel distance ($\alpha q_0$) to be associated with hole localization on the iron as the heme becomes more ruffled. The donor-to-heme-edge distance is given as $r_D$ and we take $\beta \sim 2.0$ Å$^{-1}$, which is a typical value for aromatic donor-acceptor systems when orientation effects can not be neglected (53, 56) (Ionic donors, where orientation issues are usually neglected, and covalently connected donor-acceptors, give somewhat smaller values for $\beta \sim 1.0-1.5$ Å$^{-1}$ (57).) Upon fitting the data in Fig. 4b, we find $\alpha = 1.2$ amu$^{-1/2}$, which leads to an increase of the effective tunnel distance of $\alpha q_0 \sim 1.9$, 3.6, and 4.2 Å for the three different ruffling geometries. Since these distance changes are on the order of the distance between the iron atom and the $\beta$-carbon atoms on the edge of the heme, they are self-consistent with the idea that the d-orbital hole state of the ferric heme can be delocalized anywhere from the iron atom to the heme periphery, depending upon the magnitude of the ruffling distortion, $q_0$. If the cytochrome c rate is excluded because of its differing structure and aromatic amino acids, we find $\alpha = 0.78$ amu$^{-1/2}$, which reduces the magnitude of the tunnel distance variation to 1.2 and 2.3 Å, well within the heme radius.

**Photoreduction Process.** Although transient heating of the heme acceptor states may play a role (36, 58) as discussed in the SI (Fig. S12 and Table S13), it seems likely that the actual electron transfer takes place while the heme is in an excited electronic state when there is a short lived “hole” in the porphyrin $\pi$-electron system with a lifetime, $\tau_{eg} \sim 100$fs (59, 60). If electron
transfer from a nearby aromatic donor amino acid takes place with a characteristic heme reduction time that is on the order of $\tau_r \sim 1$ ns, it results in a quantum yield of $\Phi = \frac{\tau_{eg}}{\tau_r} \sim 10^{-4}$. Given that the heme Soret band absorption cross-section is $\sim 10^{16} \text{ cm}^2$, this leads to an overall photoreduction cross-section that is close to the measured value of $\sim 10^{20} \text{ cm}^2$.

The heme excited state can undergo non-radiative relaxation via an intermediate state with one electron transferred from the porphyrin $\pi^*$ to the iron centered orbitals (61, 62). In the intermediate state, the half filled $\pi$ orbital will be delocalized on the porphyrin periphery, allowing for an increased electron transfer rate from the environmental electron donor to the heme. In the electronic ground state, the ruffling deformation tends to localize the ferric Fe $d_\pi$ hole-state on the iron atom (17) and analogous effects are expected for the $\pi$-orbital hole created by photoexcitation. For example, one can imagine that ruffling increases $\tau_r$ (by localizing the hole state at the iron and reducing the donor-acceptor overlap at the heme periphery). This leads to a reduction in the photoreduction cross-section, which is proportional to $\frac{\tau_{eg}}{\tau_r}$ as more ruffling is introduced to the heme. This interpretation is consistent with the suggestion by Liptak et al. that a more ruffled ground state heme will decrease the delocalization of Fe $3d_\pi$-based molecular orbitals onto the $\beta$ pyrrole carbons. This will effectively decrease the overlap integral between the “hole” in the $d_{\sigma}$-orbital and the electron donor orbital, which lowers the electron transfer rate (17). It is noteworthy that we draw the same conclusion, even if the photoreduction process is presumed to involve photoexcitation of the donor residue, where the overlap of the electronically excited donor orbital with the heme ground state d-electron hole orbital is the pertinent quantity to consider.

4.4 Conclusion The photoreduction kinetics of wt Pa cyt $c_{551}$ and its F7A mutant show a strong correlation between heme ruffling and the photoreduction cross-section. The
photoreduction rate is a reflection of the electron transfer process from a given donor to the heme(36) and therefore directly probes the effect of protein induced ruffling perturbations on the heme structure. When the even more ruffled heme of cyt c is also considered, it appears that the effect of ruffling on the electron transfer rates involves an exponential overlap dependence that varies on the length scale of the heme radius. This is consistent with the heme ruffling dependent hole delocalization hypothesis(17).

We also note that when the protein distorts the heme along the ruffling coordinate, which is a normal coordinate of the planar Fe-porphine core, the “ruffling mode” will mix differentially with other normal coordinates. In addition to introducing anharmonicity(51), this mixing can tune the mode-mode coupling resonances and allow interactions between the electron transfer partners to affect the electron transfer rate(23). Moreover, thermal excitations of the ruffling mode will probably contribute to the temperature dependence of the rate.

The observations presented here have obvious biological relevance for protein control of the electron transfer process. Because the ruffling deformation of the heme cofactor is believed to arise primarily from constraints associated with the CXXCH pentapeptide(2, 3), which lies close to the protein-protein binding site,(24) the binding event can perturb the CXXCH structure. Such a perturbation can lead to changes in the heme ruffling deformation that modulates and controls the electron transfer process. Additionally, it has been shown(3, 66) that when cyc c binds to the living mitochondrial membrane, the heme of the cytochrome relaxes to a much more planar state than when in solution. This suggests than it may be much easier for cyt c to accept electrons when it is bound in the mitochondrial membrane than when it is floating freely in the cytoplasm.
4.4 Supporting Information

Figure 4.S1 Absorption spectra of ferric and ferrous $Pa$ cyt $c_{551}$ wild type.

![Absorption spectra of ferric and ferrous $Pa$ cyt $c_{551}$ wild type.](image1)

Figure 4.S2 Absorption spectra of ferric and ferrous $Pa$ cyt $c_{551}$ F7A.

![Absorption spectra of ferric and ferrous $Pa$ cyt $c_{551}$ F7A.](image2)
Figure 4.S3 Resonance Raman spectra of *Pa* cyt c551 and its F7A mutant are compared with hh cyt c. Excitation wavelength is 413.1 nm. Power at the sample is 5 mw. The spectra are normalized with respect to the $\nu_4$ peak.
Figure 4.54 Resonance Raman spectra of ferric Pa cyt c551 wt and its F7A mutant. The excitation wavelength is at 413.1 nm and the power at the sample is 5mw. Spectra are normalized with respect to the ν₄ band at 1373 cm⁻¹. All mode positions agree very well between the two species, except γ₁₅ (751 cm⁻¹), which seems to diminish in the F7A spectrum and ν₁₁ (1560 cm⁻¹) which shifts to 1559 cm⁻¹. The mode γ₂₁ (566 cm⁻¹) shifts to 565 and increases intensity in the F7A spectra.
Figure 4.S5 Resonance Raman spectra of ferrous Pa cyt c551 wt and its F7A mutant. The excitation wavelength is at 413.1 nm and the power at the sample is 5mw. Spectra are normalized with respect to the $v_4$ band at 1361 cm$^{-1}$. All mode positions agree very well between the two species, except for modes at 396 cm$^{-1}$ and $\gamma_{11}$ (722 cm$^{-1}$), which shift to 394 cm$^{-1}$ and 720 cm$^{-1}$, respectively.
Figure 4.S6 The intensity of $\gamma_{21}$, near 565 cm$^{-1}$ in the resonance Raman spectra of ferric cyt c and $Pa$ cyt c$_{551}$ wt and F7A, is shown as a function of ruffling deformation. The relative intensity is obtained by normalizing each spectrum with respect to the intensity of its $\nu_4$ band. The three data points are roughly linear as shown by the fit (solid line).
**Figure 4.87** Comparison of resonance Raman spectra and the VCS results for ferric *Pa* cyt c_551_ wt (a) and its F7A mutant (b). The excitation wavelength for the Raman spectra is at 413.1 nm. The pump and probe wavelength used in the VCS experiment is 412 nm. The corresponding VCS oscillations are shown in the insert. The x-axis of the insert is time in picoseconds.

**4.88 Morse potential and frequency-distortion correlation.**

Generally, under external forces from surrounding constraints, the potential can be described as:

\[
U(q) = V(q) - q f_{ex}
\]  
(S8.1)

where \( f_{ex} \) is the force applied on the porphyrin along coordinate, \( q \), and \( V(q) \) is the ground state potential of the porphyrin in the absence of an external force. The equilibrium position, \( q_0 \), is
determined by \( dV(q_0)/dq_0 - f_{ex} = 0 \). Thus, for small oscillations near \( q_0 \), the frequency is 
\[
\sqrt{U''(q_0)/\mu_r} = \sqrt{V''(q_0)/\mu_r},
\]
where \( \mu_r \) is the reduced mass of the ruffling coordinate. For example, we can derive a correlation between frequency and distortion using a Morse potential. We let \( V(q) = D_e(1 - e^{-aq})^2 \) and, by setting the derivative of Eq. S8.1 to zero at the equilibrium position, \( q_0 \), we find 
\[
f_{ex} = 2aD_e(e^{-a q_0} - e^{-2a q_0})).
\]
This leads to:
\[
q_0 = -\frac{1}{a} \ln \left( \frac{1 + \sqrt{1 + s}}{2} \right),
\]
where \( s = -2f_{ex}/(aD_e) \) and exists on the domain \([-1, 0]\) so long as \( f_{ex} \) and \( q_0 \) are taken to be positive. Thus the frequency of a small oscillation at \( q_0 \) is:
\[
\omega = \sqrt{V''(q_0)/\mu_r} = \sqrt{2a^2D_e(2e^{-2a q_0} - e^{-a q_0})}/\mu_r
\]
As \( f_{eq} \) increases, the heme becomes more ruffled (\( q_0 \) increases) and the mode frequency \( \omega \) decreases (from Eq. S8.2, \( aq_0 \) resides on the domain \([0,-\ln(0.5)]\)).

Using the heme ruffling mode frequency as a function of ruffling distortion shown in Fig. 3 for cyt c, \( Pa \) cyt c\(_{551}\), and NP4(33), the parameters in Eq. S8.3 are determined by fitting the data, resulting in \( a = 0.09 \) (amu\(^{1/2}\)Å\(^{-1}\)) and \( a^2D_e/\mu_r = 3.15 \times 10^3 \) (rad/s\(^2\)). The linear nature of Eq. S8.3 for small values of \( aq_0 \) can also be deduced by completing the square and expanding to find
\[
\omega \approx \sqrt{4a^2 D_e}/\mu_r (3/4 - aq_0).
\]

The following figure shows the Morse potential curves generated using the fitting parameters. The dashed line is the potential in the absence of an external force. Additional potential curves with different values of \( f_{ex} \) are also plotted in the figure. For the black solid curves from top to bottom, we have normalized \( D_e \) to unity and found the values for \( f_{ex} = 0.012, 0.020, 0.026, 0.032, 0.033, 0.036 \) (amu\(^{1/2}\)Å\(^{-1}\)), which are determined by the ruffling distortion (\( q_0 \)) of each data point in Fig. 3 of the main text.
Softening of the $\gamma_a$ mode in cyt c unfolding.

It is necessary to note for cyt c, the frequency of $\gamma_a$ stays nearly unchanged in going from the folded to the unfolded state\(^3\). This is somewhat surprising because the ruffling distortion appears to be smaller in the unfolded state, based on the reduced intensity of the ruffling mode\(^3\). The reason for the lack of a frequency upshift upon unfolding is not precisely clear at this time, but it may be due to competing effects that arise from the coupling between the heme ruffling mode and the axial ligand modes which are much less constrained in the unfolded state. Because the Met heme ligand in the native state is replaced by a His ligand in the unfolded state, and because the two His ligands are free to undergo torsional motions in the unfolded conformation(s), they become mixed with the ruffling mode (as revealed by DFT calculations). The increased reduced mass depresses the ruffling frequency and may counteract the frequency upshift expected from the reduced ruffling distortion in the unfolded state.

In the cyt c unfolding study\(^3\), the reported frequency of $\gamma_a$ stays nearly unchanged in going from the folded ($\gamma_a = 44 \text{ cm}^{-1}$) to the unfolded state ($\gamma_a = 45 \text{ cm}^{-1}$). Although, absolute intensity measurements are only approximate, the decreased intensity of the ruffling mode upon
unfolding suggested(3) that at least one-third of the heme ruffling distortion remained in the unfolded form. This is presumably induced by the CHXXH motif, which remains intact upon unfolding. If the heme in the unfolded state has a ruffling deformation that is 33% of cyt c, it would correspond to \( \sim 1.2 \text{ amu}^{1/2} \text{Å} \), suggesting that the frequency of the ruffling mode should upshift to \( \sim 65 \text{ cm}^{-1} \) in order to be consistent with Fig. 3 of the text. The lack of the expected upshift may be due to the fact that there is ligand exchange in the unfolded state. The Met ligand in the folded state is replaced by another histidine so that a bis-His complex is formed with a very loosely packed structure. The absence of protein packing and hydrogen bonding means that the relative orientation of the distal histidines is not constrained in the unfolded state.

Using density functional theory (DFT) calculations, we investigated the ruffling mode frequency associated with the relaxed constraint on the His ligands in unfolded cyt c. The frequency calculation was performed on a geometry optimized imidazole-porphine-imidazole structure, which mimics the bis-His coordination of heme in the fully unfolded cyt c at neutral pH. The optimized structure and the calculation details are given in Fig. S9. From the calculation, we find that the heme ruffling mode is strongly coupled to the rotation of the imidazole ligands about the \( \text{N(imidazole)-Fe-N(imidazole)} \) axis, which accounts for 31% of the total kinetic energy. Additional calculations on the same structure, but with the two axial imidazole ligands held fixed, showed that the ruffling frequency up-shifted from \( 60 \text{ cm}^{-1} \) to \( 70 \text{ cm}^{-1} \) in the constrained model. This result supports the possibility that the relaxed constraints on the distal and proximal His ligands in the unfolded cyt c allows coupling between the His rotation about the \( \text{N-Fe-N} \) axis and the heme ruffling mode. This coupling increases the reduced mass of the ruffling mode and lowers its frequency in the unfolded state. The increased reduced mass of the ruffling mode in the bis-His state compensates to some degree, the upshift predicted by Fig. 3 of the main text.
Another possibility is that the ruffling distortion is not radically changed by unfolding. If the CXXCH motif is primarily responsible for the ruffling distortion(2), it may remain, even in the unfolded state.

**Figure 4.S9.** The DFT optimized Fe-porphine bis-histidine structure. Geometry optimization and harmonic frequency calculations were performed by using the DFT method at the B3-LYP level implemented in the Gaussian 09 software(63). Ahlrich’s VTZ basis set(64) was employed for the iron, whereas the moderate 6-31G(d) basis set was used for H, C,and N atoms. Normal coordinate structural decomposition (NSD) analysis was performed for both optimized structures and the major OOP distortions are shown. In the geometrically optimized structure, the two His ligands planes are nearly parallel to each other. The porphine is in a near planar structure with a small saddling distortion (0.2 amu$^{1/2}$Å).
Figure 4.S10 Overlap of *Pa* cyt c<sub>551</sub> wt (PDB#351C) and the F7A mutant crystal structure(PDB#2EXV).(42) The aromatic residues are shown in stick format (wt in red color, F7A in blue color). The heme groups of wt and F7A nearly overlap with each other except for a rotation of one of the propionate groups. The wt heme group is shown in solid stick format, while the heme group of the mutant is shown in a transparent blue color. For clarity, only the secondary structure of wt is shown. All aromatic groups for the cyt c<sub>551</sub> system are on the proximal side of the heme. As shown in the figure, the distance from Phe 34, Trp56 and Trp 77 to heme is nearly unchanged by the F7A mutation. Tyr27 moves 0.3 Å perpendicular to the heme plane in F7A, but maintains basically the same distance to the closest heme meso carbon atom, due to the bending of the meso carbon as a result of increased ruffling distortion in F7A. The distance from Tyr27 to the closest meso carbon and two beta pyrrole carbons is nearly unchanged, suggesting that the overlap between the heme π and Tyr27 π orbitals is minimally affected.

Finally, the Phe7 residue that is mutated, resides further from the heme and is about ~1.5x the distance from the heme as found for the aromatic residues in cyt c (see Table S11) where the photoreduction rate is an order of magnitude smaller. If Phe7 were the primary electron donor in *Pa* cyt c<sub>551</sub>, we would expect to measure photoreduction cross-sections that are much smaller and on the order of what we see in cyt c. The fact that this is not observed indicates that F7 is not the primary electron donor. Moreover, the orientation of the Phe7 plane is perpendicular to the heme plane(52, 55, 65), which is consistent with the conclusion that it is not an efficient electron donor in *Pa* cyt c<sub>551</sub>. 

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Table 4.S11  Summary of aromatic amino acids in hh cyt c and Pa cyt c551 and their distance to Fe and to the closest C atom on porphyrin ring. The symbols // and ⊥ are used to crudely indicate the relative orientation of the aromatic amino acid rings with respect to the porphyrin ring. The distance is calculated by averaging the distances from the two opposite atoms on the aromatic ring to the iron or nearest carbon atom on porphyrin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Aromatic amino acids</th>
<th>Distance to Fe (Å)</th>
<th>The closest Carbon atom on porphyrin</th>
<th>$x_0^*$</th>
<th>$e^{-\beta x_0}$ **</th>
</tr>
</thead>
<tbody>
<tr>
<td>hh cyt c</td>
<td>Phe82</td>
<td>7.3</td>
<td>Cα</td>
<td>4.6</td>
<td>1.01E-04</td>
</tr>
<tr>
<td></td>
<td>Tyr67</td>
<td>7.6</td>
<td>C₈</td>
<td>5.1</td>
<td>3.72E-05</td>
</tr>
<tr>
<td></td>
<td>Trp59</td>
<td>9.7</td>
<td>Pyrrole A</td>
<td>5.7</td>
<td>1.12E-05</td>
</tr>
<tr>
<td></td>
<td>Tyr74</td>
<td>13.1</td>
<td>C₇</td>
<td>10.5</td>
<td>7.58E-10</td>
</tr>
<tr>
<td></td>
<td>Phe46</td>
<td>9</td>
<td>Pyrrole D</td>
<td>5.7</td>
<td>1.12E-05</td>
</tr>
</tbody>
</table>
Phe10 9 C1 6.3 3.37E-06 Proximal, ⊥
Tyr48 9.7 Cγ 6.5 2.26E-06 Proximal, ⊥
His26 11.6 C6 10 2.06E-09 Proximal, //
Tyr97 13.9 C1 10.4 9.26E-10 Proximal, ⊥
Phe36 14.0 Cδ 10.7 5.08E-10 Proximal, //
His33 16 C8 14.0 6.91E-13 Proximal, //

Pa cyt c551

<table>
<thead>
<tr>
<th>Aromatic Amino Acid</th>
<th>Dist. (Å)</th>
<th>Type</th>
<th>σ(C2H)n</th>
<th>Term</th>
<th>Proximity</th>
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<tr>
<td>Tyr27</td>
<td>6.8</td>
<td>Cδ</td>
<td>5.2</td>
<td>3.04E-05</td>
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</tr>
<tr>
<td>Trp56</td>
<td>9.3</td>
<td>Cγ</td>
<td>6.2</td>
<td>4.12E-06</td>
<td>Proximal, //</td>
</tr>
<tr>
<td>Phe7</td>
<td>9.9</td>
<td>C1</td>
<td>7.6</td>
<td>2.50E-07</td>
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<tr>
<td>Phe34</td>
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<td>Trp77</td>
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<td>1.02E-08</td>
<td>Proximal, ⊥</td>
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</tbody>
</table>

† The distance, $x_0$, from the amino acid to the closest carbon atom on the porphyrin ring.
‡ The functional dependence assumed for the electronic overlap matrix element, with $\beta = 2 \text{ Å}^{-1}$, which is used to fit the photoreduction cross-sections, can be expressed as $V_0 e^{-\beta x_0} e^{-\beta aq_0}$. In the case of multiple electron donors, their contributions are summed so that $V_0 \left( \sum_i e^{-\beta x_{0,i}} \right) e^{-\beta aq_0}$. In the main text, we assumed that the term $\left( \sum_i e^{-\beta x_{0,i}} \right)$ was approximately the same for hh cyt c and Pa cyt c551 (i.e., the ratio is << 100). In order to justify this assumption, we have listed the values of $e^{-\beta x_0}$ for each aromatic amino acid in this column. From the listed values we find that the ratio between the values of $\left( \sum_i e^{-\beta x_{0,i}} \right)$ for hh cyt c and c551 is on the order of 3-5 and is essentially independent of whether single or multiple electron donors are considered. The ratio is 3.32, if only the closest amino acid is considered, 4.63, if all aromatic amino acids that are parallel to porphyrin plane are considered, and 4.76, if all aromatic amino acids are included. Since these values are smaller than 10, and so similar, it appears that the different arrangement and number of amino acids in cyt c compared to Pa cyt c551 does not play a significant role, justifying the assumption used in the main text.

**Vibrational heating of heme.**

It is possible that, during the excited state electron transfer, the excited vibrational temperature also plays a role. This could be one explanation why the photoreduction cross-section does not track the Soret absorption and is enhanced as the excitation is moved to the
blue(36, 58). More importantly, the similarity in the short time kinetics between wt Pa cyt c₅₅₁ and its F7A mutant (see Figs. S12 and Table S13), indicates that their electronic and vibrational decay pathways do not differ significantly. This is consistent with the idea that the difference in the ruffling distortion plays a primary role in determining the relative photo-reduction cross-sections, probably by altering the inherent quantum yield, \( \Phi = \frac{\tau_{eg}}{\tau_r} \), through reduced donor-acceptor electronic overlaps affecting \( \tau_r \), or possibly by changes in the heme non-radiative decay rate, \( \tau_{eg} \). The latter effect might arise if there was an increase in the porphyrin \( \pi^* \)-to-iron non-radiative decay rate due to more positive charge on the iron in the ruffled state.

**Figure 4.S12** The optical response as a function of excitation wavelength for the cyt c₅₅₁ and its F7A mutant. The figure displays the ps kinetic response of wt Pa cyt c₅₅₁ and its F7A mutant excited at different wavelengths ranging from 412 nm to 435 nm. The pump/probe pulse overlap produces a coherence coupling signal in the -200 fs to 200 fs region, which is removed in order to fit the kinetic response. From the fits, we determine that the vibrational relaxation time for wt Pa cyt c₅₅₁ and the F7A mutant is similar and is in the range \(~ 2-4 \) ps.
Table 4.S13 Time constants for the population dynamics of ferric Pa cyt c551 wild type and F7A obtained by LPSVD. The kinetic rates are roughly independent of wavelength.

<table>
<thead>
<tr>
<th>λ&lt;sub&gt;ex&lt;/sub&gt; (nm)</th>
<th>Decay time for wt (ps)</th>
<th>Amplitude for wt (a.u.)</th>
<th>Decay time for F7A (ps)</th>
<th>Amplitude for F7A (a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>412*</td>
<td>0.15 (τ&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>1.08</td>
<td>0.14 (τ&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>1.26</td>
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<tr>
<td></td>
<td>0.51 (τ&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>0.58</td>
<td>0.53 (τ&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>0.60</td>
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<tr>
<td>418</td>
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<td>0.49</td>
<td>0.16 (τ&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>0.50</td>
</tr>
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<td>0.60 (τ&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>0.54</td>
<td>0.53 (τ&lt;sub&gt;2&lt;/sub&gt;)</td>
<td>0.37</td>
</tr>
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<td>4.6 (τ&lt;sub&gt;3&lt;/sub&gt;)</td>
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<td>3.6 (τ&lt;sub&gt;3&lt;/sub&gt;)</td>
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<tr>
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<td>0.31 (τ&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>0.36</td>
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<td>0.52 (τ&lt;sub&gt;2&lt;/sub&gt;)</td>
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<td>0.43</td>
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<td>425</td>
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<td>0.52 (τ&lt;sub&gt;2&lt;/sub&gt;)</td>
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<td>0.56</td>
<td>2.8 (τ&lt;sub&gt;3&lt;/sub&gt;)</td>
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<tr>
<td>435**</td>
<td>2.0 (τ&lt;sub&gt;3&lt;/sub&gt;)</td>
<td>0.25</td>
<td>2.3 (τ&lt;sub&gt;3&lt;/sub&gt;)</td>
<td>0.21</td>
</tr>
</tbody>
</table>

* Because of low amplitude of ~ 4 ps (τ<sub>3</sub>) kinetics at 412 nm, the LPSVD does not generate a reasonable estimate for τ<sub>3</sub>.

**There is also no observable τ<sub>1</sub> and τ<sub>2</sub> kinetics at 435 nm.

References


63. Frisch, M. J. e. a. (2009) *Gaussian 09, Revision B01; Gaussian, Inc; Wallingford, CT*.


Chapter 5

Investigations of heme ligation and ligand switching in cytochromes P450 and P420

5.1 Introduction

The cytochrome P450 enzyme family (CYP) is composed of a broad range of heme-containing proteins that are involved in drug metabolism, toxicity, xenobiotic degradation, and biosynthesis(1). One key structural feature of these proteins is the coordination of the thiolate anion of cysteine (Cys) to the heme iron as the fifth ligand in the active P450 form(2-5). The biologically inactive conformation of a cytochrome P450 protein is typically denoted as the “P420” form and is characterized by a CO bound Soret peak near 420 nm, which is blue-shifted with respect to the peak at ~450 nm found in the active cytochrome P450. The inactive conformation can be formed from all known types of P450 using various methods(6-10). It has recently been accepted that this spectral change is due to protonation of the cysteine thiolate, resulting in thiol ligation to the heme iron(10-12). On the other hand, it has also been suggested that the P450 → P420 transition involves a ligand switch from a cysteine (Cys) to a histidine (His) ligated heme(13). The similarity of the absorption spectra between the P420 form of cytochrome P450 and other proximal histidine ligated heme proteins(14) reinforces such a correlation. Wells et al.(13) provided key evidence of histidine ligation in the CO bound form of P420 by observing a strong $v_{\text{Fe-His}}$ mode at 218 cm$^{-1}$ in the 10 ns transient Raman spectra of the P420$_{\text{cam}}$-CO photoproduct with an intensity equivalent to that of MbCO. Moreover, the equilibrium resonance Raman spectrum of the P420$_{\text{cam}}$-CO adduct is virtually identical to that of MbCO, lending further support to the histidine ligation model, at least when CO is bound.
On the other hand, heme model compound studies(4, 5) along with spectroscopic comparisons between chloroperoxidase and cytochrome P420$_{cam}$ have led to suggestions(12) that a thiolate-thiol transition might accompany reduction of the ferric P420 (even though a residual low-spin thiolate population is also observed(12)). More recently, Perera et al.(11) showed that the proximal ligand mutant (H93G) of deoxy myoglobin (Mb) can bind thiol and thioether compounds with high affinity, $K_d \sim 10 \mu\text{M}$. This conclusion is based on the observation of changes in the absorption spectra of the reduced H93G Mb mutant upon titration with tetrahydrothiophene (THT) and cyclopentathiol (CPSH)(11). However, it must be pointed out that the changes of the absorption spectra upon ligand binding are quite small and this could be due to either direct heme ligation or to perturbations of the electrostatic environment surrounding the heme(15, 16). The observed optical changes are not large enough to provide unambiguous evidence for heme ligation by either THT or CPSH.

Recently, Sabat et al.(10) suggested that thiol could be the proximal heme axial ligand in the inactive (P420) form of inducible nitric oxide synthase (iNOS). This protein is analogous to the P450 class, based upon thiolate ligation to the heme in its active state. The conclusion to exclude histidine as the proximal ligand in the 5 ns transient Raman spectra of the CO adduct was based on the absence of the expected $\sim 1 \text{ cm}^{-1}$ H/D isotopic shift for the 221 cm$^{-1}$ mode, which is usually assigned to the Fe-His vibration(17-22). Based on the absence of a H/D isotopic shift, it was concluded that the 221 cm$^{-1}$ mode observed in the inactive iNOS-CO photoproduct spectrum was neither a Fe-His nor a Fe-SH stretching mode (the latter ligation state was expected to generate an even larger isotopic shift). As a result, Sabat et al. assigned the 221 cm$^{-1}$ mode to a new mode associated with the thiol-ligated ferrous heme chromophore. However, if this assignment is correct, the 221 cm$^{-1}$ heme mode should be observed in the thiol-bound
reduced state of the inactive P420 iNOS and other reduced P420 systems or analogs. Because the Raman spectrum of reduced P420 iNOS is not available(10), we turned to the Raman spectrum of reduced P420$_{cam}$ to try and find the predicted thiol-bound heme mode at 221 cm$^{-1}$. However, the Raman spectra of reduced P420$_{cam}$ from either this or a prior(13) study does not reveal the presence of such a mode.

Therefore, in order to resolve the various characterizations of the heme proximal ligand in P420 systems, we have used resonance Raman spectroscopy to study the CO bound and ferrous forms of P420$_{cam}$ and H93G Mb; the latter, in the presence and absence of THT and CPSH. There is no 221 cm$^{-1}$ mode observed in ferrous P420$_{cam}$ or in the reduced H93G Mb with or without the THT or CPSH ligands. Moreover, it is also noteworthy that the experiments show no difference, within the resolution of $\pm 1$ cm$^{-1}$, between the resonance Raman spectra of reduced H93G Mb with or without the THT and CPSH. These observations do not yield supportive evidence that either THT nor CPSH directly ligates the heme iron of the reduced H93G Mb.

Additional experiments are presented that probe the inverse correlation of the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{CO}}$ Raman modes and are consistent with histidine as the proximal heme ligand in CO bound P420. As discussed below, we conclude that the $\sim 220$ cm$^{-1}$ modes observed in P420-CO, iNOS-CO, and H93G-CO photoproduct Raman spectra are, in fact, signatures of Fe-His ligation in the CO bound complexes.

We also construct a kinetic scheme that describes the photostationary states of P420-CO and H93G-CO and accounts well for the various Raman observations consistent with previously determined rate constants. A fundamental hypothesis underlying the kinetic model is that CO binding to heme greatly increases the “acidity” of the ferrous iron atom so that it efficiently recruits the strong sigma donating histidine ligand. Earlier work has shown that the affinity of
CO bound heme for imidazole is \(\sim 10^5\) times larger than its affinity for a weak ligand such as water(23). This concept has been used previously, both in the context of low pH MbCO ligation kinetics(24) and in prior studies of ligand switching in H93G-CO(25, 26); it is extended here to account for Raman observations in the P420 system.

### 5.2 Materials and Methods

All chemicals used in this study were purchased from Sigma Aldrich. Imidazole-free sperm whale H93G myoglobin was prepared as previously described(11, 27). High purity P420\textsubscript{cam} was prepared in the absence of camphor by pressure treatment of P450\textsubscript{cam} as previously reported (8, 9, 12). We have shown in previous studies that high hydrostatic pressure will dissociate bound substrate(28) and that the pressures required to initiate P420 inactivation are less in the absence of camphor. Thus, in order to generate a clean sample of P420, the substrate free form of the protein was used. The reduced H93G Mb and P420\textsubscript{cam} samples were prepared in 0.05 M KPi pH 7.0 buffer and the protein concentrations were adjusted to 100 µM. A small amount of saturated sodium dithionite solution (1% by sample volume) was used to reduce the samples. In order to prepare the samples of H93G Mb with thiol or thioether, either 20 mM of tetrahydrothiophene (THT) or cyclopentanethiol (CPSH) in ethanol stock solution was added to the reduced H93G Mb solution(11). The final concentration of THT and CPSH in the H93G Mb solution was 200 µM, which should lead to >95% binding with <1% bis-thiol formation, assuming the \(K_d=10 \, \mu\text{m}\) as taken from the work of Perera et al.(11).

Resonance Raman spectra were obtained using a standard setup with 90° light-collection geometry and a single grating monochromator model SP-2500i, Princeton Instruments, Acton, MA. An optical polarization scrambler was inserted in front of the monochromator to obtain the
intensity of the scattered light without bias from the polarization-sensitive grating. The monochromator output was coupled to a thermoelectrically cooled charge-coupled detector (PIXIS 400B, Princeton Instruments). To improve detection in the low-frequency region of Raman shifts, an interferometric notch filter (Kaiser Optical Systems, Ann Arbor, MI) was used to extinguish the elastically and quasi-elastically scattered laser light. Samples were excited with a 413.1 nm laser line generated by a krypton laser (Innova 300, Coherent) using a power of 11 mW at the sample or with a 442 nm laser line from a HeCd laser (Melles Griot) at powers up to 32 mW. In order to study the photolabile CO adducts, a cylindrical quartz cell with 10 mm diameter was mounted to a home-built spinning system and used for the Raman measurement. The spinning speed was set at 6000 rpm for all experiments except static measurements. All Raman spectra were frequency calibrated using pure fenchone with ~ 1 cm⁻¹ spectral resolution.

**Results**

Resonance Raman spectra of deoxy H93G and its THT and CPSH adducts are compared with ferrous P420cam in Fig. 1. The corresponding high frequency region (1300 cm⁻¹ - 1700 cm⁻¹) region, including the ν₄, ν₃, ν₂ and ν₁₀ bands, is shown in Fig. S2 of the Supporting Information. All spectra in Fig. 1 are normalized to the ν₇ band. No differences are observed in the Raman spectra of reduced H93G Mb, H93G(THT) Mb and H93G(CPSH) Mb, although the absorption spectra in the Soret region show subtle, but clear, differences(11) (see also Fig. S1, Supporting Information). The concentrations of THT and CPSH used in these measurements should lead to ~ 95% binding according to K_d =10 μM as reported by Perera R. et al.(11). Based on prior Raman studies of ligand binding to the H93G Mb mutant(29), we expect to observe changes in the resonance Raman spectra when ligand binding to the heme takes place. Given the fact that there is no observable change in the resonance Raman spectra upon THT or CPSH binding, it
seems possible that these thioether and thiol compounds might be binding to a site in the H93G protein that is close enough to affect the Soret band shape and position (~2 nm shift), but perhaps they are not replacing the heme water ligand that is normally present in reduced H93G Mb.

**Figure 5.1.** Low frequency resonance Raman spectra of reduced H93G Mb, its THT and CPSH adducts, and ferrous P420$_\text{cam}$. The excitation wavelength is 413 nm and the laser power at the sample is 11mw for the H93G samples, while for the P420 sample the excitation is 20mw at 413nm (blue), 25 mW at 420 nm (cyan) and 32mw at 442 nm (magenta). The sample cell is spinning at 6000 rpm. All spectra are normalized to the $v_7$ band. The 492 cm$^{-1}$ feature also appears in Fig. 3 and is not assigned to a $v_{\text{FeCO}}$ mode.

The Raman spectra of reduced P420$_\text{cam}$ and the H93G derivatives also show no evidence of a mode near ~220 cm$^{-1}$. Sabat et al. (10) observed a Raman mode at 221 cm$^{-1}$ in the 5 ns photoproduct Raman spectrum of the inactive iNOS P420-CO adduct and, because a H/D isotopic shift was not detected, they assigned the 221 cm$^{-1}$ mode to a heme vibration activated by thiol ligation. However, if thiol is ligated to the heme in the reduced state, one would expect this mode to be present in the equilibrium Raman spectrum of the reduced P420 sample (i.e., the equilibrium species should display essentially the same modes, although slightly shifted, when compared to the 5 ns transient photoproduct species). Note that the P420 sample was probed at several wavelengths (413 nm, 420 nm(13), and 442 nm) and there is no evidence of a mode near
220 cm\(^{-1}\). The absence of a 221 cm\(^{-1}\) mode in the Raman spectrum of the equilibrium reduced P420\(_{cam}\) and the H93G P420 analog samples does not support its assignment to a thiol-bound reduced heme mode\((10)\). On the other hand, if the usual assignment of this mode to the Fe-His vibration is made, its observation in the 10 ns transient Raman spectra provides strong evidence for a heme-histidine bond in the CO-bound forms of P420\((13)\) and, by analogy, iNOS\((10)\). Such an assignment is also consistent with previous transient Raman studies of the H93G-CO photoprodut\((26)\).

**Figure 5.2.** Resonance Raman spectra of CO bound H93G, H93G (CSPH), and H93G (THT) excited at 413 nm. The incident laser power at the sample is 11mw. The sample is spinning at 6000 rpm. All spectra are normalized to the \(\nu_7\) band.

The low frequency resonance Raman spectra of CO bound H93G, H93G(CSPH), and H93G(THT) Mb, excited at 413 nm, are shown in Fig. 2. All spectra are normalized to the \(\nu_7\) band. There is essentially no difference between the three samples, except for small changes in the relative amplitudes of the 507 cm\(^{-1}\) and 522 cm\(^{-1}\) modes, which are associated with the Fe-CO stretching frequency. The broad mode at 492 cm\(^{-1}\) seen in both Figs. 1 and 2 is not isotopically sensitive (Fig. 3) and therefore not assigned to a Fe-CO mode. The spectra of the
corresponding high frequency region, including the ν₄, ν₃, ν₂ and ν₁₀ bands, are displayed in Fig. S3 of the Supporting Information and are identical for all three samples.

**Figure 5.3.** Resonance Raman spectra of the ν_{Fe-CO} and ν_{CO} modes of $^{12}$CO and $^{13}$CO bound H93G, H93G (THT) and H93G (CPSH). The low frequency Fe-CO stretching region is in the left panels. The high frequency CO stretching is in the right panels and the $^{13}$CO – $^{12}$CO difference is shown in blue. Excitation wavelength is 413 nm and laser power at the sample is 11 mw. The sample was in a cell spinning at 6000 rpm. Dashed and dotted lines indicate ν_{FeCO} modes of H93G(His)CO and H93G(H₂O)CO respectively.

Figure 3 shows the resonance Raman spectra for the ν_{Fe-CO} and ν_{CO} modes of the $^{12}$CO (black) and $^{13}$CO (red) adducts of H93G, H93G(THT), and H93G(CPSH) Mb. The lower frequency Fe-CO stretching region is shown in the left panels, while the higher frequency CO stretching is displayed in the right panels. The isotopic shifts confirm that there are two peaks corresponding to the ν_{CO} stretching. For the $^{12}$CO sample there is a strong ν_{Fe-CO} peak at 522 cm⁻¹ that shifts to 517 cm⁻¹ and a broader feature at 507 cm⁻¹ where the shift is less obvious, but
becomes apparent upon fitting the data as discussed in the Supporting Information (Fig. S4). The corresponding $v_{\text{CO}}$ stretching modes are located at 1960 cm$^{-1}$ and 1942 cm$^{-1}$, respectively. The $v_{\text{CO}}$ Raman frequencies agree very well with the infrared measurements reported previously (25). The shift of the 507 cm$^{-1}$ mode is associated with the histidine bound Fe-CO mode and is not so easily seen upon isotopic labeling due to its breadth and weaker resonance enhancement. (Note that the Soret absorption band of CO bound heme is blue-shifted by approximately 5 nm when a weak ligand such as water replaces imidazole (30). This means that, based on the Raman excitation profile of MbCO (16), the resonance enhancement at 413 nm will favor the Fe-CO mode of the water bound heme relative to that of the histidine bound population). There is also interference from the broad feature near 492 cm$^{-1}$, as seen in Fig. 1 and in the fit to the CO bound lineshape (Fig. S4). This peak has been previously reported for WT deoxyMb (31, 32) and its H93G mutant (25) and, since it shows no isotopic shift, it is not assigned to a Fe-CO mode. On the other hand, there is photolytic activity in the region near 507 cm$^{-1}$ (vide infra), so we are confident that it represents the position of a Fe-CO oscillator. Upon $^{13}$CO substitution, the respective $v_{\text{CO}}$ modes, 1960 cm$^{-1}$ and 1942 cm$^{-1}$, show clear shifts down to 1915 cm$^{-1}$ and 1897 cm$^{-1}$, respectively.

Figure 4 shows the resonance Raman spectra of the same samples as in Fig. 3, but it demonstrates the effect of photolysis when the spinning cell is stopped. The spectra in black have the sample in the quartz cell spinning at 6000 rpm, while the red spectra are accumulated in a static cell. The panels on the left side show the 460-550 cm$^{-1}$ $v_{\text{Fe-CO}}$ stretching region, while the panels on the right side display the 1870–2000 cm$^{-1}$ $v_{\text{CO}}$ stretching bands. As expected, the $v_4$ bands demonstrate that the ratio of the CO dissociated (5C) to the CO bound (6C) species is increased in the static cell (see Fig. S5 in Supporting Information). In the static-cell spectra, the
intensities of the 507 cm\(^{-1}\) band and the 1942 cm\(^{-1}\) band decrease simultaneously relative to the 522 cm\(^{-1}\) and 1960 cm\(^{-1}\) bands. Thus, we associate the 507 cm\(^{-1}\) \(v_{\text{Fe-CO}}\) peak with the 1942 cm\(^{-1}\) \(v_{\text{CO}}\) peak as belonging to the same FeCO species. A similar association holds for the 522 cm\(^{-1}\) and 1960 cm\(^{-1}\) peaks. Moreover, the data indicate that the 507/1942 cm\(^{-1}\) FeCO species has a slower CO geminate rebinding rate (and thus a smaller relative population in the photostationary state of the static cell) than the species at 522 cm\(^{-1}\) and 1960 cm\(^{-1}\).

**Figure 5.4.** Resonance Raman spectra of CO bound H93G with and without THT/CPSH. Spectra in black are taken with the sample spinning at 6000 rpm, while the red spectra are with a static cell. Panels on the left side show the 460-550 cm\(^{-1}\) \(v_{\text{Fe-CO}}\) stretching band and the difference spectra in blue reveal the presence of the 507 cm\(^{-1}\) mode because of its increased photolysis compared to the 522 cm\(^{-1}\) species. Panels on the right side display the \(v_{\text{CO}}\) stretching band and the analogous increased photolysis of the 1942 cm\(^{-1}\) species compared to the 1960 cm\(^{-1}\) species. The excitation wavelength is 413 nm and the laser power at the sample is 11 mw.
Figure 5.5. (a) Resonance Raman spectra of the P420 $^{12}$CO and $^{13}$CO adducts at $\lambda=413$nm. The power at the spinning sample cell is 11mw. The $\nu_{\text{Fe-CO}}$ region is on the left and the $\nu_{\text{CO}}$ is on the right with the $^{13}$CO-$^{12}$CO Raman difference spectra shown in blue. (b) Resonance Raman Spectra of P420 CO. Spectra in black are taken with the sample spinning at 6000 rpm, while the red spectra are with a static cell.

The resonance Raman spectra of the P420$_{\text{cam}}$$^{12}$CO and P420$_{\text{cam}}$$^{13}$CO adducts, showing the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{CO}}$ peaks, are displayed in Fig. 5a. To our knowledge, these are the first $\nu_{\text{Fe-CO}}$ and $\nu_{\text{CO}}$ frequency correlations that have been made for a P420 system. The $\nu_{\text{CO}}$ difference spectrum between $^{13}$CO-$^{12}$CO is shown in blue. For $^{12}$CO, the $\nu_{\text{Fe-CO}}$ stretching peak is found at 494 cm$^{-1}$, while the $\nu_{\text{CO}}$ mode is at 1965 cm$^{-1}$. The $\nu_{\text{CO}}$ mode from the resonance Raman spectrum matches the IR measurements very well (33, 34). In Fig. 5b we show the stationary vs. spinning cell comparisons where a loss of Fe-CO and CO band intensities is observed in the stationary cell. Again for P420, as observed in the H93G adducts, the ratio of the CO dissociated (5C) to the CO bound (6C) species is increased in the stationary cell. This is evidenced by the appearance of a $\nu_4$ band at 1361 cm$^{-1}$ in Fig. S5 (Supporting Information) that confirms the presence of
significant 5C photoproduct (rather than a 4C photoproduct)(31). There is no sign of a mode near ≈220 cm\(^{-1}\) in the photostationary state data (Fig. S5). Since this mode is clearly observed and assigned to \(\nu_{\text{Fe-His}}\) in the 10 ns transient Raman spectrum(13), its absence in Fig. S5 is attributed to a ligand switch between His and another ligand with a rate that is faster than the cw photoexcitation and escape to solution. It should also be noted that the \(\nu_4\) band is located at 1354 cm\(^{-1}\) in the 10 ns transient spectrum(13) and this band is shifted to 1361 cm\(^{-1}\) in Fig. S5. This is also consistent with a ligand switch. A kinetic scheme that accounts for these observations, along with the previously observed kinetic rates for CO binding to P420(35), is given below.

5.3 Discussion

![Figure 5.6](image-url)

**Figure 5.6.** Correlation plot of \(\nu_{\text{FeCO}}\) and \(\nu_{\text{CO}}\) with data from ref. 29-32. The red color open squares are for histidine (or imidazole) ligated heme systems. The open green triangles represent thiolate ligated heme systems. The open blue diamonds represent heme systems with a weak or absent proximal ligand. Three solid lines labeled 5C/6C(water), His, and Cys are the lines fitted to the data points. The solid blue and red dots are data points for H93G(CO) and P420(CO) from this work. The notation H93G(H\(_2\)O)CO and H93G(His)CO correspond to the histidine and water ligated populations of H93G-CO observed in the H93G Raman spectra.

The Raman spectra of the CO adducts of the H93G Mb mutant with and without added THT and CPSH are presented in Figs. 3 and 4. From the variation in photoactivity, we conclude that the \(\nu_{\text{Fe-CO}}\) modes at 507 cm\(^{-1}\), and 522 cm\(^{-1}\) are associated with the \(\nu_{\text{CO}}\) modes found at 1942 cm\(^{-1}\).
cm$^{-1}$ and 1960 cm$^{-1}$, respectively. These data points, along with others(30, 36-38), are plotted on a $\nu_{\text{Fe-CO}}$ vs $\nu_{\text{CO}}$ correlation diagram shown in Fig. 6. This allows us to compare the CO adducts of P420$_{\text{cam}}$ and the H93G compounds with the large data base of other CO bound heme species that have been studied using the correlation method(16, 36).

The $\nu_{\text{Fe-CO}}$ and $\nu_{\text{CO}}$ modes typically follow an inverse $\pi$ back-bonding relationship(36) as shown in Fig. 6. Back-donation of iron d$_{\pi}$ electrons to the CO $\pi^*$ orbitals strengthens the Fe-CO bond and weakens the C-O bond. Trans ligands with a stronger $\sigma$ donation will weaken the Fe-CO bond more than expected, due to the change in $\pi$ backbonding resulting from sigma donor competition for the iron d$_{\pi}$ orbital(37). Thus, CO adducts with a strong trans ligand lie lower on the plot. For the same reason, CO adducts with weaker trans ligand lie higher on the plot (e.g., the water-ligated FePPIX-CO data point(30) is denoted by the arrow). Mb variants with a neutral trans His ligand and different distal pocket mutations are spread along the line labeled His. The position of the various CO adducts on the line reflects the polarity of their distal binding pocket. CO adducts with distal residues that produce strong H-bond interactions, like V68N, or the Mb “closed” distal pocket (A$_1$) state(16) lie higher on the line, whereas those with nonpolar distal residues, like H64V or the Mb “open” distal pocket (A$_0$) state(16), lie lower on the line. The 507/1942 cm$^{-1}$ modes of the H93G MbCO complex are assigned to 6-coordinate low-spin histidine bound forms with a “closed” distal pocket, while the 522/1960 cm$^{-1}$ modes are assigned to a water bound form(25) (In principle, the 522/1960 cm$^{-1}$ modes could also result from a five coordinate CO bound species; however, the photostationary state conditions do not produce a 4C photoproduct $\nu_4$ band. Rather, a $\nu_4$ band at 1359 cm$^{-1}$ is observed, which is consistent with the assignment(25) of water as the ligand trans to CO in these complexes).
The ν\textsubscript{Fe-CO} and ν\textsubscript{CO} modes of cytochrome P420\textsubscript{cam} are found at 494 cm\textsuperscript{-1} and 1965 cm\textsuperscript{-1}. These values agree with independent Raman and IR data\cite{13, 33}. The 494/1965 cm\textsuperscript{-1} point is found on the inverse correlation diagram at a position that is consistent with a trans His ligand and an open distal pocket. This supports the assignment of histidine as the proximal ligand in P420\textsubscript{cam}-CO, but it is not fully conclusive because of recent work on model ferrous porphyrin complexes in thioether solvents that has suggested thiol complexes may have a similar back-bonding correlation\cite{37}.

Interestingly, as seen in Fig. 5, the Fe-CO and CO frequencies of P420 do not change upon photoexcitation in a stationary cell even though a significant photoproduct population is created (as evidenced by the ν\textsubscript{4} band at 1361 cm\textsuperscript{-1} observed in Fig. S5). Analogous to the H93G system, the P420-CO shows no indication of a Fe-His mode under the photostationary conditions (Fig. S5). This suggests that, as for H93G-CO\cite{25, 26}, there is a rapid loss of the His ligand upon CO photodissociation, i.e., \( k_{\text{Ex}} \gg k_{\gamma} (k_{\text{out}}/k_{\text{BA}}^{H}+k_{\text{out}}+k_{\text{Ex}}) \) in Scheme I below. Moreover, there must be a rapid reset rate, \( k_{\text{Re}} \), to the initial His bound ligation state (A\textsubscript{H}) following CO binding.

In contrast to H93G-CO, water does not appear as a photostationary state ligand (L) in P420-CO, although it probably participates as an intermediate in the ligand exchange process denoted by \( k_{\text{Ex}} \) in Scheme I. Assuming water functions as a transient ligand (L) in the photocycle, it is potentially detectable as a trans ligand in the CO bound state, A\textsubscript{L}, when the system is driven into a photostationary state equilibrium. In such a case, we might expect to see intermediates with frequencies (analogous to the 522/1960 cm\textsuperscript{-1} modes) that appear on the upper line in Fig. 6. However, the CO geminate recombination rate and geminate yield are very large for P420\textsubscript{(29)}, possibly exceeding those observed for CooA\textsubscript{(39)}. (The missing amplitude in the
early kinetic work(30) makes precise evaluation difficult, but the actual geminate amplitude for CO binding may approach 99\%). This has the effect of reducing the effective rate of \( C_L \) formation from \( A_H \), as well as the rate of \( A_L \) formation from \( A_H \) via the \( B_H-B_L \) channel in Scheme I. Under this condition very little \( A_L \) population will exist in the photostationary state (this is discussed in more detail in the Supplementary Information).

Scheme I: Kinetic model for photostationary states in H93G-CO and P420-CO with \( H \) indicating a proximal histidine ligand and \( L \) indicating a water (or, on longer timescales a thiol) proximal ligand. The H93G-CO sample reveals populations of \( A_H \), \( A_L \), and \( C_L \) while only \( A_H \) and \( C_L \) populations are observed in P420-CO (unless the states \( A_H \) and \( A_L \) have identical FeCO frequencies). This kinetic scheme is reduced to an effective three state system and analyzed more completely in the Supporting Information.

The other possibility to account for the fixed positions of \( \nu_{Fe-CO} \) and \( \nu_{CO} \) in the stationary cell (Fig. 5b) would be for the state \( A_L \) in Scheme I to have Fe-CO and CO frequencies that are identical to the histidine-ligated CO bound state (\( A_H \)). In principle, the latter possibility might be realized if thiol (but not water) was the ligand (\( L \)). However, this would appear to be a highly coincidental situation and would also require very rapid protein rearrangements. Ultimately, we can use the \( \nu_7 \) band as a reference to determine that the strength of the Fe-His mode in the 10 ns
transient spectra of P420-CO is equivalent to that of MbCO(13). This observation is the “smoking gun”, demonstrating that thermal equilibrium must favor the $A_H$ state in the P420$_{cam}$ system.

In the myoglobin H93G mutant, the native proximal ligand is replaced by Gly and the only possible candidates for histidine ligation are His97 on the proximal side and His64 on the distal side. The binding of His64 was previously excluded by resonance Raman spectra of the CO bound H64V/H93G double mutant, which is very similar to that of H93G MbCO(25). Based on transient Raman spectra that detects the 220 cm$^{-1}$ Fe-His mode, His97 was assigned as the prime candidate to be the $trans$ ligand when CO binds and acidifies the heme iron(26). When CO is photolyzed, the time resolved step-scan infrared data indicate(26) that the histidine ligand is replaced by water on a time-scale that is faster than $\sim 10^6$ s$^{-1}$ and that the histidine ligand does not rebind until CO bimolecular rebinding takes place(25). Thus, in the static cell, the population of the His bound form will be less than that in the spinning cell because the continuous photolysis leads to a larger proportion of the fast rebinding water bound species. The water bound heme has a lower proximal barrier and rebinds CO much more rapidly than the His bound heme(40). This is why only the $\nu_{CO}$ and $\nu_{FeCO}$ peaks, associated with the water bound heme population ($A_L$), are observed in the photostationary state when the spinning cell is stopped.

The model for the CO photolysis and H$_2$O-histidine ligand exchange in the H93G system in Scheme I is very similar to the ligand switch model presented in an earlier study(25), but measurements of H93G-CO kinetics(29) suggest that CO escape into solution must compete with the rate for histidine exchange with water in the pocket. The photostationary equilibrium spectra for H93G-CO display strong evidence that water ligates to the CO bound heme as suggested by earlier work(25). Moreover, the rate of histidine recruitment by the water bound CO state ($A_L$)
to form $A_H$ is on the order of, or smaller than, the rate of $A_L$ and $C_L$ production from $A_H$ (see Supplementary Information). The different photostationary state behavior of H93G compared to P420 can be traced to the geminate rebinding rate of $A_H$ in the respective systems and the fact that H93G retains a distal barrier that significantly slows its geminate rebinding compared to P420. We also note that the photostationary populations of $A_H$ and $A_L$ in Scheme I appear favor the $A_L$ state in H93G MbCO, even under spinning conditions. More details of the kinetic analysis in spinning and stationary conditions can be found in the Supporting Information.

The frequencies of the $\nu_{\text{Fe-CO}}$ and $\nu_{\text{CO}}$ modes in H93G MbCO are unaffected by the addition of THT or CPSH. Comparison of the absorption spectra of H93G deoxyMb in 200 µM CPSH and THT solution to the pure H93G deoxyMb shows a clear difference, as can be seen in Fig. S1. These results are consistent with prior work (11), but one can interpret the small absorption spectral change as THT and CSPH binding to the protein, close enough to affect the heme electrostatic environment (15, 16), yet without direct ligation to the heme. While it is conceivable that both the thiol and thioether compounds bind to heme and precisely mimic the histidine and/or water-bound heme ligation states, it seems much more likely that these compounds are not actually ligating the heme iron. Rather, they could have a binding site nearby, close enough to account for the 2 nm shift in the Soret band, which provides the only evidence that these ligands are binding to the reduced H93G Mb system (recall from Figs 1, 2, S2, and S3 that there is no binding effect registered in the heme-specific resonance Raman spectra). A non-heme binding site for THT and CPSH is also consistent with the observation of two H93G MbCO species. Two H93G MbCO states (histidine and water) are observed in the Raman spectra as described above. If THT and CPSH were binding to the heme iron in the expected 1:1 stoichiometry, one would expect that a single set of $\nu_{\text{Fe-CO}}$ and $\nu_{\text{CO}}$ modes would be observed.
In Fig. 2, the 522 cm$^{-1}$ peak shows a somewhat lower relative intensity compared to the 507 cm$^{-1}$ peak when THT and CPSH are added to H93G MbCO. This indicates that the His bound form, characterized by the 507 cm$^{-1}$ peak intensity, has more relative population when the CPSH or THT are added to the solution. The system is undergoing a complex photon driven dynamics that involves competition between the photoexcitation rate, bimolecular CO entry into the heme pocket, and the rate for recruitment of His97 as an iron ligand during the time CO is bound to the heme. Ligand switching models involving histidine and water have been proposed previously in the context of both H93G and low pH Mb(24, 25, 31, 41). Upon CO binding to the water-ligated H93G Mb state, the iron seeks to bind a strong sigma donating ligand(23) and recruits His97. Depending upon the photoexcitation rate, the CO escape and entry into the pocket, and the very different geminate rebinding rates for the water and His97 bound heme(40), the two CO-bound populations will reach a photostationary equilibrium (e.g., see Scheme I). The presence of CPSH and THT in the heme pocket might be expected to modify the equilibrium between $A_L$ and $A_H$, leading to the subtle changes in Fe-CO populations observed in Figs. 2 and 3.

In Fig. 1 there is no 220 cm$^{-1}$ mode observed in the resonance Raman spectra of either reduced H93G Mb or reduced P420$_{cam}$. This indicates that, in the absence of CO, histidine is not ligated to the reduced heme. The cysteine thiol ligand is the obvious candidate for ligation in the reduced state of P420. Although there is no direct spectroscopic evidence, the thiol ligation assignment in the ferrous form of P420 has been discussed in the context of similarities with CPO(11, 12). The absence of water ligated CO bound signals in the photostationary state Raman spectra indicate that water does not form a particularly stable intermediate in P420-CO; however, it does not eliminate water as a possible transient ligand in states $C_L$ and $A_L$ of the photocycle.
Nanosecond transient Raman spectra of P420\textsubscript{cam}-CO(13), iNOS P420-CO(10) and H93G-CO(25) adducts all show a strong 220 cm\textsuperscript{-1} mode, which is very similar to the MbCO 10 nanosecond transient Raman spectra(13) and has been assigned in many heme protein systems as the Fe-His stretching vibration(17-19, 22). If the 221 cm\textsuperscript{-1} mode, observed in the 5 ns photoproduct Raman spectrum of the P420 form of iNOS-CO, was a heme mode(10) (rather than the Fe-His vibration), we should be able to observe it in the Raman spectrum of the reduced P420 samples/analogs in Fig. 1. The absence of a 221 cm\textsuperscript{-1} Raman mode in Fig. 1 is inconsistent with its assignment to a thiol ligated heme mode. This indicates that the transient photoproduct Raman spectra of P420 systems is actually revealing the presence of a proximal histidine ligand, which has been recruited in the CO bound state as shown in Scheme I. Such an analysis is consistent with the prior assignment for the 220 cm\textsuperscript{-1} mode observed in the H93G-CO system(26). Thus, in thermal equilibrium (i.e., no photoexcitation), the histidine ligated P420-CO state is favored.

Since the $\nu_{\text{Fe-His}}$ mode at ~220 cm\textsuperscript{-1} is not active in 6-coordinate CO adducts(36) and the CO dissociated population rapidly replaces the histidine ligand with water, the photostationary experiments are not able to observe a $\nu_{\text{Fe-His}}$ mode with either a spinning or a static cell. Thus, the 5 ns transient resonance Raman spectrum of P420-CO, which displays a 220 cm\textsuperscript{-1} mode similar in intensity to the MbCO photoproduct, provides the most direct evidence to assign histidine as the \textit{trans} ligand in the CO bound P420 systems(13). We suggest that the apparent absence of an isotopic shift for the 221 cm\textsuperscript{-1} mode in the inactive iNOS P420-CO photoproduct spectrum is due to the fact that the H/D shift is only expected(10) to be 0.7 cm\textsuperscript{-1}. The signal-to-noise of the transient Raman spectra(10) of the iNOS P420-CO was significantly worse than that of the control experiment on MbCO where a ~ 1 cm\textsuperscript{-1} H/D shift was observed. The peak shift
algorithm developed previously(20) indicates that a 25 cm$^{-1}$ (full width at half-height) Gaussian
band that shifts by 0.7 cm$^{-1}$ will generate a maximum-to-minimum in the Raman difference
spectrum that is 8% of the measured peak height. Since the noise level of the transient difference
spectrum between the protonated and deuterated iNOS P420-CO photoproduct exceeds this
value by approximately a factor of two(10), we do not believe that the 0.7 cm$^{-1}$ isotopic shift of
the Fe-His mode could be detected for iNOS P420 and therefore that the ~0.7 cm$^{-1}$ H/D shift is
present, but undetectable.

Finally, one must address the issue of whether there are His ligands available to undergo
ligand switching with Cys in various P450 systems. Using the protein data base, we find that
there is at least one His residue within 10 -15 Å of the heme iron in membrane bound P450 3A4,
P450cam, and iNOS as shown in Figs. S7, S8, and S9 of the Supporting Information. These
nearby histidine residues are candidates for binding to heme when P420 undergoes the tertiary
structural changes associated with the binding of CO and the loss of the thiol ligand. These
distances have implications regarding the extent and type of the conformational fluctuations that
are taking place in these systems as they become inactivated. In other heme proteins, such as
chloroperoxidase(42) and Rr-CooA(43), axial thiolate ligands have been shown to undergo a
ligand-switch to a nearby histidine following reduction of the heme iron. It has also been
reported that the nitrophorin 1 H60C mutant loses its heme thiolate cysteine ligand upon heme
reduction(44). Generally, upon reduction of the heme iron, the neutralized heme core could
trigger protonation of the thiolate ligand leading to its dissociation and the subsequent structural
rearrangements that facilitate a functional and biologically relevant ligand switch(43, 45). A
variety of thiolate-heme proteins that have evolved to become heme-sensor proteins with
functional ligand switching reactions have recently been reviewed(46) and the P450/P420 reaction has many similarities.

Dunford et al.(47) recently reported that CYP121 from M. Tuberculosis can undergo reversible conversion from its P420 form back to the P450 form when the pH is raised from 6.5 to 10.5. The P420 form dominates at lower pH, while the P450 form dominates at higher pH. These observations were interpreted using a simple two-state model(47) involving the reversible protonation of the proximal cysteine ligand. There are 4 histidine residues less than 15 Å from the heme iron in CYP121. However, the H343 residue is only 2 amino acids away from the proximal Cys ligand, forming a HXC-Fe sequence that is shared with both CYP101 and CYP51 (For most CYP51s, this sequence is conserved(48), including the CYP51 from M. Tuberculosis studied by Dunford et al.). The proximity of His343 to the heme in CYP121 might facilitate its ligation to the iron atom in the event that protonation of Cys345 leads to its dissociation in the CO bound state. Upon deprotonation at higher pH, the Cys345 thiolate will again become a strong ligand that can potentially displace His343 so that the P450 form is reversibly recovered. The reversibility of this process will depend sensitively upon the strength of the His-heme-CO ligation and the new structural motif that is formed in the P420 state. An important point is that a Cys(thiol)→His→Cys(thiolate) ligand switch mechanism provides an alternative interpretation of the pH dependent reversible P420/P450 conversion in CYP121 and would help to explain the irreversibility observed in CYP51 (assuming the P420 structure formed in CYP51 is particularly stable). Moreover, the relatively slow (0.72±0.02 s⁻¹) and pH independent transition rate that is observed for P450→P420 conversion(47) might actually be more consistent with a ligand switch process as the rate limiting step, rather than a simple thiolate protonation reaction. (In the context of Scheme I, this means that an additional column of states A_T, B_T, and C_T, where T represents
thiol, must be added in slow exchange with the photocycle states $A_L$, $B_L$, and $C_L$, where $L=\text{H}_2\text{O}$.) Additional investigations specific to the P420 forms of CYP121 and CYP51 are clearly needed to identify the proximal ligand in the CO bound forms at acid and alkaline pH.

Figure 5.7 Crystal structure of CYP51 (PDB#1EA1), CYP121 (PDB#2IJ7), CYP101 (PDB#2CPP) and CYP3A4 (PDB#3NXU). The HXC motif in CYP51, CYP121 and CYP101 are highlighted. The right bottom panel shows the loop (magenta) of the heme proximal pocket in CYP3A4. The C442 and H402 residues are shown with a space filling model. There is no HXC motif in CYP3A4.

Along with CYP121 and CYP51, CYP101 shares the HXC-Fe motif with X representing Phe, Arg and Leu, respectively. The Cys loop HXC sequences in the heme proximal pocket of these proteins are shown in Fig. 7. Here we recognize that the His residue is actively engaged in H-bonding with the nearby propionate group of the heme. Some possible proximal pocket conformational changes that would allow binding of the nearby histidines to the heme iron are depicted in Fig. 8. Examination of the helix-loop transition region in CYP101, which lies between residues G359(helix) and L358(loop), suggests that upon dissociation of the protonated Cys357, the three adjacent loop residues L358, C357, and L356, could coil into the alpha helix, leaving His355 in prime position to bind to the iron atom as depicted in Fig. 8. Moreover, we find that another histidine (H352) lies downstream in the loop region and that it can be easily positioned to recover the necessary H-bond to the heme propionate. Thus, a relatively simple
and energetically favorable conformational fluctuation, slightly extending the L-helix, could lead to the histidine-heme ligation following CO binding in CYP101.

**Figure 5.8.** The three panels on the left side show the crystal structure of CYP101 (PDB#2CPP), CYP51(PDB#1EA1), and CYP121(PDB#2IJ7). The L-helix is extended by 5 residues past the Cys ligand in the CYP51 and CYP121 structures. The helix structures on the left and right side of the proximal cysteine ligand are labeled as A and B, respectively. The red dashed line indicates the hydrogen bond between “near” propionate group and H355, H392 and H343 in the three structures, respectively. The three panels on the right show the possible CO bound P420 structures for the three proteins. The proposed structures are formed by extending the A helix by three amino acids (highlighted in red). A remnant of the B helix structure is preserved in the proposed P420-CO structure for CYP51 and CYP121. In the proposed structures, H352, R391, and Q342 replace the histidine residues of the native structures by forming a hydrogen bonds (red dashed line) with the propionate group. Larger renditions and different angles showing the possible structures underlying the ligand switch can be found in the SI.
It is interesting to compare the CYP121 and CYP51 proteins, which also share the HXC motif. CYP121 displays a reversible pH dependent P450/P420 conversion while CYP51 does not(47, 49). Evidently, CYP51 converts to its P420 form immediately upon reduction, even in the absence of CO (49), suggesting that its Cys heme ligand has a pK that is somewhat higher than for CYP121. A simple thiol/thiolate pH titration model for P420 conversion would predict that, upon raising the pH high enough (say above 10), the thiol Cys ligand in CYP51 should ultimately deprotonate and revert to a thiolate so that a reversible P420/P450 should also be observed in this system. On the other hand, the ligand switch model can easily explain the irreversible behavior. For example, if a particularly stable P420 structure is formed upon CO binding, the simple deprotonation of the Cys residue may not be enough to energetically reconfigure the protein structure and recover thiolate ligation to the heme iron. In Fig. 8 we have also shown a possible structural change for CYP51 conversion to P420. Here we find that, upon extension of the L-helix to include Cys394 and the orientation of His392 as the axial ligand, Arg391 is naturally prepositioned to form a strong H-bond with the heme propionate. Thus, the Cys(thiol)→His ligand switch in CYP51 may form a very stable alternative structure (e.g., by incorporating the Cys(thiol) into the adjacent alpha helix and forming a strong Arg391 H-bond with the heme propionate) leading to a situation that is energetically stable and not reversible by pH back-titration(47). It should also be noted that the P420 form of CYP51 displays a partial reconversion to P450 upon loss of CO and re-oxidation(49). Since the iron-histidine bond is weakened following CO dissociation and iron re-oxidation, it is evidently possible for the cysteine thiolate residue to successfully compete to once again become a heme ligand in the ferric state of CYP51. In contrast to CYP51, CYP121 undergoes a reversible pH titration between P420 and P450(47, 49) [22 ref Munro22]. The structures that we found for the P420-CO
state in this system appeared to have adequate but less satisfying H-bonding to the heme propionate suggesting that it might have less energetic stabilization and therefore be more likely to undergo reversible transitions following pH titration.

Although many P450 systems share the HXC-Fe motif, some do not and their potential histidine ligands are not found as close to the proximal heme ligation site. Two important examples discussed above are iNOS and CYP3A4, where a larger tertiary structure change is necessary to bring a histidine close enough for heme ligation to occur. Figures 7, S7, and S9 [?? Substitute S8 CYP101 and 51 in larger scope than is done in fig 8?] (Supporting Information) show nearby histidine residues that are ligation candidates for these systems. For iNOS, H661 (~10 Å away) and H407 (~16 Å away) are potential candidates for a ligand switch with Cys415. For CYP3A4, the H402 (~14 Å away) is a possibility for ligand switching with Cys442 as seen in the lower right quadrant of Fig. 7. The fact that a strong 220 cm$^{-1}$ mode is observed in the nanosecond transient Raman spectra of iNOS-CO indicates that, even for proteins without the HXC-Fe motif, a histidine ligand can possibly be recruited to form the final P420 state following CO binding. We also note that, in systems without the obvious HXC-Fe motif, it may be possible that other strong sigma donating nitrogenous ligands can act as a substitute for histidine binding to the CO ligated heme.

In summary, the conversion of the P450 thiolate to thiol appears to be an important step in the conversion of P450 to P420. However, upon CO binding the 10 ns transient Raman spectra demonstrate that a nearby histidine is involved in a ligand switching equilibrium with the cysteine thiol. All indications are that this equilibrium favors a histidine bound P420CO ground state in CYP101. When there are His residues near the heme, as in the HXC-Fe loop, only relatively small tertiary structural changes are needed for the ligand switch (e.g., incorporation of
C and X into the nearby helix structure can properly position the His for heme binding). The structures that are formed in these P420-CO states may have variable stability. In some cases, the process might be reversible with pH titration (e.g., CYP121), depending upon the strength of the alpha helix interactions, the His-Fe-CO bond formation, and the heme propionate H-bond that is formed. On the other hand, if the P420-CO states are less stable, reversibility via pH titration would be more likely. If the ligand switch requires a very large tertiary structural change, the process would be likely to proceed at a much slower rate and may not be easily reversible (for entropic reasons), even upon back-titration of the thiol to the thiolate species.

5.4 Conclusion

We have used resonance Raman spectroscopy to study P420cam and the H93G Mb mutant, with and without the addition of THT and CPHS compounds. There is no evidence from the vibrational spectra to indicate that THT or CPHS ligate the heme iron. There is no 220 cm\(^{-1}\) Fe-His mode observed in the reduced samples, which indicates that histidine is not the heme ligand in the ferrous state. Thiol is the likely heme ligand in the thermally equilibrated ferrous state of the P420 systems, while water appears as the ligand in the reduced H93G system. The transient Raman spectra of the CO bound species indicate that a Fe-His bond is formed when CO binds to P420 systems and acidifies the heme iron. A histidine ligand for P420-CO is also indicated by the position of the \(\nu_{\text{Fe-CO}}\) and \(\nu_{\text{CO}}\) frequencies on the inverse correlation plots although, strictly speaking, thiol cannot be ruled out by this correlation (37) (??ref Vogel/Spiro??). On the other hand, the invariance of the residual P420 FeCO frequencies when photostationary photoproduct states are formed indicates that the equilibrium between \(A_H\) and \(A_L\) in Scheme I favors the histidine ligated state, \(A_H\), unless a thiol ligated state, \(A_L\), yields exactly the same frequencies. The photostationary state Raman spectra of H93G MbCO indicate that, following CO binding to
the water ligated heme, the rate for histidine recruitment is \( \sim 10^4 \text{ s}^{-1} \) (see Supporting Information). Several P450 systems offer a common HXC-Fe motif that may facilitate Cys-His ligand switching, while other P450 systems must undergo slower and larger tertiary changes in order for the acidic iron of the CO-bound heme to replace the weakly bound thiol ligand with a nearby histidine or with some other strong sigma bonding donor.

The above considerations bring into focus some motivations for trying to better understand the P450-P420 reaction. For example, if a reversible helix-loop transition triggered by CO photolysis underlies the P450-P420 conversion in even a sub-set of P450 systems, they would present interesting models for the study of protein conformational transitions using time-resolved spectroscopies. It is also conceivable that reversible transitions of this type might play a functional role in the regulation of the monoxygenase function. Finally, if it is possible to engineer the destabilization of the P420 form of the protein without similarly destabilizing the P450 form, one might envision P450 mutants that are more robust and less likely to convert to the P420 form. Mutations that remove propionate H-bonding in the P420 form (but not in the P450 form) provide one interesting target.
5.5 Supporting Information

Figure 5.S1. Absorption spectra of ferrous H93G, and H93G in the presence of 200 μM THT or CPSH.

Figure 5.S2. High frequency region of resonance Raman spectra of deoxy H93G, and H93G in 200 μM THT and CPSH at λ=413 nm. Incident power is 11 mw. Sample was in a cell spinning at 6000 rpm.
Figure 5.S3. High frequency region of resonance Raman spectra of CO bound H93G, and H93G in 200 μM THT and CPSH at λ=413 nm. Incident power is 11 mw. Sample was in a cell spinning at 6000 rpm.

Figure 5.S4. Fits to the Fe-CO region of the Raman spectrum with $^{12}$CO (top) and $^{13}$CO (bottom). The fitting was accomplished using Origin 8.0 (OriginLab, Northhampton, MA). The increase of the mode near 556 cm$^{-1}$ (FeCO bending) in the $^{13}$CO spectrum is probably due to a Fermi resonance with an underlying heme mode at 556 cm$^{-1}$. The Fe$^{12}$CO bending mode is located near 572 cm$^{-1}$. 
**Figure 5.S5.** The left upper panels show the 200-550 cm\(^{-1}\) range of resonance Raman spectra of the H93G-CO adducts. Spectra in black were recorded when sample was spinning at 6000 rpm, while spectra in red were measured when the sample was in a stationary condition. The right upper panels show the corresponding \(\nu_4\) band region that demonstrates the presence of a photolyzed population with \(\nu_4\) at 1359 cm\(^{-1}\). The lower panels show the P420-CO sample under the same conditions. It is noteworthy that the photolyzed population has a \(\nu_4\) band at 1361 cm\(^{-1}\) and that the \(\nu_{FeCO}\) band does not shift and no mode near 220 cm\(^{-1}\) is detected. This indicates that the Fe-His species, detected in the 10 ns transient studies (13), is replaced by another ligand, water is the likely prospect, on the sub-\(\mu\)s timescale and that the CO geminate recombination in P420 is much faster than for H93G (29, 35). The ultrafast CO geminate rebinding leads to only one CO bound state population being observed in the P420 system.
S6. Three-state kinetic model

Scheme I: Kinetic model for photostationary states in H93G-CO and P420-CO with H indicating a proximal histidine ligand and L indicating either a water or thiol proximal ligand. The H93G-CO sample reveals populations of \( A_H, \ A_L, \) and \( C_L \) while only \( A_H \) and \( C_L \) populations are observed in P420-CO unless the states \( A_H \) and \( A_L \) have identical FeCO frequencies. This kinetic scheme can be reduced to an effective three state system (\( A_H, \ A_L, \) and \( C_L \)). Some rates can be approximated from prior work: \( k_{\text{Ex}} \sim 5 \times 10^6 \text{ s}^{-1} \) (ref (26)); \( k_{\text{BA}} \sim 10^8 \text{ s}^{-1} \) (ref (30, 35)); \( k_{\text{in}} \sim 10^4 \text{ s}^{-1} \) (ref (29, 35)); \( k_{\text{out}} \) (P420) \( \sim 4 \times 10^7 \text{ s}^{-1} \) (ref (35)); \( k_{\text{out}} \) (H93G) \( \sim 5 \times 10^6 \text{ s}^{-1} \) (ref (29)); \( k_{\text{BA}} \) (H93G) \( \sim 0.5 \times 10^6 \text{ s}^{-1} \) (ref (29)); \( k_{\text{BA}} \) (P420) \( \sim 10^{9-10} \text{ s}^{-1} \) (ref (35)) where we have re-evaluated the missing geminate amplitude in the P420-CO system based upon recent studies of CooA(39). The significant difference in \( k_{\text{BA}} \) for H93G and P420 is attributed to the significant distal barrier present in the H93G Mb system and its absence in P420.
Here we describe a model which allows CO to escape into solution with a rate that is less than the replacement of the histidine ligand by water. The population of \( B_H \) (H93G(His)::CO) is ignored in the model because the \( \nu_{Fe-His} \) mode at 220 cm\(^{-1}\), which indicates the presence of 5-coordinate histidine bound form, was not observed in either the stationary or spinning cell conditions. It suggests that the majority of histidine dissociates and is replaced by water before CO re-enters the protein and binds. Moreover, the fact that the \( \nu_{Fe-His} \) mode is only observed in the 5-10 nanosecond transient Raman spectra \(^{(26)}\) suggests that His dissociates at rate \( k_{in} \approx 10^4 - 10^5 \text{s}^{-1} < k_{Ex} < 10^8 \text{s}^{-1} \). Under spinning conditions, the average time the sample spends in the beam per cycle is 6 \( \mu \text{s} \). Since there is no trace of H93G(His) in the spectra, it is likely that the histidine dissociation rate satisfies: \( 10^6 \text{s}^{-1} \leq k_{Ex} \leq 10^8 \text{s}^{-1}. \)

Upon photon excitation, CO escapes at a rate \( k_{out} \) or else geminately rebinds back at rate \( k_{BA}^H \). The \( B_L \) population can be ignored since \( k_{BA}^L >> k_{r}. \) The above model can be simplified to a three-state model:

Here \( k_a = k_r k_{out} / (k_{out} + k_{Ex} + k_{BA}^H) \), \( k_b = k_r k_{EX} / (k_{out} + k_{Ex} + k_{BA}^H) \), and \( k_a + k_b = k_r (k_{out} + k_{Ex}) / (k_{out} + k_{Ex} + k_{BA}^H) \).

By assuming the total population \( A_H + A_L + C_L = 1 \), we can reduce the rate equations to the following:

\[
\frac{d}{dt} \begin{pmatrix} A_H \\ C_L \end{pmatrix} = \begin{pmatrix} k_{Re} - (k_a + k_b + k_{Re}) A_H - k_{Re} C_L \\ k_a A_H - k_{in} C_L \end{pmatrix}
\]

(1)

A. Analysis of H93G CO Raman spectra

1. Solution for stationary state conditions

Under stationary state conditions, the population of each state is the solution of:

\[
\frac{d}{dt} \begin{pmatrix} A_H \\ C_L \end{pmatrix} = 0
\]

which is:
\[
\begin{pmatrix}
A_H \\
C_L \\
A_L
\end{pmatrix} = \frac{1}{k_{\tau}k_{a} + k_{i}} \begin{pmatrix}
k_{i}k_{Re} \\
k_{a}k_{Re} \\
(k_{a} + k_{b})k_{i}
\end{pmatrix}
\]

(2)

where \( k = k_{a} + k_{b} + k_{re} \).

From the stationary spectra in Fig. 4 of the main text, the \( \nu_{CO} \) peak at 1942 cm\(^{-1} \) corresponding to \( A_{H} \) is undetectable compared to the \( \nu_{CO} \) peak for \( A_{L} \) at 1960 cm\(^{-1} \). This suggests that \( A_{L} \gg A_{H} \) for H93G under stationary photon irradiation with \( k_{\gamma} \approx 10^{6} \) s\(^{-1} \). From Eq.2 we know that \( A_{H}/A_{L} = k_{Re}/(k_{a} + k_{b}) \). From the expression preceding Eq. 1, when His is ligated, we apply \( k_{BA}^{H}(H93G) \ll k_{out} \) (using rates listed in Scheme I, the quantum yield for escape is \( \approx 91\% \)), which yields \( k_{a} + k_{b} \sim k_{\gamma} \). Thus, we conclude that \( k_{Re} \ll k_{a} + k_{b} = k_{\gamma} \approx 10^{6} \) s\(^{-1} \).

The \( \nu_{4} \) bands at 1374 cm\(^{-1} \) (CO bound) and 1359 cm\(^{-1} \) (CO dissociated) are displayed in Fig. S5. Since the population of \( A_{H} \) is negligible compared to \( A_{L} \), we can ignore the \( A_{H} \) contribution to the \( \nu_{4} \) band. Thus, the intensity of these two \( \nu_{4} \) peaks weighted by the resonance Raman cross-section corresponds to the \( A_{L} \) and \( C_{L} \) the populations and \( A_{L}/C_{L} = (k_{a} + k_{b})k_{in}/(k_{a}k_{Re}) \), according to Eq. 2. Given that the resonance Raman cross-section of the 1374 cm\(^{-1} \) mode at 413 nm is roughly twice that of the 1359 cm\(^{-1} \) mode(50), we estimate \( A_{L}/C_{L} = (k_{a} + k_{b})k_{in}/(k_{a}k_{Re}) \) to be \( \approx 0.5, \approx 1, \) and \( \approx 1.5 \), for H93GCO, H93G(THT)CO and H93G(CPSH)CO, respectively. So \( k_{Re} = C_{L}/A_{L} \), \( (1+k_{b}/k_{a})k_{in} = C_{L}/A_{L}(1+k_{out}/k_{in})k_{in} \) (from the expressions preceding Eq.1). Since \( k_{in} \approx 10^{4} \) s\(^{-1} \) for MbCO and various H93G complexes(29) (at 1 mM CO and 293K), and from above estimated \( k_{Ex} \approx 5 \times 10^{6} \) s\(^{-1} \) and \( k_{out} \approx 5 \times 10^{6} \) s\(^{-1} \), we find that \( k_{Re} \approx k_{in} \approx 10^{4} \) s\(^{-1} \).

2. Solution for spinning condition

To solve Eq. 1, each population can be written as\(^{7} \)

\[
A_{1} = a_{i0} + a_{i1}e^{-k_{1}t} + a_{i2}e^{-k_{2}t},
\]

where \( A_{1} = A_{H} \), \( A_{2} = C_{L} \), and \( A_{3} = A_{L} \). And we have\(^{7} \):

\[
k_{1,2} = \frac{1}{2}(k + k_{in} \pm \sqrt{(k - k_{in})^{2} - 4k_{a}k_{Re}})
\]

(3)

Where \( k = k_{a} + k_{b} + k_{re} \sim k_{\gamma} \approx k_{Re} \). Since \( k_{\gamma} \gg k_{in}, k_{Re} \), from Eq.3, we have \( k_{1} \approx k_{in}/2k_{in} \approx 10^{4} \) s\(^{-1} \), and \( k_{2} \approx k_{\gamma} \approx 10^{6} \) s\(^{-1} \).
The resonance Raman signal contributed by each population in the sample solution is proportional to the integration of each population over the time it spends in the laser beam. The corresponding travel time of the sample through the diameter of the laser beam is designated as T. Thus we can define the time averaged population that contributes to the Raman spectra as \( \langle A(T) \rangle = \frac{1}{T} \int_0^T dt \ A(t) \). And we have (51):

\[
\begin{align*}
\langle A_H(T) \rangle &= a_{10} + a_{11}(1 - e^{-k_1 T})/k_1 T + a_{12}(1 - e^{-k_2 T})/k_2 T \\
\langle C_L(T) \rangle &= a_{20} + a_{21}(1 - e^{-k_1 T})/k_1 T + a_{22}(1 - e^{-k_2 T})/k_2 T \\
\langle A_L(T) \rangle &= a_{30} + a_{31}(1 - e^{-k_1 T})/k_1 T + a_{32}(1 - e^{-k_2 T})/k_2 T
\end{align*}
\]

(4)

For the Raman experiment under spinning condition shown in Fig. 4, T=6\( \mu \)s, \( k_1 \sim 10^4 \) s\(^{-1}\) and \( k_2 \sim 10^6 \) s\(^{-1}\) so that \( \frac{1 - e^{-k_1 T}}{k_1 T} \sim 1 \), and \( \frac{1 - e^{-k_2 T}}{k_2 T} \sim \frac{1}{6} \).

In the spinning condition, there is no significant population of \( C_L \) as evidenced by the intensity of the \( \nu_4 \) peak at 1359 cm\(^{-1}\), which is very weak (black curves in Fig. S5). So we neglect the contribution of \( C_L \), and only consider \( A_L \) and \( A_H \). From Eq. 4, and the values of the time averaged rates estimated above, the observed population ratio can be written as:

\[
\frac{\langle A_L \rangle}{\langle A_H \rangle} = \frac{a_{30} + a_{31} + a_{32}/6}{a_{10} + a_{11} + a_{12}/6}
\]

(5)

From the initial condition of eq. 4:\( \langle A_H(0) \rangle = 1 \), \( \langle C_L(0) \rangle = \langle A_L(0) \rangle = 0 \), we have \( a_{10} + a_{11} + a_{12} = 1 \), and \( a_{30} + a_{31} + a_{32} = 0 \). And from the solution\(^7\) of eq. 1, we find that \( a_{10}, a_{11} \sim 0 \), \( a_{12} \sim 1 \), and \( a_{32} \sim -k_0/k \). So Eq. 5 can be rewritten as:

\[
\frac{\langle A_L \rangle}{\langle A_H \rangle} \sim -5a_{32} \sim 5\frac{k_p}{k}
\]

(6)

From peak fitting the \( \nu_{FeCO} \) and \( \nu_{CO} \) bands in Fig. 4, we find that \( \langle A_L \rangle/\langle A_H \rangle \sim 2 \) for all three samples. So we have \( 2k \sim 2(k_a + k_b) \sim 5k_b \) or \( k_a \sim k_b \). Since \( k_{out}/k_{Ex} = k_a/k_b \) (from the expressions preceding Eq. 1), we can determine \( k_{Ex} \sim k_{out} \sim 5 \times 10^6 \) s\(^{-1}\), which is consistent with the photostationary state analysis.
From the relationships between the rates we obtained in the stationary experiment, we have $k_{\text{Re}} = k_{\text{in}} (C_L/A_L) (k_a + k_b)/k_a \sim 4 \times 10^4 \text{ s}^{-1}$ for H93G CO where $C_L/A_L \sim 2$. If we assume that $k_{\text{out}}$ and $k_{\text{Ex}}$ are not affected by addition of THT and CPSH, the $k_{\text{Re}}$ rates are $2 \times 10^4 \text{ s}^{-1}$ and $1 \times 10^4 \text{ s}^{-1}$, respectively, for H93G(THT) CO and H93G(CPSH) CO. The fact that the histidine recruiting rate may be affected by the presence of exogenous thiol/thioether ligand might be due to these ligands hindering the H93G reorganization associated with the ligand switch. On the other hand, it must be emphasized that these values are only approximations and that the generic time scale for histidine recruitment upon CO binding in the H93G system appears to be $k_{\text{Re}} \sim 10^4 \text{ s}^{-1}$, based upon the photostationary state and spinning cell Raman data.

**B. Analysis of P420 CO Raman experiment**

For P420 CO resonance Raman experiment shown in Fig. S5, no Fe-His stretching mode is observed under spinning and stationary condition. So the three state model for H93G CO is applicable for P420-CO. Additionally, comparing the Raman spectra under stationary and spinning condition, the position of $\nu_{\text{FeCO}}/\nu_{\text{CO}}$ modes stays the same, and no extra peaks at 522/1960 cm$^{-1}$ are observed under the stationary cell condition. One explanation for this observation, based on water replacing His as transient ligand after CO photolysis (in the transient photocycle), is that the population of water bound state, $A_L$, population is negligible. The other explanation, which may exist theoretically, is that after histidine dissociates, a nearby thiol, if available, might bind to the heme as the proximal ligand in $A_L/B_L/C_L$. If we assume $\nu_{\text{FeCO}}/\nu_{\text{CO}}$ modes for *trans* thiol ligand is at the same frequency as the ones with *trans* histidine ligand, this assumption can explain the observation of no $\nu_{\text{FeCO}}/\nu_{\text{CO}}$ position shift. But in P420, a histidine-thiol ligand switch requires a tertiary structure change which is likely to happen on longer time scales. A slow conversion is inconsistent with the Raman experiment which suggests that $k_{\text{Ex}}$ is faster than $10^6 \text{ s}^{-1}$. It is likely that a water molecule is a transient ligand in $A_L/B_L/C_L$, and is involved in the histidine displacement during the photocycle. In thermal equilibrium (no photons), and on longer timescales, L is meant to represent a thiol ligand.
For the P420CO system, Tian et al.\(^{(35)}\) previously found values of \(k_{\text{out}} = 4 \times 10^7\) s\(^{-1}\) and \(k_{BA}^H = 10^8\) s\(^{-1}\). However, the fast phase of the CO binding kinetics is clearly much faster than that of H93GCO. Moreover, there is much missing amplitude so it is our conclusion, based on a reinspection of the data, that the geminate amplitude is much larger than indicated\(^{(35)}\) and that the rebinding rate, \(k_{BA}^H(P420)\), is actually \(~10^9\) s\(^{-1}\) (or larger) and may even be non-exponential as found in CooACO systems\(^{(39)}\). If we again take \(k_{\text{Ex}} \sim 5 \times 10^6\) s\(^{-1}\) \((35)\) and \(k_\gamma \sim 10^6\) s\(^{-1}\), we have \(k_a = k_\gamma k_{\text{out}}/(k_{BA}^H+k_{\text{out}}+k_{\text{Ex}}) \sim 10^4\) s\(^{-1}\), and \(k_b = k_\gamma k_{\text{Ex}}/(k_{BA}^H+k_{\text{out}}+k_{\text{Ex}}) \sim 1-5 \times 10^3\) s\(^{-1}\). The population ratio of A\(_H\) and C\(_L\) are: \(\langle A_H \rangle/\langle C_L \rangle = k_{in}/k_a\), where \(k_{in}\) is the bimolecular rebinding rate. Since A\(_L\) is negligible, we fit the two \(\nu_4\) peaks in Fig. S5, which must correspond to C\(_L\)(1361 cm\(^{-1}\)) and A\(_H\)(1372 cm\(^{-1}\)) in order to find \(\langle A_H \rangle/\langle C_L \rangle\sim 1\). This ratio suggests \(k_{in} \approx k_a \sim 10^4\) s\(^{-1}\), which is in good agreement with the observations of Tian et al.\(^{(35)}\).

To find \(k_{Re}\), we use Eq. 2, the solution of the three state model under stationary state conditions. This gives \(A_L = (k_a+k_b) k_{in}/(k_{Re} k_a+k k_{in}) \ll 1\) and \(A_H \gg A_L\). Considering \(k_a \approx k_{in}\) from the above discussion, we have \(k_{Re} >> k_a+k_b = k_\gamma (k_{\text{out}}+k_{\text{Ex}})/(k_{\text{out}}+k_{\text{Ex}}+k_{BA}^H) \sim 10^4\) s\(^{-1}\). Thus, for the P420-CO system, we find that \(k_{Re}\) is faster than \(~10^4\) s\(^{-1}\) and \(k_a \sim k_{in} \sim 10^4\) s\(^{-1}\). On the other hand, insofar as \(k_{BA}^H(P420)\) approaches the values observed for other His ligated systems with no distal barrier (i.e., \(~10^10\) s\(^{-1}\)), the upper limit on \(k_{Re}\) will be further reduced.

**Some prospective His residues in P420 systems**

**Figure 5.S7.** Membrane bound P450-3A4 (PDB#3NXU) with heme and all His residues highlighted. His residues with Fe-N\(_c\) distance less than 20 Å: 14.0 Å (H402).
**Figure 5.S8.** P450cam (PDB # 2CPP) with heme and all His residues highlighted. His residues with Fe-Nε distance less than 20 Å: 10.4 Å (H361), 12.0 Å (H355), 12.5 Å (H347), 15.3 Å (H342).

**Figure 5.S9.** Rat NOS with BH4 Bound (PDB#2G6M) with heme and all His residues highlighted. His residues with Fe-Nε distance less than 20 Å: 10.7 Å (H661), 12.2 Å (H445), 16.2 Å (H407), 17.0 Å (H651), 19.0 Å (H652).
Figure 5.S10. Larger renditions and different angles showing the possible structures underlying the ligand switch for CYP101 (PDB#2CPP), CYP51(PDB#1EA1), and CYP121(PDB#2IJ7). In each figure, upper panel show the native structure, lower panel shows their possible CO bound p420 structure, correspondingly. The red dashed line indicates the hydrogen bond between “near” propionate group and H355, H392, and H343 in the native structures, and H352, R391, and Q342 in the proposed structures.
References


Chapter 6

Coherent Vibrations and Kinetic Studies of O₂ and NO binding to Tt H-NOX

6.1 Introduction

Heme nitro oxide and/or oxygen binding domain (H-NOX), is a newly discovered family of heme-based sensor proteins; widely existing in both aerobic and anaerobic bacteria. In eukaryotes, H-NOX domains form the heme domain of soluble guanylate cyclase (sGC), which is the prototype of mammalian NO sensors. In prokaryotes, H-NOX proteins appear to fall into one of two classes. One type is a stand-alone protein most often found in a predicted operon with a histidine kinase and less frequently with diguanylate cyclase domain. The other class is fused to methyl-accepting chemotaxis domains in the same open reading frame. H-NOX domains from the majority of eukaryotes and facultative aerobic prokaryotes do not bind O₂, but they bind NO and form 5C complexes that are similar to SGC-NO, whereas H-NOX proteins from obligate aerobic prokaryotes, including Tr-HNOX, bind both NO and O₂ forming stable 6C complexes. Homology to sGC as well as genomic placement suggests that H-NOX domains in prokaryotes are likely to serve as sensors for gases such as O₂ and NO. Recent results with the H-NOX from the facultative aerobe, *Shewanella oneidensis*, are consistent with this hypothesis.

The crystal structure of O₂ bound Tt H-NOX was obtained recently. The most prominent structure feature is that it contains the most distorted heme observed to date. The out-of-plane heme distortions found in Tt H-NOX show large deviations (over 2Å) from planarity. This distortion appears to be caused by van der Waals interactions in the heme cavity, with residue Pro115 making the largest contribution (Fig. 1). Pro115 is within van der Waals contact with pyrrole-D of the heme, causing the pyrrole to shift out of plane, generating a large
kink in the heme propionate group. The mutation of P115 to Ala, drastically reduced the OOP distortions as expected. \(^{(8)}\) Using normal coordinate structure decomposition (NSD) method (fig.1), we found that the heme out-of-plane (OOP) distortions are mainly along ruffling and saddling directions for 3.8 and 3.7 amu\(^{1/2}\) Å, respectively, while in the mutant these distortions is relaxed to \(~2\) and \(~1\) amu\(^{1/2}\) Å, respectively.

**Figure 6.1** Crystal structure of (a) wildtype (WT) \(Tt\) H-NOX (PDB#1U55), and (b) \(Tt\) H-NOX Fe(II) p115A-O\(_2\) complex(PDB#3EEE). The amino acids near heme distal pocket are shown in colored sticks. A hydrogen bond between carboxyl oxygen of Tyr side chain and O\(_2\) ligand are shown in a green dashed line. The O-O distance is 2.7 and 2.3 in WT and mutant, respectively. (c) and (d) show the NSD analysis result of heme OOP distortion respectively.

Similar heme OOP distortions are typically conserved for the proteins belonging to the same functional class.\(^{(9-11)}\) Doming is typically observed in oxygen storage or transport
proteins like hemoglobin(12, 13) and myoglobin(14). Ruffling is the dominant OOP deformation found in c-type cytochromes(9-11, 15) and nitrophorins(16-18), which are involved in electron and NO transport, respectively.

Although, oxygen is the most ubiquitous ligand in biologic systems, its interaction with heme proteins was rarely investigated using non-linear spectroscopic or kinetic methods due to the inherent instability of the oxy complexes under irradiation. Consequently, the mechanisms by which O₂ interacts with the heme in the process of carrying out key biologic function such as sensing and catalysis are still, for the most part, elusive.(19, 20). The heme distortion differences in the O₂ complexes of Tr H-NOX and its P115A mutant provide a unique opportunity to study distortion related function in controlling O₂ binding.

Here we use absorption, resonance Raman (RR), and vibrational coherence (VCS) spectroscopies to systematically investigate the rebinding dynamics of O₂ and NO to Tt H-NOX and its P115A mutant. VCS has the unique ability to probe the vibrational modes below 200 cm⁻¹. We have previously investigated the low-frequency modes of a variety of heme proteins, using Soret band excitation.(14, 21-29) Unlike the higher frequency modes (>200 cm⁻¹), the low-frequency modes (which have weaker force constants) are more easily distorted from equilibrium by the protein surroundings. These modes are activated in VCS when the protein induces symmetry-breaking nonplanar heme distortions(27). In addition, these modes take on a special functional significance because of their thermal accessibility. The low-frequency coherence spectra offer a unique window into how the surrounding protein environment can alter these important thermally active heme modes.

6.2 Materials and Methods
**Sample Preparation.** *Tt* H-NOX and its P115A mutant was prepared according to previously published method.(3, 30) All samples were further reduced with small amount of sodium dithionite. Samples are freshly prepared in 50 mM HEPES buffer with 50 mM NaCl at pH 7.5 before any spectroscopy measurements. Ferrous H-NOX was prepared adding excessive amount of sodium dithionite (20 fold) under Argon. Ferrous NO-bound complexes were obtained by adding 10 fold of sodium nitrite to the ferrous H-NOX solution. O$_2$-bound complexes were obtained by passing the ferrous H-NOX solution through a size exclusion gel column (Sephadex G-25, Sigma) in the air equilibrated with HEPES buffer. The absorption spectra were recorded (U-4100, Hitachi) after the preparation procedure to ensure that all chemical modifications were achieved. For VCS experiments the final concentration of protein samples was adjusted to O.D. = 1±0.05 in a 1 mm optical path length quartz sample cell at the selected excitation wavelength. The final concentrations of the samples are ~100 μM. The absorption spectra were also taken following the vibrational spectroscopy experiments to confirm the integrity of the samples during the laser exposure. All experiments were performed at room temperature.

**Optical Systems.** Resonance Raman spectra were obtained using a standard 90$^\circ$ light collecting geometry and a single grating monochromator (Acton SP2500i with 1800 g/mm UV holographic grating, Princeton Instruments). Details of the setup have been described elsewhere.(27) Samples were placed in a standard quartz cuvette (Precision Cells, Inc.) spinning at 6000 rpm and excited with ~10 mW of the 413.1 nm line from a krypton laser (Innova 300, Coherent). The femtosecond vibrational coherence spectroscopy (VCS) system has been described in detail elsewhere.(22, 31). The time resolution of this system is 70 fs. We used the open band scheme (22, 25, 31, 32) to obtain the VCS spectra. A Si photodiode collected the entire spectral
bandwidth of the probe pulse. The LPSVD data analysis method used to extract oscillatory component of the VCS signal has been described in detail elsewhere.(33)

6.3 Results and Discussion

Absorption spectra, the same protein concentration, of ferrous Tt H-NOX and its P115A mutant, along with the spectra of their NO and O₂ adducts are shown in figures S1 a and b. Steady state difference spectra (unligated protein-ligated protein); which are proportional to the difference spectra in the extinction coefficients of the corresponding proteins, are displayed in the insets of figures S1 a and b.

![Resonance Raman spectra of wild-type and P115A H-NOX with NO and O₂ adduct.](image)

Figure 6.2 Resonance Raman spectra of wild-type and P115A H-NOX with NO and O₂ adduct. The laser excitation wavelength is 413.1 nm with a power of 12 mW. All spectra were recorded with a sample spinning at 6000 rpm.

Resonance Raman spectra, obtained with 413.1 nm excitation, are shown in figure 2. The RR spectra of ferrous proteins and their oxy complexes are in good agreement with previous studies.(30) The positions of heme core “mark” band peaks (ν₄, ν₃, ν₂, ν₁₀) clearly indicate that in
ferrous samples, the heme is a 5-coordinate or 6-coordinate with water as distal ligand; whereas NO and O₂ adducts form 6-coordinate low spin species. In Ferrous samples, a strong Fe-His stretching mode is observed near 220 cm⁻¹ and associated with 5-corodinate His bound heme configuration; whereas, no such mode is observed in NO, nor in O₂ complexes. There are noticeable differences between the spectra of ferrous Tt H-NOX and its P115A mutant in the low frequency region (200-600 cm⁻¹), which is sensitive to ruffling and saddling deformations. Similar changes are seen in their O₂ and NO complexes. These differences are attributed to the relaxation of the OOP heme distortions upon mutation (mathies). Some subtle differences between the spectra of ferrous wt and P115A, and their respective NO and O₂ adducts are also observed in the high frequency region. ν₄ shifts from 1373 to 1375 cm⁻¹, and from 1373 to 1377 cm⁻¹ in NO and O₂ adducts, respectively; which might be attributed to the ruffling distortion difference in the two samples. This decrease in ν₄ upon the increase of heme ruffling distortion agrees with previous findings. There are also clear differences in ν₃, ν₂, and ν₁₀ modes between the two O₂ complexes. The Fe-O₂ stretching mode is located at 569 cm⁻¹ and 567 cm⁻¹ in the wild type and the mutant respectively.(30)

VCS measurement results of ferrous Tt-H-NOX and its mutant, as well as their NO complexes are shown in figure 4. Those of O₂ complexes are displayed in figure 5. The VCS spectra of unligated samples show good agreement with their corresponding Raman spectra in the overlapped region (200 ~ 400 cm⁻¹). The very low frequency mode can be assigned to the doming mode, and its frequency depends on the status of proximal ligand. There is typically a 6 ~ 9 cm⁻¹ difference in the position of this mode between wt and its mutant in each pair. It is possible that the mutation of Pro115, which is close to the proximal His, plays a role in modulating the frequency of the doming mode.
Figure 6.3 VCS oscillation and LPSVD generated power spectra. Blue line show the simulated oscillations for the strongest low frequency mode for each sample. The excitation wavelength for each sample is: 425 nm for ferrous widertype and P115A; 427 nm for wide-type and P115A H-NOX NO adducts.

Figure 6.4 (a) VCS oscillation and LPSVD generated power spectra nm for widetype and P115A H-NOX O₂ adducts, with excitation wavelength at 423 nm. Blue line show the simulated oscillations for the strongest low frequency mode for each sample. (b) Comparison of $\nu_{\text{Fe-His}}$ mode of oxy complexes. No normalization was perform on the data.

Kinetic measurements shown in figure 6 were performed using the same VCS setup, but the data were taken over a much longer time scale (~200 ps). Each curve in fig. 6 is typically an average of 3~4 runs. The kinetic traces ($\Delta T/T$) were fit with a simple multi-exponential decay
function that has the following expression \( \sum_{i=1}^{n} a_i \exp(-t/\tau_i) + a_{\infty} \), where \( n = 1, 2, 3 \) for ferrous samples, NO and O\(_2\) adducts, respectively. Coherence artifacts that are due to the overlap between pump and probe pulses, were trimmed before fitting the data. The results of the different fits are summarized in Table 1. The value of \( n \) is chosen to be the minimum value necessary to fit the data with an adjusted R-square higher than 0.99. For ferrous samples, both wt and P115A mutant have a very close time constant of ~ 4 ps. This rate can be assigned to vibrational cooling of the hot heme.

![Figure 6.5 Kinetic trace of wt and P115A H-NOX samples recorded with VCS setup. Excitation wavelengths are labeled in each panel. The exponential fittings for the data longer than 0.4 ps are shown in the red solid line. 1, 2 and 3 exponential decay functions are used for ferrous, NO and O\(_2\), respectively. Fitting results are summarized in Table 1.](image)

Kinetic traces of NO complexes in both proteins show a fast decay component (0.22 ps) and a slow rise with ~7 ps time constant. The fast rate might be assigned to an electronic relaxation, or fast vibrational cooling component. The slow time constant, however, is assigned
to geminate NO rebinding. Indeed, VCS spectra of NO complexes (fig.4) show the appearance of a Fe-His mode around 225 cm$^{-1}$; indicating that NO dissociates upon photo-excitation. Therefore, NO rebinding rates are $1.34 \times 10^{11}$ s$^{-1}$ and $1.60 \times 10^{11}$ s$^{-1}$ in the wild type and the mutant respectively. These values are consistent with the rates of NO rebinding to other ferrous heme proteins. More importantly, they agree very well with the rate ($1.34 \times 10^{11}$ s$^{-1}$) of NO rebinding to the homologue protein SGC(20), which is the paradigm of NO signaling in biological systems. SGC binds NO with very high selectivity and does not bind O$_2$. NO binding results in the breaking of the weak Fe-His bond and the formation of a 5C nitrosyl complex. Recent studies have shown that NO binds to the proximal side of the heme and this complex is formed via transient NO binding to the iron from the distal side(34). These events constitute the initial steps for a cascade of signals that activate catalysis of GTP to cGMP. cGMP subsequently mediates a variety of physiological processes such as blood vessel relaxation and neurotransmission(5). Our results show that geminate NO rebinding rate to the H-NOX family is independent of the extent of heme distortion and its coordination state. It is worth mentioning here that if a protein belonging to H-NOX family members that form 6C nitrosyl complexes would function as NO sensor it is likely to have a signal transduction mechanism that is different from that of SGC.

VCS spectra of O$_2$ complexes show the activation of a Fe-His mode around 217 cm$^{-1}$ following photo-excitation (figs 5a and 5b); which is a direct evidence for O$_2$ photo-dissociation in both proteins. The kinetic traces of O$_2$ complexes probed at 435 nm (fig 6C) can be fit with 3 exponentials and an offset, $a_{\infty}$ (table 1). The extracted time constants are (1.7 ps, 7.4 ps, 64 ps) and (0.6 ps, 6.6 ps, 60 ps) for the wild type and the mutant respectively. In both cases the shortest and longest time constants cannot be assigned to O$_2$ rebinding because their amplitudes have the wrong sign (table 1). Indeed the steady state difference spectra (ferrous - O$_2$ complex) shown in
the inset of figures (S1a and S1b) indicate that the change in the absorbance $\Delta A$ due to ligand photolysis should have a positive sign when probed at 435 nm. In our experiment, the $\Delta T/T$ signal that describes the decay of the photolyzed population should have a negative sign. Hence, we attribute the time constants 7.4 ps and 6.6 ps to $O_2$ rebinding in Tt-HNOS and its mutant respectively. These time constants agree very well with fastest time constant (6 ps) of $O_2$ rebinding in Mb. We did not observe sub-picosecond $O_2$ rebinding in Tt-HNOS nor in its mutant.

Table 6.1. Summary of the exponential fitting result for data shown in figure 6.4. General expression for the fitting function can be written as $\sum_{i=1}^{n} a_i \exp(-t/\tau_i)$, where $n=1, 2, 3$ for ferrous samples, NO and $O_2$ adducts, respectively. The absolute value of $a_i$ is meaningless. Its value is only meaningful when it is compared with other $a_i$'s of the same sample.

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The photolysis quantum yield of O\textsubscript{2} in Tt H-NOX and its mutants is estimated using the following procedure: Assuming linear absorption of pump and probe light, the induced absorption change at \( t \sim 0 \) in the sample is given as:
\[
\Delta A(\lambda) = \Delta \varepsilon(\lambda) J^\text{abs}_\text{pump},
\]
where \( \Delta \varepsilon(\lambda) \) is the difference of the extinction coefficient of the product and the reactant and \( J^\text{abs}_\text{pump} = J^0_\text{pump}(1 - 10^{-A_\text{pump}}) \) is the number of absorbed pump photons per unit area. \( J^0_\text{pump} \) is the incident flux integrated over the laser pulse. \( A_\text{pump} \) is the absorbance of the sample at pump wavelength (\( \lambda = 435 \text{ nm} \)). The kinetic traces of O\textsubscript{2} and NO in Tt H-NOX and its mutant (figure 6) were taken back to back using the same conditions for the pump and the probe intensities and their overlap. The absorbance of all samples was 1 OD at the probe wavelength (\( \lambda = 435 \text{ nm} \)). Thus all the samples have the same transmittance \( T_0 \) and the same \( J^\text{abs}_\text{pump} \) at \( \lambda = 435 \text{ nm} \). The details of the method that allow us to obtain the photolysis quantum of O\textsubscript{2} relative to that of NO in each protein are explained in the supplement. If we consider that the photolysis quantum yield of NO is 50% in both proteins, similar to that in Mb, we find that the O\textsubscript{2} photolysis quantum Yield is 1.4% and 7.5% in Tt H-NOX and its P115A mutant respectively. Thus, the upper limits for the quantum yield of O\textsubscript{2} photolysis are 3% and 15% in the wild type and the mutant respectively. These values are obtained by assuming that NO dissociates form both protein with unity quantum yield. These results show that O\textsubscript{2} photolysis quantum yield in Tt H-NOX is at least 5 fold lower as compared to that in the mutant; which indicates that Fe-O\textsubscript{2} bond is stronger in the wild type.

It is clear that distal pocket H-bond network, especially the H-bond between O\textsubscript{2} and Tyr-140 plays a major role in stabilizing the oxy complex in H-NOX proteins (ref); but this factor alone cannot explain why the Fe-O\textsubscript{2} bond in the Tt H-NOX is much robust against photolysis as compared to that in its P115A mutant. Indeed, the O\textsubscript{2}-Tyr140 H-bond in the wild type (2.7 Å) is
longer than that in the mutant (2.3 Å). Thus, other factors must play a role in the stabilization of the Fe-O₂ bond. We examined the effects the Fe-O-O angle and the Fe-His tilt. We found that the Fe-O-O angle is the same (∼120°) in both proteins; whereas the extent of the Fe-His tilt is in favor of stronger Fe-O₂ in the mutant(30). Thus, by elimination the only factor that might explain the robustness of Fe-O₂ bond against photo-dissociation in the wild type as compared to that in the mutant is heme OOP distortions, particularly ruffling and saddling. Due to the fact that NO photolysis quantum yield in Tt H-NOX is unknown in these value may slightly vary. But we can found that O₂ photolysis quantum yield is <2% in the wild type and it is <11% in the mutant.

6.4 Conclusion

In summary, in this work via the study of highly ruffling wild-type H-NOX and its planar P115A mutant, We have found that heme out-of-plane distortions, including ruffling and saddling, have strongly impact on heme electronic structure. VCS of the oxy-complexes provide a clear evidence for the activation of an iron-histidine mode around 217 cm⁻¹ following photoexcitation, indicating that O₂ dissociates in both proteins. The quantum yield of O₂ photolysis is found to be very low, particularly in the wild type (<2%). Geminate recombination of O₂ and NO in both proteins is very fast and highly efficient. This indicates that the distal heme pocket in these proteins is tightly packed, and forms an efficient trap, preventing the bound ligand from escaping into the solvent upon thermal dissociation. This, along with the low photolysis quantum yield, explains the unusually high O₂ affinity in Tt H-NOX and its P115A mutant as compared to that in myoglobin.

6.5 Supporting Information
Figure 6.S1 UV/visible absorption spectra of WT (a) and P115A (b) Tr H-NOX, and their NO and O₂ adducts. The highlighted points on the spectra curve are corresponding to the excitation wavelength used for VCS experiment for each sample.
References


Appendix A

Study of Cytochrome c Thin Film

A.1 Introduction

Introduction

The properties of hydration water in biological systems have been studied for over a century by a wide range of techniques. (1) The hydration layers surrounding the protein are essential for protein structure, stability, dynamics, and their biological functions. (1-5) The hydration water has different structure and dynamic compare to the bulk water. (2, 6-8) The thickness of hydration layer is at least 1.0 nm, determined using terahertz spectroscopy. (9) For many proteins, their functions require a critical hydration level. (6, 10, 11) Previous study of hydration effect with cytochrome c film revealed a excitation decay time constant change associated with increasing hydration level. (12) But more complete optical spectroscopic study, especially the thermally accessible low frequency modes below 200 cm⁻¹, is lacking.

In this work, we investigated the hydration effect using highly uniform cytochrome c (cyt c) films with low concentration of buffer residue. Using absorption, resonance Raman (RR), and vibrational coherence spectroscopies (VCS), we obtain high signal to noise spectra of high quality ferric cyt c films at different hydration levels. We found that some small but clear Raman spectra change upon changing the hydration level of the cyt c film.

A.2 Material and experimental methods

Cytochrome c film was prepared with 0.1g/mL cyt c solution with 0.05 M Kpi. 2 μL solutions were dropped onto a clean fused silica substrate, followed by a spinning-coating process, which prepared a thin, uniform cyt c film on the substation. The spinning speed of the spin-coater was gradually increased to 2000 rpm, and stay at this speed for 20 min. The film
obtained with this method has been characterized by an optical surface profiler (NewView 6000, ZYGO). The center of the film is highly uniform with an average height of 0.8 μm. The picture of the film is shown in Fig.1. The uniform area extents from the center to around 5 mm radius area. The ratio of sample and buffer concentration in the sample is ~ 1:5. Before each experiment, the film was dried with nitrogen gas for ~ 4 hours, followed by hydration process by placing the sample in a specially designed hydration chamber (shown in SI) at desired hydration level for 2 hours before each measurement. Relative humidity (RH) was controlled by a LI-610 portable Dew Point Generator (LI-COR).

![Figure A.1. Cytochrome c film prepared with spin-coating method](image)

Resonance Raman scattering signal was collected with 180° geometry. VCS signal was collected use a newly designed scanning mirror system. All spectroscopic data are collected while sample was kept in the hydration chamber. For RR and VCS measurements, film was spinning at ~120 rpm to avoid the damage and minimize photoreduction of the sample. VCS and Raman setup was described in detailed elsewhere.(13, 14) The implementation of scanning mirror system is available in SI. Each experiment was repeated twice with newly prepared film.
A.3 Result and Discussion

Absorption, Resonance Raman and VCS spectroscopies. Cyt c is a small protein whose primary biological functions is shuttling electron between complex III and complex IV on mitochondria inner membrane. The protein structure of cyt c is rigid and very stable. The absorption spectra of cytochrome c at different RH are shown in Fig.2. There is no observable change for the absorption spectra. Thus only the highest and lowest possible hydration setting was used for resonance Raman and VCS experiment. Previous study\(^{(12)}\) on thick film (O.D. ~ 0.5 at 800 nm) with uncharacterized buffer concentration showed a weak correlation between 695 nm (Fe-Met charge transfer band) and the film hydration level. Due to the weak O.D. signal at 695 nm of our thin cyt c film, we are unable to resolve such a change in O.D.

Resonance Raman and VCS measurements between the films prepared at two different RH levels agree very well. But some significant spectral differences are observed. The corresponding RR and VCS spectra are shown in Fig. 3 and 4, respectively. In RR spectra, the only spectral difference is 693 cm\(^{-1}\) peak at 95% RH (asymmetric C\(_{\alpha}\)-S stretching)\(^{(15)}\), which shifts to 689 cm\(^{-1}\) at 20% RH. This may be related to protein hydration effect. As C\(_{\alpha}\)-S is the vibrational mode of the thioether bridge between heme and protein backbone, a small frequency shift of this mode may indicate a perturbation to protein structure upon hydration.

In VCS spectra, a low frequency \(\gamma_\alpha\) mode, which has a large ruffling content,\(^{(16)}\) is observed at 44 cm\(^{-1}\) with 95% RH, and at 47 cm\(^{-1}\) with 20% RH. It is recently found that heme ruffling mode softens as ruffling distortion increase, which may be due to the anharmonicity of potential curve.\(^{(17)}\) In cyt c proteins, the highly ruffled heme distortion is primarily induced by the Cys-X-X-Cys-His segment, which anchors heme to protein backbone. The vibrational modes of this segment are highly correlated to heme modes, and play a major role in modulating
heme distortions. (16, 18, 19) This important segment is located close to cytochrome c surface and to protein binding site. (20) So ruffling mode is sensitive to protein structure and dynamics changes. As there is no obvious hydration effect in low frequency region is observed, the perturbation to protein structure is small.

![Absorption spectra of ferric cyt c film at different relative humidity.](image)

**Figure A.2.** Absorption spectra of ferric cyt c film at different relative humidity.

When the film’s spectra are compared with the solution cyt c spectrum, the most prominence difference is the photoreduction of the sample. As shown in Fig. 3, \( \nu_4 \) band near 1364 cm\(^{-1}\) indicates a small amount ferrous population. Besides photoreduction, only small intensity change is observed at 701 and 730 cm\(^{-1}\). The similarity between cyt c film and solution’s spectra suggests that that change of protein structure upon forming cyt c film may be negligible. VCS spectra clearly reveal a peak near 226 cm\(^{-1}\) which is assigned to Fe-His stretching mode, an indicator of ferrous population. This observation is consistent with the ferrous population observed in RR spectra. So the small spectral change in film comparing that of the solution is a result of photoreduction. The oscillatory pattern in the left panel shows that the damping time constant of the very low frequency mode, \( \gamma_a \), decreased drastically compared to
that of solution. This is likely due to the increased inhomogeneous broadening when the film is forming.

![Figure 3](image3.png)

**Figure 3.** Resonance Raman spectra of hydration cyt c film in comparison with spectrum of ferric cyt c solution. The excitation wavelength is 413 nm at 5 mw.

![Figure 4](image4.png)

**Figure 4.** Open-band VCS spectra of hydated cyt c film at 412 nm, in comparision with the spectra for cyt c in solution. LPSVD fits and LPSVD power spectra are shown in the left and right panels, respectively. The oscillation represents the lowest frequency mode, $\gamma_a$, which are offset from the raw data for display. $\tau$ is the damping time constant for $\gamma_a$.

**Photoreduction Kinetics.** We found that the photoreduction is inevitable in cyt c film, compared to cytochrome in solution. As shown in our previous photoreduction measurement,
there is no observable photoreduced population for ferric cyt c solution under aerobic condition. It has been found that the photoreduction of hh cyt c can only be observed in anaerobic condition. However, for cyt c film, photo-reduced population is impossible to avoid when we collected RR and VCS data. As mentioned in an earlier work by Gu et al., adding glycerol (which only has 20% oxygen solubility of that of water) to the solution increases the observable photoreduced population in aerobic condition. Thus we attributed the photoreduction observed in the film to the decreased concentration of oxygen in cyt c film.

A photoreduction experiment was carried out in order to determine the photoreduction rate in cyt c film. A crossed-beam transient absorption detection scheme was used to measure the photoreduction kinetics of cyt c film. The laser beam (at 413.1nm, krypton laser, Innova 300, Coherent) was expanded by a Thor Labs ED1-C20-MD engineered diffuser that homogeneously illuminated the sample on the substrate. The power measured after expansion is 62 mw so that the photon flux is found to be $J = 4.1 \times 10^{16}$ photons/s·cm$^2$. The absorption change of the sample due to photoreduction is recorded by a portable absorption spectrometer (Spectral Instruments, Inc. Tucson AZ), which was aligned about $\sim 30^\circ$ to the laser beam in order to avoid collecting scattering light. The photoreduction and photooxidation cross-section determined are $5.7 \times 10^{-22}$ cm$^{-1}$ and $1.2 \times 10^{-22}$ cm$^{-2}$, respectively. These values agree very well with published data for cyt c solution.

In the film, cyt c proteins were packed tightly. The diameter of cyt c protein is 4–8 Å. And each film contains $\sim$100 layers of proteins. The determined photo-reduction and photo-oxidation cross-section is very close to the value of cyt c solution under anaerobic condition. This agreement indicates that the outer layers of film, which can access oxygen and don’t contribute
to the photoreduction signal, takes up only a small population in the sample, i.e. the oxygen may penetrate only a few layers (10% of total film is ~10 layers) in the film.

![Graph showing kinetic measurements of photoreduction of cyt c film.](image)

**Figure A.5.** Kinetic measurements of photoreduction of cyt c film. \( N_r(t) \) increases with irradiation time and is fitted with \( N_r = N_r(\infty)(1 - \exp(-\sigma Jt)) \) and \( N_r(\infty) = \sigma_r/\sigma \), with \( \sigma = \sigma_r + \sigma_o \). The fitted curves are shown in red.

In summary, our spectroscopic study has found small spectral change upon decrease hydration level: 4 cm\(^{-1}\) downshift of 693 cm\(^{-1}\) peak. And there is no change in the low frequency modes region.

**A.4 Supporting information:**

**Figure 6.S1 Hydration chamber.**
References


Appendix B

Absorption and Resonance Raman Spectra of Microperoxidase-8 Imidazole Complex

This section provides the result of the control experiment, showing that guanidinium hydrochloride (GdHCl) doesn’t bind to heme when GdHCl is used as the denaturant.

Figure B1. Absorption spectra of microperoxidase-8 with and without 4M GdHCl

Figure B2. Resonance Raman spectra of microperoxidase-8 with and without 4M GdHCl. Laser power is 5 mw, \( \lambda=413\text{nm} \).
Appendix C

Figures for study of green fluorescent protein (GFP) using Vibrational Coherence Spectroscopy

Figure C1. Absorption spectra of GFP at 293k

Figure C2. VCS oscillations and spectra of GFP at 293k
Figure C3. VCS oscillations and spectra of GFP at 120k

Figure C4. Absorption spectra of deuterium GFP at 293k

Figure C5. VCS oscillations and spectra of deuterium GFP at 293k
Figure C6. Absorption spectra of GFP E222D mutant at 293k

Figure C7. VCS oscillations and spectra of GFP E222D mutant at 293k