Investigations of Ultrafast Ligand Rebinding to Heme and Heme Proteins using Temperature and Strong Magnetic Field Perturbations

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ABSTRACT OF DISSERTATION

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Abstract

This thesis is written to summarize investigations of the mechanisms that underlie the kinetics of diatomic ligand rebinding to the iron atom of the heme group, which is chelated inside heme proteins. UV-vis absorption spectroscopy and ultrafast pump-probe spectroscopy are the major investigation methods used in this thesis.

The family of heme proteins is a major object of studies for several branches of scientific research activity. Understanding the ligand binding mechanisms and pathways is one of the major goals for biophysics. My interests mainly focus on the physics of this ligand binding process. Therefore, to investigate the problem, isolated from the influence of the protein matrix, Fe-protoporphyrin IX is chosen as the prototype system in my studies. Myoglobin, the most extensively and intensively studied protein, is another ideal system that allows coupling the protein polypeptide matrix into the investigation.

A technique to synchro-lock two laser pulse trains electronically is applied to our pump-probe spectroscopic studies. Based on this technique, a two color, fs/ps pump-probe system is developed which extends the temporal window for our investigation to 13ns and fills a gap existing in previous pump-probe investigations. In order to apply this newly-developed pump-probe laser system to implement systematic studies on the kinetics of diatomic ligand (NO, CO, O₂) rebinding to heme and heme proteins, several experimental setups are utilized.

In Chapter 1, the essential background knowledge, which helps to understand the iron-ligand interaction, is briefly described.
In Chapter 2, in addition to a description of the preparation protocols of protein samples and details of the method for data analysis, three home-made setups are described, which include: a picosecond laser regenerative amplifier, a pump-probe application along the bore (2-inch in diameter) of a superconducting magnet and a temperature-controllable cryostat for spinning sample cell.

Chapter 3 presents high magnetic field studies of several heme-ligand or protein-ligand systems. Pump-probe spectroscopy is used to study the ligand recombination after photolysis. No magnetic field induced rate changes are observed in any of these ligand recombination processes within the experimental detection limit. A magnetic field dependent CO rebinding behavior is observed for the FePPIX-CO sample in 80%glycerol/20%water environment. Careful data analysis indicates that this magnetic field induced change is due to the amplitude difference of a “fast” (<10ps) response with and without the magnetic field application (the amplitude changes from ~55% at 0 Tesla to ~45% at 10 Tesla). Kinetics of CO rebinding to FePPIX in 80%glycerol at the extremes of the magnetic field intensities (0Tesla vs. 10 Tesla) can be decomposed into a ligand rebinding process plus two 5ps decays heme cooling with different amplitudes. It leads to suggest a magnetic field induced change of a short-lived heme cooling response after photolysis. Also, CO rebinding kinetics to different heme compounds demonstrates a wide range for the Arrhenius pre-factors. This work reveals that the “spin-selection rule” does not play a key role in the recombination process of CO to heme iron.

In Appendix 1, the recombination of oxymyoglobin and its mutants is investigated in the temperature range from 275K to 318K, using a home-made cryostat. Quite surprisingly, the O₂ molecule rebinds to heme iron inside myoglobin with dramatically
different behavior as the temperature is varied, depending on the protein environment. It shows little dependence (Mb), no dependence (V68W Mb mutant) and large dependence (L29W Mb mutant) in this 40K temperature window. To expand this temperature window, since the motor inside the cryostat is capable to work as low as 230K, glycerol is introduced into the protein preparation. It is observed that protein samples in a glycerol/water mixture, even with only 20% glycerol (in weight), the temperature dependences of the O₂ rebinding to heme iron are dramatically altered. The O₂ rebinding behavior also shows a high dependence on the glycerol concentration in the solution. These tendencies are consistent with previous studies of other colleagues. A comparison of kinetics in different solutions indicates that environmental viscosity must not be the only effect inducing the glycerol-dependent behavior.

Two different methods are applied to prepare the oxymyoglobin and O₂-bound V68W myoglobin mutant. These different protocols in sample preparation also affect the O₂ rebinding kinetics.

In Appendix 2, the absorption spectra of Fe-protoporphyrin IX in different monomeric complexes are investigated and compared. This work may suggest that, inside the CTAB micelles, ferrous Fe-PPIX exists in an equilibrium state of different species, CO probably can bind to Fe\textsuperscript{II}-PPIX with different \textit{trans} ligand (H\textsubscript{2}O or OH\textsuperscript{-}), and the trans effect, exhibiting while NO binds to H93G Mb mutants might also happen when NO binds to Fe\textsuperscript{II}-PPIX inside CTAB micelles.

In Appendix 3, several ultrafast kinetics studies of CO rebinding to FePPIX are listed. They might be helpful for future studies on such systems.
In Appendix 4, a series of systematic studies of the NO recombination to Fe\textsuperscript{II}PPIX is performed with temperature and environment variation. A four-state model is proposed to explain the kinetics of NO-Fe\textsuperscript{II}PPIX after photolysis.

To sum-up, two scientific devices were designed, constructed and applied to practice successfully in the protein-ligand kinetics studies. A home-made pico-second regenerative amplifier was developed in collaboration with Prof. Anchi Yu. This laser amplifier and the whole synchro-locked laser system was aligned successfully when necessary. Diatomic ligands (CO, NO and O\textsubscript{2}) rebinding to ferrous heme (Fe\textsuperscript{II}-PPIX) or heme proteins were investigated under various experimental schemes. Strong magnetic fields, mutants, temperature alteration, variance of protein environment were used as methods to perturb these protein-ligand rebinding processes.
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It is to my wife, my daughter and my families that this thesis is dedicated.
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Chapter 1

General Introduction

1.1 Iron & Iron-Ligand Interaction

Iron serves as an important trace element that is involved in many physical and chemical activities of living cells and organisms (1-3). On our planet, every living creature needs iron and it is used in a wide variety of ways. The pigeon uses iron as a “magnet” (4) to help it travel effectively long distances to specific destinations. A recent study (5) reveals that aggregates of magnetite nanocrystals (in Fe$^{3+}$ form) located in its upper beak tissue is the key to this ability. Also, iron is involved in strengthening the teeth of rats (6), which are unusually strong and capable of biting through a wide variety of materials.

Differing from those two interesting applications of iron for living creatures as mentioned above, most of its biological functions are correlated with the redox flexibility of iron atom and its ability to bind ligands as a transition metal ion. One of the characteristic features of the transition metal family is that they are able to bond with a variety of molecules or ions to build-up metal-ligand complexes through coordinate-covalent bonds. It is worth mentioning here the difference between valence bond and coordination bond. For the valence bond, both of the bonding atoms contribute one unpaired electron and the chemical bondage is localized between the pair of bonding atoms. In the case of coordination bonding, there are no localized metal-ligand bonds in
the complex. Ligands contribute both electrons to the coordinate-covalent bond. All the electrons that ligands donate to the metal center are actually occupying a three-dimensionally delocalized orbital around the central atom.

The iron atom has electronic configuration of 1s^2 2s^2 2p^6 3s^2 3p^6 3d^6 4s^2. This is sometimes also written as [Ar] 3d^6 4s^2. It commonly evolves in chemical reactions in two oxidation states, or charge states, the ferrous (Fe^{2+}) state ([Ar] 3d^6) and the ferric (Fe^{3+}) state ([Ar] 3d^5). There are five 3d orbitals (Figure 1.1), therefore 10 vacancies for those 3d electrons to occupy. For a free Fe ion, these five d orbitals are energetically identical. The possibilities of the distribution of electrons within these orbitals are equal. When ligands attached, the main influence on the iron atom is to break the degeneracy of the 3d orbitals. Crystal Field Theory (7) assumes that these ligands are structureless negative charges, and analyzes their effect on the energy levels of iron atom. Detailed description needs to be combined with means of quantum mechanics. Hereinafter a pictorial briefing is presented to illustrate the situation in the octahedral complex, the most common geometry in iron-ligand interaction.

In Figure 1.2(a), to compare the crystal field effects with the 3d_{xy} and 3d_{x^2-y^2} orbitals laying in the xy-plane, six negative charges are disposed along the Cartesian axes, equidistantly from the origin point. On the whole, an electron in a 3d_{x^2-y^2} orbital stays closer to these negative charges, therefore experiences more repulsion from the ligands than does an electron in a 3d_{xy} orbital. So, the energy of 3d_{x^2-y^2} will be higher than that of combination of two orbitals 3d_{x^2}, 3d_{y^2}. It shares the same symmetry (e.g. symmetry)
Figure 1.1 The shapes and orientation of the 3d orbitals. $3d_{xy}$, $3d_{xz}$ and $3d_{yz}$ have their lobes between the corresponding axes. $3d_{x^2-y^2}$ has its lobe along x and y axes. $3d_z^2$ can actually be decomposed to two components: $3d_{y^2-z^2}$ and $3d_{z^2-x^2}$, which have the same shapes as the others.
Figure 1.2 (a) $3d_{xy}$ and $3d_{x^2-y^2}$ orbitals orientated in the xy-plane surrounded by 6 point negative charges along the Cartesian axes equidistantly. (b) partial orbital energy-level diagram for a transition-metal ion in an octahedron of six point negative charges. The energy of $3d_{x^2-y^2}$ and $3d_{z^2}$ is $3/5 \Delta o$ higher than that of the free ion and the energy of $3d_{xy}, 3d_{xz}, 3d_{yz}$ is $2/5 \Delta o$ lower than that of the free ion.
with 3dx2-y2. Thus, in an octahedral complex, the 3dxy, 3dyz, 3dxy orbitals are energetically degenerate, whose energy is lower than that of the other two degenerate orbitals, 3dxy and 3dz2. Corresponding partial orbital energy-level diagram is listed as Figure 1.2(b). The symbol $\Delta_o$ denotes the strength of the d-orbital splitting. Analogous consideration could be applied to other ligand orientations (Figure 1.3 & Figure 1.4).

When the outer shell 6 (ferrous) or 5 (ferric) electrons of iron occupy these splitting 3d orbitals, electron pairing energy P becomes impactful. In the electron distribution for Fe$^{3+}$, the first three electrons occupy the t$_{2g}$ level anyway. If $\Delta_o < P$, the fourth and fifth electrons go in the e$_g$ level, with spins parallel to those in the t$_{2g}$ level. It’s the weak field, high-spin (S=5/2) case and notated as $t_{2g}^3 e_g^2$. If $\Delta_o > P$, the energy required to overcome the repellence within the paired spin electrons won’t compete the energy for an electron to entering the upper level. Then, the fourth and fifth electrons locate to the t$_{2g}$ level and are paired with other electrons. This $t_{2g}^5 e_g^0$ state is the strong-field, low-spin (S=1/2) case. Figure 1.5 shows the occupation of the 3d orbitals at different strength of crystal field. Both $\Delta$ and P depend upon the iron configuration and the ligands. A partial spectrochemical series listing ligands from small $\Delta$ to large $\Delta$ can be attained from classical inorganic chemistry books.

1.2 Myoglobin and Fe-protoporphyrin IX

On average, there are 3 to 4 gram irons existing in the human body. 2/3 of these irons are in hemoglobin. Another 15% of it is reserved inside ferritin, the iron-storage
Figure 1.3 Crystal field splitting diagrams of octahedral complexes. (a) a regular octahedral complex, all six ligands are identical and equidistantly from the metal ion. (b) a Jahn-Teller distorted octahedral complex, in which the two ligands trans to each other are further away than the other four. (c) distorted octahedral complex with the two bonds along the z-axis are shorter than the other four in the xy plane.
Figure 1.4 Crystal field splitting diagrams of other possible geometries of iron-protoporphyrin complex (a) a regular octahedral complex as a reference. (b) square planar complex, four coordinate (c) square pyramidal complex in which the metal iron is five coordinated.
Figure 1.5 The energy level scheme for ferrous (Fe$^{2+}$) iron showing the occupation of the 3d orbitals for strong and weak ligand fields in a distorted octahedral geometry. On the left side, for a 5-coordinate iron, such as found for deoxy Mb where L$_1$ is the proximal histidine, the electron pairing energy ($P$) is bigger than the cubic term ($\Delta_o$) of the ligand field splitting. Under this condition, electrons occupy the d$_{x^2-y^2}$ and d$_{z^2}$ orbitals to avoid the electron pairing energy, resulting in the high spin (S=2) state of the ferrous iron atom. On the right side, when a sixth ligand (L$_2$) binds to the iron atom, the large cubic term in the crystal field results in pairing of the 6 d electrons and the formation of a low-spin (S=0) ferrous state. A similar situation holds for ferric (Fe$^{3+}$) heme complexes, leading to S=5/2 for high-spin and S=1/2 for low-spin.
WEAK FIELD

\[ S = 2 \]

\[ d_{z^2}, d_{x^2-y^2}, d_{xy}, d_{xz}, d_{yz} \]

STRONG FIELD

\[ S = 0 \]

\[ d_{x^2-y^2}, d_{z^2}, d_{xy}, d_{xz}, d_{yz} \]
Protein, for future usage. The rest is found in myoglobin, cytochromes, and other proteins, including those needed for cell division and DNA synthesis. Hemoglobin, which evolved about 450 millions years ago, picks up oxygen molecules from the lungs and transports them to the tissues of the body. Each hemoglobin molecule has eight thousand atoms elaborately weaved together(8). The primary mission of this atomic networking is to safeguard its four ferrous iron atoms from oxidation. Once these ferrous ions are oxidized to ferrie state, they lose their capability to bind oxygen (9). Myoglobin takes the oxygen molecules from hemoglobin and behaves as a local oxygen reservoir for the muscle cells. These two hemeproteins share striking similarities in their structures. Hemoglobin has four individual polypeptide units, each of which envelops a prosthetic group identified as heme and structurally resembles the single polypeptide myoglobin. Due to its simplicity, myoglobin is an ideal model for understanding these two oxygen-carrier proteins(10-13).

Myoglobin (Mb) is a globular protein with a dimension of 44Åx44Åx25Å(14). It consists of 153 amino acids, forming a single polypeptide chain, and the prosthetic heme group (Figure 1.6(b)) with a ferrous iron (Fe^{2+}) atom chelated inside. Amino acid residues packed into the interior of the molecule are predominantly hydrophobic, while the water-exposed exterior is hydrophilic. In aqueous environment, these hydrophilic amino residues facilitate a hydration shell surrounding the myoglobin molecule(15, 16). The polypeptide chain forms a three-dimensionally folded structure, generating several cavities inside. The heme is embedded in the biggest cavity and divides it into the distal and proximal pockets. A histidine (His93) inside the proximal pocket binds to the iron and forms the only connection between the Mb polypeptide chain and the heme group.
Figure 1.6 Structure of oxymyoglobin. (a) Chemical structure. (b) Heme prosthetic group. (c) Xenon binding sites inside the protein. (d) Distal pocket with key amino acids shown.
Figure 1.6(d) shows the crystal structure of myoglobin, focusing on the proximity of the heme group. Other smaller cavities (for example, Xe pockets as in Figure 1.6(c)) (17, 18) provide interior pathway and play important functional role for ligand recombination (19-22) and migration (23-26). Small molecules such as oxygen (O$_2$), carbon monoxide (CO), and nitric oxide (NO) can reversibly dissociate from and bind to the iron atom from the distal side of the heme plane.

Myoglobin (Mb) has been studied extensively both experimentally and theoretically over many decades, and is often taken as a prototype of heme proteins (10, 12, 13, 27-35). Experimentally, we investigate the three main states of myoglobin: metmyoglobin, deoxymyoglobin and ligand-bound myoglobin. In Metmyoglobin, the iron atom exists in its ferric form (Fe$^{3+}$). Both 6- and 5- coordination conformations could exist, but normally a water molecule (H$_2$O) is bound to the iron atom as the sixth ligand. Other small molecules and ions such as NO, CN$^-$, OH$^-$, F$^-$, N$_3$ can also take the role, but no situation of CO or O$_2$ molecules binding to metmyoglobin has been observed. When it obtains an electron from a reducing agent in aqueous solution, the iron atom reduces to ferrous (Fe$^{2+}$) form and the myoglobin turns to deoxymyoglobin, which is 5-coordinated. At low temperature (below 100K), an intermediate state could be formed from metmyoglobin by photo-reduction with the water molecule still bound to iron atom, which is in the ferrous low spin state (36). In the normal situation there is no sixth ligand in the ferrous state and the iron atom domes from the heme plane toward the proximal histidine ligand by ~0.4Å (28) and is usually in its high spin state (S=2 for Fe$^{2+}$). Diatomic ligands (CO, NO or O$_2$), can bind to deoxymyoglobin and pull the iron atom back to the heme plane (37) and make it change to the low spin state (S=0).
Heme (Figure 1.6(a)) is iron atom chelated with protoporphyrin IX in a square planar orientation. Protoporphyrins are tetrapyrroles containing the eight side chains: four methyl (-CH3), two vinyl (-CH=CH2) and two propionate (-CH2-CH2-COO-). Those side chains can be arranged in 15 ways and the number (e.g. IX) differentiates the arrangement of these side chains. Due to its ubiquity as an active component part inside heme protein, Fe-protoporphyrin is sometimes referred to as a model compound. Nitrogen atoms from each of the four pyrrole rings are ligated to the iron atom through coordination bonds. In the octahedral geometry, the axial positions are unhindered and another two additional ligands (L1 and L2 in Figure 1.5) can also bind to the iron atom. In myoglobin, they are histidine 93 from the polypeptide chain as L1 and a small diatomic molecule (NO, CO or O2) for L2, respectively.

Fe3+-protoporphyrin IX can not be dissolved at low pH values(38, 39). In the presence of aqueous alkaline solution, dimers(40) are formed. On addition of acid in the solution (pH values decreasing), before the precipitation occurs, monomers are re-formed(41). Electronic absorption spectrum of the Soret band varies for these two monomer and dimer states(42). The explanation of this dimerization has been prevailed with an oxo-bridge linking two iron centers (H2O-[Fe3+-O-Fe3+]-OH)(43). Recently, another mechanism is proposed without the involvement of the bridging oxygen atom(44). In the later geometry, the noncovalent π-π stacking interaction(45) from the unligated porphyrin faces accounts for the dimerization. In this case, each of the Fe3+-protoporphyrin IX is high spin(S=5/2), five-coordinated.
An effective way to break the dimerization is to encapsulate the model compound inside aqueous micelles(46, 47). A large macromolecular cavity generated by the aggregation of surfactant molecules in aqueous solution helps to stabilize monomeric model hemes in various oxidation and spin states(48, 49) of the iron with different axial ligands. On the other hand, several studies(50-53) implied that highly viscous glycerol/water mixtures can monodisperse low concentrated model compound in alkaline environment (54).

1.3 UV-vis Electronic Absorption Spectrum of Heme-proteins

Figure 1.7 shows the UV-vis electronic absorption spectra of myoglobin with different redox states and spin states, as an example to illustrate the significant differences of their expression on absorption spectrum. In the linear combination of atomic orbitals theory(55, 56), quantitative analyses of the absorption spectral behavior of different state should be based on a detailed molecular orbital description of the porphyrin(56-59). Interpretation to the lowest order (60, 61) utilize a four-orbital $\pi$-electron molecular orbitals for the porphyrin ring. While the iron porphyrin in D$_{4h}$ symmetry, parts of the porphyrin electrons occupy two nearly degenerate $\pi$-binding orbitals [$a_{2u}(\pi)$, $a_{1u}(\pi)$]. When a photon is absorbed, electrons can be exited to the corresponding $\pi^*$-antibonding orbitals [$e_g(\pi^*)$], which are degenerate. These two electronic transitions account for the Soret bands [$a_{1u}(\pi)\rightarrow e_g(\pi^*)$] and Q bands [$a_{2u}(\pi)\rightarrow e_g(\pi^*)$] absorptions for hemoproteins, respectively. Further coupling of $\pi$ states with vibrational energy levels results the $\alpha/\beta$ splitting of the Q band. The $\alpha$-band is a (0, 0) transition, which results from the $v=0$ vibrational level of the ground $\pi$ electronic state to
Figure 1.7 Electronic absorption spectra of horse heart myoglobin.  
- metmyoglobin (Mb$^{3+}$), 6C water molecule, as a weak ligand is on the distal side;  
- Deoxymyoglobin (Mb$^{2+}$) 5C water molecule can’t bind to ferrous myoglobin;  
- Ferrous nitric oxide myoglobin (Mb$^{2+}$NO);  
- Ferric nitric oxide myoglobin (Mb$^{3+}$NO).
the ν’=0 vibrational level of the π* state. The β-band results from a vibronic (0, 1) transition.

There is another model to explain these π→π* electronic transitions in an elegantly simple way. In this free electron molecular orbital model(62), π orbital of the porphyrin is simplified as a one-dimensional circular orbital. Totally 18 electrons(57, 62), one from each of the twelve carbon atoms and six from the four nitrogen atoms, occupy the π orbital. A single electron’s energy on this circular orbital is confined by the Schrödinger wave equation. The energy levels are $E\ell = \frac{\ell^2 \hbar^2}{2m_e r^2}$. $\ell$ is the quantum number for orbital angular momentum along the orbital central axis. Eighteen electrons can fill the levels up to $\ell=\pm 4$, leaving the lowest unoccupied levels have angular momentum number $\ell=\pm 5$. Electron promotions from the $\ell=\pm 4$ to the $\ell=\pm 5$ energy levels initiate the Soret band (selection allowed, $L=\pm 1$) and the Q band (selection forbidden, $L=\pm 9$). Similarly, the $\ell=\pm 3$ to the $\ell=\pm 5$ excitation is responsible for an N band (allowed) and another L band (forbidden). Optical spectroscopy of hemoglobin is well explained by taking account of this qualitative model(63).

The Soret band ranges from 360nm to 460nm and Q band is between 500nm to 600nm. The absorption around ~280nm originates from π→π* transition of the aromatic amino acids. The RZ (Reinheitszhal) value, representing the ratio of optical density of the Soret band to that at ~280nm (due to amino acid residues), indicates the purity of the heme proteins. Lowering of the RZ value in the experiments sometimes is a sign of the denaturing of the heme functional group. The band from 600nm to 800nm results from charge-transfer transitions. Charge-transfer absorption occurs between porphyrin-iron,
porphyrin-axial ligands, or iron-axial ligands(63). In the infra-red region, other charge-
transfer bands exist. Water (H₂O) also absorbs in the Near-IR region, so, to study other
charge-transfer bands at longer wavelengths, aqueous samples need to be dissolved in the
heavy water (D₂O) to avoid the spectroscopic interference from the solvent molecules.

Electronic absorption spectra of nitride oxide (NO) binding to myoglobin of
different ligation, spin and oxidation states (Figure 1.7) could be used as a general
example to illustrate some intensity and wavelength difference of the Soret and Q bands
in different states: Reduction of the iron (Fe³⁺→Fe²⁺) causes the red-shifts of the Soret
band; for ferric state (Fe³⁺), in high spin (S=5/2), there is charge-transfer band at ~600-
640nm, in low spin (S=1/2), no charge absorbance in that range; for ferrous state (Fe²⁺),
in high spin (S=2), the Q band isn’t resolved to α and β bands, in low spin (S=0), α and β
absorbance are clearly resolved.

1.4 Axial Ligands: Proximal Ligands and Distal Ligands

The electronic state of the heme iron is modulated by the protein environment and
plays an important role in the function of a heme protein(64). Porphyrin is a strong-field
ligand that places iron close to its spin-state crossover point so that the field components
from the axial ligands exert an enormous effect on the spin states of the iron atom and
thus its behavior in heme proteins. In myoglobin, the protein donates an imidazole
nitrogen of histidine (His) as the fifth ligand to the iron atom. Different atoms can
undertake this ligation role with examples including a histidine(His) nitrogen(N) in the
peroxidases(65), a tyrosine(Tyr) oxygen(O) in catalase(66) and a cysteine(Cys) sulphur(S)
in cytochromes P450(67). In some cases, as an example of cytochromes c(68-71), a
electron transfer protein, it not only donates a histidine nitrogen as the fifth ligand, but also provides a methionine (Met) sulphur from the other side, functioning as the sixth ligation. In this arrangement, the heme group is firmly fixed on the protein polypeptide. The iron atom is locked to six-coordinated and low spin, both in ferric and ferrous states. Not only is the electronic state of the iron modulated by the protein environment, but also the ligand field strength can be affected. It is not always possible to predict the strength of a given ligand is strong or weak. Nevertheless, a roughly qualitative comparison can be made. Typically, His, Met, Cys\(^{-}\), Lys, Tyr, OH-, CN-, CO and NO are strong ligands. Weak ligands include Glu, Asp, water, F\(^{-}\), Cl\(^{-}\), Br\(^{-}\), and thiols (Cys\(^{-}+H^+\)).

Established by usage, these heme ligands which connect the iron atom with the rest of the protein are designated as proximal ligands. Heme proteins that have only one amino acid ligand can bind exogenous ligands on the distal side. The interaction of these exogenous distal ligands with heme proteins are extensively studied to reveal the characteristics and functions of the protein environment. Normally, neutral exogenous ligands bind to ferrous heme more tightly than to ferric heme. Negatively charged exogenous ligands preferentially bind to the positive charged ferric heme. Small diatomic molecules, mostly studied of NO(72, 73), O\(_2\)(28, 35, 74-76) and CO(77-79), can bind to hemoproteins in fulfillment of their physiological functions. Myoglobin and hemoglobin are the most widely studied heme proteins. All three diatomic molecules (NO, CO, O\(_2\)) can bind to myoglobin, and hemoglobin. Nitric oxide (NO) binds to both ferrous and ferric myoglobin(28). Ferric myoglobin-NO has the intrinsic tendency to spontaneously autoreduce and convert to the ferrous species(80). Also, a second NO molecule can react with ferric NO myoglobin and generate its ferrous complex(81). Unlike nitric oxide,
oxygen (O₂) and carbon oxide (CO) only bind to ferrous myoglobin. When O₂ binds to ferrous myoglobin(24), it pulls the proximal histidine towards the porphyrin plane and is connected to another histidine(His-64) in the distal pocket(82, 83) through a hydrogen bond. CO binds to the heme iron taking a linear Fe-C-O geometry(84), while NO has a bent architecture(85, 86). Due to their electronic structure difference, NO binding can lead to proximal ligand dissociation and form a five-coordinated NO-heme complex, but CO prefers a six-coordinated complex retaining the ligand on the proximal side (87).

Water (H₂O) and hydroxide (OH⁻) are also common ligands to ferric heme proteins. In oxidized myoglobin, water binds as a weak distal ligand. In ferrous myoglobin, water H-bonds to the histidine 64(83) in the distal pocket and plays a key role in its affect on the distal ligation(88, 89). Water ligation is weak and its affinity for ferrous (Fe²⁺) hemes is very low. Hydroxide has a negative charge on it and its affinity to the null charge of the Fe²⁺-heme is low as well. Under few circumstances, water and hydroxide ligands can bind to ferrous iron directly. A recently X-ray characterized heme, heme c₅(90, 91), brought out the observation of a water/hydroxide ligation to ferroheme (92) iron in the protein environment of cytochromes b(6)f.

1.5 Time-resolved Absorption Spectroscopy of Hemoproteins

With the advancement of new technologies, protein-ligand interactions have been studied by various techniques(93). Time-resolved spectroscopy is one of the powerful tools which can monitor the short-lived intermediate states during the protein-ligand interaction processes. These intermediate states occur transiently during the course of reaction and may not be populated under equilibrium circumstance.
Flash photolysis is a method being extensively used to initiate reactions for kinetic investigations. Q.H. Gibson is a pioneer in this field, who first applied flash photolysis to study heme proteins(94). In the flash photolysis scheme, a sudden perturbation results from the light applied to the protein system. Immediately after the external perturbation, the system occupies a non-equilibrium state and then begins to evolve back toward an equilibrium state. According Eyring’s theory, the rate constant could be written as \( k = \left( \frac{k_B T}{R} \right) \exp \left( -\frac{\Delta G}{RT} \right) \), where \( k_B \) is the Boltzmann constant (=1.381x10^{-16} \text{ ergsK}^{-1}), \( h \) is the Plank constant (=6.626x10^{-27} \text{ ergs}) and \( \Delta G \) is the Gibbs energy of the activation. If the energy barrier of a reaction is zero (\( \Delta G=0 \)), at room temperature (\( T=25^\circ C \)), the largest rate constant \( k=6.25\times10^{12} \text{ s}^{-1} \). It’s a characteristic frequency of vibrational oscillation, which needs the time resolution of the flash photolysis to the femtosecond level. In this time range, femtosecond coherence spectroscopy (FCS) is a unique method used to retrieve the structural and dynamic properties of molecules by detecting transient information carried by the third order non-linear polarizability of the molecule(31, 95). To study transition states of the protein-ligand interaction, pico- and femto-second time resolution are needed(96).

Technically, the time resolution of the ultrafast time-resolved absorption spectroscopy doesn’t depend on the detector’s response time but depends on the energy/time profile of the laser pulse used in photolysis and in subsequent observation by a spatially delayed probe pulse. It also depends on the effective rate of the system to generate the initial photolyzed state and the accuracy of the determination of zero time (overlap of pump and probe pulses in time). Another important concept of all the photochemical reactions is the quantum yield. The quantum yield of a radiation-induced
process is the probability that a defined event occurs per photon absorbed by the system. Thus, the quantum yield is a measurement of the photolysis efficiency.

In experiments for time-resolved absorption spectroscopy of hemoproteins, the bond between the heme iron and distal ligand (usually NO, CO or O₂) is broken by a photolysis laser pulse (pump laser), initiating a non-equilibrium state ensemble of the protein molecules. Thereafter, the dissociated ligands are binding back to the iron atoms. This event is monitored by a second laser pulse (probe laser), whose light transmitting intensity at certain wavelength is recorded as a function of time separation between the pump and probe laser pulses.

Based on this core concept of pump-probe scheme, several systems are developed at our lab, emphasizing on different aspects of heme protein systems.

In one laser flash photolysis experimental setup(97, 98), the pump pulse is at 532nm with pulse width of 10ns. The probe light is a cw beam and a monochromator is used to select the probing wavelength. Probing light signals are detected by a photomultiplier and recorded in real time by a digital oscilloscope. The repetition rate of the pump laser is 10Hz. A seven decade time range (10ns to 100ms) can be studied using this setup, with two temporal resolutions (10ns or 5 us).

The multi-color pump-probe experimental setup(99, 100) is used to measure the transient absorption spectra. Here, a white light continuum pulse is used as the probe. The pump pulse could be set at 580nm or 400nm with pulse width less than 50fs. The probe wavelengths range from 380nm to 540nm. Two techniques of signal detection are used in
this design. One is using photodiode array and the other is with photomultiplier tube combined with lock-in amplification. The former method is usually used in transient spectra measurement because it gives the measurement with absolute optical density. And, the latter has higher signal to noise ratio, which is ideal for single wavelength measurement. The repetition rate of the system is 250 kHz. Samples are held inside an anaerobic spinning cell. This spinning cell, which is 2 inch in diameter, can rotate up to 7000 rpm. According to the refresh-rate of different sample species, the spinning speed needs to be adjusted to ensure that, upon every pump-probe pulse pair arriving, the sample volume perturbed by the previous pulse pair is shifted outside the beam focal area so that fresh equilibrium material is interrogated.

Femtosecond coherence spectroscopy (31, 101, 102), or FCS, is used to monitor the nuclear oscillations of the heme proteins following a “protein-quake”, which is initiated by a femtosecond pump pulse. In this technique, the coherent oscillations appear along with a monotonic decaying signal. To extract weak oscillations, a high signal/noise ratio is required. So, for this system, the pump-probe pulse train is designed at repetition rate of 76 MHz. Therefore, it has a limitation that the protein system studied in this setup must be refreshed within 13ns. The time delay between the pump and probe is controlled by a stepping motor with 1 micrometer step. So, the delay between pump and probe can be controlled as multiples of 6.666fs. Detailed knowledge of this setup could be found in other thesis of our group.

Most of the data analyzed in this thesis are collected through a two-color electronically delayed pump-probe system (103). Detailed description will be extended in
next chapter. Briefly, the pump-probe pulse pair has repetition rate of 190 kHz. The
pump pulse fixed at 403 nm with pulse width ~100 fs and the probe pulse width is ~3 ps.
The wavelength of the probe pulse can be tuned between 410 nm to 450 nm. The two
pump and probe pulses can be focused onto the sample collinearly or non-collinearly. In
the collinear geometry, a long-pass filter is used to eliminate the pump leakage to the
detectors. The delay between the pump and probe pulses can be electronically controlled
from 0 ns to 16 ns. By manually adjusting the pump-delaying circuits, the time interval
between the pump and probe pulses can be extended up to 5 µs in steps of 13.2 ns. With
this system, not only kinetics of NO, but also that of O₂ and CO rebinding to heme
proteins or model compound has been studied in this thesis.
References


diaquo(protoporphyrinato IX)iron(III) cation and aquohydroxo(protoporphyrinato IX)iron(III) intercalated in aqueous detergent micelles. 2541-2543.


# Chapter 2

## Experiment Methods

### 2.1 The Synchro-lock Pump-probe Laser System

Pump-probe techniques are normally used for investigating ultrafast phenomena. First, the sample is suddenly disturbed by the pump pulse from its equilibrium situation and returns to its initial state or relaxes towards a new equilibrium state therewith. To monitor this process, a probe pulse hits the sample after an adjustable delay time and its transmission or reflection is measured. These optical properties ($S$) are directly compared to the same characteristics when the sample’s non-equilibrium relaxation is not triggered. Recording the change of these properties ($\Delta S$) at various delay time ($t$) provides the function $\Delta S (t)$. It helps to obtain information of the phenomena initiated by the pump pulses. In kinetics studies of ligand binding to heme proteins, the pump pulse triggers the ligand dissociation and simultaneously starts the clock that synchronizes the probe pulse. Notice needs to be taken that the pump pulse not only triggers the ligand dissociation but also raises the vibrational excitation of the ligand, heme and protein matrix (1-4). The probe pulse energy is usually much weaker than the pump pulse so that the change of optical properties can be detected without disturbing the object under study.

Figure 2.1 sketches the conceptual geometry for a transient absorption measurement. In this arrangement, the probe comes from the same source as pump. The pulse train from the laser is divided into two parts by a beam splitter and sent forward.
Figure 2.1 Conceptual arrangement of a pump-probe experiment setup. The probe pulse branch has an optical delay line to lengthen the path which the probe light travels. Locking Amplifier (LIA), with a mechanical chopper, is used to detect the pump-induced probing variation.
Along different tracks. The probe beam goes to a track including a delay line which is a device that increases or decreases the path length the light will travel. The delay line could be a retro-reflector sitting on a motorized stepper. Moving the retro-reflector back and forth will change the path length the probing pulse travels. It takes the light 1fs to propagate 0.3µm. Therefore, if we want to probe the sample at a certain delay after it has been pumped, the simple way is to move the retro-reflector backwards and lengthen the path correspondingly. Since the optical property changes with and without the pump perturbation are compared and measured using lock-in detection, an “ultrafast”-response photo detector is actually not required. Each data point of the transient absorption spectra at delay time $\Delta t$ is typically averaged over many pulses.

With this optical path length delay method, the dynamic range in time is limited by the distance that the delay line can span. For example, to investigate a system’s kinetic response over 10ns range, the retro-reflector should move back and forth on the rail for 1.5 meter. Compared to the pump pulse, the probe pulse travels an extra 3 meter before it hits the sample. It brings a lot of difficulties to keep the pump and probe beams overlapped both spatially and temporally on the sample. If the probe beam is not perfectly collimated, while the retro-reflect mirror steps along the rail, the divergence of the input beam keeps changing. Also, any walk-off of the probe beam from overlap with the pump beam not only decreases the signal but also distorts the data. Therefore, when obtaining data with a long optical delay experiment, proper beam collimation and alignment are extremely difficult(5).

One possible solution is to set the delay between pump and probe pulses electronically. It results from a synchronization technique of two mode-locked ultrafast
Figure 2.2  Electronically Synchrolocked Pump-probe System. The dotted black lines refer to electronic connections. The legend:

- **Beam splitter**: 
- **Faraday isolator**: 
- **Half wave-plate**: 
- **Mirror**: 
- **Focusing lens**: 
- **BBO crystal**: 
- **Glass filter**: 
- **Fast photodiode**: 
- **Slow photodiode**: 
- **Mechanical chopper**: 
- **Spinning sample cell**: 
(picosecond or femtosecond) lasers(6-8). Figure 2.2 shows our electronically
synchrolocked pump-probe system which is developed based on Coherent (Coherent, Inc.
Santa Clara, CA) ultrafast lasers (Mira 900F, and Mira 900P) and its corresponding
synchronization system (Synchro-Lock 900).

Verdi V-10/V-18 lasers are solid-state diode-pumped Nd: YVO4
lasers. The Nd: YVO4 crystal as the gain medium provides TME00 mode 1064nm
energy transition. Inside the Verdi, a LBO (Lithium Triborate, LiB3O5) crystal is used as
the doubling optics to generate a single-frequency 532nm CW laser. The powers V-10
and V-18 can provide are at 10W and 18W, respectively. These CW laser powers are
used to pump up the Ti: Sapphire crystals inside the oscillators (Mira) and regenerative
amplifiers (RegA) as the energy reservoirs for producing ultrashort laser pulses.

Mira 900 series are self-mode-locked ultrafast oscillators that use
Titanium:sapphire crystal as the gain medium. The wavelength of Mira 900 is tunable
from approximately 700nm to 1100nm with a birefringent filter (BRF). Their repetition
rate is set at 76 MHz which is defined by the laser cavity length. The mechanism referred
to modelock laser in Mira 900 is based on a spatial self-focusing effect known as Kerr
Lens effect(9-11). A brief theory of operation for the Mira 900 can be found in their
operator’s manuals. Optical schematic of the Coherent oscillators (Mira 900F and Mira
900P) is shown in Figure 2.3. CW laser from the Verdi 10 is focused (by L1) into the
Ti:Sapphire crystal. The Ti:Sapphire crystal, cut at the Brewster angle, is placed between
two concave mirrors (M3&M4). The red solid lines represent the femtosecond operation
(Mira 900F). The femtosecond laser pulses oscillate inside the laser cavity defined by the
Figure 2.3 Dual cavity optical schematic of the Coherent oscillators (Mira 900F and Mira 900P). The red solid lines represent femtosecond operation. The black dashes represent picosecond operation. L: Focusing Lens; M: mirror; OC: Out Coupler; BRF: Birefringent Filter; GTI: Gires-Tournois Interferometer.
high reflector mirror (M6) and the output coupler (OC). The two Brewster prisms (P1 and P2) provide for group velocity dispersion (GVD) compensation in the laser cavity and the birefringent filter (BRF) is used to select the central carrier wavelength. The starter is an assembly with two galvo mounted Brewster plates with butterfly geometry. The butterfly establishes the mode-locked operation. In the picosecond configuration (Mira 900P), which is represented by the black slashed lines, the cavity is confined by two end mirrors, OC and GTI mirrors. The intracavity GVD compensation inside the picosecond pulse cavity is provided by the Gires-Tournois Interferometer (GTI) mirror.

The laser beams of these two Mira’s are monitored by two 2.0GHz photodiodes which are fiber-coupled to the Synchro-Lock 900 controller. The two Mira’s are arranged in a master-slave configuration. The picosecond Mira 900P (the master) pulse train are used as the frequency reference for the whole pump-probe system. The frequency and phase differences between the master (Mira 900P) and the slave (Mira 900F) are continuously measured and thereafter minimized by adjusting the slave Mira 900F’s cavity. In the daily operation, the ML (mode lock) knob of Mira 900P is switched to β-lock as this laser is self-optimized to minimize the GVD. In counterpart, the ML knob of the Mira 900F should be set at ML because it is the slave whose laser properties need to be regulated continuously via the synchro-lock mechanism. Three cavity length actuators are built inside the femtosecond laser cavity to match the slave frequency to the reference clock: a high frequency piezo-electric transducer (PZT), a low frequency galvanometer driven delay line and a discrete step motor-driven cavity mirror translation stage. A brief
description of the locking sequence is given as follows: First, the photodiode signal from
the slave femtosecond Mira is fed into a frequency diplexer and split to two outputs: the
fundamental frequency and a 9th harmonic sequence. Each of these outputs is send to a
radio frequency mixer to compare with the system references which are generated with
photodiode signal of the Mira 900P. The references also include two parts: the
fundamental frequency and a 9th harmonic “comb” which is produced by a frequency
multiplier. The fundamental frequency difference is minimized through adjusting the
motorized cavity mirror stepper. Then, after the coarse frequency adjustment has been
made, the signal from the fundamental frequency mixer is used to drive the PZT-driven
mirror through a phase-locked loop. To reach the laser phase-locked status, an electronic
phase shifter in this fundamental phase-locked loop adjusts the phase offset of the slave
laser accordingly to keep the mixer output equal to zero. At the same time, a parallel loop
sends a slower control signal to the Galvonometer actuator to continuously center the DC
drive voltage on the PZT component. In a word, the Galvo loop compensates low
frequency drift in the Mira 900F. Also, a third loop using the signal from the 9th
harmonic frequency mixer can be initiated to drive the difference to zero and further
reduce the timing jitter. However, it sacrifices the possibility to automatically shift the
phase of the slave laser related to the master laser (Mira 900P). Therefore, this third
phase-locked loop is not activated routinely in our ordinary kinetics experiment. In
practice, it is detected that the phase shifter in the fundamental loop has the capability to
shift the phase equivalent to 16.5 ns time delay. Figure 2.4 is the time delay calibration
curve of the phase shifter in the fundamental phase-locked loop. It’s a key reference for
the pump-probe experiment using this electronic delay setup. Extreme care must be taken
Figure 2.4 Time delay calibration curve of the phase shifter in the fundamental phase-locked loop. The X-axis is the DC voltage applied on the phase shifter.
to measure and document this calibration curve. Independent calibration should be performed when necessary with a fast response oscilloscope which has a time resolution better than nanosecond. In order to check the accuracy of the calibration, the MbNO kinetics, with electronic and optical delay, respectively, was compared in Figure 2.5. The optical delay is implemented with a computerized stepper (Nanomotion II, CVI Melles Griot, Inc.), which steps in 100 µm.

The laser pulses from the two oscillators (Mira 900P and Mira 900F), act as the seed pulses, are sent into the regenerative amplifier systems to be amplified at repetition rate of 190 kHz. The femtosecond pulsed seeds are sent to the commercial product RegA Model 9000 for increasing the pulse energy. The picosecond seeds are amplified via a home-made picosecond regenerative amplifier(12). The mechanism underneath both of the two systems is the same. The following paragraphs give a full description about the architecture of the home-build regenerative amplifier system and its timing sequence and electronic controls.

This home-made system consists of:

- A Titanium:sapphire crystal pumped by a branch of the Verdi laser;
- A Q-switch used to prevent lasing action until a high level of inversion is achieved in the Ti: Sapphire crystal;
- Two cavity dumpers integrated in the cavity for the reduction of the repetition rate from 76 MHz to 190 kHz-- one for injecting a seed pulse into the cavity, the other for ejecting out the amplified pulse.
Figure 2.5 MbNO recombination kinetics performed with optical delay and electronic delay. In the optical delay, the pump-probe pulses are synchronously locked with harmonic phase shifter. Then the delay is applied through a computerized stepper with 100 um resolution.
Figure 2.6(a) is a picture of the Ti: sapphire crystal (Ti$^{3+}$:Al$_2$O$_3$) in different shapes. In Ti: sapphire crystal, the metal ion (Ti$^{3+}$) is doped into the sapphire (transparent host). Ti is 0.1% of concentration by weight. Sapphire (monocrystalline Al$_2$O$_3$) has an excellent thermal conductivity and can easily be cut into any shapes. Since it was first introduced in 1986(13-15), Ti:sapphire lasers dominated the fields of ultrashort pulse durations of a femtosecond oscillator based on this Ti:sapphire gain medium are over the 10-150fs range. The shortest pulses obtained in research laboratories have durations around 5.5 fs(16, 17). Ti$^{3+}$ 3d$^1$ electron is responsible for the lasing emission. Figure 2.6(b) shows the Ti:sapphire absorption/emission spectra. The upper-state lifetime of Ti:sapphire is about 3.2 $\mu$s, and its saturation power is very high. So a high-quality, strongly focused pump beam is necessary for the Ti:sapphire crystal.

All the electro-optic devices of Q-switch and cavity dumpers used in this amplifier are based on the acousto-optic effect. It was first predicted by Brillouin(18) in 1921 and experimentally revealed by Lucas, Biquard(19) and Debye, Sears(20). In simple description (Figure 2.6(c)), when a radio frequency excitation is applied to the PZT transducer, an acoustic wave is present in the medium material. The material is cut in shape to help the build-up of an acoustic standing wave, which produce a refractive index grating. A beam of light passing through such a refractive index grating will be partially diffracted. The acousto-optic modulators (Q-switch & cavity dumper) spatially control the laser beam.

The Q-switch (ON) is used to prevent spontaneous lasing in the cavity. This allows the Ti: sapphire crystal to store energy for the amplification of an injected seed pulse. When the Q-switch is OFF, it gives the amplified pulse a temporal window for
Figure 2.6 Key elements inside ultrafast laser cavities. (a) Picture of Ti: Sapphire (Ti$^{3+}$:Al$_2$O$_3$) crystal in different shapes. (b) Absorption and emission spectra of Ti: Sapphire crystal. (c) Acoustic-optic effect. This effect is used to design electro-optical devices such as Q-switch and cavity dumper.
certain rounds of trip and preparation to be dumped out. For the cavity dumpers, while driven by a sufficiently short RF electronic pulse, they either diffract one pulse out from the 76 MHz pulse train into the laser cavity or eject the amplified pulse, which carries most of the stored energy in the Ti:Sapphire crystal, outside the cavity. After ejection of the pulse, the Q-switch is turned back on again to allow the excitation of the Ti:sapphire crystal, preparing for the next seed pulse injected in by the cavity dumper.

Figure 2.7 shows the construction layout of the ps Ti:sapphire regenerative amplifier. The red solid line represents the amplifier cavity. It consists of one output coupler (M1), two high-reflectivity flat mirrors (M2, M3) and six high-reflectivity concave mirrors (CM1-CM6). The Ti:sapphire rod is cut at Brewster angle with 5mm in diameter and 20 mm in length. During operation, the Ti:sapphire crystal is cooled by thermal interchanging with a NESlab reservoir whose temperature is set at 16.5°C. The same cooler is used to cool down the Q-switch crystal. The Q-switch crystal is a Brewster angle oriented acoustic optical quartz (NEOS N33080-25-3-BR-I). The two cavity dumpers (CD1 &CD2) are acoustic optical fused silica Bragg cells that are 3mm in thickness and intersect the cavity also in the Brewster’s angle. The amplifier controllers include two radio frequency drives (pulse switch, APE, Berlin) at 16.5W for the cavity dumpers, another rf drive for the Q-switch (NEOS Technologies, Florida) at 10W, and a digital divider delay unit (Quantum Technology, Inc. Lake Mary, Florida) providing the precise timing scheme for all the electro-optical components. In addition to these, a wattmeter (Bird Electronic Corp, Cleveland, OH) is connected to the rf drive in series to read the output, and two fast photodiodes (Electro-optics Technology, Inc. Traverse, MI)
Figure 2.7 (a) Construction arrangement of home-built pico-second regenerative amplifier: Ti: Al₂O₃, Ti: Sapphire crystal, 20mm cylinder; M1: Output Coupler (1% at 850 nm); M2&M3: high-reflectivity flat mirror (R>99.8%); CM1-CM6: high-reflectivity concave mirror (R> 99.8%); CD1&CD2: cavity dumper, fused silica Bragg cell, 3mm; QS : Q-switch, crystal quartz. IM1&IM2: injection mirror for seed pulse. L: Convex fused silica focus lens (f=150mm). (b) Picture of the real application. The red solids outline the cavity.
are applied. One photodiode is connected to an oscilloscope to monitor the amplifier intracavity intensity, and the other one picks up the 76 MHz pulse train of the picosecond seed laser (Mira 900P) as the external trigger for the digital divider events. All the electronic controls are powered via an isolation transformer (1000W) to block electromagnetic interference.

Figure 2.8 shows the timing scheme for this home-made regenerative amplifier. A fast photodiode picks up the 76 MHz Mira 900P pulse train and sends it to the divider delay unit (DD2). The DD2 main counter first divides the clock by 4000. After that, synchronized to the newly-generated 190 kHz clock, three TTL signals with adjustable timing sequence and adjustable pulse width are sent out to trigger other electronics. The first TTL output is sent via the Out3 to the Q-switch RF driver module. The RF signal is turned off on the rising edge of the TTL pulse for a preset duration (pulsewidth of the RF signal). Meanwhile, the other two TTL pulses are generated and fed to the injection (out1) and ejection (out2) cavity dumpers, respectively. These TTL signals trigger off the RF outputs of these two driver modules. The pulsewidth of the RF output pulse is adjustable and set as 13 ns for the injection RF signal and maximum (19.8 ns) for the ejection RF signal. This arrangement ensures a whole seeding pulse could be picked up and switched into the amplifier cavity and switched out of the cavity with the minimum residues. The intra-cavity energy intensity can be monitored through a fast photodiode. It can be seen that the intensity is saturated before the ejection happened. After the ejection, there is still some residual energy left inside the cavity until the laser cavity is blocked again by the Q-switch. The ratio of the saturated energy to the residue as well as the output energy
Figure 2.8 Timing Diagram of the home-built picosecond regenerative amplifier.
could be used as an indication for optimization. In the pump-probe experiment for heme proteins, the femtosecond pulses are frequency-doubled to 403nm by a BBO (β-BaB2O4) crystal of 500µm thickness. Using a 2mm BBO crystal, the picosecond pulses are doubled to the range 410-450nm. Together with the synchro-lock technique, they constitute an automatic pump-probe system which has a 16ns dynamic range.

In the conventional pump-probe setup, the probe beam comes from the same source as the pump beam. They are usually set at a non-collinear geometry. To suppress the background light and low frequency noise of the probe, the pump is chopped before interacting with the sample. After passing the sample, only the probe beam is sent to a photodiode coupled with a lock-in amplifier. The pump light is blocked from reaching the detector, in order to ensure that the pump-induced probe light change, not the pump change itself, is measured by the lock-in amplifier. To further reject the scattered pump light hitting the detector, the polarizations of the pump and probe pulses are set to be orthogonal. The pump leakage contamination can be further suppressed if we use a so-called cascaded lock-in technique(21). However, in the newly-developed system, since the pump and probe have different wavelengths, the pump and probe beams can be delivered to the collinearly. After interaction with the sample, the pump pulses can be blocked by using an appropriate glass filter. In practice, when using the 403nm femtosecond pulses as the pump, a 3mm thick long pass glass filter GG420 (Scott North America, Inc. New York, NY) is used to filter out the pump beam. In another arrangement, when we use the picosecond laser for pumping, and set the 403nm
Figure 2.9 Absorption spectra of Glass filters used to block pump beam before the detector. (a) long pass filter to block 403nm pump pulses; (b) band pass filter to block pump pulse 435nm and let 403nm passing.
wavelength for probing, a band-pass filter 400FS10-25 (Andover Corp. Salem, NH) is used to filter the pump light from reaching the detector (Figure 2.9).

2.2 Pump-probe Spectroscopy Setup for Kinetics Studies under High Magnetic Field

This setup is designed and constructed to perform pump-probe experiment while fast spinning aqueous heme protein samples are placed in the bore of a superconducting magnet. The closed cycle helium-cooled superconducting magnet (CFM-14T-50) used in this work is manufactured by Cryogenic Limited (London, United Kingdom). This superconducting magnet (Figure 2.10) has a 52mm clear room temperature bore which is perpendicular to the top and bottom surfaces of the magnet. The region of highly homogeneous field strength is centered over a 2mm long region at the middle of the magnet bore. The maximum possible central field is 14.3 T with effective field homogeneity of 0.1% in a 10mm dsv (diameter sphere volume). An upper limit of 10T is used in these experiments. The sample interaction volume is off the bore axis by 11mm, which leads to less than a ~1% change in the magnetic field strength compared to the bore centerline. The magnetic field direction can be switched by changing the direction of the superconducting current. The magnet needs ~24 hours to cool down, prior to energizing the superconducting current. In operation, the actual temperature near the center of the bore was 2-3 °C degree lower than ambient room temperature.

The quartz sample cell is custom-made (NSG Precision Cells, Inc New York, NY) to the specifications shown in Figure 2.11(a). The sample cell is carefully centered and
Figure 2.10 (a) Picture of the pump-probe arrangement above the magnet. 
(b) Picture of the motor and its supporter underneath the magnet. 
(c) Design of the floor to support the magnet having level adjustability.
Figure 2.11 (A) The sample cell is made from quartz and the open end is typically fitted with a rubber septum cap, which is sealed with parafilm following the preparation of the sample. (B) The optical arrangement is maintained using a cut-out aluminum tube (grey) that is inserted and coaxially aligned in the 52mm magnet bore. Lenses (B1, B2) each have a 50mm focal length and are set to focus (B1) and collect (B2) the laser light and transfer it to the detector lens assembly (C). The sample cell (S) is glued onto a spinning shaft (E) that is connected to a motor below the magnet. The shaft is supported and confined by two ceramic bearings (F) on each end of a hollow aluminum cylinder (G). The larger aluminum tube insert assembly holding the lenses is fixed to a XYZ translation stage that rests on an aluminum optical table extension above the magnet. The translation stage is adjusted so that the sample resides at a point that is equidistant between the lenses B1 and B2. The laser pulses enter from the top, and are focused by B1 into the sample and then re-collimated by B2 so that they reach the detector collecting lens C. The laser pulses are then transported by a fiber-optic cable (D) to a photodiode that is external to the system. A long wavelength-pass filter is inserted in front of the detector to extinguish the 403nm pump pulse. There is only 3.2mm of clearance within the hole of the lens holding plates (B2 and C) and the spinning shaft. This is designed to be smaller than the distance between the outer edge of the sample cell and the magnet bore wall. This helps to protect the rapidly spinning sample cell from touching any surface and breaking.
Central point of the magnet bore

(a)

(b)
glued onto an adapter which is locked onto a central shaft that spins at 3500 rpm. In order to minimize field-induced changes in optical geometry, all the components inside the magnet bore are designed using non-magnetic materials. For example, ceramic bearings are supported inside an aluminum tube and used to define the position of a high-speed spinning shaft made from hard-coat anodized aluminum. This assembly is connected to a 42V DC motor (Maxon Precision Motors, Inc. Fall River, MA) that is secured to the floor below the magnet (Figure 2.10). The assembly holding the lenses is also made from aluminum and it is supported by the optical table and is suspended into the magnet bore from above. Figure 2.11(b) illustrates the geometry inside the magnet bore. In this arrangement, the optical and mechanical parts are totally decoupled to prevent mechanical vibrations from being transferred to the optical part of the system. The spinning shaft could be released from its attachment to the motor below and the entire apparatus could be pulled out of the magnet from above, in order to disconnect the optical cell and change samples. The focal sample volume is 11.1mm away from the central axis of the magnet bore. At such a position, the strength and homogeneity of the magnetic field are better than 99% of their values on axis.

The incident light (pump and probe pulses) comes from above and passes through normal focusing optics (B1) to minimize pulse distortion and deterioration of the time resolution. Fiber optics is used to collect the light after it passes through the sample, since the time resolved transmission has already been established at that point. The collection lenses and fiber optic components are supplied by Thorlabs, Inc. (Newton, NJ). The fiber is optimized at blue wavelengths and the probe laser light (435nm, 3ps, 190 KHz) is reduced in power to 80% of its original value after 2m of fiber. Fiber optic
collimation/coupling packages (Thorlabs, Inc. Newton, NJ) are used on both ends of the fiber. The package (collecting lenses and mounts) are aligned in the lab and glued in place to optimize the collimation and recovery of the ~435nm probe wavelength.

2.3 Cryostat for spinning sample cell with uniform temperature inside as low as 190K

This cryostat is designed and home-made as a supplement in the lab to the commercial cryostat CCS-150 (Janis Research, Wilmington, MA). This Janis cryostat can achieve temperature over the range from 14K to 300K in the experimental applications. However, this cryostat requires a stationary sample cell and this limitation highly affects the refreshment of the sample during the experiment. Therefore, only nitric oxide (NO) rebinding kinetics is performed inside this cryostat because NO molecules normally have a fast enough recombination to heme proteins. Previous works at room temperature demonstrate that a 2-inch spinning cell (up to 7000 rpm) can refresh the sample efficiently in our pump-probe geometry and guarantee that each pump-probe pair interacts with an assembly of protein molecules with the identical condition. Here, a new cryostat is constructed to hold this fast spinning cell at controllable ambient temperature. It is tested to hold the temperature over the range of 190K to 315K non-loaded with the stability to ±0.5K. Limited by the thermal specification of the custom-made motor (Maxon Precision Motors, Fall River, MA) working inside the cryostat, pump-probe experiments can be performed over the temperature range from 235K to 315K.

The dimension of the cryostat (Figure 2.12(a)) is 12inx7.5inx12in. The inner chamber holding the uniform temperature is 9.75inx5.125inx10in. The cryostat is made
Figure 2.12 Pictures of the home-made cryostat for spinning sample cells.
of polycarbonate, a material which works at low temperature (up to -135 °C) and is easily machined, molded and thermoformed. Polycarbonate parts can be tightly glued together by using solvents whose main ingredient is chloroform (trichloromethane). The cryostat can be decoupled into two parts: a 2.5 inch high concave base and a hood of 9.5 inch depth. The bottom plate of the concave base is a 1 inch polycarbonate plate sandwiched ¼ inch thick Cryogel Z (Aspen aerogels, Northborough, MA). The walls of this concave base are 1¼” high and ¼” in thickness. Two barbed polycarbonate tube connectors penetrating one side of the walls are designed to couple the coolant circulation tubes in and outside of the cryostat. Two methods are applied here to boost the thermal isolation of the cryostat. First, the walls of the hood (Figure 2.12(b)) are built with double layers of ¼” polycarbonate board enveloping another 1/4” vacuum layer inside. This vacuum will enhance the thermo-isolation of the chamber as well as prevent condensation on the glass windows. Second, the same Cryogel Z sandwiched plates are used to form another thermal isolation layer clung to the interior walls the hood. 1-inch holes are drilled on two opposite walls of the hood. For each of the hole, two glass windows are mounted gas-tightly from opposite surfaces to prevent vacuum leakage.

Inside the cryostat, there are a fan, a tube-fin heat exchanger and a home-made motor holder (Figure 2.12(c)). The fan (MD24Z1QDN, Comair Rotron Inc, San Diego, CA) is home-reconstructed for working under low temperature. The motor holder is made of copper, a good thermo-conducting material. Four penstocks are drilled through the base plate of the motor holder. Polycarbonate pipe adaptors are threaded into these penstocks on both ends. Soft plastic tubing, which is low-temperature resistant, connects
adaptors on the cryostat wall, on the copper plate and to the heat exchanger. Liquid coolant, which is pumped by an outside cooler, circulates inside the close loop and makes heat exchange with the air inside the cryostat (Figure 2.12(d)). The whole assembly sits on two teflon blocks which are secured to the cryostat’s bottom plate.

18 electric wires pass through the bottom of the cryostat via two gastight adaptors. 8 of these wires are used for the Maxon motor, 2 for the fan, another six lines are used with three thermistors (ON-950-44004, Omega Engineering, Stamford, CT), two for each. The other two are reserved for future use. All the electric wires and their controls are compiled together inside a metal box to prevent electromagnetic perturbation from outside (Figure 2.12 (e)). The locations of these three temperature sensors are arranged as follows: one is dangling in the air, another one is fixed on the motor holder, and the third is immersed into a small bottle of pure glycerol. Temperatures which are measured by these thermistors are read out by an 866C thermometer (Omega Engineering, Stamford, CT). To prevent condensation or frosting under low temperature on the cell, air inside the chamber is purged with nitrogen gas before experiment. Two check valves (Figure 2.12 (f)) on the top of the cryostat are designed for this purpose. A soft gasket is placed between the hood and the concave base to prevent air exchange within and outside the cryostat chamber while it is working.

Protocol to run a pump-probe experiment with this home-made cryostat is described hereinafter:

- Assemble the sample cell on the motor.
- Turn on the controls for the motor and the fan to check that they are working fine, and then switch them off.
• Put on the chamber hood, switch on the motor at the necessary speed, maximize the experimental signal by adjusting the related optics, and then turn off the motor.
• Use the six clamps to lock the hood tightly on the cryostat base, make sure the gasket is on its right position.
• Feed nitrogen gas into the cryostat for 15 minutes, both check valves need to be open, one for gas in, the other for gas out.
• At the same time, connect the vacuum valve to a vacuum pump and start to vacuum the cryostat walls, turn off the vacuum valve once a rough vacuum (0.1~10Torr) is achieved.
• Turn on the fan’s control, a small vibration can be felt by touching the cryostat. Then turn on the motor, and switch on the cooler, setting the temperature at the required value.
• Wait until all the three readings from the thermistors evolve to within 1°C difference; give some extra time for the sample to be thermally equilibrated before taking the measurement.

An important but not fully discussed issue in this thesis in the aspect of pump-probe experiment is the concept of “magic angle” between pump-probe pulses. In viscous solvent(e.g. Glycerol/water mixture), rotational diffusion of the hemoproteins affects the ligand recombination kinetics(22-24). In order to eliminate this effect in kinetics studies, one convenient way is to set the polarization directions of the pump and probe beams at an angle of 54.7° (magic angle). For all the kinetics studies performed in this thesis, the polarization of pump and probe pulses are always set at “magic angle”, no matter whether the protein samples are caged in viscous solvents or non-viscous solvents. An explicit investigation of this rotational diffusion effect on MbCO kinetics is performed by other colleagues(12). To realize this “magic angle” geometry, a half-wave plate is first placed along one of the laser beams (pump or probe beam) and rotated to reach its maximum
transmittance. After that, the wave plate is rotated for an extra 54.7° and then placed along the other laser beam to confine its polarization. This definition of polarization geometry is kept for all the setups constructed in this thesis.

### 2.4 Sample Preparation

Sample preparation is the first and the most important step for the heme-protein kinetics studies. A subtle difference in the preparation could lead to a totally different sample response in the pump-probe kinetics investigations. To explain the kinetics data well and truly, one must first prepare and understand the sample under investigation. The importance is second to none for making the right sample for the pump-probe investigation. Figure 2.13 shows the absorption spectra of some of the samples investigated in this study. For all the samples studied in this thesis, an elaborate description of sample preparation will be recorded hereinafter for further reference and comparison.

**Stock Solution.** Heme proteins and heme compounds usually are in ferric form and can be purchased as commercial products in powder. Here in this thesis, horse heart myoglobin (MetMb) and horseradish peroxidase (HRP, type VI-A) are purchased from Sigma Chemical Co. (St. Louis, MO). Hemin (Ferriprotoporphyrin IX chloride) is purchased from Porphyrin Products Inc. These samples are used to prepare stock solution without further purification. Unless specially specified, all the samples are made into extremely high-concentrated stock solutions with corresponding buffers before further treatment. Then the stock solution is centrifuged (6000 rpm, 20°C) about 20
Figure 2.13 The absorption spectra of some of the samples investigated in this thesis.
minutes longer to centrifugate possible unsolved aggregates inside with a refrigerated centrifuge (Sorvall RT6000B, Dupont).

Normally, two types of buffer are used in the heme proteins studies here: potassium phosphate (Kpi, pH 7.0, 100mM) and Borate (pH ~8.4, 100mM). Potassium phosphate is used to prepare all the HRP stock and MetMb in some of the cases. Borate buffer is used to dissolve MetMb powder for later reducing and oxygen (O₂) binding because high pH value of the environment will help to stabilize the oxymyoglobin.

Stock solution of hemin (Fe³⁺-PPIX-Cl⁻) could be prepared in two ways. In one way, protohemin chloride dissolved in aqueous NaOH solution (1M) is used. In the other way, extra amount of solid protohemin chloride powder is added directly to 1% (w/v) cetyltrimethylammonium bromide (CTAB) solution, and the resulting mixture then is allowed to equilibrate inside a water bath at 50 °C for 1 hour. The CTAB solution needs to be freshly made on every experimental day by dissolving CTAB powder in de-ionized water or pH-fixed buffer.

Stock solutions of Mb mutant V68W and L29W are provided by Professor John Olson at Rice University. The concentration of V68W mutant (ferric, Fe³⁺) is 1.0mM and that of L29W mutant (ferrous, oxygen bound) is 1.2mM. When making oxygen-bound final samples, borate buffer is used to dilute these mutants.

**Sodium Dithionite Solution.** Sodium dithionite (Na₂S₂O₄) functions as a powerful reducing agent in aqueous solutions. It is a white powder and readily soluble in water. Usually the sodium dithionite solution is 1M and only several micro liters of
solution are needed from heme reduction per experiment. The following procedure is used to generate a 1M aqueous solution of sodium dithionite.

1. Fill a 5mL glass cylindrical vial with either 1mL de-ionized water or 1mL buffer. Put a little bit more than 0.1741 gram of sodium dithionite inside a smaller plastic vessel and let the vessel sit on the bottom of the glass vial. The wall of the vessel is higher than the liquid level to prevent the immediate mixing of the sodium dithionite with the liquid.

2. Seal the vial with a septum cap and parafilm (American National Can, Menasha, WI). Remove any residual oxygen by 3-4 rounds of vacuum-degassing followed by flushing with argon. This can be achieved by attaching small-bore (~16 gage) needles, to the vacuum line and argon supply, and letting them switch back and forth to deliver the vacuum and argon through the septum seal.

3. Flip the plastic vessel and shake well to dissolve the sodium dithionite powder in the buffer. Then, 1M sodium dithionite solution is ready for use. Sodium dithionite solution prepared in this way can live for 8-10 hours with the high capability of reduction.

Sodium dithionite (Na₂S₂O₄) is a powerful reduction agent which can grab the oxygen atoms very actively. During its storage, it gradually snatches the oxygen from the air or even from humidity and some of it oxidized to sodium metabisulfite (Na₂S₂O₅). The sodium metabisulfite still has the ability to grab another oxygen and transform to sodium dithionate (Na₂S₂O₆). Once it’s fully oxidized to sodium dithionate, it can not reduce ferric iron any more, but a side effect of lowering the pH value of the solution exists. Due to this oxidization, it’s very important to seal the sodium dithionite against the
air. Sodium dithionate will decompose in hot water and in acid solutions (28) and its days as iron reducer are over once decomposed.

The chemical reaction, in which sodium dithionite reduces the ferric iron atom (Fe$^{3+}$) to ferrous form (Fe$^{2+}$) in aqueous solution, can be written as following formula:

$$2Fe^{3+} + Na_2S_2O_4 + 2H_2O \rightarrow 2Fe^{2+} + 2SO_3^{2-} + 4H^+ + 2Na^+$$

Sodium dithionite also could react with another chemical sodium nitrite (NaNO$_2$) in aqueous solution to form nitric oxide (NO) molecules.

$$2NaNO_2 + Na_2S_2O_4 \rightarrow 2NO + 2SO_3^{2-} + 4Na^+$$

Nitric oxide (NO) molecules prepared in this way could be used to produce nitrosyl myoglobin (MbNO) or other NO-bound heme proteins or model compounds.

In practice, the preparation of sodium dithionite solution could be simplified as the following: first, half fill de-ionized water or buffer in a small glass vial (1mL) and vacuum-degas the vial followed by flushing argon as previous described. Then, put excess sodium dithionite powder (maybe metabisulfite/dithionate contaminated) directly in a syringe with a small-bore needle. Use this syringe to suck water or buffer from and then inject them back to the glass vial. When using this method, since the concentration of sodium dithionite solution can not be estimated accurately, experience is needed to prepare protein samples with this dithionite solution. Also, UV-Vis absorption spectra are used to monitor if the volume of dithionite solution is appropriately applied to the sample. The absorption of active sodium dithionite peaks around 315nm. Usually, we carefully add the sodium dithionite solution to the heme protein samples in the way that the peak of
Dithionite at 315nm is about the same height as the Soret peak (~400nm-440nm) of the reduced heme proteins.

**Ascorbic Acid.** Ascorbic Acid (Vitamin C) may also be used as a reducing agent (electron donor) for preparing oxymyoglobin. Ascorbate reacts with metmyoglobin much slower than sodium dithionite does. While preparing oxymyoglobin, there is no need to degas the metmyoglobin solution. In the open air, a small amount of ascorbic acid powder is added to metMb solution and after 2 hours they completely react. Extreme care must be taken because ascorbate does not only interact with metmyoglobin but also interacts with oxymyoglobin, inducing the oxyMb→MetMb (Fe$^{2+}$O$_2$→Fe$^{3+}$) transition(29). So, it’s forbidden to shake the sample solution during the reaction. After two hours of interaction, usually two layers of different colors could be seen of the sample. Color of heme proteins are mainly characterized by the absorption of the Q band. OxyMb has two sharp peaks in the Q band: one at 543nm (the green portion of the spectrum), one at 581nm (blue portion), which gives it a bright red color. In MetMb, the peak is shifted to 505nm in the blue portion of the spectrum and a weaker peak at 628nm in the red portion and, this two combined together give a brown-red color. Therefore, only the top layer of oxymyoglobin needs delivering to the sample cell for following experiment.

**Sodium Nitrite Solution and Nitric Oxide Gas.** Either sodium nitrite (NaNO$_2$) or nitric oxide gas (NO) is used for generating NO-bound heme proteins. 1M Sodium nitrite solution needs first to be degassed, following the same procedure described above for sodium dithionite solution. After reducing the heme iron with sodium dithionite, sodium nitrite is added to react with dithionite, generating NO molecules. In some cases,
NO-bound heme proteins are made by flushing NO gas to degassed samples. In this situation, NO gas (99.0% purity, Med-tech, Medford, MA) is bubbled through degassed 1M sodium hydroxide (NaOH) to remove trace quantities of N₂O₃ and N₂O₄ that always accompany in commercial NO gas product. These contaminants, if not removed, will lower the pH value of solution and pollute it with NO₂⁻ and NO₃⁻ ions. After that, the NO gas is bubbled through a degassed buffer before introduced to the degassed sample. In water, NO reacts with oxygen and water to form HNO₂. Therefore, it’s very important to ensure all the solutions the NO gas reacts with must be degassed and oxygen free.

**HRP³⁺-NO.** Dilute the stock solution of ferric HRP to around 1 O.D. /mm at the Soret peak. Degas the diluted sample solution. Administer NO gas for 20 seconds at pressure of 1 atm.

**MbCO.** Stock solution is diluted and degassed as described above. Sodium dithionite is then added to the MetMb sample in the ratio of 1:150 (v/v) to generate the reduced species. Then, CO gas (Med-Tech, Medford, MA) is flushed slowly over the surface of the deoxyMb for about 30 minutes. When MbCO is prepared inside high concentrated glycerol/water mixture, more time is needed for CO flushing until the absorption spectrum taken by the spectrophotometer (Hitachi U-4100) indicates that the transition is completed.

**MbNO.** The volume of sodium dithionite needs to be doubled because extra dithionite is needed to generate NO molecules.

**MbO₂.** Three ways are used to prepare oxymyoglobin in this thesis. The first way involves ascorbic acid as described before. In the second way, oxymyoglobin is prepared
by adding small amount of 1M sodium dithionite solution to 1 O.D. /mm diluted MetMb solution in the ratio of 1:100 (v/v). This results in an immediate color change as the iron atoms reduced to ferrous form. Separate the deoxymyoglobin from the excess sodium dithionite by passage through a sephadex G25 column that has been pre-equilibrated with borate buffer. Once separated from dithionite, reduced myoglobin picks up oxygen from solution to form the oxy derivative. In the third way, especially for MbO2 samples in glycerol/water mixture, a pure oxygen supply is used. First, degas and reduce the Metmyoglobin into deoxyMb. Then, administer pure oxygen to the sample very shortly (< 5 seconds). Shake the solution thoroughly to have the extra sodium dithionite react with oxygen gas. Once the sodium dithionite in the sample solution is used up, the oxygen molecules in the solution will bind to the ferrous deoxyMb to generate oxyMb. Finally, bring the pressure inside down to 1 atm by piercing the seal with a small-bore needle.

Fe2+-PPIX. NO and CO binding to ferrous protoporphyrin IX are studied in this thesis. Four environments are provided to the monomeric Fe-PPIX molecules: high-concentrated glycerol/water mixture(30), Kpi buffer with 1% (v/v) CTAB micelles(31), 80% methanol/20% water mixture(32) and a glycerol-CTAB-water ternary system(33).

In CTAB micelles, 2-Methylimidazole (2MeIm), cyanide (CN¯), Pyridine (Py) and Phenol can ligate to the Fe2+-PPIX. CO molecule can bind to these proximal ligand bound Fe2+-PPIX inside CTAB micelles. To prepare these samples, powder or solution of these “proximal ligands” were added to the micelle-encapsulated Fe-PPIX solution before degassing and reducing. The CO gas is flushed on the surface of the sample solution for 30 minutes as in the preparation for MbCO.
2.5 Data Analysis

Measurement of the Pump-probe Absorption Spectroscopy.

In time-resolved absorption spectroscopy experiment, the absorptions of the probe light with and without the pump excitation are compared. The pump induced absorption change (ΔA) of the probe light are measured as

\[
\Delta A(\lambda, t) = -\log_{10} \frac{I_{\text{on}}(\lambda, t)}{I_{\text{off}}(\lambda)}
\]

Here, \(I_{\text{on}}\) and \(I_{\text{off}}\) are the detected probe photon densities with pump light on and off.

In practice, the geometries of the optics are arranged to ensure that, on the focal plane, the probe focusing area is totally included inside the pump excited area. Also, the pump and probe light intensities are controlled inside the linear-response range for the sample. Under these conditions, the induced absorption change (over the active volume) ΔA is (34)

\[
\Delta A(\lambda, t) = \Delta \varepsilon(\lambda, t) Y_0 I_{\text{pump}}^{\text{abs}}
\]

where \(\Delta \varepsilon\) is the difference of the extinction coefficients of the photoproduct and reactant, \(I_{\text{pump}}^{\text{abs}} = I_{\text{pump}}^0 (1 - 10^{-A_{\text{pump}}})\) is the total absorbed pump light intensity during the pump excitation process and \(Y_0\) is the quantum yield of the photo-excitation. Here, \(I_{\text{pump}}^0\) is the incident pump light intensity covering the probe area, \(A_{\text{pump}}\) is the sample absorbance at the pump wavelength.

ΔA could be simplified as
\[ \Delta A (\lambda, t) = \Delta \varepsilon(\lambda, t) Y_0 f_{pump}^{abs} \]
\[ \propto (\varepsilon_{product,\lambda}(t) - \varepsilon_{reactant,\lambda}) \]

In a simple pump-probe process, \( HL \xrightarrow{hv} H + L \xrightarrow{\infty} HL \), reactant hemeprotein HL’s are photolyzed immediately into H’s and L’s. Then, after waiting long enough, all the ligands L’s rebind back to H’s. The system returns to its original unperturbed state, HL. This process can be verified by comparing absorption spectra before and after the pump-probe experiment. At time \( t \), the photoproduct includes hemoproteins partially in ligated state (HL), partially in un-ligated state (H). Suppose \( N_0 \) is the number of reactant molecules. \( N_{HL}(t) \) is the number of photoproduct in HL state and \( N_H(t) \) is the number of photoproduct in H state, at time \( t \). Letting \( \tilde{\varepsilon}_{HL} \), \( \tilde{\varepsilon}_H \) represent extinction coefficient per molecule for HL and H, \( \varepsilon_{reactant} \) could be written as \( \varepsilon_{reactant} = N_0 \tilde{\varepsilon}_{HL} \) and \( \varepsilon_{product} = N_{HL}(t) \tilde{\varepsilon}_{HL} + N_H(t) \tilde{\varepsilon}_H \). Normally, Ligand L does not absorb at the probing wavelength and is neglected. \( N_0 = N_{HL}(t) + N_H(t) \). Now, \( \Delta A (\lambda, t) \) could be derived as follows,

\[ \Delta A (\lambda, t) \propto (\varepsilon_{product,\lambda}(t) - \varepsilon_{reactant,\lambda}) \]
\[ = [N_{HL}(t)\tilde{\varepsilon}_{HL,\lambda} + N_H(t)\tilde{\varepsilon}_H,\lambda] - N_0 \tilde{\varepsilon}_{HL,\lambda} \]
\[ = [N_{HL}(t)\tilde{\varepsilon}_{HL,\lambda} + N_H(t)\tilde{\varepsilon}_H,\lambda] - [N_{HL}(t) + N_H(t)]\tilde{\varepsilon}_{HL,\lambda} \]
\[ = N_H(t)(\tilde{\varepsilon}_{H,\lambda} - \tilde{\varepsilon}_{HL,\lambda}) = N_H(t)(\tilde{\varepsilon}_H - \tilde{\varepsilon}_{HL})_{\lambda} \]
\[ \propto N_H(t) \]

For this type of two-state processes, three characteristics could be summarized through these equations: (1) if there is an extinction coefficient difference between the
reactant and photoproduct, the absorption change measurement $\Delta A$ is proportional to the time dependent population of the photoproduct. This proportionality is independent to the probing wavelength $\lambda$. (2) at certain wavelength $\lambda$, the larger the extinction coefficient difference between the reactant and photoproduct, the bigger the absorption change $\Delta A$. (3) at certain time $t$, positions of extrema in the transient spectrum should overlap with the extreme value positions on the equilibrium difference spectra $(\bar{\epsilon}_H - \bar{\epsilon}_{HL})_\lambda$ between the reactant and product.

Now, let us consider a pseudo two-state process,

$$HL + L \xrightarrow{hv} H + L + L' \rightarrow HL' + L \rightarrow H + L'.$$

In this process, not only the ligated heme protein HL, but also a different ligand L’, which is readily to bind unligated heme protein H, exists within the system for investigation. Once the hemeprotein HL is photodissociated to H and ligand L, the other ligand L’ can bind to unligated H first. Then, as time evolves, the original ligand L comes back and replaces ligand L’. After long enough, all the ligand L’s will bind back to H’s and the system returns back to its starting situation. In this type of processes, the equilibrium absorption spectra before and after the experiment are identical and have the same behavior as the two-state pump-probe process. So, this process is easily but mistakenly to be treated as a two-state process described before if only the absorption spectrum is compared.

At time $t$, the photoproduct includes hemoproteins partially in ligated states (HL’ and HL), partially in un-ligated state (H). Suppose $N_0$ is the number of reactant molecules. $N_{HL'}(t)$ and $N_{HL}(t)$ are the numbers of photoproduct in HL’ and HL states and $N_H(t)$ is the
number of photoproduct in H state, at time t. Letting $\bar{e}_{HL'}$, $\bar{e}_{HL}$, $\bar{e}_H$ represent extinction coefficient per molecule for HL’, HL and H, $\epsilon_{reactant}$ could be written as $\epsilon_{reactant} = N_0\bar{e}_{HL}$ and $\epsilon_{product} = N_{HL}(t)\bar{e}_{HL'} + N_{HL}(t)\bar{e}_{HL} + N_H(t)\bar{e}_H$. Also in here, we have $N_0 = N_{HL}(t) + N_{HL}(t) + N_H(t)$.

Now, $\Delta A (\lambda, t)$ could be derived as follows,

$$\Delta A (\lambda, t) \propto (\epsilon_{product,\lambda}(t) - \epsilon_{reactant,\lambda})$$

$$= [[N_{HL}(t)\bar{e}_{HL':\lambda} + N_{HL}(t)\bar{e}_{H:HL,\lambda} + N_H(t)\bar{e}_{H,\lambda}] - N_0\bar{e}_{HL,\lambda}]$$

$$= [[N_{HL}(t)\bar{e}_{HL':\lambda} + N_{HL}(t)\bar{e}_{H:HL,\lambda} + N_H(t)\bar{e}_{H,\lambda}]$$

$$- [N_{HL}(t) + N_{HL}(t) + N_H(t)]\bar{e}_{HL,\lambda}$$

$$= N_{HL}(t)(\bar{e}_{HL':\lambda} - \bar{e}_{H:HL,\lambda}) + N_H(t)(\bar{e}_{H,\lambda} - \bar{e}_{HL,\lambda})$$

From this equation, the three characteristics described above for two-state recombination do not hold. However, at the isosbestic wavelength, where $\bar{e}_{HL':\lambda} = \bar{e}_{HL,\lambda}$, or at a wavelength where both HL and HL’ are silent, $N_H(t)$ still can be detected because under those two conditions, $\Delta A$ is proportional to $N_H(t)$.

In an extreme situation, let us suppose that L’ binds to H much faster than L replaces L’. At time $t_1$, all the unligated H’s are bound by a ligand L’. The ligand L will not replace L’ until time $t_2$, and finally all L’ will be replaced by ligand L. Here, $t_1<<t_2$. Under this simplification,

(1) When $t<t_1$, $N_{HL}=0$, $N_{HL}(t)+N_H(t)=N_0$. 

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In this early stage, $\Delta A$ isn’t proportional to $N_H$ in general unless the experiment wavelength $\lambda$ is set at the isosbestic point of ligated species $HL'$ and $HL$. However, the derivative,

$$\Delta A(\lambda, t) \propto N_{HL}(t)(\bar{\varepsilon}_{HL',\lambda} - \bar{\varepsilon}_{HL,\lambda}) + N_H(t)(\bar{\varepsilon}_{H,\lambda} - \bar{\varepsilon}_{HL,\lambda})$$

$$= [N_0 - N_H(t)](\bar{\varepsilon}_{HL',\lambda} - \bar{\varepsilon}_{HL,\lambda}) + N_H(t)(\bar{\varepsilon}_{H,\lambda} - \bar{\varepsilon}_{HL,\lambda})$$

$$= N_0(\bar{\varepsilon}_{HL',\lambda} - \bar{\varepsilon}_{HL,\lambda}) + N_H(t)(\bar{\varepsilon}_{H,\lambda} - \bar{\varepsilon}_{HL,\lambda})$$

It gives information of the rate distribution.

(2) When $t_1 < t < t_2$, $N_{HL}=0$, $N_H=0$, $N_{HL'}=N_0$.

$$\Delta A(\lambda, t) \propto N_0(\bar{\varepsilon}_{HL',\lambda} - \bar{\varepsilon}_{HL,\lambda})$$

It’s a constant which is wavelength dependent.

(3) When $t > t_2$, $N_H=0$, $N_{HL}(t)+N_{HL'}(t)=N_0$.

$$\Delta A(\lambda, t) \propto N_{HL'}(t)(\bar{\varepsilon}_{HL',\lambda} - \bar{\varepsilon}_{HL,\lambda})$$

Starting from a later time $t_2$, $\Delta A$ represents the population change of transient species $HL'$. In other words, the ligand ($L'$) recombination process is measured hereafter.

The conceptual derivation given here can not be used to analyze real experimental data. First, ligand $L$ might compete with ligand $L'$ to rebind $H$ in its geminate rebinding process. Second, the process $L'$ rebinding to $H$ might be entangled with the $L' \rightarrow L$ ligand
replacement and can not be separated temporally. And, HL’ is an intermediate state whose equilibrium absorption spectrum ($\epsilon_{HL',\lambda}$) sometime is impossible to obtain.

**Time Zero Defining: Numerical Simulation Study.**

In the conventional pump-probe system, pump and probe pulses come from the same laser source. Time zero can be defined by generating cross-correlation coherent spike or with other nonlinear optical effects. There are several significant advantages of performing the pump-probe experiments with an electronically delayed laser system. However, in the synchrolock-delayed system, the pump and probe pulses are generated from different source and each has different bandwidth. Therefore, it is difficult to use non-linear effects to determine the temporal overlap of the two pulses.

Empirically, we use the experimental signal itself to define the temporal crossing point of the two pulses, which is necessary to define time-zero in the pump-probe experiments. In our situation, the 200fs pulse is used as the pump pulse. Because the probe pulse is ~3ps in duration, we assume that the instrument function reacts to the pump as an effective $\delta$-function excitation. The observed signal is generated from the convolution of the protein reaction and the probe pulse system response. The latter is represented by the line shape of the 3ps (FWHM) probe laser pulse. During the initial rise of the signal, as the pump and probe pulses overlap, the point where the signal equals 50% of its maximum value can be assigned to time-zero to a good approximation. Figure 2.14 shows the convolution simulation of a 3ps Gaussian function with a step function which simulates a pump-induced state jump at time zero. In this ideal situation, the step function is used to mimic a protein reaction without decaying. It demonstrates that when the pump
Figure 2.14 Convolution of a step function with a 3ps Gaussian pulse: simulation for defining the time zero in two color pump-probe experiment.
and probe pulses temporally cross each other (t=0), the convolution signal equals half of its maximum value. This feature is used to set time zero in the experiment. Routinely, in the experiment, we first optimize the spatial overlap of the pump and probe pulses and get the maximum of the response signal along the time axis. Second, move the fundamental phase shift setting to position where the lock-in amplifier reading is half of the maximum value it reached in the first step. Then, start the automatic data recording process with the awareness that the “probe before pump” signal should be around zero.

Figure 2.15 displays convolutions of several different exponentials with a 3ps Gaussian that simulates the probe pulse component of the instrument function. Exponential fits using t=0 located at the half-height of the initial signal rise agree quite well with the input time-constants (τ), so long as τ is longer than ~10ps. When τ=5ps, the method overestimates the true time-constant by ~20%. The green lines indicate Gaussian probe pulses of 3ps in pulse width. The blue lines show exponential decay processes of 5ps, 10ps, 100ps and 1000ps. The small circles represent the convolved signals that would be experimentally observed under these conditions. The convolved signals are located along the time axis so that time-zero is fixed at the point where the rising signal equals one-half of its maximum value. The red lines are the fits to the convolved signals starting at the time that corresponds to the maximum value of the signal. The data are renormalized to account for “missing amplitude” by scaling the maximum value of the convolved signal to match the exponential decay function at its respective time point (this function equals unity at t=0). The extracted time constants generated by such a fitting procedure were found to be larger by 1.1ps (22%), 0.2ps (2.0%), 0.74ps (0.7%), and 3ps (0.3%), respectively. This demonstrates that use of the
Figure 2.15 Convolutions of different single exponential decay processes with a 3ps Gaussian pulse. It shows that use of the half-height of the rising signal to define t=0 works very well when the decay is longer than 10ps.
half-height of the rising signal to define \( t=0 \) works very well when the decay is longer than 10ps and overestimates the time constant by \( \sim 1 \)ps as the decay time approaches 5ps.

**Maximum Entropy Method**

Most of the kinetic data analysis is analyzed with multi-exponential fitting or maximum entropy method (MEM)(35-37). In kinetic analysis, the MEM assumes the measurement is incomplete and inexact, so

\[
I_e(t_k) = I_f(t_k) + N(t_k) ,
\]

where \( I_e \) is the experimental data, \( I_f \) is the fitting function and \( N(t_k) \) is the noise at given time. Note either \( I_f(t_k) \) or \( N(t_k) \) is unobservable. And, the noise \( N(t_k) \) is assumed to be zero-mean Gaussian with variance \( \sigma_k^2 \).

\( I_e(t) \), in the time domain, could be written as

\[
I_e(t) = \int_0^\infty g(\lambda)e^{-\lambda t} \ d\lambda
\]

With the Laplace transformation, it could be written in discrete logarithmic space like,

\[
I_e(t) = \sum_{j=1}^{N} f_j e^{-\lambda_j t} \Delta (log\lambda_j)
\]

Under the constraint,

\[
\chi^2 = \frac{1}{M} \sum_{k=1}^{M} \frac{N_k^2}{\sigma_k^2} = expected \ value
\]
The entropy, constructed as

\[ S = - \sum_{j=1}^{N} f_j \left[ \ln \left( \frac{f_j}{F_j} \right) - 1 \right] \]

to be maximized for the \( f_j \)'s. Here, \( F_j \)'s are the so-called prior distribution. They are representatives of any previous understandings that we may obtain about the rate distribution.

Totally, there are \( N \) equations (\( j=1 \) to \( j=N \)) available for maximization of the constructed entropy (\( \frac{\partial S}{\partial f_j} = 0 (j = 1,2,3,\ldots N) \)). Iteratively solving the \( N \) equations, a set of \( f_j \)'s, constitutes a distribution of rates in logarithmic rate space, can be obtained. Performing a numerical inverse Laplace transformation with this set, \( I_f(t) \), not \( I_e(t) \), is recovered. It is an optimized kinetic trace, within some reasonable value of chi-square, which has a minimally structured spectrum of frequencies for the kinetics studied.

The MEM approach makes minimal pre-assumptions concerning the kinetic evolution, and yields a kinetic rate distribution that is only based on the experimental data. However, it is worth knowing that MEM analysis can generate artifactual distributions in the presence of incomplete (truncated) data sets and it also relies on an accurate knowledge of the signal/noise in order to prevent underfitting or overfitting of the data.

Multi-exponential fittings are carried out with Origin (Ver 6.0, Northampton, MA). Two single exponential decays, with 1% amplitude random noises, are analyzed and compared on Figure 2.16. It shows that, with 1% system noise level, 5% geminate kinetic rate constant change could be differentiated both by MEM and Origin.
Figure 2.16 Two exponential decaying processes with 5% difference in time constants are compared with analysis of MEM and exponential simulation. The exponential processes are added 1% random noise on top of the data. Both MEM analysis and Origin analysis can differentiate them clearly.
References


Chapter 3

Measurement of Diatomic Ligand Recombination Kinetics to Heme Proteins in Strong Magnetic Fields

3.1 Abstract

Heme cooling signals and diatomic ligand recombination kinetics are measured in strong magnetic fields (up to 10 Tesla). We examined diatomic ligand recombination to heme model compounds (NO and CO), myoglobin (NO and O₂), and horseradish peroxidase (NO). No magnetic field induced rate changes in any of the samples were observed within the experimental detection limit. However, in the case of CO binding to heme in glycerol and O₂ binding to myoglobin, we observe a small magnetic field dependent change in the early time amplitude of the optical response that is assigned to heme cooling. One possibility, consistent with this observation, is that there is a weak magnetic field dependence of the non-radiative branching ratio into the vibrationally hot electronic ground state during CO photolysis. Ancillary studies of the “spin-forbidden” CO binding reaction in a variety of heme compounds in the absence of magnetic field demonstrate a surprisingly wide range for the Arrhenius prefactor. We conclude that CO binding to heme is not always retarded by unfavorable spin selection rules involving a double spin-flip superexchange mechanism. In fact, it appears that the small prefactor (~10⁹ s⁻¹) found for CO rebinding to Mb may be anomalous, rather than the general rule for heme-CO rebinding. These results point to unresolved fundamental issues that underlie the theory of heme-ligand photolysis and rebinding.
3.2 Introduction

Iron is a crucial trace metal that is involved in many physical and chemical activities of living cells and organisms. In one important example, it is chelated by protoporphyrin IX (PPIX) to form the heme group. Heme proteins play key roles in oxygen metabolism, both for oxygen and electron transport (e.g., hemoglobin, myoglobin, cytochrome c), and as the terminal oxidase in mitochondria (cytochrome oxidase). Recently, it has also become apparent that many heme proteins are also involved in important regulatory processes that utilize the binding of diatomic ligands as both catalytic and signaling agents\(^{(1-5)}\) For this reason, it is important to understand the mechanism of diatomic ligand binding to heme systems and to determine, for example, if “spin selection”\(^{(6-10)}\) rules or entropy production timescales\(^{(11)}\) are important in regulating this process.

When ionized, the ferrous (Fe\(^{2+}\)) iron atom has an outer shell electronic configuration of 3\(d^6\). In octahedral coordination complexes, such as the heme, the five-fold d-orbital degeneracy is lifted by the ligand field of the porphyrin nitrogens and the axial ligands. When both the axial ligands are bound, the iron d-electrons are paired in an \(S=0\) ground state. When one of the axial ligand is absent, the weaker ligand field leads to a \(S=2\) ground state\(^{(12-14)}\). Thus, for a spin-zero ligand like CO, there is reason to believe that the \(\Delta S=2\) nature of the reaction may require binding via a double spin flip superexchange mechanism that slows the reaction rate and manifests itself in significantly reduced Arrhenius prefactors, as observed for MbCO\(^{(6-8, 15)}\).
In this study we investigate the effects of large magnetic fields on the ligand binding kinetics and non-radiative relaxation of heme in glycerol solution as well as when bound to both Mb and horseradish peroxidase (HRP). HRP catalyzes the reduction of H$_2$O$_2$ to H$_2$O in two serial one electron steps and oxidizes various aromatic substrates in the process. HRP can bind NO in either its ferrous or ferric state and the fast geminate kinetics of NO rebinding to this system have been reported under ambient conditions(16). The search for magnetic field effects on diatomic ligand binding to heme in the absence of protein material focuses on ferrous iron protoporphyrin IX (FePPIX) in 80% glycerol solution, in the absence of 2-methylimidazole (2MeIm), so that water or hydroxide is the likely fifth axial ligand.

Flash photolysis is an effective way to initialize ligand dissociation and study the protein and ligand responses over many timescales(9, 17-23). During photolysis, the iron-ligand bond is broken nearly instantaneously (probably within a fraction of the ~60fs Fe-Ligand vibrational period(24-26)). In Mb, the Fe$^{2+}$ ion moves towards the His93 and out of the porphyrin plane by about 0.4 Å. Subsequently, the photodissociated ligand, L$_2$, first located inside the distal pocket, can rebind to the iron (geminate rebinding) or explore other pockets inside the protein(27) and ultimately escape to the solvent through protein fluctuations that involve the distal histidine(28-30). The diatomic ligands (O$_2$, CO and NO) undergo very different dynamical processes when rebinding to the heme in Mb(9, 31-33). Using ultrafast pump-probe spectroscopy, it is now possible to measure the photolysis quantum yields (QY) for these ligands(34) as well as to follow the optical response over a very large dynamic range in time(9, 11, 19, 20, 22, 23, 26, 32, 33, 35, 36). For heme systems, the optical response between 400-450nm includes both the ligand
rebinding kinetics as well as signals due to the spectral diffusion of the hot Soret band lineshape as it undergoes vibrational relaxation back to thermal equilibrium (37-41).

Figure 3.1 shows a log-log plot of the “survival fraction”, N(t), of the deoxy Mb photoprodut, following photolysis of NO, O₂, and CO, over the time range 3ps-10ms. One striking observation is that the three ligands have vastly different geminate rebinding yields. At room temperature, only about 4% of the CO molecules find their way back to the iron and rebind geminately (32, 42, 43). Thus, most of the CO molecules escape from Mb following dissociation. In contrast, NO rebinds almost completely via ~10-100ps geminate processes that have been analyzed intensively in several studies (9, 22, 44, 45). The dramatic difference in kinetics for CO and NO binding has been discussed by Ionascu et al. (44) and the temperature dependence of the kinetics was used to show that both the Arrhenius prefactors and the enthalpic heme binding barriers are very different for the two ligands. For example, the main rebinding channel for NO geminate recombination is temperature independent and therefore barrierless. Other recent studies, involving O₂ rebinding to Mb, reveal two well-separated geminate phases having time-constants of ~6ps and ~40ns, each with amplitudes of ~30% (19).

For many years it has been thought that spin selection rules are an important underlying reason for the different dynamical behavior of the three ligands (6, 7, 10, 46, 47). The ligand fields of heme are such that the ferrous iron lies near to the spin crossover point. Thus, a perturbation due to the presence or absence of the diatomic axial ligand causes the spin-state to change. When the second axial ligand is not bound, the ferrous heme iron is five coordinate and has a Hund’s rule high spin (S= 2) ground state. When the diatomic ligand binds, the d-electrons of the ferrous iron pair to a low spin state.
Figure 3.1 The rebinding kinetics of diatomic ligands to ferrous myoglobin, following photolysis at room temperature in aqueous solution, are displayed on a logarithmic scale. The survival probability, $N(t)$, measures the population of the deoxyMb photoproduct as ligand rebinding proceeds. The decay times for the three ligands span approximately 9 decades in time. In the case of CO, approximately 96% of the photodissociated CO molecules escape from the heme pocket into the solvent so that rebinding primarily involves a relatively slow bimolecular process. In contrast, 99% of the photolyzed NO molecules rebind geminately in two separable phases with time constants of 15ps and 170 ps. In the case of O2 there are again two geminate phases, but the second phase (~50ns) is well separated from the first (~6ps). The bimolecular yield for oxygen is in the range 40-50%.
The spin states of ligated myoglobin are: MbCO (S=0), MbNO (S= ½), and MbO₂ (S=0), where the MbO₂ complex is thought to involve Fe³⁺O²⁻ antiferromagnetic coupling(48) or triplet state spin paring in a three center π-bond(49). In the recombination process from the initial unbound state to the final bound state, several system spin states can potentially participate. Using angular momentum addition, we see that Mb-CO presents only a single initial (S= 2) and final (S=0) spin state. However, for ferrous Mb-NO, the initial total spin angular momentum could be either 5/2 or 3/2, while the final state is S =1/2. In the case of Mb-O₂, the possible initial spin states include S=3, S=2 and S=1, while the spin state is S =0 in the final ligated form.

In theoretical considerations by Harvey(6, 50) and Franzen(7), an imidazole (Im) ligated iron-porphine (FeP) system was used as a model, and the iron spin-state energy surfaces for the binding of different diatomic ligands (Im-FeP-XO where X=C, N, O) were computed. Franzen(7) used density functional theory (DFT), to calculate the optimized ground state potential energy surfaces for each of the possible spin states as diatomic ligand approached at three different proximal heme doming distances (0.0Å, 0.2Å and 0.4Å, respectively). The fastest geminate recombination rate was assigned to the transition from the lowest initial spin state to the final correlated spin state (eg. Sᵢ =1→Sᵢ =0 for MbO₂ and Sᵢ =3/2→Sᵢ =1/2 for MbNO). The slower recombination rates were explicitly linked to the higher initial spin states as they transformed sequentially into the final spin states (eg. MbO₂: 2→1→0 and MbNO: 5/2→3/2→1/2). However, recent studies of MbNO recombination as a function of temperature and distal pocket mutation(44) have definitively shown that the slower (~200ps) geminate phase for NO
binding is due to docking in the distal pocket, rather than to a spin selection channel inherent to the NO ligand.

Strickland and Harvey(6) have calibrated their DFT calculations at the level of CCSD(T) (i.e., coupled cluster single and double substitutions and triple excitations). They have also compared both pure and hybrid DFT functionals in their calculations and focused on the binding of CO and H2O to ferrous heme. Their NO binding calculations(6) suffer from the appearance of a local minimum in the quartet state (S=3/2) that yields a barrier of 1.6kcal/mol that must be surmounted in order to reach the final NO bound state doublet (S=1/2) surface. This barrier is roughly half that calculated for MbCO rebinding using the same methodology(6, 50). Such a barrier for NO binding to heme is inconsistent with the temperature-independent rates experimentally observed for MbNO and PPIXNO rebinding. Thus, at this stage, it is probably premature to use the calculated transition-state Fe-NO bond-lengths as evidence against(6) the “harpoon”(44) and the “product-like”(51) transition state models for NO binding that have been put forward previously.

For CO binding, the direct quintet-singlet transition is spin forbidden so a superexchange process, or sequential rebinding involving an intermediate S=1 triplet spin-state, is usually invoked. Several groups(6, 7, 47) have suggested that the superexchange (double spin flip) process is the likely CO binding channel and that this accounts for the slower CO rebinding reaction observed in Mb(32, 42, 43, 52) compared to the other diatoms. This possibility has also been explicitly discussed by Hopfield et al. using a spin-orbit coupling model, operating at second order, that couples the S=0 and S=2 states through the superexchange mechanism involving the S=1 state(10, 15).
These theoretical studies suggested that strong external magnetic fields (~10 Tesla) might compete with the spin-orbit coupling to mix the iron spin-states enough to influence the CO recombination process, particularly at low temperatures (10). The external magnetic field influences the quantization axis for the iron spin. Spin-orbit interactions and the internal ligand fields at the iron are dominant, but because of the competition for the spin quantization axis, the magnitude of the (spin-orbit) coupling matrix elements between the different spin configurations can be altered as the eigenstates are mixed by the magnetic field. As a result, the transitions between spin-states can be affected, leading to a potentially observable change in the ligand binding rate.

The theoretical considerations (6, 7, 10) suggest that the diatomic heme ligands (CO, NO, and O₂) will have differences in their ligand binding and spin transition rates that should be revealed in the Arrhenius rate expression

\[ k_{BA} = k_0 e^{-\frac{H_{BA}}{k_BT}} \]  

through differences in the prefactor, \(k_0\). In Eq. 1, \(k_{BA}\) represents the overall temperature dependent rate of ligand rebinding from the initially photolyzed state, “B”, to the ligand bound state, “A”, where \(H_{BA}\) is the enthalpic barrier for this process and \(k_B\) is the Boltzman constant. Thus, comparative studies of the magnetic field dependence of the rebinding kinetics of these three ligands have the potential to reveal spin-dependent effects on the rebinding rate. When such studies are carried out with extremely good signal-to-noise, there is the possibility to reveal even relatively small effects. Such
studies can yield new insights into the underlying fundamental mechanisms associated with the important life process of diatomic ligand binding in heme proteins.

The experiments presented here document our measurements of heme relaxation and diatomic ligand recombination kinetics under high magnetic fields. We are aware of only one other experimental study of this nature(15), which monitored a weak magnetic field induced heme polarization anisotropy on much longer time scales and at very low temperatures. Recent improvements in the signal-to-noise of pump-probe experiments that are used to detect coherent oscillations in heme proteins(53, 54) offer the possibility to observe relatively small magnetic field dependent kinetic effects. Although small systematic changes in the amplitude of the short time (<10ps) optical response due to vibrational relaxation were observed in some cases, no magnetic field dependent differences in the ligand binding rates were observed beyond the noise level of the system. Based on the absence of magnetic field dependent ligand binding rates, and on the measurement of a wide range of Arrhenius prefactors for CO binding in several heme systems, we suggest that either the energy gaps between the pure spin states are larger than previously thought or that a significant mixing of the these states is already taking place in the absence of the magnetic field.

3.3 Experimental Results

The NO recombination kinetics to ferric horseradish peroxidase (HRP) at a series of magnetic fields are shown in Figure 3.2(a). A maximum entropy method (MEM) was used to fit the data(55) and the rate distributions that fit the observed kinetics are presented as an insert in the figure. It can be seen that the rate distributions obtained at
Figure 3.2 (a) Recombination kinetics of ferric HRP-NO as a function of magnetic field. The sample was stabilized for 30 minutes after each field intensity change. The pump and probe wavelengths were 403nm and 420nm, respectively. Black solid lines are the MEM fits and the insert shows the MEM rate distributions as a function of magnetic field.
(b) Comparison of the ferrous MbNO rebinding kinetics with (H=10 T) and without application of a magnetic field. No difference outside of experimental error is observed. The inset shows the MEM rate distributions.
the different field strengths agree quite well. Figure 3.2(b) shows a similar experiment conducted on ferrous MbNO. The invariance of both the raw kinetic data and the MEM analysis points to the absence of a measureable magnetic field effect on the NO binding reaction in ferric HRP and ferrous Mb.

Figure 3.3 shows the geminate kinetics and the magnetic field response of the NO complex of (Fe2+)PPIX under two solvent conditions (80% glycerol and 1% CTAB). The data again clearly demonstrate that the applied magnetic field leads to no measurable effect on the heme-NO rebinding.

Figures 3.4 and 3.5 present the effects of strong magnetic fields on the binding of CO to FePPIX. The CO complex of FePPIX in the absence of 2MeIm was chosen for study because the CO binding is much faster when water (or OH⁻) is in place as the heme proximal ligand(56). The high repetition rate lasers used in these studies generate excellent signal to noise, but do not allow the study of the slower rebinding systems such as MbCO and 2MeIm-FePPIX-CO because these samples do not fully reset to equilibrium within the arrival time (5.3μs) of the pump-probe pulse pairs. The FePPIX-CO sample is studied in 80% glycerol because the spin and optical properties of FePPIX and FePPIX-CO under this condition mimic those of deoxy Mb with surprising accuracy, especially when 2MeIm is bound as the axial ligand. In the absence of 2MeIm, the optical transitions are blue shifted by ~10nm, but, as can be seen in the insert to Figure 3.6, there is a clear isosbestic point as the CO rebinds under these conditions(56).

On the insert of Figure 3.5, statistical error analysis using sets of 10 runs, each with 99 logarithmic sample points, reveals the entire kinetics in roughly 30 minutes. The
Figure 3.3 Comparison of NO rebinding kinetics to Fe$^{2+}$-protoporphyrin IX with and without an applied magnetic field. The pump is 403nm and the probe is 435nm. The upper panel (a) is for a CTAB solution and the lower panel (b) is for a glycerol solution. No difference outside of experimental error is observed.
Figure 3.4 Comparison of the CO rebinding kinetics to Fe$^{2+}$-protoporphyrin IX in 80% glycerol with and without the application of a magnetic field. The sample is pumped at 403nm and probed at 435nm. The black solid lines are the MEM fits of data and the rate distributions are displayed in the inserts. The maximum in the data trace is arbitrarily normalized to unity and represents the cut-off time beyond which data are analyzed. The time zero is taken at the half height of the rising signal.
Figure 3.5 Detailed measurements of the magnetic field dependence of FePPIX-CO in 80% glycerol pumped at 403nm and probed at 435nm. The measured rate distributions are the same as shown in Fig. 7. The amplitude of the 5ps process appears to vary systematically with applied magnetic field. The insert shows two of the kinetic traces renormalized to one at time zero using an exponential function to fit the ~5ps component. This allows a better visualization of that the amplitude of the fast response is changing rather than the rate constant(s). The error bars for the measurement are barely discernable in the figure.
standard deviation of each data point along the N(t) axis is on the order of ~1%, while the laser jitter along the time axis is less than 800fs. As shown, the application of strong magnetic fields to heme protein samples leads to no detectable effect on the kinetic rates. The peak of the kinetic data near 3ps represents the maximum value of the signal and this is the time point after which the kinetics are fit in order to generate the MEM rate distributions. By comparing kinetics, where the underlying rate is systematically varied we find that the experimental data can be used to set a limit on magnetic field-induced changes of the observed geminate kinetic rate constant, $k_g$. After including the possibility of systematic errors due to optical geometry changes and fitting procedures, this limit is found to be $\Delta k_g / k_g \sim \pm 5 \times 10^{-2}$.

As shown in Figure 3.4, following photolysis of FePPIX-CO, a magnetic field effect can be discerned in the amplitude of the early time (<10ps) optical response. Figure 3.5 explores this effect in more detail and indicates that it is systematic. The insert in Figure 3.5 shows the zero field and 10T kinetics re-normalized by fitting the “fast” ~6ps phase of the response with an exponential function and then extrapolating back to time. The magnetic field induced change in the overall kinetics is well described by a simple change in this “fast” phase amplitude from roughly ~55% at 0T to ~45% at 10T.

SRC model\(^{(11, 57)}\) plus an exponential decay were used to fit the kinetic trace of PPIX-CO in Figure 3.5 under 0T field and 10 Tesla respectively.

\[
\Delta A(t) = P_1 \int_0^\infty dx \frac{A}{2\sqrt{\pi}x} \left( e^{-(Ax-C)^2} + e^{-(Ax+C)^2} \right) e^{x^2/\sigma^2} + P_2 e^{-x^2/\tau} + P_0
\]

The fitting parameters are compared in Table 3.1. Corresponding fittings with experimental data are displayed on figure 3.6.
Table 3.1 Fitting parameters of PPIX-CO in 80% glycerol/water mixture under 0 Tesla and 10 Tesla magnetic field

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>C</th>
<th>$t_0$(ps)</th>
<th>$P_1$</th>
<th>$P_2$</th>
<th>$\tau$(ps)</th>
<th>$P_0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Tesla</td>
<td>0.93</td>
<td>1.85</td>
<td>5.6</td>
<td>0.73</td>
<td>0.72</td>
<td>5.5</td>
<td>0.27</td>
</tr>
<tr>
<td>10 Tesla</td>
<td>0.95</td>
<td>1.82</td>
<td>5.4</td>
<td>0.70</td>
<td>0.54</td>
<td>5.5</td>
<td>0.29</td>
</tr>
</tbody>
</table>
We want to stress at this point that the “fast” optical response, observed for the FePPIX-CO sample in Figures 3.4 and 3.5, certainly involves a rapid (~0.3-5ps) spectral diffusion (cooling) response of the hot deoxy FePPIX photoproduct(37), but it may also include an underlying fast component of CO rebinding, which has been previously observed at low temperature and is sometimes referred to as process I*(58, 59). We note that the observed signal at 435nm between 1-10ps also involves a convolution of the instrument function.

Because of the potential for interference between the rebinding and cooling signals, we decided to examine the kinetics at other wavelengths. Figure 3.7 shows a comparison of the 0T data in Figure 3.4 probed at 435nm (blue stars) to that (red dots) obtained at 410nm (i.e., the peak of the transient bleaching signal) using another pump-probe instrument with much better (~100fs) time resolution(56). We also include another 0T kinetic trace taken at 424nm (i.e., the peak of the transient anti-bleaching signal) using the fs/ps magnet-based system (orange triangles).

Independent examination of the spectral response of the photoexcited deoxy heme in glycerol solution reveals a bleaching signal near 424nm (Figure 3.8). This observation helps to explain why the transient absorption signals observed at 424nm for t<10ps are so much weaker than those observed at 435nm. The CO rebinding signals at 424nm on this timescale have an opposite sign (i.e., increased transient absorption) compared to the deoxy heme transient cooling signal (decreased transient absorption). Thus, at 424nm, any fast (<10ps) CO rebinding signal would tend to be cancelled by the oppositely signed cooling signal of deoxy heme whereas at 435nm the cooling and rebinding signals are additive.
**Figure 3.6** Kinetic traces of PPIXCO (pumped at 403 nm and probed at 435 nm) in different values of the magnetic field in 0 Tesla and 10 Tesla. The solid lines represent the fits using equation (2).
Figure 3.7 The CO binding kinetics of FePPIX (pumped at 403nm and probed at 435nm) taken with the fs/ps pump-probe laser system (blue stars) are compared with the kinetics taken with a 100fs time resolved system (fs/fs) probed at 410nm (-ΔA_{410} is plotted as red dots). Additional data taken with the fs/ps system pumped at 403nm and probed at 424nm are shown as the orange triangles. The data are scaled to equal values at long times in order to reveal the short time deviations arising from the instrument function when it convolves with a fast (0-5ps) thermal response and/or rebinding signal. The insert shows the spectral evolution of this sample at room temperature when probed using 100fs continuum pulses. The small solid arrows show the probe wavelengths for the data in the main figure.
Figure 3.8 Spectral responses of PPIX-CO and Deoxy PPIX in 80% glycerol/water solution. The photoexcited deoxy heme reveals a bleaching signal near 424nm. The Y-axis indicates the lock-in amplifier readings under the same pump-probe conditions for the CO bound and deoxy PPIX samples.
In an attempt to remove some of the ambiguity introduced by heme cooling signals and the fs/ps instrument response, we also carried out room temperature experiments using the FeCO infrared transition at 1954 cm\(^{-1}\) (Figure 3.9). It is clear from these experiments that approximately 10\% of the CO photolyzed population rebinds in the first 10\(\text{ps}\). However, the analysis of the \(t < 10\text{ps}\) IR data is consistent with a straightforward extension of the distributed coupling model that has been previously applied \(^{11}\) to fit the kinetics for \(t > 10\text{ps}\), so it does not appear that a separate ultrafast exponential CO rebinding phase (attributable to the I\(^*\) process) is present. This, along with fits to the data at 435nm shown in Figure 3.6 indicates that the fast exponential phase (time constant \(~5-6\text{ps}\)) is due to cooling of the deoxy heme photoproduct.

Figure 3.10 displays measurements of geminate recombination for the oxygenated L29W Mb mutant (L29WMbO2). This mutant is used because of its enhanced geminate rebinding amplitude, which is sufficient to allow reset to equilibrium between the pump-probe pulse pairs. There is a barely perceptible difference in the data trace when the 10T magnetic field is applied, but the MEM derived kinetic rate distributions shown in the insert do not show a significant variation. Thus, the small changes perceived in the O\(_2\) kinetic data do not arise from rate changes, but rather from a possible small change in the geminate amplitude. The bottom panel of the figure shows the data renormalized to unity by use of an exponential fit to the data that is extrapolated to time zero. Here the potential for a small change in the geminate amplitude is more easily visualized.
Figure 3.9 Pump-probe kinetics measurement at the $\nu$(C-O) stretch mode wavelength, 1954 cm$^{-1}$. The blue solid line is the MEM fitting of the experiment data. Corresponding rate distribution is displayed on the insert.
Figure 3.10 Comparison of L29WMbO₂ rebinding kinetics with (H=10 T) and without application of a magnetic field pumped at 403nm and probed at 435nm. The MEM rate distribution shown in the insert reveals an exponential geminate process that is independent of the magnetic field. The upper panel shows the MEM fits when the data are normalized to one at the maximum data point, which is the cut-off time beyond which data are analyzed. The time zero is taken at the half height of the rising signal. The lower panel shows the data and fits extrapolated back to unity at time zero by using an exponential fitting function.
3.4 Discussion

Under the assumption that the spin of the iron-ligand system plays an important role in determining the reaction rate, we can introduce a very simple model to estimate the perturbative effect of the applied magnetic field on the Arrhenius prefactor ($k_0$ in Eq. 1). The prefactor incorporates all of the non-enthalpic factors (such as entropic barriers, attempt frequencies, frictional effects, and spin tunneling matrix elements) that affect the reaction rate in addition to the enthalpic barrier. Here we use a simple three level system that involves a (ligand bound) ground state as well as two “excited” unbound states, one with “allowed” (a), and one with “forbidden” (f), propensity for making the spin transition to the ligand-bound ground state. In addition to spin-orbit coupling the magnetic field can perturb and alter the mixing of these states so that the amount of allowed transition amplitude from state a mixed into state f can be altered by the magnetic perturbation. Quantum admixtures of spin states differing by $\Delta S=1$ have been treated previously by Maltempo(60).

As a specific example, we can imagine the ground state to be the CO bound singlet and the “allowed” and “forbidden” states to be the deoxy triplet and quintet states, respectively. We take the allowed triplet state to be of higher energy as CO approaches the deoxy heme. The energy gap, $E_a-E_f$, between the triplet and quintet states of the equilibrium deoxy heme, denoted as $\Delta E_{af}$, is thought to be relatively small(6, 7, 61). Both “excited” states (a and f) are assumed to be far removed (i.e., by the CO binding energy) from the 6th ligand bound ground state when the nuclei are in equilibrium. The perturbation of the spin-dependent Arrhenius prefactor can be calculated as a quantum mechanical transition rate, where the applied magnetic field mixes the allowed and
forbidden states. We take $\delta B$ to be a measure of the magnetic field induced mixing that couples the allowed (a) and forbidden (f) states. The magnitude of the perturbative mixing term, $\delta B/\Delta E_{af}$, can then be estimated based on the experimental detection limits. To do this, we take CO binding in Mb to be an experimental limiting case of a “spin-forbidden” prefactor ($k_{0f} \sim 10^9 \text{s}^{-1}$) and NO binding as a limiting case for a “spin-allowed” prefactor ($k_{0a} \sim 10^{11} \text{s}^{-1}$). Application of simple perturbation theory within the subspace of the allowed and forbidden states then leads to mixing of the states and an approximate expression for the square of the perturbed probability amplitude (i.e., the perturbed prefactor, $k_0^*$). Taking $\Delta k_{0f} = k_0^* - k_{0f}$ as the difference between the perturbed and unperturbed prefactors for the “forbidden” CO binding reaction, we find

$$\frac{\Delta k_{0f}}{k_{0f}} = 2 \left( \frac{\delta B}{\Delta E_{af}} \right) \left( \frac{k_{0a}}{k_{0f}} \right)^{1/2} \cos \Delta \varphi + \left( \frac{\delta B}{\Delta E_{af}} \right)^2 \frac{k_{0a}}{k_{0f}} (2)$$

The leading term in the magnetic field perturbation carries a phase factor, $\cos \Delta \varphi$, because it involves the interference term in the Golden Rule expression for the decay rate of the magnetically perturbed initial state $|f\rangle + \left( \frac{\delta B}{\Delta E_{af}} \right) |a\rangle$ as it evolves into the ligand bound singlet ground state. The phases of the complex matrix elements involved in coupling the ground state with the “allowed” and “forbidden” states are unknown and $\Delta \varphi$ is simply the difference of these two phases. Taking $5 \times 10^{-2}$ as the detection limit for fractional change in the observed rate, and a maximum value for the phase factor ($\cos \Delta \varphi = \pm 1$) leads to $\left| \frac{\delta B}{\Delta E_{af}} \right| < 2.5 \times 10^{-3}$. A similar calculation for the minimum phase ($\cos \Delta \varphi = 0$), using the second order term, leads to $\left| \frac{\delta B}{\Delta E_{af}} \right| < 2.2 \times 10^{-2}$. Thus, a typical magnetic field induced mixing perturbation of $\delta B \sim 10 \text{cm}^{-1}$, leads immediately to
ΔE_{af} \gtrsim 500\text{cm}^{-1} \text{ for the minimum phase, while } ΔE_{af} \gtrsim 4000\text{cm}^{-1} \text{ if the phase factors are such that they maximize the prefactor change upon application of a magnetic field. Thus, insofar as spin selection rules between “pure” spin states play a role in these reactions, the experiments reported here can be used to set approximate limits on the average energy separation between the forbidden and allowed spin levels as the transition state is approached along the Fe-Ligand and heme doming coordinate\(11, 57\). Prior calculations indicate a low lying spin S=1 triplet state within 300\text{cm}^{-1} \text{ of the ground state in order to account for the Mossbauer spectra of the deoxy heme system\(62\). More recent DFT calculations actually suggest that the triplet state energy can sometimes fall below the quintet state\(6\). Thus, the size of the energy gap between the triplet and quintet states that is needed to eliminate detectable magnetic field effects is surprisingly large.

On the other hand, it remains possible that spin admixtures are present at room temperature, even in the absence of the applied magnetic field. The presence of such admixtures is consistent with early far infrared magnetic resonance measurements on high-spin ferrous heme in Mb and Hb, where a simple S=2 spin Hamiltonian was found to be insufficient to account for the data\(61\). Magnetic susceptibility measurements have been interpreted to indicate that a “pure” S=2 spin is the likely 290K ground state for ferrous heme in Mb and Hb. However, uncertainty in the magnitude of the orbital component that generates the observed μ_{eff} =5.5\mu_B\(12-14\) makes unequivocal assignment of a pure S=2 ground state difficult.

In the process of this investigation we studied samples with a variety of possible spin channels (Table 3.2). For example, the binding of NO to ferrous heme involves two potential spin transitions; one is an “allowed” first order transition (ΔS =1 with an “initial”
### Table 3.2 Spin changes associated with ligand binding to high-spin heme iron

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ligand</th>
<th>$S_L$</th>
<th>$S_f^{Tot}$</th>
<th>$S_i^{Tot}$</th>
<th>$\Delta S$</th>
<th>$k_0 (H)$</th>
<th>$k_0 (s^{-1})$</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPIX (Fe$^{2+}$, S=2) in glycerol</td>
<td>CO</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>no</td>
<td>1.5x10$^{11}$</td>
<td>11</td>
</tr>
<tr>
<td>L29W Mb (Fe$^{2+}$, S=2)</td>
<td>O$_2$</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>no</td>
<td>1.8x10$^{11}$</td>
<td>(Unpublished)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mb (Fe$^{3+}$, S=2)</td>
<td>NO</td>
<td>1/2</td>
<td>5/2</td>
<td>1/2</td>
<td>2</td>
<td>no</td>
<td>9.0x10$^{10}$</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3/2</td>
<td>1/2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPIX$^a$ (Fe$^{2+}$, S=2)</td>
<td>NO</td>
<td>1/2</td>
<td>5/2</td>
<td>1/2</td>
<td>2</td>
<td>no</td>
<td>1.2x10$^{11}$</td>
<td>43</td>
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<td></td>
<td></td>
<td>3/2</td>
<td>1/2</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HRP (Fe$^{3+}$, S=5/2)</td>
<td>NO</td>
<td>1/2</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>no</td>
<td>&gt;6.0x10$^{10}$</td>
<td>16</td>
</tr>
<tr>
<td></td>
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<td>0</td>
<td>1</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

$^a$ In equilibrium in 80% glycerol S=2, however when NO binds, L$_1$ dissociates, so that the geminate rebinding may involve a transient S=1 state.
spin, $S_i=3/2$, and a “final” spin, $S_f=1/2$) and one is a “forbidden” transition ($\Delta S=2$, with $S_i=5/2$ and $S_f=1/2$) that can proceed via either a “sequential” or a second order “superexchange” process. In fact, it has been suggested(7) that the “fast” ($\sim 10$ps) and “slow” ($\sim 200$ps) geminate recombination phases observed(33, 44, 45) for MbNO binding are due to the allowed and forbidden (sequential) spin channels, respectively.

As direct evidence to the contrary, we note that there is a single $\sim 10$ps temperature independent(44) exponential geminate phase observed in Mb mutants that have the xenon pocket blocked. This demonstrates that spin selection is not responsible for the slower geminate rebinding phase of MbNO. Moreover, the temperature dependent measurement of the rates in native MbNO demonstrates that the prefactor for both the “slow” and the “fast” phase is $\sim 10^{11}$ s$^{-1}$(44). Thus, as suggested previously, we believe that the slower geminate phase results from a small enthalpic barrier involving the return of the NO ligand from a distal docking site near the heme, probably involving the Xe4 pocket(44). The model invoking a distal pocket docking site also explains why only the relative amplitudes, but not the rates, of MbNO binding are affected by external perturbations such as glycerol containing solvents(45), pumping wavelengths(44), or mutations(44).

An analogous situation is documented in Figures3.4 and 3.5, where it can be seen that the application of the magnetic field affects the amplitude, but not the rates, of the fast optical response for PPIXCO binding. However, we must recognize that the sub-10ps optical response at 435nm contains a significant contribution from the cooling of the transient deoxy heme state as well as from any underlying CO rebinding signal that might be present. Our fits to the room temperature PPIXCO rebinding kinetics, using the IR
data to eliminate the heme cooling signals, demonstrates that a separate sub-10ps exponential CO rebinding phase is not present. Thus, the exponential response observed at 435nm is assigned to transient cooling of the deoxy heme photoproduct.

When we fit the data in Figure 3.4, using a superposition of the SRC rebinding model(11, 57) along with a separate exponential phase to account for the short time heme cooling, we find the exponential component to have a time constant of 5.5ps and an amplitude that decreases by ~10% in going from 0 and 10T (see Figure 3.6 and Table 3.1). Using the same fitting protocols, we find that the exponential phase has a vanishing amplitude when applied to a data set that is composed of a superposition of the IR response t<40ps and the optical response t>40ps. From this we conclude that the amplitude of the 5.5ps exponential heme cooling response changes as a function of applied magnetic field. Independent studies of the deoxy heme cooling using improved 100fs time resolution reveal that the cooling response at 435nm has a time constant of ~4ps (unpublished), which is in good agreement with the present results, given that the instrument response of the fs/ps laser system increases the time constants in this range by ~20%.

One possibility that would account for the observation of a magnetic field dependent cooling amplitude involves a hypothesis where the amount of vibrationally hot electronic ground state heme is slightly reduced as the applied magnetic field is increased. This would lead to a smaller deoxy heme cooling signal and it would therefore be consistent with the observed reduction in the sub-10ps exponential amplitude at higher magnetic field strength. This is an unexpected result, and additional studies are necessary in order to further evaluate this possibility. If magnetic field effects alter the ultrafast
non-radiative decay pathways associated with CO photolysis and the deoxy heme cooling process, one might generally expect that both the rates and amplitudes might be affected. However, the branching ratio for a prompt non-radiative transition into the vibrationally hot electronic ground state (following CO photolysis) could be less than unity(9). If this is the case, and/or the branching ratio is further reduced by the applied magnetic field, the observed vibrational cooling rates in the ground electronic state should remain fixed but the amplitude of this signal would be decreased. Another obvious hypothesis involves a magnetic field dependent reduction in the CO quantum yield and the cooling of a residual hot six-coordinate CO bound species. However, this hypothesis predicts an increase, rather than a decrease, in the amplitude of the sub-10ps response and we therefore exclude this possibility.

A very weak magnetic field dependence of the O2 binding reaction in Mb (L29W) may also be present. Figure3.10 shows that, although there are indications of a very small magnetic field induced change in the geminate amplitude, the MEM analysis reveals no statistically significant change in the rebinding rate distribution. In contrast to NO binding, the preliminary temperature dependent kinetic measurements of the O2 binding reaction in wild type Mb (See Chapter4) indicate that the prefactors for the two geminate phases seen in Fig. 3.1 are quite different(19). The ~5ps geminate phase has a prefactor near $10^{11} \text{s}^{-1}$, while the ~40ns geminate phase has a prefactor near $10^{9} \text{s}^{-1}$. This could indicate the presence of either a spin selection mechanism, or an entropy production timescale(11) for the photoproduct that falls in the range between 10ps and 10ns and leads to a larger entropic barrier for the slower geminate phase.
Finally, we display in Table 3.3(63-67) the geminate rebinding rates and Arrhenius prefactors for CO binding to several different heme systems. The significant differences observed for the CO geminate rate demonstrates that there is not a universal spin selection rule for CO binding that acts as a consistent limiting factor in these reactions. The net spin change upon ligand binding in these systems is the same for each of the equilibrium species ($\Delta S=2$), yet the rates are very different. We do not believe that this variation is due to non-equilibrium photoprotoduct species involving triplet states because the transient absorption spectra (e.g., see insert to Figure 3.7) indicate that a genuine $\Delta S=2$ transition is taking place over a wide range of timescales for the different samples. For example, a detailed analysis (11) of the temperature dependence of CO binding to the FePPiX model complex reveals a prefactor for CO binding near $10^{11}\text{s}^{-1}$, which is similar to the “spin-allowed” prefactor for NO binding. Other heme systems, such as microperoxidase(63), carboxymethylated cytochrome c(67), mutants of cytochrome c(68), and CooA(66, 69) all have CO geminate rebinding rates that are faster than $10^{10}\text{s}^{-1}$ (see Table 3.3), which sets a lower limit for their Arrhenius prefactors.

Additional temperature dependent studies are needed to establish the precise magnitude of these prefactors, but it is clear from the rapid rebinding kinetics of various heme systems, that CO binding is not generally being retarded by an unfavorable ($\Delta S=2$) spin selection rule. In fact, it appears that the unusually small prefactor ($\sim10^9\text{s}^{-1}$) found for CO rebinding to Mb may be anomalous, rather than the general rule for heme-CO rebinding. The possibility that these “fast” CO binding reactions take place through genuine population of a $S=1$ transition state (i.e., via a sequential rather than a
### Table 3.3 Kinetics of CO binding to selected heme proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_g$ ($10^9$ s$^{-1}$)</th>
<th>$I_g$</th>
<th>$k_{ha}$ ($10^9$ s$^{-1}$)</th>
<th>$k_0$ ($10^9$ s$^{-1}$)</th>
<th>reference</th>
</tr>
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<tr>
<td>WT Mb$^b$</td>
<td>0.006</td>
<td>4%</td>
<td>0.0002</td>
<td>1</td>
<td>29</td>
</tr>
<tr>
<td>Mb (V68W)$^b$</td>
<td>0.006</td>
<td>76%</td>
<td>0.005</td>
<td>0.88</td>
<td>Unpublished</td>
</tr>
<tr>
<td>PPIX+2MeIm(95%Gly)$^c$</td>
<td>0.83</td>
<td>20%</td>
<td>0.17</td>
<td>&gt; 0.17</td>
<td>55</td>
</tr>
<tr>
<td>NP4 (pH=5.0)$^c$</td>
<td>5.1</td>
<td>99%</td>
<td>5.1</td>
<td>&gt;20</td>
<td>Unpublished</td>
</tr>
<tr>
<td>HRP+BHA$^b$</td>
<td>2</td>
<td>90%</td>
<td>1.8</td>
<td>&gt; 1.8</td>
<td>16, 63</td>
</tr>
<tr>
<td>PPIX (95% Gly)$^c$</td>
<td>20</td>
<td>95%</td>
<td>19</td>
<td>150</td>
<td>55</td>
</tr>
<tr>
<td>MP-11 (aggregated)$^c$</td>
<td>16</td>
<td>78%</td>
<td>13</td>
<td>&gt; 13</td>
<td>62, 64</td>
</tr>
<tr>
<td>CooA$^d$</td>
<td>13</td>
<td>60%</td>
<td>8</td>
<td>&gt; 8</td>
<td>65</td>
</tr>
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<td>Cm Cyt Ce$^e$</td>
<td>63</td>
<td>27%</td>
<td>17</td>
<td>&gt; 17</td>
<td>66</td>
</tr>
</tbody>
</table>

$^a$ To extract the $k_{ba}$ from these data we used a simple three state model$^{55}$. Fitting methods for CO geminate recombination : $^b$Single exponential fit. $^c$Stretched exponential fit. $^d$Two exponential fit, only the fast component is presented here and used to estimate the value of the $k_{ba}$. $^e$Three exponential fit, only the fast component is presented here and used to estimate the value of the $k_{ba}$. 
superexchange mechanism) or that entropy production timescales and/or ligand confinement are involved in determining the prefactor will be the topic of future work.
References


Appendix 1

Oxygen Rebinding Kinetics to Myoglobin and its Mutants (V68W, L29W) with Temperature Variation in Aqueous Solutions and Glycerol Mixtures

A1.1 Experimental Methods

Sample Preparation. Detailed experimental methods are explained and discussed within Chapter 2. Here, in connection with the oxymyoglobin (and its mutants) preparation, two methods are applied here. Method 1 below corresponds to the first method of preparation for MbO₂ described in Chapter 2 and method 2 below corresponds to the third method of preparation described in Chapter 2. The second method described in Chapter 2 can not be used for low temperature studies because the glycerol solution does not pass through the G25 column.

Method 1: Ascorbic acid with air. Stock solution of metmyoglobin is diluted within a cylindrical glass vial. The concentration of the diluted sample is tested with a 1mm quartz cuvette. The final concentration is adjusted to approximate 1 O.D. at its Soret peak (409nm). Then, a tiny amount of ascorbic acid powder is added into the metmyoglobin solution. The glass vial sits in the open air stationarily for about 2 hours (for metMb) or less (for V68W Mb) until a clear dividing line within two layers of species appears. The top layer of substance is transported into the spinning cell and ready for the experiment.
Figure A1.1 Absorption spectrum of an MbO$_2$ sample prepared with method 1 and its absorption spectrum comparison before and after a series of temperature dependent kinetics experiment.
Figure A1.1 shows absorption spectrum of an MbO\textsubscript{2} sample prepared with this method and its usual spectral change after a series of temperature dependent kinetics experiment.

Method 2: Sodium dithionite with oxygen gas. Diluted metMb sample solution within the glass vial is sealed with a septum cap and parafilm. Then it is vacuumed and Argon flushed as in the standard degassing procedure. Meanwhile, assemble and seal the spinning cell, use the vacuum pump to pull out the air inside the cell. After that, transport 3 ml metMb solution into the vacuumed cell with a needle syringe piercing through the rubber sleeve stopper septum for it. Then, adding appropriate amount of sodium dithionite solution into the cell, the sample’s Soret peak moves to 435nm. Adjust the output pressure of the oxygen cylinder to the minimum value. The oxygen bubbles going through a bottle of buffer should be observed as single, inconsecutive ones, not a bubble line as in the CO flushing. Introduce this pure oxygen gas into the sealed cell promptly for about 1-2 seconds. First, it will use up all the extra sodium dithionite molecules and then, oxygen molecules (O\textsubscript{2}) bind deoxymyoglobin to form oxymyoglobin. Dramatic color change of the sample solution could be observed during this oxygen association process. After ensuring that all the deoxymyoglobin is transformed to oxymyoglobin, the last step is to use a small-bore needle, piercing through the sleeve stopper cap for less than 1 second, bring down the gas pressure inside the cell to 1 atm.

Figure A1.2 shows absorption spectrum of an MbO\textsubscript{2} sample prepared with method 2 whose Soret peak locates at 418nm instead of 415nm which appears in the preparation with method 1. Figure A1.3 compares the absorption spectra of horse heart oxymyoglobin prepared with method 1 vs. method 2.
Figure A1.2 Absorption spectrum of an MbO$_2$ sample prepared with method 2
Figure A1.3 Absorption spectra comparison of horse heart MbO$_2$ sample prepared with method 1 vs. method 2. (the absorption data were normalized to 1 at the Soret peak for illustration purposes.)
Experience and practice are needed to observe the color changing to control the oxygen flushing. However, even with over-flushing of the oxygen to the sample, which is registered as a double peaks at the $\alpha/\beta$ band (542nm & 580nm) but the Soret band peaks around 409 or 410nm (Figure A1.4), it’s not the end of the world. Keep gas-flushing and oxidize all the samples back to the met form until the double peak feature at the $\alpha/\beta$ band is disappeared, then repeat the preparation procedure described above. In several tests, no difference is observed for this refurbished sample and a freshly made sample in absorption spectra and pump-probe kinetics. It is worth to know this, especially while treating the precious V68W mutant. Figure A1.5 shows the absorption spectrum of a V68W MbO$_2$ sample prepared with method 2 and its usual spectral change after a series of temperature dependent kinetics experiment.

Myoglobin L29W was provided by Professor John Olson at Rice University in its ferrous, O$_2$ bound form. A1.6 shows the absorption spectrum of a diluted L29W MbO$_2$ sample and its usual spectral change after a series of temperature dependent kinetics experiment.

It’s very important to monitor the sample absorption spectral evolvement before and after the experiment. MbO$_2$ samples are very easy to be oxidized and change to a mixture of different species. Abnormal kinetics traces could be obtained once this happen. A lot of factors could trigger this sample evolvement. Extreme care needs to be taken here.
Figure A1.4 Absorption spectrum of an MbO₂ prepared by method 2, but over-flushed with uncertain amount of excess oxygen gas.
Figure A1.5 Absorption spectrum of an V68W MbO$_2$ mutant sample prepared with method 2 and its absorption spectrum comparison before and after a series of temperature dependent kinetics experiment.
Figure A1.6 Absorption spectrum of an L29W MbO$_2$ mutant sample and its absorption spectrum comparison before and after a series of temperature dependent kinetics experiment.
Experimental Configuration. Temperature control and experimental setup are described in Chapter 2. For all the ultrafast kinetics (3ps-13ns), the pump wavelength is set to 403nm and the probe wavelength is set to 435nm. The pump pulse energy is around 5nJ and that for the probe pulse is around 0.5nJ as they arrive at the sample. For the longer time kinetics measurement, laser flash photolysis system is used. Briefly, laser pulses (at 532nm) generated by a 10Hz Nd-doped yttrium-aluminum-garnet (YAG) laser (Continuum, Inc.), is used to excite the sample. A cw beam produced by a universal arc lamp (Oriel Instruments, Model 66021) is used to probe the sample evolution. After passing through the sample, the probe light is sent into a 0.25m monochromator (Oriel Instruments, Model 77200). Kinetic response at certain wavelength selected by the monochromator is detected by a photomultiplier (Hamamatsu, H6780) and recorded with a broad band digitizer (Lecroy 9420).

4.2 Results and Data Analysis

Kinetic models and prior work on MbO2. Zewail et al. (PNAS, Vol. 101, Issue 52, Pages 18000-18005, 2004) studied the oxygen recombination to human WT myoglobin in the same time window from picosecond to 10 nanoseconds. Basically, they investigated this O2 rebinding kinetics at room temperature with mutation variance. Intentionally or unintentionally, two its mutants (V68F and I107F) are chosen to compare with the wild type human myoglobin. In all three cases, no mutation induced difference is observed on the picosecond scale but drastic differences are on the nanosecond time scale. Their investigation led to an explanation as “bifurcation model”. In this model, O2 rebinding to myoglobin is divided into two categories: the directed population and the undirected population. The directed population will bind back within the few picoseconds and be
little affected by the protein immediate environment around the heme binding site. The undirected population recombination can be affected by thermal and intra-molecular motions on a longer time scale.

Here, in my studies, another two mutants (V68W and L29W) are chosen to compare with native horse heart myoglobin for kinetics investigation. Contrary to what they observed, this “directed population” recombination is actually affected by the mutation. A new kinetic model is needed to describe this important kinetics.

**Figure A1.7:** Temperature dependent kinetics data of MbO₂ and its oxidation product MetMb is shown. MbO₂ sample is prepared from MetMb with Method 1. All the kinetics traces are normalized at the peak values. MbO₂ Kinetics at 296K is measured first and then repeated after two other kinetics measured. A very good agreement is reached here. Equilibrium absorption spectra are compared before and after the kinetics measurement and found to be equivalent (Shown on figure A1.1). MbO₂ displays two decaying phases within the detectable temporal windows. The first fast phase decays single-exponentially and ends at 20ps. No temperature dependence of its rate is obtained within the setup detection limitation. A very weak dependence of its amplitude related to the temperature variation is observed. The second phase, which starts around 1 nanosecond, was only partially detected here due to the restriction of setup measuring range. Clear temperature dependence is shown on the early stage of this slow phase. Because of the incomplete information, no conclusion can be drawn about the amplitude dependence on temperature based on this work. The pump-probe responses of metMb are also listed in this figure. It is revealed unambiguously that there is no temperature dependence of this decay from the laser-excited state, both for the rate and amplitude. Rising edges of these two types of
Figure A1.7 Temperature dependent kinetics data of Horse Heart MbO₂ and its oxidation product MetMb.
pump-probe responses (O₂-rebinding vs. hot spices cooling) are fitted with MEM method. These two types of responses can also be differentiated by the time at which the ΔA maximum is reached (3.2ps vs. 1.7ps). Insert: experiment data displayed linearly for the MetMb for -20ps -20ps. It shows that the response is approaching the instrumental function, which is the convolution of a 200fs pump pulse and a ~3ps probe pulse.

**Figure A1.8:** Another temperature dependent kinetics study of MbO₂ from 276K to 316K in step of 10K. The sample is made with Method 1 within borate buffer (pH=8.42). Black solids are fittings for each individual kinetic trace. The discontinuities of the fits are results from two different fitting methods: from ~3ps to 400ps, the data are fitted with a single exponential decay; after 400ps, the kinetics fittings are extracted from the MEM data fittings for that range. The single exponential fittings are back-extrapolated with the fitting functions to time zero and normalized to make N (0) =1. Related fitting parameters are listed on Table 4.1. Insert: MEM fittings for kinetics from 400ps to 10ns are shown in linear-linear plot, with normalization to unit value at the starting points. It clearly shows that as the temperature increased, the slow recombination process is getting faster.

**Figure A1.9:** O₂ recombination kinetics to Horse Heart Myoglobin in 0.1M borate buffer (pH=8.42) at room temperature (T≈296K). The oxymyoglobin sample for the ultrafast pump-probe experiment (<13ns) is prepared with Method 1. The whole range data set consists of three sets of data: for the longer time (>10ns) kinetics, flash photolysis system which has 10 Hz repetition rate is used; from 3ps to 13ns, the data was taken by a synchro-lock pump-probe system which has a repetition rate of 190 kHz. The data simulation from 0ps to ~3ps is generated from back-extrapolation of the fitting of data from 3ps to 400ps, which is $N(t) = 0.72 + 0.28e^{-\frac{t}{5.35}}$. This kinetics trace, spanned 11
Figure A1.8 Temperature dependent kinetics study of MbO2 from 276K to 316K in the step of 10K. Insert: MEM fittings for kinetics from 400ps to 10ns with normalization at the starting points.
### Table A1.1 Temperature Dependence of O$_2$ rebinding to Horse Heart Myoglobin after photolysis (inside borate buffer (pH=8.42))

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>$\tau$</th>
<th>A1 (%)</th>
<th>$k_{BA}$ ($10^{11}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>276</td>
<td>5.4±0.4</td>
<td>31</td>
<td>0.57</td>
</tr>
<tr>
<td>286</td>
<td>5.5±0.3</td>
<td>30</td>
<td>0.54</td>
</tr>
<tr>
<td>296</td>
<td>5.4±0.4</td>
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<td>0.52</td>
</tr>
<tr>
<td>306</td>
<td>5.9±0.5</td>
<td>24</td>
<td>0.41</td>
</tr>
<tr>
<td>316</td>
<td>6.0±0.5</td>
<td>23</td>
<td>0.38</td>
</tr>
</tbody>
</table>
Figure A1.9 O2 recombination kinetics to horse heart myoglobin in 0.1M borate buffer at room temperature (T≈296K).
decades, shows that there are three separated recombination processes for oxygen molecules rebinding back to iron atoms. The first geminate recombination process completes within the first 20ps. There is another recombination starts around 1ns and ends around 1us. The slowest rebinding process happens in the 100’s of microsecond. The amplitudes of these three phases are 28%, 37%, 35%, respectively.

**Figure A1.10:** Glycerol concentration dependent kinetics of O$_2$ recombination to horse heart myoglobin at room temperature (T≈296K). High concentrated stock solution of metMb is made in borate buffer (pH=8.42). Pure glycerol (purity>99%) and borate buffer are added into the stock solution weighted by mass ratio to generate the diluted samples. Final oxymyoglobin samples for the ultrafast pump-probe experiment (<13ns) are prepared according to Method 2. The colored solids are single-exponential fits of the data from 3ps to 400ps. The dashed lines are back-extrapolations of the fitting functions to time zero. All data are normalized to make N(t)=1 at time t=0. Fitting parameters are listed on Table 4.2. Kinetics data detected by the flash photolysis system of O$_2$ recombination to horse heart myoglobin with no glycerol content in the buffer are transplanted on the kinetics trace of oxymyoglobin in the same buffer (no glycerol) to illustrate the complete behavior of the slow geminate phase. For the complete rebinding kinetics (red squares), the amplitudes of those three phases are 38%, 32% and 30%, respectevily.

**Figure A1.11:** Temperature dependence of MbO$_2$ recombination kinetics inside glycerol mixture. The glycerol mixture is made with 80% (weight percentage) borate buffer (pH=8.42) and 20% glycerol. Oxymyoglobin samples are made with Method 2. Colored solid lines are single-exponential fittings of the data between 3ps to 400ps. The dashed lines are back-extrapolations of the fitting functions to time zero. All data are normalized
Figure A1.10 Glycerol concentration dependent kinetics of O2 rebinding to horse heart myoglobin at room temperature (T≈296K).
<table>
<thead>
<tr>
<th>Glycerol Pt(%)</th>
<th>$\tau$</th>
<th>$A_1$ (%)</th>
<th>$k_{BA} \times 10^{11}$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.6±0.4</td>
<td>38</td>
<td>0.82</td>
</tr>
<tr>
<td>20</td>
<td>4.9±0.2</td>
<td>51</td>
<td>1.04</td>
</tr>
<tr>
<td>50</td>
<td>5.0±0.2</td>
<td>57</td>
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</tr>
<tr>
<td>90</td>
<td>4.8±0.2</td>
<td>79</td>
<td>1.66</td>
</tr>
</tbody>
</table>

Table A1.2  Glycerol Dependence of $O_2$ rebinding to Horse Heart Myoglobin after photolysis (T=296K)
**Figure A1.11** Temperature dependence of MbO2 rebinding kinetics inside glycerol mixture.

Temperature Dependence of MbO2 inside glycerol mixture

Fittings:
- $0.32 + 0.68e^{-t/4.47}$
- $0.50 + 0.50e^{-t/5.36}$
- $0.69 + 0.31e^{-t/5.09}$

Buffer: 20% glycerol / 80% borate (pH 8.42)
to make N(0)=1 at time zero. For the fast rebinding phases, no temperature dependent rate change is observed. However, the amplitude is dramatically affected by the temperature, compared with that inside the pure borate buffer. On the other hand, the buffer effect on the second decaying process is also exhibiting much bigger than that of the pure borate. It is noticeable that at temperature 275K, the second phase is hardly observable in this timing window. Either it is diminished or is delayed.

**Figure A1.12:** Experimental data of temperature dependent O₂ rebinding kinetics to mutant MbV68W in 0.1M borate buffer (pH=8.42). The protein sample is made with Method 2. Absorption spectra before and after the experiment are shown on Figure 4.2. All the kinetics data recorded are normalized at the maximum values. No temperature dependent difference is observed within the noise variation. Two fitting procedures are applied to the data. One is a single exponential (4.2ps) with a stretched exponential (1.35ns, beta=0.8). The other applied is two –exponential fitting (4.6ps, 1.29ns). Both fit the raw data set.

**Figure A1.13:** Kinetics of oxygen rebinding to V68W Mb differentiated by preparation methods. The protein samples are prepared at room temperatures (T≈295K). The whole kinetics is fitted using a single exponential plus a stretched-exponential decay. Response between 0--3ps is generated from back-extrapolation of fitting function.

**Figure A1.14:** Kinetics of oxygen rebinding to Horse Heart Myoglobin differentiated by preparation methods. Experimental data from the maximum to 400ps are fitted with a single-exponential decay. The early time responses are back extrapolated from the fitting functions.
Figure A1.12 Experimental data of temperature dependent O2 rebinding kinetics to mutant MbV68W in 0.1 borate buffer(pH=8.42).
Figure A1.13 Kinetics of O2 rebinding to V68W Mb differentiated by preparation methods.

---

Kinetics of V68W MbO2 differentiated by preparation methods.

- V68W MbO2 prepared by Method 1
- V68W MbO2 prepared by Method 2

Equations:

1. \[ N(t) = 0.34e^{-5.15t} + 0.60e^{-\frac{t}{1321}} + 0.06 \]
2. \[ N(t) = 0.57e^{-4.67t} + 0.41e^{-\frac{t}{1276}} + 0.02 \]
Figure A1.14 Kinetics of oxygen rebinding to horse heart myoglobin differentiated by preparation methods.
**Figure A1.15:** Temperature dependent recombination kinetics of Horse Heart MbO$_2$ prepared with Method 2. The sample is prepared within borate buffer (pH=8.42). Recorded data are normalized at the peak values.

**Figure A1.16:** Temperature dependent recombination kinetics of mutant V68W MbO$_2$ inside glycerol mixture. The glycerol mixture is made with 80% (weight percentage) borate buffer (pH=8.42) and 20% glycerol. The protein sample is generated through Method 2. A single exponential decay plus a stretched exponential decay is used to fit the raw data. The fitting functions are used to extrapolate kinetic trace before 3ps. All data are normalized to make N(t)=1 at time t=0.

**Figure A1.17:** Temperature dependent recombination kinetics of mutant L29W MbO$_2$ in 0.1M borate buffer (pH=8.42) after photolysis. The protein sample is diluted directly to an O$_2$ bound stock solution. Absorption spectrum before and after the temperature dependence experiments are compared in Figure 4.3. Data between 3ps and 400ps are fitted with single exponential decay. Back-extrapolation is used to normalize the kinetics to make N(t)=1 at time zero.

**Figure A1.18:** Relationship between solvent viscosity and O$_2$ recombination kinetics to horse heart myoglobin. All the protein samples are made with method 2. Solvent viscosity values are those for water/glycerol mixtures.
Figure A1.15 Temperature dependent recombination kinetics of horse heart MbO2 prepared with Method 2.
Temperature Dependent Kinetics of V68WMbO2 inside 80% borate buffer (pH=8.42)/20% glycerol Prepared by method 2

Figure A1.16 Temperature dependent recombination kinetics of mutant V68W MbO2 inside glycerol mixture.
Figure A1.17 Temperature dependent recombination kinetics of mutant L29W MbO2 0.1M borate buffer after photolysis.
Figure A1.18 Relationship between solvent viscosity and O2 recombination kinetics to horse heart myoglobin.
Table A1.3  Viscosity of Aqueous Glycerol Solutions in Centipoises/mPa s

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http://www.dow.com/glycerine/resources/table18.htm
Appendix 2

UV-Vis Absorption Spectral Studies of Ferrous Fe-protoporphyrin IX in Different Monomeric Complexes
Figure A2.1

Fe\textsuperscript{II}PPIX-CO (flushing CO gas)
Figure A2.2

O.D.

Wavelength (nm)

400nm 419nm 434nm

Met Fe$^{II}$PPIX
Met 2Melm-Fe$^{II}$PPIX (adding 2Melm)
Deoxy 2Melm-Fe$^{II}$PPIX (adding Na$_2$S$_2$O$_4$)
2Melm-Fe$^{II}$PPIX-CO (flushing CO gas)

2Melm-Fe$^{II}$PPIX-CO in Kpi buffer with 1% CTAB pH=7

Figure A2.2
Figure A2.3

- Met Fe$^{III}$PPIX
- pyridine+Met Fe$^{III}$PPIX (adding pyridine)
- bis (py)$_2$Fe$^{II}$PPIX (adding Na$_2$S$_2$O$_4$)
- pyridine-Fe$^{II}$PPIX-CO (flushing CO gas)

pyridine-Fe$^{II}$PPIX-CO in Kpi buffer with 1%CTAB pH=7
Figure A2.4

CN-Fe\textsuperscript{II}PPIX

Met Fe\textsuperscript{III}PPIX

Met CN-Fe\textsuperscript{II}PPIX (adding CN)

CN-Fe\textsuperscript{II}PPIX (adding Na\textsubscript{2}S\textsubscript{2}O\textsubscript{4})

CN-Fe\textsuperscript{II}PPIX-CO (flushing CO gas)

CN-Fe\textsuperscript{II}PPIX-CO in Kpi buffer with 1% CTAB pH=7
Figure A2.5
Fe^{II}PPIX-NO
with CN\(^{-}\) in the solution
Kpi buffer with 1\% CTAB
pH=7

Figure A2.6
Met Fe$^{III}$PPIX
Met phenol-Fe$^{III}$PPIX (adding phenol)
Deoxy phenol-Fe$^{II}$PPIX (adding Na$_2$S$_2$O$_4$)
phenol-Fe$^{II}$PPIX-CO (flushing CO gas)

Figure A2.7
Figure A2.8

- Met Fe$^\text{III}$PPIX
- Deoxy Fe$^\text{II}$PPIX (adding Na$_2$S$_2$O$_4$)
- Fe$^\text{II}$PPIX-CO (flushing CO gas)

Fe$^\text{II}$PPIX-CO in 80% glycerol
pH=12
Inside 80% glycerol:
- deoxy Fe$^{II}$PPIX
- CO-Fe$^{II}$PPIX

Inside 1% CTAB:
- deoxy Fe$^{II}$PPIX
- CO-Fe$^{II}$PPIX

Figure A2.9
Figure A2.10 Agree with Figure 1 of Gomez. et al. (JACS, 127, 50, 2005 17636). The 441nm peak in Fe$^{II}$PPIX in CTAB (Figure A1.1) is assigned to the OH$^-$ bound species.
Figure A2.11 See Figure 1C in Gomez et al. where 435nm species is seen in 50% ethanol and assigned to (OH)$_2$ bound species. The peak at 431nm does not appear in Figure A1.1 and the (OH)$_2$ species is not assigned to any peak in Figure A1.1.
Figure A2.12 Bis (OH-)\textsubscript{2} species forms slowly (20 hours) in 3M NaOH and 1% CTAB.
Figure A2.13 Two samples: one sits for 5 hours and one is photolized using a 403nm pump (2.0mW) and probed at 435nm (0.4mW). Both show evidence of bis (OH-)$_2$ species formation (Gomez. et al. JACS. 127, 50, 2005, 17634-17643). Longer waiting does not change ratio of peaks. The ligands (CO, OH-) and (OH-, OH-) are in equilibrium with CTAB so that CO is not the primary ligand after waiting long time.
**Figure A2.14** Gomez (Gomez. et al. JACS. 127, 50, 2005, 17634-17643) assigns 428 peak to H2O ligand. Test with pH, but [H2O]≈55M, so not much of a change.
Figure A2.15 Agree with Gomez (Gomez. et al. JACS. 127, 50, 2005, 17634-17643) Figure 1C (a) where 419nm peak is seen in 50% ethanol. I surmise that this must be the (H₂O)₂ complex and the peak in Figure A1.15 correlates with the 416 peak in Figure A1.1.
Figure A2.16 Like Figure A1.1 except species at 441nm (OH- bound, see Figure A1.10) is diminished. (Given high pH~10, would expect more of 441nm species. This is contrary to expectation. A consistent explanation might be that, at high concentration of OH-, another OH- binds to 441nm species and the absorption peaks moves to 431nm, which superimposes to 428nm peak)
**Figure A2.17** Some 441 nm species observed at pH 5.5.
pH dependence of Fe$^{II}$PPIX in 80% methanol/20% water

Figure A2.18
Figure A2.19 Uncertain how Sodium Dithionite reacts at pH2-3. However, 392nm Species is also observed in FeII-PPIX in CTAB (Figure A1.1). I speculate on the possibility that the species formed here may be related to the 392nm species seen in Figure A1.1. I think this is a 5-coordinate Fe$^{2+}$ form.
Figure A2.20 Compare with Figure A1.13 for 1% CTAB and 3M NaOH, which suggests OH- as ligand trans to CO. It is unclear why CO binding would select OH- vs H2O ligand. Compare with Figure A1.11, A1.8 and A1.9 where H2O is presumably bound trans to CO and absorption is at 412nm (alpha/beta bands are also different).
In 80% Methanol/Water Mixture
pH=7

Ferric Fe(III)PPIX
Ferrous Fe(II)PPIX
NO-Fe(II)PPIX

Figure A2.21
Appendix 3

How does CO rebind to Fe$^{II}$-PPIX?
PPIXCO in Magnetic Field

- 10 Tesla
- 0 Tesla

Kpi buffer with 1% CTAB, pH=7

Pump at 403nm, probe at 435nm

Figure A3.1
Figure A3.2
Ferrous Fe-PPIX in 3M NaOH (1% CTAB) pH>14

Data: DEOXYFEPPIX3_W
Model: ExpDec1

\[ \Delta A = \exp \left(-\frac{t}{t_1}\right) \]

\[ y_0 = 0.03385 \pm 0.00225, \quad A_1 = 1.42349 \pm 0.01406, \quad t_1 = 4.81252 \pm 0.07918 \]

Figure A3.3
Figure A3.4

PPIX-CO in 80% Methanol/water Mixture

Pump 403nm
Probe 435nm

2-exponential fitting

1-exponential fitting

Data: PPIXCO435NM_W
Model: ExpDec2

Chi^2 = 0.00002
R^2 = 0.99654

y0 0.6978 ±0.00122
A1 0.46331 ±0.01
A2 0.04256 ±0.00233
t1 4.18072 ±0.12638
t2 250.79929 ±37.23482

Data: PPIXCO435NM_W
Model: ExpDec1

Chi^2 = 0.0002
R^2 = 0.97066

y0 0.71182 ±0.00223
A1 0.44198 ±0.02084
t1 5.38645 ±0.34437
Figure A3.5

Pump 403nm
Probe 428nm

PPIX-CO in 80% Methanol/water Mixture

ΔA

Time (ps)

0.0 0.2 0.4 0.6 0.8 1.0

1 10 100 1000 10000
Figure A3.6

- Pump 403nm
- Probe 428nm

2MeIm-PPIX-CO in 80% Methanol/water Mixture

pH should be around 7
Figure A3.7
Figure A3.8

2MeIm-PPIX-CO in glycerol-CTAB-buffer system
1% CTAB

Pump at 403nm
Probe at 435nm

ΔA (arbitrary unit)

Time (ps)
Figure A3.9
Figure A3.10

2MeIm-PPIX-CO in glycerol-water system

Pump at 403nm

Probe at 435nm

ΔA (arbitrary unit)

Time (ps)
Appendix 4

How does NO rebind to $\text{Fe}^{\text{II}}$-PPIX?
Figure A4.1
Figure A4.2 The whole kinetics of NO rebinding to Fe\textsuperscript{II}PPIX, with and without 0.1M 2-Melm inside the sample solutions. In both cases, 85% photolyzed NO rebinds back geminately with a time constant around 10ps. Without 2Melm inside the solution, the other 15% photolyzed NO will bind back bimolecularly later around ~100µs; With 2Melm in the solution, $\Delta A$ will first increase at a time constant around 1µs then decay at time constant around 100µs. In one of my speculations, this difference results from that the 2Melm rebinds bi-molecularly to photolyzed Fe\textsuperscript{II}-PPIX faster than NO molecular. In this scheme, NO binds from the other side of the 2Melm-bound Fe\textsuperscript{II}-PPIX, and then, 2Melm leaves due to tran effect.
Figure A4.3
PPIX-NO in different media

Temperature: 296K
pump at 403nm, probe at 435nm

Figure A4.4
Temperature Dependence of PPIX-NO

Kpi buffer with 1% CTAB
pH=7
pump at 403nm
probe at 435nm

Figure A4.5
Figure A4.6

Temperature Dependence of PPIX-NO

KPi buffer with 1% CTAB
pH=7

Time (ps)

$\Delta A$
Figure A4.7

PPIX-NO
20%Kpi buffer with 1% CTAB in 80% Glycerol
pH=7
Figure A4.8

- Pump at 403nm
- Probe at 435nm

PPIX-NO in 80% glycerol

ΔA

Time (ps)
Figure A4.9

PPIX-NO in glycerol at Room Temperature
Figure A4.10
Figure A4.11

PPIX-NO
80% methanol/water mixture
pump:403nm; probe:435nm

ΔA

Time (ps)

318K

313K

275K
Figure A4.12

Fe^{II}PPIX-NO $\xrightleftharpoons{h\nu}^{<10\text{ps}}$ Fe^{II}PPIX : NO

$\xrightleftharpoons{\sim 100\text{ps}}^{<10\text{ps}}$ Fe^{II}PPIX :: NO

$\xrightleftharpoons{\sim 100\mu\text{s}}$ Fe^{II}PPIX + NO