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Time-resolved thermodynamics studies of heme signaling proteins and model systems

Audrey Mokdad
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Time-Resolved Thermodynamic Studies of Heme Signaling Proteins and Model Systems

by

Audrey Mokdad

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
Department of Chemistry
College of Arts of Sciences
University of South Florida

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Dedication

For my parents, brother and sister
Acknowledgements

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Finally, I would like to dedicate this dissertation to my parents for their support, for always encouraging me to reach high goals, to surpass myself in my studies and to seek to be the best that I could be. Without their love, encouragement, patience and listening ears, I would never have survived these years. I also dedicate this dissertation to my brother and my sister whom I have neglected over the past few years in order to achieve this goal.

Last but not least, my words can not effectively express my sincere appreciation to Jason for his love, support, patience and understanding during this difficult time that is the doctorate years.
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Time-Resolved Thermodynamics Studies of Heme Signaling Proteins

and Model Systems

Audrey Mokdad

ABSTRACT

Heme-based gas sensor proteins have the ability to sense diatomic molecules such as O₂ (FixL, EcDos or HemAT), CO (CooA, a CO-sensing protein of *Rhodospirillum rubrum*) and NO (guanylate cyclase) molecules and subsequently regulate numerous important biological processes in prokaryotic and eukaryotic organisms. The sensing function of these proteins is initiated by the binding of an effector (i.e., O₂, CO, etc…) to the heme iron which then leads to a cascade of conformational events which gives rise to changes in kinase activity, DNA-binding activity, etc…

In order to better understand the mechanism heme-based signaling, time resolved photothermal methods as well as transient optical techniques were utilized to obtain thermodynamic profiles for ligand binding/release in heme based signaling proteins including HemAT from *Bacillus subtilis* (aerotactic transducer), FixL from *Sinorhizobium meliloti* (regulation of the nitrogen fixation) and CooA from *Rhodospirillum rubrum* (transcriptional activator). In addition, a number of model systems were examined to understand the underlying thermodynamic processes involved in heme ligation. The variation of volume and enthalpy changes associated with spin state change of the iron from high-spin to low-spin where examined using the spin crossover Fe(III)(salten)(mepepy) complex. In addition, the experimental determination of the volume change due to electrostriction events were using Ru(II)(L)₃ and the Debye-Hückel equation.
Finally, different model heme proteins were studied to understand how a signal is conformationally transmitted within a heme protein matrix. Sandbar shark hemoglobin was examined as an example of a non-signaling an allosteric protein. Two different peroxidases (horseradish and soybean) which have a direct channel between the heme pocket and the solvent involving no barrier energetic for the photodissociated ligand leaving the heme pocket were examined as example of non-signaling, non-allosteric proteins. The results show that each protein has a unique thermodynamic profile to conformationally transmit signals subsequent to photodissociation of CO, even within the same class of protein (i.e. PAS domains, globins, etc...).
1.1. Introduction

Cell signaling is a complex process involving a diverse array of proteins complexes as well as various messenger molecules [1-6]. The signaling process is critical for intracellular trafficking of metabolic pathways as well as extracellular environmental monitoring. In large organisms such as plants and mammals both intra and extracellular signaling/sensing involves complex signaling cascades [7-9]. An example of the complexity in mammalian signaling is the insulin pathways. Insulin is a hormone that allows the body to store glucose as glycogen from the blood to the liver or muscle. The insulin pathway which starts from the transmission of the signal (glucose) to the release of insulin can be summarized as follows. Glucose enters the cell through a glucose transporter and enters into glycolysis and subsequent respiratory cycle where several ATP molecules are produced. Depending upon the concentration of ATP, the potassium channels (K+) will be closed which will, in turn, control the activity of calcium channels (Ca²⁺) which will remain open to allow a flow of calcium into the cell. This increase in intracellular Ca²⁺ inside the cell will cause the release of insulin [10-13]. In contrast, the signaling pathway associated with *E. coli* engages only a few proteins. The signal is activated by ligand binding to a methyl-accepting chemotaxis protein receptor and transmitted to a kinase domain via a linker peptide fragment. The activation of the kinase results in phosphorylation of a histidine kinase (CheA), which will stimulate the phosphorylation of a methylesterase kinase (CheB). In this way, the signal is transmitted to the flagella which will allow the bacteria to translationally respond to signaling event (Figure 1.1) [14, 15].
The signaling pathways in large organisms such as plants and mammals eukaryotes is more complex than small organisms such as bacteria which makes the study of bacterial systems very attractive. In mammalian cells, the signaling pathway for the transmission of a signal involves many proteins. On the other hand, the transmission of a signal for small organisms like bacteria involves only a few proteins. A good example to compare the complexity of the transmission of the signal between the cell signaling in eukaryotes and prokaryotes can be demonstrated with the insulin pathway of a mammalian and the chemotaxis pathway of *Escherichia coli* (*E. coli*).

In order to better understand the molecular basis for signal transmission, our studies focused on the investigation of conformational and thermodynamics associated with signaling in heme-based bacterial sensors. As bacterial communication shares common features with eukaryote cells, (intra/extracellular communication with others cells or organelles and/or adaptation to environmental changes), the study of bacterial proteins will unravel the salient features of the molecular mechanisms of signaling, but with a less complicated system. In bacteria, different types of signals (amino acids, pH temperatures, gas molecules, etc…) [16] can initiate signal in the sensing domain, resulting in phosphorylation and a transmission of the signal to the flagella (Figure 1.1).
The specific class of heme proteins examined in this study responds to diatomic gases including O$_2$, CO and NO. This allows for the exploitation of the well known spectroscopic signatures, water solubility and photolability of bound ligands (e.g. CO). Well-known examples of heme sensors are the Per-Arnt-Sim (PAS) domain, the globin-coupled sensor (GCS), the CooA heme binding domain, and the heme-NO-binding proteins (H-NOX) [17]. Thus, the study of soluble heme oxygen sensors provide an opportunity to study, in detail, the mechanism of small molecule binding to a sensing domain and to understand the transmission of the signal to the effector domain. This can be accomplished by photodissociating bound ligand and monitoring the response using a variety of spectroscopic and thermodynamic probes. It was anticipated that each step in
the transmission of the signal from the sensor domain to the effector domain, exhibits a conformational change with an associated variation in volume ($\Delta V$) and enthalpy ($\Delta H$), which can be studied using time-resolved photothermal methods (Figure 1.2).

Time resolved photothermal methods have proven in the past to be powerful techniques for determining the magnitudes and time scales of molar volume and enthalpy changes associated with physiological events in proteins including ligand binding, folding/unfolding and electron transfer [18-25]. In our laboratory, these methods have been used to investigate nanosecond/millisecond thermodynamics associated with ligand photolysis from heme model systems, ligand binding to myoglobin and heme copper oxidases, and to the folding/unfolding of apo-myoglobin and several photochemically ‘caged’ peptides [26-42]. In this work, time resolved photothermal methods as well as transient optical techniques were utilized to obtain thermodynamic profiles for ligand

**Figure 1.2:** Thermodynamic profiles (variation of volume and enthalpy) of the signal transmitted along the sensor, linker and kinase domain.
binding/release in heme based signaling proteins including HemAT from *Bacillus subtilis*, FixL from *Bradyrhizobium japonicum* and *Sinorhizobium meliloti*, and CooA from *Rhodospirillum rubrum*. A number of model systems were also examined to understand the underlying thermodynamic processes associated with in heme ligation in these proteins.
1.2. References


Chapter II - Methods

2.1 Photothermal Spectroscopy

Photothermal spectroscopy methods can probe both optical absorption and thermal characteristics of a photoactive sample [1]. The principle of photothermal spectroscopy is simple. The molecule studied in solution is excited via absorbing of a photon (hν) resulting in a transition from the ground state to an excited state following Fermi’s Golden Rule [21,22]. The molecule then dissipates the excess energy via nonradiative decay, emission of a photon (fluorescence) or intersystem crossing to an excited triplet state. The molecule in the triplet state can also relax to the ground state by nonradiative decay or emission of a photon (phosphorescence). The excitation and delay processes of a molecule by a photon are summarized by the Jablonski-Perin diagram in Scheme 2.1 [20]. The molecule can also relax to the ground state by photochemical events which include formation/cleavage of bonds which affect the molecular volume (van der Waals), the formation or elimination of a charges (electrostriction), change in the metal spin states and/or the release or binding of ligand. These processes also give rise to changes in volume and enthalpy, which can be analyzed by photothermal methods.

Photothermal methods probe temperature, pressure and density changes associated with the optical absorption changes of the sample [1]. Photothermal methods can be divided in two categories depending on the parameter studying: photoacoustic methods which are based on the detection of pressure waves and grating and lensing methods which are based on the detection of changes in the refractive index [1]. The following projects presented used photoacoustic calorimetry methods.
Scheme 2.1: Jablonski-Perin diagram [20].
2.1.1. **General background**

As previously described by Larsen and Miksovska [17], when a molecule dissipates excess energy to ground state subsequent to photoexcitation, the surrounding solvent will receive thermal energy due to all non-radiative processes [13,14]. The classical heat equation describes the response of the solvent to such a heat impulse is:

\[
\alpha \left( \frac{\partial T}{\partial x} \right)^2 + \left( \frac{\alpha}{k} \right) g(x, t) = \frac{\partial T}{\partial t}, \quad 0 < x < L, \quad t > 0 \quad \text{Equation 1}
\]

T(x, t=0) = F(x) correspond to the initial condition

\[
\begin{aligned}
T(x=0; t) &= T_1 \\
T(x=L, t) &= T_2
\end{aligned}
\]

where \( \alpha \) is the thermal diffusivity, \( k \) is the thermal conductivity and \( g(x, t) \) is the heat deposited per unit volume [14].

The solution of equation 1 can be written as following:

\[
T(x, t) = \int F(x') g(x', t | x', \tau = 0) dx' + \left( \frac{\alpha}{k} \right) \int g(x', \tau) G(x, t | x', \tau) dx' d\tau
\]

\[
+ \alpha \int (T_1) \frac{\partial G}{\partial x'} \bigg|_{x'=0} d\tau - \alpha \int (T_2) \frac{\partial G}{\partial x'} \bigg|_{x'=L} d\tau \quad \text{Equation 2}
\]

where the integration limit over \( x' \) are from \( x'=0 \) to \( L \) and over \( \tau \) are from \( \tau=0 \) to \( t \), \( G(x, t | x', \tau) \).

The temperature change per excitation pulse as a function of the distance from the center of the Gaussian distribution as a function of time:

\[
T(r, t) = \left( \frac{2\alpha E_a}{\pi \rho C_{ph}} \right) \left\{ \frac{\exp \left( \frac{-2r^2}{8Dt + w_0^2} \right)}{8Dt + w_0^2} \right\} \quad \text{Equation 3}
\]

For solvents such as water, the rapid volume change expansion is caused by the heating within the illuminated volume resulting in both an acoustic wave and a change in solution of the refractive index.
In the case of photoacoustic calorimetry, within the illuminated volume, the heat impulse causes a rapid change in the temperature of the solution which results in a physical volume change which generates both an acoustic wave and a change in the refractive index. Equation 2 describes the change in solvent pressure due to the change in volume:

\[ P = 2\pi f_a v_a \Delta x \rho \]  
**Equation 4**

where \( f_a \) is the frequency of the sound wave, \( v_a \) is the acoustic velocity, \( \Delta x \) is the volume displacement and \( \rho \) is the solvent density [15,16]. The change in volume of a cylinder with radius \( R \) and length \( L \) and due to an adiabatic, isobaric expansion can be expressed as following:

\[ \pi R^2 l - \pi (R + \Delta R)^2 l = \beta V \Delta T \]  
**Equation 5**

where \( \beta \) is the thermal expansion coefficient.

Assuming \( r=0 \), exponential part of the equation \( T(r,t) \) is negligible:

\[ \pi R^2 l - \pi (R + \Delta R)^2 l = \beta V \left( \frac{2a E a}{\pi \rho C_p h \omega} \right) \]  
**Equation 6**

Assuming \( \Delta R \ll R \), the change in volume is very small:

\[ \Delta R = \left( \frac{\beta}{C_p \rho} \right) \left( \frac{a E a R}{\pi h \omega} \right) \]  
**Equation 7**

Using \( \Delta x = B \Delta R \) where \( B \) is a proportional constant and equation 4:

\[ P = B 2f_a v_a \left( \frac{\beta}{C_p} \right) \left( \frac{a E a R}{h \omega} \right) \]  
**Equation 8**

For a fixed excitation system, \( f_a \) and \( R \) can be included in \( B \):

\[ P = B \left( \frac{\gamma a \beta}{C_p} \right) (a E_0) \]  
**Equation 9**

where \( E_0 \) is the total photon energy of the excitation pulse normalized to the energy.
An acoustic wave in solution is formed due to the pressure change which is described by the following equation:

\[ \nabla^2 \Psi(r,t) - \left( \frac{1}{v_s^2} \right) \frac{\partial^2 \Psi(r,t)}{\partial t^2} = -4\pi h(r,t) \quad \text{Equation 10} \]

where \( v_s \) is the speed of sound in the medium, \( \Psi(r,t) \) is the wave amplitude at the observation coordinates \( r \) and \( t \), and \( h(r,t) \) is a heat source function [15].

The solution of the equation 10 can be written as following:

\[ \Psi(r,t) = \left( \frac{1}{4\pi} \right) \int dt'' \int dr' g(r,r',t,t')h(r,t)dr' \quad \text{Equation 11} \]

where \( g(r,r',t,t') \) is a Green’s function solving the wave equation for the impulse heat function and \( h(r',t') = \delta(r')f(t') \) at \( r' = 0 \).

The acoustic wave amplitude observed at the transducer using equation 11 can then be described as:

\[ \Psi(r_0, t) = f(t - \frac{r_0}{v_s}) \quad \text{Equation 12} \]

where \( r_0/v_s \) is the propagation delay and \( 1/r_0 \) is the energy conservation associated with spherical emitters.

For a point source \( (r'=0) \) with a lifetime \( \tau \), the wave amplitude observed at the transducer can be rewritten equation 12:

\[ \Psi(r_0, t) = f_0 \left( \frac{t}{\tau} \right) \quad \text{Equation 13} \]

where \( f(t') \) is a Heaviside unit step function, \( f(t') = (h_0e^{-t'/\tau}) \theta(t') \) at the point source and \( \tau \) is the lifetime of the heat generator.

The response of the transducer which is modeled as an under-damped oscillator to the wave amplitude associated to an impulse response is written as:

\[ G(t,t'') = A \sin(\nu(t-t''))e^{-\nu(t''-\nu t_0)} \quad \text{Equation 14} \]
where \( A \) is the amplitude, \( \nu \) is the frequency of the oscillation and \( \tau_0 \) the relaxation time.

By convoluting the impulse response \( G(t, t'') \) with the acoustic amplitude \( \psi(r_0, t'') \), the following equation expresses the transducer response:

\[
V(t) = \int_{-\infty}^{t} G(t, t'') \psi(r_0, t'') \, dt'' \quad \text{Equation 15}
\]

The transducer response (piezoelectric crystal) to the wave amplitude which represents the relationship between the lifetime of the heat evolving process and the amplitude at the transducer can be expressed using equation 15 as:

\[
V(t) = \left( \frac{\hbar A}{4\pi \rho_0} \right) \left( \frac{\nu \tau}{1 + \nu \tau} \right) \times \left\{ e^{\nu \tau} - e^{-\nu \tau} \left[ \cos(\nu t) - \left( \frac{1}{\nu t} \right) \sin(\nu t) \right] \right\} \quad \text{Equation 16}
\]

where \( A \) is the amplitude, \( \nu \) is a characteristic oscillation frequency, \( \tau_0 \) is a relaxation time, \( \tau \) is the lifetime of the heat evolving process.

**Photoacoustic calorimetry method**

Photoacoustic calorimetry (PAC) has proven to be a powerful technique for determining the magnitude and time scale of conformational changes as well as reaction thermodynamics associated with photoinitiated processes [2]. The physical principle behind this method is that photoexcited molecules dissipate excess energy nonradiatively, resulting in thermal heating of the surrounding solvent. In the case of aqueous solutions this causes rapid volume expansion (\( \Delta V_{th} \)) resulting in an acoustic wave that can be detected with a sensitive piezoelectric crystal based microphone. In addition, volume changes in the system of interest resulting from conformational/solvation changes associated with a photoinitiated reaction (\( \Delta V_{con} \)) also contribute to the acoustic wave. The contributions from \( \Delta V_{th} \) and \( \Delta V_{con} \) to the total sample signal, \( S \) (acoustic amplitude) can be evaluated by examining the temperature dependence of the signal and using a calibration compound (\( S_{ref} \)). The theory and instrumentation associated with PAC have been described in detail by [1-5]. PAC has been previously employed to determine the thermodynamic profiles for conformational changes associated with the nanosecond
charge transfer of Ru(bpy)$_3$ [6], conformational changes associated with the photodissociation of CO from a wide range of heme proteins including $Bj$FixL, EcDos, horse heart myoglobin and sperm whale myoglobin [7-12].

PAC measurements are performed (as described previously [2]) by placing a quartz cuvette containing a sample in a temperature controlled sample holder (Quantum Northwest) housing a Panametrics V103 transducer. Contact between the cuvette and the detector was facilitated with a thin layer of vacuum grease. Photoexcitation was achieved with a 532 nm or 355 nm laser pulse (Continuum Minilite I frequency double/triplet Q-switched Nd:YAG laser, 6 ns pulse, <80 µJ). The acoustic signal was amplified with an ultrasonic preamp (Panametrics) and recorded using an NI 5102 Oscilloscope (15 MHz) controlled by VirtualBench software (National Instrument). The PAC data were analyzed using the two temperature or multiple temperature method detailed in the next section. (Scheme 2.2).

Scheme 2.2: Diagram of photoacoustic calorimetry instrumentation.
Multiple temperatures method:

The amplitude of the sample acoustic wave is expressed as:

\[ S_{\text{samp}} = K \cdot E_a \cdot \Phi \cdot (\frac{\beta}{C_p \cdot \rho} \cdot Q + \Delta V_{\text{con}}) \]  \hspace{1cm} \text{Equation 17}

where \( S \) is the acoustic signal, \( K \) is the instrument response parameter, \( E_a \) is the number of Einstein absorbed, \( \Phi \) is the quantum yield of the process, \( \beta \) is the thermal expansion coefficient of the solvent (K\(^{-1}\)), \( C_p \) is the solvent heat capacity (cal g\(^{-1}\)K\(^{-1}\)), \( \rho \) is the solvent density (g mL\(^{-1}\)), \( Q \) is the amount of heat returned to the solvent (kcal mol\(^{-1}\)) and the \( \Delta V_{\text{con}} \) is the nonthermal volume change and \( \Delta V_{\text{con}} \) represents conformational/electrostriction contributions to the solution volume change (mL mol\(^{-1}\)).

The amplitude of the reference acoustic wave is expressed as:

\[ S_{\text{ref}} = K \cdot E_a \cdot \Phi \cdot \left( \frac{\beta}{C_p \cdot \rho} \right) \cdot E_{hv} \]  \hspace{1cm} \text{Equation 18}

The reference converts the energy of the absorbed photon (\( E_{hv} \)) into heat with a quantum yield equal of unity (i.e., \( \Delta V_{\text{con}} = 0 \)). The volume and enthalpy are determined by taking a ratio of the amplitudes of sample to the reference (\( S_{\text{samp}}/S_{\text{ref}} \)) as a function of temperature (\( C_p, \rho \) and \( \beta \) are temperature dependents) as expressed below:

\[ \Phi \frac{S_{\text{samp}}}{S_{\text{ref}}} E_{hv} = Q + \left( \frac{C_p \cdot \rho}{\beta} \right) \Delta V_{\text{con}} \]  \hspace{1cm} \text{Equation 19}

A plot of \( \phi E_{hv} \) versus \( C_p \rho / \beta \) gives a line with a slope equal to \( \Delta V_{\text{con}} \) and an intercept equal to the released heat (\( Q \)).

Subtracting \( Q \) from \( E_{hv} \) and dividing by the quantum yield gives \( \Delta H \) for processes occurring faster than the time resolution of the instrument (<20 ns):

\[ \Delta H = \frac{(E_{hv} - Q)}{\Phi} \]  \hspace{1cm} \text{Equation 20}

The \( Q/\Phi \) values for subsequent kinetic processes represent -\( \Delta H \) for that step (i.e., heat released).

\[ \Delta H = - \frac{Q}{\Phi} \]  \hspace{1cm} \text{Equation 21}
Two temperature method:

A two temperature methods can also be used to analyze sample and reference acoustic waves. The two temperature methods take the advantage that at $\beta = 0 \ (\sim 4.1 ^\circ C)$, the wave amplitude of the reference, in aqueous solution, is equal to zero. Thus, $\Delta V_{\text{con}}$ of the sample can be extracted easily using the following equation:

$$\Phi \Delta V_{\text{con}} = E_{hv}(C_p \rho / \beta)_{10^\circ C} \cdot (A_{T=4.1^\circ C_{\text{samp}}} / A_{T=10^\circ C_{\text{ref}}}) \quad \text{Equation 22}$$

where $\Phi$ is the quantum yield, $\beta$ is the coefficient of thermal expansion of the solvent ($K^{-1}$), $C_p$ is the heat capacity (cal.g$^{-1}$ K$^{-1}$), $\rho$ is the density (g.mL$^{-1}$), $A_{T=4.1^\circ C_{\text{samp}}}$ is the amplitude of the sample at 4.1°C, $A_{T=10^\circ C_{\text{ref}}}$ is the amplitude of the reference at 10°C and $\Delta V_{\text{con}}$ represents conformational/electrostriction contributions to the solution volume change. The fraction of heat releases in the solvent is calculated using the following equation:

$$\alpha = (A_{T=10^\circ C_{\text{samp}}} - A_{T=4.1^\circ C_{\text{samp}}}) / A_{T=10^\circ C_{\text{ref}}} \quad \text{Equation 23}$$

where $\alpha$ is the fraction of heat, $A_{T=10^\circ C_{\text{samp}}}$ and $A_{T=4.1^\circ C_{\text{samp}}}$ is the amplitude of the sample at 4.1°C and 10°C, $A_{T=10^\circ C_{\text{ref}}}$ is the amplitude of the reference at 10°C.

The release of heat deposed in solution is expressed as follows:

$$Q = \alpha E_{hv} \quad \text{Equation 24}$$

where $Q$ is the heat released to the solvent.

Subtracting $Q$ from $E_{hv}$ and dividing by the quantum yield gives $\Delta H$:

$$\Phi \Delta H = E_{hv} - Q \quad \text{Equation 25}$$
Time resolution in photoacoustic calorimetry

After looking at the overlay for both sample and the reference acoustic waves, two different cases which demonstrated or not a frequency shift can be observed between the sample and the reference.

First case:

The first case is observed when no frequency shift is visible between the sample and the reference as shown on Figure 2.1. This indicates no kinetic event between ~50 ns and ~20 μs, so everything happen faster than ~50 ns or slower than ~20 μs. In this case, \( \phi \) is equal to the ratio between the amplitude of the sample and the amplitude of the reference.

![Figure 2.1: Overlap between the sample and the reference photoacoustic signals showing no frequency shift.](image)
Second case:

The second case is observed when a frequency shift is visible between the sample and the reference as shown on Figure 2.2 which indicates one or more kinetic events occurring between ~50 ns and ~20 μs. In this case, the data will be deconvoluted in order to extract the lifetime, the volume and enthalpy changes associated with each phase or event.

![Figure 2.2: Overlap between the sample and the reference photoacoustic signals showing a frequency shift.](image)

Deconvolution corresponds to the convolution between the reference acoustic wave (Tr(t)) and the exponential decay of the heat released to the solvent (H(t)) (Scheme 2.3). In order to deconvolute both reference and sample acoustic waves, noncommercial software pacw98v1 also called larsenware will be used to determine the amplitude and the lifetime of each phase. This software uses the fast iterative convolution equation:

\[ D_{j+1} = K \exp(-t_{j+1} / \tau)I_{j+1} \]  

Equation 26
where $K$ is the amplitude, $t$ is the time and $\tau$ the lifetime. After loading the sample and reference wave and normalizing them to the absorbance, the program will create a new array using the initial guess. The program will then convolute the new arrays with the reference wave, add them together and start simplex in order to find the smaller $\text{Chi}^2$.

**Scheme 2.3:** Deconvolution of photoacoustic calorimetry signals.
2.2. **Transient Absorption Spectroscopy**

Transient absorption (TA) spectroscopy follows the kinetics of fast events in real time after excitation of the system under investigation. The absorption spectrum is characteristic of the system. The changes induced in the sample after excitation, involve changes in the absorption spectrum that are recorded on the monitor. In transient absorption spectroscopy, a pump pulse will perturb the system with a redistribution of the energy of the molecule. Then, as the probe pulse goes through the sample, the change in absorption of the sample, at a specific wavelength is recorded as a function of time ([Figure 2.3](#)). Transient absorption will give us the opportunity to extract the different kinetics of the heme after excitation of the system. In addition, the results will confirm that the lifetime of the different phases extracted using photoacoustic calorimetry and matching the TA results are due to the dynamics of the heme.

TA experiments were performed by monitoring the change in intensity of light from a Xe arc lamp (Oriel) emerging from the sample followed by passage through a 1/4 m single monochromator equipped with an Oriel R928 photo-multiplier tube. The signal was amplified using either a home-built pre-amplifier (1 MHz bandwidth) or a melles griot which is part of the wide-bandwidth amplifier family used for operating photodiodes in both the photovoltaic and photoconductive modes, followed by a Stanford Instruments SR445A 350 MHz post amplifier. The signal was digitized using a Tektronix TDS7404 4 GHz digital oscilloscope. The sample was excited with the second harmonic of a Continuum Leopard I Q-switched mode-locked Nd:YAG laser (<20 ps, 20 mJ/pulse, 20 Hz) ([Scheme 2.4](#)).
Scheme 2.4: Diagram of transient absorption instrumentation.
Figure 2.3: Transient absorption spectra after excitation at 440 nm.
By using transient absorption spectroscopy will also be able to extract the entropy and enthalpy of activation of a reaction. The enthalpy of activation for the ligand bound to the heme will give us the opportunity to extract the enthalpy of activation of the dissociation of the ligand bound to the heme. Then, the complete thermodynamic profiles of the reaction can be drawn using the following equation:

\[ \Delta H = \Delta H^{\neq}_{\text{dissociation}} - \Delta H^{\neq}_{\text{binding}} \]  

Equation 27

The following Eyring’s equation will be used to calculate the entropy (\(\Delta S^{\neq}\)) and enthalpy (\(\Delta H^{\neq}\)) of activation for a ligand (CO or O₂) binding to a protein:

\[ \ln\left(\frac{k_{\text{obs}}}{k_b h/T}\right) = -\frac{\Delta H^{\neq}}{RT} + \frac{\Delta S^{\neq}}{R} \]  

Equation 28

where \(k_b\) is the Boltzmann’s constant, \(h\) is the Planck’s constant, \(k_{\text{obs}}\) is the observed rate constant and \(T\) is the absolute temperature \([18,19]\).
2.3. References


Chapter III – Model Systems

Different model systems were selected in order to understand the underlying thermodynamic processes in heme ligation. Using photothermal methods, the volume and enthalpy changes of a system (molecule or protein) after photoexcitation can be extracted using equation 9 in the previous section. The variations of the observed volume or enthalpy observed ($\Delta V_{\text{obs}}$ and $\Delta H_{\text{obs}}$) originate from two general events. 1) variations in volume or enthalpy corresponding to the photolysis of a ligand from heme proteins ($\Delta V_{\text{photolysis}}$ and $\Delta H_{\text{photolysis}}$) and 2) variations of volume and enthalpy corresponding to the conformational changes associated with the protein matrix ($\Delta V_{\text{conf}}$ and $\Delta H_{\text{conf}}$). As follows:

$$\Delta V_{\text{obs}} = \Delta V_{\text{photolysis}} + \Delta V_{\text{conf}} \quad \text{Equation 29}$$

$$\Delta H_{\text{obs}} = \Delta H_{\text{photolysis}} + \Delta H_{\text{conf}} \quad \text{Equation 30}$$

The variations in volume and enthalpy due to the photolysis event corresponding to a ligand photorelease from a heme protein has contributions from: the Fe-L bond cleavage, spin state change of the iron from low-spin to high-spin and the solvation of the ligand after its release to the surrounding solvent. These processes are summarized in Scheme 3.1
Scheme 3.1: Simple model for CO photolysis from heme proteins.

Each of the three contributions (bond cleavage, spin state change and ligand solvation) is associated with a change in volume and enthalpy as summarized in Scheme 3.2.

Scheme 3.2: Variation of volume and enthalpy for the bond cleavage CO-Fe, Iron spin state change and CO salvation [5-7].
Thus, the variation in volume and enthalpy associated with the corresponding conformational changes within the protein matrix can be extracted using:

\[
\Delta V_{\text{conf}} = \Delta V_{\text{obs}} - \Delta V_{\text{photolysis}} \quad \text{Equation 31}
\]

\[
\Delta H_{\text{conf}} = \Delta H_{\text{obs}} - \Delta H_{\text{photolysis}} \quad \text{Equation 32}
\]

Previous high pressure results of various metallocomplexes have indicated a change of volume of ~10 mL mol\(^{-1}\) for low-spin to high-spin transition. The variation was associated to the expansion of the core of the metal complex due to the expansion of the \(dz^2\) orbital [1]. Previous studies estimated that the enthalpy change of an iron from low-spin to high-spin from iron complexes was negligible at low temperature.

The first model system project was the estimation of the variation of volume and enthalpy of a photo-induced spin state change at room temperature using an iron complex with a push-pull ligand (Scheme 3.3). The iron complex chosen was the Fe(III) salten mepepy complex.

**Scheme 3.3:** Spin crossover for the iron from low-spin to high-spin.
The variations in volume and enthalpy of protein conformational changes can correspond to different contributions such as van der Waals volume, electrostriction or the variation of volume and enthalpy due to the variation of the protein salvation [2]. The following equations summarize the different contributions that can be observed for the volume and enthalpy, associated to the protein conformational changes:

\[
\Delta V_{\text{conf}} = \Delta V_{\text{vdw}} + \Delta V_{\text{electrostriction}} + \Delta V_{\text{salvation}} \quad \text{Equation 33}
\]

\[
\Delta H_{\text{conf}} = \Delta H_{\text{vdw}} + \Delta H_{\text{electrostriction}} + \Delta H_{\text{salvation}} \quad \text{Equation 34}
\]

The second model system investigated in this study involves the variation of volume associated with van der Waals volume changes versus electrostriction of two different ruthenium complexes using Debye-Hückel theory [3,4].
3.1. References


3.2. Photophysical Studies of the Trans to Cis Isomerization of the Push-Pull Molecule: 1-(Pyridin-4-yl)-2-(N-methylpyrrol-2-yl)ethene (mepepy)

3.2.1. Introduction

The study of the class of push-pull molecules (Donor (D) – π - Acceptor (A)) has improved in the past decades. Their interest comes from their nonlinear optical (NLO) properties used in high-speed optical modulators, optical storage media, and fast/ultrafast optical switches, etc. which can be exhibited by a different dipole moment in the ground-state and excited-state of the push-pull molecule [1-5].

A push-pull molecule is a molecule with two different sides linked by a conjugated double bond, one side has an electron-withdrawing substituent and the other one has an electron-donating substituent. Typically, functional groups like amino, dialkylamino, ether, or oxide (O\(^2\)) form the electron-donating substituent while nitro, carbonyl, and cyano groups are employed as the corresponding acceptor group [6, 7]. This enhances the polarizability of the double bond region allowing for additional polarization to be induced in the presence of an electric field. A good example is chromophore molecules. Push-pull molecules are very polarized and the configuration with a donor and receptor side facilitates the molecular switch between the cis and trans molecule, by decreasing the rotational barrier. The configuration push-pull also stabilizes the double bond by given a less double bond character [1].
In addition to their nonlinear optical properties, push-pull molecules which are liganded to different transition metal complexes can change photolytically the spin state of the complex [8-13]. The push-pull molecule must have some properties such as functional group which can coordinate to the metal complex and imply a shift in the basicity after photoexitation. The complex will have two different configurations, a low-spin electron configuration which will be more basic than the high-spin configuration after photoconversion of the ligand. Actually, these complexes are now synthesized and their optical and magnetic are known. For instance, complexes of the Fe\textsuperscript{II}\((L_4)(X_2)\) type in which \(L\) is the photoisomerizable push-pull ligand and of the Fe\textsuperscript{III}\((L_4)(L')(X)\) type in which \(L'\) is the push-pull ligand. The first studies were focused on 4-sterylpyridine (Stpy; 1-phenyl-2-(4-pyridyl)ethane) as well as several phenyl derivatives of this ligand in order to photoinduce the spin crossover [10-12]. A good example of the light-induced spin-state changes, is the Fe\textsuperscript{II}(trans-Stpy)\(_4\)(NCS)\(_2\) complex which can induce a high-spin to low-spin transition thermally around 190K by a trans to cis isomerization of the Stpy [10].

An idea was to synthesize these complexes which exhibit ligand-driven, light-induced spin-state changes at higher temperatures. For instance, [Fe\textsuperscript{III}(salten)(mepepy)]BPh\(_4\) (salten = 4-azaheptamethylene-1,7-bis(salicylideneiminate); mepepy= 1-(pyridin-4-yl)-2-(N-methylpyrrol-2-y1)ethene; BPh\(_4\) = tetraphenyl borate) has been shown to exhibit a high-spin to low-spin transition at room temperature under visible irradiation [11-13]. The difference with Fe\textsuperscript{II}\((L_4)(X_2)\) type complexes discussed above is that they have four photoisomerizable ligands, the Fe\textsuperscript{III}\((L_4)(L')(X)\) type complexes (e.g., Fe\textsuperscript{III}(salten)(mepepy)) have only a single isomerizable ligand which is used to alter the ligand field strength. In the case of the mepepy complex, the mepepy will be linked to an Fe(II) complex which has an active spin crossover center. The spin-state transition is triggered by a light-induced a trans to cis isomerization of the ligand. A shift in electron density from the N-methylpyrrol moiety to the pyridine unit will be observed (see Figure 3.1 for a structural diagram of mepepy).
An example of a push-pull molecule is the mepepy complex (Figures 3.1). The cis/trans configuration of the mepepy ligand has two sides conjugated by a double bond: a pyridine forms the electron-withdrawing substituent and a N-methylpyrrole forms the electron-donating substituent. The mepepy ligand was the first molecule observed with a spin change from a high-spin to a low-spin after excitation associated with a change in energy [11]. The electron properties associated with the energetics of the cis/trans photoisomerization is still not known very well even if the cis/trans photoisomerization has been demonstrated to alter the ligand field of the chromophore. The trans conformation of mepepy shows an optical spectrum with absorption maxima at ~353 nm ($\varepsilon = 22800 \text{ M}^{-1} \text{ cm}^{-1}$) and 241 nm ($\varepsilon = 7800 \text{ M}^{-1} \text{ cm}^{-1}$) in acetonitrile. These absorption spectra have been displayed to be $\pi$ to $\pi^*$ transitions [11, 12]. After photoisomerization, a slight hypsochromic shift is observed in the absorption band at 353 nm associated with a nearly 50% decrease in the extinction. While only modest changes in extinction of the
241 nm band are showed. Time-resolved photothermal and computational methods were used in order to study the energetics in addition to the potential energy surfaces associated with the cis/trans photoisomerization of the mepepy complex after excitation.

3.2.2. Materials and Methods

3.2.2.1. Synthesis of mepepy

The procedure described in reference 1 was followed in order to prepare the trans-mepepy ligand. A solution under an argon atmosphere was prepared by adding 905 μL of 4-picoline in 4 mL of anhydrous dimethylformamide (DMF) to a suspension of 370 mg of sodium hydride (60% dispersion in mineral oil) in 10 mL of anhydrous DMF. After stirring the solution at 60°C for 2 h, a solution of 1.01 g of N-methylpyrrole-2-carboxyaldehyde in 6 mL of anhydrous DMF was added to the red anionic solution and then stirred at 60°C overnight. A yellow solid was extracted after been poured onto ice and filtered. The filtrate was then concentrated and purified using a silica gel column with a mixture of solvent hexane:ethyl alcohol at different ratio 2:1, 1:1 and 1:2. The purity of the compound was confirmed by NMR spectroscopy.

The quantum yield, Φ, associated with the isomerization of the mepepy from the trans to cis form was determined by irradiating, for a total of 35 mins, a cuvette at the wavelength used to perform the PAC measurement, which is 355 nm. The cuvette contained 0.19 mM mepepy in water with 1% dimethyl sulfoxide (DMSO). The absorption spectra were recorded every 5 mins. A control was also used by performing the same experiment with 0.8 mM azobenzene. The following equation was used in order to determine the quantum yield Φ of the mepepy by comparing the value of the quantum yield with the value already known of the azobenzene:

\[
\frac{\Delta A_{0-35}^{\text{mep}}}{\Delta A_{0-35}^{\text{azo}}} = \left\{ \frac{\Phi^{\text{mep}} \Delta \varepsilon^{\text{mep}} C_0^{\text{mep}}}{\Phi^{\text{azo}} \Delta \varepsilon^{\text{azo}} C_0^{\text{azo}}} \right\} \quad \text{Equation 36}
\]
where $\Delta A_{0-35}^{\text{mep}}$ and $\Delta A_{0-35}^{\text{azo}}$ are the changes in absorbance, respectively, for mepepy and azobenzene at 355 nm, $\Phi^{\text{mep}}$ is the quantum yield for the trans to cis isomerization of the mepepy, $\Phi^{\text{azo}}$ is the quantum yield for the trans to cis isomerization of the azobenzene (0.26), $\Delta \varepsilon^{\text{mep}}$ (2.2 M$^{-1}$ cm$^{-1}$) and $\Delta \varepsilon^{\text{azo}}$ (0.4 M$^{-1}$ cm$^{-1}$) are the differences in extinction coefficients, respectively, between the trans and cis forms of mepepy and azobenzene at 355 nm, and $C_0^{\text{mep}}$ and $C_0^{\text{azo}}$ are the initial concentrations. Using the equation 22, $\Phi^{\text{mep}}$ was calculated to be equal to 0.28.

3.2.2.2. Computational Methods

Computational calculations were performed by John Belof from Dr Space’s group. The quantum chemistry program GAMESS was used in order to calculate the electronic structure of the mepepy ligand [15]. The molecular properties of the azobenzene molecule which is a similar molecule to mepepy were already studied. The results were accurate in terms of isomerization studies using density functional theory (DFT) [16-20]. The calculations were done by B3LYP hybrid exchange-correlation functional with the augmented correlation-consistent double-$\zeta$ basis set (aug-cc-pVDZ) [19-22]. The effects of the solvent were mimicked by using a polarizable continuum model where the aqueous environment was imitated by surrounding the mepepy molecule into isotropic dielectric field [23]. The dielectric constant was estimated to be equal to 78.39 and the solvent radius equal to 1.3850 Å. In order to establish the minimum of the energy geometries of the trans and the cis mepepy the geometry optimizations were done in the solvation field. In addition, the charge distributions establish the molecular electric dipole moments for the optimized structures. Moreover, the ground state of the trans and cis mepepy potential energy surfaces were studied in order to find any other local energy minima. Each angle of the pyridine and pyrrole rings from a planar configuration was scanned from 0 to 180° in 10° increments along the $\alpha-\beta$ and $\gamma-\delta$ vectors leaving the conformation adopting any minimum. The spectroscopic calculations using ZINDO/CI method from ArgusLab [24], were completed by using the highest 45 occupied and lowest 45 unoccupied orbitals in the CI (SCF-CI, restricted Hartree–Fock, with STO-6G
minimal basis set). A dielectric constant of 78.3 and a cavity radius of 4.616 Å (SPCE water model) were also used for a self-consistent reaction field.

3.2.3. Results and Discussion

3.2.3.1. Optical Properties

Figure 3.2 shows the steady-state absorption spectrum of the mepepy isolated in its trans form and solubilized in water. The absorption spectrum shows two bands one at ~355 nm and a shoulder at ~425 nm. The band at 355 nm was designed to be the HOMO to LUMO transition or the π to π* transition. Figure 3.3 shows how the electron density migrated from the HOMO orbitals where the electron density is centered on the five-membered ring to the LUMO orbitals where the electron density migrated between the five and six-membered rings. Figure 3.3 also shows how the electron density of the cis form of the mepepy migrated from the five-membered ring in the HOMO to the six-membered ring in the LUMO. The band at 425 nm was attributed to the π to π* transition which corresponds to the protonation of the nitrogen on the pyridine ring of the mepepy. This hypothesis was confirmed by an experiment where a dilute NaOH solution (<0.1M) was added to the mepepy solution in water. The decrease of the band at 425 nm was followed on the absorption spectrum (Figure 3.2).
Figure 3.2: Optical absorption spectra of the steady-state of mepepy in water (containing <1% DMSO) (solid line) and aqueous 0.01 N NaOH (containing <1% DMSO; dotted line). 

\[ [\text{mepepy}] = 270 \text{ mM}. \]
Figure 3.3: Diagrams of the molecular orbital viewing the HOMOs and LUMOs related with the observed $\pi$ to $\pi^*$ transitions of the trans (left) and cis (right) isomers of mepepy.

Figure 3.4 demonstrates that after illumination of the mepepy and photoisomerization from the trans to cis form of the mepepy, a decrease in the molar extinction coefficient of the $\pi$ to $\pi^*$ transition of the protonated and deprotonated forms of the mepepy is observed associated with a minor hypsochromic shift of the energy.
Figure 3.4: *Top Panel:* UV-Vis spectra of the mepepy equilibrium before and after steady-state photolysis (from 10 to 70 mins). The sample was solubilized in deionized water containing <1% DMSO.

*Bottom panel:* UV-Vis spectra of the trans-mepepy equilibrium before and after 5, 10, 15, and 20 laser pulses ($\lambda_{\text{excit}} = 355$ nm, $\sim 100$ $\mu$J/pulse, and $\sim 7$ ns fwhm).
3.2.3.2. Theoretical Structural Analysis

Figures 3.1 and 3.6 represent the different trans and cis structures of mepepy. Figure 3.5 also shows the different energy minima located along the potential energy surface between the trans and cis structure of the mepepy. The details of the geometric are summarized in the Tables 3.1 and 3.2.

It has been demonstrated that the trans form of the mepepy has two minima, a global and a local minimum, which are separated by an energy barrier equal to 9.12 kcal mol$^{-1}$ and give to the trans potential energy surface unique characteristics (Figure 3.6). Only one minimum was found for the cis form where the potential energy surface was described as a shallow basin which will give the opportunity of the equilibrium structure to fluctuate out of plane into the solvated ground state. The percentage of population for each isomer after photoisomerization could not be found.

An energy difference of 8.24 kcal mol$^{-1}$ was found between the cis and the global trans minimum form of the mepepy to compare to the 7.17 kcal mol$^{-1}$ energy difference between the cis and the local trans minimum form. The difference in the dipole moment between the cis and the two different trans form of mepepy were calculated after evaluating the electrostatic moments. A difference of 0.424 D was found between the cis and the global trans minimum form and 0.0398 D between the cis and the local trans minimum. While the relative populations of the isomers after a photoisomerization event are unknown, these determined differential values place an upper and lower bound to the true equilibrium differences. The cis potential energy surface is a shallow basin with a single minimum, where it can be expected that the equilibrium structure will fluctuate out of plane in the solvated ground state. However, unique characteristics of the trans potential energy surface were found, most notably the existence of two ground-state minima separated by an energy barrier, herein referred to as the global and local trans states (geometrically shown in Figure 3.5). Complete determination of the isomerization mechanism for mepepy (which is not determined in this work) is required to know whether the transitions from the excited state to the ground state favor one isomeric trans form over the other. The minimum energy barrier between the cogeneric trans states is 9.12 kcal mol$^{-1}$, whereas the thermal energy $kT$ at 300 K is only 0.596 kcal mol$^{-1}$. 

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Table 3.1: Configurations of the Global Energy-Minimized Geometric cis- and trans-mepepy.

<table>
<thead>
<tr>
<th></th>
<th>Cis isomer</th>
<th>Trans isomer</th>
<th>Cis isomer</th>
<th>Trans isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha \beta \gamma$ (deg)</td>
<td>126.647</td>
<td>126.490</td>
<td>1.34764</td>
<td>1.35337</td>
</tr>
<tr>
<td>$\beta \gamma \delta$ (deg)</td>
<td>129.507</td>
<td>127.365</td>
<td>1.44983</td>
<td>1.43931</td>
</tr>
<tr>
<td>$d_{\alpha \beta}$ (Å)</td>
<td>1.48689</td>
<td>1.46157</td>
<td>90.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

The bond angles and distances are with respect to the labels in Figure 3.1 and $\sigma$ is the angle between the pyridine and pyrrole planes.

Table 3.2: Calculated Energy and Dipole Moment of Global Minima cis- and trans-mepepy Isomers associated with the Alternate Local Minimum along the trans Potential Energy Surface.

<table>
<thead>
<tr>
<th>Isomer</th>
<th>Energy (kcal mol$^{-1}$)</th>
<th>Molecular dipole moment (D)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis</td>
<td>$-335330.35$</td>
<td>4.12</td>
</tr>
<tr>
<td>global trans</td>
<td>$-335338.59$</td>
<td>3.69</td>
</tr>
<tr>
<td>local trans</td>
<td>$-335337.51$</td>
<td>3.08</td>
</tr>
</tbody>
</table>
Figure 3.5: Ground-state energy surfaces of the trans / cis mepepy as a function of the pyridine and pyrrole ring angles as defined by deviation from planar configuration. See text for details.

Figure 3.6: Structure of the global and local trans geometries (left and right, respectively).

3.2.3.3. Photothermal Studies

The overlay of the reference Fe4SP and the mepepy PAC signals at 35°C are shown in Figure 3.7. No shift is observed for the photoacoustic signals between the reference Fe4SP and the isomerization of the trans to cis mepepy which demonstrate that all the conformational changes appear to be faster than < ~ 50 ns which is the time response of the PAC instrument. After plotting, the equation $\phi E_{hv}$ versus $(\rho C_p/\beta)$ (equation 9), the volume and the enthalpy can be extracted as it was mentioned before.
Their profiles are summarized Figure 3.8 with a $\Delta V = 0.9 \pm 0.4$ mL mol$^{-1}$ ($1.5 \text{Å}^3$/molecule) and a $\Delta H = 18 \pm 3$ kcal mol$^{-1}$.

**Figure 3.7:** Overlay of the normalized acoustic wave of the reference Fe4SP and the trans-mepepy in water containing <1% DMSO at 35°C. Both absorbencies at the excitation wavelength (355 nm) were 0.4.
The change in the molar volume is the addition of two different contributions. The first contribution is a volume change due to a variation of the van der Waals volume of the \textit{trans} to \textit{cis} isomerization of the mepepy. The van der Waals volume change for the \textit{trans} to \textit{cis} isomerization of the mepepy was calculated to be equal to \(\sim 9\ \text{mL}\ \text{mol}^{-1}\) with the \textit{cis} conformation having the lower molar volume.

The second contribution is a variation of the structure of the solvent around the mepepy molecule which is a result of a change in the ground state dipole moment and/or in a change of the overall charge of the mepepy. The following equation will give an estimation of the volume change due to a change in electrostriction:

\[
\Delta V_{el} = - \left( \frac{\mu^2}{r^3} \right) k_T \left\{ \frac{(\varepsilon + 2)(\varepsilon - 1)}{(2\varepsilon + 1)^2} \right\} \left( \frac{N_A}{4\pi \varepsilon_0} \right) \tag{Equation 37}
\]

where \(\mu\) is the dipole moment, \(r\) is the cavity radius of the molecule (mepepy), \(k_T\) is the compressibility of the solvent, \(\varepsilon\) is the solvent’s dielectric constant, \(N_A\) is Avogadro’s number, and \(\varepsilon_0\) is the vacuum permittivity [25, 26]. The variation of the volume due to the electrostriction was calculated to be \(\sim -0.2\ \text{mL}\ \text{mol}^{-1}\) using the variation of the dipole moment for the \textit{trans} to \textit{cis} isomerization of the mepepy equal to 0.0398 D, a molecular radius equal to \(\sim 5\ \text{Å}\) by assuming that the diameter of the mepepy molecule is \(\sim 10\ \text{Å}\), \(k_T\) is equal to \(4.22 \times 10^{-10}\ \text{Pa}^{-1}\) (for water at 25°C) and \(\varepsilon\) equal to 78.4.

The total variation adding the two contributions was calculated to be equal to \(\sim -9.2\ \text{mL}\ \text{mol}^{-1}\). The experimental variation of volume was estimated to be equal to \(\sim 0.9\ \text{mL}\ \text{mol}^{-1}\), which is considerably different from the theoretical calculated value. The difference between the theoretical and experimental value shows that the interactions between the solvent and the \textit{trans} and \textit{cis} mepepy forms are significantly different. Figure 3.3 shows more electron density on the nitrogen of the pyridine in the \textit{trans} form of the mepepy than the \textit{cis} form. This observation can explain that a water molecule will have less probability to form an H-bond with the \textit{cis} form than the \textit{trans} form of the mepepy pyridine group. Van Eldik et al. [27] have demonstrated that a bond cleavage will show
an expansion in molar volume of ~ 5 mL mol\(^{-1}\) which is consistent with the expansion equal to \(\Delta V_{\text{H-bond}} \approx 9\) mL mol\(^{-1}\), between the isomerization of the \textit{trans} to \textit{cis} mepepy molecule upon the H-bond cleavage.

The variation of enthalpy was compared to the results from computational studies. The variation of volume between the \textit{cis} form and the global energy minima from the \textit{trans} form of the mepepy shows a difference of ~ 8 kcal mol\(^{-1}\). This value is significantly different from the experimental value which was calculated from PAC results equal to 18 kcal mol\(^{-1}\). The difference can also be explained by the cleavage of an H-bond between a water molecule and the mepepy pyridine group in terms of energy. Previous studies have shown that the energy of an hydrogen bond between a water molecule and a pyridine is equal to ~ 6-8 kcal mol\(^{-1}\) which is very close to the 10 kcal mol\(^{-1}\) (difference between the theoretical and experimental value) and confirms that the variation of enthalpy is consistent with the cleavage of an H-bond between a water molecule and the nitrogen of the mepepy pyridine group upon the \textit{trans} to \textit{cis} isomerization [28-32]. The thermodynamic profile is summarized in Figure 3.8.

\[\Delta H = \sim 18 \text{ kcal mol}^{-1}\]
\[\Delta V = \sim 1 \text{ mL mol}^{-1}\]

\textit{Figure 3.8:} Thermodynamic profile for the photoinduced isomerization of the \textit{trans} to \textit{cis} mepepy in aqueous solution.
3.2.4. Conclusion

To summarize the results presented for the trans to cis isomerization of the mepepy, the cis form exhibits one energy minimum to compare to the trans form which exhibits two energy minima separated by 9 kcal mol$^{-1}$ and corresponds to two different conformations of the trans form of the mepepy. The different trans conformations might help to vary the photoinduced spin-state transitions of the metal complexes coordinated to the mepepy ligand. Secondly, it has also been demonstrated by computational studies that the dipole moments between the trans and the cis form of the mepepy are very similar which show a very restraint change in the electronic structure of mepepy to compare to azobenzene molecules or other push-pull molecules. Finally, the mepepy pyridine group can form an H-bond with the solvent which suggest that the thermodynamics of the trans to cis isomerization of the mepepy can be altered by the nature of the solvent. For instance, a solvent which can form an H-bond with the mepepy pyridine group will favor the trans form by up to 10 kcal mol$^{-1}$. 
3.2.5. References


3.3. Photothermal Studies of the Room Temperature Photoinduced Spin State Change in the Fe(III)(Salten)(Mepepy) Complex

3.3.1. Introduction

The ability to modulate the spin-state within a transition metal complex has enormous implications for the design of novel magnetic materials. Of key interest is the ability to switch between metal spin-states using either optical or thermal triggering at room temperature. A number of metal complexes have now been synthesized utilizing isomerizable ‘push-pull’ ligands in which the field strength of the ligand can be modulated either thermally or through photonics. These complexes are of the Fe$^{II}$(L$_4$)(X$_2$) type or Fe$^{III}$(L’$_4$)(L)(X) type in which L is the push-pull ligand. One such complex utilizes L = 4-sterylpyridine (Stpy) (1-phenyl-2-(4-pyridyl) ethane) as well as several phenyl derivatives to photo-induce the spin crossover [1-3]. It has been shown that the Fe$^{II}$(trans Stpy)$_4$(NCS)$_2$ complex undergoes a thermally induced high-spin to low-spin transition centered near 190 K [1]. Subsequent photoexcitation of this complex imbedded within a cellulose acetate substrate results in a trans to cis isomerization of the Stpy which also induces the high-spin to low-spin transition at 140K. The drive to produce transition metal complexes with photo-induced spin crossovers at high temperatures has led to the synthesis complexes such as [Fe$^{III}$(salten)(mepepy)]BPh$_4$ (salten = 4-azahexamethylene-1,7-bis(salicylideneiminate; mepepy= 1-(pyridin-4-yl)-2-(N-methylpyrrol-2-yl)-ethene; BPh$_4$ = tetraphenyl borate) (Figure 3.9). This complex has been shown to exhibit a photo-induced high-spin to low-spin transition at room temperature [2-4].
Figure 3.9: Structure of the Fe(III)(salten)(mepepy) complex before and after photolysis. Distances: a=2.035 Å, b=1.977 Å, c=1.965 Å, d=1.888 Å, e=1.892 Å and f=2.022 Å [16].

The mepepy ligand associated with the Fe(III)(salten)(mepepy) complex (Figure 3.10) is a push-pull type molecule that exhibits either a cis or trans conformation and contains an electron-withdrawing pyridyl group and an electron-donating N-
methylpyrrole group, separated by a isomerizable double bond. Although the trans to cis photo-isomerization process is effective in modulating the ligand field strength due to changes in basicity much less is known about the electronic properties and energetics of this process.

![Structure of the Trans and Cis isomers of mepepy.](image)

**Figure 3.10: Structure of the Trans and Cis isomers of mepepy.**

Recently, our lab has utilized photoacoustic calorimetry (PAC) and computational methods to probe the energetics associated with the trans to cis photo-isomerization of mepepy in aqueous solution. These studies revealed a volume change of $0.9 \pm 0.4$ mL mol$^{-1}$ and an enthalpy change of $18 \pm 3$ kcal mol$^{-1}$ which were attributed to the loss of a hydrogen bond between a solvent water molecule and the pyridyl ring of mepepy associated with the trans to cis isomerization [5]. A number of studies have been reported in which the enthalpies of the spin crossover have been measured at low temperatures on powder samples or single crystal using differential scanning calorimetry (DSC). Collectively these studies indicate very small enthalpies (i.e., < 1 kcal mol$^{-1}$) for the spin state transition that are coupled to minor structural changes. For example, the thermal induced spin-state transition in an Fe(II) tris(2-(2'-pyridyl)benzimidazole) complex which occurs with a transition temperature of 140K exhibits an enthalpy change of only 0.6 kcal mol$^{-1}$ (low-spin to high-spin) and a corresponding increase in unit cell volume. More recently, thermal and photoinduced spin crossover in novel 3D metal organic
materials was examined in both bulk materials and thin films. These studies also revealed very small enthalpy changes ranging from ~ 1 to ~ 4 kcal mol\(^{-1}\) at temperatures below 300K [6-8].

However, to date, no studies have been reported for the volume and enthalpy change of a spin crossover in solution at room temperature. Here photoacoustic calorimetry (PAC) has been used to study the energetics following the spin crossover of the Fe(III)(salten)(mepepy) complex by resolving the magnitudes and timescales of molar volume and enthalpy changes associated with, first, the photoisomerization of the \textit{trans} to \textit{cis} conformers of the mepepy ligand and secondly, the spin crossover of the iron in order to characterize the physical properties of the metal complex.

### 3.3.2. Materials and Methods

\textit{Synthesis of Mepepy}:

The synthesis of the \textit{trans} mepepy ligand has been previously reported by Mokdad et al. [5].

\textit{Synthesis of the Fe(III)(salten)(mepepy)BH}_4\textit{ complex}:

- \textit{Synthesis of the salten ligand}:

  Under reflux, 11 mL salicylaldehyde was added to 7.5 mL 1,7-diamino-4-azaheptane in 50 mL of methanol. The solution was refluxed for 10 minutes under constant stirring. A yellow/orange oil was collected and, after evaporation of the solvent, a yellow solid was obtained (Saltenptn).

- \textit{Synthesis of the Fe(III)(salten)Cl}:

  Under reflux, a solution of 1.7 g of Saltenptn in 20 mL of methanol was stirred at 60\(^\circ\)C and was treated with a solution containing 0.63 g of iron chloride in 25 mL
methanol. The solution was stirred for 10 minutes at 50ºC, and then for 15 minutes at 60ºC. To the yellow solution, 3 mL of triethylamine was added. The solution was then stirred for two hours at 60ºC. The solution was cooled to room temperature. The solution was filtrated and the crystals were washed with methanol and diethyl ether. Brown crystals of Fe(III)(salten)Cl were collected.

- Synthesis of the Fe(III)(salten)(mepepy) Complex:

A solution of 77.3 mg of mepepy in 0.5 mL methanol was added to a solution of 90 mg Fe(III)(salten)Cl in 4 mL methanol. Under reflux, the solution was stirred at 60ºC for 4 hours. The solution was then placed in ice and filtered to remove insoluble materials. To the resulting solution was added a solution containing 71.9 mg of sodium tetraphenylborate in 1 mL methanol. The solution was stirred under reflux over night at 60ºC. The solution was then placed in ice, filtered and the solid material was washed with 3-4 mL of cold methanol, ~15 mL of water and ~25 mL of ether. The solution was then dried with a vacuum for 6 hours. A brown solid was collected. The purity for the compound was verified by mass spectra and UV where the UV spectrum matched the one published by Sour et al. [2].

Quantum yield:

The quantum yield, \( \Phi \), associated with the isomerization of the trans to cis mepepy was determined by irradiating a cuvette containing ~0.2 mM mepepy in both 50:1 and 6:1 (V:V) acetonitrile:water at 355 nm excitation (the wavelength used to perform the PAC measurements). The solution was irradiated for a total of 35 minutes, and the absorption spectra were recorded every 5 minutes. The same experiment was performed with ~0.8 mM azobenzene as a control. The value of \( \Phi \) was then determined using the following equation:

\[
\left( \frac{\Delta A_{0-35}^{\text{mep}}}{\Delta A_{0-35}^{\text{azo}}} \right) = \left\{ \left( \Phi^{\text{mep}} \Delta \epsilon^{\text{mep}} C_0^{\text{mep}} \right) / \left( \Phi^{\text{azo}} \Delta \epsilon^{\text{azo}} C_0^{\text{azo}} \right) \right\} \quad \text{Equation 38}
\]
where $\Delta A_{0.35}^{\text{mep}}$ and $\Delta A_{0.35}^{\text{azo}}$ are, respectively, the changes in absorbance at 355 nm for mepepy and azobenzene, $\Phi^{\text{mep}}$ is the quantum yield for the mepepy trans to cis isomerization, $\Phi^{\text{azo}}$ is the quantum yield for the trans to cis isomerization in azobenzene (0.26), $\Delta \varepsilon^{\text{mep}}$ (3,000 mM$^{-1}$ cm$^{-1}$) and $\Delta \varepsilon^{\text{azo}}$ (550 mM$^{-1}$ cm$^{-1}$) are the differences in extinction coefficients at 355 nm between, respectively, for the trans and cis isomers of mepepy and azobenzene, and $C_0^{\text{mep}}$ and $C_0^{\text{azo}}$ are the initial concentrations. Using this procedure $\Phi^{\text{mep}}$ was determined to be 0.28. The quantum yield associated with the corresponding Fe(III)(salten)(mepepy) photo-induced spin state was determined using the same procedure with $\Delta \varepsilon^{\text{Fe(III)(salt)(mepepy)}}$ (22 mM$^{-1}$ cm$^{-1}$) giving a value of 0.40.

Photoacoustic calorimetry method:

The principles behind PAC have been reviewed in detail in Chapter II. A stock solution of Fe(III)(salten)(mepepy) was prepared in acetonitrile (the Fe(III)(salten)(mepepy) complex is not soluble in water). Samples for PAC were prepared by diluting the stock solution from 85% to 99% acetonitrile:water with increment of 1 is used to vary the term of thermal expansion coefficient (see below). A calorimetric reference (Fe(3+)tetrakis(4-sulphonatophenyl)porphyrin) = Fe4SP was prepared under the same solution conditions as the sample. The absorbance of the Fe(III)(salten)(mepepy) complex and the reference Fe4SP at the excitation wavelength of 355 nm were both $\sim$ 0.4. The sample was stirred for all acquisitions and seven laser pulses were averaged per trace.

The experiments were performed using a Continum MiniLite I frequency triplet Nd:YAG laser (355 nm, $\sim$ 7 ns FWHM, $\sim$ 100 $\mu$J/pulse, 1 Hz). The acoustic waves were detected using a Panametrics V103 detector coupled to a Panametrics preamplifier and recorded using a PicoScope (50 MHz transient digitizer).
Determination of $C_p\rho/\beta$ for Acetonitrile-Water Mixtures

The $C_p\rho/\beta$ term is temperature dependent in aqueous solution but, in organic solvents, the variations of the $C_p\rho/\beta$ term with temperature is negligible. For mixed solvent systems (such as the acetonitrile-water solutions to be used here), $C_p\rho/\beta$ can be determined by comparing acoustic amplitudes of a reference molecule solubilized in water to those obtained in the mixed solvent system. The mixed solvent $C_p\rho/\beta$ values were determined by performing PAC measurements on ~ 10 μM Fe4SP in acetonitrile-water mixtures used for the sample measurements. The value of $C_p\rho/\beta$ for each ratio of acetonitrile:water was then determined using the following equation:

$$(C_p\rho/\beta_{\text{Fe4SP} \text{ acetonitrile:water}}) = (C_p\rho/\beta_{\text{Fe4SP} \text{ water}}) \times (S_{\text{Fe4SP} \text{ acetonitrile:water}} / S_{\text{Fe4SP} \text{ water}})$$

Equation 39

where $S_{\text{Fe4SP} \text{ acetonitrile:water}}$ and $S_{\text{Fe4SP} \text{ water}}$ are, respectively, the acoustic amplitudes for the reference in acetonitrile:water and neat water, respectively.

3.3.3. Results

The steady-state absorption spectra of the Fe(III)(salten)(mepepy) complex and that of the trans form of mepepy solubilized in 85% (volume percent) acetonitrile:water are displayed in Figure 3.11.
The optical spectrum of the Fe(III)(salten)(mepepy) complex displays transitions centered at ~ 350 nm (ε ~ 22 mM⁻¹ cm⁻¹) and at ~ 475 nm. The 350 nm transition corresponds to that of the mepepy ligand with the exception of significantly lower molar extinction coefficients (~ 2 mM⁻¹ cm⁻¹ for the mepepy) while the 475 nm band likely arises from a charge transfer transition between the iron and the mepepy ligand. The ~350 nm absorption band of the mepepy ligand can be assigned primarily from a transition between the HOMO, with electron density centered primarily on the five membered ring, and LUMO orbitals with electron density distributed between the five and six membered rings (i.e., π to π* transition) (Figure 3.11) [5]. Upon steady state illumination of the Fe(III)(salten)(mepepy) complex, photoisomerization of the trans to cis form takes place within the mepepy ligand which results in a low to high-spin state change of the iron, giving rise to a hypsochromic shift (~10 nm) in the energy of the HOMO to LUMO transition as well as a considerable decrease in the molar extinction coefficient (Figure 3.12).
Figure 3.12: Steady-state optical absorption spectrum of Fe(III)(salten)(mepepy) photolysis over time.

An overlay of the PAC signals associated with the trans to cis isomerization for the mepepy ligand, Fe(III)(salten)(mepepy) complex and the Fe4SP reference compound at 35°C in 85% acetonitrile:water are displayed in Figures 3.13-3.15. The photoacoustic signals for the trans to cis isomerization of both mepepy and Fe(III)(salten)(mepepy) complex do not show any shifts in frequency relative to the calorimetric reference. This indicates that the ΔH and ΔV take place within the response time of the PAC instrument, i.e., < ~50 ns. From a plot of φEhυ/(Cpρ/β) (equation 9), the volume and enthalpy changes are determined to be ΔV = 0.7 ± 0.3 mL mol⁻¹ and ΔH = 33 ± 9 kcal mol⁻¹ for the trans to cis isomerization of the mepepy ligand (Figure 3.14) and ΔV = 0.9 ± 0.3 mL mol⁻¹ and ΔH = 37 ± 9 kcal mol⁻¹ for the mepepy isomerization and corresponding spin-state transition associated with the Fe(III)(salten)(mepepy) complex (Figure 3.16).
3.3.4. Discussion

Mepepy ligand:

The observed volume change associated with the trans to cis isomerization of mepepy in acetonitrile:water solutions may have contributions from changes in van der Waals volume of the mepepy molecule after isomerization as well as to electrostriction effects associated with changes in overall charge due to changes in the ground-state dipole moment. The volume change due to electrostriction can be estimated using the following equation [9,10]:

\[
\Delta V_{el} = -\left(\Delta \mu^2/r^3\right) \kappa_T\left\{(\varepsilon + 2)(\varepsilon - 1) / (2\varepsilon + 1)^2\right\}(N_A / 4\pi\varepsilon_0)
\]

Equation 40

where \(\Delta \mu\) is the change in dipole moment upon isomerization, \(r\) is the cavity radius of the molecule, \(\kappa_T\) is the compressibility of the solvent, \(\varepsilon\) is the solvent’s dielectric constant, \(N_A\) is Avogadro’s number, and \(\varepsilon_0\) is the vacuum permittivity. Using the previously calculated dipole change for the trans to cis isomerization of 0.0398 D [5], a molecular radius of \(~5\) Å (this assuming the mepepy molecule sweeps out a sphere of diameter \(~10\) Å), \(\kappa_T\) of \(6.9 \times 10^{-10}\) Pa\(^{-1}\) (85% V:V acetonitrile:water) or \(\kappa_T\) of \(7.5 \times 10^{-10}\) Pa\(^{-1}\) (for 98% V:V acetonitrile:water) and \(\varepsilon = 35.97\), the electrostriction volume change was calculated to be \(~-0.29\) mL mol\(^{-1}\) for 85% acetonitrile:water and \(~-0.32\) mL mol\(^{-1}\) for 98% acetonitrile:water. An average of the volume change due to electrostriction is estimated to be \(~-0.3\) mL mol\(^{-1}\) over the solvent range used here (85% to 99% acetonitrile). The corresponding van der Waals volumes change between the trans and cis isomers was previously estimated to be \(~-9\) mL mol\(^{-1}\)[5]. Giving a total \(\Delta V\) (i.e., van der Waals volume changes and the electrostriction change) of \(~-9.3\) mL mol\(^{-1}\) which is significantly lower than the observed \(\Delta V = 0.7\) mL mol\(^{-1}\) indicating additional contributions to the volume change.
Figure 3.13: Overlay of the normalized acoustic wave of the reference Fe4SP and mepepy in 85% (V/V) CH$_3$CN:H$_2$O at 35°C. The absorbance of the sample and reference at the excitation wavelength (355 nm) was ~0.4.

Figure 3.14: Plot of $\Phi E_{hv}$ vs. $C_{p}/\beta$ for mepepy photoisomerization. The slope of these lines provides that change in molar volume ($\Delta V = \text{slope}/\Phi$ in mL mol$^{-1}$) and the intercept provides the enthalpy ($\Delta H = (E_{hv} - Q)/\Phi$ in kcal mol$^{-1}$).
The $\Delta V_{obs}$ for the *trans* to *cis* isomerization in acetonitrile:water mixtures is similar to the volume change previously observed for the *trans* to *cis* isomerization of mepepy in neat water ($\Delta V \approx 0.9 \text{ mL mol}^{-1}$) [5]. The change in molar volume in this case was rationalized by the loss of a hydrogen bond between a solvent water molecule and the N atom associated with the pyridine of the mepepy. In water, the *trans* form of mepepy H-bonded exhibits an absorbance at 425 nm which is not present in basic solutions. This band arises from a HOMO to LUMO transition for a mepepy molecule containing a hydrogen bonded/protonated N atom on the pyridyl unit. Upon photoisomerization to the *cis* isomer the electron density on the N atom is reduced resulting in a loss of the H-bond between a water molecule and the mepepy pyridyl group. Van Eldik et al. have suggested that on average bond cleavage reactions increase the molar volume of $\approx 5$-10 mL mol$^{-1}$ which together with the electrostriction volume and van der Waals volume changes accounts for the observed volume change [11]. Thus, it is apparent that *trans* conformation of mepepy solubilized in acetonitrile:water retains the protonation/hydrogen bonding to the pyridyl N atom and that this interaction is lost upon isomerization to the *cis* conformation.

The corresponding changes in enthalpy result from the energy difference between the *cis* and *trans* form of mepepy as well as enthalpy changes associated with solvation including H-bond formation/cleavage. Previous computational results have demonstrated an energy difference between the *cis* and the global *trans* isomers of $\approx 8$ kcal mol$^{-1}$ [5]. The $\Delta H$ obtained from PAC measurements for the transition between the global *trans* and *cis* conformations in acetonitrile:water is 33 kcal mol$^{-1}$ and includes the enthalpy of the isomerization, H-bond cleavage and any additional solvation effects (i.e., $\Delta H_{PAC} = \Delta H_{\text{isom}} + \Delta H_{\text{H-bond}} + \Delta H_{\text{solv}}$). Taking into account the enthalpy difference between the two mepepy conformers, the enthalpy associated with solvation changes and H-bond formation/cleavage is $\approx 25$ kcal mol$^{-1}$. Previous studies have demonstrated that the energy of an H-bond between water and pyridine is close to 6-8 kcal mol$^{-1}$ [12-14]. In contrast, Gilli et al. studies suggest a positive charge-assisted H-bond ($(+)\text{CAHB}$) including $[\text{N}\cdots\text{H}^+\cdots\text{N}]^+$ have an average H-bond energy of $\approx 16.4$ kcal mol$^{-1}$ while an $[\text{N}\cdots\text{H}^+\cdots\text{O}]^+$ interaction has an average H-bond energy of $\approx 15.2$ kcal mol$^{-1}$ [15]. Thus, the remaining
25 kcal mol\(^{-1}\) observed for the mepepy isomerization may be attributed (within the ±9 kcal mol\(^{-1}\) uncertainty) to the loss of an (+)CAHB between the mepepy pyridyl group and a water molecule which, in turn, is H-bonded at an acetonitrile solvent molecule.

**Fe(III)Salten Mepepy Complex:**

The PAC results indicate that the mepepy trans to cis isomerization and associated spin crossover of the Fe(III)(salten)(mepepy) complex also occurs in < ~ 50 ns as evident from the lack of frequency shift between the PAC signals of the complex and the calorimetric reference. From a plot of \(\varphi E_b\) versus \((C_p\rho/\beta)\) (Figure 3.16), the volume and enthalpy changes are determined to be \(\Delta V = 0.9 \pm 0.3 \text{ mL mol}^{-1} (1.5 \text{ Å}^3 / \text{molecule})\) and \(\Delta H = 37 \pm 10 \text{ kcal mol}^{-1}\). The observed volume change has two contributions from the trans to cis isomerization of the mepepy as well as the spin crossover of the center iron ion. In order to extract the volume change associated with the spin crossover, the volume change associated with the mepepy isomerization must be subtracted from the overall volume change. However, as the N-atom of the pyridine group is coordinated to the iron atom (precluding H-bonding interactions with the solvents only the previously estimated van der Waals and electrostriction contributions are included i.e., ~ -9.3 mL mol\(^{-1}\). Thus, the volume change of the iron spin is then estimated to be ~ 10.2 mL mol\(^{-1}\).

Interestingly, high pressure studies of several Ni and Fe ligand complexes have suggested that volume change associated with a low-spin to high-spin transition is on the order of ~ 10 mL mol\(^{-1}\) [11]. This value was attributed to expansion of the metal-ligand core as well as electron repulsion between the 3d\(_{z^2}\) orbitals and ligand σ orbitals. Since the Fe(III)(salten)(mepepy) complex examined here undergoes a high-spin to low-spin a \(\Delta V\) of ~ -10 mL mol\(^{-1}\) would be expected. The difference observed between the previously reported metal complexes and the Fe(III)(salten)(mepepy) may be due to significant distortion of the complex following the central iron spin state change. The average of the Fe···O and Fe···N bond distances found in the Cambridge structural database for the Fe(III)(salten)(mepepy) complex are indicated in Figure 3.9 [16]. The bond distances between the Fe···O and Fe···N demonstrate that the axial Fe···N bond to the
secondary amine has a higher average bond length indicating a lower bond energy. Thus, one possibility for the difference in the observed $\Delta V$ associated with spin state changes in metal complexes and that of the Fe(III)(salten)(mepepy) complex may be the loss of coordination between the central iron ion and the N atom associate with the salten amine group. Pixton et al. calculated a $\Delta V \sim 24$ mL mol$^{-1}$ for the cleavage of a Fe$^{-}$N bond between a histidine and heme [17], while a $\Delta V \sim 20$ mL mol$^{-1}$ was observed by Laverman et al., for the cleavage of the Fe-NO bond in NO-MetMyoglobin [18]. The release of the salten N-group would also result in solvent hydrogen bonding in the mixed acetonitrile-water system. The volume change for hydrogen bond formation has been estimated to be on the order of -10 mL mol$^{-1}$ [20]. Considering the Fe-N bond cleavage step the overall $\Delta V_{\text{obs}} = \Delta V_{\text{Fe-N}} + \Delta V_{\text{N-H}} + \Delta V_{\text{HS-LS}}$ (after subtracting $\Delta V_{\text{mepepy}}$) which, after rearranging, gives $\Delta V_{\text{HS-LS}} = \Delta V_{\text{obs}} - \Delta V_{\text{Fe-N}} - \Delta V_{\text{N-H}} = 10$ mL mol$^{-1} - 20$ mL mol$^{-1} + 10$ mL mol$^{-1} \sim 0$ mL mol$^{-1}$. Thus, the overall process following the photoexcitation of the Fe(III)(salten)(mepepy) complex can be summarized by the following processes: 1) trans to cis isomerization in the mepepy ligand, 2) cleavage of the Fe$^{-}$N bond, 3) formation of hydrogen bond between a water molecule and the secondary amine and 3) spin-state change of the iron. The lack of any significant volume change due to the spin state transition is consistent with the fact that, other than the loss of the Fe-N coordination, the iron retains all of the other ligands precluding any electronic repulsion between the Fe 3d$_{x^2}$ orbital and solvent molecules. This hypothesis is also consistent with volumetric studies of the volume change of spin crossover within the crystal forms of various iron complexes. These results show an average of volume change between 2-3 Å$^3$ which correspond to a volume change of only 1-2 mL mol$^{-1}$[20,21].
Figure 3.15: Overlay of the normalized acoustic wave of the reference Fe4SP and the 
Fe(III)(salten)(mepepy) complex in 85% (V/V) CH₃CN:H₂O at 35 °C. The absorbance of the 
sample and reference at the excitation wavelength (355 nm) was ~0.4.

Figure 3.16: Plot of ΦEₜ vs. Cₚρ/β for Fe(III)(salten)(mepepy) complex photoisomerization. 
The slope of these lines provides that change in molar volume (ΔV = slope/Φ in mL mol⁻¹) 
and the intercept provides the enthalpy (ΔH = (Eₜ− Q)/Φ in kcal mol⁻¹).
The corresponding enthalpy change has contributions from the trans to cis isomerization of the mepepy ligand as well as the spin state change of the iron and any changes in core conformation, \( \Delta H_{\text{obs}} = \Delta H_{\text{mepepy}} + \Delta H_{\text{Fe-N}} + \Delta H_{\text{N-H}} + \Delta H_{\text{HS-LS}} \). The theoretical enthalpy value for the trans to cis isomerization of the mepepy ligand was calculated to be \( \sim 8 \text{ kcal mol}^{-1} \) [5]. Subtracting this value from the observed enthalpy gives \( \sim 29 \text{ kcal mol}^{-1} \) for the subsequent Fe-N bond cleavage, hydrogen bond formation and corresponding spin state change (i.e., \( \Delta H_{\text{obs}} - \Delta H_{\text{mepepy}} = \Delta H_{\text{Fe-N}} + \Delta H_{\text{N-H}} + \Delta H_{\text{HS-LS}} \)). Previous studies have further demonstrated that hydrogen bonds between water and pyridine have a bond energy on the order of \( \sim 6-8 \text{ kcal mol}^{-1} \) [12-14] while those between water and dimethylamine are on the order of 5-6 kcal mol\(^{-1}\) [23]. By taking into account the formation of H-bond between a water molecule and the secondary amine (exothermic), the remaining difference between the theoretical and experimental values is equal to \( \sim 35 \text{ kcal mol}^{-1} \) (i.e., 35 kcal mol\(^{-1}\) = \( \Delta H_{\text{obs}} - \Delta H_{\text{mepepy}} - \Delta H_{\text{N-H}} = \Delta H_{\text{Fe-N}} + \Delta H_{\text{HS-LS}} \)). Finally, the enthalpy of dissociation of an Fe\(\cdots\)N bond from Fe-NH\(_3\) type complexes is on the order of \( \sim 30 \text{ kcal mol}^{-1} \) [27] which results in a \( \Delta H_{\text{HS-LS}} \) of \( \sim 5 \text{ kcal mol}^{-1} \) which is negligible when consider the experimental uncertainty.
3.3.5. Conclusion

In summary the results presented here demonstrate several key features of the *trans* to *cis* isomerization of mepepy and photo-initiated spin crossover of the Fe(III)(salten)(mepepy) complex. First, the mepepy ligand exhibits a volume change equal to $\Delta V = \sim 0.7 \pm 0.3 \text{ mL mol}^{-1}$ which is associated with the loss of H-bond between a water and the mepepy pyridyl group. The enthalpy change, $\Delta H = \sim 33 \pm 10 \text{ kcal mol}^{-1}$, is associated to the cleavage of a positive charge-assisted H-bond between the water and/or acetonitrile and the protonated mepepy pyridinium group in addition to the loss of H-bond between a water and mepepy pyridyl group (Figure 3.17). Subsequent PAC studies of the Fe(III)(salten)(mepepy) complex suggest that the volume ($\Delta V = \sim 0.9 \pm 0.3 \text{ mL mol}^{-1}$) and enthalpy ($\Delta H = \sim 37 \pm 10 \text{ kcal mol}^{-1}$) changes are consistent with a mechanism in which the Fe(III)(salten)(mepepy) complex undergoes a *trans* to *cis* isomerization of the bound mepepy ligand, followed by the cleavage of an Fe–N bond associated with the salten ligand and high-spin to low-spin transition on the central iron (Figure 3.18). The secondary amine of the salten ligand forms hydrogen bond with a solvent water molecule subsequent to release from the iron coordination.
**Figure 3.17:** Thermodynamic profile for the photoinduced isomerization of the \textit{trans} to \textit{cis} mepepy in acetonitrile:water solution.

\[
\Delta H = -33 \text{ kcal mol}^{-1} \\
\Delta V = -1 \text{ ml mol}^{-1}
\]

**Figure 3.18:** Thermodynamic profile for the photoinduced Fe(III) salten mepepy complex in acetonitrile:water solution.

\[
\Delta V = -0.9 \text{ mL mol}^{-1} \\
\Delta H = -37 \text{ kcal mol}^{-1}
\]
3.3.6. References


3.3. Time Resolved Photoacoustic Calorimetry and Debye-Hückel Theory: Determining Electrostriction Associated with Excited State Ru(II)(L)₃ Complexes

3.3.1. Introduction

Thermodynamic and kinetic profiles are essential to understand reaction mechanisms associated chemical and biological processes. Reactions of molecules in solution are accompanied by changes, in bond length, charges, solvent coordination, etc… which will be reflected by a variation of the volume of the solvent. One of the challenges is to interpret the origin of the change by isolating the individual contributions to the reaction. The volume change of a molecule associated with a chemical reaction can be expressed as the sum of the change of the van der Waals radii and the change in solvation as the sum of electrostriction according to:

\[ \Delta V^o = \Delta V_{\text{intr}} + \Delta V_{\text{solv}} \quad \text{Equation 41} \]

where \( \Delta V_{\text{intr}} \) represents changes in bond lengths and angles between the reactants and the products which can be approximated by changes in the van der Waals volume and \( \Delta V_{\text{solv}} \) which considers volume changes associated with electrostriction due to changes in overall charge between the reactants and products [1]. To calculate \( \Delta V^o \), quantitative structure activity relationship (QSAR) can be used for the estimation of \( \Delta V_{\text{intr}} \) while computational methods including molecular dynamics (MD) simulations and quantum mechanical calculations can be used to estimate \( \Delta V_{\text{solv}} [2,3] \).

Molecular volume changes can be determined using MD experiments to establish the thermodynamic volume of solvated molecules (\( \Delta V_{\text{solv}} \)) along with time-dependent volume changes in the condensed phase [4,5]. Principally MD simulations calculate
isothermal-isobaric data for the solvent-solute system for a specific length of time to determine the volume of the system. The solute molecule is then removed from the solvent and the volume is then recalculated after equilibrium is achieved. The difference of these two volumes calculated from the initial and final states of the molecule corresponds to the thermodynamic volume ($\Delta V_{\text{solv}}$) of a solute. Unfortunately, MD simulations become more expensive with larger molecules or proteins as the time to perform the MD simulation increases with the size of the molecule. Experimental, $\Delta V_{\text{intra}}$ and $\Delta V_{\text{solv}}$ can be obtained by examining reaction volume changes as a function of ionic strength and using the Debye-Hückel equation.

The Debye-Hückel equation, originated from Peter Debye and Erich Hückel [6], expresses the activity coefficients of ions in solutions. Different interactions between ions show discrepancies even at very low concentration. These discrepancies between ideal solutions and dilute solution containing electrolytes are often caused by the interactions occurring between different ions. The Debye-Hückel equation calculates the activity coefficients which are proportional to the concentration of the electrolytes and include the energy of interaction between ions in solution. The Debye-Hückel equation is summarized below:

$$ V_i = V_i \ + \ (z_i^2 \ Av / 2b) \ \ln(1 + b\sqrt{I}) \quad \text{Equation 42} $$

where $V_i$ is the apparent partial molar volume of the ionic species expressed as a function of the actual ionic strength $I$ of the solution, $V_i$ is the partial molar volume at infinite dilution, $z_i$ is the ion charge, $b = 1.2(\text{kg/mol})^{1/2}$ and $A_v = 1.874 \ \text{cm}^3 \ \text{kg}^{1/2} \ \text{mol}^{3/2}$ at $25 \ ^\circ \text{C}$ [7].

In order to verify if the solvent excluded volume can be experimentally determined using the Debye-Hückel equation, the total volume change of a complex or molecule determined using PAC will be plotted versus the ionic strength of the solution. The partial molar volume at infinite dilution ($V_i$) and the ion charge ($z_i$) will be extracted. This hypothesis will be verified by using two ruthenium complexes, Tris-(2,2'-bipyridine)-ruthenium(II) (Ru(bpy)$_3$) and Tris-(1,10'-phenanthroline)-ruthenium(II)
(Ru(phen)$_3$), as the photochemical and photophysical properties of these two complexes have been extensively investigated [8-12].

Ru(bpy)$_3$ and Ru(phen)$_3$ complexes were of interest since they are known to absorb light in the visible region with a high intersystem crossing yield. They are also soluble in water and can include a functional group on the ligand that can be protonated to induce a proton transfer in the excited state [13]. After photoexcitation of the ruthenium complexes, a charge is delocalized from the metal to the ligand (bipyridine or phenanthroline) via a metal to ligand charge transfer (MLCT) with a lifetime between a few picoseconds to a few nanoseconds followed by intersystem crossing to the triplet state will follow and a slow return to the ground state with a lifetime around 600 ns for Ru(bpy)$_3$ and 900 ns for Ru(phen)$_3$ [14-16]. As the charge transfer between the metal and the ligand is reversible, the results ($\Delta V_i^+$ and $z_i$) for the Debye-Hückel equation between the fast and slow phases can be compared.

In addition, the two constants $A_v$ and $b$ from the Debye-Hückel equation will also be verified as they are dependent of the solvent and the ionic strength of the solution. This can be accomplished since the charge that is transferred is know.
3.3.2. Materials and Methods

Sample Preparation:

Ru(bpy)$_3$ and Ru(phen)$_3$ were purchased from Sigma-Aldrich and Fe$^{3+}$tetrakis(4-sulfonatophenyl porphine (Fe(III)4SP) was obtained from Frontier-Scientific, Inc. The samples of Ru(bpy)$_3$ and Ru(phen)$_3$ were solubilized in either 10mM, 50mM, 100mM, 150mM, 200mM, 250mM, 500mM, 750mM, 1M, 1.5M and 2M NaCl$_{aq}$ at pH 7.0.

The samples for PAC studies were placed in a 1cm quartz cuvette and sealed with a septum cap and subsequently purged for several minutes with argon. Optical spectra of the two ruthenium complexes were obtained using a Shimadzu UV-2401PC UV-Vis spectrophotometer.

3.3.3. Results

The UV-Vis spectra of Ru(bpy)$_3$ and Ru(phen)$_3$ with different concentrations of NaCl$_{aq}$ are similar with peaks maxima at 425 nm and 454 nm for Ru(bpy)$_3$ and at 421 nm and 448 nm for Ru(phen)$_3$. Thus, high concentrations of NaCl$_{aq}$ do not have a significant impact on the electronic structure of the Ru(bpy)$_3$ and Ru(phen)$_3$.

An overlay of PAC traces and fits for both Ru(bpy)$_3$ and Ru(phen)$_3$ and the calorimetric reference compound are displayed in Figure 3.19. The graphs show a frequently shift between sample and reference acoustics signals which indicates other kinetic events occurring between ~50 ns and ~20 μs in addition to events occurring in < 50 ns. In order to calculate the solvent excluded volume of both Ru(bpy)$_3$ and Ru(phen)$_3$, different concentrations of NaCl$_{aq}$ (10mM, 50mM, 100mM, 150mM, 200mM, 250mM, 500mM, 750mM, 1M, 1.5M and 2M) at pH=7 were compared with the purpose of probing electrostatic interactions upon excitation. The ΔVs calculated for each concentration, after deconvolution of the PAC traces, plotted as a function of ionic strength and fit to the Debye-Hückel equation (Scheme 3.5) to extract V$_i$ (partial molar volume at infinite dilution) and z$_i$ (the ion charge).
The variations in volume and enthalpy in the different buffers after excitation for both Ru(bpy)$_3$ and Ru(phen)$_3$ are very similar. After deconvolution of the frequently shift between sample and reference acoustic signals, a biphasic relaxation is observed for both Ru(bpy)$_3$ and Ru(phen)$_3$. The biphasic relaxation is attributed to a prompt phase $<50$ ns subsequent to the excitation and a slower phase with $\tau \sim 550$ ns for Ru(bpy)$_3$ and $\sim 900$ ns for Ru(phen)$_3$. The $\Delta H$ and $\Delta V$ associated with these processes are obtained after plotting $(S/R)E_{nu}$ versus $C_{p}/\beta$ (as per equation 9) (Figure 3.21). The average of these results between the different concentrations of NaCl$_{aq}$ are summarized in Table 3.3 for Ru(bpy)$_3$ and Table 3.5 for Ru(phen)$_3$. The processes occurring in $<50$ ns, between the different concentrations (10mM to 2M) of NaCl$_{aq}$, reveal a $\Delta H$ between 43 ± 4 kcal mol$^{-1}$ and 59 ± 1 kcal mol$^{-1}$ and a $\Delta V$ between -2.2 ± 0.1 mL mol$^{-1}$ and -0.5 ± 0.1 mL mol$^{-1}$ for Ru(bpy)$_3$ (Table 3.3). The prompt phase for Ru(phen)$_3$ gives similar results with a $\Delta H$ between 44 ± 1 kcal mol$^{-1}$ and 53 ± 0.3 kcal mol$^{-1}$ and a $\Delta V$ between -1.8 ± 0.1 mL mol$^{-1}$ and -0.5 ± 0.03 mL mol$^{-1}$ (Table 3.5). The slow phases give also similar results between Ru(bpy)$_3$ and Ru(phen)$_3$, with a $\Delta H$ between -43 ± 2 kcal mol$^{-1}$ and -55 ± 3 kcal mol$^{-1}$ and a $\Delta V$ between 1.6 ± 0.2 mL mol$^{-1}$ and 0.2 ± 0.1 mL mol$^{-1}$ for Ru(bpy)$_3$ (Table 3.3), and a $\Delta H$ between -43 ± 3 kcal mol$^{-1}$ and -54 ± 4 kcal mol$^{-1}$ and $\Delta V$ between 2.1 ± 0.2 mL mol$^{-1}$ and 0.6 ± 0.05 mL mol$^{-1}$ for Ru(phen)$_3$ (Table 3.5). These results also demonstrate that the variation of solution ionic strength has a slight affect the $\Delta V$ and $\Delta H$ of the charge transfer between the metal and the ligand.

After fitting the volume data to the Debye-Hückel equation, $V_i$ and $z_i$ can be extracted for Ru(bpy)$_3$ (Table 3.4) and Ru(phen)$_3$ (Table 3.6). The results show similarity between Ru(bpy)$_3$ and Ru(phen)$_3$, with, respectively, $V_i = -2.1 / -1.9$ mL mol$^{-1}$ for the prompt phase and 1.7 / 1.9 mL mol$^{-1}$ for the slow phase. $z_i$ also shows similarity between Ru(bpy)$_3$ and Ru(phen)$_3$, with respectively, 1.5 / 1.5 in the prompt phase and 1.4 / 1.3 in the slow phase.
3.3.4. Discussion

The PAC results which take into account the quantum yield (0.96) for the nonradiative decay of the metal ligand charge transfer (MLCT) states for Ru(bpy)$_3$ and Ru(phen)$_3$, show a biphasic relaxation, subsequent to excitation. The summarized results for Ru(bpy)$_3$ gives, for the prompt phase, a $\Delta H$ between 43 ± 4 kcal mol$^{-1}$ and 59 ± 1 kcal mol$^{-1}$ associated with a $\Delta V$ between -2.2 ± 0.1 mL mol$^{-1}$ and -0.5 ± 0.1 mL mol$^{-1}$ for the different concentrations (10mM to 2M) of NaCl(aq) at pH = 7. The slow phase (~ 550 ns) gives a $\Delta H$ between -43 ± 2 kcal mol$^{-1}$ and -55 ± 3 kcal mol$^{-1}$ and a $\Delta V$ between 1.6 ± 0.2 mL mol$^{-1}$ and 0.2 ± 0.1 mL mol$^{-1}$ (Table 3.3). These results can be compared with the $\Delta V$ and $\Delta H$ results already known and published for the Ru(bpy)$_3$ in water [17]. Miksovska and Larsen have shown that the $\Delta V$ and $\Delta H$ associated with the formation of the MLCT state for Ru(bpy)$_3$ at pH = 6 is equal to -3.5 ± 0.5 mL mol$^{-1}$ and 44 ± 2 kcal mol$^{-1}$ and the subsequent decay is equal to 3.2 ± 0.6 mL mol$^{-1}$ and -50 ± 3 kcal mol$^{-1}$, respectively. These results are also similar to the results reported by Borsarelli [18] and Goodman [19] as well as Cherry [20] who calculated the variation of volume and enthalpy of Ru(bpy)$_3$ from the emission maxima.
Figure 3.19: Overlay of the acoustic waves of the Ru(bpy)$_3$ (bleu dashed line), the reference Fe(III)4SP (black solid line), the fit (green dotted line) and the residue (red dashed and fit line). Ru(bpy)$_3$ in 10mM NaCl$_{aq}$ (pH 7.0) at 34°C.
Figure 3.20: Overlay of the acoustic waves of the Ru(phen)$_3$ (purple dashed line), the reference Fe(III)$_4$SP (black solid line), the fit (green dotted line) and the residue (red dashed and fit line). Ru(phen)$_3$ in 10mM NaCl$_{aq}$ (pH 7.0) at 34°C.
Table 3.3: Variations of the volume and enthalpy for Ru(bpy)$_3$ for different concentrations of NaCl$_{(aq)}$ (between 10mM to 2M) at pH=7.

<table>
<thead>
<tr>
<th>Conc (mM)</th>
<th>$\tau_1$ (ns)</th>
<th>$\Delta V_1$ (mL mol$^{-1}$)</th>
<th>$\Delta H_1$ (kcal mol$^{-1}$)</th>
<th>$\tau_2$ (ns)</th>
<th>$\Delta V_2$ (mL mol$^{-1}$)</th>
<th>$\Delta H_2$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM</td>
<td>1</td>
<td>-1.9 ± 0.4</td>
<td>43 ± 4</td>
<td>540</td>
<td>1.4 ± 0.2</td>
<td>-43 ± 2</td>
</tr>
<tr>
<td>50mM</td>
<td>1</td>
<td>-2.2 ± 0.1</td>
<td>53 ± 1</td>
<td>350</td>
<td>1.1 ± 0.2</td>
<td>-50 ± 2</td>
</tr>
<tr>
<td>100mM</td>
<td>1</td>
<td>-1.7 ± 0.1</td>
<td>54 ± 0.1</td>
<td>460</td>
<td>1.3 ± 0.2</td>
<td>-55 ± 2</td>
</tr>
<tr>
<td>150mM</td>
<td>1</td>
<td>-1.3 ± 0.1</td>
<td>50 ± 0.9</td>
<td>400</td>
<td>1.6 ± 0.2</td>
<td>-45 ± 3</td>
</tr>
<tr>
<td>200mM</td>
<td>1</td>
<td>-1.2 ± 0.2</td>
<td>52 ± 0.2</td>
<td>390</td>
<td>1.2 ± 0.2</td>
<td>-52 ± 3</td>
</tr>
<tr>
<td>250mM</td>
<td>1</td>
<td>-1.2 ± 0.2</td>
<td>56 ± 0.1</td>
<td>450</td>
<td>0.8 ± 0.1</td>
<td>-48 ± 1</td>
</tr>
<tr>
<td>500mM</td>
<td>1</td>
<td>-0.7 ± 0.1</td>
<td>57 ± 1</td>
<td>470</td>
<td>0.6 ± 0.3</td>
<td>-55 ± 3</td>
</tr>
<tr>
<td>750mM</td>
<td>1</td>
<td>-1.1 ± 0.2</td>
<td>55 ± 2</td>
<td>640</td>
<td>0.5 ± 0.4</td>
<td>-54 ± 7</td>
</tr>
<tr>
<td>1M</td>
<td>1</td>
<td>-0.6 ± 0.1</td>
<td>59 ± 1</td>
<td>690</td>
<td>0.3 ± 0.2</td>
<td>-55 ± 3</td>
</tr>
<tr>
<td>1.5M</td>
<td>1</td>
<td>-0.5 ± 0.1</td>
<td>58 ± 0.2</td>
<td>670</td>
<td>0.2 ± 0.1</td>
<td>-55 ± 2</td>
</tr>
<tr>
<td>2M</td>
<td>1</td>
<td>-0.5 ± 0.1</td>
<td>51 ± 0.9</td>
<td>770</td>
<td>0.2 ± 0.1</td>
<td>-49 ± 2</td>
</tr>
</tbody>
</table>
The $\Delta V$ of Ru(bpy)$_3$ increase with ionic strength of NaCl$_{aq}$ (Table 3.3) from $-1.9 \pm 0.4$ mL mol$^{-1}$ to $-0.5 \pm 0.1$ mL mol$^{-1}$ due impart to solvent electrostriction where the charge delocalizes from the metal to ligands after excitation. The results are in accord with previous studies of the formation of the MLCT state of the Ru(bpy)$_3$ $[19, 21]$. The negative charge after excitation is delocalized over the three bipyridines around the ruthenium ion. Goodman and Herman demonstrated that the small contraction is due to a shortening of $\sim 0.01$Å of the metal-ligand bond $[19]$. Borsarelli et al. show no dependence between the salt composition of the solution and the volume change of the MLCT formation. They confirm an internal rearrangement of the ruthenium complex after excitation $[22]$. In our studies, the same salt NaCl was chosen and the variation of the volume change of the MLCT formation was studied as a function of the solution ionic strength. It is not clear why increasing the ionic strength will give rise to an expansion. One explanation is that at low ionic strength, only a few positive charged atoms will have an electrostriction interaction around the Ru(bpy)$_3$ complex. Increasing the concentration of NaCl$_{aq}$, produces more Na$^+$ ions which will have a greater electrostriction interaction around the ruthenium complex thus increasing the contraction. The fact that the charge transfer between the ruthenium and the ligand is a partial charge transfer and not a total charge transfer might be the cause of the expansion and not the expected contraction observed when the ionic strength increases.

For the slow phase, the reverse phenomenon is observed. The slow phase exhibits a $\Delta V$ between $1.6 \pm 0.2$ mL mol$^{-1}$ and $0.2 \pm 0.1$ mL mol$^{-1}$ (Table 3.3) which is equal and opposite to the $\Delta V$ observed in the prompt phase. As explained earlier for the prompt phase, the larger the solution ionic strength, the less change in volume is observed. When the Ru(bpy)$_3$ complex relaxes to the ground state through a nonradiative deactivation, the metal-ligand bond will increase and the overall negative charge on the ligands recombine with the Ru(III) ion relaxing the electrostriction.

Similarly, $\Delta H$ which is between $43 \pm 4$ kcal mol$^{-1}$ and $59 \pm 1$ kcal mol$^{-1}$, is equal and opposite to the prompt phase, and shows a small dependence between the $\Delta H$ and the ionic strength of NaCl$_{aq}$. 

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A plot of $\Delta V$ versus the ionic strength of the solution can be plotted in the Debye-Hückel equation and both $V_i$ and $z_i$ can be extracted: $V_i = -2.1 \text{ mL mol}^{-1}$ for the prompt phase and $1.7 \text{ mL mol}^{-1}$ for the slow phase and $z_i = 1.5$ and $1.4$ respectively for the prompt and slow phase (Figure 3.21). The ion charge ($z_i$) of Ru(bpy)$_3$ which was calculated from the Debye-Hückel equation is equal to $1.5 \pm 0.2 \text{ mL mol}^{-1}$ for the prompt phase which is explained by a charge delocalization from the ruthenium atom to the bipyridine ligands (MLCT). An ion charge equal to $1.4 \pm 0.2 \text{ mL mol}^{-1}$ was calculated for the slow phase which correspond to the relaxation to the triplet state to the ground state through a nonradiative deactivation with a lifetime of $\sim 600$ ns for Ru(bpy)$_3$ as demonstrated previously [14,15]. Within experimental error, the results for $z_i$ can be considered close to 1 indicating that there is one charge transfer occurring in the prompt and slow phase. The results obtained for the excluded volume change are $V_i = -2.1 \text{ mL mol}^{-1}$ for the prompt phase and $1.7 \text{ mL mol}^{-1}$ for the slow phase. The results are summarized in Table 3.4.
$\Delta V = -2.1 \pm 0.1 \text{ mL mol}^{-1}$

$Z_1 = 1.5 \pm 0.2$

$\Delta V = 1.7 \pm 0.1 \text{ mL mol}^{-1}$

$Z_2 = 1.4 \pm 0.2$

Figure 3.21: Plot of $\Delta V$ versus $\ln(1+ b \sqrt{I})$ for the 1st phase (dark blue square) and 2nd phase (green dot) from Ru(bpy)$_3$ between 10mM and 2M NaCl(aq) (pH 7.0) which gives an intercept equal to the partial molar volume at infinite dilution $V_i^\circ$ (mL mol$^{-1}$) and the slope is equal to $(z_i^2 A_v / 2b)$, which will give the ion charge $z_i$.

Table 3.4: Summary of $V_i^\circ$ and $z_i$ for Ru(bpy)$_3$ from fits to the Debye-Hückel equation.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta V^\circ$ (mL mol$^{-1}$)</th>
<th>$z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st phase</td>
<td>$-2.1 \pm 0.1$</td>
<td>1.5 $\pm$ 0.2</td>
</tr>
<tr>
<td>2nd phase</td>
<td>$1.7 \pm 0.1$</td>
<td>1.4 $\pm$ 0.2</td>
</tr>
</tbody>
</table>

The experimental electrostriction volume change can be extracted from Scheme 3.4, as the solvent excluded volume changes were experimentally determined to be $\Delta V_{VDW} = -2.1 \text{ mL mol}^{-1}$ for the prompt phase and $1.7 \text{ mL mol}^{-1}$ for the slow phase, and the total volume change was calculated between $-1.9 \text{ mL mol}^{-1}$ and $1.4 \text{ mL mol}^{-1}$. The volume change due to electrostriction is then $\Delta V_{el \, Ru(bpy)_3} = -1.9 + 2.1 = 0.2 \text{ mL mol}^{-1}$ for
the prompt phase and $\Delta V_{el} \text{Ru(bpy)}_3 = 1.4 - 1.7 = -0.3 \text{ mL mol}^{-1}$ for the slow phase at 10 mM NaCl and $\Delta V_{el} \text{Ru(bpy)}_3 = -0.5 + 2.1 = 1.6 \text{ mL mol}^{-1}$ for the prompt phase and $\Delta V_{el} \text{Ru(bpy)}_3 = 0.2 - 1.7 = -1.5 \text{ mL mol}^{-1}$ for the slow phase at 2 M NaCl. The calculation for the experimental electrostriction change are opposite in sign and similar in magnitude between the fast and the slow phases for the Ru(bpy)$_3$.

The experimental electrostriction change can be compared to a theoretical value using the Drude-Nernst equation [23]. The Drude-Nernst equation describes the contraction of electrolytes around an ion [24]. Using the classical Drude-Nernst equation for electrostriction [25]:

$$\Delta V_{el} = -Bz^2 / r \quad \text{Equation 43}$$

where B is a constant with a calculated value in water of $4.175 \text{ Å mL mol}^{-1}$, r is the radius expressed in Angström's and z is the charge, the electrostriction volume change for Ru(bpy)$_3$ and Ru(phen)$_3$ can be calculated [26]. The Drude-Nernst equation gives for Ru(bpy)$_3$ where $r = 4.8\text{Å}$ [27], a $\Delta V_{el} = -3.5\text{mL mol}^{-1}$. The theoretical calculation of the change in electrostriction shows a higher value than the experimental value. This discrepancy which is higher at low ionic strength may be due to solvent effects associated with an increase in the concentration of Na$^+$ and Cl$^-$ ions that are not account in the theoretical value. At low ionic strength, the change in overall volume is mostly due to a van der Waals volume change as the metal-ligand bond shortening and only a few Na$^+$ atoms will interact with the overall negative charge of the ruthenium complex as explained earlier.
The results for the Ru(phen)$_3$ are similar to those obtained for Ru(bpy)$_3$ with a prompt phase associated with a $\Delta H$ between $44 \pm 1$ kcal mol$^{-1}$ and $53 \pm 0.3$ kcal mol$^{-1}$ and a $\Delta V$ between $-1.8 \pm 0.1$ mL mol$^{-1}$ and $-0.5 \pm 0.03$ mL mol$^{-1}$. The slow phase (~850 ns) gives a $\Delta H$ between $-43 \pm 3$ kcal mol$^{-1}$ and $-54 \pm 4$ kcal mol$^{-1}$ associated with a $\Delta V$ between $2.1 \pm 0.2$ mL mol$^{-1}$ and $0.6 \pm 0.05$ mL mol$^{-1}$ (Table 3.5). As explained earlier, Ru(phen)$_3$ behaves like Ru(bpy)$_3$ also showing that increase the solution ionic strength of creates a shortening of the metal-ligand bond in the prompt phase associated with a small electrostriction of the Na$^+$ atoms to the overall negative charge of the ligand around the ruthenium atom. As for Ru(bpy)$_3$, the variation of enthalpies show a very small dependence of the ionic strength change. This phenomenon is reversed in the slow phase data (Table 3.5).
Table 3.5: Variations of the volume and enthalpy for Ru(phen)$_3$ for different concentrations of NaCl(aq) (between 10mM to 1.5M) at pH=7.

<table>
<thead>
<tr>
<th>Conc (mM)</th>
<th>$\tau_1$ (ns)</th>
<th>$\Delta V_1$ (mL mol$^{-1}$)</th>
<th>$\Delta H_1$ (kcal mol$^{-1}$)</th>
<th>$\tau_2$ (ns)</th>
<th>$\Delta V_2$ (mL mol$^{-1}$)</th>
<th>$\Delta H_2$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM</td>
<td>1</td>
<td>-1.8 ± 0.1</td>
<td>50 ± 0.8</td>
<td>762</td>
<td>2.1 ± 0.2</td>
<td>-45 ± 1</td>
</tr>
<tr>
<td>50mM</td>
<td>1</td>
<td>-1.7 ± 0.2</td>
<td>44 ± 1</td>
<td>930</td>
<td>1.8 ± 0.3</td>
<td>-50 ± 2</td>
</tr>
<tr>
<td>100mM</td>
<td>1</td>
<td>-1.6 ± 0.06</td>
<td>49 ± 0.4</td>
<td>959</td>
<td>1.2 ± 0.2</td>
<td>-51 ± 1</td>
</tr>
<tr>
<td>150mM</td>
<td>1</td>
<td>-1.3 ± 0.1</td>
<td>51 ± 0.8</td>
<td>840</td>
<td>1.1 ± 0.2</td>
<td>-47 ± 2</td>
</tr>
<tr>
<td>200mM</td>
<td>1</td>
<td>-1.1 ± 0.02</td>
<td>51 ± 0.1</td>
<td>650</td>
<td>1.0 ± 0.1</td>
<td>-46 ± 0.8</td>
</tr>
<tr>
<td>250mM</td>
<td>1</td>
<td>-0.6 ± 0.04</td>
<td>53 ± 0.3</td>
<td>774</td>
<td>1.3 ± 0.4</td>
<td>-43 ± 3</td>
</tr>
<tr>
<td>500mM</td>
<td>1</td>
<td>-1.1 ± 0.05</td>
<td>53 ± 0.5</td>
<td>590</td>
<td>0.9 ± 0.3</td>
<td>-43 ± 3</td>
</tr>
<tr>
<td>750mM</td>
<td>1</td>
<td>-0.5 ± 0.03</td>
<td>52 ± 0.3</td>
<td>990</td>
<td>0.8 ± 0.4</td>
<td>-54 ± 4</td>
</tr>
<tr>
<td>1M</td>
<td>1</td>
<td>-0.6 ± 0.1</td>
<td>46 ± 1</td>
<td>700</td>
<td>1.3 ± 0.6</td>
<td>-46 ± 6</td>
</tr>
<tr>
<td>1.5M</td>
<td>1</td>
<td>-0.5 ± 0.02</td>
<td>52 ± 0.3</td>
<td>815</td>
<td>0.6 ± 0.05</td>
<td>-45 ± 1</td>
</tr>
</tbody>
</table>

The excluded volume change ($V_i$) was calculated to be equal to -1.9 mL mol$^{-1}$ for the prompt phase and 1.9 mL mol$^{-1}$ for the slow phase and the ion charge ($z_i$) to be equal to 1.5 ± 0.2 mL mol$^{-1}$ and 1.3 ± 0.4 mL mol$^{-1}$ for the prompt and slow phase, respectively (Figure 3.22). Similar to Ru(bpy)$_3$, the ion charge can be considered to be close to 1 for the fast and slow phases within experimental errors. As for Ru(pby)$_3$, the theoretical volume change can be calculated using the classical Drude-Nernst equation for Ru(phen)$_3$ where $r = 4.8\text{Å}$ [27], therefore $\Delta V_{el}$ is equal to -3.5 mL mol$^{-1}$. Finally, as it was shown earlier for Ru(bpy)$_3$, the volume change due to electrostriction effects can be extracted
from Drude-Nernst equation, which will give respectively, $\Delta V_{el \text{ Ru(phen)}3} = -1.8 + 1.9 = 0.1$ mL mol$^{-1}$ for the prompt phase and $\Delta V_{el \text{ Ru(phen)}3} = 2.1 - 1.9 = 0.2$ mL mol$^{-1}$ for the slow phase at 10mM NaCl and $\Delta V_{el \text{ Ru(phen)}3} = -0.5 + 1.9 = 1.4$ mL mol$^{-1}$ for the prompt phase and $\Delta V_{el \text{ Ru(phen)}3} = 0.6 - 1.9 = -1.4$ mL mol$^{-1}$ for the slow phase at 1.5M NaCl. As for Ru(bpy)$_3$, the calculation for the experimental electrostriction change are opposite in sign and similar in magnitude between the fast and the slow phases for Ru(phen)$_3$. As with the Ru(bpy)$_3$ complex, the theoretical calculation of the change in electrostriction shows a higher value relative to the experimental value. This may also be due to solvent effects associated with the increase of the concentration of ion in solution not accounted in the theoretical calculation.

**Figure 3.22**: Plot of $\Delta V$ versus $\ln(1+b\sqrt{I})$ for the 1$^{st}$ phase (purple square) and 2$^{nd}$ phase (orange dot) from Ru(bpy)$_3$ between 10mM and 1.5M NaCl$_{(aq)}$ (pH 7.0).
Table 3.6: Summary of $V^\circ$ and $z_i$ for Ru(phen)$_3$ from fits to the Debye-Hückel equation.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta V^\circ$ (mL mol$^{-1}$)</th>
<th>$z$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1$^{st}$ phase</td>
<td>$-1.9 \pm 0.1$</td>
<td>$1.5 \pm 0.2$</td>
</tr>
<tr>
<td>2$^{nd}$ phase</td>
<td>$1.9 \pm 0.2$</td>
<td>$1.3 \pm 0.4$</td>
</tr>
</tbody>
</table>

3.3.5. Conclusion

The confirmation that the Debye-Hückel equation can be used to calculate the solvent excluded volume of molecules, complexes and/or proteins was validated using two ruthenium complexes. These results show a charge transfer between the ligand and the metal for Ru(bpy)$_3$ and Ru(phen)$_3$ close to one as demonstrated in previous studies. In addition, the excluded volume change was calculated to be equal to $\sim -2.1$ and $-1.9$ mL mol$^{-1}$ for the prompt phase and $\sim 1.7$ and $1.9$ mL mol$^{-1}$ for the slow phase, for Ru(bpy)$_3$ and Ru(phen)$_3$ respectively.
3.3.6. References


Chapter IV- Signaling Proteins

4.1. Introduction

Heme proteins are a diverse class of metalloproteins that participate in a wide array of physiological processes including electron transfer (cytochromes), oxygenation (monooxygenases), hydrogen peroxide degradation (peroxidases, catalases), small molecule sensing (FixL, PAS domain sensors, and HemAT sensors), transcription regulation (CooA type proteins), energy transduction (heme/copper oxidases, cytochrome $bc_1$, etc.), oxygen transport and storage (hemoglobins and myoglobins) and polymer synthesis/degradation (lignan peroxidase, etc.) [1-4].

The versatility in heme protein functionality is derived largely from the structure of the surrounding protein matrix. The nature of the proximal iron ligand modulates the degree of ligand reactivity/binding affinity through electron back donation into $\pi$-accepting ligands. Distal heme pocket residues influence the orientation as well as the lability of ligands through H-bond interactions, hydrophobicity, etc. The protein tertiary structure also provides conduits for ligand access to and from the heme active site. These ligand channels provide ready access from the solvent to the distal heme pocket and can be modulated by conformational changes resulting in ‘gated’ ligand access. Examination of the crystal structures of a wide range of heme proteins with structurally variable distal heme pockets reveals complex networks of open pockets and access channels through which the gaseous ligands must traverse in order to bind to the heme iron [5-13].

Metalloproteins are a generic class of protein that contains a metal ion cofactor where iron and/or copper are the most common metal ions. Heme proteins are a subclass of iron metalloprotein that contain a prosthetic group which consists of an iron centered in a large heterocyclic organic ring or protoporphyrin IX. Heme proteins are one of the most widely distributed metalloproteins in nature. The protoporphyrin ring is formed with four
pyrrole rings linked by methene bridges and the iron can be in the ferrous (Fe2+) or the ferric (Fe3+) oxidation state. The protoporphyrin IX is linked to the protein via different amino acids. The most common heme is the heme b, although other hemes also important including heme a, c and o (Scheme 4.1).

Heme groups are linked to the protein with an axial ligand(s) binding derived from amino acids. The fifth axial position is typically a histidine. The proximal side or sixth position can be occupied by an external ligand (gas molecules). Heme protein sensors can reversibly bind small diatomic ligands including O₂, CO and NO [14-20]. Studies of the kinetics of ligand binding are important in order to understand the biological function of the heme protein. Ligand binding/release to the heme, the exchange of one internal/external ligand from the heme can result in activation of the protein (Scheme 4.2). The dissociation of the Fe-ligand bond can activate the process and the transmission of the signal. The signal will be transmitted along the protein by conformational change of the protein to the effector domain which will have an appropriate response to the external signal. The bond between the iron and the ligand can be photodissociated with high quantum yield. This photodissociation is not a physiological event but provides an initiation point for the process of the transmission of the signal along the protein.
Scheme 4.1: Major hemes
There are four classes of heme-based sensors:

- PAS domain

These proteins contain a heme inside a “four over one” helical fold (Figure 4.1). The proteins FixL and EcDos are two examples of the PAS domain which are oxygen sensor. The first example is the protein FixL from *Rhizobium meliloti* which was the first heme
oxygen sensor discovered and has a 55kDa PAS domain which can bind an oxygen molecule. The FixL protein is an oxygen-binding hemoprotein and regulates the expression of nitrogen fixation in Rhizobia. In the presence of $O_2$, the kinase activity of FixL is inhibited. In the absence of $O_2$, FixL will transmit a signal to FixJ via a phosphorylation-dephosphorylation reaction with FixJ controlling the expression of other regulatory genes, including nifA, which regulate the transcription of genes required for symbiotic nitrogen fixation \cite{25,26}. Secondly; the EcDos from \textit{E. coli} has a 17kDa heme binding PAS domain and a 90kDa phosphodiesterase domain. The protein exhibits phosphodiesterase activity and is likely to modulate the cyclic-di-GMP second messenger.

- Globin-Coupled sensor

\textbf{Figure 4.2: GCS domain (PBD entry 1or6)}

The myoglobin-like domain of globin coupled sensors is a recently discovered non-PAS domain protein. The heme is inside a “three over two” helical fold (Figure 4.2). HemAT which is an aerotactic transducer, is the best example for the myoglobin-like domain as it was the first myoglobin-like domain discovered in Archaea. This is also the first heme from the globin coupled sensor family in bacteria \cite{21-24}. 

95
HemAT also responds to O₂ molecule like FixL or EcDos. The two class examples of aerotaxis transducers: HemAT-\textit{Hs} from the salt-loving archaeon \textit{Halobacterium salinarum} with 489 residues and involved in the aerophobic response and HemAT-\textit{Bs} from the soil bacterium \textit{Bacillus subtilis} with 432 residues and involved in the aerophylic response. HemAT is a dimer where its N-terminal is close to sperm-whale myoglobin and its C-terminal is close in structure to the cytoplasmic signaling domain of Tsr, a methyl accepting chemotaxis protein from Escherichia Coli.

- CooA protein

\textbf{Figure 4.3: CooA domain (PBD entry 2hkx)}

CooA from \textit{Rhodospirillum rubrum} is a CO-sensing transcriptional activator (Figure 4.3). CooA which is close to the cAMP-receptor proteins (CRP) is a CO sensor process which activates expression of genes that govern the oxidation of CO to CO₂ [27].
• Heme nitric oxide binding (HNOB)

**Figure 4.4: HNOB domain (sGC) (PDB entry 1xbn)**

The soluble guanylyl cyclase (sGC) is a NO-sensing and catalyzes the formation of cyclic guanosine monophosphate (cGMP) (intracellular second messenger) from GTP (Figure 4.4). sGC is a heterodimeric enzyme composed of a mainly alpha-helical domain and has a proximal histidine binding to the heme.
4.2. References


4.3. Evidence for fast conformational change upon ligand dissociation in the HemAT class of bacterial oxygen sensors

4.3.1. Introduction

Globin coupled sensors (GCS) represent a unique class of oxygen sensing heme proteins found in both archaea and bacteria [1]. In comparison, unlike the more omnipresent PAS domain sensors found in bacteria, archaea and eukaryotes, which include an α/β type fold with an anti-parallel β-barrel structure that encapsulates the sensor element, GCS proteins contain an N-terminal heme sensor domain similar to a myoglobin (Mb) type fold [1-4]. These proteins also contain a C-terminal domain homologous to the cytoplasmic signaling domain of various eukaryotic type chemoreceptors. The first two members revealed in the GCS class of sensor were HsHemAT from the archaea Halobacterium salinarum and BsHemAT from the gram positive prokaryote Bacillus subtilis [2]. Both HsHemAT and BsHemAT share structural homology with the globin family. Specifically, both proteins have conserved His (F8), Pro (C2), and Phe (CD1) corresponding to amino acids Pro 56, His 123, Phe 69 (BsHemAT numbering). In addition, both proteins participate in aerotaxis responses in their respective organisms. HsHemAT has been implicated in an aerophobic response while BsHemAT is involved in an aerophylic response [2,5]. HsHemAT also undergoes methylation-dependent adaptation via CheR methyltransferase.

A recent crystal structure of the BsHemAT heme domain (BsHemATHD) demonstrated a myoglobin-like heme sensing domain with a more traditional ‘three over two’ helical fold typical of globins that is structurally distinct from PAS-domain heme sensors [6]. The crystal structure also revealed a dimer in the asymmetric unit similar to the EcDosH [7]. Similar to the FixL heme domains, structural differences between liganded and unliganded forms was modest. The most significant changes were the rotation of the proximal His (His 123) and displacement of a distal Tyr residue (Tyr 70).
These results suggest that structural changes associated with the signaling event are quite small or that larger transient conformational changes are not detected by X-ray structures.

Like other heme oxygen sensors, conformational changes associated with ligand binding/dissociation to heme sensing moieties are thought to initiate signaling in the GCS. Thus, characterizing those conformational changes is critically important to understand the signal transduction process. We have utilized transient absorption spectroscopy and time-resolved photoacoustic calorimetry (PAC) to examine the thermodynamics and conformational dynamics following CO photodissociation from Fe(2+)BsHemATHD.

### 4.3.2. Materials and methods

#### 4.3.2.1. Protein expression, isolation and purification

The open reading frame corresponding to the N-terminal 180 amino acids heme binding domain of HemAT (BsHemATHD) was amplified from *B. subtilis* genomic DNA (ATCC 23857D-5). The primers employed for PCR were

5′ATGTTATTTAAAAAAGAC3′ (forward) and 5′AAACGCTTCAAGGACAAGCAG3′ (reverse). The resulting PCR product was subsequently amplified and cloned into pDEST17 (Invitrogen) using Gateway technology (Invitrogen) according to the manufacturer’s instructions. The primers used for amplification were

5′GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATCGAGGGACGAATGTTATT TAAAAAAGAC3′ (forward) and

5′CCCCCACCACCTTTGTACAAGGAAGCTGGGT TTAAACAACGCTTCAAGGACAAGC AG3′ (reverse). The resulting plasmid was named pHemAT<sub>180</sub> and contains an N-terminal six residue histidine tag. The integrity of the HemAT open reading frame was verified by sequencing in the Washington State University LBB1.

pHemAT<sub>180</sub> was established in *Escherichia coli* strain BL21(DE3) and was used to express the HemAT histidine-tagged N-terminal domain protein. This strain also
contained pHPEX3 which expresses the hemin transporter ChuA (a kind gift from Professor D.C. Goodwin (2004), Auburn University. Protein Expr. Purif. 35, 76–83). The expression strain with two plasmids was maintained under dualampicillin (pHemAT180) and chloramphenicol (pHPEX) selection. For protein expression, “Terrific Broth” (Current Protocols in Molecular Biology, Ausubel et al., Eds.) was inoculated to $A_{560} = 0.05$ and incubated with shaking at 37°C until $A_{560}$ increased to 0.6–0.8. Protein expression was induced by addition of 0.5 mM IPTG and hemin was added to a final concentration of 50 μM. The temperature was reduced to 25°C and incubation was continued for 15 h.

Following induction and expression the cells were collected by centrifugation and resuspended in lysis/wash buffer (50 mM Na phosphate pH 8, 300 mM NaCl, 20 mM imidazole), lysed by two passages through a French pressure cell operated at 12 000 psi and cleared by centrifugation. The dark red supernatant was then applied to a Ni$^{2+}$-NTA column (Qiagen) and the column was washed extensively with buffer. Protein was eluted from the column with a 20–300 mM imidazole gradient in buffer. Protein fractions were pooled then concentrated and exchanged into other buffers using a centrifugal concentrator (Amicon Ultra). Protein purity was monitored by SDS–PAGE, MALDI-TOF Mass Spectrometry (WSU LBB2) and UV–Vis spectroscopy.

4.3.2.2. Sample preparation

Samples for PAC were prepared by diluting $B$sHemATHD from an ~150 μM stock into a buffer containing 50 mM sodium phosphate (pH 8.0) and 100 mM NaCl (the protein is predominantly in the homodimeric state under these conditions). The deoxy form of the protein was formed by placing the oxy form of $B$sHemATHD in a quartz optical cuvette that was then sealed with a septum cap and purged with Argon. Dithionite was added from a buffered stock solution to give a final concentration of ~100 μM. The CO-bound form was obtained by saturating solutions of the deoxy $B$sHemATHD with CO resulting in a final solution CO concentration of 1 mM (1 atm pressure). The protein
concentration for PAC samples was ~75 μM while those for transient absorption were ~25 μM. The equilibrium binding of CO to deoxy BsHemATHD was performed by titrating aliquots of CO saturated buffer (1 mM) into a solution containing ~10 μM deoxy BsHemATHD. The change in absorption at 420 nm was monitored and plotted versus [CO]. The $K_a$ value for CO binding was obtained using:

$$\Delta A_{420\text{nm}} = K_a \Delta \varepsilon_{420}[\text{ BsHemATHD}]_0[\text{CO}] / (1 + K_a[\text{CO}])$$  \hspace{1cm} \text{equation 30}

where $\Delta \varepsilon_{420\text{ nm}}$ is the change in molar extinction at 420 nm (obtained from the fit) and $[\text{ BsHemATHD}]_0$ is the initial concentration of deoxy $\text{ BsHemATHD}$. Equilibrium UV–Vis spectra were obtained using a Shimadzu UV2401 spectrophotometer.

4.3.3. Results

The recombinant heme domain of $\text{ BsHemAT}$ displays an optical spectrum with a Soret maximum at ~410 nm and visible bands at 578 nm ($\alpha$-band) and 538 nm ($\beta$-band) (Figure 4.5). Upon deoxygenation the Soret band shifts to 425 nm with a broad visible band centered at 555 nm. Incubation of the deoxy protein in the presence of CO results in a Soret band at ~418 nm and visible bands at 573 nm and 535 nm. These spectral changes were utilized to determine the association constant for the recombinant $\text{ BsHemATHD}$ (Figure 4.6). Fitting a plot of $\Delta A_{420\text{ nm}}$ versus CO concentration to a single binding equilibrium yielded a $K_a$ value of $(1.5 \pm 0.5) \times 10^6$ M$^{-1}$ consistent with previous studies of this construct (Alam and Larsen, unpublished results) but is slightly lower than that determined previously by Zhang et al. who reported a value of $6 \times 10^6$ M$^{-1}$ [9].
Figure 4.5: Equilibrium optical absorption spectra of the $B_s$HemATHD: as isolated (red dash line), deoxy$B_s$HemATHD (blue dot line) and CO$B_s$HemATHD (black solid line). $B_s$HemATHD concentration: $\sim$10 $\mu$M in 50 mM sodium phosphate (pH 8.0) and 100 mM NaCl.
Figure 4.6: Equilibrium CO binding titration for BsHemATHD. Absorbance changes were measured at 420 nm. Sample concentrations are those described in Figure 4.5.

Preliminary results [21] on the HemAT demonstrated that the heme domain has two distinct O₂ binding components. In addition, the O₂ rebinding is biphasic with a dissociation constant equal to $K_d = 1$-$2 \ \mu$M, associated with a rate constant $k_{O2} = 50$-$80 \ \text{s}^{-1}$, and a dissociation constant $K_d = 50$-$100 \ \mu$M associated with a rate constant $k_{O2} = 2000 \ \text{s}^{-1}$ for both the first phase and second phase, respectively. In contrast, the CO binding and rebinding to the heme is monophasic. The association rate constant is $k'_{CO} = 0.2$-$0.5 \ \mu$M$^{-1}$ s$^{-1}$ which is very close to the rate constant of the whale myoglobin $k'_{CO} = 0.51 \ \mu$M$^{-1}$ s$^{-1}$.
Figure 4.7 and Figure 4.8 represent the single-wavelength kinetic trace for the O$_2$ and CO rebinding to the heme. In order to calculate the rate constant of the O$_2$ binding to the heme, the CO-bound form of BsHemAT was photodissociated in presence of O$_2$ in the solution. As the rate constant for O$_2$ rebinding to the heme is faster than the rate constant for CO, O$_2$ will bind first and the rate constant for O$_2$ binding to the heme can be estimated. As O$_2$ is in larger excess, the rate constant was assumed to be pseudo first order and the trace was fit to a single exponential decay. The rate constant of O$_2$ binding was estimated to be 0.00264 s$^{-1}$. This concludes that the rate constant of O$_2$ is more than six times faster than the rate constant of CO binding. As it was mentioned earlier, Phillips et al. [21] demonstrated a biphasic relaxation of O$_2$ rebinding with two dissociation rate constant equal to k$_{O2} = 50$-80 s$^{-1}$ and k$_{O2} \approx 2000$ s$^{-1}$. The biphasic relaxation for the O$_2$ rebinding the heme demonstrated by Phillips’ group, is different to the monophasic relaxation estimated in this paragraph. This divergence can be due to a difference in the preparation of the HemAT heme domain.

The following Eyring’s equation was used to extract the activation energy of O$_2$ binding. The activation of enthalpy was calculated equal to 8 kcal mol$^{-1}$ and the entropy of energy equal to -20 kcal mol$^{-1}$. Using the following Phillip’s equation, the rate constant of the O$_2$ dissociation was estimated.

$$k'_{O2} = \frac{(k_{obs} \times k'_{CO} \times [CO]) - k'_{CO} \times [CO])}{[O2]} \quad \text{Equation 31}$$

where $k_{obs}$ is the rate constant of O$_2$ rebinding, $k'_{CO}$ is the rate constant of CO rebinding, [CO] is the concentration of CO in solution and [O$_2$] is the concentration of O$_2$ in solution. Phillip’s equation gave a rate constant of the O$_2$ dissociation equal to $k'_{O2} = 166.9 \mu$M$^{-1}$s$^{-1}$.

Finally using the following Eyring’s equation: $R\ln(h \times k'_{O2} / k_B T) = \Delta S^\# - \Delta H^\#(1/T)$, the enthalpy of dissociation of O$_2$ was estimated equal to 15.5 kcal mol$^{-1}$ and the entropy of dissociation equal to 4.4 cal mol$^{-1}$K$^{-1}$ (Figure 4.9) [22,23].
Photolysis of the CO bound BsHemATHD results in the formation of a five coordinate high spin heme complex which decays back to the pre-flash CO bound complex with a pseudo first-order rate constant of 59 s$^{-1}$ (the corresponding second-order rate constant is $5.9 \times 10^4$ M$^{-1}$ s$^{-1}$ with a solution of CO concentration equal to 1 mM). This value is close to the one reported by Zhang et al. ($4.3 \times 10^5$ M$^{-1}$s$^{-1}$) suggesting that the difference in $K_a$ values for CO between Zhang et al. and the present study is due to the values of $k_{	ext{off}}$ (0.07 s$^{-1}$ vs. 0.04 s$^{-1}$ from Zhang et al. and the present study, respectively) [9]. In addition, the kinetic optical difference spectrum obtained equal to $\sim$4 μs subsequent to photolysis displays a bathochromic shift relative to the equilibrium optical difference spectrum suggesting an un-relaxed heme pocket geometry subsequent to photolysis (Figure 4.8). Since the concentration of transient five coordinate heme decays mono-exponentially the un-relaxed heme pocket must persist for $\sim$milliseconds. From the temperature dependence of the recombination rates the activation enthalpy and entropy were found to be 6 kcal mol$^{-1}$ and -28 cal mol$^{-1}$ K$^{-1}$, respectively (Figure 4.10). The PAC results, which can probe molar volume and enthalpy changes over a time scale from $\sim$50 ns to $\sim$20 μs [8], reveals a $\Delta H$ of $-19 \pm 5$ kcal mol$^{-1}$ and $\Delta V$ of 4 ± 1 mL mol$^{-1}$
occurring in <50 ns subsequent to CO photolysis (Figure 4.11). No additional kinetic events were observed within the PAC time scale. The value of $\Phi$ for CO photolysis from the ferrous form of $B$sHemATHD has previously been determined to be $0.90 \pm 0.1$ (Alam and Larsen, unpublished results).

Figure 4.8: Top panel: Single wavelength transient absorption trace for CO rebinding to $B$sHemATHD.

Bottom panel: Overlay of the kinetic (squares, obtained 4 ms subsequent to photolysis) and equilibrium (solid line) difference spectra (deoxy$B$sHemATHD minus CO$B$sHemATHD).
Figure 4.9: Eyring plot for O$_2$ recombination to deoxy $B$sHemATHD.

Figure 4.10: Eyring plot for CO recombination to deoxy $B$sHemATHD.
Figure 4.11: *Top panel:* Overlay of photoacoustic traces for CO-BsHemATHD (solid line) and the Fe$^{3+}$ tetraakis-(4-sulphonatophenyl)porphyrin calorimetric reference (dotted line). *Bottom panel:* Plot of $\phi E_{hv}$ vs. $(C_p \rho / \beta)$, which gives an intercept equal to the heat, $Q$, returned to the solvent giving $\Delta H = (E_{hv} - Q) / \Phi$ (kcal mol$^{-1}$) and $\Delta V = \text{slope} / \Phi$ (mL mol$^{-1}$).
4.3.4. Discussion

The asymmetry of monomer units observed in the X-ray crystal structure of the CN$^-$ bound and free forms of Fe$^{2+}$ BsHemATHD provided the basis for an initial signaling mechanism [6]. The X-ray structure reveals that the conformation of one monomer within the asymmetric dimer contains a Tyr 70 located within the distal heme pocket and within H-bonding distance to the heme bound ligand while in the other monomer Tyr 70 is rotated $\sim$100° out of the distal pocket. This is accompanied by a 14° rotation of the F helix which also affects the orientation of the proximal histidine (His 123). It has been further suggested that the presence of two conformations within the homodimer accounts for the observed high and low affinity binding sites for O$_2$ and biphasic O$_2$ recombination kinetics with the high affinity site (faster rebinding phase) that in which Tyr 70 is located within the distal pocket and the low affinity site (slower rebinding phase) that with the solvent exposed Tyr 70 [9]. In contrast, CO rebinding, subsequent to photolysis, exhibits monophasic kinetics suggesting that Tyr 70 does not significantly influence the stability of the Fe$^{2+}$–CO complex (Figure 4.12).
More recent resonance Raman results have also been used to construct models for both O₂ and CO binding to the heme domain of BsHemAT that involve H-bond formation between the bound ligand and Thr 95 [10-12]. The resonance Raman data indicates multiple conformations in the O₂ bound form of the protein as evident by multiple Fe²⁺–O₂ bending modes in the wild-type sensor domain (554 cm⁻¹, 566 cm⁻¹ and 572 cm⁻¹). These were assigned to two ‘open’ conformations (566 cm⁻¹ and 572 cm⁻¹ bands) which are responsible for the lower oxygen affinity (higher O₂ off-rates) and a ‘closed’ conformation (554 cm⁻¹ band) with a higher O₂ affinity. In the Thr 95A mutant only one
Fe$^{2+}$-$\text{O}_2$ bending mode is observed at 569 cm$^{-1}$. Examination of the X-ray structure for the deoxy form of the protein reveals that the distance between the Thr 95 hydroxy group and the heme iron is $\sim$7.2 Å and this residue must move $\sim$4 Å to move within the $\sim$3 Å required for an H-bond with the bound ligand. Thus, in the case of O$_2$ binding, in which H-bonding occurs between Thr 95 and the bound oxygen molecule, a significant conformational change must take place that relocates the Thr 95 residue.

In contrast, resonance Raman results indicate that CO binding does not result in H-bonding between Thr 95 and the bound CO since no shifts were observed in the Fe$^{2+}$–CO bending mode in the Thr 95Ala mutant. However, FTIR results by Pinakoulaki et al. [13] suggest that the CO bound form of the sensor domain contains both non-H-bonded and strongly H-bonded conformers (as determined from $\nu_{\text{C-O}}$) in both wild-type and Thr 95Ala mutants. However, mutation of Tyr 70 to Phe perturbs the non-H-bonded $\nu_{\text{C-O}}$ (shifts from 1967 cm$^{-1}$ to 1962 cm$^{-1}$) and produces a moderately H-bonded conformation ($\nu_{\text{C-O}} \sim$ 1931 cm$^{-1}$). In a second FTIR study Pinakoulaki et al. [14] proposed a second CO binding site on the proximal side of the heme group near Tyr 133 giving rise to a $\nu_{\text{C-O}} \sim$ 2069 cm$^{-1}$. Photolysis results in a slight increase in intensity of this mode indicating an increase in site occupancy. It was proposed that this site can serve as a conformational gate for ligand access to the heme.

The transient difference spectrum as well as the PAC results, indicate that, photodissociation of CO results in a non-equilibrium heme environment subsequent to photolysis and that no further conformational changes take place between $\sim$50 ns and $\sim$20 µs. In addition, the fact that the decay of the five coordinate heme is monophasic further indicates that the non-equilibrium heme pocket persists on the ms time scale. Formation of the non-equilibrium five coordinate conformation also gives rise to enthalpy and molar volume changes of $-19 \pm 5$ kcal.mol$^{-1}$ and $\Delta V$ of $4 \pm 1$ mL.mol$^{-1}$, respectively. The observed volume and enthalpy changes may have contributions from photocleavage events localized at the heme ($\Delta V_{\text{heme}}$ and $\Delta H_{\text{heme}}$), H-bond changes in the distal heme pocket (as described above) ($\Delta V_{\text{distal}}$ and $\Delta H_{\text{distal}}$), ligand migration to non-
heme binding pockets (ΔV_{NHBP} and ΔH_{NHBP}) and/or more global conformational changes in the heme sensing domain (ΔV_{conf} and ΔH_{conf}) as follows:

\[ ΔV_{obs} = ΔV_{heme} + ΔV_{distal} + ΔV_{NHBP} + ΔV_{conf} \quad \text{Equation 44} \]

\[ ΔH_{obs} = ΔH_{heme} + ΔH_{distal} + ΔH_{NHBP} + ΔH_{conf} \quad \text{Equation 45} \]

Upon photolysis of the BsHemATHD the Fe^{2+}CO bond is cleaved (ΔH_{FeC} ~ 15 kcal mol\(^{-1}\)) and the heme iron undergoes a change in spin state (from low-spin to high-spin, ΔH_{LS-HS} ~ <1 kcal mol\(^{-1}\)) giving an ΔH_{heme} ~ 15 kcal mol\(^{-1}\) [8]. In addition, if CO diffuses out of the heme pocket and into the bulk solvent on this time scale an additional ΔH_{CO-solv} ~ 5 kcal mol\(^{-1}\) would also contribute putting ΔH_{heme} on the order of ~20 kcal mol\(^{-1}\). The ΔH_{distal} contribution would likely arise from H-bond interactions between the bound CO and distal pocket residues as observed in the FTIR studies described above. The similarity in vibrational frequencies between the COMb and COBsHemATHD indicates similar H-bond strengths which, for COMb, occur between the bound CO and a distal His residue [15,16]. The H-bond energy between CO and the imidazole ring of His 64 in COMb has been calculated to be ~5 kcal mol\(^{-1}\) making ΔH_{distal} roughly this value (i.e., for a single CO–H bond). The ΔH_{NHBP} term is more difficult to estimate since it is not clear to what extent the non-heme binding pocket in BsHemATHD is occupied at room temperature and within ~20 μs subsequent to photolysis. Assuming similar extinction coefficients for the CO stretch of the heme bound CO and the CO bound in the non-heme binding pocket proposed by Pinakoulaki et al. [14] the non-heme binding site represents roughly 2% of the total protein bound CO. Upon steady-state photolysis this population increases by ~1.5 % which would not be detectable by PAC. The low occupancy of this site subsequent to photolysis suggests that a majority of the photodissociated CO leaves the heme pocket on a ~20 μs time scale. Thus, the ΔH_{NHBP} term is likely to be negligible (i.e., outside of the PAC detection range). Using the relation ΔH_{obs} = 25 kcal mol\(^{-1}\) + ΔH_{conf} allows for the estimation of ΔH_{conf}. The fact that the observed enthalpy is -19 kcal.mol\(^{-1}\) indicates an additional exothermic process of ~ -44 kcal mol\(^{-1}\) is taking place in <50 ns, which must be due to a
more global conformational change within the heme domain and/or distal residue–ligand interactions. These results are summarized in Figure 4.13 where the thermodynamic profile demonstrated the proposed conformational change.

![Figure 4.13: Thermodynamic profiles for the conformational change associated with the Fe(2+)BsHemATHD photodissociation (PDB entry 1or6).](image)

Similar arguments can be made for the molar volume changes in which the estimated changes are: $\Delta V_{\text{heme}} = \Delta V_{\text{FeC}} + \Delta V_{\text{LS-HS}} = \sim 5 \text{ mL mol}^{-1} + \sim 10 \text{ mL mol}^{-1}$ and for CO diffusing from the heme pocket into the solvent: $\Delta V_{\text{CO-solv}} \sim 11 \text{ mL mol}^{-1}$ giving a total $\Delta V_{\text{heme}} \sim 26 \text{ mL mol}^{-1}$ [6]. Once again, based upon a low occupancy for the non-heme binding site the $\Delta V_{\text{NHBP}}$ is assumed to be negligible. The contribution of $\Delta V_{\text{distal}}$ to $\Delta V_{\text{obs}}$ is also difficult to estimate. As an upper limit the value would be that assigned to the Fe–C bond cleavage since typical bond cleavage processes have a $\Delta V$ of $\sim 5 \text{ mL mol}^{-1}$. However, since the H-bonding is located in the distal heme pocket rather than
exposed to the solvent the actual contribution is likely to be much smaller than this value. With this in mind $\Delta V_{\text{obs}} = 26 \text{ mL mol}^{-1} + \Delta V_{\text{conf}}$ and since $\Delta V_{\text{obs}} = 4 \text{ mL mol}^{-1}$, $\Delta V_{\text{conf}}$ is estimated to be $-22 \text{ mL mol}^{-1}$ (36.5 Å$^3$ / molecule).

The $\Delta V_{\text{conf}}$ and $\Delta H_{\text{conf}}$ terms could arise from (1) a change in overall charge distribution on the protein (i.e., change in net protein dipole leading to solvent reorganization), (2) formation of one or more salt-bridge interactions (the release of electrostricted water molecules upon salt-bridge formation results in volume increases) and/or (3) increases in the solvent accessible van der Waals volume of the protein upon photolysis. Examination of the crystal structures for the Fe(3+)BsHemATHD and CN–Fe(3+)BsHemATHD reveals that ligand binding results in a change in the solvent accessible surface (SAS) of $-1282 \text{ Å}^3$ (772 mL.mol$^{-1}$ per dimer) (i.e., the unliganded protein has a larger SAS than that of the CN-bound protein) which is 1.8% of the total SAS of the dimer. Changes in solvation associated with the change in SAS could account for the observed volume and enthalpy changes provided a similar change in SAS occurs in the COFe(2+)BsHemATHD to Fe(2+)BsHemATHD since surface residues are likely to experience significant changes in solvent exposure subsequent to the change in SAS.

The photothermal results further demonstrate clear differences in the dynamics of CO release from either horse heart Mb or the heme domain of FixL. In the case of FixLH from *B. japonicum* photolysis of CO results in an endothermic (12 kcal mol$^{-1}$), relatively small contraction (-1 mL mol$^{-1}$) occurring in $<50$ ns that is followed by a second relaxation with $\tau \sim 150$ ns and $\Delta H$ and $\Delta V$ of 5 kcal mol$^{-1}$ and 5 mL mol$^{-1}$, respectively [17]. The fast phase dynamics were attributed to solvent perturbations arising from possible salt-bridge reorganization (Glu 182-Arg 227) subsequent to ligand release while the slow phase was attributed to ligand escape to the solvent. In the case of horse heart Mb, two thermodynamic phases are also observed upon photolysis with a small endothermic contraction (7 kcal mol$^{-1}$ and -3 mL mol$^{-1}$) occurring in $<50$ ns (similar to *BjFixLH*) which has been suggested to arise from solvation of charge formed by disruption of a salt-bridge between one of the heme propionate groups and Lys 45 (Arg 45 in sperm whale Mb) [18-20]. A corresponding slow phase with $\tau \sim 600$ ns and $\Delta H$ and
ΔΨ of 8 kcal mol$^{-1}$ and 4 mL mol$^{-1}$ is also observed that has been partly attributed to the salt-bridge reformation.

The extent to which conformational differences between ligation states participate in signal transduction is unclear. This is due to the fact that, to date, only oxygen activity of HemATs in whole cells (either *Halobacter salinarum* or *B. subtilis*) have been examined due to the fact that an assay for signaling activity in vitro has not been developed. Thus, the extent to which differences between the various H-bond networks involving CO and O$_2$ actually contribute to the signaling initiation/propagation is unclear. Both changes in heme conformation as well as perturbations to the distal H-bonding sites upon ligand binding are likely to contribute to signal initiation/propagation.

### 4.3.5. Conclusion

In summary, both transient absorption and PAC reveal a fast conformational transition associated with ligand release from *Bs*HemATHD. A conformational transition results in a non-equilibrium deoxy form of the protein that persists during the ligand rebinding phase. The thermodynamics of this transition are quite distinct from the PAS domain *Bj*FixL heme domain suggesting quite different signaling mechanisms between the GCS and PAS domain sensors.
4.3.6. References


4.4. Thermodynamics of conformational changes coupled to CO photodissociation from the CO-sensing transcriptional activator CooA

4.4.1. Introduction

Heme protein sensors that are regulated by the binding of diatomic molecules such as O₂, CO or NO are more and more studied in order to understand their mechanisms. CooA, a transcriptional activator, found in the purple non-sulfur photosynthetic bacterium, *Rhodospirillum rubrum*, was the first protein showing a heme as a prosthetic group for transcriptional activation [1]. Previous studies utilized CO as a probe to study the biochemical and biophysical properties of heme protein, because it was thought to have no physiological relevance. Recently, it has been shown that when CO binds to the heme, it involves a physiologically activation of CooA. The activated CooA is responsible of transcriptional regulation for the expression of Coo operons that allow *R. rubrum* to grow using CO as its sole energy source [2].
Figure 4.14: Ribbon structure of CooA. Heme pocket amino acid residues, Pro 2 and His 77 are displayed (PDB entry 1ft9).

Figure 4.14 represents the structure of the reduced form of CooA identified by Lanzilotta et al. [3]. CooA is a homodimer with 221 amino acid residues in each subunit (24.6 kDa). Each subunit contains a protoheme that can sense CO. The amino acid sequence analysis using X-Ray crystallography confirmed that CooA is a member of the cyclic adenosine monophosphate (cAMP) receptor protein (CRP) and fumarate and nitrate reductate protein (FNR) transcriptional regulators family [3-5]. The folding of reduced CooA is very similar to a global transcriptional regulator in *E. coli* another member of the CRP family. The b-type heme containing the sensor domain (amino-terminal region) and DNA-binding domain (carboxy-terminal region), which are linked together with a hinge region (130-140), form the monomer of the CooA [6,7]. The DNA binding domain is a helix-turn-helix (HTH) motif which recognizes the binding site of the DNA target [8]. Transcriptional activation occurs when CooA binds to the DNA binding site, thereby controlling the growth of the *Rhodospirillum rubrum*. The transcriptional activator can be active only when CO binds to the heme sensor of CooA.
[6,7,9,10]. The activation of CooA is unique. Lanzilotta et al. also show that the N-terminal of the proline of one subunit will bind with the heme of the other subunit. CooA is the first and only metalloprotein showing a proline residue binding to the metal of a heme. The heme in CooA has different axial ligands in the resting and activated forms. The ferric form of CooA shows the 6-coordinate with Pro 2 in the distal side and Cys 75 in the proximal side. After reduction of the iron to the ferrous form, Cys 75 is replaced by His 77. Then, in presence of CO under anaerobic conditions, CO replaces Pro 2 in the distal side. The different steps during the activation of CooA are summarized in Scheme 4.3.

Scheme 4.3: The schemes for the change in the coordination structure of the heme in wild-type CooA with associated lifetimes.
Using optical absorption spectra, Nakajima et al. show that intermediate 2 is formed in a fast process after reduction of intermediate 1 [11]. Intermediate 3 is within 40 μs after reduction of intermediate 1, formed after an elongation of the Fe-S bond (sulfur atom) from the Cys 75 binding to the heme and/or the protonation of the thiolate residue from the Cys 75 [12,13]. The ligand Cys 75 will then exchange with the ligand His 77 in 2.9 ms, which corresponds to the slower process and form intermediate 4. This slow step implies a large conformational change of the protein in order to allow the exchange of the ligand. This event was verified by X-Ray crystallography where the crystal structure of intermediate 4 shows a distance of 4.8 Å between the Cys 75 and the iron from the heme [3]. This exchange of ligand between Cys 75 and His 77 requires a relative flexibility of the polypeptide chain in the proximal side of the heme. This is confirmed by looking at the residues around the heme and the different possibilities to form hydrogen bonds. Only two residues can form a hydrogen bond between each other (Gly 43 and Met 76) in CooA, compared to an average of seven hydrogen bonds found in Cytochrome c and Myoglobin where no ligand exchange occurs [11]. It was also reported that the ligand exchange between Cys 75 and His 77 is regulated by a hysteresis in the reduction (-320 mV) and the oxidation (-260 mV) potentials of the heme [14,15]. In presence of CO and in anaerobic conditions, Pro 2 on the heme’s distal side is replaced by CO to form intermediate 5. Photolysis of CO leads to intermediate 6. Uchida et al. demonstrate a biphasic geminate recombination of CO to the heme within ~70 ps (~60%) and ~300 ps (~30%) after photolysis to form intermediate 7 [16]. They also show that the geminate recombination of CO has a yield of ~90% in the 1.9 ns after photolysis. This hypothesis was confirmed by Rubtsov et al [17] and Kumazaki et al. [18]. To compare, myoglobin shows a geminate recombination of CO of only 4% [19]. This difference between CooA and myoglobin can be explained by a crowded heme pocket of CooA which operates as a barrier to the release of CO to the solvent. Finally, Pro 2 will rebind to the heme and form intermediate 8 in milliseconds after the geminate recombination of CO to the heme [20].
Conformational changes associated with ligand binding/dissociation to the heme are thought to initiate signaling in heme sensor families. Therefore, the characterization of those conformational changes is essential in order to understand the signal transduction process. We have utilized time-resolved photoacoustic calorimetry (PAC) to examine the thermodynamics and conformational dynamics following CO photodissociation from Fe(2+)CooA (Figure 4.15).

**Figure 4.15:** Structural diagram of the CO photodissociation from the heme domain of CooA.

### 4.4.2. Materials and Methods

*Sample preparation:*

Samples for PAC were prepared by diluting CooA into a buffer containing 25 mM MOPS and 0.1M NaCl (pH 7.4). The CO-bound form was obtained by saturating a solution of oxy CooA with CO resulting in a final CO concentration of 1 mM (1 atm pressure). CO will bind the heme after reduction of the Fe(III) form of CooA. The deoxy form of the protein was formed by placing the oxy form of CooA in a quartz optical cuvette that was then sealed with a septum cap and purged with Ar. A freshly prepared solution of sodium dithionite was added from a buffered stock solution to give a final
concentration of protein for PAC samples of ~9.5 μM. Optical spectra of the various species were obtained using a Shimadzu UV-2401PC spectrophotometer.

4.4.3. Results and Discussion

A hypothesis suggested that CooA is activated after the conformational change of the amino acids chain following the dissociation of Pro 2 due to the binding of CO to the heme. It has been demonstrated that this hypothesis is wrong by Nakajima et al. [14] and Thorsteinsson et al. [21] who used different truncated CooA mutants to show that the CooA mutants behave as an activated wild-type CooA. This confirms that the proline dissociated to the heme, initiates the CO-dependent activation but is not necessary to the activation of CooA.

It has also been demonstrated by Resonance Raman (RR) spectroscopy that the Fe-His 77 bond in CooA is weak compared a Fe-His 93 in myoglobin. The weakness of this bond might explain, after CO binding, the displacement of the heme in the interior of the protein into an adjacent cavity associated with a movement of the C-helix which might be part of the activation process of CooA [22,23]. The movement of the C-helix will allow the DNA-binding site to reorient in order to interact with the DNA and activate the transcription. In fact, recent DFT studies by Xu et al. demonstrated that $\nu_{FeC}/\nu_{CO}$ values show a weak hydrogen bond of the imidazole from the His 77, which would favor the movement of the heme [24].

It has also been observed that CO binding to the heme inactivated involves a reorientation of the C-helix in-vcluding the entire heme pocket and the amino acid terminus [3, 25-27]. Ibrahim et al. predict using UVRR spectroscopy a displacement of 2.05 Å of the heme inside the heme pocket [20]. Yamamoto et al. confirmed using $^1$H NMR that the ligand Pro 2 is displaced by at least 4 Å from its initial location after CO binding to the heme [28]. This confirms the bent conformation of the protein due to a large conformational change of the C-helix to a high affinity form following the CO
binding to the heme. Coyle et al. demonstrate that CO, when binding to the heme, interacts with residues from other subunit such as Ile 113, Gly 117 and Leu 120 and a residue from the same subunit, Leu 116 involving a small rotation of the two C-helices and the two hemes [22]. This theory confirms the hypothesis from Akiyama et al. which show a rotation of ~ 8° between both subunits using X-Ray spectroscopy [29]. Kubo et al. show, using UVRR spectroscopy, that displacement of the C-helix following the CO binding to the heme disrupts a hydrogen bond between the charged residues Arg 118 from subunit A and Asp 72 from subunit B. This disruption creates a more constricted and negative electrostatic field around the protein. Kubo et al. also confirm the findings of Coyle et al. [22] that movement of Trp 110 inside the protein follows the conformational changes associated with the C-helix. Finally, Lanzilotta et al. [3] identified a salt-bridge between two residues from the hinge region, Arg 138 from subunit A and Glu 59 from subunit B. Movement of the C-helix following CO binding to the heme disrupts this salt-bridge, which was confirmed by Ibrahim et al., using UVRR spectroscopy, signifying that the hinge can bend and facilitate the rearrangement of the C-helix in order to bind to the DNA site [20].

CooA displays an optical spectrum with a Soret maximum at ~422 nm and visible bands at 540 nm (α-band) and 570 nm (β-band) in the oxidized (activated) form (Figure 4.16). The protein was reduced with sodium dithionite in the presence of CO resulting in a Soret band bathochromic shifted at ~424 nm and visible bands at 540 nm and 570 nm.
Puranik et al. calculated the overall quantum yield following the complete photodissociation of CO and the release of CO to the solvent using the quantum yield of myoglobin [30]. The quantum yield for CooA was calculated equal to 0.02 [31].

PAC as discussed previously, can probe molar volume and enthalpy changes over a time scale from ~50 ns to ~20 μs [32]. Figure 4.17 displays an overlay of PAC traces for CO-CooA and the calorimetric reference compound obtained in 25 mM MOPS and 0.1M NaCl (pH 7.4) at 14°C. The fact that no frequency shifts are observed between sample and reference acoustics signals indicates no kinetic events between ~50ns and ~20μs. Moreover, the difference in amplitudes observed between the sample and the reference indicates that the photodissociation of CO involved conformational changes in the protein associated with changes in energy and volume between ~50ns and ~20μs. A plot of $(S/R)E_{hv}$ versus $C_p\rho/\beta$ (equation 9) reveals a $\Delta H$ and $\Delta V$ associated with CO
photodissociation from the Fe(II)CooA, equal to $\Delta H \sim -400 \pm 30$ kcal mol$^{-1}$ and $\Delta V \sim -11 \pm 4$ mL mol$^{-1}$ occurring (Figure 4.18).

**Figure 4.17:** Overlay of the acoustic waves for photolysis of CO from CooA (purple dotted line) and the reference Fe(III)4SP (black solid line).
Figure 4.18: Plot of $(S/R)^* E_{hν}$ versus $C_p \rho / \beta$ for CO photolysis from CooA 25 mM MOPS and 0.1M NaCl (pH 7.4) between 8°C and 32°C which gives an intercept equal to the heat, $Q$, returned to the solvent giving $\Delta H = (E_{hν} - Q)/\Phi$ (kcal mol$^{-1}$) and $\Delta V = \text{slope}/\Phi$ (mL mol$^{-1}$).

The observed volume and enthalpy variations may have contributions from photocleavage events localized at the heme ($\Delta V_{\text{heme}}$ and $\Delta H_{\text{heme}}$), and/or conformational change of the protein:

\[
\Delta H_{\text{obs}} = \Delta H_{\text{heme}} + \Delta H_{\text{conf}} \quad \text{Equation 46}
\]

\[
\Delta V_{\text{obs}} = \Delta V_{\text{heme}} + \Delta V_{\text{conf}} \quad \text{Equation 47}
\]

The volume and enthalpy changes for CO photodissociation from CooA were reported. PAC results for CO photodissociation give an average $\Delta H$ of $-400 \pm 30$ kcal mol$^{-1}$ associated with an average $\Delta V$ of $-11 \pm 4$ mL mol$^{-1}$ for CooA in 25 mM MOPS and 0.1M NaCl (pH 7.4). These results can be compared to volume and enthalpy changes for CO photodissociation from Fe(II) porphyrin model systems. Photolysis of CO from an
Fe(II)heme gives rise to three contributions to $\Delta H$ and $\Delta V$: the cleavage of the Fe-CO bond, spin state change of the iron from the low spin to the high spin configuration and solvation of CO as it diffuses away from the heme. Thus, the total changes of enthalpy and molar volume can be rewritten as:

$$\Delta H_{\text{heme}} = \Delta H_{\text{Fe-CO}} + \Delta H_{\text{LS-HS}} + \Delta H_{\text{COsolv}} \quad \text{Equation 48}$$

$$\Delta V_{\text{heme}} = \Delta V_{\text{Fe-CO}} + \Delta V_{\text{LS-HS}} + \Delta V_{\text{COsolv}} \quad \text{Equation 49}$$

Upon photolysis, the variation in enthalpy for a CO photodissociation from an iron porphyrin has been estimated to be $\Delta H \sim 14 \text{ kcal mol}^{-1}$ where $\Delta H_{\text{Fe-CO}} = 17 \text{ kcal mol}^{-1}$ (cleavage of Fe$^{2+}$CO bond), $\Delta H_{\text{LS-HS}} = <1 \text{ kcal mol}^{-1}$ (heme iron going from low-spin to high-spin) and $\Delta H_{\text{COsolv}} = -3 \text{ kcal mol}^{-1}$ (diffusion of CO out of the heme pocket and into the bulk solvent) [33,34].

Using the above relation $\Delta H_{\text{obs}} = \Delta H_{\text{heme}} + \Delta H_{\text{conf}}$ where $\Delta H_{\text{heme}} \sim 14 \text{ kcal mol}^{-1}$ and $\Delta H_{\text{obs}} \sim -400 \text{ kcal mol}^{-1}$, $\Delta H_{\text{conf}}$ can be estimated equal to $-414 \text{ kcal mol}^{-1}$ and indicate an additional exothermic process taking place in $<50 \text{ ns}$ after CO photolysis. The result of $\Delta H_{\text{conf}}$ must be due to a more global conformational change of the protein. The overall thermodynamic profile for CO photodissociation is summarized in Figure 4.19.
The variation of volume for CO photodissociation from an iron porphyrin can be estimated in the same way: $\Delta V_{\text{Fe-CO}} = -5 \text{ mL mol}^{-1}$ (increase of the solvent accessible area), $\Delta V_{\text{LS-HS}} = -10 \text{ mL mol}^{-1}$ (increase of the electron density associated with the 3d$_x^2$ orbital of the iron) and $\Delta V_{\text{COsolv}} = -20 \text{ mL mol}^{-1}$ [40]. The three contributions give a $\Delta V_{\text{heme}} = -35 \text{ mL mol}^{-1}$. Once again, based on the above equation, $\Delta V_{\text{obs}} = \Delta V_{\text{heme}} + \Delta V_{\text{conf}}$ where $\Delta V_{\text{heme}} = 35\text{ mL mol}^{-1}$ and $\Delta V_{\text{obs}} = -11 \text{ mL mol}^{-1}$, $\Delta V_{\text{conf}}$ can be estimated to be $-46 \text{ mL mol}^{-1}$ (73 Å$^3$ / molecule).

The results of $\Delta V_{\text{conf}}$ and $\Delta H_{\text{conf}}$ can take place first, if there is a change in overall charge distribution on the protein (i.e., change in net protein dipole leading to solvent reorganization), secondly if there is formation of one or more salt-bridge interactions (the
release of electrostricted water molecules upon salt-bridge formation results in volume increases) and/or thirdly if there is an increase in the solvent accessible van der Waals volume of the protein upon photolysis.

Until now, the X-ray crystal structure of the Fe(2+)CooA is the only form found in the literature. As the X-ray crystal structure of the COFe(2+)CooA is not yet solved, the changes in solvation, associated with the change in the solvent accessible solvent (SAS) between the CO liganded and unliganded protein, can not be calculated in order to confirm the experimental results. However, the surface residues are likely to experience significant changes in solvent exposure subsequent to the conformational change following the photodissociation of CO to the protein.

The photothermal results of horse heart myoglobin, the heme domain of FixL or the heme domain from the BsHemAT show large difference associated with the photodissociation of CO from the protein. The results from the heme domain of FixL from Bradyrhizobium japonicum show a biphasic relaxation after the photodissociation of CO. Subsequent to the CO release, the fast phase, with a $\Delta H$ of 12 kcal mol$^{-1}$ and $\Delta V$ of -1 mL mol$^{-1}$, is associated to the reorganization of the solvent following a perturbation of the salt-bridge between Glu 182 and Arg 227. The slow phase with a lifetime $\tau \sim 150$ ns, a $\Delta H$ of 5 kcal mol$^{-1}$ and $\Delta V$ of 5 mL mol$^{-1}$ is associated to the escape of the CO molecule to the solvent [35]. In the case of horse heart myoglobin, a biphasic relaxation is also observed after photodissociation of CO. The first phase with a $\Delta H$ of 7 kcal mol$^{-1}$ and $\Delta V$ of -3 mL mol$^{-1}$ was associated to the solvation of a charge after cleavage of the salt bridge between the Lys 45 (Arg 45 in sperm whale myoglobin) and the heme propionate groups. The slow phase with a lifetime of $\tau \sim 600$ ns, a $\Delta H$ of 8 kcal mol$^{-1}$ and $\Delta V$ of 14 mL mol$^{-1}$ was partly associated to the reformation of the salt-bridge [36-38]. Finally, in the case of BsHemAT, a monophasic relaxation was observed following the photodissociation of CO. The results, associated with a $\Delta H$ of -19 kcal mol$^{-1}$ and $\Delta V$ of 4 mL mol$^{-1}$, are consistent with an increase of the SAS of the protein after comparing the X-ray structure of the BsHemAT between the CN liganded and unliganded forms of the protein [39].
The large $\Delta H$ and $\Delta V$ following the photodissociation of CO to the heme domain of CooA is likely associated to the displacement of the heme into the interior of the protein with the reorientation of the C-helix involving the entire heme pocket and the amino acid terminus. This movement will allow the DNA-binding site to reorient in order to interact with higher affinity to the DNA. In addition to the event, the disruption of a hydrogen bond between two charged residues Arg 118 from the subunit A and Asp 72 from the subunit B due to the reorientation of the C-helix is also involved in the large $\Delta H$ and $\Delta V$. This disruption is in accord with the contraction of the solvent around the protein. Finally, the large $\Delta H$ and $\Delta V$ is also associated to the disruption of the salt-bridge between two residues Arg 138 from the subunit A and Glu 59 from the subunit B following the movement of the C-helix. All these different events upon photolysis of the CO are likely to contribute to the signal initiation/propagation and activation of CooA (Figure 4.20).

Figure 4.20: Detail of the two heme-binding domain of CooA showing the Pro 2, His 77, the two salt-bridges: Asp 72 / Arg 118 and Glu 59 / Arg 138 (PDB entry 1ft9).
4.4.4. Conclusion

In summary, PAC results reveal a fast conformational change associated with the photodissociation of CO from CooA. The conformational change results in a large enthalpy and volume change which were associated to a reorientation of the C-helix in addition to the disruption of a salt-bridge. The thermodynamics results of the CooA are different from the results of the globin coupled sensor (GCS) HemAT and the PAS heme domain \( BJ \)FixL. Although the CooA is a full protein compared to the \( BJ \)FixL or HemAT heme domain protein, the results show a faster change following the photodissociation of CO implying different signaling mechanisms between proteins from the same family such as PAS domain, or compared to different family such as GCS.
4.2.5. References


4.5. Photothermal studies of Carbon Monoxide Ligand Photodissociated from FixL Sinorhizobium meliloti

4.5.1. Introduction

In general, nitrogen is necessary for plant growth. The development of plants is dependent on its symbiotic relationship with Rhizobium which will activate nitrogenase and fix nitrogen. The activation of the nitrogenase is possible in restrictive conditions such as low levels of oxygen [1]. In the biological oxygen sensors domain, FixL is well known because it is involved in the regulation of nitrogen fixation gene expression in Rhizobia [2]. FixL is comprised of three different domains: a transmembrane domain, a sensor domain and a kinase domain [1]. The kinase domain is similar to other sensor histidine kinases and is formed by ~240 amino acid residues [3,4]. On the other hand, the sensor domain is different compared to the other sensor histidine kinases. The sensor domain also called the heme domain is formed by ~150 amino acid residues [5] and activates the kinase domain by autophosphorylation of His 285 with adenosine triphosphate (ATP) after photodissociation of the oxygen from the ferrous heme (low tension of oxygen) [6,7]. FixL will then control the activity of FixJ, which is the transcriptional activator, by transferring the γ-phosphate group. FixJ will then activate the gene nifA and fixK transcription [7]. Finally, these genes are responsible for activating the proteins that will generate the production of other genes necessary to the fixation for nitrogen [7].

FixL is a monomer with two distinct domains where the N-terminal PAS heme domain is the heme based oxygen sensor and the C-terminal is the histidine kinase domain [2,3,6,8]. The sensor heme domain is a single iron (III) protoporphyrin IX held into place by four α-helices and five β-strands [6]. The ligand binding center is a high-spin five-coordinate in the absence of an exogenous ligand and is low-spin six-coordinate when an exogenous ligand is bound to the heme [9]. The heme is surrounded by a
hydrophobic pocket where the imidazole from a histidine is the fifth ligand of the heme and the distal side is crowded by hydrophobic groups such as Ile 209, Leu 230 and Val 232. It has been proposed that O₂ association to the sensor heme domain can involve conformational changes that can be transmitted to the kinase domain. FixL from *Bradyrhizobium japonicum* (*Bj*) and *Synorhizobium meliloti* (*Sm*) also named *Rhizobium meliloti* (*Rm*) are the two most studied FixLs. The crystal structure of FixL from *B. japonicum* and *S. meliloti* are very similar [10-14]. The crystal structure of FixL from *B. japonicum* with different ligands binding to the heme for instance CO, NO, O₂ is described in the literature [15,16]. In our knowledge, no crystal structure of FixL from *S. meliloti* with bound ligand has been published. Even if the heme pocket between *Bj*FixL and *Sm*FixL are similar, the nomenclature is different between both of them, for instance, the histidine linked on the proximal side of the heme pocket has for nomenclature His 200 in *Bj*FixL and His 194 in *Sm*FixL (Figure 4.21).
Tanaka et al suggested that the residues Arg 200, Arg 208, Ile 209, and Ile 210 on the salt-bridge and the hydrogen-bond network contribute to the stabilization of the $O_2$ binding to the heme. They also demonstrated that the residue Arg 214 is involved in the regulation of the kinase activity [15]. Following $O_2$ fixation to the sensor domain, the heme goes from a high-spin five coordinate to a low-spin six coordinate which results in a decrease in the activity of the kinase. A decrease in the activity of the kinase is also observed after the binding of CO or NO to the heme, involving a change of the iron from high-spin to low-spin six coordinate. This implies that the iron spin state change of the heme is involved in the kinase activity [17,18]. When $O_2$ is photodissociated from the heme sensor, the kinase domain is undergoes changes which activate FixJ. Two different pathways have been proposed based on the crystal structures of the heme domains in order to activate FixJ. First, Gong et al. have observed in $Bj$FixL, a rearrangement of the hydrogen bond network between the heme 6,7 propionates and Arg 206 and His 214.

**Figure 4.21: Heme domain of the SmFixL (PDB entry 1ewo).**
amino acids residues due to the flattening of the heme after the binding of the ligand [11,12]. On the other hand, Miyatake et al. [19,20] have observed in SmFixL, a very crowded distal pocket which implies that O2 can not bind to the heme without interacting with three residues: Ile 209, Leu 230 and Val 232. This implies then that the steric repulsion between Ile 209 and O2 causing conformational changes of the distal pocket of SmFixL, and subsequently transmission of the signal and regulation of the kinase activity (Figure 4.21). Mukai et al. [21] have also suggested that Ile 209 and Ile 210 play an important role in the signal transduction between the heme sensor and the kinase. They showed that the conformational changes associated with the binding of O2 due to steric repulsion regulated the kinase activity. Balland et al. showed that Arg 220 from BjFixL is important in the signal transduction as it interacts with O2 bound to the heme. The displacement of Arg 220 inside the heme or the fixation of a strong ligand in that position is not responsible for the conformational changes of the heme but only the formation of a strong hydrogen-bonding network between Arg 220 and the ligand O2 [22]. Energetic profiles for BjFixL by Miksovska et al. demonstrated a biphasic relaxation after photodissociation of CO from the heme. The first phase involved a contraction of the solvent associated to the change in charge distribution after the reorganization of the salt-bridge between Glu 182 and Arg 227 or a possible reorientation of the Arg 206 after photodissociation of CO from the heme. The second phase with a lifetime of 150 ns was contributed to an expansion of the solvent due to the ligand released to the solvent [9]. Ayers and Moffat, and Cusanovich and Meyer suggested that the signal originated from the heme domain can be propagated through the linker to the kinase by quaternary structural changes via a distortion of the β-sheet [23,24]. Finally, Reynolds et al. demonstrated the importance of the subunit Arg 200 (R200 for SmFixL nomenclature) in the stabilization of the kinase inhibition related to the oxy form of the SmFixL [25]. They showed a clear relation between the H-bond of the R200 and the 6-propionate heme group that stabilized the inactive form of SmFixL due to O2 binding the heme (Figure 4.21).

To further investigate, the importance of the H-bond between the R200 subunit and the heme 6-propionate group, time resolved photothermal methods (including
photoacoustic calorimetry) were used to determine energetic profiles on R200 mutants on fast (\(\sim\) ns-ms) time scales \[25\]. The thermodynamics and conformational dynamics following CO photodissociation from different Fe(2+)SmFixL were studied using photoacoustic calorimetry (PAC) by resolving the magnitudes and timescales of molar volume and enthalpy changes associated with physiological processes. We can utilize the binding of small molecules, such as carbon monoxide, to determine the mechanism through which ligands leave the heme pocket after photodissociation and how ligand photodissociation activates the nitrogenase through transmitting the signal to the kinase by conformational changes of the protein. The binding of carbon monoxide to the reduced heme will form a six coordinate low spin iron complex. Although the CO-bound ferrous form of SmFixL is not a physiologically active form of the enzyme, studies of CO photodissociation can provide insights into the mechanism of the transmission of the signal into the kinase including the role of relevant amino acids in the transmission. In this report, the thermodynamic profiles for the ligand escaping from the heme active site of the heme domain in SmFixLH, the SmFixL wild type (SmFixLWT) and four different mutants (R200A(Alanine), R200Q(Glutamine), R200E(Glutamate), R200H(Histidine)) in Tris buffer is presented in order to have a range of polarities and H-bond abilities to compare to the wild-type SmFixL. Another mutant, I209M(Methionine) was also studied as Mukai et al. showed that the conformational changes associated with Ile 209 subunit is involved in the kinase activity (Figure 4.21) \[21\]. To date, no thermodynamic profile for the photo-release of CO molecule from a wild type FixL has been described in the literature. Previous thermodynamic studies done by Miksovska et al. determined a thermodynamic profile of the heme domain of the BjFixL after CO photodissociation but nothing on SmFixL was done \[9\].
4.5.2. Materials and Methods

Sample preparation

Samples for PAC were prepared by diluting SmFixL protein into a buffer containing 20 mM Tris (pH 8.0). The deoxy form of the protein was formed by placing the oxy form of SmFixL in a quartz optical cuvette that was then sealed with a septum cap and purged with Argon gas. A fresh dithionite solution was added from a buffered stock solution to give a final concentration of ~6 μM. The CO-bound form was obtained by saturating solutions of the deoxy SmFixL with CO resulting in a final solution CO concentration of 1 mM (1 atm pressure). The protein concentration for PAC samples was ~5 μM while those for transient absorption were ~9 μM.

4.5.3. Results

The different SmFixL wild type and mutants (R200A, R200Q, R200E, R200H) display similar optical spectra regardless of the different mutations with a Soret maximum at ~418 nm and visible bands at ~543 nm and ~577 nm in the oxidized form (Figure 4.22, 4.23).
Figure 4.22: Equilibrium optical absorption spectra of SmFixLWT (top panel) and SmFixLR200Q (bottom panel) as isolated (red dash line), reduced SmFixL (blue dot line) and reduced CO bound SmFixL (black solid line). SmFixLWT and SmFixLR200Q concentration: ~5 μM in 20 mM Tris (pH 8).
**Figure 4.23**: Equilibrium optical absorption spectra of SmFixLH as isolated (red dash line), reduced SmFixLH (bleu dot line) and reduced CO bound SmFixLH (black solid line).

*SmFixLH* concentration: ~5 μM in 20 mM Tris (pH 8).

The different proteins can be reduced with sodium dithionite, resulting in a Soret band bathochromically shifted to ~434 nm and a broad visible band at ~566 nm. The binding of CO to the ferrous enzymes results in a Soret band at ~424 nm and visible bands at ~542 nm and ~572 nm. The mutants I209M and SmFixL heme domain show a different optical spectrum only in the oxidized form. The mutant I209M displays a Soret band at ~434 nm and a broad visible band centered at ~557 nm and SmFixLH displays a Soret band at ~400 nm and a broad visible band centered at ~510 nm. The Soret and Q bands wavelengths for the wild type, R200A, R200Q, R200E, R200H, I209M mutants and heme domain SmFixLH are summarized in Table 4.1. The fact that the optical absorption spectra of the various forms of SmFixLWT and mutants (R200A, R200Q,
R200E and R200H) are independent of the nature of the mutations associated with a range of polarities and H-bond abilities indicates that the salt-bridge between the heme 6,7 propionates and R200 within the distal pocket near the heme group does not have a significant impact on the electronic structure of the heme group.

**Table 4.1:** Summary of the Soret and Q bands wavelengths for wild type, R200A, R200Q, R200E, R200H, I209M mutants and heme domain of SmFixLH.

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</tbody>
</table>

Photolysis of CO bound to SmFixLWT and the five different mutants (R200A, R200Q, R200E, R200H and I209M) probed at 450 nm results in the formation of a five coordinate high-spin heme complex which decays back to the pre-flash CO bound complex (Figure 4.24) with monophasic relaxation kinetic. The first-order rate constant obtained were \(~2.68 \times 10^2\) s\(^{-1}\) for SmFixLWT and \(~2.63 \times 10^2\) s\(^{-1}\), \(~2.74 \times 10^2\) s\(^{-1}\), \(~2.42 \times 10^2\) s\(^{-1}\), \(~2.47 \times 10^2\) s\(^{-1}\) and \(~3.02 \times 10^2\) s\(^{-1}\) for the mutants R200A, R200Q, R200E, R200H and I209M, respectively (the corresponding second-order rate constant is
26.8 M$^{-1}$ s$^{-1}$, 26.3 M$^{-1}$ s$^{-1}$, 27.4 M$^{-1}$ s$^{-1}$, 24.2 M$^{-1}$ s$^{-1}$, 24.7 M$^{-1}$ s$^{-1}$, 30.2 M$^{-1}$ s$^{-1}$ with a solution CO concentration of 1 mM) (Table 4.2). The different rate constants associated to the CO rebinding to the SmFixL wild type and the five different mutants are very similar to each other. The rate constant associated with CO rebinding to SmFixLI209M is slower which might be associated to the fact that the methionine can form a hydrogen bond between CO molecule and the sulfur group compare to the alkyl group in isoleucine which has a steric repulsion with CO.

**Figure 4.24:** Single wavelength transient absorption data for CO recombination to SmFixLWT, SmFixLR200A, SmFixLR200Q, SmFixLR200E, SmFixLR200H and SmFixLI209M at 25°C. Excitation wavelength was 532 nm (<20 ps, 20 mJ/pulse, 20 Hz). Sample solution conditions are the same as those reported in **Figure 4.22.** Time scale: 40ms.
Table 4.2: Summary of the rate constant associated with CO rebinding to wild type, R200A, R200Q, R200E, R200H, I209M and heme domain of SmFixL.

<table>
<thead>
<tr>
<th>FixL</th>
<th>t₁ (s)</th>
<th>k₁ (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTA</td>
<td>0.0268 ± 0.0001</td>
<td>37.3</td>
</tr>
<tr>
<td>R200A</td>
<td>0.0263 ± 0.0001</td>
<td>38</td>
</tr>
<tr>
<td>R200Q</td>
<td>0.0274 ± 0.0001</td>
<td>36.5</td>
</tr>
<tr>
<td>R200E</td>
<td>0.0242 ± 0.0001</td>
<td>41.3</td>
</tr>
<tr>
<td>R200H</td>
<td>0.0247 ± 0.0004</td>
<td>40.5</td>
</tr>
<tr>
<td>I209M</td>
<td>0.0302 ± 0.0004</td>
<td>33.1</td>
</tr>
</tbody>
</table>

Table 4.3: Summary of the CO rebinding rate constant for BjFixLH, truncated BjFixLH and SmFixLH.

<table>
<thead>
<tr>
<th>CO rebinding rate constants</th>
<th>BjFixLH140-270</th>
<th>BjFixLH151-256</th>
<th>SmFixLWT, R200A, R200Q, R200E, R200H and I209M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10.2 ± 0.3 s⁻¹</td>
<td>17.3 ± 0.1 s⁻¹</td>
<td>Between 33 to 41 s⁻¹</td>
</tr>
</tbody>
</table>

Table 4.3 summarized the rate constant for CO rebinding to the heme for the BjFixLH₁₄₀₋₂₇₀, BjFixLH₁₅₁₋₂₅₆, SmFixLWT and the five different mutants R200A, R200Q, R200E, R200H and I209M. BjFixLH₁₄₀₋₂₇₀ show a CO rebinding rate constant equal to 10.2 ± 0.3 s⁻¹, BjFixLH₁₅₁₋₂₅₆ equal to 17.3 ± 0.1 s⁻¹ and SmFixLWT and the five different mutants between 33 to 41 s⁻¹. The results for SmFixLWT and the five mutants show a faster CO rebinding rate constant than the one observed for BjFixLH₁₄₀₋₂₇₀ and BjFixLH₁₅₁₋₂₅₆. These results indicate that the full protein SmFixL accelerated the
rebinding of CO and they also demonstrate that the mutation of R200 and I209 does not affect the rebinding of CO to the heme to compare to the full protein SmFixL wild type.

Figure 4.25: Overlay of the acoustic waves for the photolysis of CO from SmFixLWT (top panel), SmFixLR200Q (bottom panel) (cyan dotted line) and the reference Fe(III)4SP (black solid line).
Figure 4.25 displays an overlay of PAC traces for CO-SmFixLWT, CO-SmFixLR200Q and the calorimetric reference compound obtained in 20 mM Tris buffer, pH 8.0. The fact that a frequency shift is observed between sample and reference acoustic signals indicates kinetic events occurring between ~50 ns and ~20 μs. The deconvolution of the acoustic wave between the sample and the reference demonstrates four different phases for the SmFixLWT and the five different mutants. The reaction volume and enthalpy changes were calculated using an Φ of 0.86 determined by Rodgers et al. [16].

The plot of (S/R)E_{hv} versus C_{pρ/β} (as per equation 9) reveals a ΔH and ΔV associated with CO photodissociation from the Fe(II)SmFixLWT of -9 ± 8 kcal mol^{-1} and 10 ± 3 mL mol^{-1}, respectively for the first phase, 18 ± 16 kcal mol^{-1} and -18 ± 6 mL mol^{-1}, for the second phase, -35 ± 20 kcal mol^{-1} and 11 ± 6 mL mol^{-1}, for the third phase and 31 ± 25 kcal mol^{-1} and -9 ± 8 mL mol^{-1}, for the fourth phase (Figure 4.26). In order to understand the energetic impact of the salt-bridge between the R200 and the heme 6,7 propionates group compared to the wild type, different mutants were synthesized by Dr Reynolds’s group. The ΔH and ΔV associated with CO photodissociation from the Fe(II)SmFixLR200A are -7 ± 6 kcal mol^{-1} and 15 ± 2 mL mol^{-1}, respectively for the first phase, 63 ± 20 kcal mol^{-1} and -6 ± 4 mL mol^{-1}, for the second phase, 51 ± 20 kcal mol^{-1} and 28 ± 6 mL mol^{-1}, for the third phase and 18 ± 11 kcal mol^{-1} and -10 ± 3 mL mol^{-1}, for the fourth phase. The ΔH and ΔV associated with CO photodissociation from the Fe(II)SmFixLR200Q are -26 ± 13 kcal mol^{-1} and 17 ± 2 mL mol^{-1}, respectively for the first phase, 62 ± 8 kcal mol^{-1} and -19 ± 1 mL mol^{-1}, for the second phase, -43 ± 5 kcal mol^{-1} and 8 ± 0.9 mL mol^{-1}, for the third phase and 9 ± 3 kcal mol^{-1} and -7.6 ± 0.6 mL mol^{-1}, for the fourth phase (Figure 4.26). The ΔH and ΔV associated with CO photodissociation from the Fe(II)SmFixLR200E are -10 ± 5 kcal mol^{-1} and 21 ± 3 mL mol^{-1}, respectively for the first phase, 31 ± 14 kcal mol^{-1} and -21 ± 2 mL mol^{-1}, for the second phase, -47 ± 6 kcal mol^{-1} and 10 ± 1 mL mol^{-1}, for the third phase and 43 ± 10 kcal mol^{-1} and -5 ± 2 mL mol^{-1}, for the fourth phase. The ΔH and ΔV associated with CO photodissociation from the Fe(II)SmFixLR200H are -39 ± 5 kcal mol^{-1} and 4.7 ± 0.7 mL mol^{-1}, respectively for the first phase, 74 ± 7 kcal mol^{-1} and -7 ± 1.1 mL mol^{-1}, for the
second phase, $-49 \pm 5$ kcal mol$^{-1}$ and $3.9 \pm 0.7$ mL mol$^{-1}$, for the third phase and $75 \pm 5$ kcal mol$^{-1}$ and $-4.3 \pm 0.7$ mL mol$^{-1}$, for the fourth phase.

**Figure 4.26**: Plot of (S/R)* $E_{hv}$ versus $C_p \rho / \beta$ for CO photolysis from SmFixLWT (top panel) and SmFixLR200Q (bottom panel) in 20 mM Tris (pH 8) between 10°C and 34°C.
Since it has been demonstrated that O$_2$ can not bind to the heme without interacting with three residues: Ile 209, Leuv230 and Valv232, a mutant I209M was synthesized by Dr Reynolds’s group in order to have the energetic profiles associated with the conformational changes of the distal pocket of SmFixL due to the changes in the interaction between I209M and O$_2$, and understand how the transmission of the signal is affected. The $\Delta$H and $\Delta$V associated with CO photodissociation from the Fe(II)SmFixLI209M are -54 ± 3 kcal mol$^{-1}$ and 3 ± 0.4 mL mol$^{-1}$, respectively for the first phase, 84 ± 6 kcal mol$^{-1}$ and -3.7 ± 1 mL mol$^{-1}$, for the second phase, -30 ± 13 kcal mol$^{-1}$ and 5.7 ± 2 mL mol$^{-1}$, for the third phase and 14 ± 5 kcal mol$^{-1}$ and -7.8 ± 0.8 mL mol$^{-1}$, for the fourth phase. The values of $\Delta$H and $\Delta$V, obtained for the four different phases for SmFixLWT, mutants (R200A, R200Q, R200E, R200H and I209M) and SmFixLH, are summarized in Table 4.4.

Given that the crystal structure of SmFixL does not exist for the full protein, BjFixL was used in order to establish the tryptophan positions. Two tryptophanes for one subunit of BjFixL were observed, one at residue 119 (Trp 119) and another at residue 178 (Trp 178). Fluorescence of SmFixLWT and the five different mutants in the oxy and CO bound form were examined. The overlays of the SmFixLWT and SmFixLR200Q results for the oxy and CO bound forms are summarized in Figure 4.27. Previous work by Lakoxicz demonstrated the tryptophan emission spectra exhibits a blue shift when the tryptophan moves from an aqueous environment to a hydrophobic environment, the wavelength maxima of the SmFixL was ~330 nm [26]. The overlay between the oxy and CO bound forms of the SmFixLWT and SmFixLR200Q does not show any blue shift but a decrease in the intensity when CO is bound to the heme in comparison to O$_2$ binding. This indicates that the tryptophan residue does not move to a hydrophobic environment but might rotate.
Figure 4.27: Fluorescence of SmFixLWT and SmFixLR200Q in the oxy and CO bound form.
4.5.4. Discussion

In order to understand the role of residues R200 and I209, different mutants of SmFixL full protein were examined using photothermal methods and compared to SmFixL wild type and the heme domain.

Key et al. suggested, using Time-Resolved crystallography, that within 1 \( \mu s \) after photolysis, BjFixL has relaxed to a conformation which is identical to the deoxy form [27]. They also demonstrated that the transmission of the signal after photodissociation of CO is not restricted to a single region of the heme but to an ensemble of regions for instance the movement in the FG loop or in the \( \beta \)-sheet distal of the heme as well as the movement of the Arg 200. They also showed that after photodissociation of CO, different events are observed in the crystal structure of BjFixL such as the doming and displacement of the heme, and the collapse of the hydrophobic residue (Leu 236, Ile 215 and Ile 238) in the distal pocket in order to replace the space left after CO leaves the heme pocket. Furthermore, the displacement of the 6 propionate group and the FG loop residues Pro 212, His 213 and Ile 216 was notable as well as the conformational changes of the proximal histidine and the Fa-helix or the backbone atoms of the H and I \( \beta \)-strands with the Leu 236 and Val 253 on the surface of the protein. Due to the similar nature of BjFixL and SmFixL, these conformational changes may also be observed after the photocleavage of CO in SmFixL. The difference in volume and enthalpy observed after photodissociation of CO are summarized below.

As it was explained earlier, PAC results can probe molar volume and enthalpy changes over a time scale from \( \sim 50 \) ns to \( \sim 20 \) \( \mu s \) [28]. An overlay of PAC traces for SmFixLWT, SmFixLR200Q and the calorimetric reference compound obtained in 20mM Tris at pH = 8 at 34°C is displayed in Figure 4.24. A frequency shift is observed between sample and reference acoustic signals which, indicates different kinetic events between \( \sim 50 \) ns and \( \sim 20 \) \( \mu s \) in addition to the events occurring before \( < 50 \) ns. The variation of volume and enthalpy observed after photodissociation of CO from the heme may have contributions from photocleavage events contained in the heme (\( \Delta V_{\text{heme}} \) and \( \Delta H_{\text{heme}} \)), and/or conformational change of the protein:
\[ \Delta H_{\text{obs}} = \Delta H_{\text{heme}} + \Delta H_{\text{conf}} \quad \text{Equation 46} \]
\[ \Delta V_{\text{obs}} = \Delta V_{\text{heme}} + \Delta V_{\text{conf}} \quad \text{Equation 47} \]

PAC results associated with volume and enthalpy changes for CO photodissociation to \textit{Sm}FixLWT, the five different mutants and the \textit{Sm}FixLH show a quadriphasic relaxation. The results are summarized in Table 4.4. The prompt phase shows a \( \Delta H \) and \( \Delta V \) associated with CO photodissociation from the Fe(II)\textit{Sm}FixLWT of -9 ± 8 kcal mol\(^{-1}\) and 10 ± 3 mL mol\(^{-1}\), respectively, 18 ± 16 kcal mol\(^{-1}\) and -18 ± 6 mL mol\(^{-1}\), for the second phase, -35 ± 20 kcal mol\(^{-1}\) and 11 ± 6 mL mol\(^{-1}\), for the third phase and 31 ± 25 kcal mol\(^{-1}\) and -9 ± 8 mL mol\(^{-1}\), for the fourth phase. In order to understand the energetic impact of the salt-bridge between the R200 and the heme 6,7 propionates group to compare to the wild type, different mutants (R200A, R200Q, R200E and R200H) were synthesized by Dr Reynolds’s group. The \( \Delta H \) and \( \Delta V \) associated with CO photodissociation from the Fe(II)\textit{Sm}FixLR200A are -7 ± 6 kcal mol\(^{-1}\) and 15 ± 2 mL mol\(^{-1}\), respectively for the first phase, 63 ± 20 kcal mol\(^{-1}\) and -6 ± 4 mL mol\(^{-1}\), for the second phase, 51 ± 20 kcal mol\(^{-1}\) and 28 ± 6 mL mol\(^{-1}\), for the third phase and 18 ± 11 kcal mol\(^{-1}\) and -10 ± 3 mL mol\(^{-1}\), for the fourth phase. The \( \Delta H \) and \( \Delta V \) associated with CO photodissociation from the Fe(II)\textit{Sm}FixLR200Q are -26 ± 13 kcal mol\(^{-1}\) and 17 ± 2 mL mol\(^{-1}\), respectively for the first phase, 62 ± 8 kcal mol\(^{-1}\) and -19 ± 1 mL mol\(^{-1}\), for the second phase, -43 ± 5 kcal mol\(^{-1}\) and 8 ± 0.9 mL mol\(^{-1}\), for the third phase and 9 ± 3 kcal mol\(^{-1}\) and -7.6 ± 0.6 mL mol\(^{-1}\), for the fourth phase. The \( \Delta H \) and \( \Delta V \) associated with CO photodissociation from the Fe(II)\textit{Sm}FixLR200E are -10 ± 5 kcal mol\(^{-1}\) and 21 ± 3 mL mol\(^{-1}\), respectively for the first phase, 31 ± 14 kcal mol\(^{-1}\) and -21 ± 2 mL mol\(^{-1}\), for the second phase, -47 ± 6 kcal mol\(^{-1}\) and 10 ± 1 mL mol\(^{-1}\), for the third phase and 43 ± 10 kcal mol\(^{-1}\) and -5 ± 2 mL mol\(^{-1}\), for the fourth phase. The \( \Delta H \) and \( \Delta V \) associated with CO photodissociation from the Fe(II)\textit{Sm}FixLR200H are -39 ± 5 kcal mol\(^{-1}\) and 4.7 ± 0.7 mL mol\(^{-1}\), respectively for the first phase, 74 ± 7 kcal mol\(^{-1}\) and -7 ± 1.1 mL mol\(^{-1}\), for the second phase, -49 ± 5 kcal mol\(^{-1}\) and 3.9 ± 0.7 mL mol\(^{-1}\), for the third phase and 75 ± 5 kcal mol\(^{-1}\) and -4.3 ± 0.7 mL mol\(^{-1}\), for the fourth phase. It has been demonstrated that O\(_2\) can not bind to the heme without interacting with three residues: Ile 209, Leu 230 and
Val 232, thus a mutant I209M was studied in order to have the energetic profiles associated with the changes of the distal pocket of SmFixL due to the different interaction between Ile 209 and O₂. The ΔH and ΔV associated with CO photodissociation from the Fe(II)SmFixLI209M are -54 ± 3 kcal mol⁻¹ and 3 ± 0.4 mL mol⁻¹, respectively for the first phase, 84 ± 6 kcal mol⁻¹ and -3.7 ± 1 mL mol⁻¹, for the second phase, -30 ± 13 kcal mol⁻¹ and 5.7 ± 2 mL mol⁻¹, for the third phase and 14 ± 5 kcal mol⁻¹ and -7.8 ± 0.8 mL mol⁻¹, for the fourth phase.
Table 4.4: Summary of photoacoustic results for the wild type, R200A, R200Q, R200E, R200H, I209M and heme domain of *SmFixL*.

<table>
<thead>
<tr>
<th></th>
<th>$\tau_1$ (ns)</th>
<th>$\Delta V_1$ (mL mol$^{-1}$)</th>
<th>$\Delta H_1$ (kcal mol$^{-1}$)</th>
<th>$\tau_2$ (ns)</th>
<th>$\Delta V_2$ (mL mol$^{-1}$)</th>
<th>$\Delta H_2$ (kcal mol$^{-1}$)</th>
<th>$\tau_3$ (ns)</th>
<th>$\Delta V_3$ (mL mol$^{-1}$)</th>
<th>$\Delta H_3$ (kcal mol$^{-1}$)</th>
<th>$\tau_4$ (ns)</th>
<th>$\Delta V_4$ (mL mol$^{-1}$)</th>
<th>$\Delta H_4$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1</td>
<td>10 ± 3</td>
<td>-9 ± 8</td>
<td>190</td>
<td>-18 ± 6</td>
<td>18 ± 16</td>
<td>512</td>
<td>11 ± 6</td>
<td>-35 ± 20</td>
<td>1495</td>
<td>-9 ± 8</td>
<td>31 ± 25</td>
</tr>
<tr>
<td>R200A</td>
<td>1</td>
<td>15 ± 2</td>
<td>-7 ± 6</td>
<td>100</td>
<td>-6 ± 4</td>
<td>63 ± 20</td>
<td>510</td>
<td>28 ± 6</td>
<td>51 ± 20</td>
<td>1510</td>
<td>-10 ± 3</td>
<td>18 ± 11</td>
</tr>
<tr>
<td>R200Q</td>
<td>1</td>
<td>17 ± 2</td>
<td>-26 ± 13</td>
<td>92</td>
<td>-19 ± 1</td>
<td>62 ± 8</td>
<td>285</td>
<td>8 ± 0.9</td>
<td>-43 ± 5</td>
<td>1252</td>
<td>-7.6 ± 0.6</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>R200E</td>
<td>1</td>
<td>21 ± 3</td>
<td>-10 ± 5</td>
<td>120</td>
<td>-21 ± 2</td>
<td>31 ± 14</td>
<td>429</td>
<td>10 ± 1</td>
<td>-47 ± 6</td>
<td>1575</td>
<td>-5 ± 2</td>
<td>43 ± 10</td>
</tr>
<tr>
<td>R200H</td>
<td>1</td>
<td>4.7 ± 0.7</td>
<td>-39 ± 5</td>
<td>95</td>
<td>-7 ± 1.1</td>
<td>74 ± 7</td>
<td>365</td>
<td>3.9 ± 0.7</td>
<td>-49 ± 5</td>
<td>1608</td>
<td>-4.3 ± 0.7</td>
<td>75 ± 5</td>
</tr>
<tr>
<td>I209M</td>
<td>1</td>
<td>3 ± 0.4</td>
<td>-54 ± 3</td>
<td>115</td>
<td>-3.7 ± 1</td>
<td>84 ± 6</td>
<td>497</td>
<td>5.7 ± 2</td>
<td>-30 ± 13</td>
<td>1493</td>
<td>-7.8 ± 0.8</td>
<td>14 ± 5</td>
</tr>
</tbody>
</table>
The results can be compared to volume and enthalpy changes for CO photodissociation from Fe(II) porphyrin model systems. Photolysis of CO from Fe(II)heme gives rise to three contributions to \( \Delta H \) and \( \Delta V \) as it was already explained earlier: cleavage of Fe-CO bond, spin state change of the iron from the low-spin to the high-spin configuration and solvation of CO as it diffuses away from the heme. Thus, the total changes of enthalpy and molar volume can be summarized as:

\[
\Delta H_{\text{heme}} = \Delta H_{\text{Fe-CO}} + \Delta H_{\text{LS-HS}} + \Delta H_{\text{COsolv}} \quad \text{Equation 48}
\]

\[
\Delta V_{\text{heme}} = \Delta V_{\text{Fe-CO}} + \Delta V_{\text{LS-HS}} + \Delta V_{\text{COsolv}} \quad \text{Equation 49}
\]

Upon photolysis, the variation in enthalpy for CO photodissociation from an iron porphyrin has been estimated to be \( \Delta H \sim 14 \text{ kcal mol}^{-1} \) where \( \Delta H_{\text{Fe-CO}} = 17 \text{ kcal mol}^{-1} \) (cleavage of Fe\(^{2+}\)CO bond), \( \Delta H_{\text{LS-HS}} = <1 \text{ kcal mol}^{-1} \) (heme iron undergoes from low-spin to high spin) and \( \Delta H_{\text{COsolv}} = -3 \text{ kcal mol}^{-1} \) (diffusion of CO out of the heme pocket and into the bulk solvent) [29-31].

Using the above relation \( \Delta H_{\text{obs}} = \Delta H_{\text{heme}} + \Delta H_{\text{conf}} \) where \( \Delta H_{\text{heme}} \sim 14 \text{ kcal mol}^{-1} \) and \( \Delta H_{\text{obs}} \sim -9 \text{ kcal mol}^{-1} \) for \( Sm\text{FixLWT} \), \( \Delta H_{\text{conf}} \) can be estimated and indicate an additional exothermic process taking place in <50 ns equal to \sim -23 \text{ kcal mol}^{-1} \). Using the same relation, \( \Delta H_{\text{conf}} \) for the five different mutants and \( Sm\text{FixLH} \) can also be estimated. For \( Sm\text{FixLR200A} \), \( \Delta H_{\text{obs}} \) equal to \sim -7 \text{ kcal mol}^{-1} which gives a \( \Delta H_{\text{conf}} \sim -21 \text{ kcal mol}^{-1} \). \( Sm\text{FixLR200Q} \) shows a \( \Delta H_{\text{obs}} \) equal to \sim -26 \text{ kcal mol}^{-1} which gives a \( \Delta H_{\text{conf}} \sim -40 \text{ kcal mol}^{-1} \). \( Sm\text{FixLR200E} \) shows a \( \Delta H_{\text{obs}} \) equal to \sim -10 \text{ kcal mol}^{-1} which gives a \( \Delta H_{\text{conf}} \sim -24 \text{ kcal mol}^{-1} \). \( Sm\text{FixLR200H} \) shows a \( \Delta H_{\text{obs}} \) equal to \sim -39 \text{ kcal mol}^{-1} which gives a \( \Delta H_{\text{conf}} \sim -53 \text{ kcal mol}^{-1} \). \( Sm\text{FixLI209M} \) shows a \( \Delta H_{\text{obs}} \) equal to \sim -54 \text{ kcal mol}^{-1} which gives a \( \Delta H_{\text{conf}} \sim -68 \text{ kcal mol}^{-1} \). Finally, \( Sm\text{FixLH} \) shows a \( \Delta H_{\text{obs}} \) equal to \sim 8.8 \text{ kcal mol}^{-1} (Table 4.5) which gives a \( \Delta H_{\text{conf}} \sim -5.2 \text{ kcal mol}^{-1} \). These results of \( \Delta H_{\text{conf}} \) must be due to a more global conformational change within the heme domain.
In the same way, the variation of volume for a CO photodissociation from an iron porphyrin can be estimated using the three following contributions: $\Delta V_{\text{Fe-CO}} = \sim 5 \text{ mL mol}^{-1}$ (increase of the solvent accessible area), $\Delta V_{\text{LS-HS}} = \sim 10 \text{ mL mol}^{-1}$ (increase of the electron density associated with the $3d_z^2$ orbital of the iron) and $\Delta V_{\text{CO solv}} = \sim 20 \text{ mL mol}^{-1}$ \[32\]. The three contributions give a $\Delta V_{\text{heme}} = \sim 35 \text{ mL mol}^{-1}$. Once again, based on the above equation, $\Delta V_{\text{obs}} = \Delta V_{\text{heme}} + \Delta V_{\text{conf}}$ where $\Delta V_{\text{heme}} = 35 \text{ mL mol}^{-1}$ and $\Delta V_{\text{obs}} = 10 \text{ mL mol}^{-1}$, $\Delta V_{\text{conf}}$ can be estimated to be $\sim -25 \text{ mL mol}^{-1}$ for $\text{Sm FixLWT}$. As for $\Delta H_{\text{conf}}$, $\Delta V_{\text{conf}}$ can also be estimated for the five mutants and $\text{Sm FixLH}$. $\text{Sm FixLR200A}$ shows a $\Delta V_{\text{obs}}$ equal to 15 mL mol$^{-1}$ which gives a $\Delta V_{\text{conf}} \sim -20 \text{ mL mol}^{-1}$. $\text{Sm FixLR200Q}$ shows a $\Delta V_{\text{obs}}$ equal to 17 mL mol$^{-1}$ which gives a $\Delta V_{\text{conf}} \sim -18 \text{ mL mol}^{-1}$. $\text{Sm FixLR200E}$ shows a $\Delta V_{\text{obs}}$ equal to 21 mL mol$^{-1}$ which gives a $\Delta V_{\text{conf}} \sim -14 \text{ mL mol}^{-1}$. $\text{Sm FixLR200H}$ shows a $\Delta V_{\text{obs}}$ equal to 4.7 mL mol$^{-1}$ which gives a $\Delta V_{\text{conf}} \sim -30 \text{ mL mol}^{-1}$. $\text{Sm FixLI209M}$ shows a $\Delta V_{\text{obs}}$ equal to 3 mL mol$^{-1}$ which gives a $\Delta V_{\text{conf}} \sim -32 \text{ mL mol}^{-1}$ (Table 4.4). Finally, $\text{Sm FixLH}$ shows a $\Delta V_{\text{obs}}$ equal to 21 mL mol$^{-1}$ (Table 4.5) which gives a $\Delta V_{\text{conf}} \sim -14 \text{ mL mol}^{-1}$. 
Table 4.5: Summary for photoacoustic results for BjFixLH, truncated BjFixLH and SmFixLH.

<table>
<thead>
<tr>
<th></th>
<th>Fast phase (&lt; 50 ns)</th>
<th>Slow phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔV (mL mol⁻¹)</td>
<td>ΔH (kcal mol⁻¹)</td>
</tr>
<tr>
<td>BjFixLH140-270</td>
<td>14 ± 3</td>
<td>-1.4 ± 0.8</td>
</tr>
<tr>
<td>BjFixLH151-256</td>
<td>25 ± 4.0</td>
<td>4.9 ± 0.4</td>
</tr>
<tr>
<td>SmFixLH</td>
<td>21 ± 6</td>
<td>8.8 ± 0.9</td>
</tr>
</tbody>
</table>

The ΔV\text{conf} and ΔH\text{conf} results can be attributed to (1) a change in overall charge distribution on the protein (i.e., change in net protein dipole leading to solvent reorganization), (2) formation of one or more salt-bridge interactions (the release of electrostricted water molecules upon salt-bridge formation results in volume increases) and/or (3) an increase in the solvent accessible van der Waals volume of the protein upon photolysis. The results from the SmFixLH exhibit a monophasic relaxation after the photodissociation of CO which is associated with a ΔH of 8.8 kcal mol⁻¹ and ΔV of 21 mL mol⁻¹. These results are very different from the heme domain of FixL from Bradyrhizobium japonicum (BjFixLH_{140-270}), which show a biphasic relaxation after the photodissociation of CO. Subsequent to the CO release, the fast phase, with a ΔH of 14 kcal mol⁻¹ and ΔV of -1 mL mol⁻¹, was associated with the reorganization of the solvent following a perturbation of the salt-bridge between Glu 182 and Arg 227. The slow phase with a lifetime \( \tau \sim 150 \) ns, a ΔH of 5 kcal mol⁻¹ and ΔV of 5 mL mol⁻¹ was associated to the escape of the CO molecule to the solvent [35]. On the other hand, the results for SmFixLH are similar to the results from the even further truncated heme domain BjFixLH_{151-256} which was truncated at both ends of the polypeptide chain compared to
\textit{Bj\textsubscript{FixLH}$_{140-270}$}, with 11 amino acid residues deleted from the N-terminus and 14 amino acid residues deleted from the C-terminus (Table 4.5) and where $\Delta H$ is equal to 5 kcal mol\textsuperscript{-1} and $\Delta V$ equal to 25 mL mol\textsuperscript{-1}. Miksovska et al. concluded that the truncation of 11 amino acid residues at the N-terminus and 14 amino acid residues at the C-terminus of the PAS heme domain from \textit{Bj\textsubscript{FixL}} will induce changes which were associated with the protein surface that accelerates the release of ligand from the protein and/or change in the salt-bridge interactions [9]. The results obtained for the variation of volume are similar between \textit{Bj\textsubscript{FixLH}$_{140-270}$} and \textit{Sm\textsubscript{FixLH}} but the variation of enthalpy is almost a factor of two less than both of them. This might be due to the fact that the salt-bridge does not involve the same amino acids for all of the studied proteins. The salt-bridge in \textit{Sm\textsubscript{FixLH}} is between R200 and the heme propionate, while in \textit{Bj\textsubscript{FixLH}}, it is between the amino acid R206 and the heme propionate. The variation of volume and enthalpy in \textit{Bj\textsubscript{FixLH}} reported by Miksovska et al. [9] are for the salt-bridge formed between E182 and R227.

The results for the changes in volume and enthalpy associated with \textit{Sm\textsubscript{FixLWT}}, \textit{Sm\textsubscript{FixL}R200A}, \textit{Sm\textsubscript{FixL}R200Q} and \textit{Sm\textsubscript{FixL}R200E} are very similar to each other with a $\Delta H \sim$ -12 kcal mol\textsuperscript{-1} and $\Delta V \sim$ 15 mL mol\textsuperscript{-1}. This indicates that the mutation from an arginine to an alanine, a glutamine or a glutamic acid residue does not destabilized the salt-bridge between the heme propionate and Arg 200. On the other hand, \textit{Sm\textsubscript{FixL}R200H} shows a significant difference after photodissociation of CO with a $\Delta H$ of $\sim$ -33 kcal mol\textsuperscript{-1} and $\Delta V$ of 4.7 mL mol\textsuperscript{-1}. The mutation from an arginine to a histidine residue shows a volume change smaller but a higher exothermic change. This difference might be due to the fact that the amino acid histidine is shorter and larger than the amino acid arginine producing a difference in the reorganization of the salt-bridge after photodissociation of CO with the 6,7 propionate group.
The results associated with the mutant SmFixLI209M show a ΔH of ~ -54 kcal mol\(^{-1}\) and ΔV of 3 mL mol\(^{-1}\) which differ from SmFixLWT. This may indicate that the steric repulsion between Ile 209 and the CO molecule is less than with Met 209. This might be due to the fact that the sulfur group on the methionine can hydrogen bond with the CO molecule instead of the repulsive interactions between the alkyl group from the Ile 209 and the CO molecule binding the heme.

The second phase for BjFixLH occurs around ~150 ns with a ΔH of 5 kcal mol\(^{-1}\) and ΔV of 5 mL mol\(^{-1}\) was associated by Miksovska et al. to the CO molecule leaving the heme pocket and entering the solvent after CO photodissociation. The results for the variation of volume and enthalpy for the second relaxation of the SmFixLWT and the five different mutants are summarized page 157 (Table 4.4). SmFixLWT shows a ΔV\(_{\Pi}\) equal to -18 mL mol\(^{-1}\) and a ΔH\(_{\Pi}\) ~ 18 kcal mol\(^{-1}\). SmFixLR200A shows a ΔV\(_{\Pi}\) equal to -6 mL mol\(^{-1}\) and a ΔH\(_{\Pi}\) ~ 63 kcal mol\(^{-1}\). SmFixLR200Q shows a ΔV\(_{\Pi}\) equal to -19 mL mol\(^{-1}\) and a ΔH\(_{\Pi}\) ~ 62 kcal mol\(^{-1}\). SmFixLR200E shows a ΔV\(_{\Pi}\) equal to -21 mL mol\(^{-1}\) and a ΔH\(_{\Pi}\) ~ 31 kcal mol\(^{-1}\). SmFixLR200H shows a ΔV\(_{\Pi}\) equal to -7 mL mol\(^{-1}\) and a ΔH\(_{\Pi}\) ~ 74 kcal mol\(^{-1}\). Finally, SmFixLI209M shows a ΔV\(_{\Pi}\) equal to -3.7 mL mol\(^{-1}\) and a ΔH\(_{\Pi}\) ~ 84 kcal mol\(^{-1}\). The results associated with the mutants SmFixLR200A, SmFixLR200Q, SmFixLR200E and SmFixLR200H show difference in volume and enthalpy changes compared to the SmFixLWT. The change in volume is similar for SmFixLR200Q and SmFixLR200E to the SmFixLWT, this indicates that the mutation does not affect the salt-bridge interaction and the release of the CO to the solvent. In contrast, changes for SmFixLR200A and SmFixLR200H are unlike SmFixLWT. The dissimilarity between SmFixLR200H and SmFixLWT confirms the previous hypothesis concerning the fact that the histidine is shorter and larger than the arginine which implying different interaction for the salt-bridge between R200 and heme propionate and different conformational changes after photodissociation of CO and release of CO to the solvent. The results for SmFixLR200A are more in accord with the fact that the mutation from an arginine to an alanine will not form a salt-bridge with the heme propionate (negative charge) as an alanine is neutral to compare to the arginine which has a positive charge. The second phase ΔV and ΔH for SmFixLI209M also confirm also the previous hypothesis where the
sulfur group on the methionine might form a hydrogen bond causing a steric attraction instead of steric repulsion as demonstrated in SmFixLWT consequently different conformational change are associated with the release of CO to the solvent.

For each mutants, the next two phases vary from the wild type. For the SmFixLWT, these variation of volume and enthalpy may be associated to different events such as the conformational changes of the proximal histidine and the Fa-helix or the backbone atoms of the H and I β-strands at the residue Leu 236 and Val 253 on the surface of the protein as well as the displacement of the 6 propionate group and the FG loop residues Pro 212, His 213 and Ile 216. The results for the variation of volume and enthalpy for the third and fourth relaxation which occurs with a lifetime τ ~ 400 ns and 1500 ns for the SmFixLWT and the five different mutants are summarized below (Table 4.4). SmFixLWT shows a ΔV_{III} equal to 11 mL mol^{-1} and a ΔH_{III} ~ -35 kcal mol^{-1}. SmFixLR200A shows a ΔV_{III} equal to 28 mL mol^{-1} and a ΔH_{III} ~ 51 kcal mol^{-1}. SmFixLR200Q shows a ΔV_{III} equal to 8 mL mol^{-1} and a ΔH_{III} ~ -43 kcal mol^{-1}. SmFixLR200E shows a ΔV_{III} equal to 10 mL mol^{-1} and a ΔH_{III} ~ -47 kcal mol^{-1}. SmFixLR200H shows a ΔV_{III} equal to 3.9 mL mol^{-1} and a ΔH_{III} ~ -49 kcal mol^{-1}. SmFixLI209M shows a ΔV_{III} equal to 5.7 mL mol^{-1} and a ΔH_{III} ~ -30 kcal mol^{-1}. Finally, the results for the variation of volume and enthalpy for the last relaxation show a ΔV_{IV} equal to -9 mL mol^{-1} and a ΔH_{IV} ~ 31 kcal mol^{-1} for SmFixLWT, a ΔV_{IV} equal to -10 mL mol^{-1} and a ΔH_{IV} ~ 18 kcal mol^{-1} for SmFixLR200A, a ΔV_{IV} equal to -7.6 mL mol^{-1} and a ΔH_{IV} ~ 9 kcal mol^{-1} for SmFixLR200Q, a ΔV_{IV} equal to -5 mL mol^{-1} and a ΔH_{IV} ~ 43 kcal mol^{-1} for SmFixLR200E, a ΔV_{IV} equal to -4.3 mL mol^{-1} and a ΔH_{IV} ~ 75 kcal mol^{-1} for SmFixLR200H and, a ΔV_{IV} equal to -7.8 mL mol^{-1} and a ΔH_{IV} ~ 14 kcal mol^{-1} for SmFixLI209M. The results indicate that the third and fourth phase of SmFixLR200A and SmFixLI209M show the largest difference from SmFixLWT and confirm the previous hypothesis. SmFixLR200A which does not form a salt-bridge with the heme propionate show the third phase involves an endothermic reaction where the wild type and the other mutants show an exothermic reaction confirming a different conformational change and a difference in the transmission of the signal. In addition, the changes in volume and enthalpy for the third phase of SmFixLI209M provide that the steric attraction between
I209 and the CO molecule instead of a steric repulsion result in changes in the transmission of the signal along the protein compared to the wild type.

To conclude, each of the mutation for the residue R200 or I209 indicate different thermodynamic profiles after photodissociation of CO. The mutation that largely affected the thermodynamic profiles where $Sm$FixLR200A, $Sm$FixLR200H and $Sm$FixLI209M. The $Sm$FixLR200A demonstrated that by mutating a positive charge residue (arginine) to a neutral residue (alanine), the salt-bridge can not form and contributing to a different thermodynamic profile for the transmission of the signal. The different thermodynamic profiles for mutant $Sm$FixLR200H can only be explained by the fact that the new residue is shorter and larger due to the pyridine group compared the arginine but should still form a salt-bridge with the heme propionate because it has a positive. This confirms that the residue R200 is very important in the transmission of the signal. Finally, the mutation of an isoleucine to a methionine changes the steric repulsion with the CO molecule to an attractive interaction which alters the conformational change after photodissociation of CO. This also confirms that the residue I209 is also very important in the transmission of the signal after photodissociation of CO.

These results are consistent with observations by Reynolds et al. [25] who demonstrated using EPR and Resonance Raman spectroscopy that the autoxidation rates are faster for $Sm$FixLR200A and $Sm$FixLR200E compared to $Sm$FixLR200Q, $Sm$FixLR200H and $Sm$FixLWT. This means that the loss of the polarity in the mutant $Sm$FixLR200A is incapable of forming a salt-bridge with the 6-propionate group. Therefore, neutralizing the negative charge on the heme propionate. Thereby having a different thermodynamic profile associated to a different transmission of the signal.
In Figure 4.28, the overall thermodynamic profile for CO photodissociation from SmFixLWT is summarized.

![Thermodynamic profile diagram](image)

**Figure 4.28:** Thermodynamic profiles for CO photorelease from Fe(2+)SmFixLWT.

Previous PAC studies of CO photodissociation from both sperm whale and horse heart myoglobin indicate biphasic relaxations. In the case of horse heart myoglobin, the fast relaxation occurring <50 ns is associated with a ΔH of 7 kcal mol\(^{-1}\) and with a small contraction ΔV of -3 mL mol\(^{-1}\) which is attributed to the formation of a geminate pair and the cleavage of a salt-bridge between Arg 45 and the 6-propionate of the heme active site. The second relaxation occurring ~700 ns after CO photolysis gives a ΔH of 8 kcal mol\(^{-1}\) associated with a ΔV of 14 mL mol\(^{-1}\) which is attributed to the reformation of the salt-bridge. In the case of sperm whale myoglobin the fast relaxation <50 ns gives rise to a ΔH = -2.2 kcal mol\(^{-1}\) and a ΔV = -10 mL mol\(^{-1}\). The second relaxation which has a
lifetime of ~700 ns has a $\Delta H = 14.6$ kcal mol$^{-1}$ associated with a contraction $\Delta V = 5.8$ mL mol$^{-1}$. The differences between these proteins are due primarily to a Lys residue in sperm whale Mb in place of Arg 45 in horse heart Mb which affects the salt-bridge with the heme propionate [33-35]. In the case of BsHemAT, a monophasic relaxation is observed following the photodissociation of CO. The results, associated with a $\Delta H$ of -19 kcal mol$^{-1}$ and $\Delta V$ of 4 mL mol$^{-1}$, are consistent with an increase of the SAS of the protein after comparing the X-ray structure of the BsHemAT between the CN liganded and unliganded forms of the protein [36]. Finally, PAC results reveal a fast conformational change associated with the photodissociation of CO from CooA. The conformational change results in a large enthalpy and volume change which were associated to a reorientation of the C-helix in addition to the disruption of a salt-bridge and hydrogen bonds.
4.5.5. Conclusion

In summary, PAC reveals a quadriphasic relaxation for $Sm$FixLWT, and the five different mutants ($Sm$FixLR200A, $Sm$FixLR200Q, $Sm$FixLR200E, $Sm$FixLR200H and $Sm$FixLI209M) associated with the photodissociation of CO. The thermodynamics confirm that the residue R200 and I209 are very important in the transmission of the signal and a mutation involves different conformational changes which might induce a different signal transmitted at the end. The results for the $Sm$FixLH show a monophasic relaxation associated with a fast disruption of the salt-bridge and release of the CO to the solvent.
4.5.6. References


Chapter V – Model Proteins

Two model proteins were undertaken in order to help the analysis of the variation of volume and enthalpy results their contribution to cell signaling.

The first model protein project was the analysis of the sandbar shark hemoglobin as the hemoglobin is an allosteric protein (Figure 5.1). The binding of oxygen to one subunit will stimulate the conformational change of the subunit. As the four subunits are linked together by amino acids chain, the conformational change of one subunit will increase of the oxygen affinity of the other subunits. By studying an allosteric protein, the communication between proteins can be better understood. In the case of the sandbar shark hemoglobin focus was given to subunit interactions.

Figure 5.1: Hemoglobin (PDB entry 1bzo)
Also two peroxidases, the horseradish peroxidase and the soybean peroxidases, were employed to probe the energetic profile of a ligand, CO molecule, leaving the heme pocket to the solvent as peroxidases have a direct channel between the heme pocket and the solvent (Figure 5.2). Since no energetic barrier exists for the ligand release from the pocket, the mechanism of the ligand migration between the heme distal pocket and the solvent will be drawn. Giving insight to the energetic mechanism of how a ligand gains access to the heme distal pocket.

Figure 5.2: Direct channel in soybean peroxidase (PDB entry 1fhf).
5.1. Kinetic Properties of Polymorphic Hemoglobin from the Sandbar Shark Hemoglobin (*Carcharhinus plumbeus*)

5.1.1. Introduction

Hünefeld discovered in 1840 the oxygen-carrying protein called hemoglobin [1]. These globular proteins transport oxygen from the lungs to the rest of the body. Usually in mammals, hemoglobins are formed by four subunits, each composed of a tightly associated chain protein which contains a pocket with the iron heme group. Each hemoglobin consists of two \( \alpha \) and two \( \beta \) subunits non-covalently bound together. The four subunits are linked together by salt-bridges, hydrogen bonds and hydrophobic interactions.

When oxygen binds to the heme, it causes the heme to go from a conformation called T-state to a conformation called R-state. This transition of the heme involves the imidazole of the histidine to move and induce at the same time a strain in the protein helix, which will be transmitted to the other subunits. These conformational changes of the other subunits facilitates further the binding of oxygen by the other subunits effectively increasing their binding affinity; hence the cooperative, behavior of the protein. CO molecule will affect the binding of oxygen to heme, as the binding affinity for CO is 200 times greater than its affinity for oxygen [2].

In humans, the two \( \alpha \) and two \( \beta \) subunits of the hemoglobin consist of 141 and 146 amino acid residues, respectively, (also called \( \alpha_2\beta_2 \)) [20,21]. The subunits are the same size and structurally identical. The total molecular weight of the hemoglobin is 68 kDa. In general, fish hemoglobins have similar properties as mammalian hemoglobins including molecular weights, aggregations states, positive cooperativity of ligand binding, they show a Bohr effect (Root effect) and exhibit heterotropic responses to allosteric affectors [3]. Fish hemoglobins have adapted to environmental changes and have adapted their functional reactivity including heterogeneity in ligand binding between \( \alpha \) and \( \beta \) subunits, loss of cooperativity at low pH and a large Bohr effect [4-7].
The sandbar shark (Figure 5.3), or *Carcharhinus plumbeus*, is part of the requiem shark family Carcharhinidae. This class of shark can be found in the Atlantic Ocean and the Indo-Pacific [22].

![Sandbar Shark](image)

**Figure 5.3: Photographs of the sandbar shark [23].**

The sandbar shark is one of the biggest coastal sharks in the world and has a very high distinguishable first dorsal fin. Sandbar sharks are related to the dusky shark, the bignose shark, and the bull shark [22]. The color of its body can be from a bluish to a brownish grey to a bronze, with a white or pale underside. Females are longer than males and can grow up between 2 and 2.5 meters compared to 1.8 meters for the male. They can swim alone or in groups which are only composed by the same sex in shallow coastal and in deeper waters (200 meters) [22]. At night, at dawn, and at dusk are usually the time of the day that they are the most active. Sandbar shark are found around the world in temperate to tropical waters: from Massachusetts to Brazil (western Atlantic) [22].

Despite the importance of the species, very little is known on the biochemistry of the communication between the subunit of fish hemoglobin. The current study is an initial analysis of the kinetic and thermodynamic processes of the respiratory system of the sandbar shark with a focus on hemoglobin.

Like heme oxygen sensors, conformational changes associated with ligand binding/dissociation to hemoglobin are thought to initiate conformational change in the hemoglobin. Thus, characterizing those conformational changes is critically important to understand the signal transduction process. We have utilized transient absorption
spectroscopy and time-resolved photothermal methods to examine the thermodynamics and conformational dynamics following CO photodissociation from sandbar shark hemoglobin. Time resolved photothermal methods are particularly important since they can provide energetic profiles on fast (~ ns-ms) time scales. Here, photoacoustic calorimetry (PAC) has been used to probe the ΔH and ΔV profiles subsequent to CO photodissociation from sandbar shark hemoglobin. By examining the time-resolved thermodynamics associated with small molecule dissociation, the energetic mechanism through which the signal is transmitted along the protein can be determined. Theses studies can provide insights into the mechanism of the signal migration between the different subunits which are relevant to understand how a signal is transmitted in general inside a protein.

5.1.2. Materials and Methods

Sample preparation

Samples for PAC were prepared by diluting sandbar shark hemoglobin into a buffer containing 50 mM sodium phosphate (pH 6.0, 7.0 and 8.0). The deoxy form of the protein was formed by placing the oxy form of sandbar shark hemoglobin in a quartz optical cuvette that was then sealed with a septum cap and purged with Argon gas. A fresh dithionite solution was added from a buffered stock solution to give a concentration of ~13 μM for the reduced from of the sandbar shark hemoglobin. The CO-bound form was obtained by saturating solutions of the deoxy sandbar shark hemoglobin with CO, resulting in a final solution of CO concentration of 1 mM (1 atm pressure). The protein concentration for PAC samples was ~10 μM while those for transient absorption were ~1.6 μM.
5.1.3. Results

The recombinant heme domain of sandbar shark hemoglobin displays an optical spectrum with a Soret maximum at ~411 nm and visible bands at 577 nm (α-band) and 542 nm (β-band) (Figure 5.4). The hemoglobin was then reduced using a freshly prepared solution of dithionite resulting in a bathochromic shift of the Soret band to 431 nm with a broad visible band centered at 556 nm. The binding of CO to the ferrous hemoglobin results in a Soret band at ~419 nm and visible bands at 568 nm and 538 nm. The liganded and deoxy forms give a spectrum of the sandbar shark hemoglobin nearly identical to the mammalian hemoglobin A (HbA) spectrum with a Soret maximum at 430 nm and a broad visible band centered at 560 nm.

![Steady-state optical absorption spectra of sandbar shark hemoglobin](image)

**Figure 5.4:** Steady-state optical absorption spectra of sandbar shark hemoglobin (as isolated (red dotted line), deoxy sandbar shark hemoglobin (blue dashed line) and CO sandbar shark hemoglobin (black solid line) in 50mM Tris (pH=7). [Sandbar shark hemoglobin] = ~10 μM.

In order to examine the effects of the pH on the photo-release of CO from sandbar shark hemoglobin, PAC data was obtained for sandbar shark hemoglobin with the same concentration of Tris (50 mM) but at different pH = 6, 7 and 8. The values of ΔH and
ΔV, obtained from deconvolution of the acoustic waves, are summarized in Table 5.1. Figure 5.5 displays an overlay of PAC traces for CO-sandbar shark hemoglobin and the calorimetric reference compound obtained in 50mM Tris, pH 7.0. The fact that a frequency shift is observed between sample and reference acoustic signals indicates kinetic events occurring between ~50 ns and ~20 μs. A plot of (S/R)E_ν versus C_ρp/β (as per equation 9) reveals a triphasic relaxation with a ΔH and ΔV associated with CO photodissociation from the Fe(II)sandbar shark hemoglobin. At pH = 6, the prompt phase (<50 ns) shows a variation of volume and enthalpy equal to 6.6 mL mol⁻¹ and 19 kcal mol⁻¹ respectively. The intermediate phase at 0.7 μs shows a volume expansion of 12.5 mL mol⁻¹ and enthalpy of 48 kcal mol⁻¹ and the slow phase at 2 μs shows a volume contraction of -12 mL mol⁻¹ and enthalpy of -22 kcal mol⁻¹. At pH = 7, the prompt phase (<50 ns) shows a variation of volume and enthalpy equal to 5.5 mL mol⁻¹ and 17 kcal mol⁻¹ respectively. The intermediate phase at 0.7 μs shows a volume expansion of 22 mL mol⁻¹ and enthalpy of 48 kcal mol⁻¹ and the slow phase at 2 μs shows a -30 mL mol⁻¹ and -8 kcal mol⁻¹ (using a Φ = 0.21 calculated using the quantum yield of myoglobin).

Table 5.1: Variation of volume and enthalpy of the CO photodissociation at pH= 6 and 7.

<table>
<thead>
<tr>
<th></th>
<th>Fast phase</th>
<th>Intermediate phase</th>
<th>Slow phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>τ₁ (μs)</td>
<td>ΔV₁_conf (mL mol⁻¹)</td>
<td>ΔH₁_conf (kcal mol⁻¹)</td>
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<tr>
<td>pH = 6</td>
<td>&lt;50ns</td>
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<td>19</td>
</tr>
<tr>
<td>pH = 7</td>
<td>&lt;50ns</td>
<td>5.5</td>
<td>17</td>
</tr>
</tbody>
</table>
Figure 5.5: Overlay of the normalized acoustic wave of the reference Fe4SP and the sandbar shark hemoglobin in 50mM Tris at 10 °C at pH=7. The absorbance of the sample and reference at the excitation wavelength (532 nm) was ~0.4.

Photolysis of the CO bound sandbar shark hemoglobin at 440 nm results in the formation of a five coordinate high-spin heme complex which decays back to the pre-flash CO bound complex (Figure 5.6, 5.7 and 5.8) and displays a biphasic relaxation kinetic with a pseudo first-order rate constant of $\sim 1.1 \times 10^3$ s$^{-1}$ and $\sim 1.3 \times 10^2$ M$^{-1}$s$^{-1}$ at pH = 6, $\sim 2 \times 10^3$ s$^{-1}$ and $\sim 2.2 \times 10^2$ M$^{-1}$s$^{-1}$ at pH = 7, $\sim 3.4 \times 10^3$ s$^{-1}$ and $\sim 3.1 \times 10^2$ M$^{-1}$s$^{-1}$ at pH = 8 for the fast and slow phases respectively (the corresponding second-order rate constant is $\sim 1.1 \times 10^6$ s$^{-1}$ and $\sim 1.3 \times 10^5$ M$^{-1}$s$^{-1}$ at pH = 6, $\sim 2 \times 10^6$ s$^{-1}$ and $\sim 2.2 \times 10^5$ M$^{-1}$s$^{-1}$ at pH = 7, $\sim 3.4 \times 10^6$ s$^{-1}$ and $\sim 3.1 \times 10^5$ M$^{-1}$s$^{-1}$ at pH = 8 for the fast and slow phases respectively with a solution CO concentration of 1 mM) (Table 5.2).
Figure 5.6: Top panel: Single wavelength transient absorption trace for CO rebinding to sandbar shark hemoglobin (440nm) obtained at pH=6 in 50mM Tris.

Bottom panel: Overlay of the transient kinetic in the Soret region (black squares, obtained 15 ms subsequent to photolysis) and equilibrium (blue empty round) difference spectra (deoxy sandbar shark hemoglobin minus CO sandbar shark hemoglobin). Spectra were constructed from single wavelength date obtained 5μs subsequent to photolysis.
Figure 5.7: Top panel: Single wavelength transient absorption trace for CO rebinding to sandbar shark hemoglobin (440nm) obtained at pH=7 in 50mM Tris.

Bottom panel: Overlay of the transient kinetic in the Soret region (black squares, obtained 15 ms subsequent to photolysis) and equilibrium (bleu empty round) difference spectra (deoxy sandbar shark hemoglobin minus CO sandbar shark hemoglobin).
**Figure 5.8:** *Top panel:* Single wavelength transient absorption trace for CO rebinding to sandbar shark hemoglobin (440nm) obtained at pH=8 in 50mM Tris.  
*Bottom panel:* Overlay of the transient kinetic in the Soret region (black squares, obtained 15 ms subsequent to photolysis) and equilibrium (bleu empty round) difference spectra (deoxy sandbar shark hemoglobin minus CO sandbar shark hemoglobin).
Table 5.2: Variation of volume and enthalpy activation of CO photodissociation associated with their rate constant.

<table>
<thead>
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<th>pH</th>
<th>$k_1$ (ms$^{-1}$)</th>
<th>$\Delta H_{1}^\neq$ (kcal mol$^{-1}$)</th>
<th>$\Delta S_{1}^\neq$ (cal mol$^{-1}$K$^{-1}$)</th>
<th>$k_2$ (M$^{-1}$s$^{-1}$)</th>
<th>$\Delta H_{2}^\neq$ (kcal mol$^{-1}$)</th>
<th>$\Delta S_{2}^\neq$ (cal mol$^{-1}$K$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>6</td>
<td>1.1x10$^3$</td>
<td>5.6 ± 1</td>
<td>-0.06 ± 0.003</td>
<td>1.3 x10$^2$</td>
<td>18 ± 4</td>
<td>-0.1 ± 0.01</td>
</tr>
<tr>
<td>7</td>
<td>2 x10$^3$</td>
<td>2.2 ± 1.1</td>
<td>-0.05 ± 0.003</td>
<td>2.2 x10$^2$</td>
<td>-38 ± 5</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>8</td>
<td>3.4 x10$^3$</td>
<td>-2 ± 0.4</td>
<td>-0.04 ± 0.001</td>
<td>3.1 x10$^2$</td>
<td>-6.6 ± 1</td>
<td>-0.02 ± 0.003</td>
</tr>
</tbody>
</table>

These results are different than that of HbA which decays biphasically with a pseudo first order rate constant $\sim 1.4 \pm 0.11 \times 10^7$ s$^{-1}$ and $\sim 1 \pm 0.4 \times 10^4$ M$^{-1}$s$^{-1}$ for the fast and slow phase, respectively, at pH = 7.7 [8]. In the case of sandbar shark hemoglobin, the fast phase decay was attributed to the geminate recombination of CO to the originating subunit. This is slower by an order of magnitude of three compared to the CO geminate recombination of HbA. The second’s slower phase decay was attributed to geminate recombination of CO by another subunit of the same hemoglobin. This phase is also slower by an order of magnitude of two when compared to HbA.

The sandbar shark hemoglobin shows a linear dependence between the rate and the solution pH. In addition, the kinetic optical difference spectrum obtained $\sim 15$ ms subsequent to photolysis displays a bathochromic shift relative to the equilibrium optical difference spectrum suggesting an un-relaxed heme pocket conformation subsequent to photolysis at pH = 6, 7 and 8 (Figure 5.6, 5.7, 5.8). The transient difference spectra of sandbar shark hemoglobin display a maximum at 439 nm and a minimum at 421 nm at pH = 6, 7 and 8, respectively which is similar to the transient absorption difference spectrum of HbA which display a maximum at 436 nm and a minimum at 420 nm [8]. Since the concentration of transient five coordinate heme decays bi-exponentially, the un-relaxed heme pocket must persist for $\sim$ milliseconds. From the temperature dependence
of the biphasic recombination rates the activation enthalpy and entropy for the fast and slow phases were found to be 5.6 ± 1 kcal mol⁻¹ and -0.06 ± 0.003 cal mol⁻¹ K⁻¹ at pH = 6, 18 ± 4 kcal mol⁻¹ and -0.1 ± 0.1 cal mol⁻¹ K⁻¹ at pH = 7, 2.2 ± 1.1 kcal mol⁻¹ and -0.05 ± 0.003 cal mol⁻¹ K⁻¹ at pH = 8 for the fast phases, -38 ± 5 kcal mol⁻¹ and 0.08 ± 0.02 cal mol⁻¹ K⁻¹ at pH = 6, -2 ± 0.4 kcal mol⁻¹ and -0.04 ± 0.001 cal mol⁻¹ K⁻¹ at pH = 7, -6.6 ± 1 kcal mol⁻¹ and -0.02 ± 0.003 cal mol⁻¹ K⁻¹ at pH = 8 respectively for the slow phase (Figure 5.6). The results are summarized in Table 5.2.

![Figure 5.9: Eyring plot for CO recombination to deoxy sandbar shark hemoglobin at pH=6.](image)

Figure 5.9: Eyring plot for CO recombination to deoxy sandbar shark hemoglobin at pH=6.
Figure 5.10: Eyring plot for CO recombination to deoxy sandbar shark hemoglobin at pH = 7 (top panel) and 8 (bottom panel).
Figure 5.11 corresponds to the photolysis of CO bound sandbar shark hemoglobin at 440 nm at different temperatures (17°C and 33°C) and pH (6, 7 and 8). The results show that rate constant increases with increased of temperature as well as decreased pH (Table 5.2).

Figure 5.11: Single wavelength transient absorption data for CO recombination to sandbar shark hemoglobin at 17°C (Top panel) and 33°C (Bottom panel). Excitation wavelength was 532nm (<20 ps, 20 mJ/pulse, 20 Hz).
Kinetic optical difference spectra relative to the equilibrium optical difference spectrum obtained ~15 μs subsequent to photolysis at pH = 6 and 7. Figure 5.12 displays a bathochromic shift. Changes in this difference spectra as a function of time give indication of the relaxation state of the heme pocket. From this, we know that the five coordinate heme does not relax to the un-relaxed heme pocket within the time frame of the measurement and must persist for at least 1 ms after photolysis of the CO.
Figure 5.12: Transient difference spectrum overlaid on equilibrium deoxy minus CO-bound difference spectrum at pH = 6 and 7.
5.1.4. Discussion

By understanding the thermodynamics following CO binding to fish hemoglobin and the different energetic profiles associated with the transmission of the signals along the different subunits, we might be able to do a correlation with human hemoglobin. In the present work, we study the kinetic and thermodynamic profiles for the dissociation and rebinding of CO to the heme of the sandbar shark hemoglobin. The CO recombination results show a much lower affinity for the sandbar shark hemoglobin for CO than HbA. This is consistent with results exhibiting a liganded T-state conformation which are similar to the previous studies on different fish hemoglobins [9-11]. Previous studies have shown that after the photodissociation of CO to the heme (Scheme 5.1), different time relaxations are observed (Table 5.3). The first relaxation is a geminate recombination occurring at ~ 50-70 ns. The second relaxation ~ 0.7-0.8 \( \mu \text{s} \) corresponds to the geminate recombination of CO which escapes to the heme pocket and rebinds to another subunit of the protein in addition to the R to T relaxation of one subunit of the protein called tertiary relaxation. The third relaxation ~ 20 \( \mu \text{s} \) corresponds to the R to T relaxation of the hemoglobin called quaternary relaxation. The fourth relaxation ~ 190 \( \mu \text{s} \) was assigned to the bimolecular rebinding of CO molecule to the R forms of the hemoglobin. The last relaxation ~ 3.8 ms was designated to the bimolecular rebinding of CO molecule to the T forms of the hemoglobin.
**Scheme 5.1:** R and T allosteric states of the hemoglobin.

**Table 5.3:** Different time relaxations of human hemoglobin.

<table>
<thead>
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<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relaxation</td>
<td>40 ns</td>
<td>0.7-0.8 μs</td>
<td>20 μs</td>
<td>190 μs</td>
<td>3.8 ms</td>
</tr>
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</table>

The transient difference spectra for pH = 6, 7 and 8 indicate that photodissociation of CO results in a non-equilibrium heme environment between the T and R forms subsequent to CO photolysis. In addition, the fact that the decay of the five coordinate heme is biphasic and the transient absorption difference spectra is red-shift (Figure 5.6, 5.7 and 5.8) indicate that the non-equilibrium heme pocket persists on the milliseconds time scale.
The PAC results can probe molar volume and enthalpy changes over a time scale from ~50 ns to ~20 μs [12]. An overlay of PAC traces for CO-sandbar shark hemoglobin and the calorimetric reference compound obtained in 50 mM Tris at pH = 6 and 7 at 10°C (Figure 5.5) reveal a frequency shift between sample and reference acoustic signals which indicating different kinetic events between ~50 ns and ~20 μs in addition to the events occurring before < 50 ns. The variation of volume and enthalpy observed after the photodissociation of CO from the heme may have contributions from photocleavage events localized in the heme (ΔV_{heme} and ΔH_{heme}), and/or conformational change of the protein:

\[
\Delta H_{\text{obs}} = \Delta H_{\text{heme}} + \Delta H_{\text{conf}} \quad \text{Equation 46}
\]

\[
\Delta V_{\text{obs}} = \Delta V_{\text{heme}} + \Delta V_{\text{conf}} \quad \text{Equation 47}
\]

PAC results (Table 5.2) for CO photodissociation to sandbar shark hemoglobin give an average of the variation of volume and enthalpy at pH = 6 and 7 associated with a triphasic relaxation. At pH 6, the prompt phase (<50 ns) shows an average volume expansion and enthalpy equal to 6.6 mL mol\(^{-1}\) and 19 kcal mol\(^{-1}\) respectively. The intermediate phase at 0.7 μs shows a 12.5 mL mol\(^{-1}\) expansion and 48 kcal mol\(^{-1}\) and the slow phase at 2 μs shows a contraction of -12 mL mol\(^{-1}\) and -22 kcal mol\(^{-1}\). At pH = 7, the prompt phase (<50 ns) shows a variation of volume and enthalpy equal to an expansion of 5.5 mL mol\(^{-1}\) and 17 kcal mol\(^{-1}\) respectively. The intermediate phase at 0.7 μs shows a 22 mL mol\(^{-1}\) and 48 kcal mol\(^{-1}\) and the slow phase at 2 μs shows a -30 mL mol\(^{-1}\) and -8 kcal mol\(^{-1}\). These results can be compared to volume and enthalpy changes for CO photodissociation from Fe(II) porphyrin model systems. Photolysis of CO from Fe(II)heme gives rise to three contributions to ΔH and ΔV: cleavage of Fe-CO bond, spin state change of the iron from the low-spin to the high-spin configuration and solvation of CO as it diffuses away from the heme.
Thus, the total changes of enthalpy and molar volume can be summarized as:

\[ \Delta H_{\text{heme}} = \Delta H_{\text{Fe-CO}} + \Delta H_{\text{LS-HS}} + \Delta H_{\text{COsolv}} \quad \text{Equation 48} \]

\[ \Delta V_{\text{heme}} = \Delta V_{\text{Fe-CO}} + \Delta V_{\text{LS-HS}} + \Delta V_{\text{COsolv}} \quad \text{Equation 49} \]

Upon photolysis, the variation in enthalpy for a CO photodissociation from an iron porphyrin has been estimated to be \( \Delta H \sim 14 \text{ kcal mol}^{-1} \) where \( \Delta H_{\text{Fe-CO}} = 17 \text{ kcal mol}^{-1} \) (cleavage of Fe\(^{2+}\)-CO bond), \( \Delta H_{\text{LS-HS}} = <1 \text{ kcal mol}^{-1} \) (heme iron undergoes from low-spin to high spin) and \( \Delta H_{\text{COsolv}} = -3 \text{ kcal mol}^{-1} \) (diffusion of CO out of the heme pocket and into the bulk solvent) [13-15].

Using the above relation \( \Delta H_{\text{obs}} = \Delta H_{\text{heme}} + \Delta H_{\text{conf}} \) where \( \Delta H_{\text{heme}} \sim 14 \text{ kcal mol}^{-1} \) and \( \Delta H_{\text{obs}} \sim 19 \text{ kcal mol}^{-1} \) at pH = 6 and 17 kcal mol\(^{-1}\) at pH = 7, \( \Delta H_{\text{conf}} \) can be estimated and indicate an additional endothermic process taking place in <50 ns equal to \sim 5 \text{ kcal mol}^{-1} \) at pH = 6 and \sim 3 \text{ kcal mol}^{-1} \) at pH = 7. The result of \( \Delta H_{\text{conf}} \) must be due to a more global conformational change within the heme domain.

The variation of volume for a CO photodissociation from an iron porphyrin can be estimated in the same way: \( \Delta V_{\text{Fe-CO}} = \sim 5 \text{ mL mol}^{-1} \) (increase of the solvent accessible area), \( \Delta V_{\text{LS-HS}} = \sim 10 \text{ mL mol}^{-1} \) (increase of the electron density associated with the 3d\(_{z^2}\) orbital of the iron) and \( \Delta V_{\text{COsolv}} = \sim 20 \text{ mL mol}^{-1} \) [5]. The three contributions give a \( \Delta V_{\text{heme}} = \sim 35 \text{ mL mol}^{-1} \). Once again, based on the above equation, \( \Delta V_{\text{obs}} = \Delta V_{\text{heme}} + \Delta V_{\text{conf}} \) where \( \Delta V_{\text{heme}} = 35 \text{ mL mol}^{-1} \) and \( \Delta V_{\text{obs}} = 6.6 \text{ mL mol}^{-1} \), \( \Delta V_{\text{conf}} \) can be estimated to be \sim 28.4 \text{ mL mol}^{-1} \) at pH = 6 and at pH = 7 \( \Delta V_{\text{obs}} = 5.5 \text{ mL mol}^{-1} \) which correspond to \( \Delta V_{\text{conf}} \sim -29.5 \text{ mL mol}^{-1} \).

The results of \( \Delta V_{\text{conf}} \) and \( \Delta H_{\text{conf}} \) can be though to arise from (1) a change in overall charge distribution on the protein (i.e., change in net protein dipole leading to solvent reorganization), (2) formation of one or more salt-bridge interactions (the release of electrostricted water molecules upon salt-bridge formation results in volume increases) and/or (3) an increase in the solvent accessible van der Waals volume of the protein upon
photolysis. Hofrichter et al. studied human hemoglobin using nanosecond absorption spectra [16]. They explained that the relaxation I (< 50 ns) is also accompanied by a spectra change which indicates a structural change of the hemoglobin which can result by a collapse of the globin due to the escape of the CO to the solvent. This observation can be compared to the sandbar shark hemoglobin. The variation of volume and enthalpy ($\Delta V_{\text{conf}} = \sim -30 \text{ mL mol}^{-1}$ and $\Delta H_{\text{conf}} = \sim 3 \text{ kcal mol}^{-1}$ at pH 6 and 7) related to the relaxation I is associated with the CO geminate recombination to the heme from which it was photodissociated but also with the CO solvation into the bulk solvent which will imply a contraction of the bulk solvent due to the hole leaving by CO from protein matrix.

The second relaxation which corresponds to the relaxation II which occurs between 0.7 and 0.9 $\mu$s was associated by Hofrichter et al. to a pure tertiary change in addition to the CO geminate recombination to another subunit other than the one from which it was photodissociated [16]. The results show a $\Delta V_{\text{conf II}} = \sim 12.5 \text{ mL mol}^{-1}$ at pH 6 and $\Delta V_{\text{conf II}} = \sim 22 \text{ mL mol}^{-1}$ at pH 7 and $\Delta H_{\text{conf II}} = \sim 48 \text{ kcal mol}^{-1}$ at pH 6 and 7 corresponding to the variation of volume and enthalpy of the conformational change from R to T-state of the subunit. The difference in $\Delta V_{\text{conf}}$ between pH 6 and 7 observed can be due to the fact that the electrostriction of the hemoglobin is not the same between pH 6 and 7.

Previous studies demonstrate that the relaxation from the R-state to the T-state of a subunit is associated with the movement of the Fe-His bond. The tilted Fe-His configuration relaxation is associated with the moving in plane of the iron. Moreover, previous studies show that the strain associated with CO binding show no movement out of plane of the iron which indicates a rapid transfer of the binding strain to the new conformation of the Fe-His interface. To compare, the conformation associated with O$_2$ binding to the heme show a remaining tilted conformation of the $\alpha$ chain.

The third phase which corresponds to the relaxation III and occurs around $\sim 20 \mu$s was associated by Hofrichter et al. to a pure quaternary change from R to T state in
addition to a very small CO geminate recombination to another subunit than the one from which it was photodissociated [16]. In shark hemoglobin, this relaxation was faster by an order of 10 to compare to the HbA. This conformational change was associated with a \( \Delta V_{\text{conf III}} = \sim -12 \text{ mL mol}^{-1} \) and \( \Delta H_{\text{conf III}} = \sim -22 \text{ kcal mol}^{-1} \) at pH 6 and \( \Delta V_{\text{conf III}} = \sim -30 \text{ mL mol}^{-1} \) and \( \Delta H_{\text{conf III}} = \sim -8 \text{ kcal mol}^{-1} \) at pH 7. These results show that the relaxation R to T-state quaternary transition is associated to a contraction of the solvent in addition to an endothermic reaction.

In Figure 5.13, the overall thermodynamic profile for CO photodissociation at pH 6 and 7 is summarized.
Figure 5.13: Thermodynamic profile for CO photolysis from (Fe²⁺)sandbar shark hemoglobin at pH = 6 and 7.

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To compare with the horse heart Mb, two thermodynamic phases were observed upon photolysis occurring in <50 ns. The prompt phase coupled with a small endothermic contraction with $\Delta H = 7 \text{ kcal mol}^{-1}$ and $\Delta V = -3 \text{ mL mol}^{-1}$ has been related to a solvation of charge formed by disruption of a salt-bridge between one of the heme propionate groups and Lys 45 (Arg 45 in sperm whale Mb) [17-19]. The slow phase with a lifetime around \sim 600 ns was associated to a $\Delta H = 8 \text{ kcal mol}^{-1}$ and $\Delta V = 14 \text{ mL mol}^{-1}$ and partly attributed to the salt-bridge reformation.

5.1.5. Conclusion

In summary, transient absorption displays a biphasic relaxation kinetic which correspond to the CO geminate recombination to the same subunit and to another subunit. PAC reveals a triphasic relaxation associated with the CO geminate recombination, a tertiary transition for one subunit and the R to T quaternary transition of the hemoglobin. The thermodynamics of this transition are similar to the transition in the human hemoglobin with the exception of the third relaxation which is faster for the shark hemoglobin.
5.1.6. References


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5.2. Photothermal Studies of CO Photodissociation from Peroxidases from Horseradish and Soybean

5.2.1. Introduction

Plant peroxidases are one of the most diverse families of heme proteins in the biological world. This class of proteins participates in the oxidation of a large array of organic and inorganic compounds via heme-catalyzed reduction of H₂O₂ to water [1]. The general reaction for the catalytic cycle of plant peroxidases involving the degradation of various peroxides is described in Scheme 5.2 where AH and A⁺ represent the reduced substrate and its oxidized radical product respectively and compounds I and II are a high valent Fe⁴⁺=O porphyrin π-cation radical and Fe⁴⁺=O heme intermediaries, respectively [1-3].

**Scheme 5.2: General reaction for the catalytic cycle of plant peroxidases**

\[
\text{Fe}^{3+} - \text{porp} + \text{H}_2\text{O}_2 \rightarrow \text{Compound I} + \text{H}_2\text{O} \rightarrow \text{Compound II} + \text{A}^+ \rightarrow \text{Fe}^{3+} - \text{porp} + \text{A}^+ + \text{H}_2\text{O}
\]

Plant peroxidases are divided into three families. Class I peroxidases consist of the intracellular peroxidases, examples of which include yeast cytochrome c peroxidase (CCP) and ascorbate peroxidase (AP) while class II peroxidases include the secretory fungal peroxidases, which are involved in the degradation of lignin (e.g., lignin peroxidases (LiPs) and manganese-dependent peroxidase (MnPs)). Finally, class III peroxidases consist of the secretory plant peroxidases including horseradish and soybean peroxidase [3,4].
Figure 5.14: Ribbon structures of horseradish peroxidase (PDB entry 1hch) (top) and soybean peroxidase (PDB entry 1fhf) (bottom). Key distal heme pocket amino acid residues, Arg 38, His 42 and Phe 41 are displayed on the left.
Crystal structures are now available for a number of plant peroxidases revealing similar structural architectures. Class III plant peroxidases contain three domains (distal heme binding B domain, proximal heme binding F domain and the D domain) derived from a single glycosylated polypeptide chain of approximately 300 residues, a single iron (III) protoporphyrin IX active site and two bound calcium ions (see Figure 5.14). The role of the two calcium ions is to stabilize the protein structure and modulate the catalytic activity of the enzyme [4,5]. Within the different members of the peroxidase family, the secondary structure is predominantly α-helical, containing on average ten helices (A-J). The penta-coordinate heme active site also contains a central iron exhibiting a quantum-mechanical admixture of spin states (high-spin $S = \frac{5}{2}$ and low-spin $S = \frac{3}{2}$) [6,7]. The heme-binding pocket of these proteins contain five highly conserved amino acids consisting of Arg 38, Phe 41 and His 42 on the distal side and Asn 247 and His 142, on the proximal side with His 142 serving as the fifth ligand of the Fe(III) protoporphyrin IX [8]. Finally a common feature of the peroxidase family is the presence of a direct access channel from the heme active site to the surrounding solvent. Thus, small molecules such as the H$_2$O$_2$ substrate can readily diffuse into the heme active site.

Horseradish peroxidase (HRP) and soybean peroxidase (SBP) are two members of the class III peroxidases. HRP is not only important in the physiological function of the root system of horseradish (involved in the degradation of H$_2$O$_2$) but also has biotechnology applications including immunoassay diagnostics, chemical synthesis and bioremediation [9]. SBP has important biotechnological applications as well including biosensors and biocatalysts [10]. HRP and SBP are structurally related with ~ 60% sequence similarity and share analogous catalytic mechanisms [3,2,8,10]. The structures of HRP and SBP share the class III structural topology of 13 α-helices and two small β-sheet regions [2,4]. However, SBP exhibits a much higher thermal stability and a more open access channel to the heme distal pocket. The ligand access channel of SBP also contains a binding site for Tris molecules which resembles to the secondary substrate binding site associated with HRP [2].
Comparison of the distal pocket of HRP, SBP and the myoglobins (sperm whale and horse heart) reveals an active site of HRP and SBP that is larger (i.e., void volume) than the active site of the myoglobins consistent with the fact that the heme pocket of plant peroxidases can bind a variety of relatively large organic substrates. The heme pockets of both peroxidases and globins also contain a distal His residue (His E7 in globins, His 42 in HRP and SBP) that can modulate the binding of diatomic ligands to the heme iron. Unlike globins, peroxidases contain an Arg residue (Arg 38) in place of the distal phenylalanine found in globins. Both His 42 and Arg 38 have been shown to play a key role in peroxidase enzymatic activity [12,22]. The role of Arg 38 in modulating CO binding/release in ferrous peroxidase was demonstrated using an R38L mutant of HRP, which gives CO association/dissociation rates similar to those observed for the globins. This is partly due to differences in distal pocket polarity between globins and peroxidases as well as differences in hydrogen bonding [23]. While the heme bound CO of myoglobin can only form one hydrogen bond (between CO and His 42), the corresponding CO in HRP can form a hydrogen bond with either the positively charged guanidinium group of Arg 38 or to His 42 [22,18]. It has also been observed that the proximal imidazole ligand has a strong hydrogen bond with Asp 243 which alters the imidazolate character [21]. This, in turn, alters the electronic structure of bound ligands such as CO as evident by a 1932 cm\(^{-1}\) CO stretching band and a 490 cm\(^{-1}\) Fe-C stretching mode. The hydrogen bonding interactions between CO and His 42 give rise to pH dependent dissociation kinetics. At low pH, the rate constant for CO dissociation decreases due to a protonation of His 42, while the rate constant for CO association is pH independent [24,25].

Of the many time-resolved optical methods typically employed in the study of heme protein dynamics, time resolved photothermal methods are particularly important since they can provide energetic profiles on fast (~ ns-ms) time scales. Here, photoacoustic calorimetry (PAC) has been used to probe the \(\Delta H\) and \(\Delta V\) profiles subsequent to CO photodissociation from Fe(II)HRP and Fe(II)SBP. By examining the time-resolved thermodynamics associated with small molecule dissociation, the energetic mechanism through which ligands gain access to the heme distal pocket can be determined. Although the CO-bound ferrous forms of HRP or SBP are not physiologically relevant, theses
studies can provide insights into the mechanism of ligand migration between the heme distal pocket and the bulk solvent which are relevant to physiological substrate entry into the enzyme’s active site.

5.2.2. Materials and Methods

Sample Preparation:

Horseradish peroxidase and soybean peroxidase were purchased from Sigma-Aldrich and Fe(III)tetrakis(4-sulfonatophenyl)porphine (Fe4SP) was obtained from Frontier-Scientific, Inc. HRP and SBP were used without further purification. HRP and SBP were solubilized in either 50 mM, 100 mM, 150 mM Tris (pH 7.0), 50 mM Tris/100 mM NaCl (pH 8.0), 50 mM (pH 7.0) or 0.2 M (pH 7.0) phosphate buffer. Samples for PAC studies were placed in a 1cm quartz cuvette sealed with a septum cap and subsequently purged with argon. The proteins were then reduced with a freshly prepared solution of sodium dithionite and purged with CO to obtain the HRP-CO or SBP-CO samples. Optical spectra of the various species were obtained using a Shimadzu UV-2401PC spectrophotometer.

5.2.3. Results

Both HRP and SBP display similar optical spectra regardless of buffer conditions with a Soret maximum at ~407 nm and a broad visible band centered at ~505 nm in the oxidized (resting) form (Figure 5.15). Both proteins can be reduced with sodium dithionite, resulting in a Soret band bathochromically shifted to 423 nm and a visible band at 555 nm. The binding of CO to the ferrous enzymes results in a Soret band at ~423 nm and visible bands at 550 nm and ~575 nm. The fact that the optical absorption spectra of the various forms of HRP and SBP are independent of the nature of the buffer system employed indicates that Tris binding within the distal pocket near the heme group of SBP does not have a significant impact on the electronic structure of the heme group.
Figure 5.15: Top panel: Equilibrium optical absorption spectra of horseradish peroxidase as isolated (red solid line), reduced HRP (blue dashed line) and reduced CO bound HRP (black dotted line). HRP in 50 mM Tris and 100 mM NaCl (pH 8.0).

Bottom panel: Equilibrium optical absorption spectra of the soybean peroxidase as isolated (red solid line), reduced SBP (blue dashed line) and reduced CO bound SBP (black dotted line). SBP in 50 mM Tris and 100 mM NaCl (pH 8.0).
Figure 5.16 displays an overlay of PAC traces for CO-HRP, CO-SBP and the calorimetric reference compound obtained in 0.2 M phosphate buffer, pH 8.0. The fact that no frequency shifts are observed between sample and reference acoustics signals indicates no kinetic events occurring between ~50 ns and ~20 μs. However, differences in amplitudes indicate photolytic events occurring in < ~50 ns. A plot of (S/R)$E_{hv}$ versus C$_p$/β (as per equation 9) reveals ΔH and ΔV associated with CO photodissociation from the Fe(II)HRP and Fe(II)SBP of 18 ± 7 kcal mol$^{-1}$/6 ±1 mL mol$^{-1}$ for HRP and 20 ± 9 kcal mol$^{-1}$/2.4 ± 0.6 mL mol$^{-1}$ for SBP (using a Φ = 0.7 for both HRP and SBP [28]). In order to examine the effects of a bound Tris molecule on the photo-release of a CO from SBP, PAC data was obtained for SBP and HRP with differing concentrations of Tris (50 mM, 100 mM and 150 mM). A buffer system consisting of 50 mM Tris and 100 mM NaCl was also utilized in order to probe for the effects of electrostatic interactions upon CO photodissociation. In all cases the acoustic waves of the CO-HRP and CO-SBP overlap in frequency with the reference indicating only fast events occurring within the PAC time scale (i.e., < ~50 ns). The values of ΔH and ΔV, obtained from amplitude differences, are summarized in Table 5.4 and 5.5.
Figure 5.16: Overlay of the acoustic waves for the photolysis of CO from HRP (top panel) (bleu dotted line), SBP (bottom panel) (green dotted line) and the reference Fe(III)4SP (black solid line). HRP and SBP in 50 mM Tris and 100 mM NaCl (pH 8.0) at 22°C.
The $\Delta H$ and $\Delta V$ values associated with CO-HRP photodissociation are quite similar regardless of the nature of the buffer system with the average values of the enthalpy and molar volume changes of $\Delta H$ of $16 \pm 6$ kcal mol$^{-1}$ and a $\Delta V$ of $8.2 \pm 0.6$ mL mol$^{-1}$ ($\sim$13Å$^{-3}$/molecule). In contrast, the molar volume and enthalpy changes observed for CO-SBP photodissociation are dependant upon the solution buffer conditions. In 50 mM phosphate buffer, pH 7.0, $\Delta H$ and $\Delta V$ are found to be -9 kcal mol$^{-1}$ and 4 mL mol$^{-1}$, respectively, while in 200 mM phosphate buffer the enthalpy value increases to 20 kcal mol$^{-1}$. In Tris buffer with concentrations ranging from 50 mM to 150 mM the average values of $\Delta H$ and $\Delta V$ for SBP are found to be $6 \pm 5$ kcal mol$^{-1}$ and $5.7 \pm 0.3$ mL mol$^{-1}$ ($\sim$8Å$^{-3}$/molecule), respectively. For solutions containing 50 mM Tris and 100 mM NaCl a $\Delta H$ $20 \pm 5$ kcal mol$^{-1}$ and a $\Delta V$ to $9.1 \pm 0.3$ mL mol$^{-1}$ are observed similar to what is observed in 0.2 M phosphate (Figure 5.17).

![Figure 5.17](image.png)

**Figure 5.17:** Plot of $(S/R)^* E_{hv}$ versus $C_p \rho / \beta$ for CO photolysis from HRP (bleu square) and SBP (green dot) in 50 mM Tris (pH 7.0) between 6°C and 34°C.
5.2.4. Discussion

One of the key features associated with heme proteins is the mechanism through which local heme pocket dynamics modulates both the affinity of the heme-protein complex for a specific ligand and ligand specificity [29-37]. Examination of the crystal structures of a wide range of heme proteins with structurally variable distal heme pockets reveals complex networks of open pockets and access channels through which the gaseous ligands must traverse in order to bind to the heme iron. The thermodynamic profiles associated with CO photo-release from heme proteins can provide key mechanistic insights into this key process by providing detailed energetic maps associated with ligand exit/entry into the protein matrix.

**COHRP Photodissociation**

In the case of COHRP, photodissociation gives rise to an average $\Delta H$ of 16 ± 6 kcal mol$^{-1}$ associated with an average $\Delta V$ of 8.2 ± 0.6 mL mol$^{-1}$ regardless of buffers conditions (phosphate and Tris) occurring in < ~ 50 ns. The observed $\Delta H$ and $\Delta V$ values can be divided into contributions due to heme-CO dissociation and to the subsequent protein/solvent response. Photolysis of CO from an Fe(II)heme in the absence of protein involves cleavage of the Fe-CO bond, change in spin state of the heme iron from the low-spin to high-spin configuration and solvation of CO as it diffuses into the bulk solvent. Thus, the total changes in enthalpy and molar volume for this process can be expressed as:

\[
\Delta H_{\text{heme}} = \Delta H_{\text{Fe-CO}} + \Delta H_{\text{LS-HS}} + \Delta H_{\text{COSolv}} \quad \text{Equation 48}
\]
\[
\Delta V_{\text{heme}} = \Delta V_{\text{Fe-CO}} + \Delta V_{\text{LS-HS}} + \Delta V_{\text{COSolv}} \quad \text{Equation 49}
\]

where $\Delta H_{\text{Fe-CO}} \sim 17$ kcal mol$^{-1}$, $\Delta H_{\text{LS-HS}} \sim 0$ kcal mol$^{-1}$ and $\Delta H_{\text{COSolv}} \sim -3$ kcal mol$^{-1}$ giving a $\Delta H_{\text{heme}} \sim 14$ kcal mol$^{-1}$ for photolysis [38,39]. In the case of CO-HRP, enthalpy changes associated with the protein response subsequent to photolysis also contribute to the total $\Delta H$ giving $\Delta H_{\text{Tot}} = \Delta H_{\text{str}} + \Delta H_{\text{heme}}$ where $\Delta H_{\text{str}}$ represents any protein
conformational/solvation changes associated with the CO photodissociation and $\Delta H_{heme}$ represents the variation in enthalpy for photolysis of CO from the heme group. From the results in Table 5.4, $\Delta H_{str} = \Delta H_{obs} - \Delta H_{heme} \sim 2$ kcal mol$^{-1}$ which is within the uncertainty of the PAC measurements.

The corresponding variation in molar volume can also be expressed as:

$$\Delta V = \Delta V_{str} + \Delta V_{heme} \quad \text{Equation 50}$$

where $\Delta V_{heme}$ is composed of the same three contributions as for the variation in enthalpy. Previous studies of various model compounds have provided estimates for the changes in volume for these processes as follows: $\Delta V_{Fe-CO} \sim 5$ mL mol$^{-1}$ (the increase is due to changes in the solvent accessible area of the newly created molecules), $\Delta V_{LS-HS} \sim 10$ mL mol$^{-1}$ (repulsion of solvent ‘ligands’ due to an increase in electron density associated with the heme iron $3d_z^2$ orbital) and $\Delta V_{CoSolv} \sim 20$ mL mol$^{-1}$ give a value for $\Delta V_{heme} \sim 35$ mL mol$^{-1}$. Again, using the PAC results provided in Table 5.4 gives, $\Delta V_{str} \sim 8$ mL mol$^{-1}$ – 35 mL mol$^{-1} = -27$ mL mol$^{-1}$ [40]. Assuming the protein matrix can accommodate the structural changes associated with the low-spin to high-spin transition as well as the cleavage of the Fe-CO bond $\Delta V_{str}$ reduces to $\sim -12$ mL mol$^{-1}$.

The changes in enthalpy support a mechanism through which CO migrates out of the distal heme pocket and into the bulk solvent subsequent to photo-cleavage in $\sim <50$ ns. Such rapid ligand escape is consistent with the presence of a direct channel linking the heme group to the bulk solvent. These results further indicate that this ligand access channel does not contain any significant kinetic barriers for CO escape. The corresponding changes in molar volume, $\Delta V_{str} \sim -12$ mL mol$^{-1}$, cannot arise simply from CO migration into the bulk solvent. In general volume contractions may arise from 1) electrostriction in which a charged amino acid becomes solvent exposed subsequent to CO release, 2) an overall decrease in the van der Waals volume of the protein and/or 3) input of a solvent molecule(s) into a protein cavity. Examination of the results in Table 5.4 reveals no significant changes in either $\Delta V$ or $\Delta H$ as a function of solution ionic strength ruling out electrostriction contributions to $\Delta V_{str}$. The magnitude of the volume decrease is, however, consistent with the input of a single water molecule into the protein.
 subsequent to CO migration to the bulk solvent resulting in a decrease in solvent molar
volume by $\sim -18$ mL mol$^{-1}$ (i.e. $\Delta V_{\text{H}_2\text{O}} \sim 18$ mL mol$^{-1}$). Although there is no data
concerning the changes in van der Waals volumes of HRP upon ligand
association/release in solution which precludes any estimates of the protein contribution
to $\Delta V_{\text{str}}$ the similarity between the molar volume of water and the value of $\Delta V_{\text{str}}$ suggests
water input as the major contribution to the molar volume change upon CO photorelease.

**Table 5.4: Summary of PAC results for CO photo-release from ferrous HRP.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta V$ (mL mol$^{-1}$)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
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<tbody>
<tr>
<td>50mM Tris</td>
<td>9.3 ± 0.4</td>
<td>16 ± 6</td>
</tr>
<tr>
<td>100mM Tris</td>
<td>9.6 ± 0.6</td>
<td>11 ± 6</td>
</tr>
<tr>
<td>150mM Tris</td>
<td>8.9 ± 0.3</td>
<td>21 ± 3</td>
</tr>
<tr>
<td>50mM Tris + 100mM NaCl</td>
<td>7.7 ± 0.6</td>
<td>14 ± 8</td>
</tr>
<tr>
<td>0.2M Phosphate</td>
<td>6 ± 1</td>
<td>18 ± 7</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>8.2 ± 0.6</strong></td>
<td><strong>16 ± 6</strong></td>
</tr>
</tbody>
</table>
Table 5.5: Summary of PAC results for CO photo-release from ferrous SBP.

<table>
<thead>
<tr>
<th>SBP</th>
<th>△V (mL mol⁻¹)</th>
<th>△H (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 M Phosphate</td>
<td>4 ± 0.7</td>
<td>-9 ± 3</td>
</tr>
<tr>
<td>0.2M Phosphate</td>
<td>2.4 ± 0.6</td>
<td>20 ± 9</td>
</tr>
<tr>
<td>50mM Tris</td>
<td>5.6 ± 0.3</td>
<td>10 ± 5</td>
</tr>
<tr>
<td>100mM Tris</td>
<td>6.4 ± 0.3</td>
<td>4 ± 5</td>
</tr>
<tr>
<td>150mM Tris</td>
<td>5.1 ± 0.3</td>
<td>4 ± 4</td>
</tr>
<tr>
<td>Average</td>
<td>5.7 ± 0.3</td>
<td>6 ± 5</td>
</tr>
<tr>
<td>50mM Tris + 100mM NaCl</td>
<td>9.1 ± 0.3</td>
<td>20 ± 5</td>
</tr>
</tbody>
</table>

Entry of a water molecule into the protein would also be expected to contribute to △Hstr. The simplest estimate of △H_{H₂O-protein} is the enthalpy of vaporization for water which arises from a water molecule leaving the bulk solvent to the vapor phase. For water △H_vap = 10.6 kcal mol⁻¹ which does not appear in △H_{str} estimated in the previous section [41]. However, △H_vap assumes a water molecule breaking hydrogen bonds with neighboring water molecules in the bulk solvent and entering an environment in which the water molecule is isolated (i.e., vapor phase). In the context of the protein matrix this would be equivalent to a water molecule leaving the bulk and entering a hydrophobic pocket which is thermodynamically unfavorable. In contrast, a water molecule breaking hydrogen bonds between itself and neighboring water molecules from the bulk water,
entering the protein and forming new hydrogen bonds with amino acids would result in a negligible change in enthalpy, as observed.

![Diagram of heme structure](image)

**Figure 5.18:** Illustration of the differences in heme structure between CO bound (shown in yellow) and deoxy HRP.

Examination of the X-ray structures for the ferrous and ferrous CO bound forms of HRP reveal very similar heme structures (see Figure 5.18) with slight differences in heme propionate positions. Thus, from the X-ray structures it is not clear what heme conformational dynamics drive the input of a water molecule into the entrance channel to the heme distal pocket. Either these changes are quite subtle or there are additional dynamics which occur in solution that are not observed in the crystals. The structures do reveal the addition of a water molecule near the Gln 176 in the ferrous form consistent with the photothermal results.
Photodissociation of CO from ferrous HRP has also been previously studied using PAC by Feis and Angeloni [42]. These authors also obtained a monophasic relaxation within the prompt heat phase subsequent to photolysis. However, the change in volume associated with this relaxation was found to be $\Delta V = 29.6 \text{ mL mol}^{-1}$ and the enthalpy change was $\Delta H = 35 \text{ kcal mol}^{-1}$. Although the values of $\Delta H$ and $\Delta V$ obtained previously are nearly twice those obtained in the present study, similar conclusions were drawn, i.e., that the observed change in volume is due primarily to the displacement of CO to the surrounding solvent.

**Previous PAC Studies of COHRP Photolysis**

Feis and Angeloni have also been previously studied the photodissociation of CO from ferrous HRP using PAC [42]. The results, they obtained, are also a monophasic relaxation within the prompt heat phase subsequent to photolysis, although the change in volume and enthalpy associated with this relaxation was calculated equal to be $\Delta V = 30 \text{ mL mol}^{-1}$ and $\Delta H = 42 \text{ kcal mol}^{-1}$, respectively. Feis and Angeloni also concluded a CO migration into the bulk solvent with no significant energetic barriers. The origin of the disparities in the change of volume and enthalpies (almost a factor of three) observed our studies and the studies by Feis and Angeloni is not clear. Feis and Angeloni explain their large change in enthalpy (42 kcal mol$^{-1}$) to the cleavage of Fe-C bond energy. The theoretical value of the cleavage of Fe-C bond energy is equal to 35 kcal mol$^{-1}$ which was obtained for a gas phase (CO)(imidazole)Fe-porphyrin model system using molecular dynamics coupled with density functional theory (MD-DFT) [46]. More recent studies on the CO-Myoglobin using MD-DFT suggest a Fe-C bond cleavage energy of less than 10 kcal mol$^{-1}$ [47]. Moreover, previous studies in our laboratory demonstrated a photodissociation of CO from Fe(II)tetrasulphonatophenyl porphyrin with both a water or imidazole as a proximal ligand in addition to the study of the photodissociation of CO to the ferrous microperoxidase-11 equal to 12-17 kcal mol$^{-1}$ for Fe-C bond cleavage[48]. These results are in the range of the change in enthalpy presented in the study.
One of the differences between Feis and Angeloni studies and these studies is the PAC instrumentation used to generate these results. Feis and Angelino used two different PAC detectors [42]. One detector holds the sample; the other one holds the reference. The laser is divided in two in order to excite both sample and reference at the same time. The PAC instrument used in this study employed only one detector. The sample which has to be anaerobic in this case is studied first. Then the reference will replace the sample and the acoustic wave as function of temperature will be collected in the same way than the sample. The sample and the reference are studied in the same detector holder as the instrument response factor K must be removed in order to take the ratio of the sample and reference signals as demonstrated in equation 9. In this only case, the interface between the sample or reference and the detector holder is the same which imply that the factor K is the same for both sample and reference signals. By using two different detectors holder for the sample and the reference as Feis and Angeloni studies, the instrument response factor is not the same which imply that the ratio between the sample and the reference is incorrect. This can explain the difference by almost a factor of three between Feis and Angeloni results and the results presented here.

COSBP Photodissociation

The thermodynamics of CO photodissociation from SBP (Table 5.4 and 5.5) are distinct from those observed for CO-HRP and are dependent upon the solution conditions (Table 5.6 and 5.7). In the presence of 50 mM phosphate buffer, pH 8 $\Delta H_{str}$ is found to be $\sim -23$ kcal mol$^{-1}$ with a corresponding $\Delta V_{str}$ of -16 mL mol$^{-1}$ while at higher ionic strength (0.2 M phosphate) $\Delta H_{str} \sim 6$ kcal mol$^{-1}$ and $\Delta V_{str} \sim 18$ mL mol$^{-1}$. As with HRP, the changes in molar volume are consistent with the input of a water molecule into the heme distal cavity subsequent to CO photolysis regardless of solution ionic strength. However, in the case of HRP, $\Delta H_{str} \sim 0$ presumably due to the formation of additional hydrogen bonds between the water molecule and amino acid(s) within protein that compensate, energetically, for the loss of hydrogen bonding between the entering water molecule and neighboring water molecules in the bulk solution. This is clearly not the
case in SBP solubilized in phosphate buffer for which $\Delta H_{\text{str}} \neq 0$ suggesting distinct hydrogen bonding interactions within SBP. In addition, the differences in enthalpy between the high and low ionic strengths would suggest that the hydrogen bonding between the entering water molecule and the protein matrix is influenced by local charges. At higher ionic strength the value of the enthalpy change is only slightly lower than $\Delta H_{\text{vap}}$ for water indicating minimal H-bond formation while at lower ionic strengths $\Delta H_{\text{str}}$ is quite exothermic. Examination of the crystal structures reveal three Arg residues which are likely involved in stabilization of water clusters within the heme distal pocket (Arg 31) and/or the ligand access channel near the heme edge (Arg 173 and Arg 175). Since these residues are likely to be charged within the pH range used here (pH 7 - 8) changes in solution ionic strength could significantly impact hydrogen bond formation between the Arg side chains and nearby water molecules.

Table 5.6: Summary of $\Delta H_{\text{str}}$ and $\Delta V_{\text{str}}$ associated with CO photo-release from ferrous HRP.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta V_{\text{str}}$ (mL mol$^{-1}$)</th>
<th>$\Delta H_{\text{str}}$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50mM Tris</td>
<td>-11</td>
<td>2</td>
</tr>
<tr>
<td>100mM Tris</td>
<td>-10</td>
<td>-3</td>
</tr>
<tr>
<td>150mM Tris</td>
<td>-11</td>
<td>7</td>
</tr>
<tr>
<td>50mM Tris + 100mM NaCl</td>
<td>-12</td>
<td>0</td>
</tr>
<tr>
<td>0.2M phosphate</td>
<td>-14</td>
<td>4</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>-12</strong></td>
<td><strong>2</strong></td>
</tr>
</tbody>
</table>
Table 5.7: Summary of $\Delta H_{str}$ and $\Delta V_{str}$ associated with CO photo-release from ferrous SBP.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$\Delta V_{str}$ (mL mol$^{-1}$)</th>
<th>$\Delta H_{str}$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 M phosphate</td>
<td>-16</td>
<td>-23</td>
</tr>
<tr>
<td>0.2 M phosphate</td>
<td>-17</td>
<td>6</td>
</tr>
<tr>
<td>50 mM Tris</td>
<td>-14</td>
<td>-4</td>
</tr>
<tr>
<td>100 mM Tris</td>
<td>-14</td>
<td>-10</td>
</tr>
<tr>
<td>150 mM Tris</td>
<td>-15</td>
<td>-10</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>-14</strong></td>
<td><strong>-8</strong></td>
</tr>
<tr>
<td>50 mM Tris + 100 mM NaCl</td>
<td>-11</td>
<td>6</td>
</tr>
</tbody>
</table>

The presence of Tris also affects the observed thermodynamics associated with CO release in SBP. The values of $\Delta H_{str}$ and $\Delta V_{str}$ for CO-SBP in Tris and Tris + NaCl are -8 kcal mol$^{-1}$/-14 mL mol$^{-1}$ and 6 kcal mol$^{-1}$/-11 mL mol$^{-1}$, respectively. In both cases the observed volume changes are contractions with magnitudes within the range of the molar volume of water molecule similar to both HRP and SBP solubilized in phosphate buffer while the corresponding changes in enthalpy are distinct. Although the values of $\Delta H_{str}$ for SBP solubilized in Tris buffer are dependent upon solution ionic strength suggesting that the hydrogen bonding interactions involving water uptake are dependent upon charged groups they are distinct from those measured for SBP solubilized in phosphate buffer in the absence of Tris. This indicates that the presence of Tris also affects the hydrogen bonding interactions between bound water molecules and the protein matrix.
Examination of the X-ray structure of SBP (Figure 5.19) reveals a water cluster in the distal heme pocket near a Tris molecule. Since the experiments were carried out in Tris buffer at pH=8.0 and pKa of Tris is 8.1, ~ 50% of the Tris molecules are protonated (R-NH$_3^+$) resulting in a charge center near the water cluster. At higher ionic strengths it is likely that protonated Tris molecules will form ion pairs with the Cl$^-$ ions resulting in charge neutralization of the bound Tris. Since hydrogen bonding is an electrostatic interaction, changes in charge density near hydrogen bonding sites may have a significant influence on the enthalpy of H-bond formation accounting for the difference in enthalpy between the SBP in Tris and Tris + 100 mM NaCl.

![Figure 5.19: Illustration of the heme active site of ferric SBP showing the position of the bound Tris molecule.](image-url)
It is of interest to note that $\Delta H_{\text{str}}$ is the same for SBP photolysis in the presence of either Tris 100 mM NaCl or 0.2 M phosphate buffer. Since there is no evidence of phosphate binding to SBP this would suggest that water uptake upon CO photolysis for SBP is not influenced by the presence of a neutral Tris molecule but rather the presence of a charge in distal heme pocket. In addition, the fact that $\Delta H_{\text{str}}$ is 6 kcal mol$^{-1}$ for SBP and $\sim 0$ kcal mol$^{-1}$ for HRP suggests a different hydrogen bonding network for the bound water molecules between the two proteins. This is somewhat consistent with the X-ray structures for the oxidized forms of both proteins which indicate differences in the solvent access channel with that of SBP being more open than that for HRP. The thermodynamic profiles of HRP and SBP are summarized in Figure 5.20.
Figure 5.20: Thermodynamic profiles for CO photorelease from ferrous HRP and SBP under the various solvent conditions.
Miksovska et al. have studied CO photodissociation from globin type peroxidase, dehaloperoxidase from A. ornata, using photoacoustic calorimetry [43]. The relatively small molar volume and enthalpy changes associated with CO photorelease from this enzyme, $\Delta V = 9.4 \pm 0.6$ mL mol$^{-1}$ and $\Delta H = 8 \pm 3$ kcal mol$^{-1}$, were attributed to the high flexibility of His 55 in the distal heme binding site and associated with the alteration of hydrogen bonding networks. Interestingly, ligand escape to the surrounding solvent was reported to occur within 50 ns upon photodissociation suggesting that a rapid ligand escape to the surrounding solvent is also a characteristic for enzymes with peroxidase activity.

**Comparison with Myoglobin**

The PAC results for CO-HRP and CO-SBP are also quite distinct from those observed for the globin class of proteins. Previous PAC studies of CO photodissociation from both sperm whale and horse heart myoglobin indicate biphasic relaxations while both HRP and SBP display monophasic relaxations. In the case of horse heart myoglobin, the fast relaxation occurring <50 ns is associated with a $\Delta H$ of 7 kcal mol$^{-1}$ associated with a small contraction $\Delta V$ of -3 mL mol$^{-1}$ which is attributed to the formation of a geminate pair and the cleavage of a salt-bridge between Arg 45 and the 6-propionate of the heme active site. The second relaxation occurring ~700 ns after CO photolysis gives a $\Delta H$ of 8 kcal mol$^{-1}$ associated with a $\Delta V$ of 14 mL mol$^{-1}$ which is attributed to the reformation of the salt-bridge. [44,45]. In the case of sperm whale myoglobin the fast relaxation <50 ns gives rise to a $\Delta H = -2.2$ kcal mol$^{-1}$ and a $\Delta V = -10$ mL mol$^{-1}$. The second relaxation which has a lifetime of ~700 ns has a $\Delta H = 14.6$ kcal mol$^{-1}$ associated with a contraction $\Delta V = 5.8$ mL mol$^{-1}$ [44,45]. The differences between these proteins are due primarily to a Lys residue in sperm whale Mb in place of Arg 45 in horse heart Mb which affects the salt-bridge with the heme propionate.

Transient docking sites, energy barriers between transient states and even kinetics of ligand migration were studied by computation in order to offer the pathways of the ligand entry or exit into sperm whale Mb [29-34]. For example, room temperature
molecular dynamics (MD) simulations were used by Ruscio et al. [35] to probe not only the trajectories for CO diffusion from the heme group to the bulk solvent but also trajectories for the diffusion of CO from the bulk solvent into the protein matrix and approaching the heme active site. The results of these MD studies by Ruscio demonstrated in terms of ligand escape subsequent to Fe-CO bond cleavage, that the pathways is primarily involving two Xe atom docking sites, namely Xe4 and Xe1 which is consistent with previous time resolved X-ray studies of the photodissociation of CO from the ferrous protein [36,37].

Ligand migration pathways, in the case of the peroxidases, have not yet been examined either computationally or using X-ray crystallography to probe Xe binding sites comparable to those observed in Mb. Only three populations of CO were found using a recent step-scan FTIR study of the CO stretching frequency subsequent to CO photolysis [49]. One of these populations reveals a transient ‘surface bound’ state and the other two reveal different conformers of the heme bound CO. The FTIR signals show that the decay for these three populations has the same rate constants with no evidence of transiently docked CO to sites within the protein matrix even if the time resolution was only ~ 15 ms. Using the CASTp algorithm, the interior cavities of both SBP and HRP were studied and demonstrated a large internal cavities (1,332 Å³ in HRP and 1,569 Å³ in SBP) surrounding the heme active site which are in direct contact with the bulk solvent (see Figure 5.21) [50]. Additional internal cavities were also found in both HRP and SBP but these cavities do not appear to have a direct connection with the distal heme pocket which implies that it is not likely to be accessible to CO after photolysis. In contrast, sperm whale Mb demonstrated several internal cavities (the largest is 1,182 Å³) which have directly access to the distal heme pocket. These cavities provide not only transient docking sites but also ligand escape channels similar to those identified in the MD simulations discussed earlier. These analyses demonstrated that unlike Mb, the photolysis of both COHRP and COSBP results in a rapid migration of CO from the heme pocket into the bulk solvent which in the same time allowing one or more water molecules to enter through the same heme access channel (Figure 5.22).
Figure 5.21: Illustration showing the largest internal cavities in horseradish peroxidase and sperm whale Mb using CASTp.
Figure 5.22: Summary of the pathway of CO after photolysis in HRP and SBP with the view of the heme pocket for Soybean peroxidase down the direct channel. CO is bound to the heme iron (a). Then CO is photodissociated from the heme iron (b). In the first fifty nanoseconds, CO leaves the heme pocket to the surrounding solvent by the direct channel and two water molecules are input of into the ligand access channel (c).
5.2.5. Conclusion

In summary, the results presented here demonstrate unique thermodynamics for CO release from heme peroxidases that reflect the open access channel between the bulk solvent and the heme active site. The results are consistent with water molecules being input into the substrate channel upon CO release with varying degrees of hydrogen bonding depending upon the nature of the protein. In the case of HRP, the results suggest a single water molecule being taken up upon CO release and this uptake is not affected by the nature of the solvent. In contrast, CO photorelease from SBP is dependent upon the presence of Tris docked within the heme distal pocket as well as the solution ionic strength. The Tris molecule affects the hydrogen bonding network of the associated water molecules. In addition, the data suggest that it is the charge on the Tris molecule that is most influential. These results are clearly distinct from the globin class of proteins, e.g., myoglobin, highlighting the role of the protein matrix in modulating the energy barriers to ligand access to the distal heme pocket.
5.2.6. References


Chapter VI – Conclusion

6.1. Summary and Conclusion

The research presented in this dissertation is mainly focused on the understanding of the transmission of the signal in signaling proteins using time-resolved thermodynamic methods. Cell signaling is a complex system of communication and is involved in the development and coordination of the cell. The ability of the cell to sense and respond to a signal will be implicated in the development and the immunity of the cell. Some diseases like cancer, diabetes or autoimmunity can increase if an error appears in the transmission of the signal. Thus, by understanding the mechanism of cells signaling, more efficient treatments for diseases might be developed. As the signaling pathway in large organisms such as eukaryotes is more complex than small organisms, for instance, bacteria, the study of bacterial systems is very attractive. The communication in bacteria is similar to eukaryote cells (extra/intracellular communication with others cells or organelles and/or adaptation to environmental changes). Thus, the study of the signal, activation and transmission of bacteria will be similar but simpler. Different types of signals (amino acids, different pH or temperatures, or gas molecules) can bind to the sensor domain in bacteria, involve a cascade of phosphorylation and transmit the signal to the flagella which will then reply to the signal and will tumble if the signal is repellent or go forward if the signal is attractant.

The different ligands that can bind the heme sensor and activate the different steps involved in the transmission of the signal, are gas molecules such as O₂, CO and NO. The study of soluble heme oxygen sensor will give an opportunity of understanding in detail the mechanism of small molecule binding to a sensing domain and the transmission of the signal to the effector domain. Time-resolved photothermal methods as well as transient optical techniques were used to obtain thermodynamic profiles for ligand binding/release in heme based signaling proteins including HemAT from Bacillus subtilis, FixL from
Bradyrhizobium japonicum and Sinorhizobium meliloti, and CooA from Rhodospirillum rubrum. Furthermore, a number of model systems were also observed to understand the underlying thermodynamic processes in heme ligation. To summarize, this dissertation has contributed to the following:

The first project presented in the model systems is the study of the mepepy. The results for the trans to cis isomerization of the mepepy reveal one energy minimum for the cis form and two energy minima separated by 9 kcal mol\(^{-1}\) for the trans form which correspond to two different conformations of the trans form of the mepepy. Computational studies demonstrated that the dipole moments between the trans and the cis form of the mepepy are very similar which illustrate a very restraint change in the electronic structure of mepepy to compare to azobenzene molecules or other push-pull molecules. Finally, it has been demonstrated that the mepepy pyridine group can form an H-bond with the solvent which is lost after isomerization of the mepepy from the trans to cis form.

The second project for the model systems demonstrate several key features of the trans to cis isomerization of mepepy and spin crossover of the iron in the Fe(III)(salten)(mepepy) complex. Foremost, a volume change equal to \(\Delta V = 0.7 \pm 0.3\) mL mol\(^{-1}\) was calculated for the mepepy ligand and was associated to the loss of H-bond between a water and the mepepy pyridyl group. The enthalpy change associated to the isomerization to the trans to cis mepepy in the mixture acetonitrile/water is equal to \(\Delta H = 33 \pm 10\) kcal mol\(^{-1}\) and associated to the cleavage of a positive charge-assisted H-bond between the water and/or acetonitrile and the protonated mepepy pyridinium group in addition to the loss of H-bond between a water and mepepy pyridyl group. PAC results demonstrated that the volume change (\(\Delta V = 0.9 \pm 0.3\) mL mol\(^{-1}\)) and the enthalpy change (\(\Delta H = 37 \pm 10\) kcal mol\(^{-1}\)) of the Fe(III)(salten)(mepepy) complex are related to the spin crossover of the iron associated with an expansion of the Fe(III)(salten)(mepepy) complex into the solvent and to the cleavage of a Fe\(^{2+}\)N bond with the formation of a
hydrogen bond between a water molecule and the secondary amine. The spin change of the iron is estimated to be equal to $\Delta V = \sim 0 \text{ mL mol}^{-1}$ and $\Delta H = \sim 0 \text{ kcal mol}^{-1}$.

The last model system project was the use of the Debye-Hückel equation in order to calculate the solvent excluded volume of molecules, complexes and/or proteins. The theory was validated using two ruthenium complexes. These results confirm a charge transfer between the ligand and the metal for Ru(bpy)$_3$ and Ru(phen)$_3$ close to one. As well, the excluded volume change was calculated and equal to $ \sim -2.1$ and $-1.9 \text{ mL mol}^{-1}$ for the prompt phase and $\sim 1.7$ and $1.9 \text{ mL mol}^{-1}$ for the slow phase, for both Ru(bpy)$_3$ and Ru(phen)$_3$ respectively.

Different signaling projects were studied in order to understand the transmission of a signal along a protein. The first signaling project is the study of BsHemAT from *Bacillus subtilis*, a GCS domain sensor and an aerotactic transducer. In summary, both transient absorption and PAC data reveal a fast conformational transition associated with ligand release from BsHemATHD. The conformational transition results in a non-equilibrium deoxy form of the protein which persists during the ligand rebinding phase. The thermodynamics of this transition are quite distinct from the PAS domain BjFixL heme domain suggesting quite different signaling mechanisms between the GCS and PAS domain sensors.

The second signaling project is the study of CooA from *Rhodospirillum rubrum*. CooA is a transcriptional activator. PAC results reveal a fast conformational change associated with the photodissociation of CO from CooA. The conformational change demonstrated a large enthalpy and volume change which were associated to a reorientation of the C-helix in addition to the disruption of a salt-bridge.

The last signaling project is the study of full domain wild-type SmFixL associated with five different mutants from two different critical amino acids (R200 and I209M) which are supposed to be involved in the transmission of the signal and also the study of
SmFixL heme domain. SmFixL from Sinorhizobium meliloti is involved in the regulation of the nitrogen fixation gene expression in Rhizobi. PAC results reveal a quadriphasic relaxation for the different SmFixLWT, and the five different mutants associated with the photodissociation of CO. These thermodynamic results confirm that the residues R200 and I209 are very important in the transmission of the signal and if a mutation is taking place, it might involve different conformational changes which might induce the transmission of a different signal. The results for SmFixLHD reveal a monophasic relaxation which is different to the results from BjFixLHD and suggest a fast release of the ligand to the solvent.

Finally, two different model protein systems were studied in order to help understand of the transmission of the signal along a protein. The first model protein system is the study of the sandbar shark hemoglobin from Carcharhinus plumbeus as hemoglobin is an allosteric protein. The transient absorption result displays a biphasic relaxation kinetic. The first relaxation was attributed to the CO geminate recombination to the same subunit and the second relaxation to the recombination to another subunit. PAC demonstrated a triphasic relaxation where the first phase was associated to CO geminate recombination, the second phase to the tertiary transition from R to T for one subunit and the third phase to the R to T quaternary transition of the globin. The rate of the relaxation are similar to the human hemoglobin with the exception of the third phase which is faster in the shark hemoglobin.

The second model protein project was the study of two peroxidases: the horseradish and soybean peroxidase which participates in the oxidation of a large array of organic and inorganic compounds via heme catalyzed reduction of H₂O₂ to water. Unique thermodynamics for CO release from heme peroxidases which reflect the open access channel between the solvent and the heme active site are revealed with the PAC results. These results are consistent with the input of water molecules into the channel upon CO release. In the case of HRP, a single water molecule is being input into the channel upon
CO release which is not affected by the nature of the solvent. In contrast, in the case of SBP, the CO photorelease is dependent upon the presence of a Tris molecule docked within the heme distal pocket as well as the solution ionic strength. These results are clearly distinct from the globin class of proteins, e.g., myoglobin, highlighting the role of the protein matrix in modulating the energy barriers to ligand access to the distal heme pocket.

The different results which are presented in Table 6.1 reveal that the thermodynamic profiles are different for each protein. The results for the PAS domain are different to the GCS domain data. The results collected for the FixL protein which belongs to the PAS domain family, shown one, two or four different phases depending on the type of FixL (Bj/FixL or Sm/FixL) and on the type of Sm/FixL mutation (Sm/FixLR200A...). These results are different to compare to HemAT protein which belong to the GCS domain family and shown only a monophasic relaxation. CooA from the CooA domain is different to the results obtained for the GCS (HemAT) or the PAS heme domain (Bj/FixL or Sm/FixL). Although CooA is a full protein to compare to the Bj/FixL or HemAT heme domain protein, the results shown faster change following the photodissociation of CO implying different signaling mechanisms with the protein from the PAS domain or GCS domain. The results presented with Sm/FixL which is a full protein showed four different phases to compare to CooA which was also the study of a full protein demonstrating once again a difference of the transmission of the signal between the proteins. Bs/HemAT which is a myoglobin like domain should show similar thermodynamic profile to compare to myoglobin as the two heme domain present similarity. The results show very different thermodynamic profile with a monophasic relaxation obtained with Bs/HemAT and a biphasic relaxation with myoglobin.

To conclude, inside the PAS domain family, the results presented are different, which demonstrated that even inside the same family and with the same gas molecule CO, the transmission of the signal is different and each protein replies to a signal differently. All the data conclude that each protein is unique, has a unique way to respond
to a signal and its own thermodynamic profile following the transmission of the signal even if the protein belongs to the same family.

The results summarized in the Table 6.1 were all collected in Dr Larsen’s laboratory and the results in blue were collected by the Author.
Table 6.1: Summarized of the variation of volume and enthalpy for different heme-based sensor proteins.

<table>
<thead>
<tr>
<th>Sample</th>
<th>&lt;50 ns</th>
<th>Intermediary Phase 1</th>
<th>Intermediary Phase 2</th>
<th>Slow Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔH</td>
<td>ΔV</td>
<td>τ</td>
<td>ΔH</td>
</tr>
<tr>
<td>Fe4SP-2MeIm</td>
<td>17 ± 3</td>
<td>21 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FixLH</td>
<td>12 ± 3</td>
<td>-1 ± 0.5</td>
<td>150</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>tFixL</td>
<td>21 ± 0.7</td>
<td>4 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SmFixL (full protein)</td>
<td>-9 ± 8</td>
<td>10 ± 3</td>
<td>190</td>
<td>18 ± 16</td>
</tr>
<tr>
<td>SmFixLR200A</td>
<td>-7 ± 6</td>
<td>15 ± 2</td>
<td>100</td>
<td>63 ± 20</td>
</tr>
<tr>
<td>SmFixLR200Q</td>
<td>-26 ± 13</td>
<td>17 ± 2</td>
<td>92</td>
<td>62 ± 8</td>
</tr>
<tr>
<td>SmFixLR200E</td>
<td>-10 ± 5</td>
<td>21 ± 3</td>
<td>120</td>
<td>31 ± 14</td>
</tr>
<tr>
<td>SmFixLR200H</td>
<td>-39 ± 5</td>
<td>4.7 ± 0.7</td>
<td>95</td>
<td>74 ± 7</td>
</tr>
<tr>
<td>SmFixLI209M</td>
<td>-54 ± 3</td>
<td>3 ± 0.4</td>
<td>115</td>
<td>84 ± 6</td>
</tr>
<tr>
<td>SmFixLH</td>
<td>8.8 ± 0.9</td>
<td>21 ± 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcDos</td>
<td>11 ± 0.6</td>
<td>-2 ± 1</td>
<td>150</td>
<td>-28 ± 0.9</td>
</tr>
<tr>
<td>ConA (full protein)</td>
<td>-414</td>
<td>-46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HemAT-Bs</td>
<td>-20 ± 5</td>
<td>4 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HH Mb</td>
<td>7 ± 3</td>
<td>-1 ± 0.6</td>
<td>607</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>Sandbar Shark Hb (pH=6)</td>
<td>19</td>
<td>6.6</td>
<td>0.6</td>
<td>48</td>
</tr>
<tr>
<td>Sandbar Shark Hb (pH=7)</td>
<td>17</td>
<td>5.5</td>
<td>0.6</td>
<td>48</td>
</tr>
</tbody>
</table>
6.2. Future Directions

In order to better understand signaling proteins in general and how a signal is transmitted along the protein, the author proposes different directions for the signaling protein projects and for the model system projects which will help the understanding of the thermodynamic processes in heme ligation.

- Model system project:
  - In order to confirm the $\Delta V$ and $\Delta H$ of the spin crossover of the iron, another system should be tried, for instance the Fe$^{3+}$ tetrakis-(4-sulphonatophenyl) porphyrin (Fe4SP) with the mepepy ligand linked in its fifth position.
  - In order to confirm the results obtained for the Debye-Hückel equation, the results should be checked by a computational method.
  - The Debye-Hückel equation should be also tested with another ruthenium complex than the Ru(bpy)$_3$ and Ru(phen)$_3$, for instance, the Ruthenium(II)bis(2,2′-bipyridine)(4,4’-dicarboxy-2,2′-bipyridine) (Ru(bpy)$_2$(dcbpy)) in order to confirm the hypothesis stating that the excluded volume change can be calculated using the Debye-Hückel equation. This ruthenium complex will also confirm that the $\Delta V$ is ionic strength dependent but the $\Delta H$ is independent and that the excluded volume change does not dependent on the size of the ruthenium complex.
  - Myoglobin should be examined with the Debye-Hückel equation and should confirm that the Debye-Hückel equation can be used to find the excluded volume change of proteins, as myoglobin is well studied and known.
  - Another model with a total charge separation should be tested instead of the ruthenium complexes which have a partial charge separation after photoexcitation.
- **Signaling protein project:**
  
  - *Bs*HemAT protein should be analyzed after attaching the linker to the heme domain in order to draw a thermodynamic profile of the transmission of the signal until the linker. Then the kinase domain should be attached to the heme domain and the linker to draw the complete thermodynamic profile of the full protein.
  
  - HemAT protein from *Halobacterium salinarum* should also be studied to compare both proteins *Bs*HemAT and *Hs*HemAT and examine if the thermodynamic profile is similar or different in the same family.
  
  - *Bs*HemAT should also be studied for signal transmission using NO as the signal activator. As *Bs*HemAT was already studied with the gas molecule O₂ and CO, these results can be compared with the same protein, and demonstrate the effect that different gas molecules have on the thermodynamic profiles.
  
  - A NO sensor protein should also be examined in order to be able to compare the thermodynamic profiles between the four different heme-based sensors (PAC, GCS, CO sensor with CooA and NO sensor with the HNOB family). For instance, GCs, a second messenger, mononucleotide activator or Rsp2043_Rhsp, a second messenger, dinucleotide activator would be a good start.
  
  - *Sm*FixL protein should be analyzed using O₂ gas molecule to compare the thermodynamic profiles between CO and O₂ for signal transmission. As FixL is inhibited when O₂ binds the heme domain, the thermodynamic profiles of the transmission of the signal between an activator and inhibitor signal can be compared.
  
  - Finally, the thermodynamic profile of myoglobin (Mb) with different mutations should also be examined, for instance, mutation of the Lys 45 for the horse heart Mb or Arg 45 for the sperm whale Mb, in order to observe the difference in the thermodynamic profiles when CO is released to the solvent.
About the Author

Audrey Mokdad received her Bachelor’s degree in Chemistry from the University of Caen, France in 2000. In 2001, she finished her Master’s degree in Chemistry with a major in organic chemistry and in 2003, her Master’s degree in chemical engineering. While, in the Bachelor and Master’s program in France, she did several internships with domestic and international companies such as Rhodia, GlaxoSmithKline or L’Oreal. In 2004, Audrey was admitted to the Ph.D. program at the University of South Florida and joined Dr. Randy W. Larsen’s research group. Her research focuses on the time resolved thermodynamic studies in heme signaling proteins and model systems. She is co-authored of three scientific publications and is currently preparing several other manuscripts. She has presented her research at local, regional and national conferences, including Annual Raymond N. Castle Student Research Conference, Florida Annual Meeting and Exposition of the American Chemical Society (FAME), Florida Inorganic Mini-Symposium (FIMS), Southeast Regional Meeting of the American Chemical Society (SERMACS), Annual Meeting of the Biophysical Society conference and American Chemical Society National Meeting (ACS).