ULTRAFAST PROTEIN CONFORMATION DYNAMICS

DISSERTATION

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By

Justin J. Link, M.S.

* * * * *

The Ohio State University

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Dissertation Committee: Approved by
Professor Dongping Zhong, Adviser
Professor Bruce Patton
Professor Fengyuan Yang
Professor Chenglong Li

Graduate Program in Physics
ABSTRACT

Proteins are dynamical in nature. Their ability to function relies on their overall flexibility. Conformational fluctuations occur on many timescales yet, it is the ultrafast dynamics that are still not well understood. This dissertation is a systematic investigation of ultrafast protein conformational dynamics studied with ultrafast laser spectroscopy coupled with site-directed mutagenesis. Using a single intrinsic tryptophan as the local optical probe, we strived to develop optical techniques that would allow one to see in real time ultrafast protein conformational dynamics. Two methods were explored, each viewing the conformational dynamics in a different light. The first method involved resonance energy transfer (RET) between the known energy transfer pair of tryptophan and heme in proteins. RET is highly dependent upon the orientation and distance between the tryptophan and heme, thus, obtaining information of the RET characteristics will allow us to view relative distance changes between different conformations of proteins. The other method explored investigates the local environmental conformational changes in the vicinity of both the tryptophan and heme. This method utilizes ultrafast transient absorption to observe the absorption change of the ground-state of heme or
tryptophan due to environmental response of the protein after perturbation, also known as the Stark effect.

*Sperm whale* myoglobin was chosen as the first model system to study due to the wealth of knowledge available. It also only contains two intrinsic tryptophans and one prosthetic heme, both of which are chromophores. Site-directed mutagenesis was used to create over 20 single tryptophan mutations in an effort to globularly map out the ultrafast conformational dynamics. Characterization of the RET in mutants of different oxidation states was first completed using fluorescence up-conversion. Conformational dynamics were induced via photodissociation of CO from myoglobin and studied via both fluorescence up-conversion and ultrafast transient absorption. The former involved the implementation of an innovative 3-beam pump probe experiment where the latter utilized visible pump with UV-visible probing. The ultrafast transient-absorption was used in an effort to observe the ground-state tryptophan response and local heme environmental response upon photolysis of carboxy-myoglobin. The ultrafast conformational fluctuations upon ligand dissociation were determined to be extremely small and the mutation points of tryptophan were not sensitive to the fluctuations in myoglobin.

The second model system studied for the ultrafast conformational dynamics of proteins was the well studied *horse heart* cytochrome c (Cyt c). Similar studies were
performed and we observed, upon photolysis of the methionine-Fe bond, a proteinquake on the ultrafast regime. The ground-state absorption change of tryptophan was observed using ultrafast transient-absorption. Upon the success of the experiment, 11 single tryptophan mutations were created in the hope to globularly map out the ultrafast conformational dynamics. Unfolding experiments on Cyt $c$ were also studied by fluorescence up-conversion in efforts to elucidate the initial steps from unfolded to folded structure. Characterization of the unfolding curve was complete for several redox states of Cyt $c$ using resonance energy transfer.
Dedicated to my parents!
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February 12, 1980.................................. Born - Cleveland, Ohio

2002............................................. B.S. Physics

        Xavier University, Cincinnati, OH

2007............................................. M.S. Physics,

        The Ohio State University, Columbus, OH

2002-present.................................. Graduate Teaching and Research Associate,

        The Ohio State University, Columbus, OH

PUBLICATIONS


FIELDS OF STUDY

Major Field: Physics
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CHAPTER 1

INTRODUCTION

Biology is a beautiful beast evolved over millions of years. The human comprehension of the subject is currently and may never be complete but the quest for knowledge is higher than ever. Scientists’ ultimate goal is to fully and intimately decipher nature’s secrets. One of the hottest mysteries today involves the intricacies of proteins. Proteins are considered the building blocks of life in all species while each has its own unique structure and function. It is the intimate coupling of the protein’s structure to its function that is so intriguing [1]. In many biological systems a localized small structural change is converted to a higher order conformational change of protein and extends spatially to mesoscopic dimensions to elicit a biological function [2, 3]. In this quest for elucidation of this very process, many theories and techniques have been developed.

Two of the most common techniques to study the protein’s structure are X-ray crystallography and nuclear magnetic resonance spectroscopy (NMR). X-ray
crystallography is able to determine structure by observing a diffraction pattern from a protein crystalline ensemble. It is by a tedious analysis of the diffraction pattern that structural information can be obtained. This process is excellent for a determination of a stable state of a protein, typically the ground state, but it is argued that it may not be a good physiological representation of the protein’s structure due to the fact that proteins are in aqueous environments in nature. NMR on the other hand, uses the perturbations of the system seen through nuclear magnetic resonance to determine an ensemble of structures. The sample preparation in NMR solves the physiological issue with X-ray crystallography in that the protein is in an aqueous solution yet it is limited by the size of the protein, typically less than 700 residues, due to the difficulty in the spectral analysis.

Although the predominant static X-ray crystallography and NMR methods are powerful tools used to determine protein structures, a complete picture of the functionality of proteins cannot be studied. For the analysis of functions of proteins, one must understand the kinetics and dynamics hidden within the protein’s function. The context in which biochemical processes occur is essential to defining mechanisms and functions. This is where techniques of spectroscopy come to life. Optical spectroscopy, in particular, offers important advantages in versatility, specificity and time resolution. Techniques such as absorption, linear dichroism, circular dichroism, fluorescence, and vibrational excitation have been developed to probe specific chromophores for molecular
properties that may be correlated with biomolecular structure and function. Optical spectroscopies not only enable researches to obtain structural information about equilibrium species, but they also enable researches to follow biological reactions in real time, under conditions that mimic those in organism, thereby allowing characterization of mechanistically significant structural intermediates [4].

One of the difficulties with optical spectroscopy of proteins is the lack of ability to obtain site-specific information due to the lacking of chromophores that can be used to probe proteins. Absorption and fluorescence techniques can be utilized to study proteins but the site specific spatial detail is difficult to study due to the minimum number of ideal systems. One method to alleviate such issues is to attach an extrinsic dye to the protein. This allows for a very large quantum yield but it is argued that the extrinsic nature of the dye may induce conformational changes that are not desired as well as not give site-specific spatial resolution in the protein. On the other hand, the use of intrinsic amino acids and prosthetic groups preexisting in the protein has the appeal that structural perturbations are kept to a minimum when utilized correctly. Proteins are made up of twenty basic amino acids, three of which are the naturally occurring aromatic amino acids (phenylalanine, tyrosine, and tryptophan). Each has their own unique absorption and fluorescence characteristics. Of the three, tryptophan (Trp or W), is the most utilized as an optical probe due to the fact that it has the largest quantum yield and furthest red
absorption and fluorescence features. Many proteins possess multiple tryptophans and site-specific information cannot be observed on such ensemble studies with absorption and fluorescence. However, coupled with the powerful molecular biology technique of site-directed mutagenesis [5], site-specific probing of the protein can be accomplished [6-8].

The nature of proteins themselves is extremely dynamical. They exist in nature in aqueous solutions, therefore are not merely static structures. With the increasingly number of protein structures solved via x-ray crystallography and NMR, speculation directly between structure and function relationships of proteins have been made. However, it has become evident that the relationship between structure and function needs more than a static understanding for interpreting the molecular mechanism of protein functions. The missing bridge between structure and molecular mechanism of protein function is the dynamics at which the protein moves [9]. It is imperative to include time-dependent protein behaviors into the structure-function paradigm in order to better understand proteins in action. Biologically relevant motions of proteins can span more than fifteen orders of magnitudes from the femtosecond regime to seconds [10]. For a true understanding of the complex nature of protein dynamics, one must approach the problem by dissecting the process into simple processes and decipher their respective time scales. This approach can be difficult due to the fact that the simple processes can be convoluted on the same times scales, thus the need for a multidisciplinary approach.
Many classes of proteins have been studied since the advent of the science; however, one group of proteins has been seen as the model systems, heme proteins. Proteins that contain the cofactor heme (an iron centered porphyrin in our case) are classified as heme proteins. They were first described by their $O_2$ transport properties of hemoglobin in the blood of numerous animals and by which the color of the protein could be removed and crystallized as hemin [11], which in itself is considered a chromophore. Heme proteins have many different functions that range from electron transfer [12, 13], metal ion storage [14], ligand sensing [15-18] and transport [18, 19], and substrate oxidation [20, 21]. These proteins play integral parts in complex biological functions as steroid biosynthesis [22], aerobic respiration [23], and even apoptosis or programmed cell death [24]. Heme proteins have played important roles in establishing our understanding of chemical underpinnings of protein structure and function [25]. They have been important as model systems to structural biology [26], protein folding [27, 28], and the treatment of sickle cell anemia [29].

Two heme proteins in particular have served as the model systems of biophysics. Myoglobin and cytochrome $c$ are referred to as ‘the hydrogen atoms of physics’. Although these two proteins have been studied for decades, their dynamics are still not yet fully understood. Ultrafast dynamics of heme proteins have been studied since 1978 [30] and are still being studied today. These dynamics have not been truly elucidated and
it is the purpose of this dissertation to add pivotal insight into the ultrafast dynamics of heme proteins, namely myoglobin and cytochrome c with the techniques of ultrafast fluorescence spectroscopy and transient absorption coupled with the modern molecular biological techniques. In all experiments performed, some type of photo excitation or photodissociation is used to perturb the system from equilibrium. It is this perturbation that induces the conformational relaxation of the protein. Studies that have been utilizing this photodissociation were started by the pioneering microsecond flash photolysis experiment by Gibson in 1956 [31]. The protein dynamics have been studied all the way down to the ultrafast regime, where Green et. al. performed the first ultrafast optical spectra of heme proteins in 1978 [30]. Since then, many efforts have been made to further understand and describe the model systems of Mb and Cyt c ligand dynamics and conformational fluctuations. It is with this knowledge that the science field hopes to imply basic principles learned to other proteins.

Chapter 2 describes the experimental methodology with an emphasis on femtosecond-resolved fluorescence up-conversion and femtosecond transient absorption. Protocols to prepare samples used in this dissertation will be given in each individual chapter and attached appendices. A popular optical technique to infer protein conformation change is utilizing resonance energy transfer (RET). The theory of the RET is described in detail here.
In chapter 3, myoglobin is studied using several ultrafast techniques. In an effort to view in real time ultrafast protein conformational fluctuations, a new technique is developed and its successes and shortfalls are discussed. To develop the new technique, myoglobin mutants in three different oxidation states must be characterized. Site-specific information is gained by creating mutants of myoglobin with a single tryptophan. This site-specific information is used to globally map out the protein conformational dynamics. Ultrafast transient absorption coupled with photodissociation is also used to obtain a further understanding of the globular protein ultrafast dynamics.

Chapter 4 illustrates our studies completed on the heme protein cytochrome $c$. Femtosecond transient-absorption is used to determine the response of horse heart cytochrome $c$ in different redox states after photo excitation. The response is then interpreted in a manner that has never been published before. Further investigation is then described with the effort to globally map out the ultrafast protein conformational dynamics.

Chapter 5 discusses experiments designed to illustrate the protein unfolding of cytochrome $c$ using femtosecond fluorescence up-conversion coupled with resonance energy transfer. Both successes and shortcomings of the experiments are discussed.
CHAPTER 2

EXPERIMENTAL METHODOLOGIES

2.1 Femtosecond laser spectroscopy

All experimental measurements in the current studies were completed by using the femtosecond-resolved fluorescence up-conversion and transient absorption techniques. Experimental setup is illustrated in Figure 2.1 [32]. To obtain ultrafast temporal resolution, the pump-probe methodology is utilized. Synchronization of the photo induced perturbation is created by a relatively weak (70-150 nJ) and short pump-pulse (110 fs). The sample’s spectroscopic response was probed by another short and weak probe (gate) pulse. Dynamic response of the sample is created by a repetition of pump pulse followed by a temporally delayed probe pulse. Temporal resolution is limited by the spatial separation between optical path of the pump and probe pulse pair.
Figure 2.1: Schematic representation of the experimental setup with both the fluorescence up-conversion and the transient absorption configurations. The dashed line is for the transient-absorption probe pathway. F, filter. MM, movable mirror. MP, movable parabolic mirror. PD, photodiode. Millennia, Tsunami, Spitfire, Evolution 30, and SSA are the pump laser, femtosecond oscillator, two-stage amplifier, amplifier’s pump laser, and single shot autocorrelator, respectively. [32]
2.1.1 Fluorescence Up-Conversion Methodology

The fluorescence up-conversion experiment utilizes the pump probe methodology where the pump pulse excites the fluorophore to its excited state while the probe pulse detects the time-dependent fluorescence intensity with femtosecond resolution. Briefly, the femtosecond pulse after the two-stage amplifier (Spitfire, Spectra-Physics) has a temporal width of 110 fs centered at 800 nm with a pulse energy of more than 2 mJ and a repetition rate of 1 kHz. When the movable mirror (MM) is placed between the two optical parametric amplifiers (Fig. 2.1), half of the laser energy was used to pump an optical parametric amplifier (OPA-800C, Spectra Physics) to generate signal (1,289 nm) and idler (2,109 nm) beams. The latter was mixed with the residual fundamental (800 nm) in a 0.2-mm-thick β-barium borate (BB) crystal (type I) to generate a femtosecond pulse at 580 nm. This femtosecond pulse, compressed through a pair of prisms with double paths to reach a temporal resolution of 60 fs, was frequency-doubled to generate our pump wavelength at 290 nm by another 0.2-mm-thick BBO crystal. The 290 nm pump is used for the excitation of tryptophan, the experiment’s optical probe. The pump pulse energy typically was attenuated to 100-140 nJ before being focused into motor-controlled moving sample cell. The fluorescence emission was collected by a pair of parabolic mirrors and mixed with a gating pulse from another half of fundamental beam
(attenuated) in a 0.2-mm BBO crystal through a noncollinear configuration. The up-
converted signal ranging from 310 to 360 nm was detected by a photomultiplier coupled 
with a double-grating monochromator. The instrument response time under the current 
noncollinear geometry is between 400 and 500 fs as determined by the up-conversion 
signal of Raman scattering of water ~320 nm. For all studies, the pump-beam 
polarization was set at a magic angle (54.7°) with respect to the acceptance axis (vertical) 
of the up-conversion crystal, and the polarization of the gating beam was set parallel to 
this axis through a half-wave plate [6].

2.1.2 Femtosecond Transient-Absorption Methodology

The femtosecond transient-absorption experiment utilizes the pump probe 
methodology where the pump pulse initiates a perturbation/reaction in the sample cell 
and the probe pulse captures transient absorption of molecular species as a function of the 
delay time. The theory of transient absorption is now being presented. The Beer’s law of 
the absorption describes that absorption of the incident light (I_o) passing through a unit 
cell is directly proportional to the concentration of the light absorbing species in the cell. 
Light transmitted out (I_t) through the cell obeys the following relationship with the 
incident light (I_o):
Absorption \( = -\log \frac{I}{I_o} = \varepsilon cl \) \hspace{1cm} \text{Eq. 2.1}

where \((\varepsilon)\) is the molecular extinction coefficient (a constant for a particular molecular species at a given wavelength), \((c)\) is the concentration of the molecular species and \((l)\) is the path-length of the sample cell.

The transient absorption \( A(\tau, \lambda) \) at a given wavelength \( \lambda \) is defined as the ratio:

\[
A(\tau, \lambda) = -\log \frac{I_t(\tau, \lambda)}{I_o(\tau, \lambda)} \\
\text{Eq. 2.2}
\]

where \(I_t\) and \(I_o\) are the probe intensities measured with and without the pump pulse, respectively. \(A(t, \lambda)\) is obtained by two subsequent acquisitions. \(I_{t, \text{pump off}}\) is the transmitted light through the sample with no pump, i.e., there are no photoexcited states present and \(I_{t, \text{pump on}}\) is the transmitted light right after excitation. Comparing the pump off and pump on intensities with Eq. 2.1, Beer’s law of the absorption for transient absorption can be expressed as:

\[
\text{Absorption} = - \left[ \log \frac{I_{\text{pump on}}}{I_o} - \log \frac{I_{\text{pump off}}}{I_o} \right] \\
\text{Eq. 2.3}
\]

During the data acquisition, the difference in absorption in the absence or presence of the pump beam was measured with respect to the delay time between the pump and probe [33].
The experimental setup is as described. Briefly, the femtosecond laser pulse after the Spitfire Ti:Sapphire amplifier (Spectra-Physics) has a temporal width of 110 fs with energy of ~2 mJ per pulse and a repetition rate of 1 kHz. The laser beam is then equally split to pump two optical parametric amplifiers (OPA-800C, Spectra-Physics) by moving the movable mirror (MM) in Fig. 2.1 out of the beam path. The pump pulse at 400 nm was generated by direct doubling of the fundamental 800 nm from the first OPA through a 0.2-mm-thick β-barium borate (BBO) crystal. The various probe wavelengths from 500 to 700 nm were generated by mixing of the idler or signal with the fundamental from the second OPA through another BBO crystal (0.2 mm). Certain wavelengths were further doubled by a third BBO crystal (0.2 mm) to generate UV probing wavelengths from 280 nm to 310 nm. The movable parabolic mirror (MP) in Fig 2.1 must be moved out of the beam path. The pump beam polarization was set at the magic angle (54.7°) with respect to the probe beam. The sensitivity of the transient-absorption method can reach $10^{-4}$-$10^{-5}$ of absorbance change.
2.2 Förster Resonance Energy Transfer

2.2.1 Introduction

Upon the excitation of a fluorophore, there are many pathways to return to the ground state such as fluorescence, quenching, photobleaching, and resonance energy transfer (RET) [34]. Conformational dynamics is often estimated through the change of distance between two specific sites and RET offers ability to observe these changes, therefore, it can be considered a molecular ruler. RET was born in 1948 by the German scientist Theodor Förster to describe the energy transfer from a dipole-dipole interaction [35, 36]. It describes the radiationless energy transfer between two chromophores, one as the energy acceptor and the other known as the energy donor. It has been utilized in both single molecule and ensemble studies on proteins as a molecular ruler to infer protein conformational change [37-44]. RET offers an experimental approach to the determination of molecular distances in the range 10-100 Å through measurements of the efficiency of energy transfer between a donor and an acceptor located at two specific sites. RET is also a sensitive technique for detection of global structural alterations because of the sensitivity due to the inverse sixth power dependence of the transfer efficiency on the donor-acceptor distance [45].
2.2.2 Theory

Resonance energy transfer due to dipole-dipole interaction between a donor (D) and an acceptor (A) is a function of the spectroscopic properties of the two fluorophores and the physical characteristics of the solvent as represented in the classic Förster formulation by:

\[ R_0^6 = 8.79 \times 10^{23} \Phi_D \kappa^2 n^{-4} J \, \text{Å}^6 \]  

Eq. 2.4

where \( R_0 \) is the distance at which transfer depopulates the donor excited state at the same rate as all other deexcitation processes, including emission of fluorescence, \( n \) is the refractive index of the solvent, \( \Phi_D \) is the quantum yield of the donor in the absence of acceptors, \( J \) is the overlap integral between the emission spectrum of the donor and the absorption spectrum of the acceptor, and \( \kappa^2 \) is the orientation factor of the donor-acceptor system [41]. The rate of energy transfer is determined by:

\[ k_{RET} = \frac{1}{\tau_D} \frac{R_0^6}{r^6} \]  

Eq. 2.5

Where \( \tau_D \) is the donor lifetime and \( r \) is the distance between the donor and acceptor.
2.2.3 Index of Refraction: \( n \)

In Forster’s theory the index of refraction, \( n \), appears because of the interaction between point dipoles bathed in a continuous isotropic medium. In most experimental situations where RET is applied, the medium between the donor and acceptor is neither isotropic nor continuous. Nevertheless, assuming and average value in the 1.33-1.6 range will cause errors that are at the most 10% due to the \( n^{-2/3} \) dependence of \( R_0 \). The most frequently used values are 1.34 and 1.4 [46].

2.2.4 Quantum Yield: \( \Phi_D \)

The quantum yield is defined by the number of light quanta emitted by a donor divided by number of quanta absorbed by it. For RET to occur, the quantum yield must be sufficiently large. In proteins, the quantum yield can be determined by the comparison of the donor fluorescence in the absence of the acceptor and a known quantity. In the case of most protein calculations, the known quantity is that of tryptophan \( (\Phi_{Trp}=0.14) \) [47]. The quantum yield of the donor can be defined by [47]:

\[
\Phi_D = \frac{S_{protein}}{S_{Trp}} \cdot \frac{c_{Trp}}{c_{protein}} \times 0.14
\]

Eq. 2.6
Where $S$ is the area under the steady state fluorescence curve and $c$ is concentration. Concentration can be found by Beer’s law and the known molar extinction coefficient.

### 2.2.5 Integral Overlap: $J$

The integral overlap ($J$) can be defined as:

$$
J = \frac{\int F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda}{\int F_D(\lambda) d\lambda} \quad (\text{M}^{-1} \text{cm}^3)
$$

(\text{Eq. 2.7})

where $F_D(\lambda)$ is the donor fluorescence without acceptor per unit wavelength interval and $\varepsilon_A(\lambda)$ is the molar extinction coefficient of the acceptor alone at wavelength $\lambda$ [34]. The donor fluorescence needs not to be normalized. The molar extinction coefficient of the acceptor at wavelength $\lambda$ can be difficult to measure correctly, it truly depends on the known molar extinction coefficient at a know wavelength and using Beer’s law (Eq. 2.1) to determine $\varepsilon_A(\lambda)$ having the absorption spectra $A(\lambda)$. 
2.2.6 Orientation Factor: $\kappa^2$

The orientation factor ($\kappa^2$) is described as the relationship between the dipole of the acceptor and the dipole of the donor. It has a minimum value of 0 and a maximum value of 4 and an average value of $2/3$. It is a function of the angle ($\alpha_{DA}$) defined by the direction of the transition moments $\mathbf{D}$ and $\mathbf{A}$ and of the angles ($\alpha_D$, $\alpha_A$), that the two vectors form with the translation vector $\mathbf{T}$ connecting the centers of the two oscillators illustrated here in Figure 2.2 and defined as Eq. 2.8:

$$\kappa^2 = (\cos \alpha_{DA} - 3 \cos \alpha_D \cos \alpha_A)^2$$  \hspace{1cm} \text{Eq. 2.8}

Figure 2.2: Schematic of dipoles involved in energy transfer. $\mathbf{D}$ is the donor vector, $\mathbf{A}$ is the acceptor vector, and $\mathbf{T}$ is the translation vector [41].
The orientation factor ($\kappa^2$) can also be back calculated if the RET rate ($\tau_{RET}$), donor lifetime ($\langle \tau_D \rangle$), donor-acceptor distance ($r$), quantum yield ($\Phi_D$), index or refraction ($n$), and integral overlap ($J$) can be determined. By combining Eq. 2.4 and Eq. 2.5 and solving for the orientation factor, we get:

$$
\kappa^2 = \frac{\langle \tau_D \rangle r^6 n^4}{\tau_{RET} \Phi_D J \times 8.79 \times 10^{23}} \quad \text{Eq. 2.9}
$$
CHAPTER 3

ULTRAFAST DYNAMICS OF SPERM WHALE MYOGLOBIN

3.1 Introduction

Protein dynamics have been studied on different systems, however, there is one that has served as the model system, myoglobin. Myoglobin corresponds to the ‘hydrogen atom of physics’ in that it is the most well known and studied of all proteins. It was the first protein to be solved by x-ray crystallography in 1958 [26] and researchers have been using this protein as a model system for protein dynamics ever since. It is typically found in muscle tissue of vertebrates where they serve a role in oxygen storage and transport [19]. It has the ability to reversibly bind other diatomic molecules such as CO, NO, and CN [18, 19, 48, 49]. Myoglobins exhibit a low rate of autoxidation and a high affinity for O₂ [50]. Arguable the most studied species of myoglobin is from the sperm whale. Sperm whale myoglobin (Mb) consists of 153 amino acids ordered in eight α-helices (Figure 3.1). Mb contains two intrinsic tryptophans in the seventh and fourteen positions and a prosthetic heme group which is covalently bound to the proximal histidine (H93) and is closely involved with the distal histidine (H64). Our goal is to
develop a technique that can see in real time ultrafast protein conformational dynamics, therefore, we must use a model system to base our new technology upon. In order to do so, several experiments must be completed to characterize the static physiological nature of the protein. Two such methodologies will be used, one utilizing femtosecond up-conversion while the other using ultrafast transient absorption. Both experiments will be coupled with the powerful biological technique of site-directed mutagenesis to gain site-specific detail in the protein with the aspiration to globularly map out the protein conformational dynamics.
Figure 3.1: X-ray structure [51] of sperm whale myoglobin (PDB:1MBD) with the prosthetic heme group colored in purple covalently linked to the proximal histidine H93 colored in cyan. Also shown is the tryptophan W7 residue (colored yellow) as an optical for detecting protein conformation dynamics. The yellow spheres represent point mutations of W14, W12, and W15 from left to right.
3.2 Ultrafast Studies of Resonance Energy Transfer in Myoglobin

Conformational dynamics is often estimated through the change of distance between two specific sites and resonance energy transfer offers the ability to observe these changes, therefore, it can be considered a molecular ruler. RET, described in detail in chapter 2, has been used in studies of myoglobin for many decades. Warburg and Negelein first showed in 1928 that energy transfer could occur from the aromatic amino acids to the heme in heme protein [52]. Although much information is available in literature concerning the topic, several issues have arisen. Some studies have used the RET between an extrinsic dye and heme [53, 54] while others have used the intrinsic chromophore tryptophan and prosthetic heme group [42, 54-61]. These studies have evaluated all the necessary terms for the RET analysis yet have overlooked a few key points that are necessary for the correct interpretation of the data involved. Most studies attribute the orientation factor to the average over all space, which is 2/3, this may be an important neglect of the orientation factor. The intention of this chapter is to address issues such as the orientation factor and validity of x-ray structure of proteins and how they are interpreted as physiological representations of proteins. It is the goal of this entire section to develop a technique that uses RET as a molecular ruler to see in real time the ultrafast protein structural fluctuations.
3.2.1 A-helix and Local Conformational Fluctuations

Myoglobin (Mb) has two intrinsic tryptophans in the structure and a prosthetic heme. Tryptophan is considered an energy donor and heme is an energy acceptor in the RET process. Both of these Trps are located in the A-helix (W7 and W14) and have been successfully observed in RET with the heme [54, 58-61]. For preliminary studies, we have attempted to describe the RET from each individual Trp (W7 and W14) as well as two mutants (W12 and W15) of Mb located in the A-helix, Fig. 3.1, and use RET as a molecular ruler to infer local protein conformational fluctuations.

3.2.2 Materials and Methods

All of the femtosecond-resolved measurements in this section were carried out by using a fluorescence up-conversion. The integrated experimental setup has been described in chapter 2. The excitation wavelength was 290 nm and fluorescence gating were at five wavelengths: 310, 320, 330, 340, and 360 nm. Sperm whale myoglobin is a 17.7-kDa globular protein consisting of eight helical segments, namely A-H, as shown in Fig. 3.1. Among the 153-aa residues, there are two intrinsic tryptophan residues, W7 and W14, in the A-helix. In order to achieve site-specific detection, only one Trp residue in
each mutant is needed. As the first step, the tryptophan was removed and replaced by tyrosine one at a time, the two mutant proteins W7Y (W14) and W14Y (W7) were obtained. The plasmid pMb122 [62], containing the sperm whale myoglobin gene, was generously provided by Stephen Sligar’s group at the University of Illinois at Urbana-Champaign. To place a single tryptophan in other positions of the A-helix, both tryptophans (W7 and W14) were mutated out. The double mutation of W7YW14F was prepared, as also reported recently by another group [63]. This mutant is well overexpressed and stable. The incorrect folding structures of other double mutations if using other residue combinations such as W7YW14F, W7YW14Y, and W7FW14Y were noted and not used [63]. The mutant W7YW14Y was tested and the expression yield was low.

Triple mutations were designed carefully to place tryptophan in other positions by using the double mutations W7YW14F as a template. Careful design of the mutants was used and all mutants were carefully analyzed by absorption, fluorescence, and circular dichroism by Luyuan Zhang et al. of the Zhong group [6]. All of the mutants were expressed in Escherichia coli and purified mainly following the procedures described in Ref. 62 and in Appendix A. To achieve Apo-Mb mutants, the heme was removed following the procedures described in Ref. 64. For each mutant, more than 70 mg of protein were prepared. For Holo-Mb, the buffer used was 20 mM Tris 1mM EDTA pH
8.0 and for Apo-Mb, the buffer was 10 mM sodium acetate at pH 6.1. The protein concentration used in femtosecond-resolved studies was 200 µM. For quantum yield studies, the concentration was less than 10 µM and a 1 cm quartz cuvette was used. All Apo-Mb samples were made fresh and were stable for 2-3 days in 4°C. All of the femtosecond-resolved fluorescence transients were collected at 22°C and in the ferric (Fe$^{3+}$) state unless stated otherwise. Other states, deoxyMb (Fe$^{2+}$) and MbCO, were created according to Appendix C. To ensure that no change in the protein quality occurred during data acquisition, the protein absorption was taken before and after the experiment. The protein was also placed in a custom designed rotating cell made of quartz of 1 mm thickness to minimize possible photobleaching. DeoxyMb and MbCO data was taken in a 1 mm quartz cuvette that was moved rapidly to minimize photo bleaching.

### 3.2.3 Results and Discussion

#### A. Femtosecond-Resolved Transient Dynamics

Four mutations have been created in Mb and have studied using fluorescence upconversion (Fig. 3.2). The first step is to understand the single point mutations of W7
and W14, therefore Fig. 3.3 illustrates the femtosecond transients for W7 both Holo-Mb and Apo-Mb. As shown, the transient dynamics are dependent upon the fluorescence gating wavelength. One major relaxation process is protein solvation which has been studied in completeness for the aforementioned mutations of Mb [6]. From the Apo-Mb transients, we can see that there is a long lifetime in the nanosecond region. This is due to the fact that the energy acceptor (heme) is removed and the long component comes from the intrinsic fluorescence of the donor, in this case W7. The difference in the transients as a function of wavelength is the manifestation of the local solvation around the tryptophan. The intrinsic lifetime of the tryptophan itself does not change. In this case, the donor average intrinsic lifetime of W7 and W14 are 2.8 and 2.6 ns, respectively [161].

When the heme is retained within the protein as the energy acceptor, the transient dynamics change drastically. This situation where the energy donor and acceptor are present opens a fast channel for population deactivation. We monitor here the fluorescence decay as a function of time and we can interpret it as the solvation, intrinsic lifetime, and resonance energy transfer. Even in Holo-Mb, there is a slight difference in the transients for 310, 330, and 340 nm. This difference is again a manifestation of the local solvation dynamics. It is only in the 340 nm transient that the net solvation effects can be ignored and the contributing dynamics are purely due to RET and intrinsic
fluorescence lifetime. As mentioned previously, the transient can be deconvoluted and
the intrinsic lifetime of tryptophan contribution can be subtracted out, if present, to obtain
only the RET time, here it is 111 ps, consistent with published results [58]. Another
noteworthy point is that the transients decay to zero which illustrate that all excited
molecules transfer energy to the acceptor through RET. There are no conformations in
which the RET does not exist ($\kappa^2 \neq 0$) in any transient that completely decays to zero.

The remaining mutations of the A-helix of Mb are shown in figure 3.4. As
described before, the wavelength dependence of the transients is a manifestation of the
local solvation around the tryptophan. The intrinsic lifetime contribution of the
tryptophan has been removed in all of the shown transients (~10%). (Fig. 3.4 Top) The
RET time for W14, which is closer to the heme and further along on the alpha helix (Fig.
3.1), has an energy transfer of 22 ps, consistent with published results [58]. For the triple
mutant W12, where the histidine is replaced by a tryptophan, the RET time is 212 ps.
The W12 mutant is interesting and is the only mutant where the steady state fluorescence
peak for Holo-Mb at 337 nm is significantly red-shifted compared to that of Apo-Mb at
328.3 nm. This could possibly be attributed to the fact that the Apo-Mb and Holo-Mb
structures may not be the same. During the process of removing the heme, the folding of
Apo-Mb may adopt a different structure due to the hydrophobic interaction, leading to a
blue-shift of Trp emission at 328.3 nm. As you can see, the transient still has some fast
component at 350 nm that was still from the solvation. However, the RET time is 212 ps as determined from the emission of 360 nm.

Figure 3.4 bottom illustrates W12, W15, and W14 all together for a comparison. The W15 mutation is created when an alanine is replaced by the tryptophan. In this case, the energy transfer time is multiple exponential, however, the transient can be fit using a stretch model. The stretched time constant is 119.7 ps and $\beta=0.671$. The average time, $<\tau>=\frac{\tau}{\beta}\Gamma\left(\frac{1}{\beta}\right)$, is found to be 158 ps for W15. The fact that the transient cannot be fit using a single exponential decay may give some insight into the conformation of this mutant. The fact that the transient can be fit to a stretched exponential model infers that there exists heterogeneity of multiple conformations. At this position, the Trp could be very mobile and causes a multiple exponential decay of energy transfer, mainly from the orientation factor.
B. Steady State Analysis

With the RET times now measured for the mutants of myoglobin, we can focus further on the Förster energy transfer theory. The terms in Eq. 2.10, quantum yield of the donor ($\Phi_D$), integral overlap of the donor fluorescence with the acceptor absorption ($J$), the distance from donor to acceptor ($r$), must now be solved for using various techniques. The orientation factor can also be solved using molecular dynamics simulations. Each term must be meticulously calculated so that the concept of RET as a molecular ruler can be utilized. The index of refraction, $n$, for the current experiments is 1.33. All of the RET theory is addressed thoroughly in chapter 2 and equations will be referred to as in chapter 2.

The energy donor in Mb is the intrinsic Trp, this is the beauty of the experiment. Using the naturally occurring amino acid in site-directed mutagenesis gives site specific information on the conformational dynamics of the protein. To determine the quantum yield of the intrinsic Trp ($\Phi_D$), we must remove the energy acceptor, the prosthetic heme, thus creating Apo-Mb. The quantum yield is determined by Eq. 2.6. In using Eq. 2.6, one must be careful to use an extremely dilute concentration ($OD_{290nm}<0.1$), this is in the linear range of the fluorescence of most fluorescence instruments. The linear range is defined by the peak of the fluorescence being directly proportional to the concentration.
Another point of concern is to continually use fresh Trp in water due to the fact that we are using the Trp as a known control. To determine the concentration of the sample, a known molar extinction coefficient must be available. Several papers have addressed the molar extinction coefficient of Apo-Mb [19, 66, 67], however, we will use the values given in reference 67. For the wild type sperm whale myoglobin, there are two tryptophans and three tyrosines, therefore, for the single mutants (W7Y and W14Y), there are one tryptophan and four tyrosines, this is the same for the triple mutants as well because they begin with the template of W7YW14F. The molar extinction coefficient for the Apo-Mb mutants at 280 nm was 11,460 M⁻¹cm⁻¹. Another concern is the purity of our Apo-Mb. It have been seen that we have < 5% Holo-Mb contamination in our sample and this is minute in the overall calculation. The final point of concern is that of Raleigh scattering at such a small concentration. This scattering was taken into account and subtracted out, it has a λ⁻⁴ dependence and is addressed in Appendix D. The values we measured for W7 and W14 are 0.20 and 0.17 respectively.

In order for RET to occur, the integral overlap of the donor fluorescence with the acceptor absorption (J) must exist. We can see from figure 3.2 that the donor fluorescence (Apo-Mb) does indeed overlap the acceptor absorption (Holo-Mb). The integral overlap is calculated by Eq. 2.7. Points of concern are the purity of the states. When the experiments were complete, the protein was in mixture states. These mixture
states only produced a 3% error, which is well within experimental error, in the integral overlap from the purely ferric state. The percentage of Holo-Mb contaminating the Apo-Mb was also minute and taken into account. The J values for W7 and W14 are 4.71E-14 and 4.11E-14 M⁻¹cm³, respectively. These values are on the same order of magnitude as published results [58, 59].

The distances (r) between the energy donor (Trp) and acceptor (heme) were calculated two ways. One was directly from the crystal structure, PDB:1MBD, and the other was using a molecular dynamics simulation technique. In both cases, the distance was from the center of the indole ring of Trp to the iron center of the heme. Calculations from the crystal structure were directly calculated using PyMol, however were only found for W7 and W14 from the wild type structure. Crystal or NMR structures do not exist yet for the mutants studied. The distance from crystal structure for W7 and W14 were 21.2 and 15.0 Å respectively. Analysis of over 150 PDB structures of sperm whale myoglobin were checked for the distance values and all were averaged, excellent agreement occurred with the stated values.

Molecular dynamical studies were also performed to determine the distance between the Trp and heme in the single point mutants W7 and W14. The structure used was PDB:1MBD, it was chosen due to the history of use for our colleagues who did the
MD studies. Detailed analysis of the MD simulations can be found in reference 68. No mutations were performed on the crystal structure and the trajectories were then ran for 33 ns and it was determined that the distances were 23.2 and 16.1 Å for W7 and W14 respectively.

The orientation factor can also be determined using the crystal structure as well as the molecular dynamics trajectories for the mutants of Mb. In both cases, the dipoles of the Trp and heme must be well defined. Tryptophan has two excited singlet states, the \(^1\)L\(_a\) and \(^1\)L\(_b\). They have perpendicular transition moments and the \(^1\)L\(_a\) state has a larger static dipole [69]. In polar solvent, the \(^1\)L\(_a\) state lies below \(^1\)L\(_b\) and the observed fluorescence is dominated form the \(^1\)L\(_a\) emission. The photophysics of tryptophan has recently been characterized and the internal conversion of \(^1\)L\(_b\) to \(^1\)L\(_a\) has been shown to occur ultrafast, in less than 100 fs [44, 70]. The Trp dipole is accepted to be at -38° [71], relative to the center of the indole bridge (CE2 & CD2) to the carbon CD1 of Trp (long axis of the molecule). The transition dipole of the heme is little less defined. Literature states that there may be several orientations of the heme inside the protein, the normal (N) and disordered (D) [59, 60, 71-74]. The top of Figure 3.5 illustrates the possible values for the orientation factor for all possible angels and orientations of the heme transition dipole. This plot will here on be referred to the fish plot for the resemblance to a fish. Values ranging from 45 to 65° [75], 50 to 70° [60], and 50 to 60° [59] relative to
the α-meso axis of the heme have been reported. The most recent accepted values of 50-60° for the heme transition dipole is shaded in the fish plot to further emphasize the results. To determine the orientation factor from the crystal structure, the value of 55° has been used [60] and plotted on the fish plot. A program was written by a colleague in the Zhong group, Jeffrey A. Stevens, that calculated the orientation factor using the given dipoles for a crystal structure. Values for W7 and W14 from the crystal structure were determined to be 0.16 and 0.73 respectively. The molecular dynamics results for the mutants W7 and W14 are 0.2 and 0.63 respectively. By using the results aforementioned and Eq. 2.9, the experimental value of the orientation value can be determined and is also illustrated in Figure 3.5 (Bottom). Experimental values were calculated with the distances between donor and acceptor from crystal structure. A summary of all the data is listed in Table 3.1.

C. Discussion

Fig. 3.5 (Bottom) shows the results for the orientation factor for W7 and W14 as well as the methodology obtained. For both mutants, the values obtained by crystal structure and molecular dynamics are extremely similar. This arises from the fact that the molecular dynamics simulations begin from the crystal structure itself. In the analysis of
W7, when the system is heated up in the MD simulations, not much fluctuation is seen, even in a 33 ns trajectory and a value of 0.20 is obtained compared to 0.16 from crystal structure. However, the value calculated from experiment, 0.87, is drastically different. Two points are to be considered: (1) W7 is near the N-terminal of the protein, Fig. 3.1, where the protein is considered to be floppy. The opportunity for the Trp to rotate around leads to larger value of $\kappa^2$ than defined by the crystal structure. (2) The local orientation of the Trp is different from that of the crystal structure, an orientation that gives the value of 0.87 rather than 0.16. In both cases, the crystal structure and subsequent MD simulations do not represent the physiological representation of the true structure for W7.

In the case of W14, the Trp is buried inside the protein and further away from the N-terminus, Fig. 3.1. The fact that it is buried signifies a more rigid orientation than W7. Once again, both the crystal structure and MD simulation values are similar, 0.73 and 0.63 (obtained by averaging the trajectory over 33 ns), respectively. An interesting characteristic of W14 is noticed in the MD simulations, Fig. 3.5 (Middle). It appears that for the first 7.5 ns, the orientation of W14 fluctuates and for the remaining of the trajectory, it appears to come to a stable conformation, one that shows consistency with the value obtained from the crystal structure. The value for W14 from experiment, 0.69, agrees with both the crystal and MD simulation values. The results for W14 lead us to believe that the crystal structure may give a good physiological representation of the local
protein conformation. Note as well that the experimental orientation values are slightly different from the averaged 2/3 that is frequently used in literature. Arguments have been made to use 2/3 because the rotation time of the Trp would be faster than the RET, therefore, it would sweep many different conformations thus requiring an averaged value [58]. As we have shown, this is not the case and it implies that upon close examination of RET means that one must calculate the orientation factor and not just imply that it is the averaged value of 2/3.

The other two mutants, W12 and W15, have to be treated in a little different manner. It is evident that RET does exist and each have characteristic times of 212 and 158 ps respectively, however, analysis of the orientation value cannot be completed due to a structural issue. In order to obtain the quantum yield, integral overlap, distance, and orientations between the Trp and heme, a fundamental assumption needs to be made. The structure of the protein with (Holo-Mb) and without the energy acceptor heme (Apo-Mb) must be the same. One method to test this fact is by analysis of the steady state fluorescence peaks. Steady state fluorescence contains information on the structural characteristic of the protein. It is the integration of all the kinetic rates occurring in the protein. In case of solvation time, where water is moving around the Trp, it is typically less than 100 ps [6]. The fluorescence peak of Holo-Mb will be blue shifted if the RET time is shorter than the solvation time and if the time is longer than solvation, the peaks
will be extremely similar. If there is a red-shift of the Holo-Mb to that of Apo-Mb, there is a structural difference between the two. Table 3.2 shows the steady state fluorescence peaks for all four mutants both Apo- and Holo- Mb. Both W7 and W14 steady state fluorescence peaks signify that the Apo- and Holo-Mb structure are similar, however, W12 and W15 seem to have different structures between their Apo- and Holo-Mb forms due to the red-shifted Holo-Mb fluorescence peak. Due to the conformation difference between Apo- and Holo-Mb for W12 and W15, a full analysis of RET is not possible. However, this does point out that although the pH 6.1 conformation of Apo-Mb is considered the native or correctly folded structure of Mb, it is evident this is not entirely the case. When the protein is denatured in the process to remove the heme and then refolded, there appears to be some type of rotating of the A-helix. The Trp in the 12 and 15 positions do not have the same local conformation in the Apo-Mb structure to that of the Holo-Mb, mainly due to the hydrophobic interaction of Trp.

Data for three different states of Mb was also taken, ferric-, deoxy-, and MbCO. Figure 3.6 illustrates the femtosecond-resolved fluorescence transients of W14 in all three states at 350 nm gated wavelength. It is evident that all three species have extremely similar RET time, however, when the overlap integrals are calculated, there is a difference between the states, this is sensible because the absorption spectral characteristics of each species are different, Fig. 3.7. For W14, the J value of ferric is
4.11E-14, deoxy is 5.4E-14, and MbCO is 3.72E-14 (M⁻¹cm³). This is contrary to some literature [42, 59] that state the J values do not change according to the state of the myoglobin. Since we determined that the J values change yet have shown that the RET rates are extremely similar, something has to be accounting for the difference to make the rates the same. We propose that the heme dipole does not change and is static, especially between the ferric and MbCO state. In the deoxy state, the heme is in a domed formation where the iron is 0.3-0.36 Å out of plane and may alter the transition dipole slightly [76, 77]. Note well that in this experiment, we are not photodissociating the CO from the Mb, this is all ‘static’ analysis of ultrafast dynamics. The distance between the Trp and heme is also assumed to be similar; therefore, the major contributor is proposed to be the orientation factor. The J value for deoxyMb is 31% different from that of ferric and leads to a 24% difference in the orientation factor. For MbCO, the J value differs from that of ferric by 9.5% and leads to a 10% difference in the orientation factor. These changes, especially those of deoxyMb are significant, therefore leading one to believe that the orientation between the Trp dipole and that of the heme dipole are significantly different in the different states of Mb. Temperature data for several mutants were also taken and the RET times changed for W7 mutant while the W14 mutant did not considerably change between 4, 10, 22, and 40°C. This further illustrates the flexibility of the W7 mutant.
As we have illustrated here, the use of RET can be used as a ‘molecular ruler’ in the fact that information can be inferred about the local conformation in the protein. Although some mutants may not be analyzed according to distance or orientation value, insight into their structure can be gained by the analysis of their RET rates compared to those mutants in close proximity. The A-helix is the starting point of our analysis and serves as our model system for studying RET in myoglobin.
Figure 3.2: Steady-state absorption and fluorescence spectra of ferric sperm whale myoglobin mutant W7. Arrows indicate the excitation wavelength $\lambda_{pu}$ of 290 nm and the five gated fluorescence emissions: 310, 320, 330, 340, and 360 nm. Note: W7 has only one tryptophan, W14 was replaced with a tyrosine. ApoMb is simply the removal of the heme prosthetic group from HoloMb.
Figure 3.3: Femtosecond-resolved fluorescence transients of both W7 (Top) Holo-Mb (intrinsic lifetime of Trp is subtracted) and (Bottom) Apo-Mb (only one tryptophan present) gated at 310, 330, and 340 nm. As one can see, with the removal of the energy acceptor heme (Apo-Mb), the transient dynamics are considerably longer than with the presence of the energy acceptor (Holo-Mb). Therefore, the 111 ps decay seen in Holo-Mb can be attributed to the RET time.
Figure 3.4: Femtosecond-resolved fluorescence transients of Holo-Mb mutants (Top) W14 and (Middle) W12. The energy transfer time is 20 and 212 ps respectively. (Bottom) A comparison of the mutants W12, W15, and W14 RET transients is illustrated as well.
Figure 3.5: Representation of results of the orientation factor $\kappa^2$ W7 and W14 as well as type of measurement. (Top) Fish plot representing the possible orientation of the energy acceptor (heme) dipole angle for mutants W7 and W14. N and D represent normal and disordered orientations of the heme transition dipole respectively. The shaded region is to be argued the possible angles associated with the dipole in heme [59, 60]. (Middle) Data represented for kappa squared in the molecular dynamics trajectory, 0-20 ns shown. (Bottom) All orientation factors shown for W7 and W14 as well as each method achieved. The green crystal structure data correspond to the transition dipole of heme to be 55° and is shown in the top figure as green data points. The molecular dynamics values are achieved by averaging the orientation factor for a 33 ns trajectory.
Figure 3.6: Femtosecond-resolved fluorescence transients of Holo-Mb in different states. Note: Although the states of myoglobin change, the resonance energy transfer rates do not appear to change drastically.
Table 3.1: Summary of values determined for the A-helix mutants of Mb.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$\tau_{RET}$ (ps)</th>
<th>$\Phi_D$</th>
<th>$J$ (M$^{-1}$ cm$^3$)</th>
<th>$&lt;\tau_D&gt;$ (ns)</th>
<th>$r$ (Å)</th>
<th>$\kappa^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Crystal Structure</td>
<td>Molecular Dynamics</td>
</tr>
<tr>
<td>W7</td>
<td>111</td>
<td>0.20</td>
<td>$4.71 \times 10^{-14}$</td>
<td>2.8</td>
<td>21.2</td>
<td>23.2</td>
</tr>
<tr>
<td>W14</td>
<td>22</td>
<td>0.17</td>
<td>$4.11 \times 10^{-14}$</td>
<td>2.6</td>
<td>15.0</td>
<td>16.1</td>
</tr>
<tr>
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<td>212</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>W15</td>
<td>158</td>
<td>--</td>
<td>--</td>
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<td>--</td>
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</tbody>
</table>

Table 3.2: Steady state fluorescence emission peaks of A-helix mutants for both Apo- and Holo-Mb. Apo-Mb peaks are from Ref. 6.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Apo Fluorescence Peak (nm)</th>
<th>Holo Fluorescence Peak (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W7</td>
<td>332.6</td>
<td>328</td>
</tr>
<tr>
<td>W14</td>
<td>326.9</td>
<td>327</td>
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<td>337</td>
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<tr>
<td>W15</td>
<td>327.7</td>
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Figure 3.7: Steady state absorption characteristics of W14 ferric (Fe\(^{3+}\)) in black, deoxy (Fe\(^{2+}\)) in red, and MbCO (carbon monoxide bound to iron in heme) in blue. Data is presented as molar extinction coefficients normalized at the Soret peak for each state according to Ref. 19, therefore are independent of aromatic amino acids present in the protein. Wavelengths of peaks are given. Note: Data is for a single point mutation where one tryptophan is removed and replaced with a tyrosine.
3.2.4 B-H-helices and Local Conformational Fluctuations

Upon the success of the A-helix mutations, further studies were designed in effort to map out myoglobin’s global conformational fluctuations. To achieve site-specific conformational dynamics, site-directed mutagenesis was used to create ten mutations of Mb, Fig. 3.8. The mutants W22 (A22W), W48 (H48W), W57 (A57W), W71 (A71W), W74 (A74W), W84 (A84W), W95 (T95W), W113 (H113), W125 (A125), and W144 (A144W) were all created from the W7YW14F template of sperm whale Mb as was described earlier. All mutants were studied using femtosecond up-conversion to determine the resonance energy transfer in each mutant just as before. Preliminary data analysis is shown in Table 3.3.

For each mutant, the RET times have been determined, quantum yield values were determined for those mutants that had sufficient sample to make Apo-Mb. Unfortunately, no crystal structures have been studied for any of the mutations used in this experiment. In order to determine any information on the distance between donor and acceptor as well as the orientation factor, a mutation must be created in SwissPDB and MD simulations were carried out to 15 ns trajectories by a colleague Tanping Li, data not shown. In each case, the average distance between the donor and acceptor is calculated as well as the orientation value. In order to determine the orientation value.
from experiment, the average distance from MD simulations was used. Once again, the donor lifetime was calculated using data published in Ref. 6.

Most of the mutants have a RET time of less than 80 ps. Several mutants, W57, W113, W125, and W144 have RET times greater than 100 ps. In mutants W22, W48, and W57, the orientation factor obtained from experiment and MD simulations are similar, yet while the remaining mutations differ drastically. Taking a similar argument as in the A-helix discussion, these results lead us to believe that for W22, W48, and W57, the structure from the MD simulation has a high possibility to accurately represent the physiological structure of that particular Mb mutant, at least with respect to the local conformation of the Trp and heme orientation. Several mutations have extremely low experimental orientation factors as well, which is surprising for their associated RET times. All Trp donor lifetimes were calculated using methods found in reference 65. Further analysis and interpretation of this data is currently underway.
Figure 3.8: X-ray structure of *sperm whale* myoglobin (PDB:1MBD) with all of the Trp mutations in the B-H-helices, represented as yellow spheres. Each mutant only contains one Trp.
<table>
<thead>
<tr>
<th>Mutant</th>
<th>$\tau_{\text{RET}}$ (ps)</th>
<th>$\phi_D$</th>
<th>$J$ (cm$^3$ M$^{-1}$) /10$^{-14}$</th>
<th>$&lt;\tau_D&gt;$ (ns)</th>
<th>$r$ (Å)</th>
<th>$\kappa^2_{\text{Exp}}$</th>
<th>$\kappa^2_{\text{MD}}$</th>
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<td>W22</td>
<td>55</td>
<td>0.16</td>
<td>7.15</td>
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<td>0.21</td>
<td>6.75</td>
<td>1.57</td>
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<td>0.2</td>
</tr>
<tr>
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<td>0.7†</td>
<td>--</td>
<td>5.92</td>
<td>4.48</td>
<td>10</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>W71</td>
<td>20†</td>
<td>--</td>
<td>5.92</td>
<td>4.48</td>
<td>10</td>
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<tr>
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<tr>
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<td>5.69</td>
<td>2.46</td>
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<td>1.89</td>
<td>21</td>
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<td>6.91</td>
<td>21</td>
<td>--</td>
<td>--</td>
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<tr>
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<td>0.04</td>
<td>0.8</td>
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</tbody>
</table>

Table 3.3: Summary of values determined for the B-H-helix mutations of Mb for RET (Preliminary Data).  ($\tau_{\text{RET}}$: Resonance Energy Transfer Time, $\phi_D$: Quantum Yield, J:Integral Overlap, $<\tau_D>$:Trp lifetime, r: Distance from Trp to Heme calculated from molecular dynamics trajectory, $\kappa^2_{\text{Exp}}$: Experimental Value of the orientation value, MD: Molecular Dynamics Value of the orientation value), † signifies that data needs to be revisited.
3.2.5 Resonance Energy Transfer in Different Oxidation States of Myoglobin

Conformational changes between the three oxidation states of myoglobin are small but real. Our major points of interests are the conformational differences between the deoxy and CO bound states of Mb. Several studies have illustrated both the static [76, 77] and dynamic fluctuations [78] between the two states. One of the significant structural differences between the two states for our study is the doming of the heme in the deoxy state. The iron in the porphyrin ring is out of plane by ~0.3-0.36 Å. Literature has not illustrated how this doming effect would alter the heme transition dipole for the study of RET at this point, this is a concern for us. Also, Kachalova et al. [76] have proposed a scissor like motion of the E and F helices on the order of 0.4 Å. These conformational differences observed from the static x-ray structures, although small, are pivotal for the function of the protein. Schotte et al. [78] performed a breakthrough study on Mb using picosecond time-resolved x-ray crystallography. They suggest that the transient conformational changes are far more dramatic than the structural differences between the carboxy and deoxy states. We have the aspirations of seeing similar effects using optical spectroscopy.

We have attempted to us RET as a molecular ruler to see, in real time, the conformational fluctuations of Mb. To create a perturbation of the structure,
photodissociation of the CO bound to the myoglobin can be used. It is the goal of this entire experiment to develop a technique that uses RET as a molecular ruler to see in real time the ultrafast protein structural fluctuations. To do so, we created an ultrafast experiment in which three laser beams were to be used.

Several mutations of Mb (W14, W22, W71, W74, W84, and W95) have been analyzed in different oxidation states of Mb. In order to isolate a single response and know that it is attributed to a single event, there must not be any convolution with any other event. These mutations were selected due to their fast ferric RET times, 20~100 ps. The photodissociation of the CO occurs within 50 fs [79] and the Trp rotamer dynamics occur greater than 500 ps [69], therefore, the RET dynamics are isolated. Because the difference in structure we expect to observe after the photodissociation of CO is between deoxy and that of CO-bound structures, it is only the difference in RET times between those two oxidation states that are considered. Each mutant has its own characteristic RET times in deoxy and MbCO. Some mutations do not have a significant difference, while others, such as W95 exhibit a rather large difference in RET times for the two oxidation states, Fig. 3.9. It is this difference between the deoxy and MbCO transients that we will try to detect using the three beam experiment. It is important to note here that all RET times in Fig. 3.9 are determined from the static structures of deoxy and MbCO, it is not the static structures that will produce the signal at which we are
considering, they are merely a guide to decipher where to begin to look for a large change at a certain time. It has been shown that the transient conformational changes are larger than those shown in static crystal structures [78]. To check the validity of the experiment, we must have a control. This control must be well understood and possess similar RET times in both oxidation states, mutant W14 serves as our control. The RET times for deoxy and MbCO are 18.5 and 16.8 ps respectively. Our primary mutants of interest were those located in the E or F helix, W71, W74, W84, and W95 (Fig. 3.8). Each mutant was scanned for their characteristic RET times in both oxidation states and it was found that the W95 mutant had the largest difference, 25.7 and 37.9 ps respectively (Fig. 3.9). The W95 mutant was also primarily used with the expectation of its location being at the end of the F-helix would observe the largest fluctuation change.

This is a typical pump-probe experiment where a fixed delay time is set between the 290 nm pump for Trp and the 800 nm gating pulse for up-conversion while the third beam of 400 nm will be delayed appropriately to induce photodissociation of the CO bound to Mb. The fixed delay between the pump and gating pulse is determined by the largest change between RET times for deoxy and MbCO, for W95 it was 31.4 ps. The photodissociation beam is then delayed from that fixed delay pair (RET pair). The time window between the photodissociation and RET pair can be from 100 fs up to ~3 ns. It is important to note that we are attempting to use RET to gain insight on the ultrafast
conformational dynamics created after the photodissociation, not necessarily watch the full conformational change of the protein from MbCO to deoxy. We have no preconception of the signal characteristics obtained other than there would be some time dependence or possible oscillation.

In performing the experiment, unfortunately, we did not observe any significant signal although the idea is very novel. Several issues can be attributed to the unsuccessful attempt of this experiment. The first to consider is the technological difficulties of the experiment. Synchronizing three beams is difficult and nonlinear aberrations appeared frequently. The second is that we want to achieve as much photolysis as possible but truly, at best, only about 1/6 of the protein absorbs the laser beam. In efforts to improve the number of CO dissociated, the power of the beam was varied from 50-200 nJ. This proved to be a struggling point, as the power increased, photobleaching of the sample became more prevalent. The third is that the model system chosen is also a point of concern. In principle, the three beam experiment should work and has the potential to revolutionize the study of ultrafast protein conformational dynamics, especially in heme proteins, if the correct system is chosen. The mutants chosen for Mb were originally designed to probe the protein hydration [6], not the conformational dynamics. As shown below in Chapter 4, the dissociation of CO in Mb only cases very little global conformation changes, unlike cytochrome c, which induces a
noticeable conformation change after photolysis of a covalent bound heme with the protein.
Fig. 3.9: Femtosecond-resolved fluorescence transients of W14 (Top) and W95 (Bottom) mutants of myoglobin in deoxy and CO bound states for RET with lifetime subtracted, 350 nm gated fluorescence.
3.3 UV-Vis Transient Absorption Probing of Myoglobin Dynamics

3.3.1 Introduction

A protein consists of an assembly of amino acids held together by strong covalent bonds along its backbone and a multitude of much weaker, non-covalent cross-connections. These amino acid assemblies create a distribution of charge with a given set of spatial coordinates which generates an electric field in the overall matrix and thus, proteins can be viewed as simple distributions of charge. The distributions of charge are not evenly distributed in the protein matrix and in that it also undergoes significant charge restructuring in interacting with a functional partner or in response to conformational change [162]. This redistribution of charge would in turn, change the internal electric field of the protein matrix. This change in internal electric field, or the so-called Stark effect in proteins, could be observed by a ground-state absorption change of Trp [126-128].

Ultrafast dynamics of heme proteins have been extensively studied by various methods [80-95]. The relaxation occurs on multiple time scales and is very nonexponential and heterogeneous. In an effort to observe the globular protein conformational changes upon photoexcitation as described before, wild type, a double mutant that removed all tryptophan (W7YW14F), and 17 single tryptophan mutants of
Mb were studied, Fig. 3.10. Two redox states (ferric and deoxy) as well as a CO bound state of Mb were analyzed using ultrafast transient-absorption, discussed in chapter 2. A visible pump beam of 400 nm was used to photo excite the heme and a range of probe wavelengths in the UV (290, 297, and 310 nm) and visible (550, 575, 590, and 610 nm) were used to detect both the ground-state tryptophan absorption change and the heme relaxation (Fig. 3.11). The different regions of probing wavelengths are designed to probe different aspects of the protein. For the 290 and 297 nm probing wavelengths, we hope to view the ground-state absorption of Trp, which will be convoluted with the heme dynamics due to its UV absorption characteristics. To deconvolute the signals, we probe at 310 nm as well as the visible wavelengths. These, because of no tryptophan absorption at this wavelength, will hopefully give us only the heme’s response and its local environment conformational dynamics, thus allowing for the deconvolution in the UV region to determine the sole times associated with the ground-state Trp response. The use of the single Trp mutations is to determine local conformational fluctuations at each position and then induce the globular protein conformational dynamics appropriately. Just as before, we expect a significant signal response from those mutations in positions where conformational fluctuation is ‘large.’ We expect that the E and F-helices mutations would supply the best opportunity to observe any dynamical fluctuations, same as before. To unequivocally demonstrate that the signal would be due to Trp response, a control
mutant was created where no Trp residues exist, W7YW14F. The major contribution to this experiment is the novel probing at the UV wavelengths; therefore, data will be presented only from the UV perspective.

3.3.2 Materials and Methods

The mutations of sperm whale myoglobin were purified as described above. For both steady-state and time-resolved experiments, the protein was dissolved in 20 mM Tris 1mM EDTA pH 8.0. For ferric experiments, the sample was used without further purification. DeoxyMb was prepared by purging the ferric protein with high-purity nitrogen to remove oxygen and then reducing it with 1:5 ratio of the protein to sodium dithionite [96, Appendix C]. Creation of CO bound Mb was performed by briefly purging CO into the cell, the color immediately changed and absorption was taken to verify the state. The protein concentration was maintained to be 200 μM and all femtosecond experiments were carried out at room temperature with a moving 1-mm quartz cuvette to avoid heating and sample degrading. To ensure that there was no change of the protein quality and heme redox states, the absorption spectra of the samples were verified before and after time-resolved experiments. Time-resolved experiments were carried out using femtosecond transient-absorption method as detailed in chapter 2.
The sensitivity of the transient-absorption method can reach $10^{-4}$-$10^{-5}$ of absorbance change.
Figure 3.10: Schematic representation of the Mb mutations studied using transient-absorption (PDB:1MBD). Note: Each mutant contains only a single Trp, represented by the yellow spheres. Wild type (contains W7 and W14) and a control (contains no Trp, W7YW14F) were also studied.
Figure 3.11: Steady-state absorption spectra of ferric, deoxy, and CO bound Mb. Arrows mark two probing regions of visible and UV wavelengths (λ_{pr}) with Soret band excitation at 400 nm (λ_{pu}). Note that the UV probing of 290 and 297 nm also detects ground-state tryptophan dynamics.
3.3.3 Results and Discussion

A. Femtophysics of Ferric and Deoxy Myoglobin

Both ferric and deoxy myoglobin do not have diatomic ligands associated with the heme. There is a water molecule attached to the iron in the ferric form but photodissociation is not observed. Figure 3.12 illustrates the femtosecond-resolved transient absorption for the ferric and deoxy myoglobin mutant W95 probed in the UV at 290 nm. The dynamics of all mutants studied are extremely similar; therefore, only one mutant is shown. Both in the UV and visible probing regions, two distinct times are observed, ~700 fs and ~5.6 ps for ferric and 0.3 and 3 ps for deoxy Mb. These decay times are independent of mutants, which is quite interesting but not surprising. It appears that since the decay rates are independent of wavelength, the ground-state Trp absorption, which is being probed in the UV 290 and 297 nm along with the heme, is not detected or is dominated by the heme spectroscopic responses. These characteristic times [48, 97] have attributed to an initial internal conversion followed by vibrational cooling of the hot heme group for both ferric and deoxy Mb. Excellent agreement with the published data gives us comfort that our experiment and sample are correct. Meanwhile, it shows that only ‘pumping’ the heme will not cause protein conformation change even though some energy may dissipate into the protein.
B. Femtochemistry of CO Bound Myoglobin

In the presence of CO, myoglobin can bind the diatomic molecule and upon excitation with a 400 nm laser, photolysis occurs. The quantum yield of dissociation is said to be unity and bimolecular rebinding occurs on the millisecond time range while there is no appreciable geminate recombination [48]. In this case, the dynamics are surprisingly independent of Trp mutants as well, however, we see an overall nanosecond response consistent in each mutant and for each wavelength probed. Figure 3.13 illustrates the femtosecond-resolved transient absorption of representative mutants W7YW14F, W14, W57, and W74 with 400 nm excitation and 290 nm probe. The transients show an initial ultrafast positive decay in ~300 fs and then a dominant negative signal of bleaching. This is quite interesting and has not been reported before. Once again, since the dynamics at 280-297 nm are nearly the same as that of 310 nm, data not shown, we are inferring that the ground-state Trp response is not seen. This observation shows again that the fluctuations near the Trp are too small to create any substantial response. The observed nanosecond response is attributed to that of the local relaxation around the heme. This is contrary to some results that have determined that the complete globular relaxation after CO to deoxy in less than 100 ps [98]. However, evidence in time-resolved x-ray diffraction has been reported that suggests the existence of a nanosecond relaxation of heme toward the deoxy configuration [99]. Significant protein
conformational changes have also been reported within 1 ns determined by time-resolved Laue x-ray diffraction [125]. Our results support the evidence of a slow (~ns) relaxation of the heme or its local environment with a longer ~200 ns relaxation. Heme environment changes in the nanosecond region include the loss of the CO-bound ligand, the Fe displacement from the heme plane, the proximal histidine displacement toward the F helix, the swing of the distal histidine toward the bound CO location, and the location of the photodissociated ligand. Other smaller features indicate changes at the heme and displacement of the E and F helices [125]. It has been suggested that the motions of the distal E-helix and of the CD-turn are attributed to the 100-300 ns relaxation time. Local rearrangements around the heme such as heme tilting, iron motion out of the plane occur promptly on the picosecond time scale. Over the same delayed time range, CO is observed to migrate from a cavity distal to the heme known to bind xenon (Xe4) to another such cavity proximal to the heme (Xe1). It was proposed that the extended relaxation of the globin moiety reflects reequilibration among conformational substates known to play an essential role in controlling protein function [99].
C. Discussion

Upon good agreement with literature for heme relaxation in the ferric and deoxy states of myoglobin, we have determined that our system, both experimental set up and protein sample is correct. Although we have presented here a conformational fluctuation on the nanosecond scale that has not been seen before through femtosecond-resolved transient absorption, once again, we were not able to globally map out protein conformational fluctuations in real time. One particular reason seems to be that the perturbation induced by the photolysis of CO in Mb is very small although the perturbation continues from CO diffusion inside the protein and its escape from site to site. It appears, that if we want to achieve our goal of globally mapping out ultrafast protein conformational fluctuations, we must switch systems. We must chose a system that has a much larger conformational change, or chose better mutations that are closer to the perturbation sites. Another well studied heme protein is cytochrome c and, unlike myoglobin, the iron in heme is covalently bound to the protein through two amino acids, and upon photo excitation in the ferrous state, one of the bonds is broken and a proteinquake could be created. Downstream relaxation may be detectable via femtosecond-resolved transient absorption.
Figure 3.12: Femtosecond-resolved transient absorption of ferric and deoxy myoglobin mutant W95 upon 400-nm excitation probed at UV 290 nm. Characteristic decays of ferric Mb are 0.7 and 5.6 ps. Characteristic decays of deoxy Mb are 0.3 and 3 ps.
Figure 3.13: Femtosecond-resolved transient absorption of MbCO for mutants W7YW14F, W14, W57, and W74 upon 400-nm excitation and probed at UV 290 nm. Characteristic dynamics of MbCO are the initial ultrafast photodissociation of CO, ~3 and ~20 ps heme response and the long ns response of the local heme environment perturbation. Note that all mutants exhibit similar behavior, even the mutant without Trp thus all dynamics are attributed to the ground-state absorption change of heme.
3.4 Future Work

Although myoglobin has been studied for decades, new and exciting results are frequently elucidated. In a recent review [100], site-directed mutagenesis was used to follow the CO dissociations through the Xe cavities of myoglobin. Tryptophan mutations were created to alter the dissociation of CO to determine the pathway of the ligand upon release to solvent. Mutations such as L29W placed the large indole ring in the initial ‘docking site’ for photodissociated ligands where mutants I28W, V68W, and I107W blocked the Xe4 cavity and L89W, L104W, and F138W blocked the Xe1 cavity. We truly believe that upon investigation of these mutations with either RET or femtosecond-resolved transient absorption would illustrate the ultrafast protein conformational relaxations that are so arduously trying to see in real time. The important revelation concerning these mutations is that the crystal structure for I28W, V68W, I107W, and F138W were solved. It is with this new structural information that we can examine the conformational relaxations via RET. The missing piece to the puzzle before was the lack of knowledge concerning the distance between the donor and acceptor, it is now available.

Another recent study has observed conformational switching between two well defined substates of a myoglobin mutant, L29I. The conformational switching involves
motion of the distal histidine/E helix that changes the location of the imidazole side group of the histidine [95]. It is with single point mutations sensitive to this motion that the opportunity to see the ultrafast conformational dynamics in real time.
CHAPTER 4

ULTRAFAST PROTEINQUAKE DYNAMICS IN CYTOCHROME c

4.1 Introduction

Kinetic studies of heme proteins have been playing a critical role on the development of conceptual framework in protein dynamics such as heterogeneity, substates, and energy landscape [80-85]. The classic heme-ligand dynamics have been extensively studied in various heme proteins on ultrafast time scales [86-92] and also applied to address important questions of protein folding [27, 101-106], allosteric regulation [76, 107, 108], and even glass transition[80, 109, 110]. Although a variety of biophysical techniques have been used to characterize protein motions [111-123], the direct probing of global conformation relaxation has been difficult and only recently was the picosecond-resolved x-ray diffraction on myoglobin relaxation reported [78, 124, 125]. In analogy to recent mapping of hydration dynamics around protein surfaces [6, 7], we can directly map out global protein fluctuations using an optical probe placed one at a time at a desired position with site-directed mutations. Two potential molecular probes
have recently been reported: One is intrinsic amino acid tryptophan (W or Trp) by measurement of UV absorption changes [126-128] and the other is nitrile-derivatized amino acids by detection of IR absorption changes [129-131].

Cytochrome c (Cyt c), a model heme protein, has been widely used to study folding and unfolding [27, 101-106] with novel initiation methods of fast temperature jump [106], electron transfer between two redox states (ferric Fe$^{3+}$ and ferrous Fe$^{2+}$) [101], and ligand dissociation under certain denaturation conditions [103]. Figure 4.1 shows the x-ray structure of horse heart Cyt c (PDB:1HRC), an $\alpha$-globular protein with a prosthetic heme group ligated with a proximal H18 and a distal M80 and covalently linked with C17 and C14 residues [132]. The protein only contains a single tryptophan residue (W59), which is ideal for specifically probing local conformation relaxation. Figure 4.2 shows the absorption spectra of the protein in two redox states. Two ultrafast studies of the heme dynamics in Cyt c have been recently reported [90, 117]. One studied ferrous Cyt c with excitation at the B band $S_2$ state (400 nm) and probing in the visible region (450-650 nm) [90]. The other investigated both ferric and ferrous Cyt c with the Q-band $S_1$ state excitation (550 nm) and probing by resonance Raman and transient absorption methods in the B-band region (385-445 nm) [117]. Both studies focused on the local heme site and elucidated a series of time scales of heme-ligand
Figure 4.1: X-ray structure [132] of horse heart cytochrome c (PDB:1HRC) with the prosthetic heme group covalently linked with four residues, M80, H18, C17 and C14. Also shown is the W59 residue as an optical probe for detecting protein conformation dynamics.
Figure 4.2: Steady-state absorption spectra of ferric and ferrous cytochrome c. Arrows mark two probing regions of visible and UV wavelengths ($\lambda_{pr}$) with B-band excitation at 400 nm ($\lambda_{pu}$). The inset shows the weak absorption of heme-M80 charge-transfer state at 695 nm in the ferric state. Note that the UV probing from 297 nm to 280 nm also detects ground-state tryptophan dynamics.
dissociation, vibrational cooling and ground-state recovery. However, the critical protein conformational relaxation induced by the heme dynamics was not yet investigated.

In this work, we systematically examine the heme dynamics and resulting protein conformational relaxations at the B-band excitation with femtosecond resolution. The main goal here is to determine the time scale(s) of protein conformational relaxation caused by the proteinquake from the epicenter of heme-ligand photolysis. With a wide range of probing wavelengths (Fig. 4.2) from visible (700 nm) to UV (280 nm), we are able to unequivocally determine the different heme excited-state dynamics in the ferrous and ferric states. By monitoring the absorption changes of ground-state tryptophan (W59) with UV light, we can follow protein conformational relaxations. To further ascertain the tryptophan detection, we mutated W59 to phenylalanine (F) with site-directed mutagenesis. These results not only elucidate the unique heme-ligand dynamics in Cyt c but also provide a direct way to probe protein conformational fluctuations triggered by the local heme reaction in widely studied heme proteins.
4.2 Materials and Methods

Wild-type horse heart cytochrome $c$ was purchased as a lyophilized powder from Sigma with 99% purity or purified using plasmid pJRhrsN, generously provided by Prof. Jon Rumbley (Univ. of Minnesota, Duluth), by following the published procedures with our modification [133, 134, Appendix B]. The mutant protein of W59F was designed and purified with the same procedures. For both steady-state and time-resolved experiments, the wild-type protein was dissolved in 0.1 M potassium phosphate buffer at pH 6.5. For the mutant, we used 25 mM sodium phosphate buffer at pH 7.6. For ferric experiments, the sample was used without further purification. Ferrous Cyt $c$ was prepared by purging the ferric protein with high-purity nitrogen to remove oxygen and then reducing it with 1:5 ratio of the protein to sodium dithionite [96]. The protein concentration was maintained to be 400-500 $\mu$M and all femtosecond experiments were carried out at room temperature with a moving 1-mm quartz cuvette to avoid heating and sample degrading. To ensure that there was no change of the protein quality and heme redox states, the absorption spectra of the samples were verified before and after time-resolved experiments. Time-resolved experiments were carried out using femtosecond transient-absorption method as detailed in chapter 2.
4.3 Results and Discussion

4.3.1 Steady-State Absorption Characterization

Figure 4.2 illustrates the steady-state absorption spectra of both ferric and ferrous cytochrome c. In both the ferric (Fe$^{3+}$) and ferrous (Fe$^{2+}$) forms of Cyt c, the iron of the heme is six-coordinated and is bound to the protein through Met80 and His18 amino acids. Ferric Cyt c has a B-band peak at 410 nm and a Q-band peak at 529 nm with molar extinction coefficients of 110,000 and 11,400 cm$^{-1}$M$^{-1}$ respectively. In the ferric state, there is also an experimentally significant band at 695 nm with a molar extinction coefficient of ~800 cm$^{-1}$M$^{-1}$. This 695 nm band is attributed to the charge transfer between the Met80 and Fe$^{3+}$ in the heme and signifies if correct ligation has occurred upon folding of the protein. In the ferrous form of Cyt c, the B-band peak is 415 nm while the Q-band is split into 521 and 550 nm with molar extinction coefficients of 133,000, 15,400, and 28,000 cm$^{-1}$M$^{-1}$ respectively. All calculations were obtained by normalization of the absorption spectrum to the B-band molar extinction coefficients published by Butt and Keilin in 1962 [160].
4.3.2 Femtosecond-Resolved Transient Absorption Dynamics

Figures 4.3 and 4.4 show the transient-absorption (TA) dynamics of wild-type ferrous and ferric Cyt c, respectively, upon 400-nm excitation and probed by a series of wavelengths from 280 nm to 700 nm. All fitting results using multiple exponential decay functions are given in Table 4.1 and 4.2. Both states show quite different dynamical patterns. With the UV probing (310-280 nm), we expect to detect the initial excited-state dynamics and the TA signals for the two states do show drastic differences. The ferrous state shows an initial ultrafast decay and then a dominant negative signal of bleaching and recovery. The ferric state exhibits nearly all positive TA signals, simply reflecting the dynamics of excited states. In the visible region (700-530 nm), the ferrous Cyt c simply shows either positive or negative signals while for the ferric state the transients show complex TA signals with mixed positive decay and negative formation dynamics. Clearly, the two redox forms of Cyt c have distinct excited-state dynamics and different protein responses.
Figure 4.3: Femtosecond-resolved transient absorption of the ferrous state upon 400-nm excitation probed from UV 280 nm to visible 700 nm. The top inset shows the fitting transients probed by visible wavelengths for comparison. The middle inset gives deconvolution of 290-nm UV transient into the heme and tryptophan dynamics. The bottom inset shows deconvolution of 280-nm UV transient into initial excited-heme dynamics, ground-state heme-ligand recombination, and protein conformation relaxation by tryptophan detection. Clearly, all UV transients show an initial ultrafast decay in less than 100 fs and then a negative bleaching and recovery signal.
Figure 4.4: Femtosecond-resolved transient absorption of the ferric state upon 400-nm excitation probed from UV 280 nm to visible 700 nm. The inset shows the fitting transients probed by visible wavelengths for comparison. Clearly, all UV transients (310-280 nm) show positive decay dynamics, drastically different from those in Fig. 3 for the ferrous state, while in the visible region (700-530 nm), transients are convoluted by a systematic mixture of decay and formation dynamics.
Fitting: The typical fs-resolved transient-absorption data shown in Figs. 3 and 4 and were fitted by:

\[ I_\lambda(t) = \sum_i A_i e^{-t/\tau_i} \quad (i = 1, 2, 3, \ldots) \]

where the terms associated with positive \( A_i \) describe the decays, and the ones associated with negative \( A_i \) describe the formations. The time constants are in picoseconds.

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Table 4.1: Fitting parameters for the sixfold redox state of Cyt c upon 400 nm excitation.
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Table 4.2: Fitting parameters for the fivefold redox state of Cyt c upon 400 nm excitation.
A. Photochemistry of Ferrous Cyt c, Impulsive Proteinquake Formation and Global Conformation Relaxation.

As shown in Fig.4.3, in the UV region, the transients show an ultrafast initial positive decay in ~100 fs and then suddenly change to a negative signal. This dynamic pattern clearly shows a photochemical reaction of heme-ligand dissociation. The ultrafast sign switching from positive to negative signals results from the transformation from sixfold to fivefold coordination of the ferrous state. The initial positive signal results from the absorption difference of ground-state bleaching and excited-state formation of sixfold-coordination ferrous state. After heme-ligand dissociation, the heme transforms from sixfold to fivefold coordination and the negative signal mainly depends on their absorption difference, indicating that the fivefold coordination ferrous Cyt c has weaker absorption. This observation is completely different from that in ferric Cyt c (Fig.4.4 and see below) which shows all positive signals in the UV region, thus clearly distinguishing two different excited-state dynamics in the two states. The observation of ligand dissociation is consistent with recent studies using visible light probing by Negrerie et al. [117] at the Q-band excitation and Wang et al. [90] at the B-band excitation which indicated that photodissociation of heme-M80 occurs in about 40 fs.

The impulsive heme-ligand bond breakage in less than 100 fs instantaneously generates significant structural perturbation, resulting in an ultrafast proteinquake
formation [81] at the local heme site which immediately spreads outward and induces global protein conformation fluctuation. At 310 nm, we observed ground-state heme formation in 4 ps (76%) and 13 ps (23%). This cascaded formation dynamics represents vibrational cooling in a few picoseconds and then complete ground-state heme-M80 recombination and recovery in 13 ps (also see visible-light probing below). Significantly, in the region of 297-280 nm, besides the formation signal of heme-M80 in 7 ps we observed an extra long-time formation dynamics in ~40 ps. This long-time signal was not observed at 310 nm nor at any visible wavelengths (see below). This signal is from the dynamic changes of ground-state W59 absorption. Because tryptophan has nearly no absorption at wavelengths longer than 300 nm, we did not observe this long-time component in any transients probed at longer wavelengths than 300 nm. Below 300 nm, we observed this long-time component at all wavelengths we probed, 297, 295, 290, 285, and 280 nm. This result is significant and shows that the protein conformation recovery induced by the impulsive proteinquake is ultrafast and only takes ~40 ps, a timescale of only about three times longer than the local heme relaxation (13 ps).

The conformation relaxation of myoglobin, induced by heme-CO dissociation, has been extensively studied by various methods [80-92, 93-95]. The relaxation occurs on multiple time scales and is very nonexponential and heterogeneous. The initial proteinquake caused by dissociation is not very strong but the perturbation continues
from CO diffusion inside the protein and its escape from site to site. Recently, an ultrafast substate conformation switching was observed to occur even in 50 ps [95]. However, using tryptophan as a local probe and with more than fifteen mutations, we recently did not observe significant conformation relaxation of myoglobin after photolysis in an attempt to map out global relaxation, as also confirmed by others [98]. Clearly, the results depend on the probe locations and the extent of perturbation. In Cyt c, the situation is different. The residue M80 is a part of the protein and is covalently linked to the backbone of a close loop. The impulsive dissociation significantly shakes the protein and the formed quake is strong enough to cause the noticeable conformation fluctuation. The probe W59 is linked to a distant loop but points inside the hydrophobic region and forms a hydrogen bond with a propionate of the heme. Around W59, several residues, for example, L35, T40, L64, Y67 and I75, from different helices and loops are in proximity. Significantly, we observed the complete heme recovery in 13 ps after the full recombination in 7 ps but the absorption recovery of W59, representing the relaxation of its neighbor protein environment/conformation, only needs about 40 ps. Comparing with myoglobin, this relaxation process is ultrafast. One of the main reasons is because the heme-M80 recombination occurs ultrafast in 7 ps while in myoglobin the dissociated CO molecule does not recombine back and causes continuous local conformation perturbations over wide time scales.
It should be pointed out that the detected negative signal at 290 nm has two contributions. The main signal (85-90% of the total negative amplitude) is from the absorption difference of sixfold and fivefold coordination ferrous states. The molar extinction coefficient is about 15,000 M$^{-1}$cm$^{-1}$ at 290 nm for the sixfold coordination ferrous state [135] and our negative signal shows that the extinction coefficient for the fivefold one is smaller, consistent with the trend observed for myoglobin [136]. The other part of the signal (10-15%) is from the ground-state absorption change of tryptophan in different protein conformations, before and after dissociation, a process of the Stark effect. The molar extinction coefficient of tryptophan is about 3,770 M$^{-1}$cm$^{-1}$ at 290 nm [19]. Thus, the observed negative signal (10-15%) indicates that the tryptophan absorption after dissociation shifts to the blue side, resulting in decrease of tryptophan absorption at 290 nm. Using the extinction coefficient difference of several thousands for sixfold and fivefold coordination ferrous myoglobin at 290 nm [136], the observed 10-15% signal indicates that the extinction coefficient change of tryptophan is about several hundreds, which means that the tryptophan absorption spectrum shifts to the blue side in less than 1 nm.

To further ensure that the long-time component is from the tryptophan signal, not from the heme dynamics, we performed the mutation studies of W59F to remove the single W59. Figure 4.5 shows the results probed at 290 nm for both the wild type and the
Figure 4.5: Femtosecond-resolved, normalized transient absorption signals of the wild-type and mutant (W59F) ferrous Cyt c probed at 290 nm. Here we focus on the long-time components. In the mutant, the long-time component is clearly absent while in the wild type, the long-time component in ~42 ps is apparent, which is from the ground-state absorption changes of W59 due to the conformation fluctuations caused by the proteinquakes.
mutant. Cleary, the long-time component in ~40 ps is completely absent in the mutant but the transient still has the initial ultrafast positive signal of dissociation in ~100 fs and the negative signal of heme-M80 recovery in 7 ps. Similarly, we did not observe any long-time component in ~40 ps at other UV wavelengths. This mutation control experiment unambiguously proves that the observed 40-ps dynamics in the wild-type protein is from W59, a ground-state dynamics reflecting the conformation changes induced by the strong perturbation of the heme-M80 dissociation. Therefore, W59 has enough sensitivity to probe the conformation changes caused by the proteinquake, unlike in myoglobin where the perturbation is weaker and the probe may not be at desired positions.

In the visible region (Fig. 4.3), we observed systematic decay and formation signals but all faster than 7 ps. As shown in Fig. 4.2, above 560 nm, there is nearly no ground-state absorption and thus, the positive decay signals are dominant. For 700 nm, 640 nm (not shown) and 610 nm, we observed two ultrafast decay components of 0.18-0.78 ps and 4.5-5 ps besides a minor nanosecond component (<5%, no heme-M80 dissociation or recombination). At 575 nm, we only observed a single-exponential decay of 5.7 ps. At 560 nm, we observed a rise component in 1.8 ps and a decay component of 5.5 ps. At 550 nm around the Q-band absorption peak, we observed a cascaded ground-state formation signal in 1.1 and 6.7 ps. Finally, at 540 nm, due to weaker ground-state
absorption we observed one rise component in 1.0 ps and two dominant decay components again in 0.56 and 6.3 ps.

With combination of UV and visible probing, the dynamics of ferrous state of Cyt c is clear. Figure 4.6 illustrates the dynamical processes involved in the ferrous state of Cyt c upon heme excitation. The photodissociation of heme-M80 is the dominant channel and the bond breakage occurs in less than 100 fs. After ligand dissociation, it seems that the fivefold coordination ferrous state is formed in vibrationally hot ground state although the formation in electronically excited state could be possible. The cascaded vibrational cooling occurs within 7 ps, accompanying with heme-M80 recombination. The complete relaxation of the local heme site takes 13 ps. A series of proteinquake formations by initial impulsive bond breakage in less than 100 fs and subsequent local environment cooling and the heme-M80 rebinding within 7 ps all induce significant protein conformation changes. These collective structural relaxations take 42 ps to completely dissipate energy to the tryptophan environment and have the conformation fully recovered.
Figure 4.6: Schematic representation of a series of snapshots of cascaded heme and protein dynamics for the ferrous state upon excitation at $t=0$. The impulsive bond breakage of heme-M80 occurs in about 40 fs, resulting in proteinquake formation, represented by a series of concentric circles. At about 7 ps, the residue M80 rebinds back to the central iron of the heme after a series of vibrational cooling, including local structural relaxation. During this time, the heme is highly distorted and the proteinquakes spread outward and shake global conformation. At 13 ps, the proteinquakes at the local heme site are fading away and the heme environment is fully relaxed. However, the proteinquakes around the probe Trp take 42 ps to disappear and the perturbed protein conformation fully recovers on the time scale of about three times longer than the local heme recovery.
B. Photophysics of Ferric Cyt c and Ultrafast Energy Dissipation

As shown in Fig. 4.4, in the UV region, we basically observed all positive signals, totally different from those for the ferrous state (Fig. 4.3), with two systematic decays for all transients from 310 nm to 280 nm with two time constants of 0.14-0.46 ps and 0.51-4 ps, reflecting gradual relaxation from initial higher to cascaded lower energy states detected by longer to shorter wavelengths. These results strongly suggest that the excited Cyt c do not proceed to heme-ligand dissociation and the observed dynamics represent excited-state cascade relaxation. Otherwise, the dissociation would result in instantaneous transformation from sixfold to fivefold coordination and cause sudden absorption changes (see above for the ferrous state). At 310 nm, we also observed a small negative formation signal in about 6 ps but the absent observation of formation signals in other UV wavelengths (297-280 nm) indicates dominant excited-state absorption and the similar time scale of ~4 ps for the ground-state recovery. It should be emphasized here that we did not observe any long-time signal (τ ≥ 20 ps) at 297-280 nm by Trp-absorption change, which also indicates no heme-ligand dissociation and no significant conformation perturbation. Negrerie et al [117] recently reported extensive studies of the ferric Cyt c upon the Q-band (S1 state) excitation by both time-resolved TA and resonance Raman detection. By observation of the absent strong B-band absorption at 390-400 nm for fivefold coordination ferric Cyt c and of different Raman spectra
between ferric and ferrous states, they concluded that photolysis in ferric Cyt c does not occur and the heme-M80 bond is not broken. Such a conclusion is directly shown here by our UV probing. The observation of no heme-M80 dissociation and no long-time conformation relaxation further indicates that the energy dissipation in the ferric state is ultrafast and mainly occurs at the local heme site. There are no significant structural changes in the local heme site of ferric Cyt c and thus no noticeable protein conformational change occurs. Thus, we did not observe any dynamics of W59 in the ferric state. Therefore, if we do not observe W59 ground-state dynamics, it indicates no significant structural perturbation and no heme-M80 dissociation.

In the visible region (700-530 nm), we observed a series of nonexponential cascaded vibrational cooling, conformation relaxation, and final ground-state recovering. From 700 to 590 nm, we basically observed two decay components in hundreds of femtoseconds (0.3-0.7 ps) and a few picoseconds (3-5 ps), mainly reflecting hot ground-state cooling. At 700 nm, we also observed a small negative formation signal in about 6 ps. As shown in Fig. 4.2, such formation was probed by a weak absorption band of heme-M80 charge transfer [137]. For the other visible wavelengths of 575-530 nm, because the Q-band absorption becomes evident (Fig. 4.2) we observed systematic evolution of decay and formation signals: At 575 nm two decays (0.38 and 4.7 ps) and one main formation (0.77 ps); at 560 nm, two decays (0.14 and 4.9 ps) and two
formations (1.2 and 9.2 ps); at 550 nm, one decay (3.6 ps) and two formations (1.4 and 9.1 ps); and finally at 540 and 530 nm, two formation components of 1.3-4 ps and ~11 ps.

Combining these results from UV to visible probing, the photophysics of ferric Cyt c is clear. The internal conversion from the B-band to the Q-band and the ground state is ultrafast, occurring in hundreds of femtoseconds (< 250 fs) detected mainly in the UV region. The internal conversion from S\textsubscript{1} to the ground state occurs on the similar time scale [117]. The resulting molecules are formed in the highly vibrationally-excited ground state and a series of energy dissipation follows: initial intramolecular and subsequent intermolecular vibrational redistributions and then local conformation relaxation. The vibrational energy dissipation is highly nonequilibrium with multiple exponential decays but all on ultrafast time scales. The longest decay time of cooling processes is 4.9 ps observed at 560 nm. The dissipated energy into the surrounding protein causes local conformation fluctuation and such conformational relaxation inversely perturbs ferric heme Q-band absorption [138]. The complete conformational relaxation takes ~11 ps as detected at 530 nm by the full recovery of ground-state ferric Cyt c.

The observed time scales of 4.9 ps cooling and 11 ps recovery with the B-band excitation are a little longer than those of ~4 ps TA decay and ~7 ps temperature decay
observed by Negrerie et al. [117] at the Q-band excitation. Our results are also in good agreement with recent MD-simulation results observed by Bu and Straub [139] on vibrational energy relaxation of heme cooling upon 420-nm excitation. They observed biphasic distributions of 1.5 ps (60%) and 10.1 ps (40%), corresponding to two dissipation mechanisms: The fast component is due to the energy transfer via the coupling between heme and collective motions of the protein mainly through covalent bonds with M80, H18, C14 and C17 residues and through hydrogen bonds with Y48, T49, N52, T78 residues and neighboring water molecules. The slow phase is due to energy transfer via nonbonded collisional contacts with nearby heme-pocket residues such as R38, T40, G41 and F46 and solvent. The observed multiple cascade relaxation in a few picoseconds here seems more complex than the simulations and the observed longest 11 ps dynamics reflects the total relaxation of the local heme pocket.

4.4 Conclusions

In this chapter, we reported here our critical use of UV probing of the local heme dynamics and induced global conformation relaxation in ferric and ferrous states in cytochrome c upon heme excitation. The two redox states show drastically different heme and protein dynamics, which must be due to the different number of electrons in the
d-orbitals of the central iron, resulting in different energetics, coupling and dissociative or bound character of excited-state potentials. In the ferrous state, the dissociative channel is dominant and occurs in less than 100 femtoseconds, faster than the internal conversion rate, which is observed in the ferric state in hundreds of femtoseconds. Both fivefold coordination ferrous and sixfold coordination ferric states were mainly formed in the vibrationally hot ground state and the cooling processes were observed within 7 ps. However, full recovery of the local heme environment takes a little longer, 11 ps for ferric and 13 ps for ferrous states.

One significant observation by UV probing is direct probing of conformation relaxation caused by proteinquakes which are created by impulsive local heme-ligand dissociation in the ferrous state. Such cascade dynamics from the local heme site to global protein conformation are completed in 42 ps, faster than expected. Unlike in other heme proteins such as myoglobin, the dissociation of carbon monoxide from the heme site causes continuously weak structural perturbation without any prompt rebinding, leading to protein relaxation on multiple time scales [100, 140]. With violent proteinquakes, the conformation relaxation could occur on much longer time scales (ns-μs) and proteins could even unfold [101, 123]. Here, the ultrafast proteinquake dynamics must be due to the prompt bond-breaking and bond-remaking processes in 7 ps as well as the local structural integrity with another three covalent connections between the protein
and the heme group. Using the powerful UV probing of ground-state tryptophan absorption perturbation, as proved here by mutation work and also shown in recent studies of retinal charge translocation in bacteriorhodopsin [126] and electron transfer in photosynthesis [128], we can now directly map the protein conformation dynamics after the shaking by proteinquakes with global site-directed mutations.

4.5 Future Work and Considerations

Due to the rather excited and surprising results displayed by the above experiment on Cyt c, further experimentation is being pursued. With the hope to dissect the globular protein conformational changes upon photolysis of the M80-Heme bond, 11 point mutations of Trp in Cyt c (A15W, G23W, F36W, G45W, A51W, E66W, K72W, G77W, F82W, T89W, and T102W) have been carefully designed and successfully expressed as earlier described and in Appendix B. Figure 4.7 illustrates the various positions and mutants. Each point mutation is created with the W59F template where W59 is removed and a phenylalanine takes its place, overall protein yield varied between mutants from 10 mg/mL to 35 mg/mL. It is important to note that the original plasmid pJRhrsN obtained from Prof. Jon Rumbley (Univ. of Minnesota, Duluth) is already a double mutation, H26N and H33N. These mutations were created to prevent misligation in folding.
experiments [133]. The template W59Y was also created and considered for use as the template but the expression was low compared to that of W59F, also, the biological function of W59F is found to be indistinguishable from wild type [155]. An extensive literature search was performed concerned with prior knowledge of mutations in Cyt c in an attempt to pick mutation sites that have already been shown to fold correctly and conserve biological function [133, 146-159]. Steady-state absorption, fluorescence, and circular dichroism have been taken for each mutant in order to determine if any structural perturbations of the mutations exist. Preliminary results have shown that the overall structure is not significantly affected; however, mutants W51, E66, G77, and F82 do not possess the characteristic 695 nm charge-transfer band for fivefold Cyt c. This has not been reported in literature and is still under investigation.

Initial time-resolved transient absorption experiments in the two redox states have been performed and data analysis is still underway. Preliminary results show that mutants W51, W77, and W82 do exhibit a ground-state absorption change of tryptophan upon photodissociation of the Met80 ligand in the reduced form of Cyt c (Fig. 4.8). W51 has a 25 ps response (8%), W77 exhibits a 25 ps component (7%), and W82 has a 27.3 ps component (15%). Both W77 and W82 mutants are within a few amino acids of the Met80 (Fig. 4.7) which is dissociated upon the 400 nm photoexcitation and may be sensitive to bond breaking and subsequent protein quake. W51 is in the proximity of the
loop containing the W77 mutant and also may be illustrating motion of the loop nearby induced by the proteinquake. Further investigation is currently being performed.

A fairly new method to induce protein conformational dynamics has been found recently. When the iron in the prosthetic heme group is replaced with zinc-II, photodissociation of the axial ligands of heme will induce protein unfolding in less than 5 ns and will later refold [123, 141-144]. The mutations recently created for Cyt c will allow us to probe the ultrafast perturbations after the photodissociation of the ligands. The photodissociation of the ligands initiates a possible proteinquake that creates large conformational change downstream. It is in the early ps to ns region that we are interested in and are quite enthusiastic that this experiment can be used to elucidate conformational dynamics that have never been seen before. The ultrafast motion of the protein is still not well understood and we hope to elucidate the initial ultrafast globular conformational change upon photolysis of the axial ligands.
Figure 4.7: X-ray structure [132] of horse heart cytochrome $c$ (PDB:1HRC) with the prosthetic heme group covalently linked with four residues, M80, H18, C17 and C14 as shown in Fig. 4.1. Also shown are 11 Trp point mutations, illustrated as yellow spheres, which are the optical probes for detecting protein conformation dynamics.
Figure 4.8: Femtosecond-resolved, normalized transient absorption signals of ferrous Cyt c mutants W51, W77, and W82 probed at 290 nm. Here we focus on the long-time components. All three mutants show long times of ~25 ps which is from the ground-state absorption changes of Trp due to the conformation fluctuations caused by the proteinquakes.
CHAPTER 5

PROTEIN UNFOLDING DYNAMICS OF CYTOCHROME C

5.1 Introduction

As seen in the previous two chapters, protein conformational dynamics can be observed and characterized using ultrafast techniques. In an effort to further illustrate the power of these techniques, another systematic approach was completed to study conformational dynamics induced by a strong proteinquake or thermodynamically driven process. In some instances, a strong proteinquake can induce major conformation changes in a protein, at times even lead to protein unfolding. Thermodynamically driven processes between unfolded and folded protein states can also be utilized to induce conformational change, as is the case with cytochrome c. The question as how proteins fold and unfold is a longstanding, unresolved question, one that is still highly debated today. The issue has been approached through many novel methods, one of which is the triggering of protein folding in the model system of proteins, cytochrome c. The difficulty of these experiments is to synchronize the folding with a detection method.
Until the last decade, folding kinetics were studied almost exclusively using stopped-flow techniques. In this experiment, protein folding is initiated by rapidly mixing a chemically denatured protein solution with a buffer to dilute the denaturant. The kinetics of folding are then monitored with one of several optical spectroscopic methods. Similarly, unfolding can be initiated by mixing a native protein solution with concentrated denaturant. Stopped flow experiments have yielded an enormous amount of valuable information but the fundamental drawback is the poor time resolution [163]. The initial protein conformational dynamics are completed within the dead time of the experiment [164-167], typically a few milliseconds; therefore, another technique is needed to observe the initial protein conformational dynamics.

Rapid initiation of protein folding can be classified into three categories, ultrarapid mixing methods, temperature or pressure jump, and photochemical triggering or photodissociation. The first fast-folding study used photodissociation of carbon monoxide from denatured cytochrome c [103, 145, 168]. This utilizes the fact that CO binds much more strongly to the heme of the denatured protein than to the heme of the native protein. Photodissociation of CO initiates folding because the CO-free protein is much more stable (Fig 5.1). Folding can also be triggered by a photo-induced electron transfer reaction [28, 105, 169, 170]. The subsequent dynamics can be studied by several
optical techniques such as circular dichroism, fluorescence up-conversion, and transient absorption.

The model by which proteins fold and unfold is also highly debated as to whether it is a two-state, simply comprised of only the folded and unfolded protein states, or a three-state folding pathway where folding intermediates exist. To address these issues, we have attempted a systematic approach to both characterize the folding/unfolding curve in different derivatives of Cyt c as well as induce protein conformational change upon photodissociation of CO. The characterization of the unfolding curve is performed with fluorescence up-conversion while utilizing the biomolecular ruler RET. The energy transfer pair is once again the intrinsic Trp and the prosthetic heme. Different derivatives of Cyt c were characterized as a function of the denaturation agent guanidine hydrochloride (GuHCl). The understanding of the unfolding curve illustrates that when at 4 M GuHCl, photodissociation of the carbon monoxide triggers the thermodynamically driven folding reaction (Fig. 5.1). We attempted to initiate the protein folding in a similar manner and gate the folding process using the biomolecular ruler resonance energy transfer and femtosecond up-conversion, this is a continuation of the 3-beam experiment described before (see section 3.3.5).
Figure 5.1: Illustration of the fluorescence-detected guanidine hydrochloride unfolding curves for ferric Cyt c (dashed), ferrous Cyt c (solid), and ferrous Cyt c under CO environment [145].
5. 2 Materials and Methods

Wild-type horse heart cytochrome c was purchased as a lyophilized powder from Sigma with 99% purity. For both steady-state and time-resolved experiments, the wild-type protein was dissolved in 0.1 M potassium phosphate buffer at pH 6.5. Denaturation was performed via titration with the denaturing agent ultra high purity 8M guanidine hydrochloride (GuHCl). For ferric experiments, the sample was used without further purification. Ferrous Cyt c was prepared by purging the ferric protein with high-purity nitrogen to remove oxygen and then reducing it with 1:5 ratio of the protein to sodium dithionite [96]. The CO bound was created by briefly purging the sample with CO. The protein concentration was maintained to be ~200 μM and all femtosecond experiments were carried out at room temperature with a moving 1-mm quartz cuvette to avoid heating and sample degrading. Steady state absorption was measured before and after the experiment to confirm integrity of the sample. Time-resolved experiments were carried out using femtosecond up-conversion method as detailed in chapter 2.
5.3 Results and Discussion

To address the concern of the folding and unfolding model of Cyt c, we have characterized the RET times for the different redox states along the unfolding pathway. The unfolding curves for the three different states of Cyt c are illustrated in Figure 5.1 [145]. In deciphering the model observed, we took femtosecond-resolved up-conversion data both the ferric and ferrous+CO states in different concentrations of GuHCl. The data was fit to a two state model and a titration unfolding curve was generated. Attempts were done with ferrous Cyt c but no sample was stable above 2M GuHCl. We believe that the unfolding nature of the protein exposes the iron in the heme to solvent and unfortunately, we could not get 100% oxygen out of the sample cell, therefore, oxidation occurred within 10 minutes of denaturation.

An important issue to denote here is the expected signal. In any case for folded protein, the energy donor, W59, is in the immediate vicinity of the energy acceptor, heme, Fig 4.1. Since the RET rate is inversely proportional to the sixth power of distance, chapter 2, the RET rate is expected to be extremely fast for the folding vs. unfolded state of the protein, where the W59 is further away from the heme. Figure 5.2 illustrates the femtosecond up-conversion transients of ferric Cyt c for different concentrations of GuHCl, from 0 to 3 M. Figure 5.3 illustrates the femtosecond up-
conversion transients of ferrous Cyt c in the presence of CO for different concentrations of GuHCl, from 1 to 6 M. To generate the unfolding curves for each state, all one has to do is roughly fit the data and take the area under the curve. Fig. 5.4 illustrates our fitting curve generated for our experiment. The unfolding curve corresponds well to published data. As one can see from the transient fits, the two state model applied does not fit appropriately, therefore, it is believed that the folding and unfolding pathway consists of an intermediate state. At low concentrations of GuHCl, it is believed that the protein begins to become somewhat loose and floppy, thus creating an intermediate state of unfolding.

The attempt to use RET as a molecular ruler to see in real time the protein conformational fluctuations upon CO photodissociation at 4 M GuHCl was unsuccessful. This method has been explored before by Jones et. al. [103], however, their excitation source is slightly different that the one used here. They used a 400 nm dissociation beam from a 10 ns pulsed laser at 10 Hz with a power of 10 mJ/pulse [103]. They stated that at best, only one-tenth of the laser power was absorbed by the protein. With this energy and their experimental conditions, it was determined that they had a significant excess number of photons/protein (~80 photons/heme) [103] which lead to a high probability of photodissociation. In our experiment, we use a 400 nm dissociation beam of 500 nJ and repetition rate of 1 kHz, this supplies 1x10^{12} photons/pulse. With a concentration of 200
μM and path length of 1mm, and beam width of ~0.5 mm, we have ~2.4x10^{13} proteins within the beam volume. This experimental setup is a major concern because we do not have near enough photons/protein to induce significant photodissociation of the CO to induce protein folding. Another other point of concern is the fact that we are using a repetition rate of 1 kHz. This implies that the time between the photodissociation pump is only 1 ms. In most pump probe experiments, one of the fundamental implications of using the 1kHz laser, the sample must return to equilibrium within the time of pump beams. Unfortunately in this case, the folding of Cyt c occurs on a much longer time scale (~several ms). The convolution of the sample between folded and unfolded states creates a tremendous difficulty, one that cannot be ignored or overcome.

5.4 Future Work and Considerations

Building upon the successful characterization of the unfolding curve as seen above, potential studies of interest would be to generate the unfolding curve for the mutations previously created for Cyt c, see section 4.5. An interesting investigation would be to determine if there would be a slight difference in the unfolding curve due to the fact that the Trp is in different locations throughout the protein, see figure 4.7. We suspect that the decay rates would be different for each mutant due to the change in
distances between the Trp and the heme group, similar to that of Mb. If the folding curves are slightly different, it may be possible to determine the initial parts of the protein that first begin to unfold.
Figure 5.2: Femtosecond-resolved fluorescence transients of ferric cytochrome c gated at 350 nm for various concentrations of guanidine hydrochloride (GuHCl).
Figure 5.3: Femtosecond-resolved fluorescence transients of ferrous+CO cytochrome c gated at 350 nm for various concentrations of guanidine hydrochloride (GuHCl).
Figure 5.4: Guanidine hydrochloride unfolding curves of ferric (red) and ferrous under CO environment (black) cytochrome c obtained by fluorescence up-conversion.
MYOGLOBIN PURIFICATION PROTOCOL

Growth Protocol:

Day 1-Transformation
1. Turn heat block on to 42°C with a little water in each well.
2. Defrost TB1 cell and DNA plasmid on ice for 15 minutes.
3. Mix 100 µl of TB1 and 1 µl of DNA in a cooled ependorf tube and incubate on ice for 30 min.
4. Heat-shock the TB1 and plasmid DNA mixture for 45 seconds in the heat block at 42°C.
5. Incubate on ice for 2 minutes.
6. Add 0.5-1 ml of LB to the tube and mix.
7. Incubate the tube at 37°C for 1 hour (put on an angle in the incubator/shaker)
8. Spread 200 µl of the TB1 and plasmid DNA on a LB+Ampicillin plate and spread.
9. Once the solution is absorbed into the plate, place plate upside down 37°C incubator overnight.

Day 2- Preparing Overnight Culture
1. Place 1 ml of autoclaved LB+Amp (100 µg/ml) in a sterile test-tube.
2. Dilute a single colony from overnight plate into the test-tube.
3. Grow for 3-5 hours at 37°C at 225 RPM.
4. Dilute 0.5 ml of growth into overnight culture (50 ml LB in 250 ml Erlenmeyer flask with the wider mouth and 50 µl of 100 µg/ml ampicillin).

Day 3- Larger Cell Culture Growth
1. Check OD, once it reaches 1.9-2.0 (~16 hours of growth), can inoculate the overnight culture.
2. Add 1 ml of 200 µg/ml Amp into 1 liter of LB in 2 liter flask.
3. Add 10 ml of overnight culture into the flask.
4. Incubate at 225 RPM at 37°C overnight.

Day 4- Harvesting Cell and Cell Lysis
1. After OD reaches 2.0 then begins to drop to 1.7 (time varies according to mutant: 20 hours to 36 hours), harvest the cell.
2. To harvest, centrifuge the culture for 10 minutes at 4°C at 4k RPM in A-10 rotor.
3. Discard supernatant.
4. Continue this process till all the culture has been harvested.
5. Combine the pellets into one bottle by dissolving in cold autoclaved ddH₂O and centrifuge at 5k RPM for 5 minutes.
6. Flash freeze the pellet with liquid nitrogen and store in -80°C if not moving onto purification immediately.

Protein Purification Procedures:

Day 1- Cell Lysis
1. Defrost the cell pellet in a water bath.
2. Weight the pellet.
3. Add 4 ml of 2x lysis buffer for every gram of pellet.
   Lysis Buffer (Final concentration 1x):
   - 50 mM Tris, pH 8.0
   - 1 mM EDTA, pH 8.0
   - 150 mM NaCl
   - 5% Glycerol
   - 0.5 mM Dithiothreitol (DTT)
   - 1 mM p-Toluene Sulfonyl Acid
4. Add 20 units DNase I 0.75 units RNase A and 2 mg Lysozyme per ml of lysis buffer.
5. Mix well and stir overnight in 4°C.

Day 2- Sonication, Salt Cut, & Size Column
1. Sonicate lysis solution in 50 ml plastic tubes on ice (Settings are 50% duty cycle, output control of 2, and 2-3 minutes on the Branson Sonifier 450A with the microtip).
2. Spin down the culture at 8.5k RPM in A-10 rotor for 45 minutes.
3. Prepare Sephadex G-50 column with 20 mM Tris, 1 mM EDTA pH 8.0.
4. Measure the supernatant with a graduated cylinder and discard pellet.
5. 55% Salt Cut=351g of ammonium sulfate/liter of supernatant. Add slowly over 45-60 minutes while stirring in 4°C.
6. Stir an additional 45-60 minutes.
7. Spin at 7.2k RPM for 45 minutes in A-10 rotor.
8. Measure supernatant with graduated cylinder for next salt cut.
9. 95% Salt Cut = 271 g of ammonium sulfate/liter supernatant. Add slowly over 45-60 minutes while stirring in 4°C.
10. Stir for an additional 45-60 minutes.
11. Spin at 7.2k RPM for 45 minutes in A-10 rotor.
12. Discard supernatant and save pellet.
13. Dissolve pellet with minimal amount of 20 mM Tris 1mM EDTA pH 8.0, do not vortex or disturb to make bubbles.
14. Spin at 4k RPM A-10 rotor for 2 min.
15. Load onto Sephadex G-50 size column (only 5% of column volume you should load)

Day 3- Gel, Concentrate, Anion Exchange Column, Gel, Concentrate, Polishing Column, Gel and freeze
1. Run a 15% SDS-PAGE gel and check purity.
2. Concentrate purest fractions down to ~20 ml with ultrafiltration device from Amicon using YM10K filter.
3. Run anion exchange column (Whatman DE52, 20 mM Tris pH 8.4).
4. Collect at 120 drops/fraction.
5. Clean the column with 2 M NaCl, 0.5 M NaOH, ddH2O, buffer.
6. Run another 15% SDS-PAGE gel and check purity.
7. Concentrate purest fractions with either Amicon ultrafiltration device or 5000 MWCO centrifuge filters, depending on the volume.
8. Run polishing column (Bio-Rad BP-60 Size Column, 20 mM Tris 1mM EDTA pH 8.0).
9. Collect at 80 drops/fraction (Can leave this run overnight).

Day 4- Gel & Freeze
1. Run a 15% SDS-PAGE gel and check that only one band is visible to your eye.
2. Combine and concentrate the purest fractions in a 5000 MWCO centrifuge filter.
3. Flash freeze sample when desired concentration is reached (typically ~400 μM) and store in -80°C freezer in Ependorph tubes.
APPENDIX B

CYTOCHROME c PURIFICATION PROTOCOL

Growth Protocol:

Day 1-Transformation
1. Turn heat block on to 42°C with a little water in each well.
2. Defrost BL21 (DE3) cell and DNA plasmid on ice for 15 minutes.
3. Mix 50 µl of BL21 (DE3) and 50 ng of plasmid DNA in a cooled ependorf tube and incubate on ice for 30 min.
4. Heat-shock the BL21 (DE3) and plasmid DNA mixture for 45 seconds in the heat block at 42°C.
5. Incubate on ice for 2 minutes.
6. Add 0.5 ml of LB to the tube and mix.
7. Incubate the tube at 37°C for 1 hour at 225 RPM (put on an angle in the incubator/shaker).
8. Spread 200 µl of the BL21 (DE3) and plasmid DNA on a LB+Ampicillin plate and spread.
9. Once the solution is absorbed into the plate, place plate upside down 37°C incubator overnight.

Day 2- Overnight Culture
1. Remove plate in the morning (~16 hours growth) and place in 4°C.
2. Dilute a single colony into 50 ml TB+Amp (100 µg/ml) in 250 ml flask.
   TB (Terrific Broth) (per liter)
   12 g Tryptone
   24 g Yeast extract
   50 ml of 2x Salt Solution (0.34 M KH₂PO₄, 1.44 M K₂HPO₄)
   20 ml of 20% Glycerol solution
   880 ml ddH₂O
3. Grow at 37°C, 220 RPM for ~16 hours.
Day 3- Innoculate
1. Overnight culture should be a pink color.
2. Spin overnight culture at 4k RPM for 5 minutes in A-10 rotor in autoclaved centrifuge bottle.
3. Discard supernatant and suspend pellet with fresh TB+Amp.
4. Aliquot into prepared TB+Amp (100 µg/ml), can use 2 liters in 4 liter flasks.
5. Grow for 50 hours at 30°C 225 RPM.

Day 4- Continue to Grow

Day 5- Harvest Cell
1. Culture should be a pink/reddish brown/orange color depending on expression.
2. Spin for 4k RPM for 10 minutes in JA8-1 rotor.
3. Discard supernatant and save pellet.
4. Combine all pellets down to single bottle (do not store more than 2 liters of growth together).
5. Flash freeze with liquid nitrogen and place in -80°C.

Protein Purification Procedures:
Day 1- Cell Lysis
1. Defrost the cell pellet in a cool water bath.
2. Weight the pellet.
3. Add 2 ml of lysis buffer for every gram of pellet.
   Lysis Buffer:
   - 50 mM Tris pH 8.0
   - 1 mM EDTA, pH 8.0
4. Add 2 mg/ml Lysozyme and final concentration of 0.01 mg/ml DNase I.
5. Mix well and stir overnight in 4°C.

Day 2- Sonicate, Spin, Salt Cut, Spin, Salt Column, Binding Column
1. Add Stock 100 mM PMSF to final concentration of 1 mM.
2. Sonicate in metal beaker with ¼” tip on Branson Sonifier 450A (Output 7, Duty cycle 90%, 15 times each 1 minute).
3. Ultracentrifuge at 35k RPM for 90 minutes.
4. Supernatant should be a deep red color.
5. 55% salt cut (351 g of Ammonium Sulfate per liter supernatant slowly added over 45-60 minutes then stir for another hour, all done in 4°C).
6. Spin down in TA14 rotor for 20 minutes at 10k RPM.
7. Load supernatant into SDG-25 desalting columns (do not load more than 25% column volume; the buffer is 25 mM NaPi pH 7.6).
8. Collect flow through in a beaker when the protein is close to the bottom.
9. Dilute solution in half with 25 mM NaPi pH 7.6.
10. Take absorption of diluted solution to determine concentration and redox state (should be more towards the ferrous state).
11. Add 5x potassium ferricyanide and mix for 30 minutes in 4°C.
12. Measure spectrum again to confirm it is in the ferric state.
13. Load overnight onto SP Sepharose Fast Flow (GE) anion exchange column, protein will bind tightly to the top).

Day 3- Elute, Gel, Absorption, Concentrate, Polish, Concentrate, Freeze
1. Wash SP column with 3 column volumes of low salt wash buffer (25 mM NaPi pH 7.6, 40 mM NaCl).
2. Elute protein via a linear salt gradient with high salt buffer (25 mM NaPi pH 7.6, 1 M NaCl) and low salt buffer.
3. Collect 60 drops/fraction.
4. Wash column with 2 M NaCl and buffer.
5. Run a 15% SDS-PAGE gel and check purity.
6. Check ratio of A_{410nm}/A_{280nm}, if greater than 4.0, collect and concentrate.
7. Load sample onto Bio-P 60 polishing size column, collect at 80 drops/fraction and let run overnight.

Day 4- Gel, Concentrate, Freeze
1. Run 15% SDS-PAGE gel and check purity.
2. Concentrate pure fractions if needed.
3. Measure absorption spectrum.
4. Aliquot into 1.5 ml ependorph tubes
5. Flash freeze in liquid nitrogen and store in -80°C.
APPENDIX C

PREPARATION OF OXIDATION STATES FOR MB AND CYT c

1. Purge water bath for 1 hour prior to purging sample
2. Purge buffer for 1 hour
   - Fill 13mm OD test tube up with buffer, as close to the top as possible
     • This may eliminate air entering into test tube when it is opened
3. Add a 5 times molarity of sodium dithionite (SD), 2NaHSO₃ (obtained from Sigma), of the protein concentration, protein concentration determined by Soret peak molar extinction coefficient and Beer’s law.
   - Make SD solution to concentration to add about 20uL SD solution to protein
4. Load sample into cell and measure concentration
   - Volume may decrease if sample evaporates during purging
     • This will change concentration
5. Purge sample for one hour
   - 5mm cells
     • Use stir bar
       - Ensures fresh sample is hitting laser every shot
   - Stir during purging
6. Add 5x SD to sample
   - Stir to mix
   - Want to add minimal volume ~20uL
     • Don't want too small as it will be difficult to add accurately
7. Keep purging for 1 more hour
   - Pull exhaust tube out slowly over the course of an hour
8. Seal sample with parafilm
9. When CO is needed for Mb, purge CO for 5-10 minutes while mixing sample, color will change to bright pink.

Note: Argon & Nitrogen used were ultra high purity together with an oxygen trap from Trigon, CO used is 99.5% CP grade.
APPENDIX D

UNDERSTANDING RALEIGH SCATTERING IN ABSORBANCE MEASUREMENTS

Absorbance is given as:

\[ A = -\log\left(\frac{I_0 - I_{\text{Absorbed by protein}}}{I_0}\right) \]

When working with dilute concentrations, we noticed a background signal that is wavelength dependent and is suspected to be from Raleigh scattering. The intensity of Raleigh scattering \( R_s \) is: \( I_{Rs} \propto \lambda^{-4} \), which shows that more the blue the wavelength the more light that gets scattered. So in our measurement, it appears that more light is being absorbed in the blue region by the protein but it is actually being scattered away. So the absorbance can now be written as:

\[ A_{\text{measured}} = -\log\left(\frac{I_0 - I_{\text{Absorbed by protein}} - I_{Rs}}{I_0}\right) \]
\[10^{-A_{\text{measured}}} = \left(\frac{I_0 - I_{\text{Absorbed by protein}} - I_R}{I_0}\right)\]

\[I_0 10^{-A_{\text{measured}}} = I_0 - I_{\text{Absorbed by protein}} - I_R\]

\[I_0 10^{-A_{\text{measured}}} + I_R = I_0 - I_{\text{Absorbed by protein}}\]

So if we let \(I_R = \text{constant} \times \lambda^4\), then we can remove the Rs term from the absorbance spectrum by adding it to \(10^{-A_{\text{measured}}}\).

\[I_0 10^{-A_{\text{protein}}} = I_0 10^{-A_{\text{measured}}} + I_R = I_0 - I_{\text{Absorbed by protein}}\]

\[10^{-A_{\text{protein}}} = 10^{-A_{\text{measured}}} + \frac{I_R}{I_0} = \left(\frac{I_0 - I_{\text{Absorbed by protein}}}{I_0}\right)\]

\[A_{\text{protein}} = -\log \left(10^{-A_{\text{measured}}} + \frac{I_R}{I_0}\right) = -\log \left(\frac{I_0 - I_{\text{Absorbed by protein}}}{I_0}\right)\]
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