ULTRAFAST PROTEIN HYDRATION DYNAMICS INVESTIGATED BY FEMTOSECOND FLUORESCENCE SPECTROSCOPY

DISSERTATION

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By

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ABSTRACT

Protein hydration dynamics is essential for many biological functions. However, understanding protein hydration dynamics has been technically challenged by the time and spatial resolution. This dissertation thus is a systematic investigation of protein hydration dynamics by integrating ultrafast fluorescence spectroscopy with the site-directed mutagenesis method. Using single tryptophan as the local optical probe, we first studied the site-specific solvation dynamics in various different conformations of the prototype peptide melittin and protein human serum albumin; both studies showed a strong correlation between local solvation dynamics and peptide/protein conformation transitions, and the critical role of hydration water in the structural integrity of the peptide/protein. To clarify the molecular interpretation of the solvation dynamics from time-resolved fluorescence studies, we also examined the local solvation dynamics at the surface of protein Staphylococcus nuclease using site-directed mutations; we replaced the local charged residues in close proximity to the tryptophan probe one at a time with an uncharged residue and did not observe significant change in the measured solvation dynamics, thus confirming that solvation correlation functions mainly measure the water dynamics in the protein hydration layer; the solvation dynamics is typically biexponential with the fast time constant resulting from local water relaxation in the hydration layer while the slow time from hydration water network rearrangement tightly coupled with protein fluctuations.
To better understand the molecular mechanism of tryptophan fluorescence behaviors in proteins, we then surveyed the tryptophan fluorescence in more than 40 proteins with the femtosecond fluorescence method and molecular dynamics simulations. We were able to identify the carbonyl- and sulfur-containing residues as able quenching groups of tryptophan fluorescence within 100-ps; the former includes glutamine and glutamate residues while the later disulfide bond and cysteine residues. We studied the protein dynamics in human thioredoxin and were able to distinguish four time-overlapping dynamical processes at its active-site and determined their respective time scales; these results elucidated the temporal evolution of hydration water motions, electron transfer reactions and local protein fluctuations at the active site of human thioredoxin, and illustrated continuously synergistic dynamics of biological functions over wide time scales. Building on our understanding of hydration dynamics in isolated proteins or protein mimics, we are applying protein hydration dynamics as a tool to address more biologically relevant questions. Preliminary results are summarized in the final chapter for our efforts to unveil the molecular mechanism of molecular recognition in calmodulin using femtosecond-resolved fluorescence spectroscopy.
Dedicated to my family!
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Proteins are the molecular machinery of cellular functions. Despite the clear awareness of the importance of protein functions, our knowledge remains rather limited in regards to the how(s) and why(s) of protein functions. At the atomic level, our understanding of protein functions have benefited in large part from high-resolution x-ray crystallography and NMR. With the enormous amount of protein structures deposited in the protein data bank (and this number increases everyday), one can indeed start to speculate about structure-function relationships. However, it has also become increasingly clear that it needs more than just a static three-dimensional structure for interpreting the molecular mechanism of protein functions. The dynamic personalities of a protein have emerged as the missing piece [1]. For instance, calmodulin (CaM) is a small bilobal calcium regulatory protein that binds and activates numerous target enzymes in response to the transient changes in intracellular calcium concentration while the CaM-binding domain of these target enzymes bears little sequence identity. The inherent conformational flexibility of calmodulin is commonly believed to be the reason that allows for the promiscuity in its interactions with target proteins and peptides [2]. Thus, it is imperative to include time-dependent protein behaviors (protein dynamics) into the structure-function paradigm in order to better understand
proteins in action. Protein dynamics are complex processes and evolve on a multidi-
mensional energy landscape with various interactions and conformations over a wide
range of time scales [3]. Typical time scales of biologically relevant motions involved
in protein dynamics span fifteen orders of temporal evolution from femtosecond to
second. To understand such complex dynamics in proteins, one needs to dissect the
process into elementary components and determine their respective time scales. Of-
ten times, many of these elementary processes are convoluted on similar time scales,
and thus a multidisciplinary approach is necessary to distinguish these processes and
to elucidate their molecular mechanisms.

Almost all protein functions take place exclusively in aqueous environment. Wa-
ter must not be simply treated as an inert environment for proteins and needs to
be included in every discussion of protein functions. Water is widely known to be
essential for life not only at the cellular levels but also at molecular levels. Cellular
functions cease to exist in the absence of water partly due to the fact that most pro-
teins are not able to perform their biological functions without water. A dry protein
will only start to regain its biological functionality when the hydration level reaches
0.2 (g of H$_2$O)/(g of protein) and fully achieve its normal activity at the hydration
level of 0.4 (g of H$_2$O)/(g of protein) [4, 5]. To function, a protein must maintain a
delicate balance between its structural stability and conformational flexibility: the
protein needs to be sufficiently stable to retain its native three-dimensional structure
while flexible enough to allow for efficient substrate recognition, chemical reaction and
product release. It remains yet to be fully understood as to how a protein, starting
from an unstructured long chain of amino acids, is able to rapidly fold into its native
conformation that is determined by its primary amino acid sequences. Nonetheless, it is commonly accepted that protein folding will not even take place without the involvement of the natural solvent–water. Changing solvent properties immediately disrupts the protein structure, causing the protein to lose its functionality completely. Numerous lines of evidence from experimental observations [6–9] and molecular dynamics simulations [10] strongly suggested that protein dynamics are primarily driven by the dynamic motions of water. More specifically, most local protein motions are slaved by the $\beta$-relaxation of water in the hydration shell while the large scale conformational changes are slaved by the $\alpha$-relaxation in bulk solvent [8]. Thus, it is necessary to understand the dynamic properties of water molecules in the hydration shell of proteins. More reasons exist to highlight the importance of understanding hydration dynamics in proteins. During the process of molecular recognition, two proteins do not interact directly but are rather mediated by the water molecules at the interface, and water-mediated contacts may carry significant information content to allow these interactions to be discriminating, making the molecular recognition highly selective [11].

Because of the significant roles of water in biology, water properties in the biological systems have been the subject of extensive investigations for over a century using a wide range of biophysical tools [4, 12, 13]. X-ray and neutron crystallography revealed highly ordered water at the interface between proteins and bulk water. While these crystallographic methods allow one to infer the amplitudes of protein internal motions from atomic temperature factors, they in general provide little information about the time scales of protein dynamics and the dynamics of water in
the hydration layer. A variety of experimental techniques, including dielectric relaxation spectroscopy [4, 13, 14], magnetic relaxation method [15–19] and quasi-elastic neutron scattering spectroscopy [20–22], have been utilized to understand the water dynamics on protein surfaces. These techniques have contributed to our understanding of hydration dynamics in proteins to a great extent. Dielectric relaxation was among the first methods to probe dynamics in protein solution and indicated that dynamics in the hydration layer of a protein is different from that in bulk water [23]. The major strength of nuclear overhauser effect (NOE) in NMR studies of hydration dynamics is in its ability to probe spatially localized hydration, but NOE suffers from insufficient temporal resolution and is only capable of measuring water dynamics in the range of 300-500 ps [24,25]. Experimental approaches without sufficient temporal resolution certainly are incapable of fully resolving the water dynamics in proteins as recent molecular dynamics (MD) simulations have convincingly shown that water motions in the hydration layers of protein surfaces are ultrafast and on picosecond time scales [26,27]. Nuclear magnetic relaxation dispersion (MRD) is another NMR-based method that selectively probes water molecules in aqueous protein solutions with a much improved temporal resolution in the range of 10-50 ps [15]; but the inherent lack of spatial resolution and the complication of hydrogen exchange often render the molecular interpretation subject of discussion. Although quasi-elastic neutron scattering (QENS) not only probes the hydration water dynamics with subpicosecond resolution but also the magnitude of water motions in space [28], the method also lacks spatial resolution and only provides a statistical average of hydration dynamics over an entire protein surface. It is more informative if experimental methods can site-specifically measure water dynamics in protein. In so doing, it becomes possible
to examine the water dynamics in the active sites of enzymes and how water modu-
lates the enzyme catalytic activity [29]. More importantly, it is also then feasible to
examine the correlation between water dynamics in the protein hydration shell and
the local structural and chemical properties of a protein [30]. Considering the fact
that water motions on protein surfaces are ultrafast and in constant exchange with
bulk water, a new approach that measures protein hydration dynamics with sufficient
temporal and high spatial resolution is more relevant and thus needed.

Recently, this laboratory and its collaborating laboratory (Dr. Zewail at Cal-
tech) have been heavily engaged in developing and improving a novel experimental
approach to protein hydration dynamics that utilizes femtosecond fluorescence spec-
troscopy with an intrinsic amino acid as the local optical probe [12, 30–37]. Fem-
tosecond fluorescence spectroscopy has the ideal temporal resolution to resolve the
ultrafast water motions in protein environments. Tryptophan is one of only three
intrinsic fluorophores among the twenty genetically encoded amino acids with tryp-
tophan being the most frequently used one among the three because of its relatively
larger quantum yield. Tryptophan is chosen over extrinsic fluorescent dye molecules
because single-residue spatial resolution can be easily achieved by engineering a tryp-
tophan residue into desired positions in proteins through site-directed mutagenesis.
Work in this dissertation is to further advance our understanding of tryptophan pho-
tophysics, to promote tryptophan as the intrinsic optical probe for studies of protein
hydration dynamics and to demonstrate that it is now possible to dissect the com-
plex protein dynamics with the powerful combination of femtosecond fluorescence
spectroscopy and modern molecular biology techniques.

Chapter 2 describes the experimental methodology with an emphasis on the femtosecond-resolved fluorescence up-conversion method. Protocols to prepare samples used in this dissertation will be given in each individual chapter. Because tryptophan typically do not emit with a single fluorescence lifetime, the standard procedure used for single lifetime fluorophores is no longer applicable to construct the time-resolved emission maxima (Stokes shift with time) and the solvation correlation function. A new method to extract the time-resolved emission maxima and to construct the solvation correlation function for a tryptophan probe (or any other fluorophores with dual fluorescence lifetimes) has been developed by this laboratory and a full account of this methodology will be given in this chapter.

In chapter 3, we report our systematic studies of hydration dynamics in three different conformations of prototype peptide melittin using single tryptophan as a molecular probe. With femtosecond resolution, we observed hydration dynamics taking place in 0.62 and 14.7 ps in a random-coiled structure of melittin. The former represents bulk-like water motion, and the latter reflects surface-type hydration dynamics in proteins. At membrane-water interface, melittin folds into an α-helical structure, and water motions are hindered at the interface to occur within 114 ps, indicating a well-ordered water structure in the hydration shell of the highly organized membrane. In an aqueous solution of high salt concentration, dielectric screening and ionic solvation promote four identical melittin monomers to aggregate, forming a tetramer. The hydration dynamics were observed to occur in 87 ps, significantly
slower than typical water motions at protein surfaces but similar to the time scales of water motions at membrane-water interfaces. The observed time scales of $\sim$100 ps likely represents the kind of water mobility appropriate for maintaining and (possibly mediating) the high-order structures of both melittin $\alpha$-helix and tetramer. These results shed light on the critical role of hydration dynamics in peptide conformational transitions and protein structural stability.

Chapter 4 continues to systematically explore the solvation dynamics in different conformations of a whole protein human serum albumin using a single tryptophan residue (W214) in the protein as the local optical probe. With femtosecond resolution, we observed a robust bimodal distribution of time scales for all conformation isomers. The initial solvation, occurring in several picoseconds, represents local water relaxation in the hydration layer while the slower dynamics in tens to hundreds of picoseconds results from the hindered water motions due to coupling with protein fluctuations. We extensively examined the local rigidity around W214 with femtosecond fluorescence anisotropy dynamics. Under the physiological pH condition, solvation dynamics occurs within $\sim$100 ps and W214 was found to have a large rotational freedom at the binding site deeply buried in a crevice, indicating the softness of the binding pocket and the large conformation plasticity of the native structure in human serum albumin. At acidic pH, the albumin molecule transforms to an extended conformation with a large charge distribution at the surface, and similar temporal behavior in solvation dynamics was observed to occur. However, at the basic pH condition, the protein opens the crevice and tightens its globular structure and significantly faster dynamics of $\sim$25-45 ps were observed. These changes in solvation
dynamics are well correlated with the conformational transitions and their structural integrity.

Chapter 5 is a full account of the studies of local hydration dynamics at the surface of protein *Staphylococcus* nuclease using site-directed mutations in order to address concerns in literature regarding the molecular interpretation of the solvation correlation function derived from time-resolved fluorescence spectroscopy. Some have contended that the slower component of the solvation correlation functions in tens of picoseconds might be predominantly from the response of protein residues instead of protein-coupled water motions in close proximity to protein surface. By comparing the local solvation dynamics in wild-type *Staphylococcus* nuclease and four carefully designed mutants, each of which has a local charged residue replaced with an uncharged one, we were able to ascertain the contribution to observed solvation dynamics by protein as relatively insignificant. Thus the solvation correlation functions mainly measure the water dynamics in protein hydration layer. These hydration dynamics in wild-type *Staphylococcus* nuclease and its mutants are also in agreement with the results of total dynamics Stokes shift and directly correlated with their local charge distribution and structural properties. The roles of protein fluctuation on the time scales of hydration dynamics will be discussed. The initial ultrafast decay of protein hydration dynamics predicted by molecular dynamics simulations was not observed experimentally though such time scales are not beyond the temporal resolution of our instrument. Thus results from molecular dynamics simulations need to be revisited, and it is likely that the discrepancy between MD simulations and experiments could
be resolved by re-evaluating the force field used in the simulations.

Chapter 6 describes our contribution to understanding of ultrafast quenching of tryptophan fluorescence in proteins. Tryptophan has been extensively utilized as a molecular optical probe to study protein dynamics and enzyme reactions. However, tryptophan is also an easy target of fluorescence quenching in proteins. Understanding the molecular mechanism of tryptophan fluorescence quenching especially in the ultrafast time regime in protein will surely make tryptophan a better molecular probe. More than 40 proteins were studied with femtosecond-resolved fluorescence spectroscopic methods. Using site-directed mutagenesis, we substituted residues at desired positions with tryptophan or altered the neighboring residues of tryptophan to screen quenching groups among amino acids and peptide bonds. We focused our attention to ultrafast quenching dynamics within 100-ps and were able to identify two ultrafast quenching groups: carbonyl- and sulfur-containing residues. The former includes glutamine and glutamate residues while the later disulfide bond and cysteine residues. Quenching by the peptide-bond carbonyl group as well as other potential residues is much less efficient and usually on times scales longer than 100 ps. These ultrafast quenching dynamics occur at van der Waals distances through intraprotein electron transfer with high directionality. Following optimal molecular orbital overlap, electron jumps from the benzene ring of the indole moiety in a vertical orientation to the LUMO of acceptor quenching residues. Molecular dynamics simulations were invoked to elucidate various correlations of quenching dynamics with separation distances, relative orientations, local fluctuations and reaction heterogeneity.
In chapter 7, we will report our complete dissection of complex protein dynamics in human thioredoxin into elementary processes and determining their respective time scales. By integrating site-directed mutagenesis with femtosecond spectroscopy, we distinguished four partly time-overlapping dynamical processes at the active site of thioredoxin. Using intrinsic tryptophan as a molecular probe and from mutation studies, we were able to confirm the negligible contribution to solvation by protein side chains and observed that hydration dynamics at the active site occur in 0.47-0.67 and 10.8-13.2 ps. With reduced and oxidized states, we determined the electron transfer quenching dynamics between excited tryptophan and a nearby disulfide bond in 10-17.5 ps for three mutants. A robust dynamics process in 95-114 ps, present in both redox states and all mutants regardless of neighboring charged, polar and hydrophobic residues around the probe, is attributed to the charge transfer reaction with its adjacent peptide bond. Site-directed mutations also revealed the electronic quenching dynamics by an aspartate residue at a hydrogen bond distance in 275-615 ps. The local rotational dynamics determined by the measurement of anisotropy changes with time unraveled a relatively rigid local configuration but implies that the protein fluctuates on the time scales of longer than nanoseconds. These results elucidate the temporal evolution of hydrating water motions, electron-transfer reactions, and local protein fluctuations at the active site, and show continuously synergistic dynamics of biological function over wide time scales.

In chapter 8, we will briefly discuss the on-going calmodulin project whereby we propose to dissect the mechanism of molecular recognition in calmodulin using time-resolved fluorescence approaches. Hydration dynamics at the four calcium-binding
sites and central linker region were studied. Preliminary results will be presented. Directions for future experiments towards the goal of this project will be discussed at the end of the chapter.
CHAPTER 2

EXPERIMENTAL METHODOLOGY

2.1 Femtosecond Laser Spectroscopy

All the time-resolved measurements in this dissertation were carried out using a femtosecond (fs) fluorescence up-conversion method. The integrated experimental setup is schematically illustrated in Figure 2.1, which also includes a high sensitivity transient absorption component. The fluorescence up-conversion method is a pump-probe approach in which two ultrashort laser pulses are needed: the pump pulse functions to instantaneously prompt the fluorophore to its excited state while the probe pulse detects time-dependent fluorescence intensity with femtosecond time resolution. Briefly, the femtosecond laser pulse (fundamental) after the two-stage amplifier (Spitfire, Spectra-Physics) has a temporal width of 110 fs centering at 800 nm with pulse energy of more than 2 mJ and a repetition rate of 1 kHz. Half of the laser energy was used to pump an optical parametric amplifier (OPA-800C, Spectra-Physics) to generate signal (1289 nm) and idler (2109 nm) beams. The latter was mixed with the residual fundamental (800 nm) in a 0.2-mm β-barium borate crystal (BBO, type I) to generate a new femtosecond pulse at 580 nm. The resulting fs pulse (580 nm) was compressed through a pair of prisms with double paths to improve the temporal
resolution to 60 fs and then frequency-doubled to generate the eventual pump pulse at 290 nm using another 0.2-mm BBO crystal. The pump pulse energy was typically attenuated to $\sim$140 nJ prior to being focused into the motor-controlled rotating sample cell. The fluorescence emission was collected by a pair of parabolic mirrors and mixed with a gating pulse from another half of the fundamental beam (attenuated) in a 0.2-mm BBO crystal through a non-collinear configuration. The up-converted signal ranging from 218 to 292 nm was detected by a photomultiplier coupled with a double-grating monochromator. The instrument response time under the current non-collinear geometry is $\sim$400-500 fs as determined from the up-conversion signal of Raman scattering of water around 320 nm. Typically, the polarization of the pump beam was set at a magic angle (54.7°) with respect to the acceptance axis (vertical) of the up-conversion crystal, and the polarization of the probe beam was rotated parallel to this axis through a half-wave plate. For the measurements of time-dependent fluorescence anisotropy, the polarization of the pump beam was set either parallel or perpendicular to the acceptance axis to obtain the parallel($I_\parallel$) or perpendicular ($I_\perp$) signals respectively. Time resolved anisotropy was then calculated using $r(t) = (I_\parallel - I_\perp)/(I_\parallel + 2I_\perp)$.

2.2 Construction of Solvation Correlation Function

Various extrinsic dye molecules have been used as optical probes for the study of solvation dynamics in solution and proteins [38–42]. These dye molecules are not ideal and direct use of the intrinsic amino acid tryptophan as a local optical probe has been recently proposed and applied to study protein hydration. The methodology to study solvation dynamics with the time-resolved fluorescence approach will be discussed in
Figure 2.1: The schematic representation of the experimental setup with both femtosecond fluorescence up-conversion and transient absorption configurations. The dashed line denotes the pathway for the probe laser in experimental configuration for transient absorption detections. F, filter. MM, movable mirror. MP, movable parabolic mirror. PD, photodiode.

detail. Figure 2.2 shows the femtosecond-resolved fluorescence up-conversion transients of aqueous tryptophan. The initial femtosecond decay at the blue side and the rise at the red side dominantly result from solvation processes. Typically, one can construct the time-resolved emission peaks and then correlation function by following the standard procedures [43]:

$$c(t) = \frac{\nu(t) - \nu(\infty)}{\nu(0) - \nu(\infty)},$$

(2.1)

where $\nu(t)$, $\nu(0)$ and $\nu(\infty)$ are time-resolved emission maximum in cm$^{-1}$. For a molecular probe with only one fluorescence lifetime, $\nu(\infty)$ usually equals to the steady-state fluorescence emission maximum $\nu_{ss}$. However, tryptophan typically has multiple fluorescence lifetimes and can be practically to be treated with two lifetimes of two different emission maxima ($\nu_1$, $\nu_2$) due to the ground state heterogeneity. Thus $\nu(\infty)$
Figure 2.2: Normalized, femtosecond-resolved fluorescence transients of tryptophan in bulk water with a series of wavelength detections. Note that the detection range was extended to the deep blue side of the 300 nm and the far red side of 460 nm.

cannot be considered to be the apparent \( \nu_{ss} \). We need to find out when the solvation is completed \((t_{sc})\) and the corresponding emission maximum \((\nu_{sc})\). A revised method was established in this laboratory to determine \(t_{sc}\) (and \(\nu_{sc}\)) from femtosecond-resolved fluorescence measurements.

All femtosecond-resolved transients in Figure 2.2 can be best fit by a sum of a series of exponential functions. These functions can be separated into two parts.
(assuming no ultrafast quenching of tryptophan fluorescence emission). One part
represents solvation processes and the other is for lifetime emissions (or population
decay). The transient signal thus is written as follows:

\[ I_\lambda(t) = I_{\lambda}^{\text{solv}}(t) + I_{\lambda}^{\text{popu}}(t) = \sum_i a_i e^{-t/\tau_i} + \sum_j b_j e^{-t/\tau_j}, \tag{2.2} \]

where the first term is for solvation and the second term for lifetime emission contributions. The coefficient \( a_i \) is positive (decay dynamics) at the blue side of the emission peak (<349 nm) and is negative (initial rise) at the red side (≥349 nm). The coefficients \( b_j \) are always positive and represent relative contributions of two lifetime emissions. The overall femtosecond-resolved emission spectra can be constructed as follows:

\[ I(\lambda, t) = \frac{I_{\lambda}^{\text{ss}} I_\lambda(t)}{\sum_i a_i \tau_i + \sum_j b_j \tau_j}, \]
\[ I(\nu, t) = \lambda^2 I_{\lambda,t}. \tag{2.3} \]

where \( I_{\lambda}^{\text{ss}} \) is the steady-state relative emission intensity at \( \lambda \). For a given \( t \), the emission spectrum can be constructed from the fluorescence transients. The resulting femtosecond-resolved emission spectra of aqueous tryptophan are shown in Figure 2.3a. These spectra \( (I(\nu, t)) \) are fitted using a log-normal function to extract the emission maximum \( \nu(t) \). Thus, the time evolution of the emission maximum can be obtained; see Figure 2.3b. After certain time \( (t_{sc}) \), solvation is completed and the emission maximum \( \nu_s(t_{sc}) \) should be equal to the apparent lifetime emission peak \( \nu_l(t_{sc}) \) (not yet reaching the steady-state emission peak), which results from a mixture of two (effective) lifetime emissions and is constructed as follows:

\[ I_{\lambda}^{\text{popu}}(\nu, t) = \frac{I_{\lambda}^{\text{popu}} I_\lambda(t)}{\sum_i a_i \tau_i + \sum_j b_j \tau_j}, \tag{2.4} \]
\[ I_{\lambda}^{\text{popu}}(\nu, t) = \lambda^2 I_{\lambda}^{\text{popu}}(\lambda, t). \]

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The time evolution of lifetime emission maximum $\nu_l$ is also shown in Figure 2.3b, and it converges with $\nu_s$ at $t_{sc}$. Substituting $\nu(\infty)$ in Eq. (2.1) with the value of $\nu_{sc}$ at the merging point gives

$$c(t) = \frac{\nu_s(t) - \nu_{sc}}{\nu_s(0) - \nu_{sc}},$$

(2.5)

where $c(t)$ finishes at $t_{sc}$. Considering the multiple-lifetime nature of tryptophan, the more accurate solution would be to subtract the apparent lifetime emission maximum $\nu_l(t)$ from the overall emission maximum $\nu_s(t)$ at any given $t$ and the resulting $c(t)$ is then written as follows:

$$c(t) = \frac{\nu_s(t) - \nu_l(t)}{\nu_s(0) - \nu_l(0)}.$$  

(2.6)

When solvation time is much short than the lifetimes, both approaches (Eqs. 2.5 and 2.6) to construct $c(t)$ give very similar result because of $\nu_l(t) \approx \nu_{sc}$. However, when solvation dynamics becomes much slower, such as in proteins, on a time scale comparable to the lifetimes, the contribution of $\nu_l(t)$ is significant and Eq. (2.6) must be used to construct $c(t)$, as is the case for all solvation dynamics reported in this dissertation. For molecular probes with single lifetime emission, Eq. (2.6) converges to Eq. (2.1) because of $\nu_l(t) = \nu_l(0) = \nu_{ss} = \nu(\infty)$.

The key step in this method is to construct both $\nu_s(t)$ and $\nu_l(t)$ and to determine $t_{sc}$; see Figure 2.3b. When the difference between two maxima $(\nu_s(t) - \nu_l(t))$ reaches within 0.5 cm$^{-1}$, we consider solvation dynamics as completed ($t = t_{sc}$). The difference between $\nu_{sc}$ and $\nu_{ss}$ purely results from the mixture of two lifetime fluorescence emissions. The time evolution from $\nu_{sc}$ to $\nu_{ss}$ could be very long. For tryptophan in water, the time-zero emission maximum ($\nu_0$) is 322.1 nm and solvation dynamics completes within 18 ps. Both $\nu_s$ and $\nu_l$ converge at 346.6 nm and the total Stokes
Figure 2.3: (a) Normalized, femtosecond-resolved fluorescence emission spectra derived from femtosecond-resolved fluorescence transients in 2.1. (b) Time evolution of overall femtosecond-resolved emission spectra $\nu_s$ and lifetime associated emission spectra $\nu_l$. Note that the two emission spectra converge at $28,848 \text{ cm}^{-1}$ (346.6 nm) and 18 ps, and solvation dynamics is completed. Both spectra continue red-shifting to reach the steady state emission until $\sim 850$ ps. The resulting hydration correlation function is shown in the insert.
shift is 2186 cm$^{-1}$. However, it reaches the steady-state emission maximum at 349 nm until $\sim$850 ps. This observation strongly indicated that the steady-state emission maximum is not relevant for the construction of the solvation correlation function. The lifetime emission spectrum has a maximum at 329.8 nm for the short lifetime component (500 ps) and 349.8 nm for the long lifetime component (3 ns), consistent with previous results [44].

The solvation dynamics of bulk water have been extensively studied. Jarzeba et al. [45] obtained a correlation function with 160 fs (33%) and 1.2 ps (67%); and Jimenez et al. [46] reported an initial Gaussian-type component (frequency 38.5 ps$^{-1} \approx 25$ fs in time width, $\sim$48%), and two exponential decays of 126 fs (20%) and 880 fs (35%). The solvation correlation function we obtained with the revised method is shown in the insert of Figure 2.3b; it can be best fit by double exponential decays integrated with an initial Gaussian-type contribution through a stretched model $c(t) = c_1 e^{-(t/\tau_1)^\beta} + c_2 e^{-(t/\tau_2)}$ where $\beta = 2$ for a standard Gaussian-type decay; the two decays are 340 fs (55%, $\beta=1.35$) and 1.6 ps (45%). The 1.6-ps component observed here, a little longer than previously reported values ($\sim$1 ps), is likely due to the strong interactions between water molecules and Zwitterionic tryptophan (pH 7.0) in the first hydration shell.
CHAPTER 3

ULTRAFAST HYDRATION DYNAMICS IN MELITTIN FOLDING AND AGGREGATION

3.1 Introduction

Understanding protein-water interactions is essential to many aspects in proteins such as protein folding and aggregation, conformation plasticity and molecular recognition, and enzymatic catalysis [12, 24, 31, 47–55]. The protein-associated water in the hydration layer plays a critical role in the protein’s structural stability, flexibility, and integrity. The dynamics of water motions directly affects protein conformational transitions such as from the denatured to native state [55, 56] or from the misfolded structure to the final protein aggregate [57, 58]. Thus, elucidation of molecular mechanisms of protein-water interactions is essential to the understanding of protein dynamics [7, 9, 31, 48]; the knowledge is also practically important as it could lead to better control over early protein misfolding and aggregation that occurs in neurodegenerative diseases [59–61]. In this chapter, we first studied water motions around tryptophan in a tripeptide Lys-Trp-Lys (KWK) with femtosecond temporal resolution; we then used melittin, a prototype peptide, to systematically examine the water dynamics in different conformational states that include a random-coiled primary
structure, a folded α-helical secondary structure, and the self-assembled tetramer.

Melittin, a peptide toxin found in honeybee venom and one of the most extensively studied archetypes of membrane proteins [62–65], consists of 26 amino acid residues (Figure 3.1). The first 20 residues are predominantly hydrophobic while the other 6 residues at the carboxyl terminus are hydrophilic under physiological conditions. This amphipathic property allows melittin to easily dissolve in aqueous solution and readily bind to membranes as well. It typically exists as a flexible random-coil monomer in water [66–68] but forms an α-helix in alcohol solvents [69–71] or during an encounter with membranes [62–65, 72–74]. In aqueous solution of high ionic strength, high peptide concentration, or high pH, four melittin monomers associate to form a tetramer [66–68, 70, 75, 76]. The X-ray crystallographic structure [77, 78] of the melittin tetramer shows a twofold symmetric configuration with two almost stereochimically identical dimers; each one consists of two antiparallel aligned α-helical monomers (Figure 3.1).

Melittin has been extensively studied to understand the interactions of peptide with biological membranes. Self-assembly of α-helical melittin monomers is believed to be essential for its lytic activity of membranes [62–64]. Its versatility to adopt different conformations has also made melittin an ideal model system for studies of protein folding [76]. A number of simulations have been carried out to examine the interactions of melittin with water and membrane [79–84]. The lone tryptophan residue (W19) has been widely used as an optical probe to study conformational dynamics through measurements of its steady-state emission [72, 85–87], rotational
Figure 3.1: X-ray crystallographic structure of melittin tetramer (upper), schematic illustration of membrane-bound melittin α-helix (middle), and melittin random coil monomer (lower) derived from one MD simulation trajectory (800 ps) through melting of the α-helical monomer at 600 °C in water. Note that W19 in melittin α-helix is highly constrained at the membrane interface. The melittin sequence is given at the bottom.
anisotropy, and fluorescence lifetimes [51, 88–90]. Tryptophan is very sensitive to microenvironment polarity, and its time-resolved fluorescence emission (Stokes shift) directly probes local solvation dynamics [12, 32, 37, 91–93]. In this study, we will use the intrinsic tryptophan (W19) in melittin to examine hydration dynamics in different conformations of melittin from a random coil, to a membrane-bound α-helix, and to a self-assembled tetramer in various aqueous solutions.

3.2 Materials and Methods

Melittin and tripeptide KWK were purchased from Sigma-Aldrich in lyophilized powder, Hepes chemicals from USB, lipid monoolein (1-oleoyl-rac-glycerol, >99% purity) from Nu Chek Prep (Elysian, MN), and all other chemical reagents from Fisher Scientific. All samples were used as received without further purification. Tripeptide KWK was dissolved in Tris (100 mM) buffer solution at pH 7.5 with a concentration of 3 mM. Melittin random-coil monomer was prepared by directly dissolving melittin powder in distilled water/HCl at pH 4 with a concentration of 1 mM while tetramer with a concentration of 3 mM was prepared in 100 mM Hepes/2 M NaCl at pH 7.5 [70, 88]. To prepare membrane-bound melittin α-helix, the lipid-water mixture was made at 20 °C consisting of 60% (w/w) lipid monoolein and 40% (w/w) water solution which contains 2 mM melittin and 20 mM TES buffer at pH 7.4. Under this condition, this mixture forms the lipidic cubic phase (Pn3m) with a diameter of aqueous channels at ∼50 Å [94,95]. All the time-resolved measurements were carried out on the femtosecond up-conversion apparatus as detailed in chapter 2.
3.3 Results and Discussions

3.3.1 Steady-State Fluorescence Characterization

The steady-state fluorescence spectra were characterized using a SPEX FluoroMax-3 spectrometer. The tryptophan fluorescence emission peaks at 349 nm in bulk water and shifts to 352 nm in 100 mM Tris buffer [37]. The emission of tripeptide KWK has a maximum at 350.6 nm. The tryptophan emission maximum in the melittin random coil monomer is at 348.5 nm, indicating a great exposure of intrinsic W19 to an aqueous environment. Upon incorporation into a membrane in lipidic cubic phase, the emission peak shifts to 341 nm, suggesting a less polar microenvironment around W19 in melittin α-helix. The emission maximum of W19 upon tetramerization of melittin further blueshift to 333.5 nm, consistent with a more hydrophobic location of W19 in the tetramer as observed by the X-ray structure [77,78]. These tryptophan emission peaks in different conformations of melittin are well correlated with the degree of hydration of its local environments.

3.3.2 Femtosecond-Resolved Fluorescence Dynamics

(a) Tripeptide KWK. Figure 3.2 shows fluorescence temporal behavior of tryptophan in tripeptide KWK, and the overall decay dynamics is similar to that of tryptophan in the same buffer [37]. Besides the two lifetime contributions ($I_{pop}(t)$), three exponential decays ($I_{sol}(t)$) were used to represent the solvation dynamics. At the blue side, the three decays occur in 0.22-0.4, 1.8-3.4, and 8-24 ps, and the first...
Figure 3.2: Normalized, femtosecond-resolved fluorescence transients of tripeptide KWK in the short (left) and long (right) time ranges with a series of gated fluorescence emissions.

ultrafast component dominates. At the red side (≥350 nm), an initial rise was observed in all transients within 0.2-0.5 ps followed by two lifetime emissions.

(b) Melittin: Random Coil Monomer. Figure 3 shows a set of femtosecond-resolved fluorescence transients of melittin random coil monomer in aqueous solution at pH 4. At the blue side, the transients exhibit two solvation components. These components decay in 0.4-1.3 and 4.4-25 ps with considerable contributions, respectively. At the red side (≥350 nm), we observed two initial rise components in all transients with the time scales of 0.24 and 1.8-4.2 ps. Compared with the results of the tripeptide
Figure 3.3: Normalized, femtosecond-resolved fluorescence transients of melittin random coil monomer in the short (left) and long (right) time ranges with a series of gated fluorescence emissions.

above, the overall solvation dynamics in melittin random coil monomer is slower.

(c) Melittin: Membrane-Bound α-Helix. Figure 3.4 shows a series of femtosecond-resolved fluorescence transients of melittin at membrane-water interfaces. Compared with the transients of the random coil monomer (Figure 3.3), the solvation dynamics becomes drastically slower. At the blue side, three solvation components were observed to occur in 0.6-4, 7-23, and 71-120 ps with similar amplitudes. At the red side (≥340 nm), we observed two initial rise components in 0.5 and 22 ps. Compared with
Figure 3.4: Normalized, femtosecond-resolved fluorescence transients of a membrane-bound melittin α-helix in the short (left) and long (right) time ranges with a series of gated fluorescence emissions. Note that the solvation dynamics drastically slows down.

those of free tryptophan in the same lipidic cubic phase [37], the first solvation component observed here slows down but the other two longer components have similar dynamics.

(d) Melittin: Self-Assembled Tetramer. We studied the solvation dynamics after melittin aggregates to form a tetramer under high-salt conditions. Because of its symmetry (Figure 3.1), all four tryptophan residues in the tetramer nearly have the same microenvironments. Figure 3.5 shows a series of femtosecond-resolved fluorescence transients of the resulting melittin tetramer. Similar to the membrane-bound α-helix
**Figure 3.5:** Normalized, femtosecond-resolved fluorescence transients of a melittin tetramer formed in high-salt aqueous solution in the short (left) and long (right) time ranges with a series of gated fluorescence emissions. Note that the solvation dynamics is drastically slow, similar to that of melittin $\alpha$-helix in Figure 3.4

In Figure 3.4, we observed significantly slow solvation to decay in 2-10 and 30-100 ps at the blue side emission. Clearly, no ultrafast decay components ($<1$ ps) were observed. At the red side ($\geq 335$ nm), we also observed two initial rise components in $\sim 0.5$ and 5 ps.

### 3.3.3 Solvation Correlation Functions

The initial decay components at the blue side and rise components at the red side of wavelength-gated fluorescence transients are signatures of solvent relaxation.
The overall femtosecond-resolved emission spectrum (FRES) was constructed using the methods detailed in chapter 2, and these results are shown in Figure 3.6 at several typical delay times. Similarly, lifetime-associated FRES was also constructed (not shown). The overall emission maxima $\nu_s$ (Stokes shifts) and lifetime-associated emission maxima $\nu_l$ were extracted by fitting each FRES to a lognormal function and are shown in Figure 3.7. Clearly, the solvation-complete time $\tau_{sc}$ and the corresponding emission maximum $\nu_{sc}$ are noticeably different from $\tau_{ss}$ and $\nu_{ss}$. For example, in melittin random coil monomer the solvent relaxation completes in 140 ps while FRES takes 950 ps to reach its steady-state emission, and the emission shift is 289 cm$^{-1}$ with the addtional 810 ps ($\nu_{sc} - \nu_{ss}$). All derived emission maxima and times for all peptide samples used in this study are summarized in Table 3.1.

**Table 3.1: Emission maxima and time constants from constructuion of time-resolved emission**

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_0$</th>
<th>$\lambda_{sc}$</th>
<th>$\tau_{sc}$</th>
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<th>$\tau_{ss}$</th>
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<tr>
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<td>350.6</td>
<td>840</td>
<td>332.70</td>
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<td>melittin random coil</td>
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<td>348.5</td>
<td>950</td>
<td>331.32</td>
<td>349.34</td>
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<tr>
<td>$\alpha$-helix</td>
<td>322.55</td>
<td>339.69</td>
<td>775</td>
<td>341.0</td>
<td>1070</td>
<td>328.04</td>
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<td>332.90</td>
<td>600</td>
<td>333.5</td>
<td>950</td>
<td>326.80</td>
<td>334.47</td>
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$^a$ All emission maxima ($\lambda$) and time constants ($\tau$) are the units of nanometers and picoseconds, respectively.

We carefully considered the contributions of vibrational relaxation to the obtained total Stokes shifts by examining FRES bandwidth change with time. Vibrational relaxation typically narrows the bandwidth of emission spectra. We noticed that the bandwidth of lifetime-associated FRES of tryptophan also changes with time, e.g., a
Figure 3.6: Representation of normalized overall FRES at several delay times, constructed from femtosecond-resolved fluorescence transients (Figure 3.2-3.5) for tripeptide KWK, melittin random coil, membrane-bound α-helix and tetramer. The dashed curves are the corresponding steady-state fluorescence emission spectra.
Figure 3.7: Femtosecond-resolved emission maxima of the overall emission spectra ($\nu_s$) and the lifetime-associated emission spectra ($\nu_l$) for all peptide conformations studied. The inset shows the entire evolution of $\nu_s$ and $\nu_l$ to reach the steady-state emission ($\nu_{ss}$); see text.
total decrease of 200 cm\(^{-1}\) was observed within 600 ps for melittin tetramer. However, we observed initial bandwidth broadening (not narrowing) of the total Stokes shift \((\nu_s)\) within several picoseconds in \(\sim100\) cm\(^{-1}\) for tetramer, \(\sim300\) cm\(^{-1}\) for \(\alpha\)-helix, and \(\sim1500\) cm\(^{-1}\) for random coil monomer and tripeptide KWK. These increases are likely due to the initial wave packet dynamics and the data analysis procedures. Although W19 experiences different water environments in three melittin conformations, the vibrational relaxation seems ultrafast within \(\sim1\) ps, similar to the vibrational relaxation dynamics of tryptophan in bulk water [33]. This conclusion is also consistent with our obtained emission maxima of \(\sim320\) nm at \(t = 0\). The emission maxima of tryptophan in proteins at the temperature of 2 K with 300-nm excitation are all \(\sim320\) nm [96]. In addition, our excitation of tryptophan at 290 nm is at the red edge of its absorption. Thus, the vibrational relaxation in our cases is ultrafast and probably minor, and the observed total Stokes shifts dominantly result from the local solvation dynamics.

The solvation correlation functions were constructed using the procedure from chapter 2, and results are shown in Figure 3.8 and summarized in Table 3.2. Clearly, solvation dynamics is drastically different for melittin of different conformational states. Specifically, for the tripeptide, the solvent response function can be described by triple exponential decays: 0.58 ps with 66% of total amplitude, 3.1 ps (27%), and 23 ps (7%). Free tryptophan in the same buffer gives a relaxation distribution of 0.52 ps (68%), 1.9 ps (23%), and 7.6 ps (9%). The first ultrafast component represents bulklike water motion. The slow solvation (3.1 and 23 ps) observed in tripeptide KWK probably results from some rigid water molecules sticking to the positively
Figure 3.8: Hydration correlation functions probed by tryptophan in tripeptide KWK and in three different conformations of melittin. The inset shows the correlation functions in the short time range. The solvation correlation function obtained for aqueous tryptophan is also shown for comparison.

charged lysine in close proximity of tryptophan.

Table 3.2: Results obtained for hydration correlation function $c(t)$ in different conformations$^a$

<table>
<thead>
<tr>
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<th>$\tau_1$</th>
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<td>0.27</td>
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<td></td>
<td>0.47</td>
<td>0.53</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ All hydration correlation functions were best fit with $c(t) = c_1 e^{-(t/\tau_1)^\beta} + c_2 e^{-t/\tau_2} + c_3 e^{-t/\tau_3}$ where $\sum_{i=1}^{3} c_i = 1$. The time constants are in units of picoseconds.
In melittin random coil monomer, the solvation dynamics of W19 can be described with double exponential decays: 0.62 ps (68%) and 14.7 ps (32%). The overall relaxation dynamics becomes slower than that of tripeptide KWK. In membrane-bound α-helical melittin, the relaxation process becomes drastically slower: 1.25 ps (35%), 14 ps (27%), and 114 ps (38%). Triple-exponential relaxation dynamics has been proposed as a result of three different types of water layers near membrane surfaces [37, 97, 98]. When the four melittin monomers aggregate to form a tetramer at high-salt concentration, the solvation dynamics also become much slower: 3 ps (47%) and 87 ps (53%). Because tryptophan solvation in high-salt solution only takes about 10-20 ps (Lu W and Zhong D, unpublished data), the 87-ps solvation time observed here must be related to the local conformation in melittin tetramer.

3.3.4 Hydration Dynamics and Conformation Transitions

(a) Hydration Dynamics and Protein Solvation. The constructed solvation correlation function is the response of the local environment around tryptophan to the sudden change of its static dipole moments from the ground state to the excited state. The response thus in principle could result from both neighboring water molecules and protein residues (and backbone) in close proximity besides a negligible contribution of ions in solution [37, 99]. Although the motions of water molecules and their associated protein residues cannot be completely decoupled, certain rotational and translational motions of small water molecules may not give rise to significant local
motions of the associated residues which are bulky and restricted by peptide structures.

For all conformations studied here, we examined the local rigidity by measuring tryptophan anisotropy dynamics. These results are shown in Figure 3.9, and all initial decay in ~100-fs is due to internal conversion between $^1L_a$ and $^1L_b$ states [33,37,100]. Interestingly, we did not observe any local wobbling motion of tryptophan in tripeptide KWK and membrane-bound melittin α-helix. The time scale of 127 ps in tripeptide KWK is the tumbling motion of the entire peptide in solution, indicating a rigid local structure probably caused by cation-π interactions between tryptophan and the lysine(s) [101–103]. The anisotropy decay of membrane-bound melittin α-helix is nearly constant, suggesting the tryptophan is confined in a highly restricted environment in proximity to membrane headgroups; see Figure 3.1. For melittin random coil monomer and tetramer, we observed a local wobbling motion of tryptophan but at least three times slower than the corresponding solvation dynamics. Thus, the local side-chain motion, if any, takes a much longer time than the observed solvation dynamics. Also, the fluorescence emission maxima of tryptophan in all different conformations are well correlated with its hydration environments. As shown in Table 1, the emission peak of tryptophan shifts from ~350 to 333 nm for a full water exposure in tripeptide KWK and random-coil monomer to partial water exposure in melittin α-helix and tetramer, respectively. Thus, the observed solvation dynamics predominantly describes the hydration dynamics of local water molecules around tryptophan in the peptides.
Figure 3.9: Time-resolved fluorescence anisotropy of tryptophan in tripeptide KWK and in the different conformations of melittin. The initial ~100-fs decay results from the ultrafast internal conversion between $^1L_a$ and $^1L_b$. Note that all observed rotational time constants are at least three times longer than the corresponding solvation dynamics.
(b) Tryptophan Sensitivity and Local Hydration. The spatial resolution in hydration studies using tryptophan as a molecular probe is determined by dipole-dipole interactions that follow a $R^{-3}$ distance dependence. Clearly, the definition of hydration layer is sensitive to spatial and temporal resolutions intrinsic to the probing method. Care must be taken to provide the dynamical time scales without convolution of the intrinsic experimental factors [12]. Zewail et al. determined the distance of the hydration layer to be $\sim 7$ Å [32, 91, 92], consistent with the value determined by the scattering methods [50]. Friedrich et al. [104] suggested a distance of 4.5 Å for the interaction range of a molecular probe with its environment using a hole-burning method. The dipole-dipole interaction at $\sim 3.5$ Å of the first solvent shell to the 7-10 Å solvent molecules drops to 12.5-4.3%. Therefore, tryptophan is able to probe water in a distance up to $\sim 7$-10 Å (about two to three layers of water molecules), which is also confirmed by recent theoretical calculations [105]. Figure 3.10 gives a schematic of local-ordered water network/clusters in the melittin $\alpha$-helix and tetramer and the probing sensitivity of tryptophan.

(c) Solvent Relaxation and Local Polarization. The fluorescence emission maximum of tryptophan is often used to indicate its location in proteins. The steady-state Stokes shift, determined by the total local polarization, is an integration of the entire solvent relaxation and does not reflect the time scales of hydration. The hydration dynamics is the rate of hydrogen-bond breaking/making and does not necessarily reflect the magnitude of the Stokes shift. For example, we observed a similar long solvation dynamics ($\sim 100$ ps) of tryptophan in tetramer and in the membrane-bound $\alpha$-helix, but the fluorescence emission maximum is 334 nm for the former and 341
Figure 3.10: Schematic representation of the local-ordered water network/cluster in the melittin α-helix (upper) and melittin tetramer (lower). The probe sensitivity is indicated by the dashed circle in diameter of ~7-10 Å. The blue color code represents different layers of water structures.
nm for the latter. Conversely, we observed a similar emission peak (~350 nm) for aqueous tryptophan and W19 in the melittin random coil monomer, but the solvation dynamics occur in 1.6 and 14 ps, respectively (Figure 3.8). Thus, in addition to qualitatively deducing local environment hydrophobicity with tryptophan emission maximum, the study of hydration dynamics is critical; it not only provides the time scales of actual water motions but also elucidates the local ordering (structure) and rigidity of hydrogen-bonded water network/clusters. Revealing these interactions at the local molecular level is central to the understanding of protein stability and flexibility, as well as conformational transitions.

**(d) Biological Water Motions and Peptide Conformational Transitions.** Melittin in water can be regarded as a denatured protein with all constituting residues projecting into aqueous environment (Figure 3.1). W19 is fully exposed to water and probes the hydration dynamics occurring at the local structure of a flexible random coil. The observed 620-fs component represents the ultrafast relaxation of bulklike water molecules including inertial/librational solvent motions while the 14.7-ps dynamics is from the relaxation of local-ordered biological water. This longer water relaxation is similar to the reported hydration dynamics on protein surfaces [12,32,91,92]. Thus, the observed time scale of 10-20 ps reflects the fluctuating nature of the ordered water on the surface of denatured melittin.

Melittin readily binds to membranes and performs a toxic function of membrane lysis. The first twenty mainly hydrophobic residues strongly interact with lipids. The last six hydrophilic residues protrude into interfacial water layers. Melittin was also
observed to form an α-helical structure in alcohol solvent [69–71]. It is believed that the aliphatic chain of alcohol reduces the hydrogen bonding between amide protons and surrounding solvent molecules, promotes the intramolecular hydrogen bonding in melittin, and stabilizes the α-helical structure [79]. Thus, the mobile water molecules as observed in the random coil disfavor the α-helix formation in solution. In the membrane-water interface, water is much more rigid and ordered. The immobilized water promotes the secondary α-helix formation at least in the C-terminus. We observed three distinct time scales of 114, 15, and 1.25 ps, representing three different types of water structures near the membrane interface: well-ordered, quasi-free, and bulklike [37]. The long component of 114-ps in solvation dynamics represents the slow interfacial water motion, critical for the stabilization of the high order structures of the α-helix and lipid.

The X-ray crystallographic structure of melittin in membrane has not yet been resolved, but extensive studies have shown that the membrane-bound melittin adopts an amphipathic α-helix [62–65]. Whether the peptide would lie down at the membrane surface or insert into the membrane highly depends on many factors such as temperature, surface curvature, and chemical identity of headgroups. For a neutral headgroup lipid and at ambient temperature, it is believed that melittin partially inserts into the membrane, as also confirmed by recent molecular dynamics (MD) simulations [82]. Compared with the solvation dynamics obtained from free tryptophan in the lipidic cubic phase water channel (108, 9.2, and 0.56 ps), the tryptophan
in the α-helix probes less bulklike water, and thus it is closer to the membrane, consistent with the fluorescence emission peaks shifting from 344 nm for free tryptophan in the water channel to 341 nm for the membrane-bound α-helix.

The emission peak at 341 nm is similar to that of typical surface tryptophan emission of proteins and also rules out the possibility of a buried W19 inside the hydrophobic core underneath polar headgroups. Our anisotropic studies (Figure 3.9) showed a highly restricted local motion, indicating that W19 locates at the interface around headgroups and well-ordered water molecules. Also, tryptophan side chains in most membrane proteins have been observed to reside preferentially at membrane interfaces most likely due to their flat rigid shape and π-electronic structure (aromaticity) [106–108]. All these facts support that the α-helix penetrates into the membrane until W19 is spatially hindered by the interfacial polar headgroups (Figure 3.1). We are also aware of the fact that W19 and ordered water could penetrate into the membrane at certain lipidic conditions (Figure 3.10).

The observed hydration dynamics here is significant and reflects local water ordering (structure) and its motion in the membrane nanochannel of 50 Å in diameter. The ordered water near the channel wall fluctuates in ~100 ps, significantly longer than water relaxation in bulk and at protein surfaces. Thus, the time scale of ~100 ps is necessary to keep the global structural stability of lipid bilayers as well as the protein structural integrity of the α-helical content in membrane-bound melittin.

At high ionic strength, ions are solvated by water molecules and such processes reduce the availability of water molecules interacting with melittin, enhancing α-helix
formation. Due to the unique sequence and structural integrity, a large hydrophobic surface is formed in the α-helical monomer (and dimer), driving tetramerization with a large hydrophobic core in the center of the tetramer. Four highly charged residues at the C-terminus form two crossing joints (Figure 3.1), and each of these two regions has eight positively charged residues. Besides the salt dielectric screening of the interchain electrostatic repulsion, water molecules solvate these charged residues and associated ions and form a highly ordered structure around two assembled regions, therefore stabilizing the local tetramer structures. The longer water relaxation observed here takes 87 ps, significantly longer than the surface hydration dynamics of most proteins which have been studied so far [30, 109]. This time scale of ~87 ps, similar to ordered-water motion observed in membrane interfaces, again highlights the importance of appropriate water mobility for the self-assembly of both lipid bilayers and high-order structures of melittin.

### 3.4 Conclusions

In this chapter, we reported our systematic studies of hydration dynamics in different conformations of melittin that include a random coil, a α-helix and a self-assembled tetramer using intrinsic tryptophan as a molecular probe. With femtosecond resolution, we observed the solvation dynamics with two time scales of 0.62 ps (68%) and 14.7 ps (32%) in a random coil monomer. The former ultrafast component mainly represents inertial/librational solvation dynamics of bulklike water molecules, and the slower one reflects surface-type hydration dynamics of proteins, indicating that a hydrogen-bonded water network/cluster was formed. As a comparison, we
also studied tripeptide KWK, and bulklike water solvation was observed. In lipidic aqueous solution, melittin folds into an $\alpha$-helical structure at the membrane-water interfaces, and we observed hydration dynamics with three distinct time scales: 1.25 ps (35%), 13.9 ps (27%), and 114 ps (38%), implying three different layers of water structures in the water nanochannel (50 Å in diameter). The slowest dynamics represents the interfacial water motion of a well-ordered water structure along the membrane surface.

In high-salt aqueous solution, salt dielectric screening and ionic solvation promote the hydrophobic core collapse in melittin aggregation and facilitate the tetramer formation. This tertiary structure is further stabilized by the strong hydrophilic interactions of charged residues and associated ions with hydrogen-bonded water molecules in the two assembled regions. The hydration dynamics was observed to occur in 3 ps (47%) and 87 ps (53%), significantly slower than water relaxation at protein surfaces but similar to water motion at membrane interfaces. Thus, the observed time scale of $\sim$100 ps represents a type of necessarily dynamical ordering of water molecules to mediate high-order structure formation of melittin in the $\alpha$-helix and self-assembled tetramer. These studies elucidate a critical role of a dynamically ordered water network/cluster on the picosecond time scales in promoting peptide conformational transitions as well as maintaining protein structural stability, flexibility, and integrity.
CHAPTER 4

ULTRAFAST SOLVATION DYNAMICS OF HUMAN SERUM ALBUMIN: CORRELATIONS WITH CONFORMATIONAL TRANSITIONS AND SITE-SELECTION RECOGNITION

4.1 Introduction

Biomolecular recognition is mediated by water motions, and the dynamics of associated water directly determines local structural fluctuation of interacting partners [7, 9, 12, 31, 48]. In the proceeding chapter, we studied the local hydration dynamics in different conformations of melittin. This chapter continues to explore the study of local water motions site-specifically in various environments, from a buried crevice to the solvent exposed surface induced by the protein conformation changes using tryptophan as the intrinsic optical probe. The correlation between hydration dynamics and protein conformational transitions will be drawn and related to the biological functions of protein human serum albumin.

The structure and hydration of human serum albumin (HSA) are important for the transport of fatty acids and for binding a great variety of metabolites, drugs, and organic compounds in the circulatory system [110, 111]. It contains a single
polypeptide chain of 585 amino acids. Under physiological conditions (pH 7), HSA adopts a heart-shaped three-dimensional structure with three homologous domains I-III (Figure 4.1); each domain consists of two subdomains A and B with 4 and 6 α-helices, respectively [112–114]. The X-ray structure shows the two halves of the albumin molecule form a 10-Å-wide, 12-Å-deep crevice that is filled with water molecules [112–114]. The single tryptophan residue (W214) in the protein is located at the binding site IIA at the bottom of the crevice (Figure 4.1).

With the change in pH, HSA undergoes reversible conformational isomerization, and pH-dependent isomers were first demonstrated by Luetscher [115] in 1939 and later systematically classified by Förster (15) in 1960. At neutral pH, the conformation of HSA is in its common physiological state, referred to as the normal form (N);
an abrupt transition occurs at a pH value of less than 4.3, changing the N form to
the so-called fast migrating form (F), and when the pH is less than 2.7, a further
transition takes place from the F form to the extended form (E). When the pH is
above 8, the N conformation changes to the basic form (B), and at a pH above 10
the structure transforms to the aged form (A).

The protein has been extensively studied in almost every aspect due to its physio-
logical importance and its potential as a drug delivery vehicle [110,111], and because
different acidic and basic isomers may have certain biological functions [116, 117].
Most studies have concentrated on its binding interactions with various ligands and
revealed that HSA is an assembly of squirmy and resilient components that frequently
change in conformation through opening and closing of major crevices. With this
“breathing” motion, HSA assimilates and releases a variety of substances during
transportation in the circulatory system. Many complex structures have also been
resolved at high X-ray resolution [112, 118, 119]. The flexibility to adopt different
conformations with independent segmental movements has also been shown through
certain independent and sequential folding/unfolding studies of individual subdo-
 mains [120,121]. Both tryptophan W214 and extrinsic dye molecules have been used
as optical probes to study the conformational dynamics through changes in fluo-
rescence lifetime, resonance energy transfer, and time-resolved anisotropy [122–126].
The significant fluorescence lifetime variations of W214 in a series of mutations in
the binding site IIA clearly demonstrated the local flexibility in human serum albu-
min [127].
4.2 Materials and Methods

HSA (essentially free of fatty acids and globulin) was obtained from Sigma-Aldrich in lyophilized powder and used without further purification. All of the other chemical reagents were purchased from Fisher Scientific. Protein stock solutions of 0.5 mM HSA were prepared by dissolving the lyophilized powder in corresponding buffers to make various isomers (N form, 10 mM sodium phosphate, pH 7.0; F form, 10 mM sodium acetate, pH 4.1; E form, 10 mM sodium acetate/HCl, pH 2; B form, 10 mM Borax, pH 9.0; and A form, 10 mM Na₂HPO₄/NaOH, pH 11.0). For all femtosecond-resolved measurements, protein solutions were used without further dilution while sample solutions were diluted to 10 µM for the measurement of steady-state fluorescence spectra. Protein stock solution at pH 11.0 was incubated at 4 °C for more than 24 hours before any spectroscopic measurements. All time-resolved measurements were carried out using the femtosecond up-conversion method as described in chapter 2.

4.3 Results and Discussions

4.3.1 Steady-State Fluorescence Characterization

Steady-state fluorescence spectra were measured using a SPEX FluoroMax-3 spectrometer. All of the protein samples were excited at 295 nm to ensure the fluorescence emission is dominantly from the single W214. At neutral pH, the emission spectrum of HSA has an emission maximum at 338 nm, similar to the surface tryptophan emission [32, 34, 91, 92] which indicates a highly polar environment with a large amount
of trapped water around W214 in the crevice, as also confirmed by the crystallographic structure [114] (Figure 4.1). The emission peak is blueshifted to 332.6 nm at pH 4.1, suggesting a less polar microenvironment surrounding W214 when the protein undergoes N→F conformational transition. At pH 2.0, though the protein structure expands to its maximum extent allowed by the internal disulfide linkages, the rearrangement of subdomain structures leads to a further blueshift in W214 emission maximum to 330 nm, indicating a more hydrophobic environment around W214 when protein conformation changes from F to E. In the slightly alkaline pH condition (pH 9.0), HSA exhibits an emission spectrum with a peak at 336 nm, indicating a slight change of local polarity around W214 upon N→B transition. At pH 11.0, no significant further shift of emission maximum was observed for the A form while the spectrum width becomes slightly narrower compared with the emission from the B form, suggesting similar local polarity.

4.3.2 Femtosecond-Resolved Fluorescence Dynamics

A series of femtosecond-resolved fluorescence up-conversion transients of HSA for different isomers are shown in Figures 4.2-4.6. More than eight transients of different emission wavelengths were collected for each HSA isomer. For each conformation, all fluorescence transients were analyzed with a global fitting strategy. Typically, four discrete exponential functions were used, two of which are the lifetime emission decays. The two fluorescence lifetimes varied depending on pH value but were fixed for all transients of a given isomer.
The fluorescence lifetimes were determined carefully because they would affect the time scale of slow solvation dynamics. A series of transients at the red side of wavelengths, from 360 to 380 nm, was obtained, and the two lifetimes, with different ratios, are well fitted for these red-side transients. The longer lifetime is determined by a conventional photon-counting measurement (from the literature), and the shorter one was obtained by fitting our 3-ns-long transients. Finally, these two lifetimes were used to fit all other transients, and all parameters from the fits are self-consistent. The longer lifetime of the N form is 6.1 ns [122], and the shorter one from our 3-ns transients was determined to be 1.4 ns. The red-side transients of the F form are nearly the same as those from the N form; thus the F form has the same two lifetimes. By comparison of red-side transients of other isomers, we determined the lifetimes of 912 ps and 4.6 ns for the E form, 1.15 and 6.1 ns for the B form, and 318 ps and 3.8 ns for the A form. However, it should be pointed out that because the time interval between two consecutive pump excitations is 2 ms and each transient was averaged over more than 1 hour, for each isomer all fluctuating configurations would be sampled over during the data acquisition. Thus, the two apparent lifetimes do not mean the presence of only two static configurations but rather mainly represent two types of temporal distributions; the system is in dynamic heterogeneity.

a. N Form. Figure 4.2 shows the femtosecond-resolved fluorescence transients of W214 at several typical wavelengths. The overall decay dynamics is significantly slower than that of aqueous tryptophan in a similar buffer solution [37, 93]. Clearly, the ultrafast decay components (<1 ps) observed in tryptophan solution were not observed at the blue side for the protein. The two exponential decays of solvation
have time constants of 4.4-9.2 ps and 104-125 ps for all the blue-side transients. At
the red side, we also observed a decay component of 51 ps with \( \sim 10\% \) of the total
amplitude, which results from the excited-state quenching of W214 by the neighboring
residue(s), as discussed below.

\textit{b. F Form.} Figure 4.3 shows the representative femtosecond-resolved fluorescence
transients in the F conformation at pH 4.1. At the blue side, the solvation dynamics
are represented by two decay components of 4.1-10.7 ps and 125-208 ps, respectively.
Similarly, we did not observe ultrafast components (\(<1\) ps) at the blue side. The
second solvation component slows down, and its percentage increases after the N→F
transition. We also observed a quenching component of 100 ps with an amplitude of
about 10\% in all the red-side transients.

c. \textit{E Form.} The femtosecond-resolved transients of the E form at pH 2 are shown
in Figure 4.4. At the blue side, the first solvation component slows down, and the
dynamics occur in 6.7-16.2 ps. The second component becomes relatively shorter
(72-120 ps) from the F→E transition. These changes certainly reflect different local
environments. The quenching contribution at the red side is reduced, and a decay
component of 46 ps with \( \sim 5\% \) amplitude was observed.

d. \textit{B Form.} Figure 4.5 shows a series of selected femtosecond-resolved fluorescence
transients of B form HSA at pH 9.0. At the blue side, two solvation components were
observed to occur in 1.1-3.1 ps and 32-45 ps, respectively. This observation is striking,
and the solvation dynamics is drastically different from those observed in the N, F,
Figure 4.2: Normalized, femtosecond-resolved fluorescence transients of the N isomer in the short (left) and long (right) time ranges, at a series of gated fluorescence emissions. Note that solvation dynamics is significantly slower than that of tryptophan in a similar buffer.
Figure 4.3: Normalized, femtosecond-resolved fluorescence transients of the F conformation in short (left) and long (right) time ranges, at a series of gated fluorescence emissions.
**Figure 4.4:** Normalized, femtosecond-resolved fluorescence transients of the E form in short (left) and long (right) time ranges, at a series of gated fluorescence emissions.
and E isomers. Both solvation time scales are significantly faster, indicating a large conformational change. At the red side, we observed a faster quenching component of 35 ps with an increasing amplitude of 10-20%.

e. A Form. Figure 4.6 shows representative femtosecond-resolved fluorescence transients in the A form under the extreme pH value of 11.0. Under such conditions, the conformation significantly changes, and at the red side, a considerable quenching component with a time constant of 29 ps and an amplitude of 37% was observed. At the blue side, solvation dynamics occurs in 1.1-3.5 ps and 15-28 ps, similar to the time scales observed in the B form, but with different amplitude percentages.

4.3.3 Solvation Correlation Functions

Following the equations we described in chapter 2, we constructed the overall and lifetime-associated femtosecond-resolved emission spectra (FRES) for all conformational isomers. Figure 4.7 shows overall femtosecond-resolved emission maxima $\nu_s$ and lifetime-associated emission maxima $\nu_l$, determined by fitting FRES to a log-normal function. Table 4.1 summarizes the derived emission maxima and times of different conformational isomers. Clearly, the complete solvation time $\nu_{sc}$ and the corresponding overall emission maximum $\tau_{sc}$ are quite different from $\nu_{ss}$ and $\tau_{ss}$, respectively. The solvation correlation functions were constructed, and the derived results are shown in Figure 4.8 and summarized in Table 4.2.
Figure 4.5: Normalized, femtosecond-resolved fluorescence transients of the B isomer in short (left) and long (right) time ranges, at a series of gated fluorescence emissions. Note that solvation dynamics becomes much faster than those reported in Figures 4.2-4.4 and also the quenching component at the red side becomes faster.
Figure 4.6: Normalized, femtosecond-resolved fluorescence transients of the A isomer in short (left) and long (right) time ranges, with a series of gated fluorescence emissions. Note the large, fast quenching component at the red side of the fluorescence emission.
Figure 4.7: Femtosecond-resolved emission maxima of the overall emission spectra ($\nu_s$) and the lifetime-associated emission spectra ($\nu_l$) for four typical conformational isomers. The insert shows the entire evolution of $\nu_s$ and $\nu_l$ to reach the steady-state emission ($\nu_{ss}$).

We carefully considered the contribution of vibration relaxation to the obtained total Stokes shifts by examining the change of FRES bandwidth with time. We observed an initial bandwidth broadening, not narrowing, of FRES, and such an increase in bandwidth is probably due to the initial wave packet dynamics. Thus, no noticeable contributions of vibration relaxation were observed, consistent with the fact that the excitation wavelength of 295-nm in this experiment is at the red edge of tryptophan absorption. We also estimated whether the residual quenching observed in the red-side transients affects the overall solvation correlation function and constructed the overall and lifetime-associated FRES for all conformational isomers without taking into account the quenching contribution in all the red-side transients. No noticeable differences in the correlation functions were observed except for the A form isomer.
### Table 4.1: Emission maxima and time constants of W214 from constructed time-resolved spectra

<table>
<thead>
<tr>
<th>isomer</th>
<th>$\lambda_0$</th>
<th>$\lambda_{sc}$</th>
<th>$\tau_{sc}$</th>
<th>$\lambda_{ss}$</th>
<th>$\tau_{ss}$</th>
<th>$\lambda_1$</th>
<th>$\lambda_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>321.88</td>
<td>334.33</td>
<td>1000</td>
<td>338.0</td>
<td>3000</td>
<td>326.90</td>
<td>340.49</td>
</tr>
<tr>
<td>F</td>
<td>321.36</td>
<td>331.70</td>
<td>1200</td>
<td>332.6</td>
<td>3500</td>
<td>326.83</td>
<td>333.03</td>
</tr>
<tr>
<td>E</td>
<td>320.30</td>
<td>328.40</td>
<td>700</td>
<td>330.0</td>
<td>1600</td>
<td>323.61</td>
<td>332.53</td>
</tr>
<tr>
<td>B</td>
<td>323.08</td>
<td>334.03</td>
<td>280</td>
<td>336.0</td>
<td>3000</td>
<td>327.02</td>
<td>336.71</td>
</tr>
<tr>
<td>A</td>
<td>327.43</td>
<td>334.05</td>
<td>150</td>
<td>336.0</td>
<td>1500</td>
<td>330.01</td>
<td>336.32</td>
</tr>
</tbody>
</table>

*a* All emission maxima ($\lambda$) and time constants ($\tau$) are in units of nanometers and picoseconds, respectively. *b* The construction of the emission spectrum at time zero ($\lambda_0$) includes the quenching component.

For simplicity, we constructed the correlation function for the A conformation by including all the quenching contributions.

As shown in Figure 4.8, conformational isomers exhibit very different solvation dynamics around W214. In the N form, the solvation correlation function is best represented by two exponential decays with the time constants of 5.0 ps with a 39% of the total amplitude and 133 ps (61%). The solvation times in the F form change to 4.3 ps (30%) and 186 ps (70%), respectively. The second long solvation dynamics slows down by about 40%. In the extended E form, the obtained solvation dynamics occurs in 6.7 ps (39%) and 108 ps (61%); the former is the longest time scale in the initial solvation, and the latter is the shortest in the second relaxation among these three (N, F, and E) conformers. For the B form isomer, the solvation dynamics have two time constants of 1.6 ps (30%) and 46 ps (70%), faster than those in the N form by a factor of 3. For the aged form A, the solvation correlation function has two exponential decay components of 2.3 ps (76%) and 27 ps (24%); the percentages are different from those obtained for the B form, and the overall solvation dynamics
Figure 4.8: Solvation correlation functions probed by tryptophan (W214) in the five conformational isomers studied. The inset shows the correlation function in the short-time range. Note the large difference upon changing from the acidic to basic condition. The solvation correlation function of the A form was constructed with the inclusion of the quenching contribution; see text.

becomes much faster.

4.3.4 Femtosecond-Resolved Anisotropy Dynamics

We also studied the femtosecond-resolved rotational dynamics by monitoring tryptophan fluorescence anisotropy changes with time. Figure 4.9 shows the anisotropy dynamics for the different isomers. All results are well fit by three exponential decays, and the total anisotropy is well expressed by a sum of three parts: \( r(t) = r_I + r_W + r_T \).
The initial ultrafast decay \((r_I)\) is about 100 fs, which is due to the internal conversion between the two electronically excited states of \(^1L_a\) and \(^1L_b\), as observed before \([33, 100, 128]\). The anisotropy for all transients initially drops to a value of \(\sim 0.15\) in such a short time, and this apparent anisotropy \((r_{app} \approx 0.15)\) is consistent with the early reported value at excitation of 295 nm \([129]\). The second component \((r_W)\) represents the local wobbling motion of W214, which reveals the local packing and backbone rigidity. The wobbling semiangle \((\theta)\) was estimated using the model of an axially symmetric oscillation about a fixed axis \([34]\):

\[
1 - \frac{r_w}{r_{app}} = \left[\frac{3\cos^2\theta - 1}{2}\right]^2
\]  

(4.1)

The resulting semiangles are given in Figure 4.9. It is striking that the normal form \((N)\) has the largest value of \(r_W\) and thus has the biggest wobbling semiangle of 24°, almost double the semiangles derived in E, F, and B conformational isomers. The last term \(r_T\) is the global tumbling motion of the entire protein, and the time scale was estimated to be about \(\sim 22\) ns \([125]\).

4.3.5 Two Distinct Time Scales and Two Types of Dynamic Motions

For all conformational isomers, we observed two distinct time scales for solvation dynamics, and these results are robust as they were also observed in other systems \([32, 34, 37, 91–93]\). The constructed solvation correlation function is the response of the local environment around tryptophan to the sudden charge rearrangement from the ground state to the excited state. In principle, the response could result from both surrounding water molecules and neighboring protein polar/charged residues.
Usually the motions of water molecules and their associated protein residues cannot be completely decoupled. However, the motion of protein residues highly depends on the local protein packing and structural rigidity. On the other hand, water molecules are much more mobile and easy to proceed to various motions under energy fluctuations. For tryptophan residues located at protein surfaces, it has been shown that the obtained correlation function is dominant from the local water cluster/network response [32,34,91,92]. For tryptophan partially exposed to protein surfaces or buried inside proteins, the response function could be from a combined contribution of water molecules and protein residues, depending on the local protein rigidity. However, it is not conceivable that the response function is solely from protein residues because water always accompanies residue fluctuations, especially at protein surfaces. Contrary to recent molecular dynamics (MD) simulations [26,130,131], we did not observe any ultrafast component (<1 ps) for all isomers. Given that the femtosecond up-conversion apparatus in this laboratory is able to resolve 200-fs solvation dynamics in aqueous tryptophan [35,37,93], this lack of sub-picosecond solvation dynamics likely indicates that the force field used in the MD simulations needs to be revisited.

The observed initial solvation dynamics, occurring in several picoseconds, represents the local librational/rotational motions of surrounding water molecules and possibly the associated (polar/charged) residues. This localized motion of water molecules has a similar time scale to the short residence time obtained in MD simulations for the equilibrium ground state. The MD simulations of residence time also gave an additional time scale in tens of picoseconds [26,131,132], reflecting long-time dynamic exchange and equilibration in the ground state. When tryptophan is excited
to the $^1L_a$ state, the local equilibrium is shifted, and the system is in a nonequilibrium state. The observed long-time solvation dynamics reflects the temporal evolution from the initial nonequilibrium configuration to the new equilibrium state around tryptophan in the excited state. During this process, dynamic exchange with bulk water always exists. The time scale to reach the new equilibrium is related to the residence time in the equilibrated excited state, which is probably similar to that found in MD simulations for the equilibrated ground state. Since the excited-state dipole ($\Delta \mu \approx 5 \text{ D}$) is larger than that of the ground state, the dynamic evolution will finally result in an increase of water molecules around tryptophan according to MD simulation results from this laboratory [27]. The shorter the residence time, the faster the system reaches a new equilibrium. Thus, the long-time component results from an integrated process with the system evolving from the nonequilibrium configuration to the new excited-state equilibrium, convoluted with dynamical exchange of the hydration water with bulk water. This slow dynamics, in fact, reflects a long-range translational motion.

### 4.3.6 Dynamics Quenching and Local Configuration

For all conformational isomers, we observed quenching of tryptophan fluorescence by neighboring protein residues. For example, at the red-side emission of 360 nm, we observed quenching time constants of 29 ps with 37% in total amplitude in A form HSA, 35 ps (18%) in B form, 51 ps (10%) in N form, 100 ps (10%) in F form, and 46 ps (5%) in E form. From the basic to acidic conditions, the quenching amplitudes systematically decrease, reflecting more rigid local structures and/or increasing
separation distances between W214 and the potential quencher(s). The observed wobbling motions in these isomers (Figure 4.9) suggests the latter is likely the case. The quenching time constants from isomers A to E follow the trend of the derived two lifetimes; the faster the quenching, the shorter the other two lifetimes. Thus, the quenching component and multiple lifetime contributions indeed reflect a distribution of dynamic heterogeneous configurations of the W214 residue. The dynamic motion of W214 occurs on a time scale longer than several nanoseconds.

Bhagavan and co-workers [127] have recently conducted a systematic examination of potential quenching residues around W214 in the N form using site-directed mutagenesis and identified R218 as the main quenching residue (Figure 4.1). They also observed the mutation of R257, a residue at a distance of more than 11 Å away from W214, can eliminate the quenching by R218, indicating a local structural change induced by the mutation. The X-ray structure shows a distance of 3.81 Å between W214 and R218; both residues are in the same α-helix in domain IIA. At such a close distance it is possible that cation-π interactions between the two residues may result [101, 103], leading to electron-transfer quenching, as suggested for other systems [133]. The observed systematic decrease of quenching magnitude (percentage) from A to E caused by R218 suggests a gradual loosening of contact between W214 and R218 in the same α-helix and a systematic local configuration change in domain IIA.
Figure 4.9: Femtosecond-resolved fluorescence anisotropy of W214 of the five conformational isomers. The initial ~100-fs decay is due to the internal conversion between $^1L_a$ and $^1L_b$ states. The derived wobbling motion is also given in terms of the semiangle ($\theta$). The long component represents the protein tumbling motion; see text.
4.3.7 Hydration and Structural Flexibility of the N Conformation

At normal physiological conditions, the heart-shaped N form HSA adopts a globular structure with multiple binding sites [118, 119]. The common warfarin-binding site is located inside a 12-Å-deep crevice in domain IIA. W214 sits at the bottom of this water-filled binding pocket. Eight water molecules are shown in the X-ray structure within a distance of 7 Å (edge to edge) from the indole ring of W214, and three charged residues of K199, R218, and E450 are within 5 Å (Figure 4.1). Although W214 is deeply buried in the crevice, the highly polar environment, composed of trapped ordered water molecules and neighboring charged residues, leads to a considerable Stokes shift of W214 with its emission peak at 338 nm, similar to that of a surface-type tryptophan [134].

In this deep binding site, W214 has the largest wobbling motion among all conformational isomers with a semiangle of 24°. Because the N-form HSA has the most tertiary structure content and W214 is buried inside the protein, the observed result is striking and indicates a flexible local protein structure, consistent with the observation of dynamic quenching of W214 fluorescence by R218. More importantly, we observed solvation dynamics around W214 in 133 ps (Table 4.2). Typically, deeply trapped water molecules in cavities or crevices have dynamic motions or residence times in the range from sub-nanoseconds to nanoseconds, as observed in other systems [24,29,38]. As discussed above, this relatively long solvation component reflects the translational motion or residence time of the local hydration water in the binding
pocket. The observed result is significant because of the intrinsic presence of water structure, although charged residue solvation may contribute due to the similar time scale of the wobbling motion of the tryptophan residue (174 ps). Thus, the binding pocket is “soft” in that the “trapped” water molecules are in fast dynamic exchange. The initial solvation component of 5 ps is also consistent with the local librational/rotational motions of trapped water molecules.

Table 4.2: Results obtained from solvation correlation functions $c(t)$ of different human serum albumin isomers $^a$

<table>
<thead>
<tr>
<th>isomer</th>
<th>$\tau_1$</th>
<th>$\tau_2$</th>
<th>$c_1$</th>
<th>$c_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>5.0</td>
<td>133</td>
<td>0.39</td>
<td>0.61</td>
</tr>
<tr>
<td>F</td>
<td>4.3</td>
<td>186</td>
<td>0.30</td>
<td>0.70</td>
</tr>
<tr>
<td>E</td>
<td>6.7</td>
<td>108</td>
<td>0.39</td>
<td>0.61</td>
</tr>
<tr>
<td>B</td>
<td>1.6</td>
<td>46</td>
<td>0.30</td>
<td>0.70</td>
</tr>
<tr>
<td>A$^b$</td>
<td>2.3</td>
<td>27</td>
<td>0.76</td>
<td>0.24</td>
</tr>
</tbody>
</table>

$^a$ Solvation correlation functions were best fitted with $c(t) = c_1 e^{-t/\tau_1} + c_2 e^{-t/\tau_2}$ where $c_1 + c_2 = 1$. The time constants are in units of picoseconds. $^b$ The time scales were obtained by constructing the correlation function with inclusion of the fast quenching components.

Both the observed solvation and the anisotropy dynamics reveal a highly flexible binding pocket. To assimilate and release various molecules in the circulatory system, the flexible structure is necessary for HSA to accommodate these ligands with different shapes and sizes. This conclusion is consistent with results revealed by X-ray structural studies that show considerable structural changes after ligand binding [112–114]. The large plasticity for the carrier protein HSA ensures efficient transport of a variety of fatty acids, drugs, and small organic molecules in the blood through constant “breathing” motions to adopt binding recognition with fast-moving
4.3.8 Conformational Transitions among Isomers

The stability, structure, and function of proteins are dependent on environmental pH values [135]. HSA undergoes a series of reversible conformational changes from acidic to basic conditions. At the neutral pH of physiological conditions, the N isomer adopts a tertiary structure with all $\alpha$-helices (Figure 4.1). Previous extensive studies [111, 136, 137] have shown that in the pH region of the N→F transition (pH 5.0-3.5) the drastic structural change takes place primarily in domain III and domain II transforms to a molten-globule-like state, whereas domain I undergoes a structural rearrangement with minor changes in the secondary structure [111,136,137]. Overall, a reduction of helical structure occurs in HSA whereas the content of sheet structure increases. In the acid-expansion region (pH < 3.5), a further noticeable reduction in the helix content takes place together with an increase in $\beta$-sheet structure, especially in domain I, resulting in a loss of interdomain and inter-subdomian contacts and a disruption of the structure in the hinge and linker regions. The albumin molecule is fully extended in the E form as its disulfide bonding structure allows. In the alkaline pH range between pH 7.4 and 9.0, the N→B transition leads to a slight reduction in helical content and a small increase in $\beta$-sheet structure. The interdomains I and II experience a tertiary structural isomerization whereas domain III does not undergo structural alternation.
Under acidic conditions, the significant reduction of the tertiary structure and extension of the three interdomains disrupt the 12-Å crevice and destroy the local hydrophobic interactions between W214 and the residues from domain IIIA. Our observations of the fluorescence emission peak at about 330 nm and the more rigid local wobbling motions of about 13° in the F and E isomers strongly suggest that W214 is confined in a hydrophobic environment with partial solvent exposure due to the structural rearrangements. HSA is a highly charged protein with more than 180 charges at pH 7, for example, 36 aspartic acids, 62 glutamic acids, 59 lysines, and 24 arginines (Figure 4.10). The disruption of salt bridges or protonation of carboxyl groups (aspartic and glutamic acids) may lead to a significant charge distribution on the protein surface, resulting in a considerable water alignment in the hydration layer. The observed solvation dynamics in 100-200 ps is consistent with the highly ordered water molecules at lipid interfaces [37,93,97,138,139]. When the solvation dynamics between isomers E and F (Figure 4.8) are compared, the faster water exchange in E is probably due to the wide protein extension with a reduced charge density at the protein surface. The initial fast decay component of 4-7 ps comes from the librational/rotational motions of these water clusters/networks. As discussed above, no ultrafast water motion in less than 1 ps was observed.

The N→F conformational transition at a similar pH value has been found to be highly conserved among different species [111]. Such conservation has led to the belief that the F conformation occurs when the albumin molecule binds to the membrane surfaces of several tissues under physiological conditions at a lower pH value [116]. The structural extension could be a necessary step for the albumin molecule to bind
membrane surfaces to reach maximum contact area and then offload ligand(s) to the target. The observed time scale in 100-200 ps is ideal for ordered water motions to match the interfacial water dynamics on membrane surfaces, which was observed recently to occur also in hundreds of picoseconds [37,93,97].

Under basic pH conditions, the tertiary and secondary structures suffer slight fluctuations. Surprisingly, we observed significant changes of solvation dynamics, and the water motion becomes much faster, occurring in 27-46 ps. The initial local water dynamics occurs in 1.6-2.3 ps. Both the solvation dynamics and the emission peak (\( \sim 336 \) nm) reveal that W214 is located close to the protein surface, and thus the 10-Å crevice must open widely to allow more mobile water molecules into the IIA binding site. The similar emission peaks, but very different solvation dynamics, between N and B or A isomers result from the fact that the total Stokes shift is an integration of the local polarity while hydration dynamics reflect the rate of the local hydrogen bond making/breaking. For a similar polar environment, the trapped water in the crevice of the N form moves much slower than the mobile water at the protein surfaces in B and A isomers. The observed increase in quenching percentages reflects a more compact structure with a shorter separation between W214 and R218. Overall, these results indicate that in the B and A isomers the protein dynamically widens the crevice entrance and concurrently allows the globule structure to achieve more selective recognition [117].
4.4 Conclusions

In this chapter, we conducted systematic studies of solvation dynamics and local rigidity in a series of reversible conformations of human serum albumin. The studies were made for the protein under different pH conditions, using the intrinsic tryptophan residue (W214) as the local optical probe, and the dynamics were probed with femtosecond time resolution. We observed a robust bimodal distribution of solvation time scales. For all conformational isomers, the initial solvation dynamics occurs in several picoseconds, reflecting local librational/rotational motions. We did not observe subpicosecond solvation dynamics for the proteins studied here even though we have shown that the femtosecond up-conversion apparatus in this laboratory is capable of resolving 200 fs solvation dynamics in aqueous tryptophan [37,93]. These findings are at odds with results from most MD simulations, and we suggest that the force fields used in simulations allow for large flexibility of the motion.

The observed second and longer-time solvation dynamics occurs in the range of tens to hundreds of picoseconds, depending on the conformers, and reflects the exchange between the site-hydrated and bulk water. The Stokes shift is an integration of solvation processes while solvation dynamics measure the rate of local hydrogen bond rearrangements. This long time scale depends on the local water structure in the crevice, known to be eight in number, and local chemical identity. The observed time scale is consistent with long residence times observed in MD simulations. However, it is also true that polar/charged protein residues may contribute to the observed total solvation dynamics to certain extent depending on local rigidity. Considering the conformational and orientational correlations reported here, hydration by the smaller
water molecules is major, as water molecules are already present in the crevice and will contribute solely or in association with residues to solvation. The result reported here for the shortening of this longer-time solvation, when HSA opens up its structure (conformer of basic pH), is consistent with the exchange being a major channel between crevice and bulk water.

Under the normal physiological conditions of neutral pH, the single tryptophan W214 in the binding site II A is buried inside a 12-Å-deep crevice. Despite this closure, we observed fast solvation dynamics of 133 ps, revealing a binding pocket capable of solvation on the fast time scale. Moreover, this globular native structure of the normal isomer shows the largest flexibility among all conformational isomers as revealed from measurement of the local wobbling motions of W214 tryptophan. The large plasticity of HSA is essential for the albumin molecule to accommodate a variety of ligands and to transport its ligands in the circulatory system. The observed time scale of \(~100\) ps is ideal for the trapped water molecules in the crevice at the binding site to maintain the local structural integrity as well as to maintain dynamic flexibility and lubrication in recognition and conformational transitions (Figure 4.10). The concept of “lock-and-key” or “induced-fit” for molecular recognition is therefore incomplete without knowledge of dynamical solvation and plasticity of the protein.
Figure 4.10: (Top) Surface map of HSA with negative (red) and positive (blue) charge distributions. The crevice is shown in the center with tryptophan W214 (yellow) at its bottom. (Bottom) Schematic representation of conformational transitions, from the contracted configuration in basic pH, to the flexible globule structure at neutral pH, and to the extended form in acidic pH. The location of W214 is indicated by the white dot in domain IIA.
CHAPTER 5

PROTEIN SURFACE HYDRATION BY SITE-SPECIFIC MUTATIONS

5.1 Introduction

Extensive studies of the time and length scales of water layers around the protein surfaces have been carried out in this laboratory and our senior collaborator Dr. Zewail at Caltech [12, 31–35, 37, 91, 92, 121, 140]. These studies were for proteins subtilisin Carlsberg [32], monellin [91], phospholipase A2 [92], melittin [34], and human serum albumin [35, 121]. A theoretical model was developed to take into account the exchange with bulk water [32, 141]. The solvation dynamics, on the time scales from femtoscond to picosecond, are consistent with residence times derived from molecular dynamics simulations (MD) [26, 131, 142, 143]. Earlier NMR studies have reported hydration dynamics (residence times) in the subnanosecond regime [15, 24, 25, 144]. However, a claim recently has been made that water motions at protein surfaces are still ultrafast, only slowing down by a factor of two to three compared with bulk water [17, 145]. It was thus suggested that the observed long-time solvation dynamics in tens of picoseconds are in large part due to protein side-chain relaxation rather than the relaxation of water [130, 145]. This issue has been addressed in detailed earlier
and reasons for the dominant observation of hydration dynamics were given [12]. To further quantify the contribution of side-chain motions to total solvation on the time scale of hydration dynamics, we studied the effect of mutation on hydration of the protein *Staphylococcus* nuclease (SNase).

Figure 5.1 shows the X-ray structure of SNase, which consists of three α-helices and a five stranded β-barrel with a total of 149 amino acids [146]. The only tryptophan residue (W140) has one edge exposed to surface and is part of a hydrophobic cluster at the C-terminus of the protein, which was found to be essential to the protein foldability, stability and activity [147]. Within 7-Å of W140, there are three charged residues: K110, K133 and E129 (Figure 5.1). We replaced each charged residue with the hydrophobic residue alanine one at a time with site-directed mutagenesis, and we also mutated K110 into the polar residue cysteine. We then measured the Stokes shift, solvation dynamics and local rigidity of wild-type SNase (WT) and its various mutants using the intrinsic W140 as the optical probe and with femtosecond temporal resolution. From these results, we determined the hydration dynamics and evaluated their dependence on local charge distribution.

5.2 Materials and Methods

SNase mutant E129A, K133A, K110A, and K110C were prepared by the method of Kunkel as described elsewhere [148]. Protein expression and purification were performed by following the protocol of Byrne *et al.* [149]. Proteins were dissolved in
**Figure 5.1:** Protein structure with sites of hydration. (Left) X-ray crystallographic structure of wild type SNase (PDB ID: 1SNO). The single tryptophan W140 (in yellow) is sandwiched between K110 and K133, with one edge exposed to the protein surface. (Right) The local configuration around W140 with three charged residues (K110, K133, and E129) in close proximity (<5 Å).

A buffer of 25 mM sodium phosphate and 100 mM NaCl at pH 7 for both steady-state and time-resolved measurements. All time-resolved measurements were carried out on the femtosecond-resolved fluorescence up-conversion apparatus as detailed in chapter 2. The protein concentration used in time-resolved studies was typically 200-300 µM. All steady-state fluorescence emission spectra were measured using a SPEX FluoroMax-3 spectrometer. Protein sample was kept in a rotating cell to minimize possible photobleaching during time-resolved measurements. We carefully measured and compared the protein fluorescence emission spectra before and after experiments to ensure that no change of the protein quality occurred during data acquisition.
5.3 Results and Discussions

5.3.1 Fluorescence Stokes Shifts and Femtosecond-Resolved Transients

Steady-state fluorescence emission spectra of all the proteins used in this study are shown in Figure 5.2. All emission peaks are at \( \sim 332.5 \pm 0.5 \) nm, and we did not observe a significant shift in emission maxima by mutations of charged residues. This observation strongly suggested that the three neighboring charged residues make negligible contribution to solvation of the excited W140. These observations further confirmed that the maximum of tryptophan emission mainly depends on its location (i.e., the extent of its exposure to surface water) and not on its neighboring residues [134]. Thus, the observed Stokes shift cannot be due to solvation dynamics of neighboring charged residues, indicating relatively immobile charged side chains. The Stokes shift is dominantly from hydration. From the X-ray structure (Figure 5.1), we find that W140 is sandwiched between K110 and K133 (4.65 and 2.99 Å, respectively), forming two cation-π interactions. K133 is only 3.50 Å from E129, resulting in formation of a salt bridge. This unique structural motif with these strong electrostatic interactions is probably the origin of local protein rigidity around W140, which is consistent with our observation; see discussions below.

For each protein, >10 fluorescence transients from the blue to red side were measured. Figure 5.3 shows the femtosecond-resolved fluorescence transients of W140 in the WT at several typical wavelengths. The overall decay dynamics is significantly slower than that of aqueous tryptophan in a similar buffer solution [37]. Clearly, the ultrafast decay components (<1 ps) observed in aqueous tryptophan were not
observed at the blue side for the protein. Besides the lifetime contributions, solvation components for all blue-side transients are well represented by a double-exponential decay with two time constants in the range of 4.9-12 and 102-130 ps. For the red-side emission, the rise occurs in the range of 1.04.8 ps and is clearly present in all transients. For the four mutants (K133A, K110C, K110A, and E129A), the transients showed similar solvation patterns from the blue side to the red side besides the different lifetime emission contributions; see Figure 5.4. Among the four mutants, K110A has the shortest decay time for the two solvation components, followed by E129A. The mutants K133A and K110C have similar temporal behaviors as the WT.

5.3.2 Solvation Correlation Functions

With the methodology in chapter 2 and published elsewhere [37], we constructed the overall and lifetime-associated, femtosecond-resolved emission spectra (FRES) for
Figure 5.3: Normalized femtosecond-resolved fluorescence transients of W140 from WT SNase on short (Left) and long (Right) time scales with a series of gated fluorescence emissions. Note that no ultrafast decay components (<1 ps) were observed.
Figure 5.4: Normalized femtosecond-resolved W140 fluorescence transients of SNase mutants K133A, E129A, K110C, and K100A on both short and long time scales. Note that only three characteristic transients from >10 gated fluorescence emission wavelengths are shown for each mutant.
WT and all four mutants. By fitting these FRES to a lognormal function, we deduced the femtosecond-resolved overall emission maxima $\nu_s$ and lifetime-associated emission maxima $\nu_l$; see Figure 5.5. The obtained total dynamic Stokes shifts are 850±50 cm$^{-1}$ for all of the proteins, and the emission maxima $\nu_s(0)$ at $t = 0$ are all $\sim$321 nm, consistent with a previous report [96] of the emission peaks of several proteins as 320 nm at 2 K and 300-nm excitation. MD simulations of the Stokes shift of tryptophans in four proteins also gave an emission maximum of $\sim$320 nm at $t = 0$ [105]. The possible contribution of vibrational relaxation is negligible [34,35,140], and thus the observed total Stokes shift is mainly from the local solvation.

Using $c(t) = [\nu_s(t) - \nu_l(t)]/\nu_s(0) - \nu_l(0)$, we constructed all solvation correlation functions, and the obtained results are shown in Figure 5.6 and summarized in Table 5.1. All solvation correlation functions can be represented by a double exponential decay. For the WT, the time scales are 5.1 ps with 46% of the total amplitude and 153 ps (with 54% of the total amplitude); for K110C, 4.2 ps (51%) and 149 ps (49%); for K133A, 3.9 ps (59%) and 157 ps (41%); for E129A, 3.5 ps (60%) and 124 ps (40%); for K110A, 3.1 ps (77%) and 96 ps (23%). Overall, all four mutants show faster temporal behaviors than the WT, and all of the longer component of solvation times are within $\sim$100-150 ps. Note that no ultrafast solvation dynamics in <1 ps were observed for the proteins studied here.
Figure 5.5: Femtosecond-resolved emission maxima of the overall emission spectra ($\nu_s$) and lifetime-associated emission spectra ($\nu_l$) for WT SNase and mutants K133A, E129A, and K110C. Inset shows the entire evolution of $\nu_s$ and $\nu_l$ to reach the steady-state emission maximum ($\nu_{ss}$). The mutant K110A has a single lifetime, and the data analysis was carried out by the conventional method (not shown).
Figure 5.6: Hydration correlation functions probed by tryptophan W140 for WT SNase and four mutants, K110C, K133A, E129A, and K110A. Inset shows the correlation functions in the short time range. Note the similarity of the time scales and the difference in amplitudes.

5.3.3 Hydration vs. Charged Side-Chain Solvation

The constructed solvation correlation function is the response of the local environment around W140 to the sudden dipole change from the ground state to the excited state. This response could in principle result from both surrounding water molecules and neighboring protein polar/charged residues (and protein peptide bonds). If the longer component of solvation dynamics came only from protein side-chain motions, we should observe significantly different solvation time scales and amplitudes of the second solvation components for all five proteins studied here. However, for the mutants K133A and K110C, we observed a long relaxation time (149 and 157 ps) similar to the WT (153 ps). Overall, the obtained solvation dynamics for the four mutants become faster with the decrease in the local charge distribution around W140 (Figure
Table 5.1: Results obtained from the hydration correlation functions $c(t)$ of wild-type SNase (WT) and four mutants

<table>
<thead>
<tr>
<th>SNase</th>
<th>$\tau_1$, ps</th>
<th>$\tau_2$, ps</th>
<th>$c_1$</th>
<th>$c_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>5.1</td>
<td>153</td>
<td>0.46</td>
<td>0.54</td>
</tr>
<tr>
<td>K110C</td>
<td>4.2</td>
<td>149</td>
<td>0.51</td>
<td>0.49</td>
</tr>
<tr>
<td>K133A</td>
<td>3.9</td>
<td>157</td>
<td>0.59</td>
<td>0.41</td>
</tr>
<tr>
<td>K129A</td>
<td>3.5</td>
<td>124</td>
<td>0.60</td>
<td>0.40</td>
</tr>
<tr>
<td>K110A</td>
<td>3.1</td>
<td>96</td>
<td>0.77</td>
<td>0.23</td>
</tr>
</tbody>
</table>

All the hydration correlation functions were fitted with $c(t) = c_1 e^{-t/\tau_1} + c_2 e^{-t/\tau_2}$, where $c_1 + c_2 = 1$.

5.6), consistent with MD simulations that show that water has longer residence times around charged residues than near hydrophobic side chains [26,49]. The observed long time scale of $\sim 100 \text{ to } 150 \text{ ps}$ is also consistent with our recent observation in the melittin tetramer and human serum albumin (E and F isomers); in both cases [34, 35], the probe tryptophan has a similar emission maxima ($\sim 332 \text{ nm}$) and charge surroundings as in SNase. Thus, the obtained solvation correlation functions essentially reflect the local hydration dynamics at the protein surfaces.

For SNase, W140 has one edge exposed to the surface. Because of the local strong electrostatic interactions of the cation-$\pi$ stacking/salt bridge and the tight packing of the hydrophobic cluster near C-terminus, the local structure is more rigid and W140 is less mobile. We studied the femtosecond-resolved rotational dynamics of W140 by the measurement of anisotropy changes with time for all of the proteins (see Figure 5.7). We observed that all anisotropy dynamics dominantly decay in nanoseconds with a small decay component at the early time. The long nanosecond dynamics represents the whole protein tumbling motion. The early small decays, on the time scales from 62
to 464 ps, result from the local wobbling motions with semiangles of 11-19° (30). The time scales of 62-464 ps do not correlate with those of solvation dynamics (Figure 5.6). All of these findings suggest a relatively small local fluctuation; the protein structure around W140 does not undergo large conformation changes in the time window of our measurements (≈1.3 ns). These results from anisotropy dynamics are consistent with those obtained above from site-directed mutagenesis. The conclusion is indeed consistent with the results of the steady-state fluorescence emission maxima (i.e., the dynamic Stokes shifts of the WT and four mutant proteins are independent of the three charged residues K110, K133, and E129). However, these findings do not support claims of recent MD simulations which would otherwise indicate a very large contribution to the total Stokes shift from K110 and K133 in SNase [105].

5.3.4 Molecular Mechanism of Surface-Water Hydration

To summarize, the studies of mutations, steady-state fluorescence, anisotropy measurements, and biphasic behavior of dynamic Stokes shifts exclude charged side-chain solvation as a significant contributor. We did not observe an ultrafast component of <1 ps for any of the proteins in this study, contrary to the initial dynamics obtained from recent MD simulations [26, 130, 131, 142]. Given that our instruments were able to resolve the 200-fs solvation dynamics of tryptophan in bulk water [33, 37], we would have observed subpicosecond solvation dynamics in the proteins if such ultrafast solvation process existed. The absence of subpicosecond solvation suggests that the
Figure 5.7: Femtosecond-resolved fluorescence anisotropy of W140 of wild-type SNase and four mutants. The initial \(\sim\)100-fs decay is due to the internal conversion between the \(^1L_a\) and \(^1L_b\) states. The small components on the picosecond time scale represent the local wobbling motion of W140 with the derived semiangles (\(\theta\)). The long component at \(\sim\)11 ns results from the protein tumbling motion.
force field utilized in these MD simulations might be too flexible and should be re-examined to account for relevant interactions between the water network and protein.

Previous MD simulations found a biphasic distribution of residence times with two discrete time scales [26,130,131,142], which remarkably resemble our observation of hydration dynamics. Two distinct residence times represent two different ways for water to escape hydration sites. The fast route is through consecutive hopping by breaking and making of the neighboring hydrogen bonds while the slow path is through direct exchange with bulk water by disrupting the local water structure: hence, the distribution in residence times [150]. Along the slow path, water has longer residence times at concave surfaces than around convex patches, and around charge residues than near hydrophobic side chains [132]. MD simulations of nonequilibrium excited-state trajectories of tryptophan in proteins from this laboratory and our collaborator Dr. Singer’s laboratory give a similar dynamic pattern of two distinct time scales in addition to a subpicosecond component (Li T, Hassanali A, Zhong D, and Singer S, unpublished data). The initial dynamics occurs in a few picoseconds and represents local water librational/rotational motions as discussed. The long-time solvation, in tens of picoseconds, is from the collective motions of local water and strongly assisted by local protein fluctuations. Only if the local protein structural rigidity is lost by conformational changes does the protein contribute to the total solvation energy in a significant way [151]. It should be noted that static spectral shifts reflect the internal and static Stark perturbation, which attractively or repulsively alters the energy levels at equilibrium, whereas dynamical solvation measures
the fluctuations in energy as a function of time [12].

Figure 5.8 shows a series of surface maps around W140 for WT and four mutants with the local surface topography and neighboring residues. The x-ray structure at 1.7-Å resolution shows around W140 four surface-water molecules sticking to three charged residues and one water molecule buried inside the protein. As also shown by recent MD simulations of water penetration in SNase [152], the buried water molecule bridges three residues, the side chain of W140 (NE1-O distance of 2.87 Å) and two backbone oxygen atoms of V104 (O-O distance of 2.89 Å) and A109 (O-O distance of 2.6 Å), by three hydrogen bonds and has a residence time that is longer than the simulation time window of 10 ns. Another recent 7-ns MD simulation [153] of surface-water properties reveals a complex hydration structure with a pronounced pentagon-pentagon ring distribution in the hydration layers that depends highly on the surface topography. Around the hydrophobic atoms, clathrate-like cage structures parallel to the protein surface were observed. Near the polar atoms, the ring structures, perpendicular to the surface, have one or two hydrogen bonds with the polar atoms. From our recent 2-ns MD simulations, we found that within 5 Å from the O atom of H$_2$O to the indole moiety (including H), there are ~17 water molecules around the ground-state W140. We did not observe significant changes of total water molecules for the different mutants, implying nearly similar polar water environments, consistent with our observation of the same Stokes shifts for all mutants and WT.

When tryptophan is excited to the $^1L_a$ state, the local equilibrium is shifted, leading the system to a nonequilibrium state. Our observed hydration dynamics with
Figure 5.8: Protein surface maps of the WT and four mutants showing the local topography and neighboring protein residues around W140. There are four water molecules sticking to three surface charged residues (K110, E129, and K133) near the probe W140 in the x-ray structure. The water molecules within 5 Å from the O atom of H₂O to the indole ring are shown from our 2-ns single-trajectory MD simulations at 295 K in aqueous solution. Note that the number of water molecules is nearly the same for all of the proteins but the structures are different. The structural landscapes are different, especially for E129A and K110A, with ruptures at the surfaces near W140.
two distinct time scales reflect the temporal evolution of two types of motions from the initial nonequilibrium configuration to new equilibrated state around the excited tryptophan. The initial hydration dynamics, occurring in 3-5 ps, represents the librational/rotational motions of these surrounding water molecules, slowing down by a factor of three to five compared with similar bulk-water motions around tryptophan, which occur in <1 ps [33, 37]. We clearly observed a correlation of the initial fast time scale and amplitude with the local charge distribution (Table 5.1). For example, we observed the fastest hydration dynamics, in 3.1 ps, for the mutant K110A with the largest amplitude (77%); for K110C, we obtained a time scale of 4.2 ps with 51%; for the WT (K110), we have the initial dynamics of 5.1 ps with 46%. Thus, from the hydrophobic (A110) to the polar (C110) and to the charged residue (K110), we observed a lengthening of the time scale, consistent with stronger interactions of local water with charges. Furthermore, we observed a decrease, not an increase, in the amplitude, suggesting that the charged/polar residue does not directly contribute to the solvation energy. These results reveal that the initial relaxation is from the local surface-water motions and that the observed variations in hydration dynamics in the mutations reflect the alternation of the local landscape and the change of the neighboring chemical identity (Figure 5.8).

The observed slow water dynamics in 100-150 ps represent the long-time collective motions, reflecting a dynamic structural change of the local water molecules. The time scale to reach the new structural configuration depends on the local protein-water interactions. Upon the sudden change of the dipole moment of W140 in the protein, besides initial librational/rotational motions, at least two local structural motions are
expected: one is the alignment of the local water network/cluster to the new excited-state \(^1L_a\) dipole moment, and the other is the increase of water molecules around W140 due to the larger excited-state dipole moment (\(\Delta \mu \sim 5D\)). Because of the longer time scale (tens of picoseconds), these overall structural changes are coupled with the protein fluctuation. These fluctuations assist the structural rearrangement of surface water and its exchange with bulk water. As such, the connection of residence time to hydration is incomplete without knowledge of fluctuations. Thus, the long-time hydration dynamics, defined here as the rate for water structure to reach the new equilibrated state of minimal free energy, is an integrated process determined by the local interactions with the protein (Figure 5.8) and assisted by its fluctuations. The coupled protein-water motions enable surface-water molecules to make structural arrangements (solvation), but these small protein fluctuations themselves do not make direct contributions to total solvation.

### 5.4 Conclusions

Our studies of protein SNase, using site-selected mutagenesis and femtosecond fluorescence spectroscopy, confirm that surface protein hydration occurs on the picosecond time scale with a biphasic behavior, consistent with observations made previously for a number of other proteins that we have studied. The initial fast component (a few picoseconds) is due to the librational/rotational motions of water near the surface, whereas the longer one (tens of picoseconds) is due to the motions of water molecules coupled to the surface and structurally modified by protein fluctuations. The evidence for dominance of hydration dynamics rather than side-chain solvation
comes from a number of observations discussed above and studies of different proteins reviewed elsewhere [12]; here, the direct evidence comes from studies of the same protein but with different side chains of different charges and structures. It would be fortuitous and less likely that mutations at three different sites would exactly cancel out the effect of hydration and protein solvation to give nearly the same Stokes shifts and correlation functions. The insignificant contribution of side-chain solvation was recently examined in experiments involving the native and denatured states [154].

The key to understanding surface hydration is the time scales involved. In a simple model (for reviews, see refs. [12, 31, 141, 155]), the time scale is related to hydrogen-bond exchanges in layered water, consistent with residence times obtained from several MD simulations [12, 142]. This can also explain slow hydration in micelles [155]. However, proteins are “rigid” on time scales of a few picoseconds while fluctuating on time scales of tens of picoseconds. This distinction of these two time scales is relevant not only for hydration but also for other protein-related interaction, such as molecular recognition [156]. The protein fluctuations assist solvation by allowing for restructuring in the new nonequilibrium state (in this case, the structure of water around tryptophan’s dipole). They themselves do not contribute to solvation by directly minimizing energy, as evidenced by the robustness of hydration time scales and Stokes shifts independent of local charge and structural changes around tryptophan. Large-amplitude side-chain motions may occur, but they are on somewhat longer time scales and may involve conformation changes; such changes contribute to solvation [151].
Unlike libration or distant rotation, the longer component of hydration dynamics directly represents interactions with the protein and is significant for biological functions (for recent reviews, see refs. [157, 158]). The hydration time scale in tens of picoseconds is ideal for many biological processes involved in molecular recognition, reactivity, and conformational intactness [12]. The robustness in slow hydration for proteins of different sequences and structures cannot be ignored. Earlier NMR work suggested that the hydration of proteins and DNA occurs in subnanoseconds (for reviews, see refs. [15, 24, 25, 144]), and it was the work from this laboratory and our collaborator (for review, see refs. [12, 34]) that pointed out that the time scale of surface hydration is much shorter than the time scales reported by NMR. However, in a more recent work published from one of the same NMR groups [17, 130, 145], hydration was claimed to occur in a few picoseconds. Similarly, in earlier MD simulations (see, for example, ref. [142]), the time scales varied from picosecond to nanosecond residence times, depending on the strength of hydrogen bonding and the location of water molecules. However with a force field and linear response theory, the claim now is that water is much more labile, although fast and slow (10%) components observed in experiments are still present, with the latter assigned to protein solvation [130]. As discussed above, the search for the large ultrafast (<1 ps) dynamics predicted by MD simulations was unsuccessful. It is possible that the flexibility imposed by MD modeling is reducing all time scales and increasing amplitudes of subpicosecond components by order(s) of magnitude. Protein fluctuations, including side chains, cannot be blind to local water hydration that is responsible for solvation and is structurally evident through X-ray and mutation studies. It is thus our conclusion that the picture based on MD simulations and emphasized in several recent publications [130, 145] needs to
be revisited, taking into consideration the approximations made regarding force field, linear response, and the exact meaning of hydration for the coupled protein-water network. Protein fluctuation-assisted interactions with surface water are an integral part of hydration dynamics, particularly at longer times.
CHAPTER 6

DISSECTION OF COMPLEX PROTEIN DYNAMICS

6.1 Introduction

Protein dynamics is a complex process evolving on a multidimensional energy landscape with various interactions and conformations over a wide range of time scales [29, 159–164]. To understand such complex dynamics, we need to dissect the process into elementary steps and determine their respective time scales. Many of these elementary processes occur on similar time scales [165], and thus multidisciplinary approaches are needed to distinguish these processes and elucidate their molecular mechanisms [29, 33, 166–169]. In this chapter, we report our direct studies of complex protein dynamics at the active site of human thioredoxin (hTrx). Using site-directed mutagenesis and femtosecond-resolved fluorescence spectroscopy, we were able to break down the complex dynamics into four elementary processes: one solvent relaxation dynamics and three electron-transfer (ET) reactions.

Figure 6.1 shows the x-ray structure of oxidized hTrx at a 2.1-Å resolution [170]. hTRx, an enzyme catalyzing dithiol-disulfide exchanges with its target proteins, is
a compact globular protein with a central core of five strands of \(\beta\)-plated sheet surrounded by four \(\alpha\) helices. The active site of hTrx consists of a highly conserved sequence W31-C-G-P-C-K36 that forms a protruding part of the structure between the second \(\beta\) strand and the second \(\alpha\) helix. The tryptophan (W31) at the active site of hTrx is the only tryptophan the protein has. The reduced hTrx involves various biological functions through a redox exchange of two cysteines (C32 and C35) with a disulfide bond in a series of target proteins including Trx peroxidase, ribonucleotide reductase and transcription factors [171,172]. Regeneration of reduced hTrx with two free sulfhydryls involves an ET mechanism from NADPH and the enzyme thioredoxin reductase [173,174]. Various structures of reduced and oxidized hTrx and their mutants and hTrx homologs from other species have been determined with x-ray crystallography [170,175,176].

The active site is fully exposed to aqueous environment, and thus all the chemical reactions occurring at the active site likely will be coupled with hydration dynamics, a process that often modulates chemical changes through local water rearrangements. In the previous chapters, we have characterized the hydration dynamics for several proteins in different structures and conformational states using the intrinsic tryptophan as the local optical probe. Thus, we will be able to determine the time scales of water motions at the active site of hTrx with high confidence. However, recent studies have reported severe quenching of tryptophan fluorescence by local environment in thioredoxin from *Escherichia coli*, calf thymus and yeast [177–179]. There are three potential quenchers to the fluorescence of W31 with quenching dynamics in subnanosecond and within 7 Å: disulfide bond (C32-C35), a peptide bond (between
Figure 6.1: Protein structure with quenching species at the active site. (Left) X-ray crystallographic structure of oxidized hTrx mutant C73S, consisting of five strands of β-pleated sheet and four α-helices. Note that the single tryptophan W31 (in yellow) is located at the protruding part of the active site structure. (Right) The local configuration in close proximity (within 7 Å) to W31 includes one charged residue (D60), a disulfide bond (C32-C35) and a peptide bond between W31 and C32.

W31 and C32), and charged residue aspartate D60. Quenching of excited tryptophan by disulfide bond has been observed in many proteins such as cutinase [180] and oxidoreductase DsbA [181]. The quenching process is commonly believed to occur through an ET mechanism with the excited tryptophan donating an electron to the disulfide bond [180,181], but the time scales of this dynamical process has never been characterized. Peptide bond and aspartate residue also quenches the tryptophan fluorescence through excited-state electron transfer. In this chapter, we report how to dissect the complex protein dynamics in hTrx, which involve local water relaxation, and various ET reactions at the active site, and determine the time scales of these processes with femtosecond time resolution.
6.2 Materials and Methods

Three hTrx mutants used in this study including C73S, C73S/D60G and D60N were designed, over-expressed and purified as described elsewhere [182–184]. Protein samples were dissolved in 50 mM Tris-HCl buffer, pH 7.5 to keep the enzyme in a monomer form. Oxidized hTrx was a result of air oxidization throughout an extensive time period of purification and storage. Reduction of hTrx was then obtained by adding fresh 10 mM DTT. For time-resolved measurements, the concentration of protein sample was typically 200-300 µM, whereas the concentration was 5-10 µM for steady-state fluorescence characterizations. All time-resolved measurements were carried out on the femtosecond-resolved fluorescence up-conversion apparatus as detailed in chapter 2.

6.3 Results and Discussions

6.3.1 Femtoscond-Resolved Transients and Active-Site Hydration Dynamics

The hTrx protein was found to easily form homodimers through an interprotein C73-C73 disulfide linkage [182]. All the studies thus focus on hTrx monomer by substituting C73 with serine (S73) to eliminate the possible formation of interprotein disulfide bridge. The steady-state fluorescence emission spectra of W31 for two hTrx mutants C73S and C73S/D60G are shown in Figure 6.2. All emission maxima are ~340 nm, similar to those of Escherichia coli thioredoxin [185, 186], indicating that W31 is located at the protein surface with great exposure to the aqueous environment, consistent with the x-ray structure. For each mutant, both reduced and
oxidized states have the same emission peak but with dramatically different fluorescence intensities. The fluorescence intensity of the reduced C73S is about twice as strong, indicating significant quenching by the disulfide bond in the oxidized hTrx.

Compared with C73S, the fluorescence intensity of C73S/D60G was found to increase by a factor of 2, suggesting severe quenching by D60. Similar fluorescence changes have also been observed for thioredoxins from other species [177, 185, 186]. The observed 3-nm difference between emission peaks of C73S and C73S/D60G are not due to changes in hydrophobicity but instead due to multiple emissions with different peaks (decay associated emission spectra).

We first studied reduced hTrx mutant C73S without disulfide-bond quenching. Figure 6.3 shows the femtosecond-resolved fluorescence transients of W31 at several typical wavelengths gated from the blue to red side of the emission spectrum.
Apparently, the overall decay dynamics is slower than that of the aqueous tryptophan in a similar buffer condition [37] and resembles tryptophan solvation on protein surfaces [32,34–36,91]. Solvation components for all blue-side transients are well described by a double-exponential decay with time constants of 0.3-1 and 2.6-12.6 ps. All transients at the red-side emission have a single rise component with time constant in the range of 0.7-1.3 ps. The two effective fluorescence lifetimes were determined to be 95 and 275 ps through global data analysis; these dramatically shortened lifetimes result from local quenching processes (see below).

With the methodology detailed in Chapter 2, we constructed the overall and lifetime-associated femtosecond emission spectra for reduced hTrx mutant C73S. Fitting these femtosecond-resolved emission spectra to a lognormal function (Figure 6.4a inset), we deduced the femtosecond-resolved overall emission maxima \( \nu_s \) and lifetime-associated emission maxima \( \nu_l \), and determined the solvation-complete time \( t_{sc} = 5 \) ps and merging emission peak \( \nu_{sc} = 29,564 \text{ cm}^{-1} \); see Figure 6.4a. The obtained total dynamic Stokes shift is 1,725 cm\(^{-1} \), and the emission maximum \( \nu_s(0) \) at \( t = 0 \) is \( \sim 320 \) nm, consistent with previous observations [96] and recent MD simulations [105]. The possible contribution of vibrational relaxation is negligible [34], and thus the observed total Stokes shift is predominantly from the local solvation. Using \( c(t) = [\nu_s(t) - \nu_l(t)]/[\nu_0(t) - \nu_l(0)] \) from chapter 2, we subsequently constructed the solvation correlation function, and the result is shown in Figure 6.4b and summarized in Table 6.1. The solvation dynamics is a double-exponential decay with time constants of 0.67 ps with 68% of the total amplitude and 13.2 ps (32%).
Figure 6.3: Normalized, femtosecond-resolved fluorescence transients of reduced hTrx mutant C73S in both the short (Left) and long (Right) time ranges with a series of gated fluorescence emissions. Note the fast solvation at the blue side, whereas the fluorescence lifetimes are significantly shortened.
Figure 6.4: Femtosecond-resolved emission spectra and hydration dynamics. (a) Femtosecond-resolved emission maxima of the overall spectra ($\nu_s$) and lifetime-associated spectra ($\nu_l$) for hTrx mutant C73S in its reduced state. (Inset) Several normalized, femtosecond-resolved overall emission spectra, and the dashed curve is the corresponding steady-state emission spectrum. (b) The deduced hydration correlation function from (a). (Inset) The femtosecond-resolved fluorescence anisotropy dynamics of W31. The initial $\sim$100-fs decay is due to the internal conversion between $^1L_a$ and $^1L_b$ states. Note the restricted local motion of W31, shown here by the apparent absence of subnanosecond decay.
Table 6.1: Results obtained from the hydration correlation functions $c(t)$ of three hTrx mutants in reduced state

<table>
<thead>
<tr>
<th>hTrx</th>
<th>$\tau_1$, ps</th>
<th>$\tau_2$, ps</th>
<th>$c_1$</th>
<th>$c_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>D60</td>
<td>0.67</td>
<td>13.2</td>
<td>0.68</td>
<td>0.32</td>
</tr>
<tr>
<td>D60G</td>
<td>0.47</td>
<td>12.7</td>
<td>0.67</td>
<td>0.33</td>
</tr>
<tr>
<td>D60N</td>
<td>0.53</td>
<td>10.8</td>
<td>0.69</td>
<td>0.31</td>
</tr>
</tbody>
</table>

All the hydration correlation functions were fitted with $c(t) = c_1 e^{-t/\tau_1} + c_2 e^{-t/\tau_2}$, where $c_1 + c_2 = 1$.

As we discussed in the previous chapters, the constructed solvation correlation function is the response of the local environment around W31 to its sudden shift in charge distribution from the ground state to the excited state. The response could result from both the surrounding water molecules and the charged/polar residues in proteins [27]. With 7 Å to W31, there is only one such residue, D60 at 3.59 Å which forms a hydrogen bond between O of the carboxyl group and N of the indole ring (Figure 6.1). We also studied hTrx mutants D60G and D60N to place W31 in hydrophobic and polar environments respectively, and obtained nearly the same solvation-complete times ($t_{sc}$), merging emission peaks ($\nu_{sc}$), and correlation functions as those shown in Figure 6.4b and Table 6.1. These results are striking and strongly suggest the charged/polar residues (D60/D60N) make negligible contribution to the total Stokes shift [36]. Therefore, the obtained solvation correlation function in Figure 6.4b represents primarily the local hydration dynamics, suggesting water motions within 13.2 ps at the active site of hTrx.
6.3.2 Dissection of active site complex quenching dynamics

(a) ET reaction with disulfide bond. In their oxidized states, the two catalytic cysteines C32 and C35 form a disulfide linkage significantly quenching excited-state W31 (Figure 6.2). Figure 6.5 shows the femtosecond-resolved fluorescence transients of W31 for several typical wavelengths gated from the blue to red side of the emission spectrum. All transients are clearly much faster than those obtained at the same wavelengths for the reduced state (Figure 6.3), see the inset of Figure 6.5 for a comparison at 360-nm emission. At the red side of emission, all transients can be globally fitted by an initial ultrafast hydration rise (0.43-0.87 ps) and three exponential decays: one is 17.5 ps with major contribution (~74%), and two minor components (26% total) are 95 ps and 615 ps. We also observed similar time constants of 15 and 10 ps with significant contributions in oxidized mutants of D60G and D60N respectively. Thus, the 10-17.5 ps is the time scale of electron transfer from excited-state W31 to the disulfide bond (C32-C35) at a distance of ~5.75-Å. The difference in reaction times is a result of local configuration changes between W31 and the disulfide bond due to mutations. One may notice that the longer component of hydration dynamics (13.2 ps) is on the same time scale of the ET reaction (10-17.5 ps) and mixed together at the blue side of emission. We were able to accurately determine the ET reaction time of 10-17.5 ps by femtosecond-resolved fluorescence gating of the fully relaxed state at the red side emission. Thus the elucidation of the time scales, even in this case $\tau_{sol} \sim \tau_{ET}$, is crucial to the understanding of complex protein dynamics.

(b) Charge separation with peptide bond. In both reduced and oxidized mutant C73S, we observed a very short lifetime of 95 ps. This short lifetime could
Figure 6.5: Four representative fluorescence transients of oxidized hTrx mutant C73S from 305 to 380 nm. Note the fast decay of quenching dynamics by the disulfide bond at the red side of emission, whereas the solvation decay components are intertwined with the quenching process at the blue side. (Inset) A comparison of the transients at 360 nm for two redox states.

be caused by potential quenching residues or peptide bond around W31. Within 7 Å to the indole ring of W31, the only possible quenching residue is D60. Figure 6.6 shows the femtosecond-resolved fluorescence transients of hTrx mutant D60G gated from the blue to red side of emission for both reduced and oxidized states. For all red-side transients in two states, we still observed short fluorescence lifetimes of 108 and 114 ps. For hTrx mutant D60N in which the charged Aspartate D60 was replaced with a polar residue Asparagine, similar short lifetimes of 105 and 109 ps were observed in both reduced and oxidized states respectively. The robust observation of 100 ps quenching dynamics, regardless of the nature of neighboring residues (charged/polar/hydrophobic), must be from a charge separation from excited W31 to adjacent peptide bond(s). Examination of the local protein structure in close
proximity to W31 revealed that the closest distance between the indole ring and its nearest peptide bond (between W31 and C32) is 3.61 Å (Figure 6.1). Peptide bond has recently been proposed as a strong quencher of excited tryptophan to interpret different lifetimes observed in proteins [187, 188]. Recent studies of a well designed cyclic peptide indicated that the quenching time could range from a few to hundreds of picoseconds, depending on local configurations [189]. The slight difference in reaction times with the peptide bond for three mutants probably results from the small alteration of their relative geometries due to mutations. Thus, the 100 ps is the ET time from excited W31 to the peptide bond at a 3.61-Å distance in hTrx.

(c) Electronic quenching with neighboring residues. At the blue side of emission, we observed the same hydration dynamics in the reduced state and a similar ET reaction dynamics (1017.5 ps) in the oxidized state of three mutants. Besides the observed robust short lifetime of 100 ps by peptide-bond quenching, there is another lifetime that varies in different mutants and in two states. Table 6.2 summarizes their lifetimes and percentages of contributions. From reduced D60 to D60G, we observed the change of the lifetime 275 ps to 1.26 ns. We attributed 275 ps to the quenching dynamics by the adjacent charged residue D60 at a hydrogen-bond distance of 3.59 Å. The 1.26 ns observed in reduced D60G is from weak quenching by the neighboring cysteine residue (C32) at a distance of 4.24 Å, similar to the lifetime we recently observed for the cysteine quenching in *Staphylococcus* nuclease [36]. In oxidized D60, the 275 ps changes to 615 ps, which must be due to the variation of local configurations with different separation distances and/or orientations upon formation of the disulfide bridge. For reduced D60N, we observed a 1.2-ns lifetime, which becomes 3.5 ns in
Figure 6.6: Femtosecond-resolved fluorescence dynamics of mutant D60G. (Upper) Four representative fluorescence transients of reduced D60G. (Inset) The dramatic lengthening of fluorescence lifetime(s) at 360 nm (and all other wavelengths) in C73S/D60G as compared with C73S. (Lower) Four representative fluorescence transients of oxidized D60G. Note that all of the transients decay faster because of the quenching by the disulfide bridge. (Inset) The transients of oxidized C73S/D60G and C73S at 360 nm for a comparison.
the oxidized state. The quenching induced by the mutation of D60N is significantly reduced, consistent with its x-ray structure, which shows that the asparagine residue flips away from W31 [170]. These quenching dynamics by the residues D60 and C32 all occur in the range from 275 ps to 1.2 ns, and the time scales depend on their chemical identities and local configurations (distance and orientation).

### Table 6.2: Results of total electron-transfer quenching dynamics of three hTrx mutants in reduced and oxidized states.

<table>
<thead>
<tr>
<th>hTrx</th>
<th>D60</th>
<th>D60G</th>
<th>D60N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>O</td>
<td>R</td>
</tr>
<tr>
<td>(\tau_s), ps</td>
<td>17.5</td>
<td>10.0</td>
<td>15.0</td>
</tr>
<tr>
<td>(c_1), %</td>
<td>74.1</td>
<td>40.2</td>
<td>54.2</td>
</tr>
<tr>
<td>(\tau_p), ps</td>
<td>95</td>
<td>108</td>
<td>105</td>
</tr>
<tr>
<td>(c_2), %</td>
<td>73.8</td>
<td>39.2</td>
<td>20.3</td>
</tr>
<tr>
<td>(\tau_r), ps</td>
<td>275</td>
<td>615</td>
<td>1264</td>
</tr>
<tr>
<td>(c_3), %</td>
<td>26.2</td>
<td>21.2</td>
<td>39.2</td>
</tr>
</tbody>
</table>

R, reduced state; O, oxidized state. \(\tau_s\), \(\tau_p\), \(\tau_r\) are quenching times by disulfide bond, peptide bond, and potential residues respectively. The sum of the population percentages \((c_i, i = 2, 3 or 1 - 3)\) for each state is 100.

### 6.3.3 Local Structure and Dynamical Heterogeneity

The anisotropy dynamics of W31 (Figure 6.4b Inset) shows a rigid local structure in the time window of 800 ps, consistent with the inflexible turn (-T30W31-) in the transition from the second \(\beta\)-sheet and the second \(\alpha\) helix (Figure 6.1). The hydrogen bonding with D60 further anchors W31 on the protein surface. In the oxidized state, the disulfide bond adds more constrains on the flexibility of the active site. However, we observed significant quenching dynamics of W31 with the adjacent disulfide bond,
peptide bond, and quenching residue(s), reflecting a distribution of dynamic heterogeneous configurations. Thus, the protein must fluctuate on a time scale of longer than several nanoseconds, a process recently called as solvent-slaved α fluctuations [6, 8, 9]. At such longer times, because the time interval between two consecutive pump excitations is 2 ms and each transient was averaged over >1 hour, all fluctuating protein conformations will be sampled. Thus, the observed several quenching processes do not mean that several static configurations are present, but they mainly represent a temporal configuration distribution; the protein is in dynamic heterogeneity. Similar anisotropy dynamics were observed even for D60G and D60N, and W31 is indeed locked in the rigid turn but slowly fluctuates with the protein.

6.4 Conclusions

We reported our studies to dissect complex protein dynamics in hTrx using site-directed mutagenesis and femtosecond-resolved fluorescence spectroscopy. Four elementary dynamical processes were found to concurrently occur within 1 ns upon excitation of W31 at the active site. Using this single intrinsic tryptophan as a local optical probe and from the measurements of three mutants with a charged, polar, or hydrophobic residue around the probe, we unambiguously determined the time scales of hydration dynamics at the active site to be 0.47-0.67 and 10.8-13.2 ps. The former time is the result of local reorientational/translational motions of water near the active site; the latter is a direct measure of surface hydration coupled with the local protein fluctuation [27]. These results are consistent with our recent studies of surface hydration dynamics in Staphylococcus nuclease [36]. In both systems, the
contribution to local solvation by protein sidechains is relatively insignificant. The observation of the longer time solvation dynamics is significant and the dynamics controls sidechain motions, a process of hydration-shell-coupled $\beta$ fluctuations [8].

We determined the quenching dynamics of excited tryptophan by a disulfide bond, a common motif of tryptophan near a disulfide bond in many proteins although the biological implication is still unknown [190]. We separated the quenching reaction from the hydration dynamics even though both processes occur on the same time scale, and observed a time scale of 10-17.5 ps for the ET quenching at a separation distance of $\sim 5.75$ Å for three mutants of oxidized hTrx. From studies of three mutants and their two redox states, we observed a robust quenching dynamics of $\sim 100$ ps in all of the proteins and this dynamics represents the electron-transfer reaction from excited tryptophan to the adjacent peptide bond at a distance of 3.61-Å, a quenching mechanism which was recently proposed to interpret various short lifetimes of tryptophan observed in proteins [187,188]. The quenching dynamics by aspartate residue at a hydrogen-bond distance of 3.59-Å in two redox states were also determined and the time scales are within hundreds of picoseconds (275-615 ps).

The key to understand complex protein dynamics is to measure the time scales of those underlying elementary processes. In hTrx, the enzyme fluctuation, slaved by bulk solvent ($\alpha$ fluctuations), takes nanoseconds or longer to recognize its target proteins and optimize the molecular recognition process. The redox reaction of dithiol-disulfide exchanges occurs on a much shorter time scale of less than nanoseconds; and the active site remains relatively immobile during this time period, as
observed in the anisotropy dynamics, to make efficient catalysis. During the electron transfer between two active sites, intermediate water molecules move ultrafast within 13 ps to reorganize hydrogen bond networks instantaneously and control sidechain flexibility ($\beta$ fluctuations) to facilitate the catalytic reactions. The redox function is completed in continuously synergistic motions over wide time scales. Thus, elucidation of complex dynamical evolution is essential for complete characterization of molecular mechanisms of biological function.
7.1 Introduction

Tryptophan (W), the most important fluorophore among amino acids, has been extensively used for decades in studies of protein dynamics by examining changes in its photophysical properties [134, 191–195]. The recent renaissance in ultrafast studies of tryptophan in proteins has established tryptophan as an ideal local optical probe for protein hydration dynamics [12, 30–37, 91, 140, 196] as well as an excellent local molecular reporter for intraprotein electron-transfer and energy-transfer dynamics [33, 166, 197–200]. Moreover, tryptophan can be engineered into desired positions using site-directed mutagenesis with single-residue spatial resolution. All these ultrafast measurements require no potential quenching of excited-state tryptophan by neighboring residues (or peptide bonds) on the picosecond time scale. However, it is known that tryptophan fluorescence is readily quenched by various amino acid residues [133] and peptide bonds [188, 201, 202]. Intraprotein electron transfer (ET) from excited indole moiety to nearby electrophilic residue(s) was proposed to be the quenching mechanism [133, 188, 201, 202]. Thus, identifying ultrafast quenching
residues and determining their time scales are critical to the understanding of protein
dynamics probed by tryptophan.

In a recent contribution from this laboratory [140], Zhang et al. described a
methodology to distinguish the ultrafast processes between local solvation and elec-
tronic quenching of tryptophan in proteins using its fluorescence dynamics with fem-
tosecond (fs) resolution. In this chapter, we report various ultrafast quenching dy-
namics within 100 ps and important quenching residues identified from our extensive
examinations of more than 40 proteins. Each protein only contains a single trypto-
phan residue. Among twenty amino acids, we observed two major ultrafast quenchers:
carbonyl- and sulfur-containing groups. The former (-C=O) includes the residues of
aspartate (D), glutamate (E), asparagine (N) and glutamine (Q) as well as the pep-
tide bond; the latter is the cysteine residue (-SH) and related disulfide bond (-S-S-).
Surprisingly, a recent extensive survey of high-resolution protein structures in Protein
Data Bank showed strong tendency of weak interactions between aromatic residues
with carbonyl oxygen atoms [203] and thiol or disulfide sulfur atoms [190,204]. These
weak contacts at a short distance of ∼3-4 Å may function to stabilize protein struc-
ture and have other functional implications [190,203,204].

The quenching of indole fluorescence by a variety of carbonyl compounds in so-
lution was well characterized before and intermolecular charge-transfer complex was
proposed [205]. In proteins, the electron donor (tryptophan) and acceptor residues
are spatially anisotropic due to steric hindrance and electrostatic interactions within
the protein architecture. Such interresidue electron transfer at short distances may
not follow exponential dependence on their separation distances. The ET rate will be affected by their relative orientations [206–208] and also by local solvation, depending on their relative time scales of the two dynamical processes [196]. Here, we systematically examine ET quenching dynamics of excited tryptophan with carbonyl- and sulfur-related groups in various local environments and determine their time scales. Molecular dynamics (MD) simulations were carried out to examine the effects of distance and orientation on the observed ET rates and to relate local protein fluctuations to the quenching dynamics. These results will without doubt extend our further use of tryptophan as a powerful fluorescence probe for ultrafast studies of protein dynamics in general.

7.2 Materials and Methods

*Sperm whale* myoglobin (Mb) mutants (triple mutation: W7Y/W14F/K34W, W7Y/W14F/K87W, W7Y/W14F/A125W and W7Y/W14F/G129W) were made possible with a generous gift of plasmid pMb122 by Prof. S. Sligar (UIUC, USA). All these mutants were expressed in *E. coli* and purified primarily using the standard protocol [209]. To avoid complication from resonance energy transfer between excited tryptophan and the heme group [199,200], we removed the heme [210] and apomyoglobin (apoMb) was used for studies. All the apoMb mutants were in a buffer of 10 mM sodium acetate at pH 6.1 for time-resolved measurements.

Human thioredoxin (hTrx) mutant C73S/D60G was kindly provided by Prof. D.A.R. Sanders (Univ. of Saskatchewan, Canada). The description of the mutant
design, protein overexpression and purification was detailed elsewhere [182,184]. hTrx mutant C73S/D60G was in 50 mM Tris buffer at pH 7.5 to keep the protein in its monomeric form. Oxidized hTrx was a direct result of air oxidization throughout an extensive time period of purification and storage. Reduced hTrx was then obtained by adding 10 mM of dithiothreitol (DTT). *Fusarium solani* cutinase was generously provided by Dr. H.A.M. Pepermans (and M. van der Burg-Koorevaar, Unilever Research, The Netherlands). A detailed account of the procedures for expression and purification of the protein can be found elsewhere [211]. The protein sample was finally dissolved in 10 mM sodium acetate at pH 4.6. *Staphylococcal* nuclease (SNase) mutant K133C was generously provided by Prof. W.E. Stites (Univ. of Arkansas, USA). SNase K133C was expressed in *E. coli* and the protein purification procedure was given elsewhere [149]. The protein sample was finally in a buffer with 25 mM sodium phosphate and 100 mM NaCl at pH 7.0.

*Chicken* calmodulin (CaM) mutant T26W/T62C was made using the plasmid generously provided by Prof. A.J. Wand (Univ. of Pennsylvania, USA). The CaM mutant was expressed in *E. coli* strain BL21 (DE3) cells grown on minimal media. We used a standard protein purification procedure [212]. The protein sample was finally in a buffer of 50 mM Hepes and 20 mM EDTA at pH 7.5 to ensure its calcium-free form (apoCaM).
All time-resolved measurements were carried out on a femtosecond up-conversion apparatus in this laboratory as detailed in chapter 2. For all time-resolved measurements, the protein concentration was typically in the range of 200-300 \( \mu \text{M} \). Steady-state fluorescence emission spectra prior to and after time-resolved experiments were measured and compared to ensure no change of the protein properties during data acquisition. We also kept the sample in various rotating cells throughout time-resolved measurements to minimize possible photo-bleaching.

Molecular dynamics (MD) simulations were conducted using GROMACS package [213] with the GROMOS96 force field [214]. We used the SPC/E water model because dynamical properties such as the diffusion constant derived from MD simulations are in good agreement with experimental observations. A quasi-Newtonian algorithm (l-bfgs) was utilized for energy minimization to relax the internal constraints for each protein before simulations. Throughout the MD simulations, the LINCS algorithm [215] was used to constrain all the bond lengths. The non-bonded pair list was generated using a 9-Å cutoff. Long-range electrostatic interactions were treated using a smoothed particle mesh Ewald (SPME) method [216,217] with a real space cutoff of 9 Å. The cutoff length for the Lennard-Jones potential was set at 14 Å. Periodic boundary conditions were implemented using a truncated triclinic box of appropriate side lengths solvated with water molecules for different proteins. The Nose-Hoover thermostat [218–220] was used to maintain the system under study at temperature of 295 K. The time step of all simulations was 2 fs and coordinates were saved every 1 ps. The initial configurations of proteins were taken from their respective crystallographic structures in Protein Data Bank (Sperm whale apoMb:
7.3 Results and Discussions

7.3.1 Ultrafast Quenching Dynamics by Carbonyl Group

From the extensive studies of protein surface hydration dynamics with more than 30 apoMb mutants in this laboratory [30], we only observed ultrafast quenching dynamics of excited tryptophan in several mutants which all contain a nearly carbonyl group. Figure 7.1 shows the femtosecond-resolved fluorescence transients of mutant G129W at three typical wavelengths from the blue to red side. Unlike the transients obtained for protein surface hydration [30,34–37,140,196], the transient at the red side of 360 nm shows an initial ultrafast decay, not formation, along with long fluorescence lifetime emissions (586 ps and 3.5 ns). Clearly, the ultrafast decay represents local quenching dynamics because solvation would cause fluorescence signal to rise initially. The ultrafast decay dynamics at the blue side of the emission peak, as shown here at 305 and 330 nm, result from a combining effect of solvation and quenching processes. Thus, the ultrafast decay obtained at the red side of the emission peak reflects more accurately the quenching dynamics of excited tryptophan. The quenching signal at 360 nm can be fitted by two decay components: 3.1 ps with 19% of the total amplitude and 82 ps (28%). By carefully examining the structure of G129W mutant (top panel in Figure 7.1), we found that the carbonyl group of residue Q128 is at a van der Waals distance from the indole ring of W129 and Q128 is thus the potential
Figure 7.2 shows the results of initial ultrafast decays at 360 nm for other three mutants of apoMb, K87W, K34W, and A125W, without inclusion of long fluorescence lifetime components. The overall pattern of transients from the blue to red side (not shown) is similar to that in Figure 7.1 for mutant G129W. For K87W, the quenching dynamics are nearly similar to those obtained for G129W with two decay components: 4.1 ps (24%) and 79 ps (28%). For K34W, the quenching dynamics is a single exponential decay of 51 ps (18%). For A125W, the quenching dynamics becomes longer with a time constant of 85 ps (29%). For more than twenty other apoMb mutants, we did not observed any ultrafast quenching dynamics within 100 ps, and the decay components at 360 nm are all longer than 200 ps [30]. We carefully examined the local structures of all three mutants derived from ground state MD equilibration, and found that the carbonyl groups of three residues Q91, E52 and Q128 are all within van der Waals contacts with W87, W34, and W125, respectively (Figure 7.2). These carbonyl-containing residues are likely the major contributor to the observed ultrafast quenching dynamics.

For hTrx, we recently reported a series of ultrafast quenching dynamics and identified one quenching process in ~100 ps which appears in both reduced and oxidized states and all mutants studied [196] Figure 7.3a shows three typical femtosecond-resolved transients of hTrx mutant C73S/D60G from the blue to red side of emission, in the reduced state where the disulfide bond (C32-C35) becomes two thiol groups. At 360 nm, the initial ultrafast dynamics can be represented by a long single exponential
Figure 7.1: (Top): Equilibrium structure of Sperm whale apomyoglobin mutant G129W from 1-ns MD simulations (left) and local configuration in close proximity to W129 with one possible quenching residue Q128 (right). (Bottom): Normalized, femtosecond-resolved fluorescence transients of apoMb G129W in short (left) and long (right) time ranges with a series of gated fluorescence emissions. Quenching of W129 fluorescence is highlighted with an ellipse at 360 nm.
Figure 7.2: Normalized, femtosecond-resolved fluorescence transients for apoMb mutants of A125W, K34W and K87W at 360 nm. Note that all long lifetime emission components are not included for clarity. These signals represent ultrafast quenching dynamics; see Table 7.1 for time scales and percentages. Also shown are local configurations around tryptophans with potential quenching residues.
decay with $\sim$100 ps (40%). Similar to apoMb, at shorter wavelengths of 305 and 330 nm, the initial ultrafast decays result from both hydration and quenching processes. Careful examination of the local structure (top panel in Figure 7.3) shows that one of the potential quenchers is the carbonyl group of intrahelical peptide bond(s). The quenching mechanism is the same as observed in the mutants of apoMb.

### 7.3.2 Ultrafast Quenching Dynamics by Sulfur-Related Residues

Figure 7.3b shows three typical femtosecond-resolved transients for oxidized hTrx at three typical wavelengths from the blue to the red side of emission: 305, 330, and 360 nm. The overall transients is apparently faster than those in reduced state (Figure 7.3a). Similarly, we observed a combination of hydration and quenching processes at the blue side and the quenching dynamics at the red side. Besides the quenching by the peptide bond as observed in reduced state with $\sim$100 ps, a major ultrafast quenching component was observed to occur with a time constant of 10 ps (40%). This quenching is due to the presence of the electrophilic intrahelical disulfide bond (C32-C35) in oxidized state as electron jumps from the indole ring to the disulfide bond.

Figure 7.4 shows the ultrafast quenching dynamics by the sulfur-containing residues at 360-nm emission for three other proteins (cutinase, SNase and apoCaM) without inclusion of the long fluorescence lifetime components. The overall pattern of femtosecond-resolved transients over entire emission wavelengths (not shown) is similar to that in Figure 7.3b. For cutinase, we further examined the disulfide-bond
Figure 7.3: (Top): X-ray crystallographic structure of oxidized hTrx (C73S/D60G) with the local configuration in close proximity to W31 with a disulfide bond (C32-C35) and a peptide bond between W31 and C32. (Bottom): Normalized, femtosecond-resolved fluorescence transients of reduced (a) and oxidized (b) hTrx at three typical gated emissions. The initial ultrafast decay highlighted at the 360-nm transient is from quenching. Note the different time scales in (a) and (b).
quenching. The single W69 is at a van der Waals distance from the interhelical disulfide bond (C31-C109) and the observed ultrafast quenching dynamics occurs in 28 ps (61%). Cysteine thiol group (-SH) also causes quenching of tryptophan fluorescence in proteins. For K133C mutant of SNase, the single W140 is in van der Waals contact with the thiol group of C133. The quenching dynamics has two decay components of 15 ps (51%) and 100 ps (36%). For T26W/T62C double mutant of apoCaM, the single W26 is at a van der Waals distance with the thiol group of C62. Similarly, the quenching dynamics can be described with a double-exponential decay of 17 ps (17%) and 100 ps (31%). Interestingly, so far no ultrafast quenching by the sulfide group (-SCH3) has been observed.

In more than 40 proteins examined so far, all ultrafast quenching dynamics are due to electron transfer from excited tryptophan to the electrophilic carbonyl group or sulfur-related residue. The results are summarized in Table 7.1 with time scales and relative quenching percentages. All quenching dynamics within 100 ps follow either a single- or double-exponential decay. The double-exponential dynamics can also be fitted by a stretched single-exponential decay, $a e^{-t/\tau^\beta}$, reflecting the dynamic heterogeneity of quenching processes. To relate observed ET quenching dynamics to donor-acceptor distances and their relative orientations, we performed MD simulations for all proteins in the ground state over a few nanoseconds and the distances listed in Table 7.1 are the possible donor-acceptor quenching separation. All the distances are about 3.5 Å for oxidized hTrx, in which the shortest separation between the indole ring of W31 and sulfur atom of the disulfide bond is ~5.3 Å. Similar donor-acceptor pairs at larger distances would result in slower quenching dynamics longer
Figure 7.4: Normalized, femtosecond-resolved fluorescence transients at 360 nm for cuti-
nase (a), nuclease (b) and calmodulin (c). Note that all long lifetime emission components
have not been included for clarity. These signals represent ultrafast quenching dynamics;
see Table 7.1 for time scales and percentages. Also shown are local configurations around
tryptophans with potential sulfur-containing quenching residues. Note the different time
scales of (a) with (b) and (c).
7.3.3 Donor-Acceptor Orientation and Electron-Transfer Directionality

For all proteins studied here, we run MD simulations over one nanosecond and examined protein fluctuations with focus on donor-acceptor distances and relative orientations. Figure 7.5 shows 1-ns trajectories of donor-acceptor edge-to-edge distances of apoMb mutants with snapshots of their relative orientations. Most of the shortest distances are about 3.4 Å at van der Waals contacts but the fluctuations are clearly large, especially for mutants of K87W and G129W. For these distances, we also distinguish relative positions where the carbonyl group is located, i.e., closer to the benzene (B) or pyrrole side (P) of the indole ring (Figure 7.5). Recent quantum chemistry calculations found that the benzene ring has net negative charge and the pyrrole ring has net positive charge in the excited state indole [105, 221]. Thus, for efficient electron transfer from the indole ring to the acceptor, the carbonyl group should be in close proximity and oriented to the benzene ring.

Among four apoMb mutants, the carbonyl group of K34W stays mostly on the pyrrole side and such a configuration is not in favor of efficient electron transfer, leading to the least quenching percentage (18%). For mutant A125W, the carbonyl group always resides on the benzene side but the quenching percentage is only 29% with a long time of 85 ps. We also calculated the excited-state minimum distance and noticed that the separation between Q128 and W125 increases by 0.5-1.0 Å, leading to slower quenching dynamics. More importantly, the low quenching percentage must
Figure 7.5: Trajectory fluctuations of edge-to-edge distances between indole ring and interresidue carbonyl oxygen atom from MD simulations for four apoMb mutants of K87W, G129W, A125W and K34W. The red dotted line (B) indicates that the carbonyl group stays closer to the benzene side of the indole ring while the blue dotted line (P) indicates that the carbonyl group resides on the pyrrole side. The green lines represent possible quenching distances. One typical quenching orientation for each mutant is given on the right side. The blue arrows show possible fluctuations, resulting in dynamic quenching heterogeneity.
result from the sideways orientation of the carbonyl group relative to the benzene ring. From the optimal molecular orbital overlap, the most efficient ET configuration is that the carbonyl group lies on top of the benzene ring. Thus, K87W, G129W and K34W have faster quenching dynamics, consistent with our observation (Table 7.1). Therefore, the observed electron transfer has directionality, following the maximum molecular orbital overlap. For G129W, we observed 47% quenching percentage because of the significant percentage of time the carbonyl group stays on top of the benzene ring. However, for K87W, we observed as large as 52% quenching percentage but over 1-ns simulations the time of the carbonyl group staying at the benzene side is less than at the pyrrole ring. Other than limited simulation time, another possible reason is the quenching from the carbonyl group of intrahelical peptide bonds.

Recent extensive analysis of 500 high-resolution protein structures revealed [203] that aromatic residues also have a tendency to interact with the carbonyl oxygen atom of peptide bonds. For an aromatic residue at position i of a protein sequence, the most possible intrahelical positions of interacting peptide bonds are i-4, i-1 and i+1. Figure 7.6 shows all distances of indole ring with peptide bonds within 4 Å. For K87W, there is significant time for the carbonyl group (i) to orientate toward the benzene ring, leading to more quenching percentage. For G129W, the contribution is minor due to extensive orientation (i-4) at the sideways pyrrole ring. For A125W, a certain quenching contribution could be from the carbonyl group (i). For K34W, all the carbonyl groups are beyond 4 Å(not shown) and the quenching contributions from peptide bonds are negligible.
Figure 7.6: Trajectory fluctuations of edge-to-edge distances within 4 Å between indole ring of tryptophan residue at position i and carbonyl oxygen atom from its neighboring peptide bonds positioned at i-4, i-1, i, i+1 from MD simulations for three apoMb mutants of K87W, G129W and A125W, hTrx in reduced state, nuclease, apoCaM, and cutinase in oxidized state. The red dotted line (B) indicates the carbonyl group closer to the benzene side of the indole ring while the blue dotted line (P) indicates the carbonyl group on the pyrrole side. The green lines represent possible quenching distances. One typical quenching orientation for each protein is given on the right side.
For all sulfur-related quenching proteins, besides the quenching from sulfur-containing residues which are presented below, we first examine the quenching possibility from the carbonyl group of peptide bonds. As shown in Figure 7.6 for reduced hTrx, the carbonyl group (i) orientates sideways and spends a large fraction of time with the benzene ring at a distance of 3.8 Å, resulting in longer quenching dynamics in 100 ps but with a significant percentage. As shown below, cysteine C32 could also contribute to quenching of tryptophan fluorescence. For SNase, the carbonyl group (i-1) partially orientates at the top of the benzene ring but with large fluctuation distances, which may result in quenching contributions to the total 87%. For apoCaM, the carbonyl group always lies on the pyrrole side and the quenching contribution is insignificant. Similarly, for cutinase the carbonyl groups (i-1 and i-4) also lie at the pyrrole side and the quenching contributions are minor. For oxidized hTrx, all the carbonyl groups are beyond 4 Å (not shown). The ultrafast quenching dynamics in 10 ps is not from the peptide bonds and is mainly from the disulfide bond.

Figure 7.7 shows the distances and orientations between the indole ring and sulfur-related groups for five proteins. Surprisingly, all sulfur-related residues are mainly located at the benzene side, resulting in significant quenching. For oxidized hTrx of the wide type and mutants, the quenching dynamics is ultrafast within 10-17 ps and the quenching percentage is 40-70%, respectively [196]. The donor-acceptor configuration is very special; the average distance is about 5.3 Å, significantly larger than van der Waals contact, but the disulfide bond is titled at top of the benzene ring. We increased our MD simulation time to 6 ns and the resulting distances and orientations are nearly invariant. This geometrical configuration is very similar to the
classic complex system of benzene with an iodine molecule [222]. In analogy to I-I bond breakage induced by charge separation from benzene to iodine, electron-transfer quenching from tryptophan to the disulfide bond leads to S-S bond cleavage. Here, the donor-acceptor orientation is ideal and electron transfer is directional. For cutinase, the configuration is drastically different. The donor-acceptor minimum distance is 3.4 Å at van der Waals contact but the disulfide bond lies sideways in an unfavorable orientation, leading to a long quenching time of 28 ps. Once again, it shows directionality for short range electron transfer. In addition, both proteins have high quenching percentages, probably due to the relatively rigid configurations and less fluctuations as also shown by our simulations (Figure 7.7).

ET reactions in protein matrix can be mediated by local solvent polarity and ET rate has been reported to increase in higher polar solvents [223]. In oxidized hTrx, the ET reaction takes place in the active site that has great solvent exposure as indicated by its crystal structure and surface-type emission peak of W31 at 340 nm. We have measured the hydration dynamics in the active site using W31 as the fluorescence probe and the active-site hydration dynamics were found to occur in 0.53-0.67 ps (70%) and 10-13 ps (30%) [196], on the time scale similar to the ET quenching reaction between W31 and the disulfide bond C32-C35. During electron transfer, the mobile water molecules at the active site can instantaneously reorganize the local hydrogen-bond network to facilitate the ET reaction. However, W69 in cutinase is shielded from the aqueous environment with its emission maximum at 332 nm and
Figure 7.7: Trajectory fluctuations of edge-to-edge distances between indole ring and sulfur atom of related residues from MD simulations for hTrx in reduced and oxidized states, cutinase in oxidized state, nuclease and apoCaM. The red dotted line (B) indicates the sulfur-related group closer to the benzene side of the indole ring while the blue dotted line (P) indicates the sulfur-containing residue on the pyrrole side. Note the extensive favorable orientation of the sulfur quencher closer to the benzene side. The green lines represent possible quenching distances. One typical quenching orientation for each protein is given on the right side. The blue arrows show possible fluctuations, resulting in dynamic quenching heterogeneity.
the solvation dynamics is expected to be longer [30]. The lack of instant solvent reorganization likely slows down the ET reaction.

For SNase, besides possible quenching by the peptide bond (i), the quenching by cysteine C133 must be dominant because the thiol group is in van der Waals contact and extensively orientates at top of the benzene ring, resulting in a significant quenching percentage of 87%. For apoCaM, the thiol group is mostly in a favorable ET orientation at top of the benzene ring, leading to a 48% quenching percentage. For reduced hTrx, the orientation between the thiol group and the benzene ring is favorable for quenching but the distance fluctuates significantly, which may result in a minor quenching contribution.

7.3.4 Dynamic Quenching Heterogeneity and Multiple Time Scales

Protein fluctuations cover a wide time range from picosecond to millisecond. The sidechain motions are controlled by hydrating water molecules on the picosecond time scale, similar to the relaxation in glasses [8], as shown by our recent extensive hydration dynamics studies [30]. Protein loops and secondary-structure segments mostly fluctuate in the nanosecond time regime. Protein local domains are slaved by bulk-water fluctuations on the time scale from nanosecond to millisecond, as recently proposed in analogy to the relaxation in glasses [8]. Thus, the quenching dynamics of tryptophan fluorescence is heterogeneous and occurs on multiple time scales. Experimentally, for a typical transient averaged in a few hours we sampled all possible quenching configurations and the resulting fs-resolved transient would show multiple
exponential decays. Here we focused on initial ultrafast quenching components within 100 ps and clearly the quenching dynamics could extend to beyond 100 ps as we fit those here as effective lifetimes.

For all proteins studied here, ultrafast quenching heterogeneity can come from a single or multiple quenching acceptors (-C=O and/or -S-) with large conformation fluctuations. For apoMb mutants A125W and K34W, we observed a single exponential decay in tens of picoseconds, by the carbonyl group only from the sidechains and not from the peptide bonds, consistent with our MD simulations (Figure 7.5 and 7.6). For G129W, the quenching is only from the residue’s carbonyl group, again not the peptide bond, but the sidechain has large fluctuations (Figure 7.5), leading to an apparent double-exponential decay. For K87W, the quenching also follows a double-exponential decay probably resulting from two carbonyl groups of the sidechain and the peptide bond (i) (Figure 7.6).

For reduced hTrx, the quenching is caused by the peptide bond (i) (Figure 7.6) and the cysteine residue C32 (Figure 7.7). As reported before [196], the 100-ps quenching dynamics was also observed in the oxidized state and other mutants. We believe that the carbonyl group is the main quencher. For both SNase and apoCaM, the dominant quenching is from the thiol group and the large conformation fluctuations result in an apparent double-exponential decay (Figure 7.7). The quenching by the peptide-bond carbonyl groups is minor (Figure 7.6). For both oxidized hTrx and cutinase, the local structures are more rigid due to the disulfide bond and the quenching dominantly follows a single-exponential decay within a few tens of picoseconds, leading to S-S
bond cleavage.

7.4 Conclusions

In this chapter, we discussed our systematic studies of ultrafast quenching of tryptophan fluorescence in proteins within 100 ps. With site-directed mutagenesis, we placed tryptophan to desired positions or altered its neighboring residues to screen potential ultrafast quenching groups among twenty amino acid residues and peptide backbones. With femtosecond resolution, we observed several ultrafast quenching residues, mainly the carbonyl group and sulfur-containing residues. The former are identified to be glutamine (Q) and glutamate (E) residues. The similar residues of asparagine (N) and aspartate (D) showed longer quenching dynamics beyond 100 ps [196], probably due to the shorter sidechain length and less flexibility. Although recent studies emphasize the peptide-bond quenching of tryptophan fluorescence [188], it seems that the time scale is mostly longer than 100 ps in proteins. The latter of sulfur-containing residues is determined to be the disulfide bond (S-S) and cysteine residue (C). The sulfide group of methionine shows a weak quenching ability probably due to less electrophilicity. The observation of ultrafast quenching by the carbonyl group and sulfur-containing residues surprisingly coincides with the recent finding of weak interactions between aromatic residues with these two quenching groups which are extensively found in high-resolution x-ray structures. Although such weak interactions no doubt increase protein stability, the ultrafast quenching to
efficiently dissipate UV energy by electron-transfer photocycle through these interaction pairs may have significant biological implications.

MD simulations were further performed to provide more quantitative understanding of the quenching dynamics. We observed strong correlations of ET quenching dynamics with donor-acceptor orientations. The electron acceptor favors its orientation to the negative-charged benzene ring of excited indole chromophore. With optimal molecular orbital overlap, the ET quenching at van der Waals distance has directionality with the acceptor on top of the benzene ring other than the sideways orientation. We also observed the correlation of sidechain flexibility with quenching heterogeneity. The dynamic heterogeneity can also be described by a stretched-exponential decay model. More flexible the protein is, more stretched the dynamics. Thus, the identification of these ultrafast quenching residues, determinations of their time scales, and elucidation of various quenching correlations with configurations from extensive screening of more than 40 proteins, are significant to the optical studies of protein dynamics and enzyme catalysis as well as to rational protein design.
Table 7.1: Ultrafast quenching of tryptophan fluorescence in proteins.

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Quenching Pair</th>
<th>Distance (Å)(^a)</th>
<th>Time (ps)(^b)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken Apocalmodulin</td>
<td>T26W/T62C</td>
<td>3.5</td>
<td>17;100(51/0.59)</td>
<td>17;31(48)</td>
</tr>
<tr>
<td>Staphylococcus Nuclease</td>
<td>K133C</td>
<td>3.5</td>
<td>15;100(33/0.66)</td>
<td>51;36(87)</td>
</tr>
<tr>
<td>Fusarium solani Cutinase</td>
<td>Wild type (Oxi.)</td>
<td>3.4</td>
<td>28</td>
<td>61</td>
</tr>
<tr>
<td>Fusarium solani Cutinase</td>
<td>W69:(C31-C109)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Thioredoxin</td>
<td>C73S/D60G (Oxi.)</td>
<td>5.3</td>
<td>10</td>
<td>40</td>
</tr>
<tr>
<td>Human Thioredoxin</td>
<td>C73S/D60G (Red.)</td>
<td>3.8</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>Human Thioredoxin</td>
<td>W31:(C32-C35)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Thioredoxin</td>
<td>W31:Peptide Bond</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm whale Apomyoglobin</td>
<td>K87W</td>
<td>3.4</td>
<td>4.1;79(31/0.63)</td>
<td>24;28(52)</td>
</tr>
<tr>
<td>Sperm whale Apomyoglobin</td>
<td>G129W</td>
<td>3.4</td>
<td>3.1;82(36/0.62)</td>
<td>19;28(47)</td>
</tr>
<tr>
<td>Sperm whale Apomyoglobin</td>
<td>A125W</td>
<td>3.4</td>
<td>85</td>
<td>29</td>
</tr>
<tr>
<td>Sperm whale Apomyoglobin</td>
<td>K34W</td>
<td>3.4</td>
<td>51</td>
<td>18</td>
</tr>
</tbody>
</table>

\(^a\) All emission maxima (λ) and time constants (τ) are the units of nanometers and picoseconds, respectively. \(^b\) The construction of the emission spectrum at time zero (λ₀) includes the quenching component.
CHAPTER 8

ULTRAFAST STUDIES OF MOLECULAR RECOGNITION BY CALMODULIN

8.1 Introduction

We have thus far garnered a solid understanding of hydration dynamics in simple protein systems or protein mimics. We have characterized the time scales of hydration dynamics in various systems and determined the physical origins of these time scales. Hydration dynamics in proteins is extremely sensitive to the local environments, and this property can be utilized for understanding biologically relevant questions in systems with more complexity. The main objective of this chapter is to elucidate the mechanism of molecular recognition in calmodulin using the methods of femtosecond-resolved hydration dynamics and resonance energy transfer.

Calmodulin (CaM) is a small protein with approximately 148 residues and highly acidic (pI≈3.7). The primary function of calmodulin in all eukaryotic cells is to act as an intracellular Ca^{2+} sensor protein that relays Ca^{2+} signal to various downstream calcium-sensitive enzymes through protein-protein interactions in a Ca^{2+}-dependent
manner [224]. Figure 8.1 shows the structures of calmodulin in three different conformational states: apoCaM (CaM free of calcium), Ca$^{2+}$-CaM (CaM saturated with calcium), and CaM-Target (Ca$^{2+}$-CaM in complexation with a target protein or protein mimic). Structurally, CaM consists of two homologous globular domains linked by a short sequence of residues. These two domains each contain a pair of helix-loop-helix Ca$^{2+}$-binding motifs, and CaM can bind four Ca$^{2+}$ ions in total. In the absence of Ca$^{2+}$, the linker sequence (D78-T-D-S81) is flexible and does not form any regular secondary structure [225,226]. When saturated with calcium, Ca$^{2+}$-CaM is capable of recognizing and binding a broad spectrum of target proteins of various different structures, which has been widely attributed to CaM’s intrinsic conformational flexibility. High resolution NMR structures of both apoCaM [225,226] and Ca$^{2+}$-CaM [227–230] suggested that the conformational flexibility originates from the dynamically disordered linker region between the C- and N-terminal domains. CaM undergoes dramatic structure rearrangement during Ca$^{2+}$-binding and target recognition. Each helix pair within the helix-loop-helix motif changes from a nearly antiparallel alignment in apo-CaM to an almost perpendicular alignment in Ca$^{2+}$-CaM. The structure change during calcium binding also exposes the normally deeply buried hydrophobic methyl groups of methionine residues; upon encountering with its target proteins, its C- and N-terminal domains come close to each other and form a hydrophobic channel with the CaM-binding domain from the target protein engulfed inside as an $\alpha$-helix; see Figure 1. CaM binds to calcium differently with and without the presence of its target proteins. In the absence of target proteins, Ca$^{2+}$ binds in a cooperative manner to the two Ca$^{2+}$-binding sites in the C-terminal domain and then to the two Ca$^{2+}$-binding sites in the N-terminal domain [231,232]. The presence
of target proteins dramatically increases the Ca$^{2+}$-binding affinity and the binding of Ca$^{2+}$ by CaM becomes totally cooperative [233, 234].

Because of its physiological implications, CaM has been extensively studied with various experimental approaches. The central linker region and hydrophobic pocket of CaM were found to be more flexible than were previously thought through the analysis of NMR relaxation data and residual dipolar couplings [228–230]. Frequency-domain fluorescence spectroscopy experiments suggested that the conformation of CaM is highly heterogeneous and this conformational heterogeneity likely is due to the intrinsic flexibility of the central linker sequence [235]. Recently, the existence of multiple conformations of CaM was directly established by Johnson and coworkers using
single-molecule fluorescence resonance spectroscopy [236–238]. Using translational-librational-screw analysis and multi-conformer models of protein disorder to refine the crystal structure, Wilson and Brunger were able to unveil a high degree of structural plasticity of CaM in their determination of the crystal structure of Ca$^{2+}$-CaM [239].

It is commonly believed that CaM’s inherent conformational flexibility directly contributes to its recognition diversity, but the molecular origin of such structural plasticity remains elusive. Water molecules in the immediate vicinity of CaM clearly play an important role in maintaining the conformational flexibility of CaM and mediating the processes of Ca$^{2+}$-binding and target protein recognition. However, a detailed account of hydration dynamics in CaM simply lacks; and we will systematically survey hydration dynamics in all four calcium-binding sites and the central linker region in three different conformational states of calmodulin: apoCaM, Ca$^{2+}$-CaM and CaM:Target. Because CaM is activated by calcium binding, understanding the functional diversity of CaM also hinges on a detailed understanding of the interaction between Ca$^{2+}$ and CaM. We will use hydration dynamics to probe whether there exists communications (allosteric regulation) between different calcium-binding sites and use resonance energy transfer experiments to determine whether the information is propagated through large scale conformational transition or small scale side chain fluctuations. Because the two globular N- and C-domains bind to calcium with dramatically different binding affinities, we can vary the calcium levels to primarily occupy the high-affinity calcium-binding domain and then measure the hydration dynamics in the low affinity calcium-binding sites; this result will be compared with that from the same calcium-binding sites when calmodulin is totally free of calcium,
which will allow us to the determine the effect of the calcium binding to the high-afﬁnity calcium-binding sites on low afﬁnity calcium-binding sites. Likewise, we will measure and compare the hydration dynamics in the high afﬁnity calcium-binding sites when the low afﬁnity calcium-binding sites are free of and occupied with calcium ions; this will allow us to probe the effect of calcium binding to low afﬁnity sites on the high-afﬁnity counterparts. For both cases, possible large scale conformation transitions will be tracked using carefully designed resonance energy transfer pairs to determine whether large scale conformational change is incurred during domain communications.

8.2 Materials and Methods

Five single tryptophan-containing calmodulin mutants (T26W, T62W, Y99W, Q135W and S81W) were designed to site-speciﬁcally measure the local hydration dynamics in all four calcium-binding sites and the central linker region; see Figure 8.2a. Figure 8.2b shows the five double mutation constructs (T26W/A57C, T62W/S81C, Y99W/L69C, Y99W/S81C and Y99W/G132C) for resonance energy experiments. All chicken CaM mutants were made using the plasmid generously provided by Prof. A.J. Wand (Univ. of Pennsylvania, USA). These proteins were over-expressed and puriﬁed using the method published elsewhere [212] and a detailed account of the protein preparation and puriﬁcation protocol can also be found in Appendix A. All the double mutants (T26W/A57C, T62W/S81C, Y99W/L69C, Y99W/S81C and Y99W/G132C) were further covalently labeled with 1,5-IAEDANS to the cysteine residue, and the readers are referred to Appendix B for a detailed description of the procedure to label CaM mutants with ≥95% labeling efﬁciencies. Calcium removal from CaM was
achieved by passing the protein solution in the presence of 10-20 mM EDTA through a Sephadex G-25 desalting column. For studies involving CaM:Target complexes, peptide fragments derived from two different CaM target proteins were used. Peptide smMLCk from the CaM-binding domain of smooth muscle myosin light chain kinase (acetyl-A-R-R-K-W-G-K-T-G-H-A-V-R-A-I-G-R-L-S-S-S-NH₂) was synthesized with solid phase peptide synthesis using automatic Fmoc-chemistry; the peptide was purified by reverse-phase HPLC on a C-18 column, and a linear gradient from 20% to 40% of buffer B (buffer B: 0.1% TFA, 90% (v/v) acetonitrile in H₂O) in buffer A (buffer A: 0.1% TFA, 1% acetonitrile in H₂O) in 30 minutes. Peptide CaMKK from the CaM-binding domain of CaM-dependent kinase kinase (acetyl-V-R-V-I-P-R-L-D-T-L-I-L-V-K-A-M-G-H-R-K-R-F-G-N-P-F-R-NH₂) was also synthesized with solid phase peptide synthesis but using manual Boc-chemistry; this peptide was purified by reverse-phase HPLC on a C-18 column, and a linear gradient from 28 to 42% of buffer B in buffer A′ (buffer A′: 0.1% TFA in H₂O). For most experiments, the buffer was 50 mM Hepes, pH 7.5. However, a buffer of 50 mM Tris-HCl, 2 mM CaCl₂ and pH 7.5 was used for the preparation of CaM:CaMKK complex because of the low solubility of peptide CaMKK in 50 mM Hepes, pH 7.5. Briefly, peptide CaMKK stock solution of 300 µM was immediately prepared prior to use in 50 mM Tris-HCl, 2 mM CaCl₂ and pH 7.5; this peptide solution was then used to titrate CaM (∼600 µM) in the same buffer condition and the complex formation was monitored by changes in the steady-state fluorescence emission maximum of tryptophan in the protein; the protein solution was considered to be saturated with peptide CaMKK when the steady-state tryptophan fluorescence emission maximum did not show further change; a minor population of unbound aggregated CaMKK was removed by centrifugation and no
noticeable change in the tryptophan fluorescence emission maximum was observed after centrifugation. The procedure to prepare the CaM:smMLCK complex is only slightly different from that of CaM:CaMKK because the tryptophan is within the sequence of the peptide. One needs to titrate the peptide solution with calmodulin protein until the steady-state fluorescence emission maximum of tryptophan shows no further change. For all single tryptophan-containing mutants, we have characterized the steady-state fluorescence emissions at various calcium levels and in the presence of the peptide CaMKK. We have characterized the energy transfer efficiencies of all five energy transfer constructs both in the absence and presence of peptide CaMKK (data not shown). All time-resolved measurements were carried out on the femtosecond-resolved fluorescence up-conversion apparatus as detailed in chapter 2.

8.3 Preliminary Results and Discussions

8.3.1 Femtosecond-Resolved Transients and Local Hydration Dynamics

Hydration dynamics in all four calcium-binding sites (T26W, T62W, Y99W and Q135W) and the central linker region were measured in three different conformational states of calmodulin: apoCaM, Ca$^{2+}$-CaM and CaM:CaMKK. The steady-state emission maxima vary from 337nm in T62W:CaMKK to 352 nm in T26W:CaMKK, indicating that these tryptophans are located at the protein surfaces with great exposure to the aqueous environments. Figure 8.3 shows the normalized fluorescence emission spectra of apoT62W, Ca$^{2+}$-T62W and T62W:CaMKK. Because all the tryptophans (except for the one in S81W) are closely located at the calcium-binding sites, the unusually long emission maxima (≥350 nm) observed in Ca$^{2+}$-T26W, T26W:CaMKK
Figure 8.2: (a) Locations of tryptophans for studying hydration dynamics. The tryptophans are colored in red, and each mutant only contains one single tryptophan. (b) Double mutation constructs for resonance energy transfer experiments. The donor positions are colored in blue while the acceptor positions in yellow. For each construct, tryptophan will be the donor, and the acceptor 1,5-IAEDANS will be covalently linked to the cysteines; see text for detail.
and Ca\textsuperscript{2+}-Q135W are likely due to the cation-π interactions between the tryptophans and calcium ions.

Figure 8.4-8.6 show the femtosecond-resolved fluorescence transients of W62 at several typical wavelengths gated from the blue to the red side of the emission spectrum in apoT62W, Ca\textsuperscript{2+}-CaM and CaM:CaMKK respectively. With the methodology from chapter 2, we were able to construct the overall and lifetime associated emission spectra; hydration correlation functions were subsequently derived. The preliminary hydration dynamics results are summarized in Table 8.1. In most cases, the hydration correlation function can be sufficiently described with a double exponential decay with time constants in 2.1-4.9 ps and 64-171 ps. The physical origin of the two time
constants has been discussed in detail in previous chapters and elsewhere [30]. The hydration dynamics represent the fluctuations of ordered water molecules inside the hydration layer: the first ultrafast dynamics in a few picoseconds is directly from the initial libration and hindered rotation of local water molecules; the second hydration dynamics represents subsequent water network rearrangement after initial relaxation and this motion is tightly coupled with protein fluctuations. For apoY99W, we found that the hydration dynamics is clearly a triple-exponential decay and more work needs to be done to understand whether the slowest component results from protein solvation. In all cases, the initial decay component in several picoseconds dominates the decay of the hydration correlation function, and this is consistent with the fact that all the tryptophan probes here are highly exposed to aqueous environments. Table 8.2 summarizes the emission maxima and other time constants. We also noticed that the fluorescence transients for some mutants need three effective fluorescence lifetimes, and this is likely due to inherent conformational flexibility of the protein.

Table 8.1: Results obtained from the hydration correlation functions $c(t)$ of apoT26W, apoS81W, apoY99W, apoQ135W and T26W in three different conformational states. $^a$

<table>
<thead>
<tr>
<th>Protein</th>
<th>$\tau_1$, ps</th>
<th>$c_1$</th>
<th>$\tau_2$, ps</th>
<th>$c_2$</th>
<th>$\tau_3$, ps</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoT26W</td>
<td>4.5</td>
<td>70</td>
<td>64</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>apoT62W</td>
<td>2.2</td>
<td>71</td>
<td>84</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$-T62W</td>
<td>2.1</td>
<td>80</td>
<td>65</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>T62W:CaMKK</td>
<td>4.9</td>
<td>92</td>
<td>108</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>apoS81W</td>
<td>3.2</td>
<td>81</td>
<td>95</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>apoY99W</td>
<td>4.3</td>
<td>77</td>
<td>68</td>
<td>19</td>
<td>780</td>
</tr>
<tr>
<td>apoQ135W</td>
<td>4.1</td>
<td>71</td>
<td>171</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ All the hydration correlation functions were fitted with $c(t) = c_1e^{-t/\tau_1} + c_2e^{-t/\tau_2} + c_3e^{-t/\tau_3}$, where $c_1 + c_2 + c_3 = 1$. 

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Figure 8.4: Normalized, femtosecond-resolved fluorescence transients of W62 in apoT62W in both the short (Left) and long (Right) time ranges with a series of gated fluorescence emissions. Note the fast hydration at the blue side.
Figure 8.5: Normalized, femtosecond-resolved fluorescence transients of W62 in Ca\textsuperscript{2+}-T62W in both the short (Left) and long (Right) time ranges with a series of gated fluorescence emissions. Note the fast hydration at the blue side.
Figure 8.6: Normalized, femtosecond-resolved fluorescence transients of W62 in T62W:CaMKkin both the short (Left) and long (Right) time ranges with a series of gated fluorescence emissions. Note the fast hydration at the blue side.
8.4 Perspectives and Future Experiments

We have presented here the overall objectives of the calmodulin project. We also have surveyed the hydration dynamics at all four calcium-binding sites and the central linker region. Clearly, more work is needed to understand the observed hydration dynamics partially listed in Table 8.1. As we discussed in the introduction section, we need to continue the survey of hydration dynamics at all four calcium-binding sites and the central linker region at intermediate calcium levels; these experiments together with results presented here will help dissect the communications between different calcium-binding sites. We also need to conduct time-resolved resonance energy transfer experiments at all calcium levels and in the presence of target protein mimic. For wild-type calmodulin, the high-affinity calcium-binding sites are within the C-terminal domain, but there have been reports that point mutations could modify the relative calcium-binding affinities between domains and even reverse the calcium-binding sequence. Experiments should be conducted to determine the calcium-binding sequence for all the mutants studied here. We also have designed several calmodulin mutants that have different fluorescent probes at the same position, and these mutants include T62W, T62C-IAEDANS, S81W and S81C-IAEDANS. Experiments need to be done with these mutants to determine whether hydration dynamics has a profound dependence on the nature of probe.
Table 8.2: Emission maxima and time constants of tryptophans in various calmodulin mutants.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Proteins</th>
<th>$\lambda_{ss}$</th>
<th>$\lambda_0$</th>
<th>$\lambda_{sc}$</th>
<th>$t_{sc}$</th>
<th>$t_{ss}$</th>
<th>$\tau_{f1}$</th>
<th>$\tau_{f2}$</th>
<th>$\tau_{f3}$</th>
<th>$\lambda_{f1}$</th>
<th>$\lambda_{f2}$</th>
<th>$\lambda_{f3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoT26W</td>
<td>348</td>
<td>323</td>
<td>339</td>
<td>350</td>
<td>460</td>
<td>1040</td>
<td>400</td>
<td>1900</td>
<td>5100</td>
<td>325</td>
<td>327</td>
</tr>
<tr>
<td>apoT62W</td>
<td>343</td>
<td>320</td>
<td>336</td>
<td>342</td>
<td>560</td>
<td>2400</td>
<td>550</td>
<td>1700</td>
<td>3740</td>
<td>325</td>
<td>327</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}-T62W</td>
<td>343</td>
<td>320</td>
<td>336</td>
<td>349</td>
<td>410</td>
<td>2200</td>
<td>510</td>
<td>1300</td>
<td>5000</td>
<td>329</td>
<td>331</td>
</tr>
<tr>
<td>T62W:CaMKK</td>
<td>337</td>
<td>320</td>
<td>331</td>
<td>337</td>
<td>580</td>
<td>520</td>
<td>300</td>
<td>650</td>
<td>329</td>
<td>321</td>
<td>343</td>
</tr>
<tr>
<td>apoS81W</td>
<td>346</td>
<td>322</td>
<td>338</td>
<td>347</td>
<td>680</td>
<td>2000</td>
<td>400</td>
<td>1800</td>
<td>6700</td>
<td>323</td>
<td>323</td>
</tr>
<tr>
<td>apoY99W</td>
<td>346</td>
<td>319</td>
<td>341</td>
<td>349</td>
<td>2900</td>
<td>2600</td>
<td>2200</td>
<td>5800</td>
<td>328</td>
<td>348</td>
<td></td>
</tr>
<tr>
<td>apoQ135W</td>
<td>347</td>
<td>320</td>
<td>339</td>
<td>342</td>
<td>1300</td>
<td>6300</td>
<td>2500</td>
<td>6700</td>
<td>326</td>
<td>349</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} All emission maxima ($\lambda$) and time constants ($\tau$) in units of nanometers and picoseconds respectively.
APPENDIX A

CALMODULIN PURIFICATION PROTOCOL

This protocol is for the preparation and purification of a 4 L cell culture.

1 Calmodulin DNA Transformation

1.1 Before starting, prepare the water bath at 42°C, preheat 2 mL of LB medium in the water bath to 42°C, and place a LB-Ampicillin plate at room temperature.

1.2 Thaw the BL21(DE3) competent cell on ice; make sure to store the competent cells on ice at all times.

1.3 Pipet 1 µL of calmodulin DNA and gently mix with 50 µL of BL21(DE3) in a 1.5 mL eppendorf tube.

1.4 Incubate the reaction on ice for 30 minutes.
1.5 Heat-shock the reactions in the 42°C water bath for exactly 45 seconds, and then place the reactions immediately back on ice and incubate for 2 minutes.

1.6 Add 0.9 mL of the preheated LB medium to the transformation reaction, and incubate the reactions at 37°C for 1 hour with shaking at 220-250 rpm.

1.7 Centrifuge the reactions with a tabletop centrifuge at 14k rpm for 1 minute, decant the supernatant and resuspend the cell pellet with 200 µL of LB medium.

1.8 Use glass spreader and rotating platform to plate 200 µL of the above cell suspension on the prewarmed LB-Ampicillin plate.

1.9 Incubate the plate overnight at 37°C, and ≥20 colonies are expected to develop on the plate after 16 hours.

2 Preparation of Escherichia coli

2.1 Prepare 50 mL of LB medium in a 150 mL flask, add 50 µL of 100 mg/mL Ampicillin, pick a single colony using a pipetman with sterile tip, pipet up and down several times to ensure cells are transferred to the medium in the flask, and incubate the culture overnight with shaking at 220-250 rpm.
2.2 The next morning, thaw IPTG; monitor the OD (optical density) of the overnight culture at 600 nm. When OD is $\geq 1.8$, add 1 mL of 100 mg/mL Ampicillin into 1 L M9 medium and then inoculate 10 mL of overnight culture into the Ampicillin-containing M9 medium.

2.3 Incubate the cultured M9 medium at 37°C with shaking at 220-250 rpm and monitor the OD.

2.4 Add 2 mL of 0.5 M IPTG to induce the cell growth when the OD is within 0.6-0.75. Note the final concentration of IPTG is always 0.5 mM.

2.5 Continue to incubate the cultured M9 medium at 37°C with shaking at 220-250 rpm for another 3-4 hours until the OD is $\geq 1.2$.

2.6 Harvest the cell culture with 50 mL centrifuge bottle for 15 minutes (A-10 rotor, 5K rpm), wash and combine the cell pellet with ice cold water, centrifuge for 5 minutes (A-10 rotor, 5K rpm), decant the supernatant and store the pellet in a 50 mL centrifuge bottle in freezer (-80°C).

3 Calmodulin Protein Purification

3.1 Thaw the cell pellet overnight on ice.
3.2 Resuspend gently the cell pellet in 40 mL of calmodulin lysis buffer (calmodulin lysis buffer: 50 mM MOPS, 100 mM KCl, 1 mM EDTA and 1 mM DTT).

3.3 Resuspend gently the cell pellet in 40 mL of calmodulin lysis buffer (calmodulin lysis buffer: 50 mM MOPS, 100 mM KCl, 1 mM EDTA and 1 mM DTT); add to the completely resuspended cell pellet 1.2 mL of 10 mg/ml lysozyme (lysozyme specific activity = 23,500 units/mg, the final concentration of lysozyme ≈ 300 µg/mL), and incubate the cell pellet suspension on ice for 30 minutes.

3.4 Sonicate the cell pellet suspension for 3-5 minutes (duty = 50%, output = 3), incubate the suspension on ice for 5 minutes and sonicate for another 3 minutes.

3.5 Centrifuge the lysate (cellular debris and fluid produced by lysis) 30 minutes (A-14 rotor, 14K rpm) at 4°C, collect carefully the supernatant with minimum disturbance to the cell pellet and store the cell pellet in the cold room until the whole purification is done.

3.6 Dilute the supernatant 2 fold with lysis buffer to a final volume of ~100 mL, add 500 µL of 1M CaCl$_2$ (the final concentration of CaCl$_2$ is ~5 mM).

3.7 Load ~50 mL of the supernatant on to the primary Phenyl-Sepharose 4B column pre-equilibrated with the loading buffer (loading buffer: 50 mM Tris-HCl, 1 mM CaCl$_2$ and pH 7.5). Note, the loading volume is determined by the capacity of the Phenyl-Sepharose 4B column, and in the current setting,
the maximum capacity of the column is only good for loading supernatant equivalent to 2 L cell culture.

3.8 Wash the column with 3-4 column volume of loading buffer, and collect the flow-through. Phenyl-Sepherose 4B is hydrophobic interaction chromatography (HIC) column, and after the extensive wash with 3-4 column of loading buffer, calmodulin protein will bind to the column through hydrophobic interaction and other unbound contaminants will be removed.

3.9 Wash the column with 4-6 column volume of high salt/wash buffer (Tris-HCl, 0.5 M NaCl, 1 mM CaCl$_2$ and pH 7.5), and collect flow-through. This will remove the loosely bound contaminants.

3.10 Wash the column with 3 column volumes of loading buffer to restore the column to low NaCl, and collect flow-through.

3.11 Elute calmodulin with elution buffer (elution buffer: 10 mM Tris-HCl, 10 mM EDTA, and pH 7.5), and collect the fractions with 100 drops/fraction.

3.12 Use protein assay (Bio-Rad Protein Assay) to identify and combine the fractions with calmodulin. Typically, the protein purity after the primary Phenyl-Sepharose 4B column is around 95%. Higher purity can be obtained by using a secondary Phenyl-Sepharose 4B column, which usually is a different column or the
same column but throughly cleaned.

3.13 Adjust the final CaCl$_2$ concentration of the combined fractions to 20 mM.

3.14 Reload the combined protein fractions to a pre-equilibrated secondary Phenyl-Sepharose 4B, repeat steps 3.8-3.10 to equilibrate and wash the column, elute calmodulin with elution buffer and collect the fractions with 100 drops/fraction.

3.15 Use protein assay (Bio-Rad Protein Assay) to identify the fractions with protein, run 15% SDS-PAGE gel to check the final purity of these fractions, and combine together the fractions with good purity ($\geq 99\%$).

3.16 Dialyze overnight the combined protein fractions against dialysis buffer (dialysis buffer: 20 mM Tris-HCl, 0.1 mM CaCl$_2$ and pH 7.0).

3.17 Aliquot in 1-mL fractions, quick-freeze with liquid nitrogen and store in freezer (-80°C).
APPENDIX B

CHEMICAL MODIFICATION OF CALMODULIN WITH 1,5-IAEDANS

The working procedure suitable for covalent attachment of the molecular probe 1,5-IAEDANS to the single cysteine-containing calmodulin mutant is outlined below. This protocol is a modification from the original one by Squier and colleagues [235]. It is expected to be working as well for conjugation of other thiol-reactive probe to proteins with limited modification.

1.1 Dissolve the protein at 250-350 µM in 20 mM Tris-HCl at pH 7.0-7.5 at room temperature. In this pH range, the protein thiol groups are sufficiently nucleophilic and react almost exclusively with 1,5-IAEDANS, while the more numerous protein amines are protonated and relatively less reactive.

1.2 Gently mix 2 mL of protein solution, 240 µL of 0.5 M EDTA and 10 ml of 20 mM Tris-HCl and 7.5 M GnHCl (guanidine hydrochloride) at pH 7.0-7.5 in a GeneMate 15ml Sterile Centrifuge Tube. It is important to make sure the final concentration of GnHCl in the mixture is ~6 M for complete denaturation of the protein. It is also optional at this stage to add 10 molar excess of TCEP for the full
reduction of disulfide bonds in the protein. If TCEP is added, the reaction mixture needs to be incubated at room temperature for \(~2\) hours with constant and gentle stirring. The reducing agent TCEP is recommended over DTT because it is not necessary to remove excess TCEP during conjugation with iodoacetamides or maleimides.

1.3 THIS STEP SHOULD BE HANDLED IN DARK. Dissolve appropriate amount (10 moles of dye for each mole of protein in the reaction mixture) of 1,5-IAEDANS in 100 \(\mu\)L of DMSO immediately prior to use, and prepare only as much dye solution as is needed. Because the reagent is extremely sensitive to light, the solution must be prepared in a dark room and be protected from illumination. Add the dye solution dropwise to the reaction mixture while it is stirring. Wrap the reaction tube with aluminum foil and allow the reaction to proceed for 8-10 hours at room temperature with constant and gentle stirring.

1.4 To get optimal degree of labeling, repeat step 1.3 one more time: dissolve appropriate amount of 1,5-IAEDANS in 100 \(\mu\)L DMSO, add all the dye solution to the reaction mixture, and leave the reaction to proceed for another 8-10 hours protected from light with constant and gentle stirring.

1.5 Add sufficient DTT (120 \(\mu\)L of 1 M DTT in this case) to the reaction mixture to consume excess 1,5-IAEDANS for 2 hours upon completion of the labeling reaction.
1.6 Separate the labeled protein with a Sephadex G-25 column, combine all the protein fractions and concentrate to the desired concentration. Measure the absorption of the protein solution, and calculate the degree of labeling using the following formula:

\[
\beta = \frac{A_{337} \epsilon_{280}^P}{A_{280} \epsilon_{337}^D - A_{337} \epsilon_{280}^D},
\]

where \(A_{337}\) and \(A_{280}\) are the respective absorbance at 337 nm and 280 nm of the absorption spectrum, and \(\epsilon_{280}^P\), \(\epsilon_{337}^D\) and \(\epsilon_{280}^D\) are the molar extinction coefficient of the protein at 280 nm, the dye at 337 nm and the dye at 280 nm respectively. Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) can also be used to determine the labeling efficiency, and the high labeling efficiency is shown in Figure B.1 for mutant Y99W/L69C-IAEDANS:
Figure B.1: The MALDI-TOF mass spectrum of calmodulin mutant Y99W-L69C labeled with 1,5-IAEDANS. The dominant peak with the mass of 17023 Da corresponds to the perfectly labeled Y99W-L69C with 1,5-IAEDANS.


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