Investigation of Electron Transfer and Solvation Dynamics in (6-4) Photolyase

THESIS

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Abstract

Electron transfer is involved in many enzymes which play crucial roles of a variety of biological functions. Mechanism of biological ET, especially construction of related theoretical models, has been investigated for around 40 years. A consensus has been reached that high efficiency ET in biomolecules is generally barrierless owing to their optimized structures and energetics through natural evolution. Among all biological ET processes, photoinduced ET (PET) has been intensely studied in photosynthetic systems and flavoproteins. These PET processes have been recognized to occur in non-equilibrium with dynamic solvation relaxation of excited-state cofactors and thus cannot be modelled by Marcus theory. Efforts on incorporation of solvation dynamics into ET rate theories have been made to account for experiment results, while experimental and computational studies, in turn, have been carried out to test the applicability of various theoretical models. So far, reaction-diffusion model, which was proposed for the first time by Sumi and Marcus, has been successfully used in photosynthetic systems to study dependence of non-ergodic PET on reaction free energy and reorganization energy. However, appropriateness of this model to account for temperature effect on ET rate, especially ET profile in cryogenic range, is still controversial.
Solvation dynamics in a broad temperature range from room temperature to 77.5K were studied by measuring steady-state and time-resolved fluorescence of the reduced cofactor FADH\(^-\) of (6-4) photolyase WT and H364A mutant. Steady-state emission spectra confirms that two major peaks arise from vibronic bands rather than from isomers due to the third peak which stands out at lower temperatures. Solvation correlation function extracted from time-resolved emission spectra reveals a relaxation process through protein active-site collective motion, which, in H364A, occurs in hundreds of picoseconds at high temperatures and slows down to nanosecond timescale at 210.5K and below. Electron transfer dynamics were fit with stretched exponential decay model. ET from FADH\(^+\) to adenine and to (6-4) photoprodut both show dichotomy of activation barrier throughout the whole temperature scale, whose mechanism, however, is still unknown. For the latter intermolecular ET, the transition in the stretching coefficient $\beta$ before and after 230K shows different mechanisms to the heterogeneity in H364A dynamics.
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Chapter 1. Introduction

1.1 Introduction to dynamic solvation of protein.

Life is indispensable of water. When a protein molecule is dissolved in water, the solvent molecules can be divided into two categories, bulk and biological water, distinguished by permittivity of water measured with dielectric relaxation spectroscopy at different frequency ranges. Biological water interacts with protein molecule and plays a crucial role in maintaining protein structure and function. Depending on its position relative to protein molecule, biological water can either be buried inside the protein or form hydration layers in the vicinity of the protein surface, the latter of which bridges protein surface and bulk water. Surface water is more mobile so that it can mediate interaction between protein and other functioning molecule, mostly potential substrates. Therefore, investigation of surface water, especially its dynamic properties, has been lasting for decades via a variety of methods.

As mentioned above, dielectric relaxation spectroscopy measures dielectric loss in a broad range of frequency from several MHz up to hundreds of GHz, and thus detects water motion occurring in well-separated timescales. However, two major problems may lead to controversy in peak assignment. On the one hand, the dielectric loss signal is often dominated by the tumbling motion of hydration and bulk water, whose wing
signal tends to interfere with other relaxation motions represented by weaker signals.\textsuperscript{13} On the other hand, for signal with multiple peaks close to each other, e.g., δ dielectric dispersion, ambiguity of peak separation and assignment still exist.\textsuperscript{14}

Neutron scattering gives not only temporal but also detailed spatial resolution of hydration water motions by probing average trajectories of hydrogen atoms.\textsuperscript{15} Observable time window of motion ranges from femtoseconds to nanoseconds, corresponding to motions spanning from low-frequency vibration to collective diffusion.\textsuperscript{16} To some extent, site specificity can be realized by deuterating particular interfering hydrogen atoms.\textsuperscript{17} Nevertheless, neutron scattering requires deuterated water or protein side chain, which may not reflect the real solvent or protein motions in pure H\textsubscript{2}O. Furthermore, neutron scattering can only detect interaction within short distance limited in the first solvation shell that extends by a spatial scale of up to 1nm, similar to the wavelength of thermal neutrons.\textsuperscript{16} Such a short-range restriction is also present in X-ray diffraction technique.\textsuperscript{18}

Nuclear magnetic resonance (NMR) determines solvation dynamics by spin-lattice relaxation time $T_1$ and spin-spin transverse relaxation time $T_2$.\textsuperscript{19,20} Besides, chemical shift of hydrogen can reveal structural information of hydration water network.\textsuperscript{21} Two methodologies are commonly used in NMR studies of solvation, i.e., nuclear Overhauser effect (NOE) which probes spin-spin coupling between water and protein protons, and magnetic relaxation dispersion (MRD) measurement of the water $^2$H and $^{17}$O atoms.\textsuperscript{22,23} Relaxation times, e.g., translational and rotational correlation time, has been
determined in large numbers of proteins, ranging from a few to tens of picoseconds. However, information gained from NMR signal is still relatively limited due to its incapability of determining sub-picosecond hydration dynamics.

Terahertz (THz) absorption spectroscopy measures absorption mostly within the frequency range of 0.3-3THz and therefore can probe solvation processes in as short as around 1ps and detect hydration water layer within the range of more than 20Å, much greater than the spatial limit allowed by neutron scattering and NMR. Such a broad spatial range contains not only static water bound proximate to protein surface in the first solvation shell but also more mobile dynamical water layers. Through determination of protein concentration dependence of change in terahertz absorption coefficient with respect to that of bulk water, an ensemble effect exerted by “THz defect” from protein absorption and “THz excess” from hydration water absorption, one can accurately determine the sizes of hydration shell.

One common disadvantage of all above methods is obvious: their results all represent ensemble solvation behavior on the whole or a large area of protein surface. In contrast, ultrafast fluorescence up-conversion technique determines local solvation dynamics with femtosecond time resolution by measuring transient fluorescence of site-specific optical probe, either intrinsic or extrinsic to the protein. After Franck-Condon excitation of the probe and before it fluoresces, energy of the excited state can be lowered through solvation (or more generally environmental) relaxation induced by dipole-dipole interactions between the excited-state probe and its surroundings,
resulting in Stokes shift of emission spectrum, whose dynamics can be described by solvation correlation function $C(t)$.\textsuperscript{5,30}

\begin{equation}
C(t) = \frac{\nu(t) - \nu(\infty)}{\nu(0) - \nu(\infty)}
\end{equation}

$v(0)$, $v(t)$ and $v(\infty)$ represent emission peak at initial moment, time $t$ and final moment at which molecules emit from equilibrium configuration of excited state.

In early times, extrinsic fluorescent dyes and synthetic amino acids were applied, e.g. fluorescein,\textsuperscript{31} eosin,\textsuperscript{32} coumarin derivatives,\textsuperscript{33-37} Aladan,\textsuperscript{38} etc. Given less perturbation to the protein conformation, the amino acid tryptophan has been sophisticatedly established as an intrinsic fluorescent probe in studying protein local solvation dynamics with distinct structural topology and environmental polarity.\textsuperscript{39} Other intrinsic probe includes native and modified cofactor of enzymes, e.g. phycocyanobilin chromophore in C-phycocyanin and allophycocyanin,\textsuperscript{40} Zn(II)-substituted porphyrin in cytochrome c,\textsuperscript{41,42} chromophore of green fluorescent protein variants\textsuperscript{43-45} and flavin cofactor in family of riboproteins.\textsuperscript{9,11} Investigation of dynamic solvation around the cofactor directly reflects motions of hydration water and protein residues in the active site and facilitates deeper understanding of structural mechanism to the corresponding enzymatic function.

1.2 Review on ET theories

1.2.1 Equilibrium ET
Electron transfer is involved in all redox reactions and plays a critical role in various processes, e.g., industrial electrolysis, photocurrent generation in solar cell, biological respiration chain, etc. Development of ET theory is therefore of great significance in understanding the above processes and improving ET efficiency associated with them by proper design and manipulation. In 1956, Rudolph Marcus proposed the famous Marcus theory assuming weak electronic coupling between donor and acceptor, and rapid dielectric relaxation of the solvent compared to ET timescale. Thermal equilibrium is maintained during the ET timescale. The non-adiabatic ET rate is expressed as follows.

\[ k_{ET} = \frac{|H_{DA}|^2}{n} \frac{1}{\sqrt{4\pi \lambda k_B T}} \exp \left( -\frac{(\lambda + \Delta G^0)^2}{4\lambda k_B T} \right) \]  \hspace{1cm} (1.2)

\( H_{DA} \) is electronic coupling between the electron donor and acceptor, \( \lambda \) and \( \Delta G \) are solvent reorganization energy and free energy of the reaction, respectively.

Another equilibrium-state ET theory was developed by Calef and Wolynes based on Kramers theory for steady-state rate of diffusive barrier crossing. The non-adiabatic ET rate can be expressed as follows.

\[ k_{ET} = \frac{D}{(2\pi k_B T / k)^{1/2}} \left[ \int_{x_1}^{x_2} e^{V(x)/k_B T} dx \right]^{-1} \]  \hspace{1cm} (1.3)
D is the diffusion constant along the harmonic potential energy surface (PES), whose energy is denoted by \( V(x) = k(x-x_1)^2/2 \), \( x_1 \) and \( x_2 \) represent coordinates of bottom of reactant PES and barrier saddle point, respectively.

1.2.2 Non-equilibrium ET

When the ET rate is comparable to solvation relaxation rate, ET may occur before solvation is completed and the system reaches thermal equilibrium, i.e., relaxation of the system cannot explore the entire phase space before ET takes place, which is statistically non-ergodic and results in the breakdown of canonical-ensemble Gaussian statistics in terms of energy gap between product and reactant. Hence, modification to the traditional Marcus theory is necessary.

Sumi and Marcus\(^{58, 59}\) separated the reorganization energy into the fast intramolecular and slow solvent relaxation energies. In their theory, diffusion-reaction formalism was applied to incorporate the solvation effect, i.e., the initial reactant state diffuses along the slow relaxation coordinate \( X \) on harmonic PES towards the transition state before ET occurs as a kinetic sink. At each \( X \), relaxation along the intramolecular vibrational coordinate, \( q \), was considered very rapid compared to ET, and therefore equilibrium on this coordinate is always maintained during the ET timescale. The two coordinates together consists of a two-dimensional PES of the reactant and product. Decay of probability distribution was formalized by a diffusion model with an ET sink (reaction-diffusion model).
\[ \frac{\partial P}{\partial t} = D \frac{\partial^2 P}{\partial x^2} + \frac{\partial}{\partial x} \left( P \frac{\partial V}{\partial x} \right) - k(X) P \]  

(1.4)

\( k(X) \) denotes ET rate as a function of slow solvent coordinate. Two types of average survival time characteristic of average survival probability of the initial state were introduced to represent ET dynamics in different scenarios.

Jortner and Bixon\(^6\) brought in effect of solvation relaxation to ET rate (equation 1.5 and 1.6), specifically through \( H_A \), the adiabatic parameter proportional to solvent longitudinal relaxation time \( \tau_L \).

\[ k_{\text{ET}} = \frac{|H_{DA}|^2}{\hbar(4\pi \lambda k_B T)^{1/2}} \exp(-\frac{E_A}{k_B T})(1 + H_A)^{-1} \]  

(1.5)

\[ H_A = \frac{2|H_{DA}|^2 \tau_L}{\hbar \lambda} \]  

(1.6)

\( E_A \) represents the activation energy of ET reaction. In the adiabatic limit where \( H_A >> 1 \), \( k_{\text{ET}} \propto \tau_L^{-1} \), which depicts a solvent-controlled adiabatic ET rate.

Walker et al\(^6\) incorporated Sumi-Marcus and Jortner-Bixon models. The ET rate can be expressed with determined reaction free energy.

\[ k(X) = \sum_n \frac{v_n^2 |\langle 0|n\rangle|^2}{\hbar(4\pi \lambda_{\text{cl,vib}} k_B T)^{1/2}} \exp\left[ -\frac{(\Delta G_{0\rightarrow n} + \lambda_{\text{cl,vib}})^2}{4\pi \lambda_{\text{cl,vib}} k_B T} \right] \]  

(1.7)

\( \lambda_{\text{cl,vib}} \) represents classical intramolecular vibrational relaxation energy, and the two multipliers in the numerator of pre-exponential factor denote electronic and vibrational coupling between reactant and product. Free energy of the reaction \( \Delta G^*_{0\rightarrow n} \) is expressed as follows.
\[ \Delta G^*_{0 \to n} = \lambda_{solv} + \Delta G^0 - 2X\lambda_{solv} + nhv \]  

(1.8)

\(\lambda_{solv}\) is reorganization energy of the slow solvation relaxation.

Diffusion-reaction model was applied by Wang et al\(^6^3\) to depict photo-induced ET rate in photosynthetic systems, with the diffusion constant \(D(t)\) calculated from solvation correlation function, \(C(t)\)\(^6^4\), determined by dynamical Stark shift of the tryptophan’s electronic absorption.

\[ D(t) = -C(t) \frac{dC(t)}{dt} \]  

(1.9)

However, the above formalism for \(D(t)\) is controversial in that the validity of this expression is still ambiguous in non-Debye type relaxation,\(^6^5,6^6\) i.e., multiple-exponential or stretched-exponential decay shown in many protein solvation dynamics.\(^5,9-11,6^3\)

Continuing with diffusion-reaction model through MD simulation, Lebard and Matyushov\(^6^7\) applied a time-dependent diffusion constant constructed by Okuyama and Oxtoby\(^6^8\) from generalized Smoluchoski equation with a harmonic potential. They also incorporated non-ergodicity correction by applying a frequency cutoff at \(k_{ET}\) and constructing ET rate-dependence for both energy gap and reorganization energy. Specifically for reorganization energy \(\lambda\),

\[ \lambda^C(k_{ET}) = \beta \int_{k_{ET}}^{\infty} C^C_X(\omega) d\omega \]  

(1.10)

where the superscript \(C\) represents Coulomb reorganization energy, and \(C_X(\omega)\) is Fourier transformation of the Stokes shift correlation function, which can be determined by our
time-resolved fluorescence experiment. However, such an equality between the reorganization energy defined in Marcus theory and that coming from Stokes shift is characteristic of ergodicity and thus not rigorous for non-ergodic ET processes. In the new model proposed later by the same group\(^6\)\(^9\)-\(^7\)\(^1\), a key parameter to reflecting loss of ergodicity and Gaussian statistics, \(\chi_G\), was introduced, which equals the ratio of \(\lambda_{\text{var}}\) arising from Gaussian width (\(\sigma^2\)) in the energy-gap law to \(\lambda_{\text{st}}\) from the Stokes shift (\(\Delta X\), difference between minima of the two potential energy surfaces) and is unity in traditional Marcus theory (equation 1.11) but larger in non-ergodic ET process.

\[
\lambda_{\text{var}} = \frac{1}{2} \beta \sigma^2 = \frac{1}{2} \Delta X = \lambda_{\text{st}} \quad (1.11)
\]

When \(\chi_G > 1\), \(\lambda'\), the reorganization energy of the “horizontal” ET transition define in Marcus theory, is then expressed below in equation 1.12.\(^7\)\(^1\),\(^7\)\(^2\)

\[
\lambda' = \frac{(\lambda_{\text{st}})^2}{\lambda_{\text{var}}} = \frac{\lambda_{\text{st}}}{\chi_G} \quad (1.12)
\]

If \(\chi_G \gg 1\), \(\lambda'\) will be fully reduced to minimize energy dissipation in form of heat and enhance the ET efficiency, which is commonly seen in many redox proteins.\(^7\)\(^1\) For non-ergodic ET, the two-parabola picture used in Marcus theory is distorted, altering the activation barrier calculated with harmonic free energy surfaces.

1.2.3 Dependence of ET rate on temperature.

Fermi-golden-rule-based semiclassical Marcus theory yields Arrhenius-type dependence on temperature.\(^7\)\(^3\) However, deVault and Chance\(^7\)\(^4\) studied ET behavior of
cytochrome oxidation in *R. spheroids* Chromatium and obtained higher barrier at high temperature range and nearly activationless ET at low temperatures. To reveal the mechanism to this observation, Hopfield\textsuperscript{75} first studied ET dependence on temperature with a semiclassical Förster-type formalism but failed in low temperature range. Less than two years later, in contrast to ET coupled with low-frequency solvent polar mode depicted in Marcus theory, Jortner\textsuperscript{76} incorporated high-frequency vibrational modes of solute, which was believed to strengthen D-A coupling and lower the activation barrier, and developed formalism of ET transition probability at different temperature ranges by applying the well-known multi-phonon vibrational transition model.\textsuperscript{77} Specifically in low temperature limit, barrierless electronic tunneling occurs from zero-point configuration of the reactants, resulting in insensitivity to temperature. Such a mechanism has been largely applied in early days to account for abnormal temperature effect on ET in photosynthetic systems which is more rapid below 77K than at room temperature.\textsuperscript{78-80}

Besides the activationless ET pathway, two more mechanisms have been proposed. One argues that coherence between hot vibrational states of reactant and product, which decays slower in low temperature range, drives the acceleration of ET.\textsuperscript{81, 82} The other is in consistence with the reaction-diffusion model. At low temperature, ET rate is controlled by rate of diffusion to the activationless optimal configuration. When then initial conformation is proximate to the optimized one, the observed decay rate of excited-state donor will speed up with low reorganization energy.\textsuperscript{83}
Given the controversy on temperature dependence of ET, we aim to clarify it by studying ET in (6-4) photolyase. Effect of coupling between ET and solvation will also be explored.

1.3 Crystal structure of (6-4) photolyase

(6-4) photolyase is a flavoprotein that repairs (6-4) lesion in double-stranded DNA (dsDNA). Figure 1.1A shows X-ray diffraction crystal structure of the active site in At. (6-4) photolyase. Several charged and polar residues surrounding the cofactor create a highly polar active site. A few static water molecules close to the active site are observed in the crystal structure, indicating more dynamical water molecules that may contribute to solvation relaxation around the cofactor. Figure 1.1B shows the crystal structure of Dm. (6-4) photolyase active site when binding 64PP DNA lesion. A key and conservative residue, His365 (His364 in At. (6-4) photolyase), was confirmed to play a crucial and indispensable role in DNA repair. This is due to the strong hydrogen bond formed between N atom in H365 and H5 atom of thymine in 6-4PP. The distance between the two atoms is 2.7 Å (figure 1.1B). Function of H364 will be further introduced in detail in section 4.1.
Figure 1.1 Close-up active-site X-ray crystal structure of (A) *Arabidopsis thaliana* (At.) (6-4) photolyase active site and (B) *Drosophila melanogaster* (Dm.) (6-4) photolyase bound to dsDNA that contains (6-4) photoproduct (64PP).
Chapter 2. Methods of Experiments

2.1 Pretreatment of protein solution

2.1.1 Pretreatment of sample solution without 64PP

WT and H364A mutant are prepared according to the Li paper and procedures listed in appendix A. 350µl of sample solution dialyzed in storage buffer within a concentration range of 150-200µM is added into a polymethyl methacrylate (PMMA) cuvette (1cm optical path length) together with excessive reducing agent dithiothreitol (DTT) at a final concentration of 20mM. Before optical experiment, the sample is purged with 99.99% Argon for 2hrs while being stirred, followed by being irradiated under white light for 30 minutes until the cofactor is fully photo-reduced to the active state FADH⁻.

2.1.2 Pretreatment of sample solution with 64PP

Synthesis of substrate, 64 Thymine-Thymine (TT) DNA lesion, is introduced in Appendix B. Solution of (6-4) photolyase and substrate DNA are mixed with a final molarity ratio of roughly 1 to 2 (enzyme to substrate) in yellow light surroundings. The mixture is then purged in dark and photo-reduced with white light according to the method described in section 2.1. For each temperature, transients at 508nm and 540nm, close to the two major peaks of steady-state emission, are measured by TCSPC. Control experiment without substrate is also carried out in the same sample solution except that
the substrate solution is replaced with same volume of ddH₂O. Each transient is then fit with multi-exponential or stretched-exponential decay model for extraction of ET rate.

2.2 Optical measurement

After the sample is treated as above, it is cooled down to desired temperature by liquid nitrogen in the sample chamber of Optistat DN (Oxford Instrument). The scheme of Optistat DN structure was presented in figure 2.1. Using time-correlated single photon counting (TCSPC, PicoQuant FluoroTime 200), time-resolved fluorescence transients of the sample were measured every 4nm from 440nm to 680nm with the pump wavelength of 372nm. The optical layout of TCSPC is shown in figure 2.2. The reconvolution model for the time-resolved signal is expressed as follows.

\[
I(t) = \int_{-\infty}^{t} IRF(t') \sum_{i=1}^{n} A_i e^{-\frac{t-t'}{\tau_i}}
\]  

(2.1)

Where IRF refers to instrumental response function of TCSPC detector. With FluoFit 4.4 (PicoQuant GmbH), each transient is fit iteratively into exponential decay model based on nonlinear least-squares error minimization algorithm.
Figure 2.1 Scheme of Optistat DN. Liquid nitrogen is injected through the liquid nitrogen inlet into the corresponding chamber and then into the sample space through a pinhole. Whole opening size can be controlled by the nitrogen flow valve. Temperature of the sample is measured and probed by the heat exchanger at the bottom of the sample space, which is connected to the ITC503 temperature controller.
Figure 2.2 Optical layout of TCSPC. The transient fluorescence is probed at magic angle (54.7°) when the sample is excited by a 372nm laser diode (peak power 789µW, repetition rate 8MHz) at vertical polarization. The emission signal is focused and cut before being gated by a Seya-Namioka type monochromator (ScienceTech Model 9030) with 2.0mm slit width for both inlet and outlet slit, and amplified by a microchannel-plate photomultiplier (MCP PMT).
Chapter 3 Temperature-Dependent Emission of (6-4) Photolyase

3.1 Introduction

In (6-4) photolyase, the cofactor FAD is selected as the fluorescent probe due to its proximity to the active site and participation in the ET process.\textsuperscript{11, 85} Hence, determination of time-resolved fluorescence of FADH\textsuperscript{-} will provide a scenario of active-site solvation dynamics during the photo-induced forward ET. At room temperature, Chang et al\textsuperscript{11} has determined solvation correlation function of three redox states of FAD cofactor in (6-4) photolyase active site. For the reduced state, three lifetimes of Stokes shift were extracted to be 1.4ps, 17ps, and 730ps with stabilization energies of 274cm\textsuperscript{-1}, 471cm\textsuperscript{-1} and 53cm\textsuperscript{-1}, the last one being assigned to active-site collective protein motion that reflects the rigid binding interaction between the protein backbone and the cofactor. Such a correlation time is longer than but in the same order of magnitude with the 274ps ET lifetime from the cofactor to the 64PP in H364A reported by Li et al,\textsuperscript{85} rendering the ET a non-ergodic process.\textsuperscript{67}

3.2 Steady state emission of WT and H364A

Each transient is fit with multi-exponential decay model. The steady-state fluorescence spectra is constructed at each wavelength by integrating the decay dynamics over time from zero to infinity.

\[
I_{ss}(\lambda) = \int_0^{\infty} \sum_{i=1}^{n} A_i e^{-\frac{t}{\tau_i}} dt = \sum_{i=1}^{n} A_i \tau_i 
\]  
(3.1)
Steady-state emission spectra of (6-4) photolyase WT and H364A at temperatures from 289K down to 77.5K are shown in figure 3.1. All the spectra was normalized at the 536nm peak. Wavelengths (λss) derived from average frequency (νss) of each spectrum are listed in table 3.1 and 3.2. For both species, it is shown that as temperature is lowered, both of the two major peaks become increasingly narrowed. Besides the two major peaks, a shoulder around 580nm that is not easily observed at room temperature stands out at low temperatures, which again arises from spectral narrowing. Through normal mode analysis, Nakai et al.\textsuperscript{88} calculated two equilibrium ground state isomers of the reduced anion FADH\textsuperscript{-}, one planar and the other bent, with different charge distributions. However, confirmation of the minor peak in our studies showed that the two major peak should not be assigned to the two isomers. Together with the temperature-dependent narrowing effect, the three peaks are all assigned to vibronic bands due to the cofactor-bath coupling. As the sample is cooled down to lower temperatures, system-bath coupling becomes weaker. Therefore, less inhomogeneous broadening of fluorescent transition is shown in emission spectra, resulting in narrower peaks.

3.3 Investigation of (6-4) photolyase solvation through time-resolved fluorescence

In many molecules, e.g., tryptophan, the emission spectra are singly peaked and can be fit with a lognormal line shape,\textsuperscript{89} from which the peak frequency can be extracted. Solvation correlation function is then constructed based on the time-dependent red shift of the peak energy. In (6-4) photolyase, however, the emission spectra are neither
mono-peaked nor can be fit with lognormal model.\textsuperscript{11, 85} Instead, \( \nu(t) \), the average frequency of spectra at each time point is calculated as follows:\textsuperscript{11}

\[
\nu(t) = \frac{\int_{0}^{\infty} \nu F(v, t) dv}{\int_{0}^{\infty} F(v, t) dv}
\]  

(3.2)

\( F(v, t) \) represents intensity of emission in frequency domain at time \( t \). \( \nu(t) \) is then applied to calculate the solvation correlation function, \( C(t) \) (see Eq. 1.7), which is then fit with the below multi-exponential model.

\[
C(t) = E_0 + \sum \Delta E_i \exp \left( -\frac{t}{\tau_i} \right) - \sum \Delta E_i
\]  

(3.3)

\( \Delta E_i \) denotes stabilization energy of each component of relaxation dynamics. \( E_0 \) is the average frequency of initial fluorescence spectra.

Temporal evolution of emission spectra are shown in figure 3.2-3.7. In WT, both the blue and red side of the spectrum red shifts in the first 5ns at 289K. From 5ns to 14ns, no significant shift is observed at the blue side, while the red side shrinks, leading to a blue shift of the spectrum in this time range. In lower temperatures down to 230K, the initial red-shift of the blue side is observable, whereas the red-side shrinkage is only constraint in the far-red side. At 210.5K and below, the spectra shows remarkably less redshift of the blue side even in early time points. Instead, only the red side expands slightly to longer wavelengths. Behavior of red-side decay at later times is similar to that observed in high temperatures, with increasingly less amplitude of narrowing at lower temperatures. In H364A, the blue side of the spectra still narrows down to the red, but
with less amplitude than in WT. At 210.5K and below, initial spectral red-shift less significantly than that in WT. From 132.5K to 77.5K, the red side of spectra directly shrinks in early period without red-shift observed in other temperatures.

Solvation correlation functions of 64WT and H364A are shown in figure 3.8 and the fitting results are presented on the corresponding curves. Due to lack of thorough understanding of the rising component, we only fit the declining segment of the correlation functions, i.e., only decay dynamics are considered. At 289K in WT, solvation occurs with a stabilization energy of 70cm\(^{-1}\) in 1.5ns, not far from the timescale of 735ps measured by Chang et al,\(^{11}\) according to which this relaxation is attributed to intrinsic collective protein motion of the active site. As temperature drops to 230K, solvation decays in a longer timescale of 2.89ns, with a smaller stabilization energy of 44.5cm\(^{-1}\). However, at 210.5K, solvation decays slightly faster in 2.18ns with a larger stabilization energy of 75cm\(^{-1}\), indicating certain transition in protein structure. Below 210.5K, solvation occurs monotonously in gradually longer timescale up to 4ns at 77.5K, while stabilization decreasing to 38.2cm\(^{-1}\), indicating less active-site flexibility at lower temperatures. In H364A, higher solvation speed in a few hundreds of picoseconds is observed from 289K to 251K, suggesting a more flexible active site in H364A than in WT. When the positively charged Histidine is replaced by Alanine, stronger electrostatic constraint is overtaken by weaker hydrophobic interaction. On the other hand, smaller volume of alanine will leave more space for the backbone to move. Such a sub-nanosecond solvation decay vanishes when the sample solution is frozen at 210.5K.
From 210.5K to 152K, solvation slows down from 1.71ns to 1.98ns with stabilization energy decreasing from 31.1cm$^{-1}$ to 19.4cm$^{-1}$. At the lowest four temperatures, initial rise (blue-shift) is observed. However, the corresponding “destabilization energy” ranges from 5-10cm$^{-1}$, trivial enough for one to surmise that active-site collective motion is completely frozen in this temperature range.

Another trend shown in solvation correlation functions of both species is the increasing initial average frequency with decreasing temperature (figure 3.8). Such cryogenic blue shift can be attributed to less solvent/protein mobility which leads to less dipole change and thus less stabilization energy during solvation. Alternatively, it is due to slow active-site relaxation that competes with ET quenching dynamics, resulting in incomplete solvation. Note that though the protein solution is observed to freeze at 210.5K, the origin of dramatic deceleration of active-site collective motion should not be attributed to crystallization of solvent because hydration water molecules, due to their strong hydrogen bonding interaction with protein, do not crystallize as easily as bulk water does.$^{90}$ Instead, such a change is possibly due to the universal dynamical transition of protein molecules occurring around 200K,$^{69, 91, 92}$ during which the anharmonic mode of protein motion is no longer observed.$^{93, 94}$
Figure 3.1 Steady-state emission spectra of A) WT, B) H364A throughout the whole experimental temperature range. The black arrows points to the direction in which temperature decreases. The red arrows points to the minor peaks.
Figure 3.2 Snapshots of WT emission spectra at 289K-230K. Three time points were picked in each temperature, i.e., 0 and 14ns, the beginning and end of the time window of all presented solvation correlation functions, respectively, and one where the correlation function reaches the minimum. This standard will be also applied in figure 3.3-3.7
Figure 3.3 Snapshots of WT emission spectra at 210.5K-152K.
Figure 3.4 Snapshots of WT emission spectra at 132.5K-77.5K.
Figure 3.5 Snapshots of H364A emission spectra at 289K-251K.
Figure 3.6 Snapshots of H364A emission spectra at 210.5K-152K.
Figure 3.7 Snapshots of H364A emission spectra at 132.5K-77.5K.
Figure 3.8 Dynamical solvation in (6-4) photolyase. Left two panels: solvation correlation function of WT and H364A at all temperatures. Each curve is labeled by a vector in the general form of \((\Delta E, \tau, T)\), i.e., (stabilization energy, solvation timescale, temperature). For H364A at 272K and 251K, correlation function shows a bi-exponential behavior with two sets of \(\Delta E\) and \(\tau\) separated by a semicolon.
4.1 Introduction

(6-4) photolyase repairs 64PP lesion of DNA. The mechanism of DNA repair was reveal by Li et al.\textsuperscript{85} After photo-excitation of the active reduced state of the cofactor FADH\textsuperscript{−}, one electron is transferred from the cofactor to the bound 64PP, followed by a proton transfer and a series of bond breakage and formation before the repair is completed. As mentioned in section 1.3, it has been confirmed that His364 is responsible for the proton transfer between its imidazole side chain and the lesion product, according to the complete loss of DNA repair activity in several H364 mutants.\textsuperscript{85} Besides ET to substrate, excited-state FADH\textsuperscript{−} has been considered to decay through mechanism of intramolecular ET from reduced flavin to adenine in our previous studies.\textsuperscript{95} Therefore, mutation of H364 residue generates a proper system to study electron transfer in two different systems, enzyme only and enzyme with substrate. Considering a relatively high yield of purification, H364A mutant was selected in this project.

4.2 Non-ergodic ET dynamics in H364A

All transients of H364A are fit with stretched-exponential decay model (Eq. 4.1),\textsuperscript{95} the average lifetime \(<\tau>\) is calculated by the equation 4.2. Transient curves of enzyme with 64PP are fit by multiple-exponential model.
\[ I(t) \propto \exp(-(t/\tau)^\beta) \quad (4.1) \]

\[ \langle \tau \rangle = \frac{\tau}{\beta} \Gamma\left(\frac{1}{\beta}\right) \quad (4.2) \]

\( \beta \) is the stretching coefficient (\( \beta < 1 \)) and reflects the dynamical heterogeneity of the investigated process coupled with solvation.\(^{96,97}\) Figure 4.1 and 4.2 show the normalized decay transients in (6-4)-photolyase-only (system 1) and (6-4)-photolyase+6-4PP (system 2) systems, whose dynamics are listed in table 4.1-4.4. Note that in system 2, we applied double-stretched-exponential decay, with one component representing ET from FADH\(^{-}\) to adenine in certain proportion of free enzyme molecules whose parameters equals that in system 1. Table 4.4 only shows parameters for ET from FADH\(^{-}\) to 64PP.

In system 1, decay of excited state of FADH\(^{-}\) occurs in an average timescale of 2.2-2.3ns at 289K and slows down by a factor of around 5 at 77.5K to \(~12\)ns. The stretching parameter \( \beta \) fluctuates around 0.9 throughout the entire temperature range. Note that the decay rate does not show remarkable change when probed at two major peaks. This is consistent with the conclusion drawn in section 3.1 that the two major peaks arise from vibronic coupling band rather than from two isomers. For the rest of this thesis, we will focus on signal probed at 540nm unless specifically mentioned. For the intramolecular ET from isoalloxazine ring to adenine, dichotomy of rate in activation energy is observed throughout the whole temperature range. Over the highest four temperatures, ET occurs after crossing an activation barrier of 8kJ/mol, three times the
thermal energy at 289K. For the lowest four temperature, ET is almost barrierless, with Ea merely half of the thermal energy at 77.5K.\textsuperscript{76}

In system 2, ET from FADH\textsuperscript{+} to 64PP substrate occurs in 253ps at 289K, consistent with the 236ps timescale reported by Li et al. At 77.5K, ET decelerates by a factor of 5 to $\sim$1.2ns. The temperature dependence of ET rate is similar to that observed in system 1, with an activation energy of 8.65kJ/mol throughout the highest five temperatures and 0.11kJ/mol for the lowest four temperatures. The stretching coefficient $\beta$ remains around 0.9 from 289K to 251K, at which the sample solution is still liquid. At 230K specifically when the sample starts to freeze while still a liquid-solid mixture, $\beta$ drops to 0.83. From 210.5K at which the sample freezes completely, $\beta$ fluctuates around 0.73, indicating more heterogeneity at low temperatures with 230K as the transition temperature. For FADH\textsuperscript{+}, the butterfly motion of the isoalloxazine ring contributes to the structural heterogeneity. At high temperatures, more rapid solvation drives transition between different conformations and thus offsets the structural heterogeneity to some extent. At lower temperatures, slower solvation cannot cause remarkable structural fluctuation within ET time scales and thus makes the structural heterogeneity dominant in cryogenic ET process. In addition, the intermolecular ET in system 2 occurs with higher heterogeneity than the intramolecular ET in system 1 due to the extra conformational contribution from the substrate.
The mechanism to the anomalous temperature-dependence of ET and the role of solvation dynamics are still unknown. Further understanding requires ultrafast laser experiment to study solvation dynamics in shorter times.
Figure 4.1 Decay of FADH* emission in (6-4) photolyase H364A probed at A) 508nm B) 540nm with pump wavelength of 370nm
Figure 4.2 Decay of FADH* emission in (6-4) photolyase H364A binding 64TT substrate probed at A) 508nm B) 540nm with the pump wavelength of 370nm.
Figure 4.3 Temperature dependence of ET timescale in system 1 (A probed at 508nm and B at 540nm) and in system 2 (C probed at 508nm and D at 540nm). Ea denotes activation energy of ET. In each panel, the upper Ea value represents the barrier at high temperature range and the lower at cryogenic range.
Table 4.1 Fitting parameters of transient decay in H364A without 64PP at 508nm

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<τ>, average lifetime, is calculated based on equation 4.2. In the last column, k is the reciprocal of <τ>. The same method is also applied to table 4.2, 4.3 and 4.4.
Table 4.2 Fitting parameters of transient decay in H364A without 64PP at 540nm

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Table 4.3 Fitting parameters of transient decay in H364A with 64PP at 508nm

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Table 4.4 Fitting parameters of transient decay in H364A with 64PP at 540nm

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Appendix A Purification protocol of At. (6-4) photolyase

A.1 PCR

1. PCR mixture is added into PCR tube. Ingredients for the mixture are 5μl of 10X reaction buffer, 45ng template DNA of (6-4) photolyase WT, 150ng top primer, 150ng bottom primer, 1mM dNTP mixture, 1μl DNA polymerase and proper volume of ddH2O to make final volume of 51μl. Put the PCR tube into PCR machine.

2. Start PCR at 95°C for 30 seconds.

3. Continue running PCR for 16 cycles. Recipe for each cycle is 95°C for 30 seconds, 55°C for 1 minute and 68°C for 12 minutes.

4. Cool down to 4°C.

5. Add 0.5μl 20U/μl DPN1 into the mixture. Incubate at 37°C for 1.5 hours.

A.2 Transformation and Purification of plasmid DNA for sequencing

1. Mix 1μl At. (6-4) photolyase DNA with 100μl XL10 Gold competent cell in an Eppendorf tube.

2. Keep on ice for 30 minutes.

3. Heat shock the mixture in 42°C water bath for 45 seconds.
4. Put the tube back on ice for 2 minutes.

5. Add 0.5ml Luria Broth (LB) solution and grow in 225rpm shaker at 37°C for 1 hour.

6. Spin the tube at 14K for 1 minute and discard the supernatant.

7. Suspend the pellet precipitate in 150μl LB and spread on LB+Kanamycin (Kan) agarose plate, Kan in concentration of 30μg/ml. This concentration will be kept for culture media in all the rest steps.

8. Place the plate at 37°C overnight.

9. Pick one colony in the above plate, spread it into 3ml LB+KAN medium in a 25ml test tube.

10. Put the test tube in a shaker at 37°C, 225rpm overnight. The culture should get cloudy the next morning.

11. Add 1.5ml culture into an Eppendorf tube, spin at 14K for 1 minute. Dump the supernatant.

12. Invert the tube to dry off trace remaining medium.

13. Use “QIAprep Spin Miniprep Kit” to purify the protein.


15. Add 250μl Buffer P2 and mix thoroughly by inverting the tube 4-6 times.
16. Add 350μl Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.

17. Spin at 14K for 10 minutes.

18. Transfer the supernatant into a QIAprep spin column.

19. Spin at 14K for 1 minute. Dump the flow through.

20. Wash the spin column with 0.5ml buffer PB. Repeat step 19.

22. Wash the spin column with 0.75ml buffer PE (with >70% ethanol). Repeat step 19 twice. Note that the second centrifugation removes the residual wash buffer.

23. Elute the column with 60μl buffer EB added toward the center of the column into an Eppendorf tube. Let it stand for 1 minute before spin at 14K for 1 minute.

24. Measure the concentration of DNA by mixing 5μl eluent with 995μl ddH2O in 1cm quartz cuvette.

25. Take the OD reading at 260nm. Calculate the concentration based on the reading.

26. Spare 10μl DNA for sequencing. Store the rest in -20°C.

A.3 DNA transformation for E. Coli. cell growth.

1. Mix 1μl At. (6-4) photolyase DNA with 100μl BL21/DE3 competent cell in an Eppendorf tube.

2. Repeat step 2-6 in A.2.
A.4 Protein expression.

1. Spray a single colony into 150ml LB+Kan medium and put it in the 225rpm shaker at 37°C overnight. This rpm is kept in the rest of steps of cell growth.

2. Inoculate 10mL overnight culture (OD @600nm around 2.0) into 1L LB+Kan medium.

3. Grow at 37°C until OD600 reaches 1.7.

4. Induce overexpression of protein with 0.2mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25°C and grow for 15-18 hours.

5. Spin the saturated cell culture at 4°C, 5K for 10 minutes, discard the supernatant.

6. Wash, suspend and transfer the pellet with 1XPBS buffer (pH) into 50ml tubes.

7. Spin at 4°C, 5K for 10 minutes, discard the supernatant.

8. Store cell pellet at -80°C.

A.5 Protein purification (6L cell pellet)

1. Thaw pellet and suspend it with 90ml (15ml/L pellet) lysis buffer with 1mg/ml lysozyme.

2. Incubate at 4°C with gentle stir for 30-60 minutes.

3. Sonicate the pellet for 2.5minutes each time with 30 seconds interval until the pellet suspension becomes translucent.
4. Spin at 4°C, 20K for 30 minutes. Transfer the supernatant into new set of centrifuge tubes and spin for another 30 minutes.

5. Load all the supernatant onto 4-5ml Ni-agarose column with the flow rate around 1ml/min.

6. Wash the column with 5 column volumes (CV) of wash buffer.

7. Elute the column with elution buffer, dilute the eluent with Heparin A buffer.

8. Load the diluted eluent onto FPLC 5ml heparin column with 1.5ml/min flow rate.

9. Wash the column for 5 CVs of heparin A buffer.

10. Elute the column in a gradient of heparin B buffer percentage from 0 to 100% for 5 CVs.

11. Collect fractions of eluent around 450nm and 580nm absorption peak.

12. Concentrate the fractions at 4°C, 5K with centrifugal filter tube (cut-off molecular weight 30KD).

13. Dialysis the concentrated solution in storage buffer for 6 hours.

14. Quick freeze the sample in liquid nitrogen and store it at -80°C.
Appendix B Synthesis of 64TT substrate

1. Dissolve the synthesized normal DNA oligomer in ddH₂O to the final concentration of 0.5mg/ml.

2. Irradiate the solution in peri dish (5cm diameter) with 254nm UV light for 1 hour.

3. Concentrate the UV-irradiated DNA in SpeedVac spin evaporator to the final concentration of approximately 20mg/ml.

4. Use 250X10mm C18 hydrophobic interaction column to purify the DNA solution (100μl for each injection) on HPLC at 30°C and the flow rate of 4.5ml/min, with two running buffer (TEAA pH7.0 as buffer A and acetonitrile as buffer B) mixed with proper volume ratios. Recipe in terms of buffer B percentage for each cycle is 7.9% for 15 minutes (separation), 30% for 3 minutes (cleaning) and 7.9% for 7 minutes (re-equilibration). Collect the 64TT fraction around the major peak of 325nm absorption. Also collect the unreacted DNA oligomer fraction around the superstrong peak during 30% B cleaning for recycling.

5. Dry the collected 64TT fractions and re-dissolve the 64TT solid in ddH₂O. Measure the concentration based on the absorption at 325nm.

6. Dry the unreacted DNA and repeat step 1 to 5.
Bibliography


