ULTRAFAST DYNAMICS OF BIOLOGICAL ELECTRON TRANSFER OVER SHORT DISTANCES

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Ting-fang He, M.S.
Ohio State Biochemistry Program

The Ohio State University
2011

Dissertation Committee:
Professor Dongping Zhong, Advisor
Professor Chenglong Li
Professor Thomas Magliery
Professor Michael Poirier
© Copyright by

Ting-fang He

2011
ABSTRACT

Photoinduced electron transfer reaction plays a pivotal role in regulating many light-switched enzyme activities such as photosynthesis and circadian rhythm. In the biological system, the donor-acceptor distance may vary from a few angstroms to tens of angstroms. For the past several decades, long distance (>14 Å) electron transfer has been well studied. However, understanding the short distance transfer is technically limited due to temporal resolution. In the dissertation, we address this issue with the use of ultrafast spectroscopy and investigate a protein model system of Desulfovibrio vulgaris flavodoxin. Using the flavin cofactor, we initiate the electron-transfer process by ultrashort laser pulse. By monitoring time-dependent laser induced fluorescence and absorption change, we unravel the associated elementary dynamics and elucidate key factors (static reaction energy, environment dynamic relaxation, and distance variant) that affect the rate of electron transfer. With the site-directed mutagenesis, we first block the electron-transfer channel by replacing the tryptophan and tyrosine donor with phenylalanine. The inert mutant thus shows the time constants of active-site solvation ranging from several picoseconds to hundreds of picoseconds. Subsequently, from the native flavodoxin, we isolate the donor of tryptophan and tyrosine adjacent to the acceptor, and determine the individual transfer rate and the complete electron-transfer cycle. At the same time, by altering the critical redox-sensitive residues, different redox potentials for the acceptor to
convert from oxidized to one-electron reduced, and from one-electron reduced to two-electron reduced states are generated. From such a great variety of driving forces, the result shows a strong correlation between the rates of electron transfer and the reaction energy. A subpicosecond dynamics is substantially slow down to a few picoseconds with a reduced energy of about 0.5-0.6 eV. Some slower processes in several picoseconds are shown strongly coupled with the active-site solvation, thereby exhibiting a decay behavior in a non-single exponential way. Surprisingly, the rate of back electron transfer is found not directly related to such energy potential change but is otherwise more sensitive to the solvation involvement throughout the cycle. In the final part, we change the donor-acceptor distance by locating the tryptophan donor at different places one at a time. From W60 to the replaced L67W, the edge-to-edge distance to the acceptor is lengthened from 3.7 Å to 8-9 Å, and the rate of electron transfer is significantly decreased from subpicosecond to nanosecond time scales. In conclusion, our study has resolved several important parameters for understanding the electron transfer occurring in several angstroms that are key to the nonequilibrated reactions in biology.
Dedicated to my family!
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Prof. Dongping Zhong, for his intellectual guidance and unwavering support. Especially, I appreciate the providing rich and stimulating academic environment in his group, with much room for independent thinking and discovery. Also, I would like to thank all the former and present lab members for the friendship and support on multiple levels. A particular thank is to Drs. Lijun Guo, Xunmin Guo, Ya-Ting Kao, and Chih-Wei Chang for the collaboration, countless help, and invaluable scientific discussion.

I gratefully appreciate Profs. Chenglong Li, Thomas Magliery, and Michael Poirier for serving on my general examination committees as well as dissertation examination committees.

My final thanks must be to my dearest family especially my twin sister, Ting-fen Ho, who is always there for me for any encounter trouble. Their constant love and freedom of family education has brought me toward what and who I am today.
VITA

December 10, 1979 .............. Born, Taichung, Taiwan

2002 .................................. B.S. Chemistry
National Tsing Hua University, Taiwan

2003-2004 ......................... Research Assistant, Institute of Biological Chemistry
Academia Sinica, Taiwan

2007 .................................. M.S. Biochemistry Program
The Ohio State University, USA

2004-present ...................... Graduate Teaching and Research Associate
The Ohio State University, USA

PUBLICATIONS


FIELDS OF STUDY

Major Field: Biochemistry Program
# TABLE OF CONTENTS

Abstract ........................................................................................................................................... ii
Dedication .......................................................................................................................................... iv
Acknowledgements ............................................................................................................................ v
Vita ...................................................................................................................................................... vi
List of Tables ....................................................................................................................................... ix
List of Figures ..................................................................................................................................... x
Abbreviations .................................................................................................................................... xiii

Chapter 1: Introduction ...................................................................................................................... 1

Chapter 2: Experimental Methodology ............................................................................................ 13
  2.1 Femtosecond Laser Spectroscopy ............................................................................................... 13
    2.1.1 Fluorescence Upconversion Methodology ............................................................................ 14
    2.1.2 Transient Absorption Methodology .................................................................................... 15
  2.2 Fitting of Reaction-Diffusion Equation ....................................................................................... 16

Chapter 3: Mapping Solvation Dynamics at the Function Site of Flavodoxin in Three Redox States ................................................................................................................................. 21
  3.1 Introduction ................................................................................................................................ 21
  3.2 Materials and Methods .............................................................................................................. 23
  3.3 Results and Discussion .............................................................................................................. 25
    3.3.1 Ultrafast Fluorescence Transients and Solvation Dynamics in Three Redox States ........... 25
    3.3.2 Femtosecond-Resolved Emission Spectra and Redox-Dependent Correlation Functions .... 27
    3.3.3 Binding Site Solvation and Protein Surface Hydration ....................................................... 31
  3.4 Conclusion .................................................................................................................................. 33
LIST OF TABLES

Table 3.1: Results of solvation dynamics at the function site of flavodoxin in three redox states........................................................................................................................................41

Table 4.1: Redox potentials and dynamics of electron-transfer cycle in oxidized flavodoxin ........................................................................................................................................61

Table 5.1: Redox potentials and dynamics of charge separation in semiquinone flavodoxin ........................................................................................................................................83

Table 5.2: Transient absorption dynamics in semiquinone flavodoxin ...................... 84

Table 6.1: The replaced tryptophan residues in the flavodoxin mutants ................. 100
LIST OF FIGURES

Figure 1.1: Marcus energy gap dependence ................................................................. 10
Figure 1.2: Two-dimensional contour energy diagram for a electron transfer reaction... 11
Figure 1.3: Timeline of electron transfer and solvation reactions. .......................... 12
Figure 2.1: Schematic illustration of upconversion and transient absorption setup ....... 18
Figure 2.2: Principle of time-resolved signal generated by................................. 19
Figure 2.3: Electronic transition describing the fluorescence and transient absorption.. 20
Figure 3.1: X-ray crystal structure of oxidized D. vulgaris flavodoxin....................... 35
Figure 3.2: Steady-state absorption and emission spectra of W60F/Y98F mutant........ 36
Figure 3.3: Normalized femtosecond-resolved fluorescence transients of W60F/Y98F.. 37
Figure 3.4: Three-dimensional representation of femtosecond resolved emission spectra of W60F/Y98F mutant in (A) OX, (B) SQ, and (C) HQ states........ 38
Figure 3.5: Surface-map representation of a snapshot of the function site of flavodoxin in (A) OX, (B) SQ, and (C) HQ states from 1-ns MD simulations.............. 39
Figure 3.6: Normalized femtosecond-resolved fluorescence transients of apoflavodoxin gated from the blue to the red side of the emission spectrum. ................. 40
Figure 4.1: (Left) X-ray crystallographic structure of oxidized D. vulgaris flavodoxin. (Right) A close-up view of the local configuration at the FMN-binding site. ................................................................. 54
Figure 4.2: (A) Normalized (transient) absorption spectra of protein-bound FMN* and anionic flavin semiquinone, and free cationic radicals of indole and phenol in organic solvents. (B) Steady-state absorption spectra of............................ 55
Figure 4.3: Normalized femtosecond-resolved fluorescence transients of FMN in flavodoxin mutants gated at 538 nm................................................................. 56

Figure 4.4: Normalized femtosecond-resolved transient absorption of flavodoxin mutant of W60F probed from 800 nm to 410 nm........................................... 57

Figure 4.5: Normalized femtosecond-resolved transient absorption of flavodoxin mutant of Y98F probed from 800 nm to 410 nm........................................... 58

Figure 4.6: Normalized femtosecond-resolved transient absorption of wild-type flavodoxin and Y98W mutant probed at a multiple wavelengths from 740 nm to 410 nm................................................................. 59

Figure 4.7: Schematic representation of the complete ET processes in the flavodoxin. .. 60

Figure 5.1: Superimposition of residue Y98 and W60 with respect to the FMN molecule in oxidized and semiquinone flavodoxin. ........................................ 76

Figure 5.2: Steady-state spectra of absorption and emission of flavodoxin semiquinone in the visible region. ................................................................. 77

Figure 5.3: Normalized femtosecond-resolved fluorescence transients of FMNH• in flavodoxin and the mutants gated at 670 nm........................................... 78

Figure 5.4: Normalized femtosecond fluorescence upconversion of FMNH• in flavodoxin gated at different wavelengths................................................................. 79

Figure 5.5: Fitting curve for flavodoxin charge separation associated with Y98 donor (dotted) and W60 donor (solid) in the. ................................................................. 80

Figure 5.6: Normalized transient absorption in the semiquinone mutants of (A) W60F and (B) Y98F................................................................. 81

Figure 5.7: Two-dimensional illustration of ET mechanism in semiquinone flavodoxin. 82

Figure 6.1: Topology of the residues to be substituted by tryptophan in flavodoxin. ...... 96

Figure 6.2: Trajectory fluctuations of edge-to-edge distance between indole moiety and flavin ISO moiety in different mutants ................................................................. 97

Figure 6.3: Normalized upconversion transients of the flavodoxin mutants displayed with short (A) and long (B) delay time................................................................. 98

Figure 6.4: Electronic coupling (Left) and tunneling time table (Right) for intraprotein ET in flavodoxin................................................................. 99
Figure B.1: Molecular scheme for riboflavin and FMN. .................................................. 117

Figure B.2: Representative of flavin mechanistic reduction and protonation. ............. 118

Figure B.3: Absorption change of oxidized FMN molecule in buffer (A) and in the W60F/Y98F mutant (B) probed at different wavelengths. .......................... 119

Figure B.4: Steady-state absorption and emission spectra of oxidized FMN molecule in buffer and in the W60F/Y98F mutant .......................... 120

Figure B.5: Normalized transient absorption of the W60F/Y98F mutant probed at different wavelengths................................................. 121

Figure F.1: Typical Nernst titration curve for one-electron reduction......................... 133

Figure F.2: Schematic diagram of pH-dependent midpoint redox potential ............ 134
# Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Å</td>
<td>angstrom; $10^{-10}$ meter</td>
</tr>
<tr>
<td>α</td>
<td>alpha; alpha helix secondary protein structure</td>
</tr>
<tr>
<td>β</td>
<td>beta; used for (1) beta sheet secondary protein structure, (2) degree of stretched exponentials, or (3) distance decay constant</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>$c(t)$</td>
<td>solvation correlation function</td>
</tr>
<tr>
<td>$d$</td>
<td>derivative (mathematics)</td>
</tr>
<tr>
<td>$D_0$</td>
<td>doublet ground state</td>
</tr>
<tr>
<td>$D_1$</td>
<td>doublet first excited state</td>
</tr>
<tr>
<td>$D(t)$</td>
<td>time-dependent diffusion coefficient</td>
</tr>
<tr>
<td>$\Delta$</td>
<td>delta; difference in two parameters</td>
</tr>
<tr>
<td>$\Delta E$</td>
<td>stabilization energy</td>
</tr>
<tr>
<td>$\Delta G^\circ$</td>
<td>standard Gibbs free energy</td>
</tr>
<tr>
<td>$\Delta G^*$</td>
<td>activation energy</td>
</tr>
<tr>
<td>$\Delta \mu$</td>
<td>change of dipole moment</td>
</tr>
<tr>
<td>$E$</td>
<td>redox potential vs NHE</td>
</tr>
<tr>
<td>$\varepsilon$</td>
<td>molecular extinction coefficient</td>
</tr>
<tr>
<td>$\varepsilon_0$</td>
<td>dielectric constant</td>
</tr>
<tr>
<td>$\hbar$</td>
<td>h-bar; Dirac constant (also called reduced Planck constant)</td>
</tr>
<tr>
<td>$h\omega$</td>
<td>molecular characteristic frequency</td>
</tr>
<tr>
<td>$H_{AB}$</td>
<td>electronic coupling matrix element between donor (reaction) and acceptor (product)</td>
</tr>
<tr>
<td>$I$</td>
<td>intensity</td>
</tr>
</tbody>
</table>
\begin{align*}
\infty & \quad \text{infinity} \\
^{\circ}\text{K} & \quad \text{degree Kelvin} \\
k_B & \quad \text{Boltzmann constant} \\
k & \quad \text{electron transfer rate} \\
k_{et} & \quad \text{electron transfer rate} \\
k_F & \quad \text{rate of spontaneous emission of radiation} \\
k_{sol} & \quad \text{solvation rate} \\
\lambda & \quad \text{lambda; wavelength} \\
\lambda_{flu} & \quad \text{wavelength of sample emission} \\
\lambda_{pr} & \quad \text{wavelength of probe pulse} \\
\lambda_{pump} & \quad \text{wavelength of pump pulse} \\
\lambda & \quad \text{reorganization energy} \\
\lambda_h & \quad \text{quantum-vibration reorganization energy} \\
\lambda_i & \quad \text{vibration reorganization energy} \\
\lambda_o & \quad \text{solvation reorganization energy} \\
n_p & \quad \text{quantum vibrational mode of product} \\
n_r & \quad \text{quantum vibrational mode of reactant} \\
p & \quad \text{population} \\
\partial & \quad \text{partial-derivative (mathematics)} \\
q & \quad \text{vibration coordinate} \\
\Phi_T & \quad \text{quantum yield conversion to the triplet state} \\
Q(t) & \quad \text{survival probability} \\
R & \quad \text{distance between donor and acceptor} \\
S & \quad \text{strength of electron-vibrational coupling} \\
S & \quad \text{solvation speed} \\
S_0 & \quad \text{singlet ground state} \\
S_1 & \quad \text{singlet first excited state} \\
S_n & \quad \text{singlet higher excited state} \\
t & \quad \text{time} \\
\tau & \quad \text{tau; time coefficient}
\end{align*}
\( \tau_F \) fluorescence lifetime

\( \tau_{et} \) electron transfer time constant and equals to the reciprocal of \( k_{et} \)

\( \tau_{sol} \) solvation time constant

\( T \) temperature

\( \tilde{\nu} \) averaged frequency

\( V \) free energy

\( X \) solvation coordinate

NON-SYMBOL

A acceptor

BBO \( \beta \)-Barium Borate crystal

CS charge separation

CR charge recombination

D used for (1) donor or (2) Debye

2D two-dimensional

3D three-dimensional

eV electron volt

EDTA ethylenediaminetetraacetic acid

ET electron transfer

FC Franck-Condon

FAD flavin adenine dinucleotide

FMN flavin mononucleotide

FMN\(^3\) FMN molecule in triplet state

FMN\(^*\) FMN molecule in electronic excited state

FMN\(^\ddagger\) FMN molecule in vibrational hot state

FMNH\(^*\) Neutral semiquinone form of FMN molecule

FMNH\(^{++}\) FMNH\(^*\) molecule in electronic excited state

FMNH\(^{++\ddagger}\) FMNH\(^*\) molecule in vibrational hot state

FMNH\(^-\) Anionic hydroquinone form of FMN molecule
FMNH⁺  FMNH⁻ molecule in electronic excited state
FRES  femtosecond resolved emission spectra
GS  ground state
HCl  hydrochloric acid
HQ  hydroquinone; two-electron reduced flavin
ISC  intersystem crossing
ISO  isoalloxazine; one moiety of flavin molecule
MD  molecular dynamics
NaCl  sodium chloride
OX  oxidized
OD  optical density
SE  stimulated emission
SQ  semiquinone; one-electron reduced flavin
TRES  time resolved emission spectra
UV  ultra-violet
WT  wild type

SCALE
f  femto-; 10⁻¹⁵
p  pico-; 10⁻¹²
n  nano-; 10⁻⁹
μ  micro-; 10⁻⁶
m  milli-; 10⁻³

fs  femtosecond
ps  picosecond
ns  nanosecond
CHAPTER 1: INTRODUCTION

Electron transfer (ET) is a very simple chemical process but closely governs the life on Earth. In nature, an immediate transfer of the electron(s) in photosynthetic reaction center converts light from the Sun to the forms of chemical energy and is stored in glucose or other types of organic compounds, ultimately supplying “food” for living creatures (1-2). In a class of blue-light receptor called cryptochrome found ubiquitously in plants and in animals, the process of ET is pivotal to generate the 24-hour life cycle of circadian rhythm and endogenously informs the body organism “time to sleep” (3-4). Furthermore, the ET reaction is also involved in repairing photo-damaged DNA and prevents some diseases like skin cancer (5). The role of ET in biology serves as an energy transmission and evolves the life using Sun power.

In biology, the electron donor and acceptor molecules are spatially separated by several to tens of angstroms (Å) and localized on the different biomolecular sites. This usually allows little chance for a direct collision between two redox partners to transfer the electron. An efficient ET mechanism is highly desired. Adversely, the unit of peptide and nucleotide has been proved not to act as an electron conductor, i.e., bridging the ET process by forming a long-lived intermediate, since it is energy cost and potentially loses the traveling direction, easily damaging the system (6-8). A breakthrough study on the
photosynthetic ET by DeVault and Chance in 1966 (9) has reported two important observations: (1) at high temperatures (>120 °K), the oxidation rate of cytochrome increased exponentially with the increasing temperature; (2) at the cryogenic low temperatures (35-100 °K) where the activation energy was simply lacking, a significantly slower rate was observed with the otherwise temperature independence. The latter is the evidence for the well-known “tunneling” behavior established from the quantum-mechanical solid state and nuclear physics, yet contrarily to the former observation. One explanation to comply both temperature dependence says that, by a fully quantized point of view, not only the electronic tunneling but also the nuclear tunneling occur, when sufficient thermal energy is provided (6, 9). Depending on the molecular characteristic vibrational frequency ($\hbar\omega$), the higher thermal energy ($k_B T$) is able to populate more vibrational levels and raises the oxidation probability. Another explanation replaces the classical view for the nuclear motions and refers to the traditional chemical theory (10). Analogous to the Arrhenius concept, the thermal energy is used to drive the system nuclei to attain the transition configuration. The quantitative energy barrier to surpass becomes a major concern. Nevertheless, no matter which describes, the motion of system nuclei is believed responsible for high-temperature dependence behavior. From the famous Fermi’s golden rule, it is the Franck-Condon (FC) factor that denotes such nuclear tunneling/motion while the first term ($H_{AB}$) is the electronic coupling matrix element of donor and acceptor (11).

$$k = \frac{2\pi}{\hbar} H_{AB}^2 \text{ FC}$$  \hspace{1cm} (1.1)
Since the definition of the FC term by a classical way is widely accepted, in the following we discuss how it is originally described and intricately modified with different models.

In the Marcus theory, the rate of electron transfer can be expressed as:

\[
k = \frac{H_{AB}^2}{\hbar} \sqrt{\frac{\pi}{k_B T \lambda}} \exp \left( -\frac{(\Delta G^o + \lambda)^2}{4\lambda k_B T} \right)
\]  

(1.2)

The $\Delta G^o$ denotes the Gibbs free energy, and $\lambda$ is the nuclear reorganization energy (10, 12). The quantity of intrinsic $\lambda$ ranges in 0.25-1.2 eV and is summed of $\lambda_i$ for the reactant bond-length and bond-angle vibrational adjustment and of $\lambda_o$ for the solvent orientation change, from the initial to final states (13). When $\lambda$ is satisfied by an exergonic $\Delta G^o (\Delta G^o < 0)$, the activation barrier vanishes and the ET rate will reach the fastest. On the other hand, both insufficient and excess $\Delta G^o$ will decrease the ET rate quadratically. Figure 1.1 shows the energy dependence of ET rate, and in the right region ET is adversely inhibited by the excess $\Delta G^o$ is the prestigious Marcus inverted reaction. The bell-shaped curve has been observed widely in different systems, but only pertaining for slow ET reactions (14-19).

Shortly later, Sumi and Marcus proposed the two-dimensional (2D) model for the fast ET reactions (20). What underlies the modification is that the presupposition in the previous ET always much slower than the solvent response is no longer hold. The 2D model briefly views the reaction with the vibration coordinate $q$ on one dimension and the solvation $X$ on the other (Figure 1.2). Since the relaxation in $q$ is much faster than in
\( X \), the model is reduced to simpler \( X \) dependence. The population change of reactant with time is described:

\[
\frac{\partial p(X, t)}{\partial t} = D(t) \frac{\partial}{\partial X} \left[ \frac{\partial}{\partial X} + \frac{1}{k_B T} \frac{dV(X)}{dX} \right] p(X, t) - k(X)p(X, t)
\]  

(1.3)

Where \( D(t) \) is the time-dependent diffusion coefficient (21),

\[
D(t) = -\frac{k_B T}{2\lambda_o} \frac{1}{c(t)} \frac{dc(t)}{dt}
\]  

(1.4)

\[
c(t) = a_1 \exp\left(-\frac{t}{\tau_1}\right) + a_2 \exp\left(-\frac{t}{\tau_2}\right) + a_3 \exp\left(-\frac{t}{\tau_3}\right)
\]  

(1.5)

\( a_1 + a_2 + a_3 = 1; \tau_1, \tau_2, \tau_3 \) is the time constants for solvation dynamics, \( V(X) \) is the free energy surface on the solvation coordinate, and \( k(X) \) represents the \( X \)-dependent ET rate in a nonadiabatic limit and defined as:

\[
k(X) = \frac{H_{AB}^2}{h} \sqrt{\frac{\pi}{k_B T \lambda_i}} \exp\left(-\frac{\Delta G^*(X)}{k_B T}\right)
\]  

(1.6)

The ultimate survival probability \( Q(t) \) is the integral population over all \( X \):

\[
Q(t) = \int p(X, t)dX
\]  

(1.7)

With
normalizing $Q(0) = 1; \ Q(\infty) = 0 \ if \ k(X) > 0 \quad (1.8)$

At one extreme that ET rate is much faster than solvation, the last term on the right-hand side of Equation (1.3) will dominate and the decay of $Q(t)$ resulting from the ET reaction is affected by $X$-diffusion itself negligibly. On the contrary, if $k(X) \to 0$ or $\tau_{et} \to \infty$ for slow ET reaction, Equation (1.3) describes the population change only impacted by $V(X)$ toward the equilibrium and in such case at any time $Q(t) \approx Q(0)$ is equivalent to 1. Beyond these two extremes, more importantly, the 2D model will predict both the nonexponentially (i.e., multi-exponentially) decay behavior of $Q(t)$ contributed from the various $k(X)$ and the ET shifting from solvent dynamics control to vibrational control (21-22).

In spite of the fact that the 2D model can fairly predict the rate of electron transfer approaching to some experimental observations, further modification is still necessary due to its commonly underestimating flaw. Such underestimation was normally observed in the barrierless ET like the charge transfer on betaine-30 molecule, at least by six orders of magnitude, and in the inverted-regime ET (15-16, 23-24). In contrarily to the pure classical nuclear motion, the “extended” Sumi-Marcus ET model incorporates the quantum vibrational mode on the product $(n_p)$ that is originated from Jortner-Bixon quantum theory (22, 24-26). The ET rate in Equation (1.6) becomes $k(n_p, X)$:

$$k(n_p, X) = \frac{H_{AB}^2}{\hbar} \sqrt{\frac{\pi}{k_B T \lambda_i}} \frac{S^{n_p}}{n_p!} \exp \left( -S - \frac{\Delta G^*(n_p, X)}{k_B T} \right) \quad (1.9)$$
The sum of ET at multiple channels \( \sum_{n_p} k(n_p, X) \) will substitute \( k(X) \) in Equation (1.3) (detailed in Appendix A). In this expression, the nonadiabatic ET transition to different \( n \)-th-level vibronic accepting modes is allowed. With each \( n \)-th coupling the activation energy differs accordingly. It should be noted that, on the reactant \( n_r = 0 \) is only considered. Since the vibrational relaxation is assumed much faster (27), the transition always occurs from the vibrationally relaxed state on the reactant \( (0 \rightarrow n_p) \). Here, the electron coupling between the reactant vibrational ground state and the product \( n \)-th vibrational state \( (H'_{AB}) \) is corrected by the electron-vibrational coupling strength \( S \):

\[
H'_{AB} = H^2_{AB} \frac{S^{n_p}}{n_p!} \exp(-S)
\]

\[
S = \frac{\lambda_h}{\hbar \omega}
\]

The \( \lambda_h \) denotes the relaxation energy in quantum vibrational mode and \( \hbar \omega \) is the molecular character frequency on the simple harmonic oscillator potentials. In summary, this hybrid model preserves the reorganization energy for the classical solvation \( (\lambda_o) \) and classical vibration \( (\lambda_i) \) with the addition of quantum vibration \( (\lambda_h) \). The energy gap is adjusted to \( \Delta G^0 + n_p \hbar \omega \) and the reaction moves, if originally in the inverted region, to the activationless or normal region. As a consequence, the extended Sumi-Marcus model is fruitful in predicting the ET rate covering the bell-shaped energy dependence curve.
However, no study has extends the application from solution to a more bulky biological system with the hybrid concept build-in.

Until now, we only focus on different perspectives to define the FC factor associated with ET rate without considering the electron penetration through insulating medium. In quantum theory, the probability to find a tunneling electron on the acceptor site is related to the square of integrated overlap of the donor and acceptor electronic wave function which also represents the strength of interaction between donor and acceptor at the transition configuration ($H_{AB}^2$). The value of $H_{AB}$ can vary depending on the intervening length (R) and bridge material, and is expressed in proportional to the exponential of ($-\beta R$). The $\beta$ is distance decay constant and ranges from 2.9-4.0 Å$^{-1}$ for vacuum barrier, 1.65 Å$^{-1}$ for water, to 1.0 Å$^{-1}$ for saturated alkane bridges (7, 14, 18, 28-29). Ultimately, the drop of coupling element with distance leads to ET rate decreasing exponentially by a slop according to the tunneling materials (10, 30). In biological systems, understanding the variant $\beta$ coupled with a meaningful R between redox pairs is key to the elucidation of tunneling pathway tied to the tertiary atomic distribution (31). Due to the fact that for repeat biological element through-bond jump is predominant over through-space (7), with the same distance $\beta$-sheet secondary structure of proteins is proven to be a better tunneling medium than $\alpha$-helix and has a small decay constant of 1.0 Å$^{-1}$ (32). A common $\beta$ within 1.0-1.4 Å$^{-1}$ is generally found in protein systems. However, early studies on intraprotein ET behavior have shown that, at a distance shorter than 20 Å, slower rates aberrant from the anticipated by 3-6 orders of magnitude were observed (8, 33).
authors attributed the deviation from the “traveling” pathway through a circuitous route connecting the donor and acceptor and thus made the rates applicable at the 2 to 3-fold extended distances. Such distance adjustment is on a basis of barrierless transfer for the reactions occurring on the timescales of nanoseconds and milliseconds. With the recent advent of femtosecond spectroscopy, faster ET reactions are being detected and it is nevertheless invalid to treat a nanosecond ET process as an activationless reaction. In this regard, a new examination of short-range ET rate is necessary.

In this dissertation, we choose the biological model system of *Desulfovibrio vulgaris* (*D. vulgaris*) flavodoxin to uncover the intraprotein ET behavior between the prosthetic flavin mononucleotide (FMN) (Appendix B) and the amino-acid residues W60 or/and Y98 (34-35) and other replaced tryptophan residues. Two goals are endeavored. One is to elucidate the ET behavior from free of solvation influence to being controlled by solvation (Figure 1.3). The other one is to characterize the ET rate on the distance aspect spanning from the van der Waals contacts up to \( \approx 18.2 \) Å (edge-to-edge) and determine how severe the ET rate is deviated compared to the activationless prediction. In Chapter 2, the experiment methodology and the practical application for the reaction-diffusion equation is described. Protocols for sample purification are outlined in Appendix C. In Chapter 3, we resolve the dynamics of flavodoxin FMN-binding site solvation. At three different oxidation states (Appendix B and Appendix D for preparation), we identify the corresponding solvation time constants and discuss the results with respect to the structures. In Chapter 4, in the oxidized (OX) flavodoxin we find the photoinduced ET
highly favorable ($-\Delta G^\circ \approx \lambda$) and occur at subpicosecond time scales. Such charge separation (CS) process is faster than the binding-site solvation and represent the condition of point I in Figure 1.3. With 11 other mutants, we characterize the complete ET cycle and determine the according time constants. Besides the charge recombination (CR) at a few picoseconds, one process of energy dissipation (cooling) upon CR is observed. In Chapter 5, we repeat the experiments in the one-electron reduced (semiquinone, abbreviated SQ) flavodoxin. Here, the CS dynamics is decreased by 5-30 times and strongly coupled with the binding-site solvation (point II, Figure 1.3). The transients are observed following the nonexponential-decay behavior. We apply the extended Sumi-Marcus 2D model and resolve the parameters such as reorganization energy and electronic coupling matrix element. The effective total $\lambda$ is about 1.2-1.3 eV making the CS in the normal regime and CR the inverted. In the conclusion part, we provide a topologic view of the overall ET mechanism along solvation and vibration coordinates. In Chapter 6, we define the electron coupling in a relation to distance. With the site-directed mutagenesis, 10 different distances between ISO moiety of FMN and the W aromatic indole ranging from 3.7 Å to 18.2 Å is generated. The resulting CS is surprised to slow down in accordance with the lengthened distance, covering the range of dynamics with four orders of magnitude. Compared to the previous study on modified cytochrome c, with similar distances the rate of ET in flavodoxin is found to be faster by 2-3 orders of magnitude but remains one-order slower relative to the theory. A detailed discussion concerning the barrier and pathway model is provided.
Figure 1.1: Marcus energy gap dependence. The logarithmic ET rate shows a quadratic increase with the reaction free energy in the normal region and a decrease in the inverted region after passing through the activationless (or barrierless) apex where $-\Delta G^0 = \lambda$. At the top of each region, the parabolic potential of reactant (R) and product (P) along the system nuclei coordinate cross at the different position (red circles) relative to the reactant bottom.
Figure 1.2: Two-dimensional contour energy diagram for an electron transfer reaction. During the $X$ diffusion, at each $X$, ET occurs along the $q$ coordinate to overcome the $\Delta G^*(X)$ energy showed by the open arrows. The C-C thick line represents the transition-state curve.
Figure 1.3: Timeline of electron transfer and solvation reactions. Assuming the solvation dynamics for the system of interest is given, the ET reaction can take place very fast at point I, slightly slower at point II or much slower at point III.
CHAPTER 2: EXPERIMENTAL METHODOLOGY

2.1 Femtosecond Laser Spectroscopy

Utilizing the fluorescence upconversion and transient absorption techniques, in this article we carry out all the femtosecond temporal-resolved measurements. The method requires an ultrashort laser pulse with sufficient energy to initiate the reaction of interest and to monitor the spectroscopic response with time. Figure 2.1 depicts the layout of the optical apparatus to generate the time-dependent signal detainted in Figure 2.2. Briefly, the femtosecond seeding pulses are generated by a diode pumped Ti:sapphire oscillator (Tsunami or Mai Tai, Spectra-Physics). A two-state amplifier (Spitfire or Spitfire Pro, Spectra-Physics) amplifies the output mode-locked pulses, producing a high-energy ultrashort pulse for the experimental use (energy: ≈2 mJ; width: ≈110 fs; repetition rate: 1 kHz; wavelength: centered at 800 nm). In this dissertation, different wavelengths other than the fundamental 800 nm are needed. Tuning of the wavelength is achievable through the non-linear optical processes of sum-frequency generation (SFG) or second-harmonic generation (SHG) in the optical parametric amplifiers (OPA800C or TOPAS, Spectra-Physics). For both techniques, the laser beam splits into two beams, one for pump and the other for probe/gate purposes. The former beam passes through a movable translational
stage, creating a path difference with respect to the other. Due to the speed of light, a 0.3-mm path difference between these two pulses is equivalent to a 1-ps delay signal after time zero. A cycle of repeating the pump-probe approach with varying delay time, for example, from 0-mm to 3-mm path difference, a 10 ps temporal profile is generated. For all studies, the pump beam polarization is set at a magic angle (54.7°) with respect to the probe or gate beam.

2.1.1 Fluorescence Upconversion Methodology

The upconversion method is particularly to determine the quenching or deactivation dynamics occurring at the S\textsubscript{1} electronic state shown in Figure 2.3 (15). Since the fluorescence intensity is directly proportional to the S\textsubscript{1} population, one photoinduced reaction can be determined by monitoring the change of fluorescence in time. Oftentimes, such reactions take place faster than nanosecond (ns) timescale and can be captured using the femtosecond pulse. The upconversion technique also allows for scanning the emission wavelength at a set of delay time to construct the time-evolved fluorescence spectra. In Figure 2.1A, the apparent sample emission is collected by a pair of parabolic mirror and mixed with the gating pulse (800 nm or 560 nm) in the 0.5-mm β-Barium Borate crystal (BBO) crystal. At different BBO rotation angle, different emission wavelength is selected by an effective sum-frequency mixing. For spectra acquisition, a computer-controlled rotation stage scanning the BBO angle is used. Subsequently, the up-converted signal is
collected by a pair of lens and sent to a photomultiplier (PMT, Hamamatsu) coupled with monochromator (Gemi-180, Jobin Yvon).

2.1.2 Transient Absorption Methodology

With the same approach to initiate the reaction as upconversion, transient absorption uses the probe pulse to monitor the group of molecules already excited and being transformed to other intermediate species. As shown in Figure 2.3, the molecules in S₁ state absorb the probe pulse transited into one higher singlet state. The intensity change of the probe pulse with time is therefore informative to the reaction dynamics taking place in S₁ state. This approach is able to capture other intermediates and used to derive the mechanism occurring in the dark channel. In Figure 2.1B, the pump and probe beams are spatially overlapped inside the sample cell. After passing through the sample, the intensity of transmitted light is detected by the photodiode and digitalized by a computer. The signal readout is the difference of probe intensity with \( I_{t, \text{pump on}} \) and without \( I_{t, \text{pump off}} \) the pump passage switched by the chopper. Obeying the Beer’s law, Equation (2.1) represents the observed absorbance change \( \Delta A \) for the readout signal. The \( I_0 \) is the intensity of the probe-pulse incident, that is determined by introducing a third beam (the reference beam) diverged from a portion of probe beam without passing through the sample.
\[
\Delta A = - \left[ \log \left( \frac{I_t}{I_0} \right)_{\text{pump on}} - \log \left( \frac{I_t}{I_0} \right)_{\text{pump off}} \right]
\] (2.1)

In the case that the probe pulse can be absorbed by the sample itself (second excitation) an unwanted noise usually occurs due to non-homogeneous illumination the light traveling through the sample. In other words, the \( I_0 \) is more fluctuated. A low concentration of sample is preferred, typically controlled with the optical density (OD) at the pump wavelength around 0.5 or below. Ideally, the sensitivity of transient absorption can reach \( 10^{-4} \) to \( 10^{-5} \) absorbance change. With such technique, one consecutive or branching reaction can be deduced by solving the traditional chemical kinetics (36).

### 2.2 Fitting of Reaction-Diffusion Equation

The reaction-diffusion equation for ET reaction is employed to resolve the values of \( H_{AB}, \lambda_i, \lambda_o, \lambda_h \) and vibrational mode \( n \) in flavodoxin. The \( V(X) \) on the reactants is defined as \( \lambda_o X^2 \) where the reaction starts from \( X = X_0 \) to the product equilibrated position \( X = 1 \). If \( X_0 = 0 \), then \( V(X) = 0 \), the reaction initiates at the solvation equilibrated condition (21). In this case, \( \lambda_o \) is the solvation energy needed for the reaction. Otherwise, a less value of \( \lambda_o X_0^2 \) is computed. Typically, the laser-excited FMN molecule will deviate from the solvational equilibrium state \( (X_0 \neq 0) \) due to the dipole-moment change of the FMN molecule, introducing the solvation energy \( \lambda_o X_0^2 \) on the reactant. The activation energy of \( \Delta G^* (n_p, X) \) in Equation (1.9) becomes (also see Appendix A):
\[ \Delta G^* (n_p, X) = \left( \lambda_0 + \Delta G^* - 2X\lambda_0 + n_p \hbar \omega + \lambda_i \right)^2 / 4\lambda_i \] (2.2)

The resulting prediction from Equation (1.7) is thus used to fit upconversion data by the software of Wolfram Mathematica. In the OX flavodoxin, \( c(t) = 0.53 \exp(-t/1) + 0.26 \exp(-t/25) + 0.21 \exp(-t/670) \) and in the SQ flavodoxin, \( c(t) = 0.75 \exp(-t/2.6) + 0.25 \exp(-t/40) \) are resolved in Chapter 3. Initial condition is set a Gaussian distribution of \( p(X, 0) \) at \( 2\lambda_o / k_BT * \exp(2\lambda_o / k_BT) \).
Figure 2.1: Schematic illustration of (A) upconversion and (B) transient absorption setup. MP, movable parabolic mirror. PD, photodiode. Unfilled arrow shows the light path for pump pulse and the filled arrow for gate/probe pulse. In (A), the black arrow after BBO crystal is the up-converted signal. In (B), the gradient arrow is the reference beam sent to the other photodiode.
Figure 2.2: Principle of time-resolved signal generated by one pump pulse (blue-yellow gradient) and one gate or probe pulse (blue) with certain delay time. For a given normalized exponential decay, the time constant is when the signal drops to that of \(1/e\) relative to the original and is indicated by the red lines.
Figure 2.3: Electronic transition describing the fluorescence and transient absorption of the molecules in $S_1$ state. Other nonradiative transition and the vibrational sublevels are not shown.
CHAPTER 3: MAPPING SOLVATION DYNAMICS
AT THE FUNCTION SITE OF FLAVODOXIN
IN THREE REDOX STATES§

§Adapted from: C.-W. Chang¶, T.-F. He¶, L. Guo, J. A. Stevens, T. Li, L. Wang and D. Zhong.

### 3.1 Introduction

Flavoproteins are ubiquitous in biology and have unique ET properties due to the rich redox chemistry of the cofactor flavin molecules (37-43). They mostly contain flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) as their cofactors and participate in a variety of biological functions such as cell apoptosis (44), detoxification (45), redox reactions (43, 46), light-driven DNA repair (5, 47), and blue-light photoreceptors (3, 5, 48). To perform these various biological activities, the dynamical motions at function sites are essential to their catalytic reactions, as we have recently observed in photolyases (47, 49). Here, we choose an ET flavoprotein of flavodoxin as a model system and systematically characterize the function-site solvation in its three redox states of OX, one-electron reduced neutral SQ, and two-electron fully reduced anionic hydroquinone (HQ). Such characterization of the local solvation in the three states is
critical to understanding how the functional ET dynamics are modulated by local protein motions in flavodoxin.

Flavodoxin is a small family of flavoproteins with FMN as the cofactor and has been recognized as the electron carrier in many bacteria (50-52). The X-ray structures in three redox states have been solved (53) and Figure 3.1 (top Left) shows the crystal structure of wild-type *D. vulgaris* flavodoxin in its oxidized state, which is constituted of a central five-stranded parallel β-sheet flanked by several α-helices. The FMN cofactor is bound with three loops at the top of the protein and is therefore solvent exposed. There are total 6 polar/charged amino acid residues (S58, T59, W60, D62, Y98, and C102) within 6 Å from the isoalloxazine ring of FMN, and FMN is sandwiched by two aromatic residues of W60 and Y98 (Figure 3.1, top Middle). In the other two redox states, the X-ray structures show a significant cis-trans peptide bond conversion between G61 and D62, resulting in the formation of a hydrogen bond between the N5 hydrogen of the isoalloxazine ring and the carbonyl oxygen of G61 (Figure 3.1, bottom panel) (53). Figure 3.1 (top Right) also shows a snapshot of our MD simulations (1 ns) and clearly the FMN moiety is inserted into the hydrophobic pocket, and the exposed dimethylbenzene ring is surrounded by surface water molecules and neighboring negatively charged residues.

Using the intrinsic cofactor FMN as the local optical probe without extrinsic labeling, we are able to map out the entire evolution of local relaxations upon excitation at the function site in three redox states. To eliminate the potential ET quenching of excited FMN from the stacked aromatic residues of W60 and Y98 in OX and SQ states (54-58),
we design a double mutation of W60F/Y98F using the redox inert residue of phenylalanine and reported the excited-state FMN lifetimes in 6 ns, 230 ps, and 800 ps for the three redox states of OX, SQ, and HQ, respectively (58). The long lifetime of 6 ns in the oxidized state provides a sufficient time window to measure various local motions (49, 59-61). By examining the femtosecond (fs) resolved fluorescence dynamics and entire emission spectra of excited FMN at the function site, we observe the very different solvation dynamics in the three states and thus reveal their different local properties, structurally and dynamically, at the function site. We also examine the solvation dynamics of the FMN binding site in apoflavodoxin without FMN, using intrinsic W60 as a local optical probe by mutation of the other tryptophan residue (W140F). The single W60 gates the entrance of the binding pocket and thus also directly report the local water and protein motions at the binding site of apoflavodoxin.

3.2 Materials and Methods

The procedures of the expression and purification for *D. vulgaris* flavodoxin wild type and its mutants are described in Appendix C (62). Upon purification the flavodoxin mutants (W60F/Y98F and W140F) contain the oxidized FMN and is then converted to either the neutral radical semiquinone form (63) (FMNH•) or the two-electron reduced anionic hydroquinone state (64) (FMNH⁻) under the anaerobic conditions (Appendix D). For fs-resolved experiments, the oxidized flavodoxin is prepared at the concentration of
50 μM in 50 mM phosphate buffer solution at pH 7. For the reduced states, the samples in 200-250 μM are mixed with the reducing agents in 50 mM MOPS buffer at pH 7 for the semiquinone and in 50 mM phosphate buffer at pH 8.3 for the hydroquinone. The FMN sample (USBioAnalyzed, more than 98% weight purity) is directly used without further purification with a concentration of 400 μM in 50 mM phosphate buffer at pH 7. Apoflavodoxin of the W140F mutant is prepared by following the well-documented trichloroacetic acid (65) procedure except that the buffer solution of 50 mM Tris-HCl at pH 7.5 at room temperature was used throughout the preparation.

All fs-resolved measurements are carried out using the fluorescence upconversion method, described in Chapter 2. Briefly, the pump wavelengths are set at 400 nm for holoflavodoxin in OX and HQ states and FMN in buffer solution, at 560 nm for holoflavodoxin in SQ state, and at 290 nm for apoflavodoxin. The pulse energy typically is attenuated to 50-100 nJ for the OX state, free FMN and apoflavodoxin, and to 140-200 nJ for SQ and HQ states. The instrument response time under the current noncollinear geometry is about 450 fs, and all data are taken at a magic angle (54.7°). The fs-resolved emission spectra at various delay times are obtained by scanning the mixing crystal angles and monochromator wavelengths simultaneously over the entire emission range. Time zero at each wavelength is carefully calibrated and a variety of delay times are appropriately chosen to reconstruct the 3D fs-resolved emission spectra.
3.3 Results and Discussion

3.3.1 Ultrafast Fluorescence Transients and Solvation Dynamics in Three Redox States

Figure 3.2 shows the steady-state absorption and emission spectra of the W60F/Y98F mutant in different redox states. The absorption spectra in three redox states are similar to those of the wild type (62), indicating that the mutant has a negligible structural perturbation and can represent wild-type flavodoxin. The corresponding weak emission spectra are shown in the thick lines. The steady-state fluorescence emission of OX state shows a peak at 515 nm with a shoulder at 545 nm while in SQ and HQ states the spectra have the significant broadening around the peak regions of 680 and 530 nm, respectively, leading to the nonlog-normal emission profiles. These structural features in emission typically result from the heterogeneous electrostatic distributions and different structural flexibility around the (partially) buried chromophore inside the protein (49, 66).

Panels A-C of Figure 3.3 show respectively several typical fluorescence transients of OX, SQ, and HQ states gated from the blue to red side of the emission spectra. As a comparison, we also measure the dynamics of FMN* in buffer solution (Figure 3.3D). All transients are taken within 3.5-ns time windows and exhibit a general trend of decay at the blue side and rise at the red side, a manifestation of typical solvation dynamics. Strikingly, the transients show significantly different decay behaviors for the three states. In OX state (Figure 3.3A), the transients at the blue side are fitted with three solvation components ranging from 0.9-1.8, 9-30, and 600-700 ps besides the long lifetime
component in 6 ns. After the emission peak, the transients show rise components in 20-30 ps. In SQ state, the transients (Figure 3.3B) are clearly slower than those in OX state. At the blue side, the transients exhibit two decay components in 3.5-5 and 25-58 ps. Due to the short lifetime (230 ps) of SQ state, the second solvation component could mix with the lifetime emission, and any further slow relaxation will not be detected. Thus, the relaxation is not completed yet before the total excited-state deactivation. At the red side, the rise components in 1.4-5 ps are clearly present. In HQ state, the solvation dynamics at the blue side become faster again in 0.9-3.5, 10-31, and 55-300 ps, similar to those observed in OX state, but with much more pronounced amplitude for the first component. The third relaxation in hundreds of picoseconds could also mix with the lifetime component of 800 ps. At the red side, we also observe a fast decay component of 35 ps (10-15%) besides the lifetime decay (Figure 3.3C), indicating a loose binding pocket to allow FMNH\textsuperscript{−} to proceed to a butterfly bending and distortion motions for fast deactivation as also observed in (6-4) photolyase (49, 67), photoreceptor cryptochrome (68) and chemical systems (58). In all the three redox states, at the far red side of the emission, the transients become fast decay again, a general phenomenon for the slow solvation in heterogeneous protein environments through various spectral tunings (49, 69).

To extract the solvation correlation function, the fs-resolved emission spectra (FRES) are typically constructed using the obtained transients at various wavelengths (70-71). However, for the irregular spectral profiles not following the standard lognormal
distribution, we have to directly measure the FRES to follow the entire spectral evolution. Instead of using the emission peaks, we calculate the averaged frequency $\tilde{\nu}$ of FRES to obtain the averaged Stokes shifts and thus derive the solvation correlation function (72).

### 3.3.2 Femtosecond-Resolved Emission Spectra and Redox-Dependent Correlation Functions

Figure 3.4 show the reconstructed three-dimensional plots of FRES (upper panel) and a few snapshots at various delay times (lower panel) in the three states. The corresponding steady-state emission spectra are shown at the bottom. In all cases, we observe minor spectral shifts but obvious emission profile changes. As we observed in photolyases, the spectra of FMN in the three states here show little shifts of the emission peaks but obvious shrinking at the blue side and broadening at the red side. Such shape changes by the spectral tuning reflect heterogeneous local electrostatics and corresponding anisotropic relaxation (49, 73). To quantify such changes of local relaxations, we calculate the averaged frequencies and use the relative stabilization energies $\Delta E(t)$, relative to the time-zero averaged energy, to represent the solvation dynamics. The derived functions then could be fitted by the following equation with three exponential decays.

$$\Delta E(t) = \Delta E_1 e^{-t/\tau_1} + \Delta E_2 e^{-t/\tau_2} + \Delta E_3 e^{-t/\tau_3} + \Delta E_\infty$$  \hspace{1cm} (3.1)
To further evaluate the local flexibility through solvation, we define an averaged solvation speed (or averaged environment reorganization rate) in terms of energy drop (in \(\text{cm}^{-1}\)) per picosecond by local relaxation (49, 61).

\[
\Delta E_1 + \Delta E_2 + \Delta E_3 + \Delta E_\infty = 0
\] (3.2)

\[
S_1 = \frac{\Delta E_1}{\tau_1}, \quad S_2 = \frac{\Delta E_2}{\tau_2}, \quad S_3 = \frac{\Delta E_3}{\tau_3}
\] (3.3)

Figure 3.5 show the derived relaxation functions in the three states, and Table 3.1 gives the summary of their time scales, stabilization energies, and solvation speeds. Clearly, the correlation functions of three redox states are significantly different.

Specifically, in OX state, the solvation dynamics occur in 1 (53%), 25 (26%), and 670 ps (21%) (Figure 3.5A). For the shallow binding pocket with the partial exposure of FMN to the protein surface, the three time scales mainly represent the local water-network relaxation, coupled water-protein fluctuation, and intrinsic collective protein motion, respectively (59-61, 74-75). From our MD simulations, about 20-25 water molecules are found within 6 Å to the isoalloxazine ring of FMN and around the entrance of the function site (Figure 3.5A). Given the loop nature of the function site, the time scales of 1 and 25 ps are consistent with the dynamics of hydrating water molecules at the protein surface observed in the other proteins (60, 76). In SQ state, the solvation drastically slows down in 2.6 (75%) and 40 ps (25%) (Figure 3.5B). The MD simulations show no obvious changes in number of neighboring water molecules (Figure 3.5B), and
the main difference is the local structures in the two redox states (Figure 3.1). The SQ protein has superior stability (77) and a tighter binding pocket due to the formation of a specific hydrogen bond between the N5 position of FMN and the backbone carbonyl oxygen of G61 (Figure 3.1), indicating a more rigid local structure (Figure 3.5B). Thus, such a structural change in rigidity directly affects the neighboring water-network flexibility and coupled water-protein fluctuations, leading to the observed slow solvation dynamics. The lack of observation of the third longtime protein relaxation in subnanoseconds is due to the short chromophore’s lifetime (230 ps).

For HQ state, the solvation becomes faster again and the time scales are 1 (83%), 21 (14%), and 180 ps (3%), comparable to those of OX state, but with a significant increase of the first fast component (Figure 3.5C). From the X-ray structure (53), our MD simulations (Figure 3.5C), and the NMR studies (78), the shallow binding pocket become looser, mainly in the regions of 56-82 residues of the 60’s loop and 91-103 residues of the 90’s loop and largely due to the repulsion between FMNH and the surrounding negatively charged residues such as D62 and D95 (Figure 3.1 and Figure 3.5C). Thus, the local hydrogen-bond network becomes more flexible, and also more water molecules can penetrate into or are trapped around the function site, resulting in a larger stabilization energy in 1 ps and suggesting a more polar environment in HQ state. Since the stabilization energy depends on the local polarization and the dipole-moment change $\Delta \mu$ between the ground and excited states and the recent experiments by Stark spectroscopy suggested similar value of dipole-moment changes in three redox states (79-81), the
observed large increase of $\Delta E_1$ (360 cm$^{-1}$) in HQ state must reflect the local environment change and in this case both the local water network and protein structure probably become more flexible. Interestingly, we observe very similar stabilization energy of $\Delta E_2$ (50-60 cm$^{-1}$) for all the three states, reflecting the same amount of relaxation energy from coupled water-protein fluctuations. Since there is no ET quenching of FMNH$^*$ in HQ state, we also examined the solvation dynamics of the wild-type protein and obtained results similar to those of the mutant (Table 3.1). The increase in stabilization energy of $\Delta E_2$ (97 cm$^{-1}$) probably results from the contributions of stacked Y98 (polar) and W60 residues, whereas in the mutant, FMN is sandwiched by the nonpolar residue of phenylalanine. The observed small $\Delta E_3$ and fast $\tau_3$ are due to the short lifetime of the wild-type protein (300 ps).

The solvation speeds in the three states (Table 3.1) are dramatically different and directly elucidate the local flexibility. The first and second solvation speeds ($S_1$ and $S_2$) systematically change from the largest in HQ state, to the middle in OX state, and to the smallest in SQ, reflecting an evolution of the hydrogen-bond network from flexible to rigid and correlating well with the local protein mobility. The third solvation speed ($S_3$) is similar, indicating an intrinsic collective motion of flavodoxin. Thus, we observe a relatively tight function site in SQ state with both rigid local water networks and inflexible side chains and a relatively loose function site in HQ state with considerable water-network flexibility and side-chain mobility, a drastic difference of the local properties for these two physiological in vivo states in cell.
We also examine the solvation dynamics of FMN in buffer solution as a control to quantify the stabilization energy (Table 3.1). We observe three time scales of 0.4 (11%), 1.4 (70%), and 11 ps (19%). Compared to bulk water (71, 82), the solvation dynamics overall is slow down, presumably resulting from the more hydrogen-bond networks and ions of the ribityl phosphate moiety. For the log-normal distribution of FRES, the relaxation energy can be derived by calculating either the emission peak shifts or averaged frequency changes (Table 3.1). We obtained a stabilization energy of 341 cm$^{-1}$ for the first two fast relaxations, similar to the values (345-360 cm$^{-1}$) in HQ state but much larger than those in OX and SQ states (111-154 cm$^{-1}$), suggesting more and looser water molecules in the function site of HQ state, less mobile water molecules in OX state, and even more rigid in SQ state.

### 3.3.3 Binding-Site Solvation and Protein Surface Hydration

We further examine the local solvation dynamics at the binding site without the cofactor FMN inside the pocket. The X-ray structure of apoflavodoxin (83) shows that the tryptophan residue W60 slightly rotates and perfectly covers the entrance of the binding pocket and lies down at the protein surface with a maximum emission peak at 343 nm. Using the mutant W140F, the single tryptophan W60 can be used as a local probe to study the surface hydration dynamics around the binding site as has been extensively used in our other studies (59). Figure 3.6 shows the final results, and the obtained time
scales and stabilization energies are also given in Table 3.1. Significantly, we observe the two similar relaxations in 1 (50%) and 18.3 ps (50%), reflecting the initial local water-network relaxations and subsequent water-protein coupling fluctuations, completely consistent with the dynamics observed in the other proteins with similar local protein properties (60, 76). The lack of the possible third relaxation is due to mixing with one short lifetime (400 ps) of W60. Compared with holoflavodoxin in OX state, the second relaxation (18.3 ps) of the water-protein coupling motion becomes obviously faster, reflecting a more flexible structure in apoflavodoxin and a more rigid binding structure in holoflavodoxin. The observed greater stabilization energy by a factor of 4-5 results from the larger change of dipole moments $\Delta \mu$ of W60, consistent with the reported values of dipole-moment changes, 1.1 D (Debye) for FMN (79) and $\approx$5 D for tryptophan (84). Thus, for the shallow and hydrophobic binding site of FMN, the local hydration dynamics probed by W60 show the fast water-network relaxations, similar both in time and energy to those of the function-site solvation probed by the cofactor FMN. Moreover, the two probes can distinguish the different water-protein coupling motions, reflecting the intrinsic different flexibility of the local structures with and without binding of the cofactor FMN.
3.4 Conclusion

We report a systematic study of the local solvation dynamics in the binding site of apoflavodoxin and in the function site of holoflavodoxin in three redox states using the intrinsic tryptophan (W60) and cofactor FMN as the local optical probes without extrinsic labeling. With site-directed mutations, we design two mutants to reach the specificity for W60 and to eliminate the potential quenching of FMN, respectively. With femtosecond resolution, we observe the distinct solvation dynamics in apoflavodoxin and in three redox states of holoflavodoxin. From the apoflavodoxin to oxidized holoflavodoxin (FMN), we observe similar fast water-network relaxations in 1 ps but different water-protein coupling fluctuations (18.3 vs 25 ps) from the two different structural flexibilities. From the oxidized to semiquinone (FMNH•) states, the local structure becomes more rigid due to the extra hydrogen-bond formation by the redox switching. The local solvation dynamics significantly slow down to 2.6 and 40 ps, a clear correlation between the local structural rigidity and water-network immobility. From the semiquinone to hydroquinone (FMNH‾) states, the solvation relaxations become faster again in 1 and 21 ps with a significant increase in total solvation energy, due to a more polar environment with more mobile water molecules in a looser and larger pocket caused by the negative repulsion between the anionic cofactor and neighboring negatively charged residues. With the long excited-state lifetime of the local probe, we also observe the intrinsic protein relaxation in hundreds of picoseconds as shown in oxidized and partially hydroquinone states.
The observed distinct solvation dynamics in three redox states are significant, reflecting the redox dependence of protein conformation plasticity and water network flexibility, ultimately relating to the biological functions. As an electron shuttle, the compact, immobile function site in the semiquinone state is ideal to dock into electron-donor partners (such as flavodoxin reductase or photosystem I) for reduction, and the loose, flexible function site in the hydroquinone state is necessary to deliver electrons to a variety of electron-acceptor partners (such as ferredoxin-NADP\(^+\) reductase or methionine synthase) with nonspecific interacting interfaces (52, 85-87). The observed correlation between the redox states and the solvation dynamics is fundamental and elucidates the intrinsic dynamical relationship between hydration networks and local protein structures. For a given redox state with a unique structure, it requires a certain water network for its stability and flexibility. In turn, the dynamics of such a specific water network controls the flexibility of the local protein structure, an intimate relationship of water-protein coupling motions. Clearly, such a relationship of redox states with protein conformation flexibility and water network mobility at the function site is essential for performing the various biological activities.
**Figure 3.1**: Top panel: (Left) X-ray crystal structure of oxidized *D. vulgaris* flavodoxin (PDB: 3FX2). (Middle) Close view of the FMN binding site embraced by two 60’s and 90’s loops. The FMN cofactor is sandwiched by two aromatic resides of W60 and Y98. (Right) Surface-map representation of a snapshot of 1-ns MD simulations, showing the function site with hydrating water molecules within 6 Å from the isoalloxazine ring and neighboring negatively charged residues (red). Lower panel: Local X-ray structures of three redox states show the loop flipping in SQ and HQ states to form a hydrogen bond with N5 of the isoalloxazine ring.
Figure 3.2: Steady-state absorption (thin line) and emission (thick line) spectra of W60F/Y98F mutant in OX, SQ, and HQ states. The excitation wavelengths were 400 nm for OX and HQ states, and 560 nm for SQ state. For SQ state, the emission spectrum at the red side was fitted with a lognormal function to extrapolate the emission longer than 800 nm as shown in the dashed line.
Figure 3.3: Normalized femtosecond-resolved fluorescence transients of W60F/Y98F mutant in (A) OX, (B) SQ, (C) HQ states, and (D) FMN in buffer solution. These transients were gated from the blue to red side of the emission spectra. Note the drastic differences in three redox states.
Figure 3.4: Upper panel: Three-dimensional representation of femtosecond resolved emission spectra of W60F/Y98F mutant in (A) OX, (B) SQ, and (C) HQ states. The intensity is scaled by a color code. Lower panel: Three snapshots of the femtosecond-resolved emission spectra at typical delay times for the three states with their corresponding steady-state emission spectra. For comparison and clarity, the steady-state emission peaks are indicated by gray dotted lines.
Figure 3.5: Upper panel: Surface-map representation of a snapshot of the function site of flavodoxin in (A) OX, (B) SQ, and (C) HQ states from 1-ns MD simulations. Only water molecules within 6 Å from the isoalloxazine ring are shown. The negatively charged residues are indicated in red. Lower panel: Solvation responses at the function site of flavodoxin in three redox states represented by the stabilization energy changes ($\Delta E$) with time. The insets show the responses at short time scale.
Figure 3.6: (A) Normalized femtosecond-resolved fluorescence transients of apoflavodoxin (W60) gated from the blue to the red side of the emission spectrum. The transients are shown at two different time scales. (B) Representative of normalized femtosecond-resolved emission spectra (FRES) at different delay times. The symbols represent the experimental data, and the solid lines are from the log-normal fitting. The dashed line is the steady-state emission spectrum. (C) Solvation response at the binding site of apoflavodoxin represented by the stabilization energy change ($\Delta E$) with time, and the inset shows the short time range.
Table 3.1: Results of solvation dynamics at the function site of flavodoxin in three redox states

<table>
<thead>
<tr>
<th>Environment</th>
<th>Probe</th>
<th>(\tau_1)</th>
<th>(\Delta E_1)</th>
<th>(\tau_2)</th>
<th>(\Delta E_2)</th>
<th>(\tau_3)</th>
<th>(\Delta E_3)</th>
<th>(S_1)</th>
<th>(S_2)</th>
<th>(S_3)</th>
<th>(\Delta E_T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavodoxin (W60F/Y98F)</td>
<td>FMN/OX</td>
<td>2.6</td>
<td>154</td>
<td>40</td>
<td>50</td>
<td>59</td>
<td>1.25</td>
<td>360</td>
<td>360</td>
<td>2.5</td>
<td>204</td>
</tr>
<tr>
<td></td>
<td>FMNH(^{\text{SQ}})/SQ</td>
<td>1</td>
<td>360</td>
<td>21</td>
<td>59</td>
<td>180</td>
<td>11</td>
<td>360</td>
<td>2.8</td>
<td>0.06</td>
<td>430</td>
</tr>
<tr>
<td>Flavodoxin (wt)</td>
<td>FMNH(^{\text{HQ}})/HQ</td>
<td>0.9</td>
<td>345</td>
<td>26</td>
<td>97</td>
<td>81</td>
<td>6</td>
<td>383</td>
<td>3.7</td>
<td>0.07</td>
<td>448</td>
</tr>
<tr>
<td>Buffer solution(^b)</td>
<td>FMN</td>
<td>0.4</td>
<td>45</td>
<td>1.4</td>
<td>296</td>
<td>11</td>
<td>80</td>
<td></td>
<td></td>
<td></td>
<td>421</td>
</tr>
<tr>
<td>Apoflavodoxin (W140F)(^c)</td>
<td>W60</td>
<td>1.1</td>
<td>594</td>
<td>18.3</td>
<td>586</td>
<td>540</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
<td>1180</td>
</tr>
</tbody>
</table>

\(^a\)The solvation dynamics were derived by calculating the averaged frequency changes with time. The time constant \((\tau)\), total stabilization energy \((\Delta E_T = \Delta E_1 + \Delta E_2 + \Delta E_3)\) and solvation speed \((S)\) are in units of ps, cm\(^{-1}\), and cm\(^{-1}\)/ps, respectively. \(^b\)The solvation dynamics derived from the peak shifts by the log-normal fitting are \(\tau_1=0.4\) ps (121 cm\(^{-1}\)), \(\tau_2=1.4\) ps (400 cm\(^{-1}\)), and \(\tau_3=11\) ps (133 cm\(^{-1}\)). \(^c\)The solvation dynamics derived from the peak shifts by the log-normal fitting are \(\tau_1=1.1\) ps (595 cm\(^{-1}\)) and \(\tau_2=18.4\) ps (610 cm\(^{-1}\)).
4.1 Introduction

Dynamical process of ET in biological systems plays a crucial role in manipulating structure-function correlations (18, 88). Short-distance ET over less than 14 Å is in particular key to many light-initiated enzymatic activities. One typical system carrying such activities is flavoprotein/flavoenzyme which commonly use flavin cofactors as a light sensor (47, 67, 89). Flavin chromophores are rich in redox property (46) and enable the flavin-containing systems to tune the potential energy and modulate various catalytic processes. Therefore, an in-depth understanding of flavin involved ET will be critical to the elucidation of their molecular mechanisms. Several studies unveiling ET dynamics in different flavoproteins and flavoenzymes have been reported, including in glucose oxidase, riboflavin (vitamin B₂)-binding protein, DNA-repair photolyase and flavodoxin (47, 54-56, 67). All the studies have shown the intraprotein ET dynamics from sub-ps to hundreds of ps and are occurring on the same timescales as protein solvation and cooling dynamics (59, 90-92). ET at such hot states is in concomitance with the local molecular relaxation and the system energy dissipation. In this contribution, we will attempt to
characterize the intimate relationship between ultrafast ET including CS and CR and the circumventive environmental relaxation in the model system of *Desulfovibrio vulgaris* flavodoxin.

Flavodoxin is one class of flavoproteins and functions as an electron shuttle through the cofactor of FMN (51). Structurally, flavodoxin consists of five-parallel β-strands surrounded by several α-helices as displayed in Figure 4.1, Left (53). The prosthetic FMN group is noncovalently yet tightly bound by a series of interactions to the apoprotein. Aromatic π-π stacking of Y98 or W60 and FMN isoalloxazine ring at van der Waals contact (Figure 4.1, Right) is one such interaction and with this sandwich conformation, the FMN-binding site is most water excluded, leaving only partial FMN moiety of dimethylbenzene ring access to solvent. In Chapter 3, we have characterized the dynamics of such surface water approximate to the binding site and the associated water-protein coupling fluctuation at a few and tens of picoseconds, respectively (93). Flavodoxin can undergo two successive electron reductions, reduced from oxidized, to one-electron neutral semiquinone, and to two-electron hydroquinone states. Under physiological condition, the second radical form features the long-lasting and functioning state. By the site-directed mutagenesis, several key residues contributing to the thermodynamic stable form of semiquinone have been identified (62, 94-96). Residue G61 (see Figure 4.1, Right), for example, is responsible for cis-trans peptide bond (G61-D62) conversion and the consequent stabilization of an unpaired electron (53, 94). Acidic residues enclosing the binding site like D95 (see Figure 4.1, Right) are believed to
suppress the formation of flavodoxin hydroquinone anion through an unfavorable electrostatic effect and retard further reduction of the semiquinone (96). Such detailed information of manipulating redox property in flavodoxin has made it a good candidate for ET study.

In this article, firstly, we show our investigation of CS dynamics in flavodoxin and different mutants by utilizing the fs temporal-resolved laser spectroscopy. Our previous study replacing both W60 and Y98 by inert aromatic F (a double mutant W60F/Y98F) has abolished the intraprotein ET in *D. vulgaris* flavodoxin, extending the FMN* lifetime from 158 fs to 6.0 ns (54, 93). Therefore, we only consider W60 and Y98 donating electron to the excited-state FMN in flavodoxin, and eliminate the possibility from the next adjacent Y100 and W140 aromatics. In keeping one or both donor residues in flavodoxin, we isolate donors of Y98 and W60 and change the binding-site environment to evaluate the redox effect. In the following, we list 11 mutants and classify into four groups: W60F and W60A leaving only Y98 as ET donor; Y98R, Y98H, Y98A and Y98F leaving only W60 as ET donor; G61A, G61V and D95N containing both Y98 and W60 donors; and Y98W and W60Y including two ET donors of W60/W98 and Y60/W60, respectively. Subsequently, we approach the transient absorption technique and deduce the following CR and cooling dynamics accordingly. In the proceeding discussion, we investigate the energy potentials for the observed CS and CR dynamics and examine a timely correlation with the local solvation and protein cooling.
4.2 Materials and Methods

The purification procedures for flavodoxin and the mutants are performed by following the same protocol as does in Chapter 3 (Appendix C) (62, 97). For fs-resolved experiments, the flavodoxin is prepared at the concentration of 60-250 µM in 50 mM phosphate buffer solution at pH 7. To remove the unbound FMN molecule, the sample is passed through a mini-desalting column (Sephadex G-25 media) right before use. During laser experiments, the sample is in aerobic condition at the room temperature. The instrument response time is about 260-300 fs and 160-420 fs for fluorescence detection and transient absorption, respectively. All experiments are taken at the magic angle (54.7°). To avoid heating and photobleaching, the sample is kept in stirring quartz cells during laser irradiation.

4.3 Results and Discussion

4.3.1 Flavodoxin and Transient Species Absorption

In Figure 4.2B, we showed the visible-region absorption spectra of oxidized flavodoxin in wild type (WT) and the mutant of Y98W. The visible spectra represent the $S_1 \leftarrow S_0$ and $S_2 \leftarrow S_0$ transition of FMN molecule bound in flavodoxin (98), and in Y98W the higher bump in 500-700 nm is attributed to the charge-transfer (CT) complex, CT$\leftarrow S_0$, formed by flavin and indole aromatic compounds (62, 99). In principle, all the absorption spectra measured at the room temperature demonstrate the electron jump originated from the
lowest vibrational mode in $S_0$ state. In some special cases, electron at high frequency mode (quantized, $h\nu > k_B T$) can also be detected, those including high-temperature measurement (91, 100) and in the process of hot-stage CR reaction (101). Usually, the detection of such hot molecules is more substantial at the long-wavelength tail close to the molecular absorption. In flavodoxin, we highlight these regions in Figure 4.2B (gray bar). In Figure 4.2A, we list the transient-absorption spectra of all the intermediate species associated with ET mechanism from Y-FMN* and W-FMN* redox pair, including flavodoxin bound FMN* (35), anionic flavin semiquinone in protein (102), and cationic indole and phenol radicals in organic solvents (35, 103).

4.3.2 Upconversion Charge Separation and the Gibbs Free Energy

All the upconversion transients of flavodoxin show the FMN* quenching dynamics in several hundreds of femtoseconds. Small amplitudes of nanosecond component (<1-3%) observed in some mutants is assigned as free FMN molecules and therefore removed for clarity (55). As shown in Figure 4.3, except the mutant of W60A, all the transients gated at the near emission peak (538 nm) are fitted with a single exponential decay. With instrument response of 260-300 fs, we show the dynamics of $\approx 157$ fs in WT, $\approx 258$ fs in Y98F and $\approx 302$ fs in W60F, well consistent with the dominant component of $\approx 158$ fs (92%) in WT, $\approx 245$ fs (85%) in Y98F, and $\approx 322$ fs (83%) in W60F, from previous study
The second component in their report could have arisen from the concern about sample freshness. In all single W60-donor mutants such as Y98R, Y98H, Y98A and Y98F transients are displayed with the quenching time of 193-258 fs, while single Y98 donor in W60F and W60A mutants decrease the dynamics by 20-40% and showing the dominant 302-341 fs (see Table 4.1). The main difference is ascribed to the lower redox potential (Appendix F) (104) of W$^{+}$/W of $\approx +1.15$ eV vs NHE (105) than Y$^{+}$/Y of $\approx +1.47$ eV vs NHE at the physiological condition (pH=7). The value of Y$^{+}$/Y is derived from the following standard Equation (4.1) (104), using $pK_{\text{ox}}=2$ (106), $pK_{\text{ox}}=10$ (107) and $E_{m,7}=+0.93$ eV (108), resulting in a little higher value than the measurable limit at 1.35 eV (107).

$$E_{m,x} = E_{m(\text{acids})} - 0.06 \log \left( \frac{1 + K_{\text{ox}}/[H^+]}{1 + K_{\text{red}}/[H^+]} \right)$$  \hspace{1cm} (4.1)$$

By employing a similar equation at the basic side in Equation (4.2), redox potentials for FMN/FMN$^{+}$ in flavodoxin can also be obtained by knowing the value for FMN/FMNH$^{+}$ at physiological pH as $E_{m,7}$ for OX/SQ in Table 4.1 (62, 94, 109-110), and the universal $pK_{\text{ox}}=0$ for N10 protonation (111) and $pK_{\text{red}}=10.5$ in flavodoxin (62).

$$E_{m,x} = E_{m(\text{bases})} - 0.06 \log \left( \frac{1 + [H^+]/K_{\text{ox}}}{1 + [H^+]/K_{\text{red}}} \right)$$  \hspace{1cm} (4.2)$$
In Table 4.1, the driving force for CS in Equation (4.3) and CR in Equation (4.4) are calculated including the 0-0 transition energy equivalent to +2.5 eV (495 nm for flavin molecule) in flavodoxin with Coulomb interaction ignored in both equations (80, 112).

\[
\Delta G_{\text{CS}}^{\circ} = E(D^*/D) - E(A/A^{*+}) - E_{0,0}(A) \tag{4.3}
\]

\[
\Delta G_{\text{CR}}^{\circ} = E(A/A^{*+}) - E(D^*/D) \tag{4.4}
\]

Within an acceptable error, the distinct pattern of flavodoxin dynamics for CS generally follows the Marcus normal-region behavior (10), but those with two ET donor mutants are not taken in account due to the difficulty to derive an accurate Gibbs free energy. In Y98W where two W stack closely on the FMN*, the very weak fluorescent signal appears beyond our pulse limit and is assumed to be much faster than 100 fs (data not shown). Dual donors of Y in W60Y apparently contribute to the slightly faster CS dynamics than one Y (resolved at \(\approx 258\) fs) but remaining the slowest among all the mutants holding two ET donors (see Table 4.1). It is not surprising that the mutant of D95N shows a similar CS rate as in WT (i.e., with comparable redox potentials), but in G61A and G61V the very negative redox potentials obviously has failed to slow down the CS dynamics. This is easily understood since the key effect of G61 mutation is to hindering the readily protonation on the one-electron reduced FMN (FMN\(^{-}\)→FMNH\(^{+}\)), our temporary CS mechanism of FMN\(^{+}\)→FMN\(^{-}\) should not consider such hindrance.
4.3.3 Ultrafast ET Mechanism

Time-resolved transient absorption of the W60F mutant at different wavelengths clearly displays the detection of various species evolving in ET mechanism (see Figure 4.4 and Figure 4.4A). Both the fastest transients probed at 800 nm and 740 nm are resolved with $\approx 485$ fs, a little slower than the upconversion observation ($\approx 302$ fs) but reasonably assigned as CS dynamics by the transient-absorption measurement (113). Probe of 540-500 nm moving toward the flavodoxin absorption tail detects two additional slower decays of $\approx 950$ fs (45-55%) and 3.7-4.0 ps (9%-40%) with the latter component more and more pronounced. The deconvolution of 510-nm transient (Figure 4.4B) demonstrates its signal superimposition from FMN*, FMN•– and Y•+ which decay at $\approx 950$ fs, and the FMN† species disappearing in $\approx 3.8$ ps. In the recovery transient, probing at 480 nm shows the uprising time at a mixture of $\approx 485$ fs and $\approx 950$ fs plus some trace of 3.96 ps ($\approx 10\%$). A dominant recovering dynamics matching FMN† cooling is not observed here and, as shown in Figure 4.4C, is a cancellation consequence with several positive signals. The transient of 410 nm where FMN* and FMN† absorption are significant (Figure 4.2A) shows major decays of $\approx 485$ fs and $\approx 950$ fs.

Transients in the Y98F mutant show the CS dynamics somewhat faster than in W60F, and recombines slightly slower at 1.27-1.78 ps. At 800 nm and 740 nm, the dominant FMN* absorption determines the CS from W60 at $\approx 370$ fs and is in a fair difference compared to the upconversion quenching (see Figure 4.5 and Figure 4.5A). Signal of W•+ was easily deconvoluted in the transient of 580 nm (Figure 4.5B) and clearly the
vanishing decay (1.61 ps) shows about three times slower than the CS, thereby an
sufficient amount accumulated and being observable up to 800 nm (Figure 4.5A vs
Figure 4.2). Like in W60F, we also observe an evolving 3.3-3.8 ps component from 540
nm to 500 nm and with the same fitting strategy containing the obtained CS and CR
dynamics (see Figure 4.5C), has isolated it with 5-40% amplitude. Figure 4.5D show the
FMN in Y98F recovering at ≈1.78 ps, a result of superimposed FMN*, FMN−, W++ and
FMN† absorption at 480 nm as described in the above W60F. Probe of 410 nm shows a
very similar transient as of 580 nm regardless the much shorter pulse and indicates the
equal dynamics for FMN− and W++ intermediates.

Transient absorption of WT in Figure 4.6 (Upper) shows several species in
associated with ET reactions from both W and Y residues. The transients probed at 740
nm and 700 nm all show the major component of ≈180 fs and a considerable ≈1.0 ps. At
580 nm, in spite of some initial fast ≈180 fs, the transient displays decay dynamics of
≈1.56 ps and represents the CR due to the paired W++ and FMN−. The other CR with Y
involved is obviously embedded in the transients from 540 nm to 500 nm and with some
of this ≈800 fs component, the decaying pattern is more like a combined Y98F and W60F
mutants (see Figure 4.4, Figure 4.5, and Figure 4.6, Upper). In WT, a quantitative
distribution between these two ET reactions is uncertain, but the recovering of ground-
state FMN at 480 nm appears a more favorable W-donor channel, leading to the observed
≈1.56 ps very comparable to the Y98F. Figure 4.6 (Lower) in Y98W also show this
similar recombination dynamics of ≈1.1 ps determined in 740-nm transient where no
obvious ultrafast component matching the upconversion FMN$^*$ decay (the black dashed line in Figure 4.6) is detected. It is noteworthy to mention that in Y98W, the cooling dynamics of 3.0-4.0 ps along with ≈1.1 ps are observed starting from 630 nm continuously to 480 nm as highlighted with grey bar in Figure 4.2B (Inset) and consistent with the raised CT absorbance. With the same reason as Y98F, the bleaching dynamics at 480 nm almost mirrors its 580-nm decay and recovers at ≈1.1 ps.

In Table 4.1, more flavodoxin mutants show that ET mechanism behaves no difference among the mutants of W60A, W60Y and W60F. Although CS in transient absorption is generally slower than in upconversion, little is distinguished among all the Y-donor flavodoxin. In transient absorption, the mutants holding only W60 donor in Y98R, Y98H, and Y98A are in accordance with upconversion CS pattern (0.32-0.37 ps), but in the electron recombining and intraprotein cooling there shows no distinct dynamics among them. The same scenario also applies to the mutants of G61A, G61V and D95N compared to WT, and display a general ≈800 fs and 1.23-1.56 ps for the respective Y and W involved CR reactions.

4.3.4 The Energetic and Solvation View

In our previous study on flavodoxin solvation, the oxidized state has showed a multiple solvation dynamics of 1.0 ps (53%), 25 ps (26%) and 670 ps (21%). The light-induced ET mechanism in flavodoxin occurs on the time window of faster than 0.5 ps in CS
followed by 0.8-1.78 ps in CR and 2.5-4.0 ps cooling, and obviously only the first solvation 1.0-ps and some in 25-ps components are involved throughout the reaction. In Figure 4.7, the reaction is initiated by an instantaneous pump of the FMN molecule to the ET channel and proceeds with three consecutive steps of CS, CR and intraprotein cooling. Mainly along the nuclear coordinate, the flavodoxin undergoes an immediate CS much faster than solvation process and produces an ion-pair species. In this exothermic CS (-0.65--0.99 eV), the occurrence of concomitant solvent reorganization is very limit and strongly indicates the CS crossover at the initial static configuration in flavodoxin. Subsequently, the newly formed ion pair experience a divergence on the solvation coordinate and is apparently accompanying the following recombination reaction along nuclear coordinate. The very exergonic CR (-1.5--1.85 eV) in competing with solvation is accomplished at the crossing point at FMN hot vibrational state, leaving certain energy to be dissipated. During the last heat dissipation, the system is drawing back to the origin in solvation coordinate.

4.4 Conclusion

We report our systematic study of flavodoxin charge separation and charge recombination with the associated vibrational cooling and solvation dynamics. By the site-directed mutagenesis, we isolate the aromatic amino Y and W donors at a comparable distance to the FMN$^+$ acceptor, and investigate the resulting ET mechanistic
dynamics contributed from each donor and from different combination of dual donors in an altered redox environment. Generally, CS in oxidized flavodoxin occurs within 0.1-0.5 ps with negligible solvation involved, and the proceeding CR at 0.8-1.8 ps competes with the circumventive solvent reorganization, yielding the vibrational unrelaxed FMN product. The produced FMN\(^{\dagger}\) then dissipates the excess heat to the protein environment in 2.5-4.0 ps. At the same time, the solvent coordinate returns back to its starting configuration. Among 12 different flavodoxin, we found the last cooling process all at similar dynamics unaffected by the mutation. In our observation of flavodoxin ET, the residue W60 appears to be an efficient ET donor compared to Y98, with ≈1.5 times faster the forward ET and slightly and moderately slower than the electron returns. Furthermore, the dual ET donors composed of two W residues in Y98W also show the fastest CS dynamics (<0.1 ps) with the electron returning in ≈1.1 ps. By changing the binding-site environment, the redox energy shows a globally positive correlation with CS dynamics but is otherwise insensitive to G61 mutation due to its role in protonating the newly formed FMN\(^{-}\) irrelevant to the current CS reaction. Surprisingly, unlike the energy tendency in CS, flavodoxin CR associated with either W or Y donor (or both) does not show any obvious dependence of the driving forces, and it is probably that the recombination reaction is too exergonic (-1.5~-1.8 eV) to be affected by small energy shift. Overall, the elucidation of underlying flavodoxin CS and CR, distant from and entering the initial solvation window, and their respective energy properties is significant and opens new page for flavin involved intraprotein ET reactions.
Figure 4.1: (Left) X-ray crystallographic structure of oxidized *D. vulgaris* flavodoxin (PDB code 2FX2). (Right) A close-up view of the local configuration at the FMN-binding site. The FMN cofactor (in yellow) is sandwiched between two neighboring aromatic residues W60 (in green) and Y98 (in orange) at the van der Waals contact. Also shown are the acidic residue D95 and the backbone carbonyl group of G61 in close proximity of FMN.
Figure 4.2: (A) Normalized (transient) absorption spectra of protein-bound FMN$^+$ (red) and anionic flavin semiquinone (dark yellow), and free cationic radicals of indole (blue) and phenol (dark purple) in organic solvents. The last profile of phenol radical cation was extracted by direct subtraction of long-lived phenoxy radical absorption from the mixture spectrum. In the transient absorption experiments, all these transient species can be detected but contribute variably, depending on the probe wavelength used as horizontally listed above. (B) Steady-state absorption spectra of wild-type (blue) and Y98W (red) flavodoxin mutant. Arrows mark the pump wavelength of 400 nm in transient-absorption experiments and the multiple probe wavelengths in probing the vibrationally hot FMN molecules (Inset).
Figure 4.3: Normalized femtosecond-resolved fluorescence transients of FMN in flavodoxin mutants gated at 538 nm. Note that, in some transients, a small long-lifetime component of less than 3% due to the presence of free FMN molecule was removed for clarity.
Figure 4.4: Normalized femtosecond-resolved transient absorption of flavodoxin mutant of W60F probed from 800 nm to 410 nm. In this mutant, Y98 serves as ET donor. The initial spike caused by nonlinear optical effects and some long-lifetime components due to the strong triplet state absorption of free FMN molecule (Appendix B) were removed for clarity. Inset A shows all the fitting transients for readily comparison. Note the fitting 480-nm transient was normalized only to one half. Inset B and C explains the transient partly attributed from the decaying FMN* (red line), the dynamics of intermediate FMN*/Y•+ (blue line), and the cooling of transient-formed FMN† (green line). Note the deconvoluted FMN recovery (light-orange line) only shows half of its actual intensity.
**Figure 4.5**: Normalized femtosecond-resolved transient absorption of flavodoxin mutant of Y98F probed from 800 nm to 410 nm. In this mutant, W60 serves as ET donor. Also, the observed initial spike and long component were removed. Inset A shows the transient absorption probed at 800 nm dominantly contributed from FMN* (red line) plus a minor signal (7%) of W•+ (blue line). Inset B shows the 580-nm transient comprised of some FMN* signal but major due to W•+ intermediate (65%). Inset C shows the deconvolution of 510-nm absorption transient with FMN*, intermediate FMN*/W•+, and FMN†. Inset D interprets the observed signal probed at 480 nm as a resulting superimposition of positively signaled FMN*, FMN*/W•+ and FMN† with negative FMN recovery.
Figure 4.6: Normalized femtosecond-resolved transient absorption of wild-type flavodoxin (Upper) and Y98W mutant (Lower) probed at a multiple wavelengths from 740 nm to 410 nm. The black dash line in the lower panel shows a simulated 100 fs decay, distinguished from 1.1 ps decay in 740-nm transient. The negative signals probed at 500 nm are not completely shown in this display. Note the transients probed at 480 nm were normalized only to one half.
Figure 4.7: Schematic representation of the complete ET processes in the flavodoxin. The scale is a relative display of driving force for each process where the pump energy is 2.5 eV.
Table 4.1: Redox potentials\textsuperscript{a} and dynamics\textsuperscript{b} of electron-transfer cycle in oxidized flavodoxin

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Donor</th>
<th>$E_{m,7}$ (OX/SQ)</th>
<th>$\Delta G^\circ_{CS}$</th>
<th>$\Delta G^\circ_{CR}$</th>
<th>$\tau_{CS}$\textsuperscript{c}</th>
<th>$\tau_{CS}$\textsuperscript{d}</th>
<th>$\tau_{CR}$</th>
<th>$\tau_{cooling}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>W60F</td>
<td>Y98</td>
<td>-0.157\textsuperscript{e}</td>
<td>-0.653</td>
<td>-1.847</td>
<td>0.302</td>
<td>0.485</td>
<td>0.95</td>
<td>3.7-4.0</td>
</tr>
<tr>
<td>W60A</td>
<td>Y98</td>
<td>0.193</td>
<td>0.32</td>
<td>1.1</td>
<td>2.6-3.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y98R</td>
<td>W60</td>
<td>0.204</td>
<td>0.32</td>
<td>1.0-1.43</td>
<td>3.2-4.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y98A</td>
<td>W60</td>
<td>0.247</td>
<td>0.37</td>
<td>1.0</td>
<td>2.5-3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y98F</td>
<td>W60</td>
<td>0.258</td>
<td>0.37</td>
<td>1.27-1.78</td>
<td>3.3-3.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>Y98,W60</td>
<td>0.157</td>
<td>0.18</td>
<td>0.8\textsuperscript{j}, 1.56\textsuperscript{k}</td>
<td>3.0-4.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G61A</td>
<td>Y98,W60</td>
<td>0.149</td>
<td>0.16</td>
<td>0.8\textsuperscript{j}, 1.23\textsuperscript{k}</td>
<td>3.0-3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G61V</td>
<td>Y98,W60</td>
<td>0.154</td>
<td>0.16</td>
<td>0.8\textsuperscript{j}, 1.23\textsuperscript{k}</td>
<td>3.0-3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D95N</td>
<td>Y98,W60</td>
<td>0.135</td>
<td>0.18</td>
<td>0.8\textsuperscript{j}, 1.51\textsuperscript{k}</td>
<td>3.0-4.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y98W</td>
<td>W98,W60</td>
<td>0.152</td>
<td>0.37</td>
<td>1.0</td>
<td>3.0-4.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W60Y</td>
<td>Y98,Y60</td>
<td>0.258</td>
<td>0.46</td>
<td>0.95</td>
<td>3.7-4.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a}Redox potential values ($E_{m,7}$) and the driving forces for CS ($\Delta G^\circ_{CS}$) and CR ($\Delta G^\circ_{CR}$) are in unit of eV. \textsuperscript{b}Time constants are in unit of ps. \textsuperscript{c}Fitting data from upconversion. \textsuperscript{d}Fitting data from transient absorption. \textsuperscript{e}From ref (109). \textsuperscript{f}From ref (62). \textsuperscript{g}From ref (94). \textsuperscript{h}From ref (110). \textsuperscript{i}The average of double exponentials of 0.341 ps (96%) and 1.82 ps (4%). \textsuperscript{j}Assigned for the cationic phenyl radical recombination. \textsuperscript{k}Assigned for the cationic tryptophan radical recombination.
CHAPTER 5: SOLVATION COUPLED ELECTRON TRANSFER IN SEMIQUINONE FLAVODOXIN

5.1 Introduction

Solvent dynamic effect on ET has drawn great attention to theoreticians and experimentalists (20-21, 26, 114). With recent development of femtosecond optical spectroscopy, fast ET reaction occurring before or in accordance with the local solvent relaxation is observed (88, 115-116). As opposed to the role of static dielectric medium, the solvents no longer control the nonequilibrated ET by the static free energy influencing the activated barrier at the intersection. In a non-diffusing limit where ET is much faster than the solvation ($k_{et} \gg k_{sol}$), the solvent configuration at the initially prepared state representing that of the transition state determines the ongoing ET rate predominately. In Chapter 4, the CS in the oxidized flavodoxin is at such condition. When the ET rate is decreased to the extent coupled with solvation, however, the transfer rate becomes sensitive to the energetics imposed by the solvent reorientational fluctuation.

At the earlier times, the quantum theory predicts the so-called adiabatic solvent-controlled ET rate ($k_{a,et}$) inversely proportional to the solvation dynamics ($\tau_{sol}$) that
limits the fastest ET for a given barrierless reaction (117-119). Without the involvement of internal vibrations, the fastest ET one can achieve is:

\[
k_{a,et} = \frac{1}{\tau_{sol}} \sqrt{\frac{\lambda_o}{16\pi k_B T}} \exp \left( -\frac{(\lambda_o + \Delta G^o)^2}{4\lambda_o k_B T} \right)
\]

(5.1)

Unfortunately, the quantum prediction has been valid for limited cases. An otherwise milder $1/\tau_{sol}$ dependence of $k_{et}$ is more observed in a wide variety of solvents. In the Sumi-Marcus 2D model, the classical motion of solvation drives the ET reaction distributing a width on the $X$ coordinate and the crossing takes place at several intersections with finite barrier (24). One distinct feature is that the exhibited $k_{et}$ not only governed by the solvation, in the case that $\tau_{sol}$ too slow to cause a large increase of $k(X)$ the fast ET can proceed along the other coordinate of classical vibrations. A shift of ET from solvation control to the vibrational control is thereof predictable, responsible for many observed $k_{et}$ being slightly or much faster than the $1/\tau_{sol}$ relaxation. In this article, we will apply the extended Sumi-Marcus ET model to treat the solvation effect on CS process in the flavodoxin system.

In biology, only few of intraprotein ET on the perspective of active-site solvation has been studied. Due to the virtue of inhomogeneity and poor degrees of freedom at the reaction center, behavior of the solvation-driven ET may be more sophisticated than in solution. It is well known that, in a buried biological cage, at least three local motions attributed from the trapped water, water-protein coupled fluctuation, and protein intrinsic
relaxation are responded (59). The dynamics for each motion differs from system to system but commonly ranges in the corresponding several picoseconds, tens of picoseconds, and hundreds of picoseconds. Accordingly, each relaxation drops system energy to some extent depending on its solvent-exposed area and the active-site rigidity. Treatment of such more than one component of solvation will employ the time-dependent diffusion in Equation (1.4) and (1.5) regardless the detailed information for each component (21). So far, only one specific application on the photosynthetic initial ET by the use of classical Sumi-Marcus ET model has been reported (88). The study has ignored the aid of product internal vibrations and resolved $\lambda$ with only 0.35 eV (Marcus inverted region). The initial validity of biological ET by the 2D model has provoked a more elaborated picture for a complete ET cycle in different systems.

In this chapter, we focus on the role of active-site solvation in modulating the ET dynamics in the flavodoxin system. The complete scheme in all the 12 samples has been discovered in Chapter 4. Here, in the semiquinone state we repeat the measurement for each elementary process but further evaluate the dynamics with respect to solvation behavior. Since the CS in the semiquinone flavodoxin is substantially slower, compared to the oxidized, the coupling with the solvation and the effect on the following recombination and cooling process will be of great interest. This chapter is organized in the following way. By upconversion, we uncover the CS dynamics in all the semiquinone samples. The quenching transients due to CS are then fitted with the extended Sumi-Marcus ET model, resolving the quantitative value for the reorganization energy, the
characteristic frequency, and the electronic coupling element. The model employment also includes the data from the oxidized flavodoxin. In the proceeding part, we report the subsequent CR and cooling dynamics determined by the transient-absorption method. In-depth discussion as how each process occurs with view of two coordinates and the influence by solvation coupling is provided.

5.2 Materials and Methods

The protocols for flavodoxin purification and photoreduction converting the sample to the semiquinone state are listed in Appendix C and Appendix D, respectively. Due to the change of redox potentials by mutation, duration of UV irradiation differs from sample to sample. Upon irradiation, the sample presents the color ranging from the dark purple to dark brown, depending on the amount being reduced. In the particular G61V mutant, only some (<20%) SQ can be converted and gives relative weak signal for the ultrafast measurement (94). Samples prepared for the time-resolved experiments are controlled at the concentration of 150 µM determined by the oxidized absorption in 50 mM MOPS buffer at pH 7.

By following the time-resolved methodologies in Chapter 2, the SQ flavodoxin under the anaerobic condition are measured at the room temperature. For upconversion and transient absorption techniques, the pump energy at the wavelength of 580 nm tuned by passing through the OPA800C (Figure 2.1) is attenuated to 100-150 nJ. The gate beam
in the upconversion is set at 800 nm, while the probe beam in the transient absorption is adjusted at 525-800 nm by TOPAS (Figure 2.1). With our laser setup, the instrument response time is about 300-380 fs for the former upconversion and 150-300 fs for the latter transient absorption. All data are acquired at the magic angle (54.7°).

5.3 Results and Discussion

5.3.1 Semiquinone Flavodoxin: Structure and Spectroscopic Properties

The crystallographic study of flavodoxin at different oxidation states has shown that, on the reduction of FMN to the semiquinone state the flavodoxin commonly experiences a large structural change in the polypeptide chain 60-64 moving away from the flavin molecule by several Å (53, 94, 110). This is outlined in Figure 3.1 (Chapter 3). Concerning the spatial separation between the ET donor and acceptor, Figure 5.1 indicates that no notable effect is introduced during reduction. The alignment of crystallographic structures of oxidized and semiquinone native flavodoxin revels the approximate distances for one electron traveling from Y98 or from W60 to the ISO moiety in these two states. The same conservation should apply to other flavodoxin with some site mutation such as D95 and Y98 as suggested from the previous studies (94, 110, 120). It is therefore fair to compare the ET in two different oxidation states without considering the structural perturbation.
Figure 5.2 displays the steady-state (part) absorption and emission spectra of the flavodoxin semiquinone in the visible region. In contrast to the neutral flavin semiquinone in the solvents (102, 121), the bound one in the protein environment is considerably red shifted with similar absorption coefficients (122). The characteristic D₁←D₀ transition (555-650 nm) (123) of neutral flavodoxin radical is peaked at 580 nm with a pronounced shoulder near 620 nm. Despite the slightly broaden in the Y98W mutant and some higher shoulder in W60F, the absorption band is generally shared among these mutants, indicating a negligible environmental change by the mutation. The right side of Figure 5.2 shows the emission of semiquinone flavodoxin a non log-normal fluorescence spectrum obtained in the double mutant of W60F/Y98F discussed in Chapter 3.

5.3.2 Ultrafast Charge Separation in Semiquinone Flavodoxin

Like in Chapter 4, the photoinduced CS of FMNH⁺ from the stacking Y98 or/and W60 aromatic residues can be initiated by the 580-nm pump pulse (56). For all samples, we detect the upconversion quenching at the 670-nm emission (Figure 5.2) between the observed solvation decaying (7 ps, 15%) in 655 nm and rising (160 fs, 76%) in 702 nm (Chapter 3). At this gating wavelength, the decay dynamics should represent the flavodoxin CS reaction and is equivalent to the survival probability Q(t) in Equation (1.7). Figure 5.3 and Table 5.1 show such upconversion transients and a distinct CS
dynamics is observed. The first component ranging in 0.47-1.93 ps (66%-44%) and the
second in 1.63-8.52 ps (34%-56%) demonstrate the obvious biexponentiality in all the
transients. In accord with the stretched fitting (124), the CS dynamics determined at 0.81-
5.37 ps show a good agreement with the corresponding averaged τ_{CS} in Table 5.1, with β
value resulting in 0.69-0.81 manifest of the nonexponentiality. The outcome pattern
discerns that Y98 in the W60 mutations has a slower transfer rate than W60 in the Y98
mutations, more pronounced than in the oxidized flavodoxin. Contrarily to the OX state,
with dual W60 and Y98 electron donors the native and mutants of D95N, G61A, and
G61V exhibit a quenching dynamics comparable to the merely W60 transfer, reaching
the overall average at 0.8-1.9 ps. In the mutant of W60Y, the providing additional Y
donor does not drastically accelerate the quenching rate, decaying at ≈5 ps even slower
than the W60A transient.

To our surprise, the transients gated at the emission other than 670 nm appear to
decay with different dynamics. As shown in Figure 5.4, the fluorescence detected at 725
nm in WT and W60F has the respective decay averaged at ≈1.54 ps and ≈7.3 ps, slower
than the corresponding 670-nm transient to some extent. Since there should exist no
solvation component contributing to the short time decay in such red emission region, the
dynamics is directly related to the CS but at a different solvation coordinate. In another
word, the 1/k(X) of WT alters from 1.46 ps to at least 1.54 ps and of W60F from 5.61 to
at least 7.3 ps. When X is at a configuration closer to the initial preparation, however, our
monitor of the bluer 652-nm emission has found the even faster decay of averaged ≈677
fs in WT and ≈3.54 ps in W60F. The former seems a more efficient CS in WT making the fast quenching but the latter is apparently mixed with the solvation component about 4-7 ps (see Chapter 3). The similar phenomenal change of gate wavelength dependence is also observed in all other mutants (data not shown).

In Table 5.1 we list the redox potentials for the flavin couple SQ/HQ accounting for the semiquinone flavodoxin CS and the reaction energy calculated by following the Equation (4.3) where $E_{0,0}$ is 2.0 eV representing the absorption shoulder at 620 nm.

When correlated with the $\Delta G^\circ_{CS}$ forces the CS quenching generally follows a normal region behavior with slower transfer rate in the Y98F mutant and much slower in W60A. If only considering the W60 donating the electron in WT and the D95N mutant, some slower CS dynamics is anticipated for the relatively more negative potential of the flavin acceptor. In upconversion, our observation of 1.2-1.5 ps quenching time has reflected that the residue Y98 present in these two samples somehow also contributes the transfer. However, such rate enhancement by the dual ET donors is not obvious in the G61 mutations, probably due to the less compact active pocket introduced by the steric alanine and valine replacement (94).

### 5.3.3 Protein Solvation Coupled Charge Separation

In this section, we employ the extended Sumi-Marcus 2D model detailed in Chapter 2 to fit the above 670-nm upconversion transients and the oxidized ones from Chapter 4. As a
result, $\lambda_l$ is $\approx 0.85$ eV for the redox pair of Y-FMN$^*$ (Y-FMNH$^*$) and $\approx 0.95$ eV for the pair containing at least one W donor. The electron-vibrational coupling $S \approx 3.86$ is the quotient of $\lambda_h \approx 0.58$ eV divided by the mean frequency $\hbar \omega \approx 0.15$ eV ($\approx 1210$ cm$^{-1}$). The solvation reorganization energy $\lambda_o$ attempting to stabilize the produced ion pair is found to have a larger quantity for any with W involved, equivalent to 0.07-0.08 eV in all W60 mutations while $\approx 0.14$ eV for all others. The laser excitation is calculated to introduce the energy on the solvation potential well with $\lambda_o(0.5)^2 \approx 0.017$-0.035 eV that is well agreed with the previously measured dynamic Stokes-shift relaxing 0.025 eV in Chapter 3. The electronic coupling $H_{AB}$ for such van der Waals contact of the donor and acceptor is generally fitted 0.028-0.029 eV ($>0.025$ eV, thermal energy at the room temperature) except the oxidized ones with dual donors of W60 and Y98 where 0.032-0.035 eV is obtained. The higher coupling may reflect either the better efficacy for CS with two donors or simply indistinguishable from the fitting for $\Delta G^\circ_{CS}$ value. Some smaller $H_{AB}$ is also obtained especially in SQ samples of G61A and Y98H at 0.023 eV and Y98A and Y98R at 0.026 eV, possibly attributed from the slightly loose stacking of the donor on the cofactor and giving rise to a longer donor-acceptor separation. Figure 5.5 illustrate the two distinct fitting curves expressing the energy gap dependence of CS with Y donor and with at least one W donor. It is obvious that the CS rate is insensitive to the minor difference of $c(t)$ between different oxidation states. The total effective $\lambda$ is $\approx 1.2$-1.3 eV with the possible fastest CS at $\approx 170$-190 fs at the apex. For the reason of CS reaction occurring at solvation unrelaxed states, the application by the Dutton’s empirical equation fails to obtain the $\lambda$ within a reasonable range for the current transients (14). By the 2D
model the fitted driving energy higher than the calculated in Table 5.1 by \(\approx 0.3\) eV may
arise from the Coulomb interaction ignored in our calculation. The integrated CS rate
from the Equation (5.2) for each transient shows a well correlation with the
biexponential-fitting average (Table 5.1) plotted in Figure 5.5 Inset.

\[ k_{CS} = 1/\tau_{CS} = \frac{1}{\int Q(t)\,dt} \quad (5.2) \]

5.3.4 The Complete Cycle of ET Mechanism

In the transient absorption measurement, the probe beam is tuned at several different
wavelengths ranging from 525 nm to 800 nm. As shown in Table 5.2 and displayed in
Figure 5.6A, the 800-nm transient in the mutant of W60F shows the biexponential decay
of 1.95 (36%) and 10.3 (64%), averaging at \(\approx 7.3\) ps, surprisingly matched with the
upconversion quenching detected at 725 nm. This allows us to assume that, at the probe
of 800 nm, the dominant FMNH\(^{**}\) is observed in the semiquinone flavodoxin representing
the CS dynamics determined by the transient absorption. All other samples are
accordingly followed and the fitted CS time constants are explicitly listed in Table 5.2. In
particular, the nearly nonfluorescent Y98W mutant is clearly observed here, dominantly
decaying at the fast timescale of 0.36 ps.
When the detection wavelength is close to the FMNH$^\cdot$ absorption edge, in Figure 5.6A one slow decay component of $\approx$11.0 ps accompanying the rise in 1-5 ps is progressively evolved from 670 nm moving toward 630 nm. Distinguished from the upconversion progression that is associated with different $k(X)$, the current distinct transients should indicate some species other than FMNH$^{**}$ being detected, since approach of transient absorption usually discerns the reaction at different solvation coordinate weakly. Such phenomenon is an analogy of that in Figure 4.4, demonstrating the capture of some vibrational hot FMNH$^\cdot$ at the electronic ground state (i.e., FMNH$^{**}$).

$$
\text{FMNH}^{**} + Y(W) \xrightarrow{\text{CS}} \text{FMNH}^- + Y^{**}(W^{**}) \xrightarrow{\text{CR}} \\
\text{FMNH}^{*\dagger} + Y(W) \xrightarrow{\text{Cooling}} \text{FMNH}^* + Y(W)
$$

Analysis by the above consecutive mechanism shows that, when CS for the W60F mutant is about $\approx$7.3 ps and cooling remains at 3-6 ps for the flavodoxin bath (Chapter 4), one slowest CR responsible for the decay of $\approx$11.0 ps is observed by the detected FMNH$^{*\dagger}$ species. The progressive pattern in 670-640 nm is mainly due to the more observed FMNH$^{*\dagger}$ beating FMNH$^{**}$ in the bluer probe wavelength. Since the rise component of 1-2 ps has been observed in the ET-inert mutant of W60F/Y98F (see Appendix B and Figure B.5), we interpret some energy dissipation proceeding prior the CS reaction also occur. At 630 nm and 525 nm, the ground-state FMNH$^\cdot$ absorption is significant. The bleaching signal of 525-nm transient followed by the recovery constants of $\approx$11.0 ps (32%), 6.8 ps
(62%), and ≈7.3 ps (6%) is a superimposed consequence of mainly FMNH∗ and FMNH↑ absorption, much like the cancellation in Figure 4.4C without the ion pair contribution.

Figure 5.6B show the relatively minor evolution of transients in the mutant of Y98F than W60F. Compared to the 800-nm decay averaged at 3.47 ps, one rise component of ≈1-2 ps and a decay of ≈5.0 ps appear to grow the transient pattern in the visible 640-690 nm. In addition to the FMNH↑↑ and FMNH↑↓ absorption, signal due to W↑↑ radical is anticipated. In our observation, this W↑↑ seems more dominant and resolves the CR at ≈1.09 ps accounting for the formation. At 630 nm, the prominent rise nearly 3-4 ps followed by a 5-ps decay shows the more significant FMNH↑↑ being detected here. Again, the recovery at 525 nm almost mirroring the 800-nm decay is a result of superimposition from the signal due to FMNH↑∗, FMNH↑↓, FMNH↑ and W↑↑ absorption.

The above strategy of resolving the complete ET mechanism is applied to all other SQ flavodoxin. In Table 5.2 we list the time constants of CS, CR, and cooling process determined by the transient absorption. In general, the CR dynamics in the W60 mutations including W60Y resolved in 9-12 ps is slightly slower than the CS reaction. On the other hand, the W60 donor in all other samples has a fast recombination of around ≈1.0 ps. It is important to note that, even we propose that in some flavodoxin where two ET donors are available the CS is moderately accelerated, only one CR value contributing to the detected W↑↑ radical is obtained. In Table 5.2, the recombination seems adversely
retarded by the more $\Delta G_{\text{CR}}^0$ energy (Table 5.2). Such inverted-region behavior not appearing in the oxidized flavodoxin may be led by some solvation involved.

**5.4 Conclusion**

In this contribution, we investigate the intraprotein ET behavior on the aspect of motions of solvation and vibration in the semiquinone flavodoxin. Analogous to the study in Chapter 4, the complete cycle is analyzed. In the upconversion, the first process of CS from W60 or Y98 to the FMNH$^{\cdot*}$ is determined on the order of several picoseconds, substantially slower than the respective oxidized CS and heavily coupled with the FMN-binding site solvation. Most of the transients due to the CS reaction decay nonexponentially and are fitted by two exponentials. Analysis by the extended Sumi-Marcus 2D model resolves the separate parameters for the reactions with W60 and with Y98 donor, total reorganization effective at 1.2-1.3 eV. The CS reaction falling in the Marcus normal region is suggested to follow the solvation-dependent behavior that is diversely prevailed in solution system. Illustrated in Figure 5.7, several ion pairs at various quantum levels (hot) at each $X$ and at different solvation $X$ are produced. The wide spreading ion products may be further stabilized to the potential bottom by the vibrational and solvational relaxation in a competence to the subsequent charge neutralization.
We determine the dynamics of CR to the residue W60 and to the Y98 at ≈1.0 ps and 9-12 ps, respectively. The former shows a similar time scale as the oxidized Y98 mutations and the latter is nevertheless much slower. This is a strong correlation that the solvation heavily coupled with the slow CS in the W60 mutations can affect the following recombination. Due to the feature of ultrafast reaction, the stabilization of ion pairs produced at different levels is not allowed. The multiple levels mainly depending on the CS solvation coupling may be pivotal to the subsequent CR process. In Figure 5.7 the Step 3 is assumed to synchronously bring both coordinates to the configuration suitable for the final (origin) state. In the transient absorption, we clearly observe the energy dissipation of the Step 4 at several picoseconds that probably accompany some minor solvation.

Here we propose the appropriate employ of extended Sumi-Marcus model and directly prove the existence of $k(\chi)$ in the biological model system of flavodoxin. Strikingly, the behavior of both exponential and nonexponential decay can be predicted by varying from little to strong couple with solvation, as in our observation of the respective oxidized and semiquinone CS reaction. In the latter case, the following recombination is significantly slow down especially when CS is slower than 4.5 ps in the current system. Such fundamental discovery will be beneficial to the future investigation of protein solvation role in controlling the biological function.
Figure 5.1: Superimposition of residue Y98 and W60 with respect to the FMN molecule in oxidized (light yellow stick, PDB accession code 3FX2) and semiquinone (dark purple, PDB accession code 4FX2) flavodoxin. Color of red, orange, and blue indicates the respective atom of oxygen, phosphorous, and nitrogen. For both flavodoxin, the closet distance of FMN ISO moiety to Y98 and to W60 side chains is 3.31 Å and 3.46 Å, respectively.
Figure 5.2: Steady-state spectra of absorption (black and gray) and emission (red) of flavodoxin semiquinone in the visible region. Absorbance spectra of WT (thick black solid line), W60F (gray solid line), Y98W (gray dashed line), Y98F (gray dashed-dot line) and G61A (black dashed line) are normalized at the absorption maxima in the 560-600 nm region. Emission is due to the flavodoxin double mutant W60F/Y98F semiquinone (Figure 3.2, Chapter 3). The arrows represent the pump wavelength at 580 nm and the upconversion fluorescence emission gated at 670 nm for the time-resolved experiments.
Figure 5.3: Normalized femtosecond-resolved fluorescence transients of FMNH$^\cdot$ in flavodoxin and the mutants gated at 670 nm. Open symbols represent the raw data for the respective sample indicated at the top right. Solid lines are the least square fitting by two exponentials.
Figure 5.4: Normalized femtosecond fluorescence upconversion of FMNH$^+$ in flavodoxin gated at different wavelengths. The long-dashed line represents the fitting curve at 670 nm from Figure 5.3. Solid lines and dotted lines are the fitting results by the exponentials.
Figure 5.5: Fitting curve for flavodoxin charge separation associated with Y98 donor (dotted) and W60 donor (solid) in the oxidized (light gray) and semiquinone (dark gray) states. For both solid lines, $\lambda_i=0.95$ eV, $\lambda_o=0.14$ eV, $\lambda_h=0.58$ eV, $\hbar\omega=0.15$ eV, and $H_{AB}=0.029$ eV. For both dotted lines, $\lambda_i=0.85$ eV, $\lambda_o=0.07$ eV, $\lambda_h=0.58$ eV, $\hbar\omega=0.15$ eV, and $H_{AB}=0.029$ eV. Inset expresses the well-correlated CS rates of flavodoxin resulting from the 2D model and from the (average of) exponentials in Table 4.1 and Table 5.1.
Figure 5.6: Normalized transient absorption in the semiquinone mutants of (A) W60F and (B) Y98F. Both samples were pumped at 580 nm and probed at the labeled wavelengths. Solid line shows the fitting result for the symbol data whereas the transient of 740 nm can be fitted the same as of 800 nm. Note in some transients the nonlinear spike near or before the time zero is removed for clarity.
Figure 5.7: Two-dimensional illustration of ET mechanism in semiquinone flavodoxin. Upon laser excitation, CS to the ion-produced state proceeds along the solvational relaxation in Step 1 followed by a quick vibrational relaxation at each solvation-dependent crossing (Step 2). At every transition (light-blue circle) several ion paired products with different quantum vibrational state are generated shown on the bottom right. Recombination of the charge (Step 3) back to the original involves both solvational and vibrational relaxation, coupled with (quantum) hot vibrational state of the final. Cooling on the final state (Step 4) closes the cycle.
<table>
<thead>
<tr>
<th>Mutants</th>
<th>Donor</th>
<th>$E_{m,7}$ (SQ/HQ)</th>
<th>$\Delta G^\circ_{CS}$</th>
<th>$\tau_{CS1}$</th>
<th>$\tau_{CS2}$</th>
<th>$\tau_{CS}^c$</th>
<th>$\tau_{CSY}^d$</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>W60F Y98</td>
<td>1.93 (44%)</td>
<td>8.52 (56%)</td>
<td>5.61</td>
<td>5.37</td>
<td>0.73</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W60A Y98</td>
<td>-0.357$^e$</td>
<td>-0.173</td>
<td>1.28 (50%)</td>
<td>5.50 (50%)</td>
<td>3.39</td>
<td>3.21</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>Y98R W60</td>
<td>-0.265$^f$</td>
<td>-0.585</td>
<td>0.47 (66%)</td>
<td>1.63 (34%)</td>
<td>0.87</td>
<td>0.81</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>Y98H W60</td>
<td>-0.262$^f$</td>
<td>-0.588</td>
<td>0.71 (64%)</td>
<td>2.92 (36%)</td>
<td>1.51</td>
<td>1.40</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Y98A W60</td>
<td>-0.304$^f$</td>
<td>-0.546</td>
<td>0.61 (67%)</td>
<td>2.60 (33%)</td>
<td>1.25</td>
<td>1.16</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>Y98F W60</td>
<td>-0.414$^f$</td>
<td>-0.436</td>
<td>0.83 (60%)</td>
<td>4.59 (40%)</td>
<td>2.31</td>
<td>2.08</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>WT Y98, W60</td>
<td>-0.443$^f$</td>
<td>0.65 (66%)</td>
<td>2.99 (34%)</td>
<td>1.45</td>
<td>1.3</td>
<td>0.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G61A Y98, W60</td>
<td>-0.359$^g$</td>
<td>0.78 (63%)</td>
<td>3.37 (37%)</td>
<td>1.75</td>
<td>1.62</td>
<td>0.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G61V Y98, W60</td>
<td>-0.299$^g$</td>
<td>N/A</td>
<td>N/A</td>
<td>1.93$^i$</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D95N Y98, W60</td>
<td>-0.395$^h$</td>
<td>0.62 (65%)</td>
<td>2.32 (35%)</td>
<td>1.21</td>
<td>1.12</td>
<td>0.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y98W W98, W60</td>
<td>-0.452$^f$</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W60Y Y98, Y60</td>
<td>1.76 (44%)</td>
<td>7.69 (56%)</td>
<td>5.07</td>
<td>4.86</td>
<td>0.76</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$Redox potential values ($E_{m,7}$) and the driving forces ($\Delta G^\circ_{CS}$) are in unit of eV. $^b$Time constant are in unit of ps. $^c$Average of the fitting by two exponentials: $\tau_{CS} = \tau_{CS1} \ast (A_{CS1} \%) + \tau_{CS2} \ast (A_{CS2} \%)$. $^d\tau_{CSY} = (\tau/\beta) \ast \Gamma(1/\beta)$ where $\tau$ and $\beta$ are the fitting results from the stretched exponentials. $^e$From ref (109). $^f$From ref (62). $^g$From ref (94). $^h$From ref (110). $^i$The data is too noisy that a single exponential can be globally fitted.
Table 5.2: Transient absorption dynamics$^a$ in semiquinone flavodoxin

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Donor</th>
<th>$\Delta G_{CR}^b$</th>
<th>$\tau_{CS1}$</th>
<th>$\tau_{CS2}$</th>
<th>$\tau_{CS}^c$</th>
<th>$\tau_{CR}^d$</th>
<th>$\tau_{cooling}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>W60F</td>
<td>Y98</td>
<td>1.95(36%)</td>
<td>10.3(64%)</td>
<td>7.3</td>
<td>11.0</td>
<td>5.0-6.8</td>
<td></td>
</tr>
<tr>
<td>W60A</td>
<td>Y98</td>
<td>-1827</td>
<td>1.87(52%)</td>
<td>7.34(48%)</td>
<td>4.5</td>
<td>8.8</td>
<td>6.5</td>
</tr>
<tr>
<td>Y98R</td>
<td>W60</td>
<td>-1415</td>
<td>0.93(74%)</td>
<td>2.9(26%)</td>
<td>1.45</td>
<td>0.95</td>
<td>3.2-5.0</td>
</tr>
<tr>
<td>Y98H</td>
<td>W60</td>
<td>-1412</td>
<td>0.93(64%)</td>
<td>3.6(36%)</td>
<td>1.89</td>
<td>0.92</td>
<td>3.5-5.0</td>
</tr>
<tr>
<td>Y98A</td>
<td>W60</td>
<td>-1454</td>
<td>0.83(43%)</td>
<td>3.06(57%)</td>
<td>2.1</td>
<td>0.9</td>
<td>3.0-5.0</td>
</tr>
<tr>
<td>Y98F</td>
<td>W60</td>
<td>-1564</td>
<td>1.9(61%)</td>
<td>5.87(39%)</td>
<td>3.47</td>
<td>1.09</td>
<td>5.0</td>
</tr>
<tr>
<td>WT</td>
<td>Y98,W60</td>
<td>1.29(67%)</td>
<td>3.4(33%)</td>
<td>1.98</td>
<td>1.13</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>G61A</td>
<td>Y98,W60</td>
<td>0.97(53%)</td>
<td>3.96(47%)</td>
<td>2.37</td>
<td>0.98</td>
<td>3.0-5.0</td>
<td></td>
</tr>
<tr>
<td>G61V</td>
<td>Y98,W60</td>
<td>0.6(52%)</td>
<td>2.59(48%)</td>
<td>1.55</td>
<td>0.99</td>
<td>3.0-5.0</td>
<td></td>
</tr>
<tr>
<td>D95N</td>
<td>Y98,W60</td>
<td>0.74(48%)</td>
<td>2.36(52%)</td>
<td>1.58</td>
<td>1.19</td>
<td>2.6-5.0</td>
<td></td>
</tr>
<tr>
<td>Y98W</td>
<td>W98,W60</td>
<td>0.36(100%)</td>
<td>0.36</td>
<td>1.0$^e$</td>
<td>2.8-4.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W60Y</td>
<td>Y98,Y60</td>
<td>1.63(30%)</td>
<td>8.98(70%)</td>
<td>6.79</td>
<td>11.9</td>
<td>5.0-7.0</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Time constant are in unit of ps. $^b$Calculated following the Equation (4.4) and is showing in unit of eV. $^c$Average of the fitting by two exponentials: $\tau_{CS} = \tau_{CS1} \cdot (A_{CS1\%}) + \tau_{CS2} \cdot (A_{CS2\%})$ for the 800-nm transient. $^d$Average of two-exponential decays (not explicitly shown here). $^e$Only one decay is obtained.
6.1 Introduction

It is known for decades that the electron tunneling behaves with a strong dependence on the traveling distance \((10-11)\). In biology, distance control is therefore of great importance to the regulation of ET involved enzyme activity and function. With the recent advent of femtosecond spectroscopy, fast ET reactions can be captured providing a great impetus on study of tunneling at shorter distance. Opposed to the long-range ET process where metal ions are the common partners, many ET reactions on short distance utilize organic molecule such as flavin and amino acid of tryptophan and tyrosine immobilized near the reaction center \((67, 125)\). The size of organic molecule is relative large and, for instance, it is \(5 \text{ by } 10 \text{ Å} \) in dimension for flavin ISO moiety greater than several bonded bridges. For this reason, contrary to most suggestions, some argue that edge-to-edge measurement between donor and acceptor should not represent the actual tunneling distance. Notably, previous studies on scrutinizing the role of distance at short range all find a necessity to increase the measured edge-to-edge distance in some way in order to explain the observed slow ET rate \((8, 33)\). In the pathway model where traveling
through a circuitous route is proposed, distance applied to the estimated ET rate extrapolated from long-range study needs to be changed drastically, for example, from 4.5 Å to 11.2 Å and 8.4 Å to 24.6 Å, accounting for the observed nanosecond-to-millisecond “barrierless” reactions (8). A drawback of the treatment is that, at least for nowadays observations, it seems inappropriate to blindly treat one ET process occurring longer than nanosecond timescale as a no-barrier crossing. If the otherwise barrier is present in the FC factor, the calculated tunneling route is obviously overestimated. In addition, the experimentally observed slower rates by 3-6 orders of magnitude may be exaggerated due to instrument limit (126-127).

The expression for the rate of one ongoing ET reaction shows as:

\[ k_{et} = \frac{H_{AB}^2}{\hbar} \sqrt{\frac{\pi}{k_B T \lambda_i}} \times (\text{FC}) \]  \hspace{1cm} (6.1)

\[ (\text{FC}) = \sum_{n_p} \frac{\left( \frac{\lambda_h}{\hbar \omega} \right)^{n_p}}{n_p!} \exp \left( -\frac{\lambda_h}{\hbar \omega} - \frac{(\lambda_o + \Delta G^o - 2X \lambda_o + n_p \hbar \omega + \lambda_i)^2}{4 \lambda_i k_B T} \right) \]  \hspace{1cm} (6.2)

The electron term \( H_{AB} \) and nuclear FC factor through \( \Delta G^o \) and \( \lambda_o \) can vary with distance at different extent. Since the latter depends how one defines dielectric constant (\( \epsilon_0 \)) for protein environment, the change of FC term on distance dependence can be unreasonably magnificent when \( \epsilon_0 \) is small (10, 15-16, 125, 128-129). A proper value of \( \epsilon_0 \) used in biological systems is still on debate (130). With this regard, we bypass the complication
by treating the FC factor unchanged and only focus on $H_{AB}$ varied with distance. Using one-dimensional square tunneling barrier model, $H_{AB}$ is predicted to depend on distance $(R)$ between donor and acceptor exponentially ($6, 131$):

$$H_{AB}(R) = H_{AB}(R_0) \times \exp \left\{ -\frac{1}{2} \beta (R - R_0) \right\} \quad (6.3)$$

Symbol $\beta$ represents the distance decay constant and defines the dropping $H_{AB}$ for $R$ larger than a finite value ($R_0$). Dutton’s line of tunneling across the protein medium reveals $\beta$ at a constant of 1.4 Å$^{-1}$ irrelevant of presence of FC barrier ($18$). By the pathway model, $\beta$ is suggested to value from 1.52 to 0.98 Å$^{-1}$ indicating 73% to 95% of bonded intervening medium responsible for observed ET rates in different proteins ($7$). For some particular studies, especially for short distance, $H_{AB}$ is shown dependent of distance in a nonexponential behavior ($11, 33, 132$). However, caution should be taken that different measurement of $R$, i.e., edge-to-edge, center-to-center, and sum of total traveling pathway, will lead to a variety of $\beta$ decay constant.

In this article, our purpose is to examine such distance dependence of ET rate on short range using femtosecond-resolved spectroscopy. We use flavodoxin from *Desulfovibrio vulgaris* as a model system and alternate the separation between FMN cofactor and $W$ locating on several different sites. By the site-directed mutagenesis a total of 10 mutants with donor-acceptor separation from 3.7 Å to 18.2 Å is obtained. We perform the MD simulation (described in Appendix E) for the individual $W$ replacement
and compute the distance fluctuating in a 1-ns time window. Experimentally, at the oxidized state we monitor the quenching dynamics of FMN fluorescence upon laser excitation in every sample. The consequent tunneling kinetics is found to decrease by four orders of magnitude (from subpicosecond to thousands of picoseconds) with distance lengthened to be around or more than 9 Å. The upconversion transients are further analyzed by the extended Sumi-Marcus ET model, same as in Chapter 5, with a special emphasis on the varying $H_{AB}$ value.

### 6.2 Materials and Methods

By following the protocols in Appendix C, we have successfully purified 10 engineered flavodoxin mutants with one W of interest adjacent to the FMN group. As listed in Table 6.1, all the mutants except Y98F have four point mutations, with one residue replaced by W and other potential ET donors of W60, Y98, and Y100 by the inert amino acid phenylalanine. For example, the sample of F101W/W60F/Y98F/Y100F is to monitor the ET process between F101W and the prosthetic FMN cofactor. Geographic localization of the W substituting residues is displayed in Figure 6.1. Upon purification the holoflavodoxin is formed at a yield some lower than the wild type. Sample concentration and the spectroscopic property are determined by the absorption in the visible region. In all the samples, the maximum absorption is peaked at 385 and 454 nm with an obvious shoulder at 480 nm, comparable to Figure 3.2 from the mutant of W60F/Y98F and
distinguished from free FMN in solution (Figure B.4). The visible circular dichroism (VCD) spectra in the 350-540 nm region can also detect the signal due to the flavodoxin bound FMN cofactor (133-134). At a sufficient concentration of about 550 µM, the four-point flavodoxin mutants all give rise to a broad signal achieving the negative maximum at the identical 380 nm and the positive at 482 nm (data not shown). Since the visible spectroscopic property represents the local environment around the FMN cofactor, the fact that these spectrum peaks are insensitive to different mutants ensures the similar tertiary composition at the FMN binding site arising from a proper folding, hence fair to be used in this article. For the femtosecond upconversion measurement, samples are prepared at the concentration of 150 µM in 50 mM phosphate buffer solution (pH=7). As detailed in Chapter 2 and others, the pump pulse is set the wavelength at 400 nm (≈100 nJ). Sample emission at 538 nm is monitored at the magic angle (54.7°).

6.3 Results and Discussion

6.3.1 Engineered Tryptophan Donors in Flavodoxin

The ET donor of W placed at the position adjacent to the FMN acceptor is mainly located approximate to or on the loop region. As indicated by the native flavodoxin, Figure 6.1 show Loop 60 and Loop 90 surrounding the ISO moiety of the FMN cofactor are aimed to perform the mutagenesis. The residue W60 at the beginning Loop 60 flanking the ISO moiety is investigated the ET behavior in the mutant of Y98F (Table 6.1). Other W
localized on the loop include the sequencing D62W, I65W, E66W and L67W, moving more distant away from the ISO ring. Following this loop wraps perpendicular to the isoalloxazine plane near the middle ring, W60 and W66 are close to the dimethylbenzene side of the FMN and others of W62, W65, and W67 are to the other uracil side (see Figure B.1). Opposed to Loop 60, Loop 90 encompasses the other face of FMN cofactor and connects the strand β4 and helix α4. The substituted D95W, F101W, G103W, and A104W are distributed on the corresponding β4-Loop 90 joint, Loop 90, Loop 90-α4 joint, and α4. The loop segment within the first two residues lies horizontally against the isoalloxazine surface and is followed by a vertical extension joining the helix. All these engineered W donors are approximate to the uracil moiety of ISO ring with an exception of W95 (Table 6.1). As a control, one more W is set distant to the ISO moiety achieved by replacing the R131 near the helix α5.

In each designed mutant, the spatial separation between the FMN cofactor and the W of interest is computed by the MD simulation shown in Figure 6.2 and Table 6.1. On a basis of Newtonian motion, multiple optimal configurations for each site mutation are resolved, responsible for the trajectory fluctuation in time. Our representation of some results in Figure 6.2 shows that, when monitoring the edge-to-edge distance between the ISO moiety and the W indole over 1-ns time window, such fluctuation behaves differently from one to one. The most stable one comes from the W60 which stacks on one face of the FMN cofactor contributed from the strong π-π interaction at the van der Waals contact. Second to the W60, the W104 donor located on the deeper helix than
W103 exhibits a steady distance to the FMN acceptor of 8.9 Å fluctuating ±4% in space. In Table 6.1 all other W on the loop region seemed allowed to move at a larger scale give rise to a higher flexibility. Among which the donors of W95 and W65 have a maximum of distance variation of 1.2 Å relative to the FMN isoalloxazine while others are within 0.8-1.0 Å difference. Throughout these mutants, a range of the donor-acceptor distance in 3.7-18.2 Å is mapped out in the flavodoxin system.

### 6.3.2 Electron Tunneling Behavior in Flavodoxin

Based on the study in Chapter 3 and Chapter 4, it is now clear that the light-induced ET in the native flavodoxin is subjected to the donors of W60 and Y98 which are adjacent to the FMN cofactor. Mutation of both W60F and Y98F drastically increases the FMN fluorescence lifetime to ≈6 ns, which abolishes ET process and resumes the lifetime comparable to the solution environment (93, 135). Meanwhile, W at the sequencing 140 and Y at 100 are thus ensured not contribute donating the electron in spite of the latter only 5.6-Å edged to the ISO moiety (Figure 6.1, top sequencing). For the current study we, nevertheless, replace the residue Y100 by the ET-inert phenylalanine in addition to the double mutation. The three-point mutant of W60F/Y98F/Y100F is characterized identical to the two-point mutant (W60F/Y98F) in regarding the spectra of absorption and fluorescence and the femtosecond-resolved upconversion (data not shown). Using the
W60F/Y98F/Y100F mutant as a template, nine W donors at the desired distance are generated and listed in Table 6.1.

Figure 6.3 show the distinct change of dynamics of FMN quenching ranging from subpicosecond to nanosecond time scales measured in these engineered mutants. Indicated by the top distance ruler, the quenching is consistently becoming slower with lengthened distance. For the distant W131, the transient exhibits the same dynamics as the W60F/Y98F/Y100F mutant (not shown) and reflects the actual ET process between FMN and W131 longer than 6 ns. Clearly, the pattern shows a manifestation of ET quenching influenced by variant donor-acceptor distance. Contrary to the canonical propose that limited the separation between FMN and W smaller than 5-6 Å in order to permit the ET reaction (125), our data show much slower decay dynamics previous not observed at longer donor-acceptor separation. Fitting of the transients employs the extended Sumi-Marcus two dimensional ET model. In our treatment, all the parameters but the electronic coupling element ($H_{AB}$) are preserved at the obtained Y98F mutant (Chapter 5). Except W66 and W62 (not shown), the fitting lines in Figure 6.3 can roughly represent the observed quenching behavior disregard some initial decay of several tens picosecond in W104 and W67. The value of $\lambda_o$ is remained at 140 meV for all the transients excluding W66 where the number of 355 meV is obtained. This raised $\lambda_o$ in W66 features its highly nonexponential decay and, however, may arise from a more vigorous structural fluctuation as computed in Figure 6.2. Overall, in the range of 3.7-6 Å a sharp decrease of quenching dynamics from 257 fs for W60, to 3.93 ps for W95, and to
≈50 ps for W101 and W65 is observed. When this donor-acceptor separation is changed within the longer 7.7-8.9 Å, the decay constants show hundreds of picoseconds time scales in W66 and W103, and nearly few nanoseconds in W104 and W67. Since the quenching rate is a sum of reciprocal of fluorescence lifetime (6 ns in flavodoxin) and ET tunneling rate, modification for the fitted $H_{AB}$ is necessary.

With the 2D model fitting, the decaying transient of the control mutant W60F/Y98F/Y100F is resolved with the value of $H_{AB}$ at 0.32 meV. This is not an actual electron coupling of donor and acceptor but results from the existing chromophoric FMN lifetime at ≈6 ns instead of infinitely long. Assuming that the FC factor remains unchanged in all flavodoxin samples, the corrected coupling element ($H_{AB,c}$) subtracting rate equation for mutant by that of ET-inert rate can be recovered. In Figure 6.4 (Left), the square of $H_{AB,c}$ obtained in different mutants is illustrated in relation to separation of donor and acceptor. Comparison of our observation on short range to the theoretical prediction (lines) reveals several important features: (i) the value of $H_{AB,c}$ decreases in accord with increasing donor-acceptor separation with a decay constant of about 1.4 Å$^{-1}$ if ignoring the first point; (ii) both $H_{AB,c}$ and the tunneling time (Figure 6.4, Right) are smaller (slower) than the predicted no-barrier crossing with the latter by about 1-1.5 orders of magnitude; and (iii) a considerable scatter of data pointes appears on both scaled $H_{AB,c}$ and tunneling time and even obvious for distance <5.5 Å. In our analysis, the FC term has minor barrier with effective λ ≈1.2 eV and $ΔG^0$ ≈1.1 eV (see Figure 5.5) adjusted along with coupling solvation, and may be responsible for certain degrees of
slower rates relative to the activationless prediction (14). However, it is striking that our observation show a significant approach to the theory compared to the study on modified cytochrome c, where is by 3-6 orders of magnitude slower, considering the edge-to-edge distance in 4.5-14.8 Å (8). Though studied on different systems, such big discrepancy should mainly attribute from the improved instrument in our measurement. In other words, the employment of pathway model in cytochrome c may lead to a severe overestimate of route distance. On the other hand, however, decay of the square of coupling with distance in Figure 6.4 show an anisotropic scattering about one β at 1.4 Å⁻¹ in flavodoxin, which matches the concept of pathway model that balances through-bond and through-space coupling (11). From the pathway model point of view, since the engineered W donors are randomly located around the FMN cofactor instead of along one outstretching peptide segment, the overall electron traveling is composed of stochastic mixing of bond and space jump. One addition/removal of single covalent or hydrogen bond is responsible for 1-2 Å fluctuation. Furthermore, if space coupling over van der Waals contact (3-4 Å) is significant which is so far not clear (11), a harsh distribution of ET rate with distance will occur. Adding together, the observed random ET rates at the distance shorter than 5-6 Å can be explained by the pathway-model essence.
6.4 Conclusion

We provide a new examination of distance-dependence ET rate on short range. With the site-directed mutagenesis coupled with femtosecond temporal resolved upconversion technique, we monitor the rate arising from the W located at different sites of flavodoxin to the FMN cofactor. In the total 10 flavodoxin mutants, the edge-to-edge separation between indole (W) donor and ISO (FMN) acceptor is spanned from 3.7 Å to 18.2 Å determined by the MD simulation. Accordingly, the rate of ET tunneling is found to be on the order of subpicosecond at 3.7 Å, of picosecond at 4.2 Å, of tens of picosecond at 5-6 Å, and of hundreds of picosecond at 8-9 Å. Beyond 9 Å the transient represents the same as the ET-inert decay and the actual ET dynamics should exceed the FMN fluorescence time. In general, the observed rate is only 1-1.5 orders of magnitude slower than the theoretical prediction, with the decay of coupling at the grossly proposed $1.4 \text{ Å}^{-1}$ in protein environment. Some barrier in the FC factor may account for the slower behavior. Additionally, the ET rate (and thus the fitted coupling element) is fluctuated around the exponential line of distance dependence, presumably due to the nature of anisotropic coupling pathway which is amplified at the distance shorter than 5.5 Å.
Figure 6.1: Topology of the residues (ball) to be substituted by tryptophan in flavodoxin (PDB code: 2FX2). Color of the ball represents the localized secondary structure. The FMN cofactor is shown in dark green stick.
Figure 6.2: Trajectory fluctuations of edge-to-edge distance between indole moiety and flavin ISO moiety in different mutants from MD simulation. The red line indicates the average value over the 1100-ps window. At the corresponding right side, the relative orientation at 1050-ps snapshot is provided.
Figure 6.3: Normalized upconversion transients of the flavodoxin mutants displayed with short (A) and long (B) delay time. The samples are pumped at the wavelength of 400 nm, and in each flavodoxin the FMN emission gated at 538 nm is quenched by the W at the above corresponding distance.
Figure 6.4: Electronic coupling (Left) and tunneling time table (Right) for intraprotein ET in flavodoxin. Solid lines with decay constant of 1.0 Å⁻¹ and 1.3 Å⁻¹ and the dashed of 1.1 Å⁻¹ are extrapolated from the long-range tunneling in the Ru-modified azurin and the coupling element is calculated with λ and -ΔG° equal to 0.7 eV (28). In the right representation, data point for W62 tunneling (at 3.9 Å) is included by showing the averaged time constant from multiple exponential fitting. Note the tunneling time for W67 is assumed about 2 ns and the corrected electron coupling is about 0.16 meV ($H_{AB,c} = 0.027$ meV²).
Table 6.1: The replaced tryptophan residues in the flavodoxin mutants

<table>
<thead>
<tr>
<th>Mutants</th>
<th>Donor</th>
<th>W Localization</th>
<th>Close ISO Moiety</th>
<th>Distance(^a) (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y98F</td>
<td>W60</td>
<td>Loop 60</td>
<td>Dimethylbenzene</td>
<td>3.7±0.2</td>
</tr>
<tr>
<td>D95W(^b)</td>
<td>W95</td>
<td>β4</td>
<td>Dimethylbenzene</td>
<td>4.2±0.6</td>
</tr>
<tr>
<td>I65W(^b)</td>
<td>W65</td>
<td>Loop 60</td>
<td>Uracil</td>
<td>5.5±0.6</td>
</tr>
<tr>
<td>F101W(^b)</td>
<td>W101</td>
<td>Loop 90</td>
<td>Uracil</td>
<td>4.7±0.4</td>
</tr>
<tr>
<td>D62W(^b)</td>
<td>W62</td>
<td>Loop 60</td>
<td>Uracil</td>
<td>3.9±0.5</td>
</tr>
<tr>
<td>E66W(^b)</td>
<td>W66</td>
<td>Loop 60</td>
<td>Dimethylbenzene</td>
<td>7.7±0.5</td>
</tr>
<tr>
<td>G103W(^b)</td>
<td>W103</td>
<td>α4</td>
<td>Uracil</td>
<td>7.9±0.4</td>
</tr>
<tr>
<td>A104W(^b)</td>
<td>W104</td>
<td>α4</td>
<td>Uracil</td>
<td>8.9±0.3</td>
</tr>
<tr>
<td>L67W(^b)</td>
<td>W67</td>
<td>Loop 60</td>
<td>Uracil</td>
<td>8.8±0.4</td>
</tr>
<tr>
<td>R131W(^b)</td>
<td>W131</td>
<td>α5(^c)</td>
<td>Dimethylbenzene</td>
<td>18.2±0.4</td>
</tr>
</tbody>
</table>

\(^a\)Edge-to-edge distance between the indole moiety and FMN isoalloxazine moiety. \(^b\)With three other mutations of W60F, Y98F, and Y100F. \(^c\)Also at the end of one loop.


APPENDIX A: REACTION-DIFFUSION EQUATION

Sumi-Marcus 2D model for ET process outlined in Figure 1.2 has one nuclear coordinate $q$ and one solvation coordinate $X$ (20-22). Free energy on the reactant ($G_r$) and product ($G_p$) are:

$$G_r(X,q,n_r) = \lambda_o X^2 + \lambda_i q^2 + n_r \hbar \omega_r$$  \hspace{1cm} (A.1)

$$G_p(X,q,n_p) = \lambda_o (X - 1)^2 + \lambda_i (q - 1)^2 + n_p \hbar \omega_p + \Delta G^o$$  \hspace{1cm} (A.2)

The $n_r$ and $n_p$ are the quantum vibrational mode with $\hbar \omega_r$ and $\hbar \omega_p$ frequency, respectively. At the transition line in Figure 1.2 where $G_r = G_p$, the coordinate $q$ can be resolved as:

$$q = \frac{\lambda_o + \lambda_i + \Delta G^o - 2X\lambda_o + n_p \hbar \omega_p - n_r \hbar \omega_r}{2\lambda_i}$$  \hspace{1cm} (A.3)

By substituting $q$ in (A.4), the activation energy is expressed in Equation (A.5):

$$\Delta G^*(X) = G_r(X,q) - G_r(X,0) = \lambda_i^2 q$$  \hspace{1cm} (A.4)

$$\Delta G^*(X,n_p) = \left(\lambda_o + \Delta G^o - 2X\lambda_o + n_p \hbar \omega_p + \lambda_i\right)^2 / 4\lambda_i$$  \hspace{1cm} (A.5)
APPENDIX B: FLAVIN PHOTOCHEMISTRY AND PHOTOPHYSICS

A flavin molecule is composed of a three-ring isoalloxazine moiety (ISO, the lower gray box in Figure B.1) and a ribityl side chain (upper gray box) constituent. Derivatives with different X entailing the ribityl chain form the molecule riboflavin when only one hydrogen atom and the molecule riboflavin-5'-phosphate, i.e., FMN, when a phosphate group is substituted. For all flavins, however, it is the ISO ring responsible for the versatility of photochemical and photophysical properties that is directly involved in various enzyme activities. For example, a reversible interconversion between different oxidation and protonation states (Figure B.2) occurring during the catalytic cycle regulates the substrate metabolism involving one-electron transfer process (42, 46). The oxidation-reduction property and the pK_a values for each conversion may differ to some extent in enzymatic or protein-complex systems. In solution, the redox potential for the couple OX/SQ FMN has been determined at -0.238 eV and the value of -0.172 eV for further reduction to HQ state (136). The former redox potential in the flavodoxin, however, is reduced to -0.143 eV and the latter is substantially enhanced to -0.440 eV (137). The pK_a for N5(H) mainly modulating the neutral and anionic flavosemiquinone is much higher than that in solution, reaching >10.5 beyond the measurable limit (109). Combing these two property shifts, the neutral semiquinone state in flavodoxin is highly
favorable and in turn features its functional state. Spectroscopically, the flavin molecule has unique absorption and emission spectrum in the visible region for the respective oxidation and protonation state, making it a special pigment presenting different colors when encountering a conversion. For this reason, the spectroscopic study serves the best way to uncover the functional dynamics involving either oxidation or protonation switch.

One of the unique flavin photophysics to be discussed is the high conversion to the triplet state existing in the oxidized flavins (138-139). In solution, the riboflavin has the quantum yield ($\Phi_T \approx 0.375$) where the fluorescence lifetime ($1/k_F$) is $\approx 5$ ns. Equation (B.1) shows that if the nonradiative intersystem-crossing (ISC) rate remains in different system, the $\Phi_T$ is changed accordingly with various emission lifetimes. In our flavodoxin system, the oxidized FMN has the slower emission rate ($\approx 6$ ns in Chapter 3) than in solution that anticipates a higher amount of triplet-state FMN molecules, which may be detected in our experiments ($\Phi_T \sim 0.46$ in flavodoxin).

$$\Phi_T = \frac{k_{ISC}}{k_F} \quad (B.1)$$

In Figure B.3, we show the transient absorption due to free and flavodoxin-bound FMN molecules in the oxidized state. For both samples, the change of positive to negative signal in the probe wavelengths from 800 nm to 480 nm demonstrates the detection of FMN* molecules followed by some stimulated emission (SE) in 620-570 nm and by the ground-state bleaching (GS) at 480 nm. Spectral shift of SE and GS in the flavodoxin
relative to the solution system is consistent with the steady-state spectra (Figure B.4). In both systems, one striking slow continuous rise is believed due to the formation of FMN$^3$ molecule and, as the normal triplet-state lifetime, decays in the millisecond timescale. In addition to the ns-ms components, some with shorter timescale are observed. Mixture of sub- to several and tens of picoseconds rising in the wavelengths of 480-550 nm (data not shown) and decaying in 570-650 nm in both samples follows an apparent solvation behavior due to SE. Besides the solvation components, in the flavodoxin a delay of 1-2 ps in the strong SE (500-570 nm) region is also observed. This probably arises from the vibrational relaxation (or internal conversion) occurring in the flavodoxin-bound FMN at the excited state (98), and also explains the rise of 1-2 ps in the 620-650 nm probe region.

We further show the transient absorption due to flavodoxin semiquinone and compare with that of the oxidized state. Contrarily to Figure B.3, all the transients in Figure B.5 show the decay or recovery around the fluorescence lifetime (250-400 ps) with no ns-ms timescales observed. This manifests that the crossing from SQ (doublet) to quartet does not exist in the flavodoxin. Moreover, the SE responsible for the corresponding 680-nm negative signal (Figure 3.2) does not appear here. In other words, only the absorption of FMNH$^{+*}$ and of FMNH$^-$ contribute to the signal. As shown in Figure B.5 Inset, one significant rise (50%) of 1-2 ps should represent the vibronic cooling of flavodoxin FMNH$^{+*}$ and is parallel to the oxidized observation.
Figure B.1: Molecular scheme for riboflavin and FMN.
Figure B.2: Representative of flavin mechanistic reduction and protonation. "R" symbolizes the ribityl chain. The values of $pK_a$ in each conversion are determined in solution and may subject to change in protein environment. In flavodoxin, only the neutral oxidized (top middle), neutral semiquinone (middle), and the anionic hydroquinone (lower right) states can be produced.
Figure B.3: Absorption change of oxidized FMN molecule in buffer (A) and in the W60F/Y98F mutant (B) probed at different wavelengths. The pump wavelength is set at 400 nm. Two sets of data were taken side-by-side with similar sample concentration at 100-150 μM.
Figure B.4: Normalized steady-state absorption and emission spectra of oxidized FMN molecule in buffer (solid line) and in the W60F/Y98F mutant (dash-dotted line).
Figure B.5: Normalized transient absorption of the W60F/Y98F mutant probed at different wavelengths. Except the transient of 610 nm where the pump wavelength is set at 550 nm all are pumped at 580 nm. Solid lines represent the multiple exponential fitting. Inset enlarges the initial 8-ps window. Note the nonlinear spikes are removed for clarity.
APPENDIX C: PROTOCOL FOR FLAVODOXIN PURIFICATION

This procedure is to prepare 6 liters of cell culture. Culture medium and materials such as pipette tip, test tube, and flask are sterilized before use.

C.1 Transforming Competent Cells

1. Briefly centrifuge the Flavodoxin DNA before use.
2. Prewarm 1000 μl of LB (Luria-Bertani) medium at 37°C.
3. Pre-heat the LB agar plate containing 100 μg/ml ampicillin at 37°C.
4. Thaw the tube containing 200 μl DH5α competent cell on wet ice.
5. Place one empty sterile 1.5 microcentrifuge tube (Eppendorf) on wet ice.
6. Gently mix the cell tube and pipette 50 μl into the chilled Eppendorf tube.
7. Pipette 1 μl DNA (>10 ng) into the tube of step 4, and tap it slightly.
8. Incubate the mix vial on wet ice for 30 minutes.
10. Place on ice for 2 minutes.
11. Add the above 950 μl LB medium to the mix vial.
12. Shake the vial in incubator at 37°C for 1 four (225 RPM).
13. Spin down the cell pellet by centrifuging for 1 minute (14000 RPM) and discard the supernatant.

14. Resuspend the cell pellet with 200 μl LB medium. (Note: Step 13 and step 14 are to concentrate the cells.)

15. Spread 100 μl from the transformation vial on the LB agar plate.

16. Invert the plate and incubate at 37°C overnight.

17. Store the remaining transformation vial at 4°C.

18. On the next day, there should appear numerous colonies on the plate. If not, repeat step 15-16 using the stored remaining transformation vial.

C.2 Cell Culture (Overexpression of Flavodoxin)

1. Using a pipette tip, select a well-separated colony from the transformation plate. Directly immerse the tip in a glass tube containing 4 ml LB medium with 100 μg/ml ampicillin. Replace the tube cap.

2. Tilt the glass tube on the tube rack. Incubate at 37°C overnight (225 RPM).

3. On the next day, inoculate 3 ml of the above culture to the 80 ml LB medium with 100 μg/ml ampicillin in a 250 ml Erlenmeyer flask.


5. In the next morning, the OD at 600 nm should reach above 1.8 which ensures the culture opacity.

6. Prepare six 1 L LB medium in 2 L Erlenmeyer flask. Add the ampicillin to each flask at the final concentration of 100 μg/ml.

7. Inoculate 10 ml of the overnight cell culture in step 5 to each 1 L LB medium.

8. Incubate at 37°C (225 RPM) for 24-48 hours.

9. Harvest the cell by centrifuging for 30 minutes at 4°C (4000 RPM).

10. Discard the supernatant.
11. Resuspend the pellet with cold deionized water (IDW).

12. Collect all cell pellets in a 50 ml Falcon tube.

13. Centrifuge the tube for 10 minutes at 4°C (6500 RPM).

14. Discard the supernatant.

15. Place the pellet tube in the -80°C freezer.

C.3 Flavodoxin Purification

To release the recombinant protein, cells are lysed and sheared physically through one cycle of freeze/thaw followed by sonication (step 1-7). The target protein is isolated by two identical ion-exchange DEAE Sephacel chromatography (80 ml/each). Buffer of 50 mM, Tris-HCl (pH=7.3, determined at the room temperature) with or without the salt is used throughout the purification. Caution should be taken that the pH value of tris buffer is highly temperature sensitive (-0.028 pH/°C). A change of temperature at the room temperature (25°C) to 4°C where normally the purification is performed will cause a significant pH increase (ΔpH=0.588). The procedure is outlined below.

1. Thaw the frozen pellet tube at the room temperature.

2. Add 80 ml of 50 mM Tris-HCl (pH=7.3). Mix with the pellet thoroughly.

3. Transfer the liquid cell suspension to a sonication beaker.

4. Immerse the cell beaker on ice bath.

5. Sonicate the cells on ice bath for 2 minutes.
6. Wait for 3 minutes.

7. Repeat Step 5 and 6 three times.

8. Remove the cell debris by centrifuging for 50 minutes at 4°C (20K RPM).

9. Pour all the supernatant into a cold Erlenmeyer flask.

10. Load the supernatant (purple color) on the first DEAE column pre-equilibrated with Tris-HCl buffer at the rate of 0.5 ml/minute.

11. Wash the DEAE column with 350 ml of the same Tris-HCl buffer.

12. Wash the DEAE column with 1000 ml of the Tris-HCl buffer containing 150 mM NaCl (low salt buffer). The flavodoxin is gradually oxidized now.

13. Elute the sample (yellow color) with the buffer of Tris-HCl containing 250 mM NaCl.

14. Collect sample fractions with \( \text{OD}_{275\text{ nm}}/\text{OD}_{455\text{ nm}} = 3-6 \).

15. Dilute the sample 1:2 with Tris-HCl buffer.

16. Load on the second DEAE column pre-equilibrated with Tris-HCl buffer at the rate of 0.5 ml/minute.

17. Wash the second DEAE column with low salt buffer extensively until the output sample \( \text{OD}_{280\text{ nm}} \) is below than 0.05 using 1-cm cuvette (typically, 500 ml of the buffer is needed).

18. Elute the sample with the same buffer as step 13 (high salt buffer).

19. Collect sample fractions with \( \text{OD}_{275\text{ nm}}/\text{OD}_{455\text{ nm}} < 4.6 \).

20. Dialyze the sample against Tris-HCl buffer (1000 ml) overnight.

21. Aliquot the sample into Eppendorf tubes and quick-freeze by liquid nitrogen. Store at -80°C.
APPENDIX D: PHOTOREDUCTION AND CHEMICAL REDUCTION

Flavodoxin can be converted to the reduced states by photoreduction (63, 140) or chemical reduction (64). The former is achieved mainly due to the flavin photochemistry occurring at the triplet state. Certain amount of riboflavin is added during the preparation. Ethylenediaminetetraacetic acid (EDTA) is a candidate exogenous reducing agent to reduce the triplet-state riboflavin. The latter chemical reduction chooses sodium dithionite (standard potential: -0.66 eV, pH=7.0) as a reducing agent, one of few that has electron affinity lower than the flavodoxin SQ/HQ couple (standard potential for WT is -0.44 eV, pH=7.0). Use of the latter to obtain the HQ flavodoxin is tricky and sometimes difficult. Since the sodium dithionite is highly active in water solution, the factors such as the concentration itself and the ratio to the flavodoxin, temperature, and pH condition will greatly affect the reducing capability. At the best condition we find by trial and error, the freshly prepared sodium dithionite is immediately used no more than 10 minutes.

D.1 Photoreduction: from OX to SQ flavodoxin

1. Dialyze the flavodoxin against 50 mM MOPS buffer (pH=7.0) overnight.

2. In a quartz purging cell, add the following: 125 μl of 407 μM flavodoxin, 18 μl of 142 μM riboflavin, 4 μl of 250 mM EDTA (pH=8.0), and 33 μl of 50 mM MOPS
buffer (pH=7.0). The storage concentration for each reagent may vary but the final concentration of 280 μM for flavodoxin, 14 μM for riboflavin, and 5 mM for EDTA should be remained. Sample volume of 180 μl-200 μl is desired.

3. Purge the cocktail solution with nitrogen gas (purity: >99.998 %) for at least 3.5 hours.

4. Irradiate the samples by a table UV lamp. Samples are completely immersed on ice bath during the irradiation.

5. Monitor the sample absorption spectrum constantly. Keep irradiation until the maximum semiquinone flavodoxin is achieved.

D.2. Chemical reduction: from OX to HQ flavodoxin

1. Dialyze the flavodoxin against 50 mM phosphate buffer (pH=8.3) overnight.

2. Prepare 180 μl of 280 μM flavodoxin in the above phosphate buffer.

3. Transfer to the quartz purging cell.

4. Purge the sample solution with nitrogen gas (purity: >99.998 %) at the room temperature.

5. Purge another test tube containing 10 ml the same phosphate buffer only.

6. At the purge time of 2.5 hour, add 0.03 g of sodium dithionite power to the purged buffer, and gently mix it well.

7. Immediately inject 20 μl of the sodium dithionite solution to the purged flavodoxin sample.

8. Purge the mix sample for another 1 hour.

9. Check its absorption spectrum to ensure the fully reduced flavodoxin.
APPENDIX E: MOLECULAR DYNAMICS SIMULATION

Given a flavodoxin 3D structure, motion of all the atoms can be computed in time by solving Newton’s equation. The position at $x_p,y_p,z_p$ for each atom is updated at every simulated time-step by computing the forces at $x_f,y_f,z_f$. The forces are of bonds and electrostatic interaction between the atoms within a certain cut-off distance. Such computational method is called molecular dynamics (MD) and can be easily executed by using the Groningen Machine for Chemical Simulations (GROMACS) software package. Length of running time depends on the sample size, the simulation temporal length and, of course, the computing hardware. In this dissertation, Gromacs Forcefield (ffgmx), a derivative from GROMOS87 force field, is used which does not include explicit nonpolar hydrogen. Although this force field is approved outdated, due to the necessity of self build-up FMN cofactor in flavodoxin, this may be the best appropriate for our needs. The general procedure for performing GROMACS and calculating the distance between residue W of interest and FMN in time is described below.

1. Given a structure PDB file of holo-flavodoxin, generate the FMN molecule topology by using PRODRG tool available online (141). This tool uses the empirical GROMOS87 force field and is only suitable for small molecules.

2. From the same PDB file, generate the apoflavodoxin topology.
3. Center the results of 1. and 2. in one dodecahedron box with a suitable size.

4. Solvate the sample box with water molecules.

5. Neutralize the system with charge counterions (19 Na\(^+\) is needed).

6. Simulate the trajectories as the following:
   
   (a) Do energy minimization with step size of 0.01 nm with the tolerance 1000 kJ mol\(^{-1}\) nm\(^{-1}\), up to 500 steps.

   (b) Run MD with step size of 0.1 fs for 0.3 ps at 30 K.

   (c) Repeat (a).

   (d) Run MD with step size of 0.1 fs for 10 ps at 30 K.

   (e) Run MD with step size of 1 fs for 30 ps at 30 K.

   (f) Attempt to run MD with step size of 2 fs for 1300 ps at 295 K.

   (g) If crashed in (f), select a trajectory at the time at least 10 ps earlier than the crash time, run MD with step size of 0.2 fs for 20 ps. Such step can be easily done by a built-in function in Gromacs 4.0.

   (h) Repeat (f).

7. Output the time-dependent distance between W residue and FMN from the result of 6(f) or 6(h).
APPENDIX F: REDOX POTENTIAL

For a given component $A$ which can be reduced to another oxidation state $A^-$ from the reducing agent, the value of redox potential $E_0$ for the couple $A/A^-$ is an indication of the electron affinity to proceed the reduction (104). For simplicity, we only consider one electron here ($e^-$). The redox potential is quantified with respect to the reference normal or standard hydrogen electrode (NHE or SHE), $1/2 H \rightarrow H^+ + e^-$, expressed in the unit of electrical potential unit (eV). The value of 0 eV is chosen for the reference electrode at any temperature. In practice, the more positive $E_0$ signifies the higher possibility the component to be reduced. The following Equations (F.1) to (F.5) show the relationship between $\Delta E_0$ and the Gibbs free energy $\Delta G^\circ$ in one complete reaction.

$$A + B \rightleftharpoons A^- + B^+ \quad (F.1)$$

$$K_{eq} = \frac{[A^-][B^+]}{[A][B]} \quad (F.2)$$

$$\Delta G = \Delta G^\circ + RT \ln K_{eq} = \Delta G^\circ + RT \ln \frac{[A^-][B^+]}{[A][B]} \quad (F.3)$$

$$\Delta G = -F \Delta E \quad (F.4)$$
\[ \Delta E = \Delta E_0 - \frac{RT}{F} \ln \frac{[A^-][B^+]}{[A][B]} \] \hspace{1cm} (F.5)

Clearly, the reaction (F.1) is spontaneous to the right when \( \Delta E_0 \) is positive. The symbol \( F \) stands for Faraday, a conversion factor from chemical to electrical potential (i.e., \( F = 96,493 \text{ C/chemical equivalent} = 96,493 \text{ J/eV equivalent since } 1\text{J} = 1\text{C/J} \)).

Replacing the \( \Delta E \) in Equation (F.5) with the individual potential \( E_{A/A^-} - E_{B^+/B} \) the equation becomes:

\[ E_{A/A^-} - E_{B^+/B} = E_{0A/A^-} - E_{0B^+/B} - \frac{RT}{F} \ln \frac{[A^-][B^+]}{[A][B]} \] \hspace{1cm} (F.6)

When the electron donor \( B \) represents the standard hydrogen electrode (pH=0) in (F.7), the famous Nernst equation is formed in Equation (F.8) and (F.9) at 29°C.

\[ E_{A/A^-} = E_{0A/A^-} - \frac{RT}{F} \ln \frac{[A^-]}{[A]} \] \hspace{1cm} (F.7)

\[ E_h = E_0 + \frac{RT}{F} \ln \frac{[A]}{[A^-]} \] \hspace{1cm} (F.8)

\[ E_h = E_0 + 0.06 \log \frac{[A]}{[A^-]} \] \hspace{1cm} (F.9)

The Nernst curve in Figure F.1 explains the value of \( E_h \) a function of the concentration ratio of \([A]\) to \([A^-]\). At the value where 50% of \( A \) is reduced, the midpoint \( E_h \) (\( E_m \)) is the
standard potential $E_0$. Typically, $E_{m,x}$ is common to express the $E_0$ value measured at pH$=x$. In many cases, the oxidized form, here refer to $A$, is a stronger acid than the conjugated reduced form. For convenience, we express the reaction of $AH \rightarrow A^-H$ with deprotonation process on both sides in (F.10).

$$
A^-H \overset{E_{m(acids)}}{\leftrightarrow} AH + e^- \\
K_{red} \uparrow \quad \uparrow K_{ox} 
$$

(F.10)

$$
A^- + H^+ \overset{E_{m(bases)}}{\leftrightarrow} A^- + H^+ + e^- 
$$

Usually, under the physiological condition, $pK_{ox} < pH < pK_{red}$, the value of $E_{A^-/A^-H}$ is obtained instead (Figure F.2). The actual $E_{AH/A^-H}$ (i.e., $E_{m(acids)}$) and $E_{A^-/A^-}$ (i.e., $E_{m(bases)}$) appear only at an extraordinary low or high pH conditions. However, they can also be derived using Equation (F.11) and (F.12).

$$
E_{m,x} = E_{m(acids)} - 0.06 \log \left( \frac{1 + K_{ox}/[H^+]}{1 + K_{red}/[H^+]} \right) 
$$

(F.11)

$$
E_{m,x} = E_{m(bases)} - 0.06 \log \left( \frac{1 + [H^+]/K_{ox}}{1 + [H^+]/K_{red}} \right) 
$$

(F.12)
**Figure F.1:** Typical Nernst titration curve for one-electron reduction. Here shows a titration for standard potential $E_0$ at 0.1 eV (the cross point) at the normal temperature.
Figure F.2: Schematic diagram of pH-dependent midpoint redox potential. Common to many redox participants, the protonation and deprotonation take place upon the reduction or oxidation. The plot shows the left plateau of $E_m$ (acids) at 0.38 eV is reached when pH is smaller than the $pK_{ox}$ of 2.5, and the right $E_m$ (bases) at 0.05 eV in very basic solution ($pK_{red}=8.2$).