Ultrafast Dynamics of Intramolecular Electron Transfer

and DNA Repair by Photolyase

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ABSTRACT

One of the detrimental effects of ultraviolet (UV) radiation on DNA is the formation of the cyclobutane pyrimidine dimer (CPD) and a less frequently occurring (6–4) photoprodut (6–4PP) between two adjacent pyrimidine rings. These UV-induced photolesions interfere with replication and transcription, and may result in mutation and cell death. Photolyase, a class of flavoprotein, uses blue light to restore the CPD and 6–4PP to two normal bases. The cofactor, flavin adenine dinucleotide (FAD), has an unusual bent configuration in photolyase and cryptochrome and such a folded structure may have a functional role in initial photochemistry. We report here our systematic characterization of cyclic intramolecular electron-transfer (ET) dynamics between the flavin and adenine moieties in four redox forms of the oxidized, neutral and anionic semiquinone, and anionic hydroquinone states. These four ET dynamics unambiguously determine only the anionic hydroquinone flavin as the functional state in photolyase due to the slower ET dynamics with the adenine moiety and a faster ET dynamics with the substrate, while the intervening adenine moiety mediates such electron tunneling for repair of damaged DNA. Utilizing ET as a universal mechanism for photolyase and cryptochrome, these results support an anionic flavin as the active state for the blue-light photoreceptor cryptochrome. With the understanding that the anionic hydroquinoid flavin is the functional redox state in photolyase, we further studied the dynamics of damaged DNA repair by photolyase. Based on the previously resolved radical
mechanism, two fundamental issues, the mechanism of cyclobutane ring splitting and the pathway of electron tunneling, were ready to be resolved. Here, we use ultrafast UV absorption spectroscopy to show that the CPD splits in two sequential steps within 90 ps. Site-directed mutagenesis reveals that the active-site residues are critical to achieving high repair efficiency, a unique electrostatic environment to optimize the redox potentials and local flexibility, and thus balance dynamics of all catalytic reactions. Besides that, we found that the adenine moiety of the unusual bent cofactor is essential to mediating all electron transfer dynamics through a super-exchange mechanism. The active-site structural integrity, unique electron tunneling pathways and the critical role of adenine enable this complex photorepair machinery to achieve the maximum repair efficiency close to unity. Finally, we revealed the mechanism of 6–4PP restoration by photolyase for the first time. Using site-directed mutagenesis and femtosecond-resolved absorption spectroscopy, we determined that the key step in the repair photocycle is a cyclic proton transfer between the enzyme and the substrate and leading to 6–4PP repair in tens of nanoseconds. These key dynamics define the repair photocycle and explain the underlying molecular mechanism of the enzyme’s modest efficiency.
Dedicated to my family!
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CHAPTER 1

INTRODUCTION
Sunlight is essential to life on earth since it can provide sufficient amounts of luminous energy. However, the ultraviolet (UV) component in sunlight damages DNA. Among various forms of UV-induced photolesions, two major forms are cyclobutane pyrimidine dimer (CPD) and a less-frequently pyrimidine-pyrimidone (6-4) photoprodut (6–4PP) (Fig. 1.1). Both photoproducts lead to mutagenesis and even skin cancer. Photolyase, a class of flavoprotein, restores damaged DNA through the absorption of blue light. Two kinds of different photolyases share similar primary sequences and folding structures but a photolyase that repairs one type of photoproduc cannot repair the other. Therefore, we classify these two enzymes as CPD photolyase and (6-4) photolyase based on their repair functions. Both photolyases noncovalently bind to a fully reduced flavin adenine dinucleotide (FADH⁻) molecule as the catalytic cofactor. During the past two decades, the crystal structures of photolyases, especially of the enzyme-substrate (CPD or 6–4PP) complexes were solved (Fig. 1.2 and 1.3). In these schemes, the damaged DNA lesions flipped out of double strand and inserted into the active sites of CPD photolyase and (6-4) photolyase.

The flavin molecule is a critical and versatile cofactor in enzymatic reactions and the flavoproteins, which usually contain flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are involved in a wide range of biological functions. Among these proteins, two examples involving photobiology are DNA-repair enzymes and blue-light photoreceptors. Unlike other flavoproteins, the FADH⁻ cofactor in photoenzyme (photolyase) and photoreceptor (cryptochrome) takes an unconventional U-shape folding configuration (Fig. 1.4A). The active state of the
cofactor is the anionic hydroquinone form (FADH\(^{-}\)) in photolyase shown in vitro\(^{44}\) or in vivo\(^{45}\), but whether the active state form is the oxidized (FAD) or anionic (FAD\(^{-}\)/FADH\(^{-}\)) states in cryptochrome is a matter of debate.\(^{30,34,35,46-50}\) To clarify the active state of catalytic flavin cofactor in photolyase, we systematically studied the photochemistry of excited lumiflavin in oxidized, anionic semiquinoid, neutral semiquinoid, and anionic hydroquinoid states. Surprisingly, our study revealed that the intramolecular ETs between the lumiflavin moiety and adenine moiety of FAD cofactor are universal among all four redox states and determined the time scales of all ET steps. These four ET dynamics unambiguously determine only the anionic hydroquinone flavin as the functional state in photolyase. Utilizing ET as a universal mechanism for photolyase and cryptochrome, these results favor an anionic flavin as an active state to induce charge relocation to cause an electrostatic variation in the active site and then lead to a local conformation change to form initial signaling.

With knowing the intramolecular ET between flavin and adenine moieties in photolyase at four redox states, we measured the dynamics of CPD restoration by photolyase (chapter 3).\(^{51}\) In previous studies, we proved that CPD photolyase restores the thymine dimer through a cyclic ET mechanism with the photocycle finishing in 700 picoseconds (ps). To reveal how the thymine dimer splits after the electron injection, we extend our detection wavelengths from visible to deep UV light to catch various CPD related intermediates and products and then measure the dynamics of all initial reactants, reaction intermediates, and final products with wild type and active-site mutant enzymes. Unlike in many theoretical studies, the anionic thymine dimer splits by a sequential
mechanism but not by any other synchronously or asynchronously concerted mechanism. The C5-C5’ bond breaks within a couple of picoseconds and C6-C6’ splits in the following with the timescale of tens of picoseconds. The unproductive back ET, which induces the reclosure of CPD ring and causes no repair of damaged DNA, is much slower than CPD ring splitting. To further elucidate the role of photolyase active-site, we designed a series of active-site mutants to identify the key residues for synergistic catalytic reactions. The active site of photolyase significantly contributes to the repair efficiency by modulating the redox properties of the flavin/CPD pair or by steric effects.

Besides the splitting mechanism and repair efficiency, another important issue about CPD repair is the electron tunneling pathway. Two main tunneling schemes have been recently proposed by theoretical calculations. One prediction suggests that the electron would tunnel through the intervening adenine moiety at a longer distance of about 8 Å with the unusual U-shape configuration of the cofactor.\(^{52,53}\) The other claims that the electron would directly travel through space at a shorter distance of 4.3 Å (Fig. 1.2).\(^{54,55}\) To reveal the electron tunneling pathway and determine its tunneling directionality, we carefully designed the ET systems in photolyase (chapter 4). With detection in more than 13 wavelengths from visible to UV light, we are able to resolve the dynamics of forward ET, back ET without repair, bond splitting, and electron return in all CPD repairs with different substrates as well as with careful determination of the enzyme/substrate binding constants and total repair quantum yields. With the semi-classical Marcus ET theory, we evaluate the driving forces, reorganization energies and electronic coupling constants of these electron tunneling processes.
Besides the CPD lesion, another important UV damaged photolesion is 6–4PP, which also interferes with replication and transcription and may result in mutation and cell death. The (6–4) photolyase, which repairs 6–4PP upon blue-light excitation, also contains a fully reduced flavin adenine dinucleotide (FADH) as the catalytic cofactor. Several hypothesis of 6–4PP repair were proposed but the detailed mechanism remained very elusive until this work. In chapter 5, we examined the repair dynamics of 6–4PP by (6-4) photolyase. With femtosecond (fs) synchronization, we followed the function evolution and observed direct electron transfer from the excited flavin cofactor to the 6–4PP in 280 ps, but surprisingly fast back electron transfer in 57 ps without repair. We found that the catalytic proton transfer between a histidine residue in the active site and the 6–4PP, induced by the initial photoinduced electron transfer from the excited flavin cofactor to 6–4PP, occurs in 481 ps and leads to 6–4PP repair in tens of nanoseconds (ns). These key dynamics define the repair photocycle and explain the underlying molecular mechanism of the enzyme’s modest efficiency.

To monitor the transient electron transfer and DNA repair reactions in real time, we need to achieve very high temporal (femtosecond) and spatial (atomic) resolution. In all femtosecond-resolved experiments, we carried out the pump-probe technique which needs two laser pulses. The first pulse is called the pump or trigger pulse, which synchronizes the ultrafast reaction. In addition, the probe/gating pulse, which travels some distance/time longer than the pump pulse, detects the progress of the reaction at certain delay time. In other words, the pump-probe setup acts as a stop-motion camera, which restores the evolution of the original chemical reaction on a fs timescale by
dividing the observation of reaction into many parts and then assembling all these snapshots. The quality of the data (signal-to-noise ratio) will be gradually increased with repetition using fresh samples each time. Specifically, the time-resolved measurements in this dissertation were carried out using fs fluorescence up-conversion and transient absorption methods. The integrated experimental setup is schematically illustrated in Fig. 1.5.64,65
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CHAPTER 2

DYNAMIC DETERMINATION OF THE FUNCTIONAL STATE IN PHOTOLYASE AND THE IMPLICATION FOR CRYPTOCHROME
2.1 Introduction

The photolyase-cryptochrome superfamily is a class of flavoproteins that utilize a conserved flavin adenine dinucleotide (FAD) as the functional cofactor. Photolyase repairs damaged DNA\textsuperscript{1,7,51,63,66} and cryptochrome performs a variety of activities such as regulating growth of plant, synchronizing circadian rhythm and sensing direction as a magnetoreceptor.\textsuperscript{35,67-73} Strikingly, the FAD cofactor in the superfamily adopts a unique bent U-shape configuration with a close distance between its lumiflavin (Lf) and adenine (Ade) moieties (Fig. 1.4A). The cofactor could exist in four different redox forms (Fig 1.4B): oxidized (FAD), anionic semiquinone (FAD\textsuperscript{−}), neutral semiquinone (FADH\textsuperscript{•}), and anionic hydroquinone (FADH\textsuperscript{−}). In photolyase, the active state \textit{in vivo} is FADH\textsuperscript{−}. We have recently showed that the intervening adenine moiety mediates electron tunneling from the Lf moiety to substrate in DNA repair.\textsuperscript{66} Because the substrate such as CPD could be either an oxidant (electron acceptor) or a reductant (electron donor), the fundamental question is why photolyase adopts FADH\textsuperscript{−} as the active state rather than the other three redox forms, and if an anionic flavin is required to donate an electron, why not FAD\textsuperscript{−} that could be easily reduced from FAD? With the unusual bent structure, is FADH\textsuperscript{−} the only redox form that can be the active state? In cryptochrome, the active state of the flavin cofactor \textit{in vivo} is currently under debate and two competitive models of cofactor photochemistry have been proposed.\textsuperscript{30,34,35,46-50} One is called the photoreduction model\textsuperscript{48,49} that the oxidized FAD is photoreduced mainly by the conserved tryptophan triad to neutral FADH\textsuperscript{•} (signaling state) in plant, then triggering structural rearrangement to form signaling. The other model\textsuperscript{30,34,35,46,47} hypothesizes that cryptochrome utilizes a
similar mechanism of photolyase by donating an electron from its anionic form (FAD$^-$ in insect or FADH$^-$ in plant) to a putative substrate that induces local electrostatic variation to cause conformation changes for signaling. Both models require ET at the active site to induce electrostatic changes for signaling. Similarly, the Ade moiety near the Lf ring could also be an oxidant or a reductant. Thus, it necessitates to understanding the role of Ade moiety in initial photochemistry of FAD in cryptochrome.

Here, we use *Escherichia coli* photolyase as a model system and systematically studied the dynamics of the excited cofactor in four different redox forms. With site-directed mutagenesis, we can mutate all neighboring potential electron donors or acceptors and leave FAD in an inert environment. Strikingly, we observed that in all four redox states the excited Lf proceeds to intramolecular ET reactions with the Ade moiety. With femtosecond resolution, we followed the entire cyclic ET dynamics, determined all reaction times and thus reveal the molecular origin of the active state in photolyase. With the semi-classical Marcus ET theory, we further evaluated the driving force and reorganization energy of every ET step in the photoinduced redox cycle to understand the key factors that control these ET dynamics. These observations may imply a possible active state among the four redox forms in cryptochrome.

2.2 Materials and methods

2.2.1 Sample preparations

The oxidized *E. coli* photolyase with His-tag was prepared as described with modifications. To ensure the flavin cofactor in photolyase is fully oxidized, the protein
is incubated in the buffer with 500 mM imidazole at 4°C for up to 24 hours before loading on a HiTrap heparin HP column (5 mL). The protein is stored in a reaction buffer at pH 7.5 containing 50 mM Tris·HCl, 100 mM NaCl, 1 mM EDTA, and 50% (v/v) glycerol. Before femtosecond-resolved experiments, we used a desalting spin column (Pierce) to remove the possible free FAD cofactor in solution and a 100 µM of holoprotein was used for the experiment. Based on the template of photolyase E109A mutant, we mutated two tryptophans (W382 and W384) near the flavin to phenylalanine and prepared this double mutant. For the anionic semiquinone (FAD⁻), we mutated two critical positions in EcPL (E363L and N378C). After purification in vitro, we immediately obtained the mutant protein in completely oxidized state. Before each experiment, 100 µM protein was immediately exchanged into a basic reaction buffer at pH 9 with 50 mM Tris-HCl, 300 mM NaCl, 1 mM EDTA, 20 mM dithiothreitol, 1 mM oligo-(dT)$_{15}$ containing cyclobutane thymine dimers, and 50% (v/v) glycerol. After purging with argon and irradiation with ultraviolet light at 365 nm (UVP, 8W), the flavin cofactor was stabilized in the FAD⁻ state under anaerobic conditions. The neutral semiquinone (FADH*) photolyase was prepared by mutation of W382F in EcPL. Besides that, we purified the photolyase wild type and stabilized it in the anionic hydroquinone (FADH⁻) state under anaerobic conditions after purge with argon and subsequent photoreduction.
2.2.2 Femtosecond laser spectroscopy

All the femtosecond-resolved measurements were carried out using the transient-absorption method. The experimental layout has been detailed in chapter 1. In the oxidized (FAD) and anionic semiquinone (FAD\textsuperscript{−}) states, the proteins were excited at 480 nm. In the neutral semiquinone state (FAD\textsuperscript{H\textsuperscript{*}}), the pump wavelength was set at 640 nm. For the anionic hydroquinone state (FADH\textsuperscript{−}), we used 400 nm as the excitation wavelength. The probe wavelengths were tuned to cover a wide range of wavelengths from 800 to 260 nm. The instrument time resolution is about 250 fs and all the experiments were done at the magic angle (54.7°). Samples were kept stirring during irradiation to avoid heating and photobleaching. The studies with the neutral oxidized FAD and FAD\textsuperscript{H\textsuperscript{*}} states were carried out under aerobic conditions while those with the anionic FAD\textsuperscript{−} and FADH\textsuperscript{−} states were executed under anaerobic conditions. All experiments were performed in quartz cuvettes with a 5-mm optical length except that the FADH\textsuperscript{−} experiments probed at 270 and 269 nm were carried out in quartz cuvettes with a 1-mm optical length. For the weak signal probed at 555 nm of semiquinoid state, a long component (~20%) was removed for clarity and this signal could be from products resulting from the excited FADH\textsuperscript{*}, with a short lifetime of 230 ps.
2.3 Results and discussion

2.3.1 Electron transfer from adenine to neutral oxidized and semiquinoid lumiflavins

The excited FAD in photolyase is readily quenched by the surrounding tryptophan residues, mainly tryptophan triads with W382.46,75-79 With a double mutation of W382 and W384 within 6-7 Å to a redox inert phenylalanine (W382F/W384F), we abolished the possible ET of FAD$^*$ with the neighboring aromatic residues and observed a dominant decay of FAD$^*$ in 19 ps (an average time of a stretched exponential decay with $\tau=18$ ps and $\beta=0.92$) as shown in Figure 2.1A ($k_{\text{FET}^{-1}}$) with a probing wavelength at 800 nm. The observed stretched behavior reflects a heterogeneous quenching dynamics, resulting from the coupling with the active-site solvation on the similar time scales.80 The dynamics in 19 ps reflects the intramolecular ET from the Ade to Lf moieties to form a charge-separated pair of Ade$^+$+ Lf$^-$. Tuning the probe wavelengths shorter than 700 nm to search for the maximum contribution of intermediate Ade$^+$, we show two typical transients in Figs. 2.1B and C probed at 630 and 580 nm, respectively. With deconvolution (Appendix A), we observed the formation of Ade$^+$ in 19 ps and decay in 100 ps. The decay dynamics reflects the charge recombination process ($k_{\text{BET}^{-1}}$) and leads to completing the redox cycle. Using the reduction potentials of +1.9 V vs. NHE from adenine81 and about -0.3 V vs. NHE in photolyase for oxidized Lf,82 with the $S_1\leftarrow S_0$ transition of FAD at 500 nm (2.48 eV) we conclude that the ET reaction from Ade to LfH$^{**}$ has a favorable, negative free-energy change of -0.28 eV. This indicates that the
ET dynamics between the Lf and Ade moieties is favorable by negative free energy changes.

Similarly, we prepared the W382F mutant in the semiquinone state (FADH•) to eliminate the dominant electron donor of W382. Without any tryptophan in proximity, we observed a dominant decay of FADH•• in 85 ps (τ=82 ps and β=0.93) probed at 800 nm (Fig. 2.2A), which is consistent with the previous report but the interpretation of 80 ps to the intrinsic lifetime of FADH•• was not correct. The lifetime of the excited FMNH•• in flavodoxin is about 230 ps, which is two times longer than that of FADH•• observed here. Using the reduction potentials of +1.9 V vs. NHE from adenine and of +0.02 V vs. NHE in photolyase for neutral semiquinoid LfH•• with the S1←S0 transition of FADH• at 650 nm (1.91 eV) we obtained that the ET reaction from Ade to LfH•• has a favorable, negative free-energy change of -0.03 eV. Thus, besides the intrinsic lifetime, the excited LfH• is likely to be quenched by intramolecular ET with Ade to form a charge-separated pair of Ade+LfH−. Taking 230 ps as the lifetime of LfH•• without ET, we derived a forward ET dynamics with Ade of 135 ps, given by an overall decay of FADH•• in 85 ps.

To probe the intermediate Ade+, we tuned the probe wavelengths to the shorter wavelengths to detect the maximum intermediate contribution. The best probing wavelength would be the one at which the absorption coefficients of the excited and ground states are equal, resulting in cancellation of the positive LfH•• signal by the negative partial LfH• formation signal, leading to the dominant rise and decay signal of
Ade\(^+\). Fig. 2.2B shows the typical signal probed at 555 nm. We observed the negative signals due to the initial bleaching of FADH\(^+\). We can regroup all three signals of LfH\(^*\), Ade\(^+\) and LfH\(^*\) into two dynamic types of transients: one represents the summation of two parts (FADH\(^*\) and FADH\(^+\)) with an excited-state decay time of 85 ps and its amplitude is proportional to the difference of absorption coefficients between the two parts. Because the FADH\(^*\) has a larger absorption coefficient (\(\varepsilon_{Lnf^*} < \varepsilon_{Lnf^+}\)), the signal flips and shows as a negative rise (Fig. 2.2B). The second-type transient reflects the summation of two parts (Ade\(^+\) and FADH\(^+\)) with a dynamic pattern of Ade\(^+\) in a rise and decay behavior and similarly the signal flips due to the larger absorption coefficient of FADH\(^*\). Kinetically, we observed an apparent rise in 20 ps and a decay in 85 ps. Fig. 2.2C shows the transient probed at 530 nm and the ground-state LfH\(^*\) recovery in 85 ps dominates the signal. Thus, the observed 20 ps dynamics reflects the back ET process and the signal appears apparent reversed kinetics, leading to less accumulation of the intermediate state. Here, the 20 ps charge recombination is much faster than the 135 ps charge separation with a driving force of -1.88 eV in the Marcus inverted region. In summary, although the neutral FAD\(^+\) and FADH\(^*\) states can draw an electron from a strong reductant and the dimer substrate can be repaired by a strong oxidant\(^{86}\) by donating an electron to induce cationic dimer splitting, the ultrafast cyclic ET dynamics with the adenine moiety in the mutants reported here or with the neighboring tryptophans in the wild type\(^{64,77}\) exclude these two neutral redox states as the functional state in photolyase.
2.3.2 Electron transfer from anionic semiquinoid and hydroquinoid lumiflavins to adenine

In photolyase, FAD\(^-\) cannot be stabilized and is readily converted to FADH\(^*\) through a proton transfer from the neighboring residues or trapped water molecules in the active site. However, in insect cryptochrome, the flavin cofactor can stay in FAD\(^-\) \textit{in vitro} under anaerobic condition and this anionic semiquinone was also proposed to be the active state \textit{in vivo}.\(^{34,46}\) By examining the sequence alignment and x-ray structures\(^{11,42}\) of these two proteins, the key difference is one residue near the N5 atom of the Lf moiety, N378 in \textit{E. coli} photolyase and C416 in \textit{Drosophila} cryptochrome. Through structured water molecules, the N378 is connected to a surface-exposed E363 in the photolyase but a hydrophobic L401 in the cryptochrome. Thus, we prepared a double-position photolyase mutant E363L/N378C to mimic the critical position near the N5 atom in the cryptochrome. With a higher pH of 9 and in the presence of the thymine dimer substrate at the active site to push water molecules out of the pocket to reduce local proton donors, we can successfully stabilize FAD\(^-\) for more than several hours in the mutant under anaerobic condition. Fig. 2.3 shows the absorption transients of excited FAD\(^-\) probed at three wavelengths. At 650 nm (Fig. 2.3A), the transient shows a 12 ps decay dynamics (\(\tau=12\) ps and \(\beta=0.97\)) without any fast component or long plateau. We also did not observe any noticeable thymine dimer repair and thus exclude the ET from FAD\(^*-\) to the dimer substrate.

The radical Lf\(^*-\) probably has a lifetime in hundreds of picoseconds as observed in insect cryptochrome,\(^{46}\) also similar to the lifetime of the radical LfH\(^*\).\(^{84}\) Thus, the
observed 12 ps dynamics must result from an intramolecular ET from Lf$^+$ to Ade to form Lf+Ade$. Such an ET reaction also has a favorable driving force ($\Delta G^0=-0.28$ eV) with the reduction potentials of Ade/Ade$^-$ and FAD/FAD$^-$ to be around -2.5 and -0.3 V vs. NHE, respectively. The observed initial ultrafast decay dynamics of FAD$^{-*}$ in insect cryptochromes in several to tens of picoseconds, besides the long lifetime component in hundreds of picoseconds, could be from an intramolecular ET with Ade as well as the ultrafast deactivation by a butterfly bending motion through a conical intersection due to the large plasticity of cryptochrome. However, photolyase is relatively rigid and thus the ET dynamics here shows a single exponential decay with a more defined configuration.

Similarly, we tuned the probe wavelengths to the blue side to probe the intermediate state FAD and minimize the total contribution of the excited-state decay components. Around 350 nm, we detected the significant intermediate FAD signal with a rise in 2 ps and a decay in 12 ps. The signal flips to the negative absorption due to the larger ground-state Lf absorption. Strikingly, at 348 nm (Fig. 2.3C), we observed a positive component with the excited-state dynamic behavior ($\varepsilon_{\text{Lf}^{-*}}$) and a flipped negative component with a rise and decay dynamic profile ($\varepsilon_{\text{Lf}^{+}}$+$\varepsilon_{\text{Ade}^-}$ < $\varepsilon_{\text{Lf}^-}$). Clearly, the observed 2 ps dynamics reflects the back ET dynamics and the intermediate FAD signal with a slow formation and a fast decay appears to reflect apparent reverse kinetics again. This observation is significant and explains why we did not observe any noticeable thymine dimer repair due to the ultrafast back ET to close redox cycle and thus prevent further electron tunneling to damaged DNA to induce dimer splitting. Thus, in the wild-
type photolyase, the ultrafast cyclic ET dynamics determines that FAD$^-$ cannot be the functional state even though it can donate one electron because the back ET dynamics with the intervening adenine moiety completely eliminates the electron tunneling to the dimer substrate. Also, the observation explains why photolyase utilizes the fully reduced \text{FADH}^– as the active state rather than anionic hydroquinoid FAD$^-$, even though FAD$^-$ can be readily reduced from the oxidized FAD.

We previously reported the total lifetime of 1.3 ns for \text{FADH}^*-7. Because the free-energy change, $\Delta G^0$, for ET from fully reduced \text{LfH}^* to adenine is 0.04 eV,\textsuperscript{66,85} the ET dynamics could occur on a long time scale. We observed that the fluorescence and absorption transients all show the 1.3 ns excited-state decay dynamics (Fig. 2.4A, $\tau$=1.2 ns and $\beta$=0.90). Similarly, we need to tune the probe wavelengths to maximize the intermediate absorption and minimize the contributions of excited-state dynamic behaviors. According to our other studies (see chapter 3),\textsuperscript{51} at around 270 nm both the excited and ground states have similar absorption coefficients. Figs. 2.4B and C show the transients around 270 nm and the intermediate \text{LfH}^* signal is positive ($\varepsilon_{\text{LfH}^*}+\varepsilon_{\text{Ade}}$) and dominant. Similarly, we observed an apparent reverse kinetics with a rise in 25 ps and a decay in 1.3 ns. With the N378C mutant, we reported the lifetime of \text{FADH}^*- in 3.6 ns (chapter 3) and taking this value as the lifetime without ET with the adenine moiety, we obtained the forward ET time in 2 ns. Thus, the rise dynamics in 25 ps reflects the back ET and this process is ultrafast, much faster than the forward ET. This observation is significant and indicated that the ET from the cofactor to the dimer substrate does not follow the hopping mechanism with two tunneling steps from the
cofactor to adenine and then to dimer substrate. Due to the favorable driving force, the electron directly tunnels from the cofactor to dimer substrate and on the tunneling pathway the intervening adenine moiety mediates the ET dynamics to speed up the ET reaction in the first step of repair.66

2.3.3 Unusual bent configuration, intrinsic electron transfer and unique functional state

With various mutations, we have revealed that the intramolecular ET from the flavin to adenine moieties always occurs with the bent configuration in all four different redox states of photolyase and cryptochrome. The bent flavin structure in the active site is unusual among all flavoproteins. The flavin cofactor mostly is in an open, stretched configuration and if any, the ET dynamics would be longer than the lifetime due to the long separation distance. We have found that the adenine moiety mediates the initial ET dynamics in repair of damaged DNA using this unusual bent structure.52,66 Currently, it is not known whether the bent structure has a functional role in cryptochrome. If the active state is FAD$^-$ in insect cryptochrome or FADH$^-$ in plant cryptochrome, then the intramolecular ET dynamics with the adenine moiety could be significant due to the charge relocation to cause an electrostatic change, even though the back ET could be ultrafast, and such a sudden variation could induce local conformation changes to form initial signaling state. On the other hand, if the active state is FAD, the ET dynamics in wild-type cryptochrome is ultrafast in about 1 ps with the neighboring tryptophan(s) and the charge recombination is in tens of picoseconds.46 Such ultrafast change in
electrostatics could be similar to the variation induced by the intramolecular ET of FAD$^+$ or FADH$^*$. Thus, the bent configuration assures an “intrinsic” intramolecular ET within the cofactor to induce a large electrostatic variation for local conformation changes in cryptochrome, which may imply its functional role.

The mystery of the active state FADH$^-$ in photolyase is finally unveiled. With the U-shape bent configuration, the intrinsic ET dynamics determines the only choice of the active state to be FADH$^-$, not FAD$^-$, due to the much slower intramolecular ET dynamics within the cofactor in the former (2 ns) than the latter (12 ps) although both anionic redox states could donate one electron to the dimer substrate. With the neutral redox states of FAD$_{ox}$ and FADH$, the ET dynamics are ultrafast with the neighboring aromatic tryptophan(s) even though the dimer substrate could donate one electron to the neutral cofactor but the ET dynamics would be not favorable, much slower than those with the tryptophans or adenine moiety. Thus, the only active state is the bent anionic hydroquinone FADH$^-$ due to the unique dynamics of the slower intramolecular ET (2 ns) in the cofactor and the faster intermolecular ET (250 ps) with the dimer substrate. These intrinsic intramolecular cyclic ET dynamics in the four redox states are summarized in Figure 2.5A.

The intrinsic intramolecular ET dynamics in the bent cofactor configuration with four different redox states all follow a single-exponential decay with a slightly stretched behavior ($\beta=0.90-0.97$) due to a close compact structure between the flavin and adenine moieties. Thus, these ET dynamics are weakly coupled with local protein relaxations. With the cyclic forward and back ET rates, we can use the semi-empirical Marcus ET
theory\textsuperscript{88-91} and evaluate the driving forces (\(\Delta G^0\)) and reorganization energies (\(\lambda\)) for the ET reactions of the four redox states. Since no significant conformation variation in the active site for different redox states is observed, we assume that all ET reactions have a similar electronic coupling constant of \(J=12\) meV. With the assumption that the reorganization energy of the back ET is larger than that of the forward ET, we solved the driving force and reorganization energy of each ET step and the results are shown in Figure 2.5B with a 2D contour plot. The driving forces of all forward ET fall in the region between +0.04 and -0.28 eV, while the corresponding back ET are in the range from -1.88 to -2.52 eV. The reorganization energy of the forward ET varies from 0.88 to 1.1 eV, while the back ET only acquires a slightly larger value from 1.11 to 1.64 eV (see Figure 2.5). These values are consistent with our findings about the reorganization energy of flavin-involved ET in photolyase (chapter 4),\textsuperscript{66} which is mainly contributed by the distortion of the flavin cofactor during ET (close to 1 eV). All forward ET steps fall in the Marcus-normal region due to their small driving force and all the back ET processes are in the Marcus-inverted region. Note that the ET dynamics of the anionic cofactors (2 and 4 in Fig. 2.5B) have larger reorganization energies than those with the neutral flavins, especially for the back ET, probably because Ade/Ade\(^-\) has the larger distortion than Ade\(^+\)/Ade or the different high-frequency vibrational energy is involved in the back ET.\textsuperscript{92}

Overall, the ET dynamics are controlled by both free-energy change and reorganization energy as shown in Fig. 2.5B. The active site of photolyase modulates both factors to control the ET dynamics of charge separation and recombination or charge relocations in every redox state.
2.4 Conclusions

We reported here our direct observation of intramolecular ET between the lumiflavin and adenine moieties with an unusual bent configuration of the flavin cofactor in photolyase in four different redox states with femtosecond spectroscopy and site-direct mutagenesis. While the neutral oxidized and semiquinone lumiflavins can be photoreduced by accepting an electron from the adenine moiety (or neighboring aromatic tryptophans) upon excitation, the anionic semiquinone and hydroquinone lumiflavins can reduce the adenine moiety by donating an electron. After the initial charge separation or relocation, all back ET dynamics occur ultrafast in less than 100 ps to close the photoinduced redox cycle. Strikingly, besides the oxidized state, all other three back ET dynamics are much faster than their forward ET processes, leading to less accumulation of the intermediate state. The key to capture the intermediate state is to find an appropriate probing wavelength to cancel out the contributions from both the excited state (positive signal) and ground state (negative signal), leaving the weak intermediate-state signal dominant.

The intramolecular ET dynamics in the four redox states with the bent cofactor configuration reveal the molecular origin of the active state in photolyase and imply a universal ET model for both photolyase and cryptochrome. To repair damaged DNA in photolyase, an electron must be transferred from the anionic flavin cofactor. The intramolecular ET dynamics resolved in this study unambiguously determine only the FADH\(^{-}\) as the active state rather than FAD\(^{-}\) due to the intrinsically slower ET (2 ns) in the former and faster ET (12 ps) in the latter, allowing a feasible, relatively fast, ET (250
ps) to the damaged-DNA substrate with the intervening adenine moiety to mediate such initial electron tunneling for repair (see chapters 3 and 4). In cryptochrome, either neutral FAD and FADH\(^*\) or anionic FAD\(^-\) and FADH\(^-\) can proceed to an ET dynamics upon blue-light excitation; for the former, the ET with the neighboring aromatic tryptophans occurs in 1 and 30 ps or with the adenine moiety in 19 and 135 ps and for the latter, the ET with the adenine moiety occurs in 12 ps and 2 ns, respectively. All back ET dynamics occur within 100 ps. Such ET dynamics induce an electrostatic variation in the active site, leading to local conformational changes to form initial signaling. Utilizing a unified ET mechanism for both photolyase and cryptochrome, it implies that an anionic redox form seems more attractive as a functional state in cryptochrome.
Fig. 2.1 Femtosecond-resolved intramolecular ET dynamics between the excited oxidized lumiflavin and adenine moieties. (A-C) Normalized transient-absorption signals of the W382F/W384F mutant in the oxidized state probed at 800, 630 and 580 nm, respectively, with the decomposed dynamics of the reactant (Lf* ) and intermediate (Ade+ ). Inset shows the derived intramolecular ET mechanism between the oxidized Lf* and Ade moieties.
Fig. 2.2 Femtosecond-resolved intramolecular ET dynamics between the excited neutral semiquinoid lumiflavin and adenine moieties. (A-C) Normalized transient-absorption signals of the W382F mutant in the neutral semiquinoid state probed at 800, 555 and 530 nm, respectively, with the decomposed dynamics of two groups: one represents the excited-state (LfH\textsuperscript{+}) dynamic behavior with the amplitude proportional to the difference of absorption coefficients between LfH\textsuperscript{+} and LfH\textsuperscript{*}; the other gives the intermediate (Ade\textsuperscript{+}) dynamic behavior with the amplitude proportional to the difference of absorption coefficients between Ade\textsuperscript{+} and LfH\textsuperscript{*}. Inset shows the derived intramolecular ET mechanism between the neutral LfH\textsuperscript{+\textsuperscript{*\textsuperscript{w}}} and Ade moieties.
Fig. 2.3 Femtosecond-resolved intramolecular ET dynamics between the excited anionic semiquinoid lumiflavin and adenine moieties. (A-C) Normalized transient-absorption signals of the E363L/N378C mutant in the anionic semiquinoid state probed at 650, 350 and 348 nm, respectively, with the decomposed dynamics of two groups: one represents the excited-state (Lf$^*$) dynamic behavior with the amplitude proportional to the difference of absorption coefficients between Lf$^*$ and Lf$^-$; the other gives the intermediate (Lf) dynamic behavior with the amplitude proportional to the difference of absorption coefficients between (Lf+Ade$^-$) and Lf$^-$. Inset shows the derived intramolecular ET mechanism between the anionic Lf$^*$ and Ade moieties.
Fig. 2.4 Femtosecond-resolved intramolecular ET dynamics between the excited anionic hydroquinoid lumiflavin and adenine moieties. (A-C) Normalized transient-absorption signals in the anionic hydroquinoid state probed at 800, 270 and 269 nm with the decomposed dynamics of two groups: one represents the excited-state (LfH\(^{-}\)) dynamic behavior with the amplitude proportional to the difference of absorption coefficients between LfH\(^{-}\) and LfH\(^{-}\); the other reflects the intermediate (LfH\(^{-}\)) dynamic behavior with the amplitude proportional to the difference of absorption coefficients between (LfH\(^{-}\)+Ade\(^{-}\)) and (LfH\(^{-}\)+Ade). Inset shows the derived intramolecular ET mechanism between the anionic LfH\(^{-}\) and adenine moieties.
Fig. 2.5 Summary of the cyclic intramolecular ET dynamics and molecular mechanisms between the lumiflavin and adenine moieties of photolyase in the four different redox states and their dependence on driving forces and reorganization energies. (A) Reaction times and mechanisms of the cyclic ET between the Lf and Ade moieties in all four redox states. (B) Two-dimensional (2D) contour plot of the ET rates relative to free energy ($\Delta G^0$) and reorganization energy ($\lambda$) for all electron tunneling steps. All forward ET reactions are in the Marcus normal region ($-\Delta G^0<\lambda$) while all back ET steps are in the Marcus inverted region ($-\Delta G^0>\lambda$).
CHAPTER 3

DYNAMICS AND MECHANISM OF CYCLOBUTANE PYRIMIDINE DIMER REPAIR BY DNA PHOTOLYASE
3.1 Introduction

The UV component of sunlight irradiation causes DNA damage by inducing the formation of cyclobutane pyrimidine dimer (CPD), which is mutagenic and a leading cause of skin cancer.\textsuperscript{3-5,93} CPD can be completely restored by a photoenzyme, photolyase, through absorption of visible blue light.\textsuperscript{1} In our early work,\textsuperscript{7,80,94} we have observed a cyclic electron-transfer (ET) reaction in thymine dimer (T\textless\textrangle\textgreater T) repair by photolyase and determined the time scale of 700 ps for the complete repair photocycle. However, the central questions of whether the splitting of the cyclobutane ring is synchronously or asynchronously concerted or stepwise and whether the cyclic electron transfer involves specific tunneling pathways were not resolved. Furthermore, the molecular mechanism underlying the high repair efficiency has not been elucidated. Here, using femtosecond spectroscopy and site-directed mutagenesis, we are able to measure the dynamics of all initial reactants, reaction intermediates, and final products with different substrates and with wild type and active-site mutant enzymes, and thus reveal the complete spatio-temporal molecular picture of thymine dimer repair by photolyase. Photolyase contains a fully reduced flavin adenine dinucleotide (FADH\textsuperscript{−}) as the catalytic cofactor and electron donor as mentioned before. Based on previous studies, a sequential repair mechanism of thymine dimer splitting is shown in Fig. 3.1. Previously, we reported the forward ET from FADH\textsuperscript{+} to T\textless\textrangle\textgreater T in 250 ps (1/\textit{k}_{\text{FET}}) and the total decay of intermediate FADH\textsuperscript{*} in 700 ps (1/\textit{k}_{\text{total}}).\textsuperscript{7,94} These dynamics usually follow a stretched-single-exponential decay behavior, reflecting heterogeneous ET dynamics controlled by the active-site solvation.\textsuperscript{7,80,95,96} However, in that study, no thymine-related species could be detected in
the visible light region and no information about the dimer splitting was obtained. To reveal how the thymine dimer splits, we extended our detection wavelengths from visible to deep UV light to catch thymine-related intermediates. To uncover how the electron tunnels in the repair, we used the different dimer substrates to follow electron tunneling pathways. Finally, to elucidate how photolyase achieves such high repair efficiency, we designed a series of active-site mutants to identify the key residues for synergistic catalytic reactions.

3.2 Materials and methods

3.2.1 CPD photolyase and mutants

The purification of *E. coli* CPD photolyase with depletion of the antenna cofactor has been reported previously. For mutant studies, we mutated a series of critical residues (E274A, R226A, R342A, N378C and M345A) at the active site, including two typical mutants of N378C near the flavin cofactor side and E274A near the substrate side, to examine enzyme activities. Mutant plasmids were constructed using QuikChange II XL kit (Stratagene) based on the plasmid of wild-type enzyme. All mutated DNA plasmids were sequenced to ensure correct results. In femtosecond UV absorption studies, 100 μM of enzyme (or 50 μM in experiments with probe wavelengths of shorter than 300 nm) was used in a reaction buffer containing 100 mM NaCl, 50 mM Tris-HCl at pH 7.5, 20 mM dithiothreitol, 1 mM EDTA, and 50% (v/v) glycerol. For some visible-light measurements, a higher enzyme concentration of 300 μM was used.
For absorption spectra of FADH$^\bullet$ and FADH$^-$ at longer than 300 nm, we directly obtained them after purification from holoenzyme with FADH$^\bullet$ and then the active form FADH$^-$ by photoactivation. The absorption spectra of FADH$^\bullet$ and FADH$^-$ at shorter than 300 nm were acquired as described elsewhere with some modifications. In short, we dissolved 10 μM of photolyase in pH 3.5 50 mM KPi buffer for 30 min. The flavin cofactor was released from the binding pocket and was completely removed by concentrating the sample solution in Amicon centrifugal filter devices (30,000 MWCO) to 10% of the volume and then restoring back to the original volume, repeated by three times. The absorption spectrum of apoenzyme was then obtained. The absorption spectra of FADH$^\bullet$ and FADH$^-$ below 300 nm were obtained by subtracting the absorption spectrum of apoenzyme from holoenzyme absorption spectra at the respective redox states.

### 3.2.2 CPD substrates

We prepared CPD substrate (T<>T) as described elsewhere with some modifications. The dinucleotide dTpdT (Sigma-Aldrich) were dissolved in 15% aqueous acetone (v/v) with a concentration of 100 OD units per ml. The argon-purged DNA solutions were irradiated on ice with a UVB lamp (302 nm, General Electric) at a distance of 2 cm for 1.5-2 hours to eliminate the distinctive 260 nm absorption peak. After irradiation, we purified the cyclobutane pyrimidine dimers by HPLC, using a C18 reversed-phase column (Grace, 250 mm×10 mm) with a 75 mM potassium phosphate buffer (pH 6.8). The potassium salt was removed later by washing and eluting with water.
twice through the same C-18 column. The final concentration of all CPD substrates used in femtosecond and steady-state repair experiments is 18 mM. For all experiments, the ratio of the substrate to photolyase is at least 60:1 and such mixing can be used for several hours for femtosecond-resolved measurements without any noticeable change of the repair dynamics.

3.2.3 Enzyme activities

We measured the enzyme activities of thymine dimer (T<>T) repair by photolyase mutants as follows. Two mixtures of samples in two cuvettes were prepared with 1 µM concentrations of the wild type and mutant photolyase (N378C or E274A) with 18 mM T<>T substrate. The two cuvettes were purged with argon and irradiated at room temperature using a white-light lamp (General Electric) with the same distance of 6 cm. We measured the change of absorption spectra of the two mixtures and recorded the absorption change at 266 nm with the illumination time. Such steady-state repair experiments were repeated for multiple times (one typical experiment is shown in Fig. 3.2). The change of absorption at 266 nm was averaged by these measurements and the slope of the average-absorption change against time is proportional to the repair quantum yield of the enzyme. By knowing the repair quantum yield of T<>T by the wild type enzyme (0.82), we then obtained the mutant repair quantum yield. In addition, we did not detect any absorption change at 266 nm in the control experiments with only T<>T at the same concentration under the same conditions.
3.2.4 Femtosecond absorption spectroscopy

All the femtosecond-resolved measurements were carried out using the transient absorption methods. The experimental layout has been detailed previously. Briefly, for all measurements, the pump pulse at 400 nm in 1 kHz was generated by the doubling of 800 nm in a 0.2 mm thick β-barium borate crystal (BBO, type I). The pump pulse energy was typically attenuated to 140-200 nJ/pulse before being focused into the sample cell. All desired probe wavelengths, from visible to ultraviolet, were generated from optical parametric amplifiers (OPA-800C and TOPAS, Spectra-Physics). The instrument response time is about 250 fs and all experiments were done at the magic angle (54.7°). Samples were kept fast stirring during irradiation to maintain the fresh complex concentration as well as to avoid heating and photobleaching. All enzyme reactions in the femtosecond-resolved measurements were carried out under anaerobic conditions.

3.3 Results and discussion

3.3.1 Sequential splitting mechanism of the cyclobutane ring.

Figure 3.3 shows a striking pattern of the transient absorption signals of the complex of E. coli CPD photolyase with substrate T<>T, probed at fifteen wavelengths. At 430 nm, the signal is the summation of all three flavin species (FADH\(^-\)\(^*\), FADH\(^*\) and FADH\(^-\)) and decays to zero upon completion of repair. From 335 nm in the UV region, we captured the formation and decay of thymine-related intermediates and from 300 nm we clearly observed the long-component formation of final repaired thymines (insets B-E in Fig. 3.3). By knowing the dynamics of FADH\(^-\)\(^*\) and the absorption coefficients of
FADH* and FADH– (inset A in Fig. 3.3), only with the sequential model shown in Fig. 3.1 and not any other synchronously or asynchronously concerted schemes of thymine splitting and electron return,100-102 we can systematically fit all the absorption transients from visible to UV [see Appendix B]. The results are shown in deconvolution of various species in insets B-E and the related absorption coefficients in inset A of Fig. 3.3, and thus we obtain the entire dynamics of thymine dimer splitting.

3.3.2 Dynamics of ring splitting, back electron transfer and electron return

Our data indicate that in contrast to the computational reaction scheme models101,102 the thymine dimer splits by a sequential pathway. The first-bond C5-C5’ breakage (1/k_{sp1}) finishes in much less than 10 ps, consistent with the theoretical prediction of a nearly barrierless process.101-109 Also, the driving force of direct back ET between T<>T– and FADH* is quite large (~2.0 eV)85,110 which falls in the Marcus inverted ET region and should occur in hundreds of ps. Thus, the slow formation (k_{FET}) and ultrafast decay (k_{sp1}) result in negligible accumulation of T<>T– population and a close-to-unity efficiency of C5-C5’ splitting. In the following, we observed the formation and decay of T-T– intermediate after the first-bond C5-C5’ breakage (Fig. 3.1 and insets B, D and E in Fig. 3.3). The decay dynamics in 87 ps mainly represents the second-bond C6-C6’ splitting. Given the total repair quantum yield as 0.82,111-113 the forward ET branching as 0.85, and the yield of C5-C5’ breaking as 1.0, the branching of C6-C6’ splitting is 0.96. Therefore, the second-bond breakage finishes in 90 ps (1/k_{sp2}) which is much longer than that of theoretical calculations, and the back ET without the second-
bond splitting occurs in 2.4 ns ($1/k_{\text{BET}}$), which is much slower than the report of recent time-resolved study with sub-nanosecond resolution. After the second-bond cleavage, the signal of $T^-$ forms after C6-C6’ ring splitting and decays in 700 ps ($1/k_{\text{ER}}$ and inset C of Fig. 3.3), reflecting the fact that the electron return from $T^-$ to FADH$^*$ is completely decoupled from the second-bond breaking. This is confirmed by the observation of final products of two repaired thymine molecules, which are formed in two sequential steps in 90 and 700 ps upon the initial electron injection (see insets D and E).

### 3.3.3 Active-site mutation and repair efficiency modulation.

The repair efficiency (0.82) of thymine dimer by photolyase is higher than those (0.004-0.41) of all chemical model systems synthesized so far, indicating that the amino acids in the active site must significantly contribute to the repair efficiency by modulating the redox properties of the flavin/CPD pair or by steric effects. To examine how the protein active site controls the higher repair efficiency, we mutated a series of residues (E274A, R226A, R342A, N378C and M345A) at the active site and here showed two typical mutants, N378C near the cofactor side and E274A near the substrate side, which make critical contributions to the repair efficiency (Fig. 3.4A). We systematically studied these two mutants by probing from visible to UV. Four typical results are shown in Fig. 3.4B-D at 800, 620, 270 and 266 nm. The final results of forward ET, back ET, second-bond splitting, and electron return are shown in Fig. 3.5B and Table 3.1 with the measured total repair quantum yields in Fig. 3.5A and Table 3.1. Both mutants exhibit the lower quantum yields of 0.69 for N378C and 0.40 for E274A, resulting from a
combination of two-step quantum yields, forward ET relative to lifetime emission ($k_{LT}$) and second-bond splitting relative to back ET (two pairs of dashed lines in Fig. 3.5B). Both mutants modulate the ET redox potentials, N378C for FADH$^-$ at the cofactor side and E274A for T$<>$T at the 5’ side, leading to longer forward ET times and thus resulting in the lower first-step quantum yields. Furthermore, the backward ET processes significantly become faster, reducing the chance for the second-bond splitting and again causing a decrease in the second-step splitting quantum yields. For N378C, we obtained the same second-bond splitting time in 90 ps as the wild type, consistent with the fact that the mutation affects only the cofactor. For E274A we observed a faster second-bond splitting time of 30 ps, probably due to the destabilization of the splitting transition state by the mutant of E274A that abolishes two hydrogen-bonds with T$<>$T at the 5’ side (Fig. 3.4A). The observation of thymine dimer repair by mutant E274A also excludes any possibility of proposed proton transfer(s) between E274 with T$<>$T during repair that has been suggested based on theoretical consideration.$^{102,118}$

3.4 Conclusion

We reported our direct observation of ultrafast sequential splitting dynamics of the cyclobutane ring in a few and 90 picoseconds and identification of unique electron tunneling pathways in dimer repair. Such identification reveals the critical functional role of the adenine moiety as an efficient electron-tunneling mediator in the unique bent U-shape conformation of flavin cofactor. During the repair, the back electron transfer without the second-bond splitting tremendously slows down to 2.4 ns to maximize the
repair channel and the electron return after the repair is in 700 ps, completely decoupled from the ring splitting. Thus, to maximize the repair quantum yield and balance the four elementary processes between the forward ET and lifetime emission, and between the second-bond splitting and back ET, the active-site electrostatics of photolyase must contain the appropriate functional groups to optimize redox potentials and active-site mobility for electron tunneling.\textsuperscript{7,80,109} Clearly, the active-site environment in photolyase seems ideal for CPD repair and is well optimized over the course of evolution. Any mutation would break the delicate balance of the four processes and is unlikely to speed up the forward ET and slow down the back ET.\textsuperscript{89,119} The best combination of the four processes is shown in Fig. 3.6, a photocycle for the maximum repair of thymine dimer by photolyase on the ultrafast time scale.\textsuperscript{120}
Table 3.1 Fitting parameter with quantum yield of CPD repair by wild type and mutant photolyases

<table>
<thead>
<tr>
<th></th>
<th>&lt;τ_{FET}&gt;</th>
<th>&lt;τ_{life}&gt;</th>
<th>&lt;τ_{sp}&gt;</th>
<th>&lt;τ_{BET}&gt;</th>
<th>&lt;τ_{ER}&gt;</th>
<th>B_{FET}</th>
<th>B_{SP}</th>
<th>Φ_{repair}</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>250</td>
<td>1300</td>
<td>90</td>
<td>2400</td>
<td>700</td>
<td>0.85</td>
<td>0.96</td>
<td>0.82</td>
</tr>
<tr>
<td>N378C</td>
<td>1300</td>
<td>3600</td>
<td>90</td>
<td>1300</td>
<td>560</td>
<td>0.73</td>
<td>0.94</td>
<td>0.69</td>
</tr>
<tr>
<td>E274A</td>
<td>580</td>
<td>1100</td>
<td>30</td>
<td>50</td>
<td>77</td>
<td>0.65</td>
<td>0.62</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*aThe time constants are given in picoseconds. Lifetime: radiative decay of FADH\(^*-\) in the absence of T<>T; FET: forward electron transfer from FADH\(^*\) to substrate; sp: bond breakage of C6-C6'; BET: back electron transfer without repair; ER: electron return after repair; B_{FET}: efficiency of FET; B_{SP}: efficiency of CPD splitting; Φ_{repair}: quantum yield for CPD repair by photolyase.
Fig. 3.1 A proposed sequential repair scheme of CPD. After the forward electron transfer (FET, reaction rate $k_{\text{FET}}$) from FADH$^\bullet$ to thymine dimer upon light excitation, the repair channel includes splitting of two bonds of C5-C5' ($k_{\text{sp1}}$) and C6-C6' ($k_{\text{sp2}}$) in thymine dimer accompanied with unproductive back electron transfer ($k_{\text{BET}}$) without repair. The subsequent electron return ($k_{\text{ER}}$) after complete ring splitting finishes the reaction photocycle. $k_{\text{total}}$ is the overall decay rate of intermediate state FADH$^\bullet$ after the initial charge separation.
Fig. 3.2 Steady-state measurements of repair efficiency of T<>T by mutant photolyase. Under the same conditions, a reference repair of T<>T by wild-type photolyase was conducted. Difference absorption spectra of T<>T repair by the wild type and N378C mutant relative to their time-zero absorption spectra, respectively, were taken from 30 to 240 min illumination under white light and anaerobic conditions. Multiple independent experiments were taken to obtain an average value of absorption at 266 nm with visible-light illumination time. Given the quantum yield of 0.82 for T<>T repair by the wild type, the repair efficiency of N378C mutant is calibrated by the change of distinctive 266-nm absorption.
**Fig. 3.3** Femtosecond-resolved transient absorption dynamics of reactants, various intermediates and products involved in repair of thymine dimer. The repair dynamics are probed systematically from 800 to 260 nm and shown are the typical results in UV region with a distinct pattern. Inset A: The absorption coefficients of all species involved in repair. Insets B-E: Transient absorption signals probed at 335, 300, 270 and 266 nm. These dynamics are systematically fitted by total flavin-related species (FADH\(^+\) + FADH\(^*\) + FADH\(^-\), dashed red), thymine dimer intermediate T-T\(^-\) (dashed cyan), thymine anion (dashed dark red) and thymine products (dashed dark yellow).
Fig. 3.3 continued
Fig. 3.4 Effect of active-site mutations on repair dynamics. (A) X-ray structure of the active site of *A. nidulans* photolyase with two critical residues of N386 (N378 in *E. coli*) and E283 (E274 in *E. coli*). The hydrogen-bonding distances of the two residues with FADH$^-$ and CPD are also shown, respectively. (B–D) Femtosecond-resolved absorption signals of the repair of damaged CPD by the wild type and two mutants (N378C and E274A) probed at 800 and 620 nm (B), 270 nm (C), and 266 nm (D). Insets in (B) and (C) show the deconvolution of various species’ contributions of N378C mutant probed at 620 and 270 nm, respectively, while the inset in (D) for E274A mutant probed at 260 nm.
Fig. 3.5 Quantum yields and various reactions times of four elementary steps for wild type and various mutants of CPD photolyase. (A) The overall repair quantum yields (QY) of the two mutants N378C and E274A were measured, relative to the known wild-type one (0.82), by monitoring the formation of thymine bases at 266-nm absorption with certain visible-light irradiation of the enzyme-substrate solution. (B) The reaction times of each elementary step in CPD repair by the wild type and two mutants with our measured total QY from (A). The vertical dashed lines represent the two-step repair efficiency of the FET to lifetime emission (LT) and the second-bond splitting (SP2) to BET. The ER is decoupled from the CPD splitting.
Fig. 3.6 Complete photocycle of CPD repair by photolyase. All resolved elementary steps of CPD (thymine dimer) repair, showing the complete repair photocycle on the ultrafast time scales and the elucidated molecular mechanism.
CHAPTER 4

ELECTRON TUNNELING PATHWAYS AND ROLE OF ADENINE IN REPAIR OF CYCLOBUTANE PYRIMIDINE DIMER BY DNA PHOTOLYASE
4.1 Introduction

Photolyase, which contains an active cofactor flavin adenine dinucleotide in fully reduced form (FADH\textsuperscript{-}), repairs CPD through an electron transfer (ET) mechanism upon excitation of blue light.\textsuperscript{1,7,121,122} The complex structure of \textit{A.nidulans} photolyase with thymine dimer (T<>T) after \textit{in situ} repair is shown in Figure 4.1.\textsuperscript{12} With femtosecond (fs)-resolved spectroscopy, we recently followed the entire repair dynamics from the reactants, to intermediates, and finally to products and thus resolved the complete repair photocycle by photolyase (Fig. 3.6).\textsuperscript{51} Briefly, the excited FADH\textsuperscript{-} donates one electron to CPD in 250 picoseconds (ps). The cyclobutane pyrimidine ring in anionic CPD breaks sequentially in less than 10 ps and in 90 ps, respectively, and the electron from the repaired anionic thymine (T\textsuperscript{-}) returns to FADH\textsuperscript{•} in 700 ps, finishing the photocycle and restoring the damaged DNA. The futile back ET, parallel to the ring splitting, occurs very slowly in 2.4 nanoseconds, favoring the repair channel. However, a key question remains: how the electron tunnels between excited FADH\textsuperscript{-} and T<>T in the forward ET and between FADH\textsuperscript{•} and T\textsuperscript{-} in the electron return. Two main tunneling schemes have been recently proposed by theoretical calculations.\textsuperscript{52-55} One prediction suggests that the electron would tunnel through the intervening adenine moiety at a longer distance of about 8 Å with the unusual U-shape configuration of the cofactor.\textsuperscript{52,53} The other claims that the electron would directly travel through space at a shorter distance of 4.3 Å (Fig. 4.1).\textsuperscript{54,55} Thus, to reveal the electron tunneling pathway and determine its tunneling directionality, we carefully design the ET systems in photolyase, examine their dynamics and finally elucidate the electron tunneling routes.
The proposed studies will provide new insights into protein electron transfer at a short distance (<10 Å) although numerous long-range protein ETs have been carried out in the past two decades. Some long-distance ETs use certain protein residues as electron donating/accepting intermediates, especially aromatic residues of tryptophan and tyrosine, to complete an electron hopping process and most ETs employ protein matrix to facilitate electron tunneling via a super-exchange mechanism. The electron tunneling pathways in proteins usually consist of covalent bonds, hydrogen bonds, and spatial protein architectures with secondary structures of α-helices and β-sheets. These ETs typically occur at long distances and their ET rates are much faster than those by simply tunneling through space or in aqueous solution. For photolyase studied here, the ET is at a short distance within 10 Å with two possible tunneling pathways: one is shorter but with no molecular mediation like tunneling in space and the other has a longer distance with an intervening molecule in the pathway. Thus, which route does the electron take and how does this step affect the repair function?

To answer these critical questions, we use a series of (deoxy)uracil-modified dimers (Fig. 4.3) (U<>T, T<>U and U<>U) and perform a complete series of fs-resolved studies from visible to UV light region. With detection in more than 13 wavelengths from visible to UV light, we are able to resolve the dynamics of forward ET, back ET without repair, bond splitting, and electron return in all CPD repairs with different substrates as well as with careful determination of the enzyme/substrate binding constants and total repair quantum yields. With the semi-classical Marcus ET theory, we evaluate the driving
forces, reorganization energies and electronic coupling constants of these electron tunneling processes. These results elucidate the electron tunneling pathways and the function role of intervening adenine, and provide the molecular basis of the high repair efficiency.

4.2 Materials and methods

4.2.1 Sample preparation

The purification of *E. coli* CPD photolyase with depletion of the antenna cofactor has been reported previously in chapter 3. For all fs-resolved experiments with UV detection, 100 μM of the enzyme (or 50 μM in experiments with the probe wavelengths at shorter than 300 nm) was used in a reaction buffer containing 100 mM NaCl, 50 mM Tris-HCl at pH 7.5, 20 mM dithiothreitol, 1 mM EDTA, and 50% (v/v) glycerol. For all visible-light measurements, a higher enzyme concentration of 300 μM was used.

We prepared various CPD substrates (T<>T, T<>U, U<>T and U<>U) as described previously (Fig. 4.2). The final concentration of all CPD substrates used in fs-resolved experiments is 18 mM. For all these studies, the ratio of the substrate to photolyase is at least 60:1 and such samples can be used for several hours for fs-resolved measurements without any noticeable change of the repair dynamics.

4.2.2 Enzyme activities

We quantitatively measured the enzyme activities of various substrate (T<>U, U<>T, U<>U and T<>T) repair by photolyase. First, we determined the dissociation
constants (or binding constants) of photolyase-CPD complexes (E·S). We prepared several sets of mixtures in cuvettes with 1 µM wild-type photolyase with excess concentrations of T<>T (T<>U, U<>T, or U<>U), respectively. Then, the cuvettes were irradiated at room temperature under an UV-light lamp (UVP, 365 nm) at the same distance of 6 cm. The inset in Fig. 4.3A shows a typical repair process with absorbance increasing over time and displays gradual formation of T and U bases. Fig. 4.3A shows typical steady-state repair measurements for four substrates. For each substrate repair, we plotted a set of the absorbance changes against time with different substrate concentrations. Given [S]>>[E], a small amount of substrate repair does not change the substrate concentration. Thus, the resulting slopes (m) will be directly proportional to the binding complex concentrations, [E·S]. For any two concentrations of the substrate, we can solve their binding complex concentrations (and the dissociation constant) as follows.

\[
K_d = \frac{([E]_1 - [ES]_1)[S]_1}{[ES]_1} = \frac{([E]_2 - [ES]_2)[S]_2}{[ES]_2}
\]

\[
\frac{[ES]_1}{[ES]_2} = \frac{m_1}{m_2}
\]

To be more accurate, after solving binding complex concentrations, we actually plotted the binding complex percentage ([ES]/[E]) against substrate concentration and using the following equation fit the dissociation constant, \(K_d\).

\[
\frac{1}{[ES]/[E]} - 1 = \frac{K_d}{[S]}
\]

Fig 4.3B shows the fitting results for four substrates and the derived four dissociation constants are 5.0x10^{-4}, 4.7x10^{-4}, 4.8x10^{-4} and 1.5x10^{-3} M for T<>T, T<>U,
U<>T and U<>U, respectively. The first three substrates have similar dissociation constants ($\sim 5 \times 10^{-4}$ M) and for U<>U, the dissociation constant is the largest by a factor of 3.

By knowing the dissociation constants and under the same conditions, then we can obtain the repair quantum yields, for example, from Fig 4.3A, by comparing the slopes of T<>U, U<>T, and U<>U with the T<>T repair one and taking account of the binding-complex concentrations. Knowing the repair quantum yield of T<>T to be 0.82, we obtained the repair quantum yields of 0.88, 0.86 and 0.84 for T<>U, U<>T and U<>U, respectively. We have not observed any absorption change at 266 nm in control experiments with only substrates (T<>U, U<>T and U<>U) at the same concentrations under the same conditions. Note that the obtained repair quantum yields for T<>U, U<>T and U<>U are larger than those reported in early literatures and the new values here are more accurate due to the careful determination of dissociation (or binding) constants and also consistent with the measured various reaction rates as shown below.

### 4.2.3 Femtosecond absorption spectroscopy

The fs-resolved measurements were carried out using the transient absorption method. The experimental layout has been detailed elsewhere and chapter 3.
4.3 Results and discussions

4.3.1 Electron injection, adenine mediation and tunneling pathway

Figures 4.4-4.6 show femtosecond-resolved absorption dynamics of T<>U, U<>T and U<>U repair by photolyase with detection wavelengths from visible 710 nm to UV 266 nm to map out the complete dynamic evolution of the repair. In the visible region from 710 to 425 nm, we detected only flavin species.

At 710 nm, only excited state FADH\(^{+}\) has absorption and thus the transients directly reflect the electron injection dynamics from excited cofactor to CPD substrates. These dynamics were the stretched parameters of 0.87, 0.89 and 0.92 (\(\beta\)) for T<>U, U<>T and U<>U, respectively. In our earlier studies of T<>T repair,\(^7,51\) we observed a decay of 170 ps with \(\beta=0.71\). As pointed out elsewhere,\(^6,26,80,120,134\) the stretched-exponential dynamic behavior results from the modulation of ET reactions by active-site solvation. The active-site solvation occurs in a few picoseconds to sub-nanoseconds. Thus, the longer ET dynamics has a smaller parameter \(\beta\) and more stretched behavior, indicating more coupling between active-site motions and electron tunneling.

Using \(<\tau>=(\tau/\beta)\Gamma(1/\beta)\) and taking excited FADH\(^{-}\) lifetime as 1.3 ns contributions in to considerations, we further obtained the average time scales of 212, 80, 60 and 69 ps and the final ET times of 250, 85, 63, and 73 ps (Table 4.1) for T<>T, T<>U, U<>T and U<>U, respectively. Thus, the ET dynamics (rates) from the excited FADH\(^{-}\) to four CPD substrates vary in decreased order of 5\(^{\prime}\)-U<>T-3\(^{\prime}\) > U<>U > T<>U > T<>T. This observation is significant and indicates that the electron tunnels toward ending at the 5\(^{\prime}\) side, not the 3\(^{\prime}\) side, of the dimers. U<>T and T<>U have similar reduction potentials,
but the ET time lengthens by more than 35% from 63 ps of U<>T to 85 ps of T<>U, i.e., the rate decreases to 74%. The theoretical studies showed similar binding configurations\textsuperscript{52} for U<>T and T<>U and thus the observed differences in ET rates should not come from the electronic couplings because of the similar donor-acceptor separation. Also, such changes of ET rates, if caused by the electronic coupling, could lead to a distance increase of 0.3-0.4 Å, which was not observed in the simulations\textsuperscript{52} and is also unlikely in structural and electrostatic interactions.\textsuperscript{12,17}

The uracil base has a reduction potential higher than thymine by \textasciitilde0.11 V.\textsuperscript{81} Although the electronic interactions between T and U in the covalent CPD species delocalize the CPD states and result in similar redox properties of U<>T and T<>U, the U moiety without a methyl group at the C5 position at the 5’ side should have a larger electron affinity than the T moiety. Thus, if the adenine moiety is involved in electron tunneling, our results indicate that the local configuration and orientation favors the electron tunneling to the 5’ side, rather than the 3’ side (Fig. 4.1), although the distances from the adenine to the two sides are nearly equal\textsuperscript{12}, 3.1 and 3.2 Å, respectively. The larger ET rate of U<>T vs. T<>U also excludes the electron tunneling pathway directly from the o-xylene ring of the flavin to the 3’ side of the dimer (Fig. 4.1).\textsuperscript{54} Thus, the electron does tunnel through the adenine moiety and this conclusion will also be separately obtained from the ring splitting dynamics as shown below.

Given that the reduction potentials of adenine and FADH\textsuperscript{−} is -2.52 V and +0.08 V vs. NHE, respectively, and assuming the S1←S0 transition of FADH\textsuperscript{−} at 500 nm (2.48 eV), the intramolecular electron hopping from the isoalloxazine ring to adenine in
FADH$^*$ is unfavorable due to the free energy. At the absence of substrate, we did not observe any fast decay of FADH$^*$ and the excited-state dynamics is in nanoseconds.$^7,51$ Thus, we can rule out the possibility of direct two-step electron hopping between the isalloxazine ring and the substrates bridged by adenine in a total of 63-85 ps. If the two-step hopping did occur, the first-step hopping from the isalloxazine ring to adenine should happen at least in 63-85 ps, assuming the second-step hopping from the adenine moiety to substrates occurs instantaneously with a short separation distance of 3.1 Å. With the substrates, even though the redox properties of FADH$^*$ could be affected, it is unlikely that the first-step ET reaction from the isalloxazine ring to adenine, if it occurs, drastically speeds up from nanoseconds to tens of picoseconds upon the binding of substrates. Thus, the electron tunneling from the isalloxazine ring to substrates must be mediated by adenine through a super-exchange mechanism, which matches the observation in chapter 2.

We can estimate the ET times for the two proposed ET pathways. Both tunneling routes are a hybrid of structural and chemical configurations. We first need to determine the initial location of the tunneling electron in FADH$^*$. Some calculations showed that the electron density in the HOMO molecular orbital of the ground state and in LUMO of the excited state is mainly localized at the pyrimidine and pyrazine rings in FADH$^{52,53,135}$ and such a minor change of electron distributions upon excitation leads to a small variation of dipole moments of the two states, as observed to be about 1-2 D in recent experiments.$^{80,136}$ The other calculations claimed the electron in LUMO localized at the o-xylene ring,$^{54,55}$ resulting in a huge change of dipole moments of the two states in more
than 10 D, which is contrary to experimental observations.\textsuperscript{50,107} Thus, the tunneling electron starts at the side of the pyrimidine and pyrazine rings and here we assume the N10 (Fig. 4.3) position in FADH\textsuperscript{–*} as the initial starting point for the two potential ET pathways. The first route is from N10 to C10\textquotesingle through a covalent bond in 1.5 Å, then from C10\textquotesingle to passing through the adenine in 3.6 Å and finally to reaching the 5\textquotesingle side of the dimer in 3.1 Å (Figs. 4.1 and 4.3). The total tunneling length is 8.2 Å. The second pathway is from N10 to C8\textquotesingle through four covalent bonds in 5.7 Å and then to the 3\textquotesingle side of the dimer in 4.3 Å (Figs. 4.1 and 4.3). The total tunneling length in this second route is 10 Å, although the distance between the isoalloxazine ring and 3\textquotesingle-side of the dimer is the shortest at 4.3 Å. Thus, using the empirical ET formula below,\textsuperscript{88,137,138} we can estimate the ET times for the two hybrid tunneling pathways,

\[
\log k = 13 - A - 3.1 \frac{(\Delta G + \lambda)^2}{\lambda}, \quad [4.4]
\]

\[
A = \beta (r - r_0) \log e = 0.434 \beta (r - r_0)
\]

where \( k \) is the ET rate in s\textsuperscript{–1}, \( A \) is the electronic coupling term, \( \Delta G \) is the total free energy of the reaction in eV, \( \lambda \) is the reorganization energy in eV, \( \beta \) here is the empirical ET parameter in Å\textsuperscript{–1} and \( r \) is the separation distance in Å. \( r_0 \) is the van der Waals distance at 3.0 Å for calculating the long tunneling distance.

For different tunneling configurations, the \( \beta \) parameters are different.\textsuperscript{90} For tunneling through covalent bonds, we could use \( A=0.434 \beta r \) assuming \( r_0=0 \) and \( \beta=0.71 \) Å\textsuperscript{–1}. For tunneling in proteins mediated by different structures, \( \beta=1.0-1.4 \) Å\textsuperscript{–1}, while for tunneling directly through space, \( \beta=2.9-4.0 \) Å\textsuperscript{–1}. Thus, for the first hybrid
pathway, we obtain $A=2.07-2.71$ and for the second hybrid route, $A=3.39-4.02$. For the T$\leftrightarrow$T dimer, the free energy $\Delta G$ is -0.44 eV and the reorganization energy $\lambda$ is 1.21 eV (see below). Thus, we obtained the ET times of 389 ps-1.7 ns for the first pathway and of 8.1-34.7 ns for the second route. The measured ET time for T$\leftrightarrow$T is 250 ps, closer to the estimated value of 389 ps through the first hybrid tunneling pathway. Thus, the electron tunnels through the covalent bond N10-C10’ at 1.5 Å and then from C10’ to the 5’ side of the dimer in 6.7 Å mediated by adenine nearly in the middle pathway. Using the measured ET time of 250 ps, we obtained $\beta=0.83$ Å$^{-1}$ for the tunneling from C10’ to the 5’-side dimer due to the mediation by the adenine moiety. Thus, from the C10’ position to the 5’-side dimer, the nature of tunneling is more likely toward the covalent bond tunneling ($\beta=0.71$ Å$^{-1}$) by 3.6 Å from C10’ to adenine (N6 atom in Figure 4.2) and 3.1 Å from adenine to the 5’-side dimer. Such a hybrid tunneling pathway mediated by the adenine moiety is also observed in 6-4 photoproduct repair by 6-4 photolyase. In that system, the first pathway to the 5’ side of the 6-4 photoproduct through adenine is nearly the same as in the dimer repair, but the separation between C8’ to the nearest 3’ side of the 6-4 photoproduct in the second route increases to 6.3 Å. However, the forward ET has a similar dynamics of 280 ps due to the first unique hybrid tunneling pathway through the adenine moiety in the unusual U-shape configuration of FADH$^-$ at the active site. Thus, the adenine moiety plays a critical role on mediating electron tunneling in repair of damaged DNA through a super-exchange mechanism.

With the measured forward ET dynamics, we can determine the electronic coupling constant ($J$) and related free energy for different substrates. We can rewrite Eq.
4.4 in a semi-classical ET expression.

\[
k_{ET} = \left( \frac{4\pi^3}{h^2 \lambda k_B T} \right)^{0.5} J^2 \times 10^{-1.1 |\Delta G_{ET} + \lambda|^3 / \lambda}
\]

[4.5]

where \( h \) and \( k_B \) are Planck and Boltzman constants, respectively, and \( T \) is temperature in Kelvin.

For four different substrates, the coupling constant (\( J \)) and the reorganization energy (\( \lambda \)) can be considered as constants. Knowing the redox potential \( T<>T/T<>T^- \) to be -1.96 V vs. NHE, thus the free energy \( \Delta G \) is -0.44 eV for forward ET between FADH\(^-\)\( ^* \) and \( T<>T \) (Table 4.2). Interestingly, both reduction potentials of \( T \) and \( T<>T \) are ~0.11 V lower than \( U \) and \( U<>U \) in organic solvents.\(^{81,110}\) Thus, the free energy of forward ET between FADH\(^-\)\( ^* \) and \( U<>U \) is around -0.55 eV. Given the reported reorganization energy of \( \lambda \) to be 1.2-1.4 eV in flavoprotein ETs\(^{139-141}\) and by consideration of a larger \( J \) and a smaller \( \lambda \) in the forward ET than in the electron return, we finally obtained the free energy for ET with \( U<>U \) to be -0.59 eV, \( \lambda=1.21 \) eV and \( J=3.0 \) meV. The coupling constant of 3.0 meV is in a good agreement with the theoretical calculation\(^{52}\) which assumed the electron tunneling through adenine instead of space. Using the obtained \( \lambda \) and \( J \) values, we obtained the free energy (\( \Delta G \)) of -0.61 eV for \( U<>T \) and -0.57 eV for \( T<>U \) (Table 4.2). The difference of 40 meV for \( U<>T \) and \( T<>U \) probably, in fact, results from the preferred electron tunneling directionality to the 5’ side rather than 3’ side of the dimer, assuming the same electronic coupling constants for both ETs.
4.3.2 Sequential ring splitting, back electron transfer and maximum quantum efficiency.

With understanding of the forward ET dynamics probed at 710 nm, we tuned the probe wavelength to 625 nm to detect the flavin intermediate FADH$^\bullet$ and to 425 nm to detect the flavin product FADH$^\circ$ (Fig. 4.4-4.6). To detect the substrate-related intermediates and products, we extended the probe wavelengths to UV region from 360 to 266 nm and observed a series of striking features of transients. All these transients can be fit systematically only with the sequential model shown in Fig. 3.1 and the deconvoluted transients probed at 266, 270, 300, 335 and 620 nm are shown in A-E insets in Figs. 4.4-4.6. The final results are listed in Table 4.1 and the resulting reaction times of all elementary steps are also shown in Fig. 4.7A.

After charge separation, the anionic CPD could follow two routes: repair of the dimer by breaking of two C-C bonds and back ET to the original ground state without repair. Experimentally, we did not observe any signals of T$\ll$U, U$\ll$U, U$\ll$T at all wavelengths, indicating that the C5-C5’ bond breaks instantaneously, leading to the trace accumulation of CPD anions. Our fitting results give an upper limit of less than 10 ps for the C5-C5’ bond breaking, otherwise we should have observed noticeable signals of CPD anions in UV region. Thus, the splitting of the C5-C5’ bond has little activation barrier (Fig. 4.7B) and occurs ultrafast, consistent with recently extensive theoretical calculations. On the other hand, the back ET of these uracil-containing CPD anions without any bond breaking has a driving force around -2.0 eV$^{81,110}$ and should also occur within hundreds of ps in the Marcus inverted region, comparable with that T$\ll$T$\circ$. Thus,
the back ET from intact CPD anions could not compete with the C5-C5’ bond breaking, leading to the complete evolution along the first bond splitting.

At wavelengths shorter than 360 nm, we observed the anionic intermediates of T^-U, U^-U and U^-T (insets A, B and D of Figs. 4.4-4.6). Following the C5-C5’ bond breaking within a few picoseconds, the dynamics of T^-U, U^-U and U^-T decay in 64, 31 and 30 ps, representing the total dynamics (rates) of both the C6-C6’ bond breakage of anionic CPD intermediates and futile back ET after the C5-C5’ splitting. Given the splitting branching of T^-U, U^-U and U^-T as 0.94, 0.88 and 0.90 (Table 4.1), respectively, the T^-U takes the similar bond splitting time of 75 ps as T^-T in 90 ps, but the C6-C6’ bond in U^-U and U^-T splits in a much faster time scale of 35 ps. These splitting times are much longer than our recent theoretical values (less than 1 ps) of the C-C bond splitting in a CPD model system in bulk water. Clearly, the second C6-C6’ bond breaking has a longer time with T and a shorter time with U at the 5’ side. Thus, after the C5-C5’ bond breakage, the excess electron mainly remains at the 5’ side because the T moiety at the 5’ side with a methyl group at the C5 position can significantly stabilize the anionic radical and thus has a longer time of the C6-C6’ splitting. This observation also shows the electron tunneling toward the 5’ side of the dimer, which also must pass through the adenine moiety. According to the transition-state theory, we estimated the activation energy of ~0.174 eV (4.0 kcal/mol) for T^-T bond breaking, ~0.170 eV (3.9 kcal/mol) for T^-U bond splitting, and ~0.152 eV (3.5 kcal/mol) for U^-T and U^-U bond cleavage (Table 4.2). Thus, the stabilization in T^-T by the methyl group(s) at the C5 (and C5’) position leads to ~0.022 eV (0.5 kcal/mol) more for the
activation of the bond splitting than U'-U. On the other hand, before the C6-C6’ splitting, the futile back ET results in the intermediates to reclose the ring by formation of the C5-C5’ bond again and to return the original ground state (Fig. 4.7B). The splitting of C6-C6’ bond in the neutral ground state after the back ET is probably minor. The splitting of the CPD lesion on the neutral surface (Fig. 6B) is unlikely and the recent theoretical studies showed a free energy of larger than 1.7 eV required to lengthen and break the C5-C5’ bond.\textsuperscript{109,142} Thus, the free energy for back ET after the C5-C5’ breaking must be around -0.34 eV (Fig. 6B). Assuming the reorganization energy to be the similar as the forward ET, 1.21 eV, the back ET thus switches to the Marcus normal region (Fig. 4.7B). From the measured quantum yields (Table 4.1), we obtained the back ET time scales of 1175, 315 and 260 ps for T^-U, U^-T and U^-U, respectively, much shorter than that for T^-T in 2.4 ns. Thus, the back ET dynamics (rates) change in a decrease order of 5'-U'-U-3' > U^- -T > T^-U > T^-T (Fig. 6A). Using the same $\lambda$ and J values of 1.21 eV and 3 meV, respectively, we obtained the free energy of the back ET to be -0.22 eV for T-T', -0.29 eV for T^-U, -0.42 eV for U^-T, and -0.44 eV for U^-U (Table 4.2). Although thymine has a reduction potential lower than uracil by 0.11 V, which would lead to a larger driving force and a faster back ET, it seems that the methyl group at C5/C5’ position significantly stabilizes the anion intermediate, resulting in the less driving force and strategic slowdown of the back ET. With T at the 5’ side, the stabilization energy is about 0.15 eV by comparison of T^-U vs. U^-U, while T at the 3’ side the stabilization energy is about 0.02 eV from U^-T vs. U^-U, indicating that the electron is mainly localized at the 5’ side with a partial distribution on the 3’ side and the methyl group at the C5’ position.
has some stabilization effect. Our recent high-level calculation\textsuperscript{101,109} showed that the electron is delocalized between two bases and even solvated by neighboring water molecules. As shown above, the methyl group also stabilizes the transition-state free energy $\Delta G^\dagger$ of $T^-T$ by 0.02 eV, compared with $U^-U$, to slow down the C6-C6’ breaking. Thus, the methyl group(s) at the C5 (C5’) position slows down both rates of the bond splitting and futile back ET. However, the free energy due to the stabilization by the methyl groups in $T^-T$ is 0.22 eV higher than in $U^-U$, well exceeding its lower reduction potential, leading to a much longer back ET time (2.4 ns), and strongly favoring the splitting channel for a high repair quantum yield.

The total repair quantum yield is the product of two-step yields: forward ET branching and complete splitting branching. As shown in Table 4.1, the forward ET with T<>T has the longest time scale of 250 ps and thus has the smallest first-step branching of 0.85. However, due to the longest back ET time of 2.4 ns even with the longest C6-C6’ bond splitting time of 90 ps, the second-step splitting branching is highest, 0.96, among all four substrates. Thus, the highest second-step splitting branching compensates the lowest first-step forward ET branching, still leading to a high total quantum yield of 0.82. Similarly, for three other uracil-substituted substrates, the forward ET is faster and results in a larger first-step branching of 0.94-0.95, but the splitting branching becomes relatively smaller, 0.88-0.94, due to the faster back ET process. However, the total quantum yields are similar in the range of 0.84-0.88. Thus, for all four CPD substrates, the total quantum yields are optimized and all lies in the range of 0.82-0.88, a very high repair efficiency of close to 1 for the complex repair photomachinery.
4.3.3 Electron Return, Catalytic Restoration and Repair Photocycle

After a sequential breakage of both C-C bonds, the cyclobutane ring is repaired but the electron needs to return semiquinone FADH$^*$ to complete the photocycle and restore the catalytic state of FADH$. In UV region, we did observe the signal of T$^-$ and U$^-$ intermediates around 300 nm (inset C of Figs. 4.4-4.6) and products of T and U in various substrates (insets A-D of Figs. 4.4-4.6). The (T+U)$^-$ and (U+U)$^-$ have faster return dynamics in 185 and 210 ps than (T+T)$^-$ in 700 ps, while (U+T)$^-$ has the longest electron return time of 1220 ps. The electron-return dynamics are much slower than the ring splitting and thus both processes are decoupled. From the four measured electron-return times, the U at the 3’ side has faster charge recombination and the T at the 3’ side has slower electron-return dynamics. After the cyclobutane ring splits and is repaired, the electron mostly stays at the 3’ side. From the x-ray structure, several water molecules, polar/charged residues, and high reduction FADH$^*$ are all around the 3’ side, which probably stabilizes and solvates the electron.

Thus, the electron-return dynamics for the four substrates vary in a decrease order of $5’$-T+U$^-$-3’ > U+U$^-$ > T+T$^-$ > U+T$^-$ (Fig. 4.7A). Again, the direct electron hopping from T$^-$/U$^-$ to the adenine moiety is unlikely due to the positive free energy of +0.3/+0.4 eV. However, the back electron tunneling from T$^-$ at the 3’ side passing the adenine-mediated pathway again to final FADH$^*$ has a large driving force of -2.26 eV$^{81,85}$ (Table 4.2) and such tunneling is feasible in hundreds of ps and probably in the Marcus inverted region. Using Eq. 4.5 for the electron-return dynamics of T+T$^-$ and U+U$^-$ ($\Delta G$ around -2.15 eV) and by consideration of a smaller J and a larger $\lambda$ in the
electron return than in the forward ET, we obtained the electronic coupling and reorganization energy of 2.6 meV and 1.37 eV for return tunneling, and the free energy of -2.12 eV for U+U−. The reorganization energy is slightly larger than that of the forward ET (1.21 eV) because the electron return is in the Marcus inverted region (Fig. 6B) and the donor-acceptor pairs of both reactions are different, FADH−*/T<>T for the forward ET and T−/FADH* for the electron return. The smaller coupling value, compared with the forward ET (3.0 meV), indicates the electron tunneling favors the 5’ side over the 3’ side as observed. Also, the excess driving force of T− (the lower reduction potential) over U− slows down the tunneling rate. Using the obtained J and λ values, we obtained a smaller free energy of -2.10 eV for T+U− and a larger free energy of -2.32 eV for U+T− (Table 4.2). Comparing T+U− with U+U− and T+T− with U+T−, the U at the 5’ side slows down the electron return due to the strong electron affinity of U to hinder the electron tunneling back to FADH*, effectively, leading to more negative free energies, but in the inverted ET region.

The electron return within nanoseconds is necessary and essential to complete the photocycle for further DNA repair by photolyase. Also, the longtime stay of the extra electron in repaired DNA is harmful and could induce other new damage.143,144 All these dynamics of the elementary steps involved in photorepair machinery are well optimized by evolution. As shown in Fig. 3.6 for the complete photocycle and in Fig. 4.7 for all reaction dynamics and molecular mechanism, we completely mapped out the entire evolution of catalytic repair process. Photolyase uses a photon and an electron to repair damaged DNA with no net change in the redox state of the flavin cofactor through a
cyclic ET mechanism. Four elementary steps of forward ET, back ET, two C-C bond splitting, and electron return all occur ultrafast and in synergy to maximize the repair quantum yield. DNA is damaged by UV irradiation but repaired by photolyase with visible blue light. The cyclic ET between the enzyme and the damaged DNA catalyzes the repair reaction with an ultrafast photocycle.

4.4 Conclusions

We reported here our systematic characterization and analyses of three electron transfer processes and cyclobutane ring splitting involved in repair of damaged DNA by photolyase. With femtosecond resolution and (deoxy)uracil-substituted substrates of cyclobutane pyrimidine dimers at the 5’ and 3’ sides of DNA, we completely mapped out the entire dynamic evolution of repair processes for four different T<>T, T<>U, U<>T and U<>U combinations. We resolved all the dynamics of four elementary reactions of the forward ET with one electron injection from the cofactor into the substrate, sequential cyclobutane ring splitting of two single carbon-carbon bonds, futile back ET after the first carbon-carbon splitting to return to the original ground state by reclosure of the ring, and final electron return after the repair to the cofactor to restore the catalytic active state and complete the photocycle. These dynamics significantly depend on the locations of T and U at the 5’ or 3’ side of DNA and thus provide unique and strong evidence for determination of the electron tunneling pathways for the forward ET and final electron return. As shown in Fig. 4.8 for CPD photolyase and 6-4 photolyase (see chapter 5), the forward ET ends on the 5’ side of DNA with a hybrid of tunneling configurations. The
electron passes through the critical intervening adenine moiety of the unusual bent configuration of the cofactor flavin through a super-exchange mechanism. All atoms in the adenine-mediated pathway are in van der Waals contact with their neighboring ones, but the through-space tunneling pathway obviously has a vacuum-type gap, regardless of the distances, in both photolyases. For CPD photolyase, the electron return after the repair starts at the 3’ side, shifting from the 5’ side during splitting, and follows the reverse direction through the mediated adenine (Fig. 4.8). Both electron tunneling pathways have unique, high directionality.

We determined all time scales of four elementary steps. The forward ET occurs in tens to hundreds of picoseconds. The cyclobutane ring splitting in a sequential way is ultrafast within tens of picoseconds. The futile back ET to the original ground state without repair takes hundreds of picoseconds to nanoseconds. After repair, the electron takes the similar time scales to restore the initial catalytic active state of the cofactor flavin. Using the Marcus ET theory, we obtained all ET free energies, reorganization energies and electronic coupling constants for three ET processes. The forward and back ET is in the normal region and the electron return is in the inverted region. For the hybrid tunneling pathways mediated by the adenine moiety, the reorganization energies and electronic coupling constants are relatively large, 1.21-1.37 eV and 2.6-3.0 meV, respectively. Clearly, through evolution, the active-site configuration and electrostatics must enable the cyclobutane ring splitting to occur as fast as possible to exceed all other processes. The unique bent configuration of the cofactor assures the critical adenine position to mediate all three ET processes in hundreds of picoseconds to nanoseconds,
slower than the ring splitting, in a delicate balance of time scales. These ET times are not too slow to decrease the efficiency of the forward ET and not too fast to increase the futile back ET channel. Such structural integrity, unique electron tunneling pathways and the critical role of adenine ensure a high repair quantum yield in a synergistic way to optimize the forward ET, futile back ET and ring splitting in this complex photorepair machinery, and finally achieve a maximum repair efficiency close to unity.
Table 4.1 Results of reaction times, efficiencies of the elementary steps and overall repair quantum yields of various damaged substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>QY</th>
<th>β</th>
<th>$&lt;\tau_{\text{FET}}&gt;$</th>
<th>$B_{\text{FET}}$</th>
<th>$&lt;\tau_{\text{BET}}&gt;$</th>
<th>$&lt;\tau_{\text{SP}}&gt;$</th>
<th>$B_{\text{SP}}$</th>
<th>$&lt;\tau_{\text{ER}}&gt;$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;&gt;T</td>
<td>0.82</td>
<td>0.71</td>
<td>250</td>
<td>0.85</td>
<td>2400</td>
<td>90</td>
<td>0.96</td>
<td>700</td>
</tr>
<tr>
<td>T&lt;&gt;U</td>
<td>0.88</td>
<td>0.87</td>
<td>85</td>
<td>0.94</td>
<td>1175</td>
<td>75</td>
<td>0.94</td>
<td>185</td>
</tr>
<tr>
<td>U&lt;&gt;T</td>
<td>0.86</td>
<td>0.89</td>
<td>63</td>
<td>0.95</td>
<td>315</td>
<td>35</td>
<td>0.90</td>
<td>1220</td>
</tr>
<tr>
<td>U&lt;&gt;U</td>
<td>0.84</td>
<td>0.92</td>
<td>73</td>
<td>0.95</td>
<td>260</td>
<td>35</td>
<td>0.88</td>
<td>210</td>
</tr>
</tbody>
</table>

*All times are in units of picoseconds. Here β is the stretched parameter, and QY is the overall repair quantum yield of damaged substrates calculated by $QY = B_{\text{FET}} \times B_{\text{sp}}$ or by direct measurement. $^b<\tau_{\text{FET}}>$ is calculated from $[((\tau/\beta)\Gamma(1/\beta))^{-1} - (1/\tau_1)]^{-1}$. τ is the observed time scale of FADH$^*$ decay at 710 nm and $\tau_1$ is 1.3 ns determined in the absence of substrate. $^cB_{\text{FET}}$ and $B_{\text{sp}}$ are efficiencies of the forward ET and the ring splitting and calculated by $B_{\text{FET}} = (\tau_{\text{FET}})^{-1}/(\tau_{\text{FET}}^{-1} + \tau_1^{-1})$ and $B_{\text{sp}} = (\tau_{\text{sp}})^{-1}/(\tau_{\text{sp}}^{-1} + \langle \tau_{\text{BET}} \rangle^{-1})$, respectively.*
**Table 4.2 The derived free energies of all elementary steps in repair of various substrates**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\Delta G_{\text{FET}}$</th>
<th>$\Delta G_{\text{BET}}$</th>
<th>$\Delta G_{\text{BET}}^\dagger$</th>
<th>$\Delta G_{\text{ER}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;&gt;T</td>
<td>-0.44</td>
<td>-0.22</td>
<td>0.174</td>
<td>-2.26</td>
</tr>
<tr>
<td>T&lt;&gt;U</td>
<td>-0.57</td>
<td>-0.29</td>
<td>0.170</td>
<td>-2.10</td>
</tr>
<tr>
<td>U&lt;&gt;T</td>
<td>-0.61</td>
<td>-0.42</td>
<td>0.152</td>
<td>-2.32</td>
</tr>
<tr>
<td>U&lt;&gt;U</td>
<td>-0.59</td>
<td>-0.44</td>
<td>0.152</td>
<td>-2.12</td>
</tr>
</tbody>
</table>

All free energies are in units of eV. The reduction potentials of several involved species are $E(FADH^*/FADH^-) = +0.08 \text{ V vs NHE}$ in the presence of substrate binding to photolyase, $E(T/T^-) = -2.18 \text{ V}$, $E(U/U^-) = -2.07 \text{ V}$ and $E(T<>T/T<>T^-) = -1.96 \text{ V}$. The 2.48 eV for the $S1\leftrightarrow S0$ transition at 500 nm is used in calculation of $\Delta G_{\text{FET}}$. The pre-exponential factor A in calculation of $\Delta G_{\text{sp}}^\dagger$ from $k = A \exp(-\Delta G_{\text{sp}}^\dagger/(k_B T))$ is assumed to be $10^{13} \text{ s}^{-1}$. 
**Fig. 4.1** X-ray structure of the enzyme–substrate complex. A complex structure of *A. nidulans* photolyase (light-blue ribbon) containing the catalytic cofactor FADH⁻ (yellow stick) and duplex DNA (orange and pink ribbons) with a repaired CPD lesion (orange stick). A close-up view shows the relative positions of the catalytic cofactor FADH⁻ and the repaired substrate with various distances for two potential tunneling pathways. The adenine moiety of FADH⁻ was in van der Waals contact with both 5’- and 3’-sides of CPD.
Fig. 4.2 Chemical structures of various damaged substrates and flavin adenine dinucleotide.
Fig. 4.3 Determination of dissociation constants and repair quantum yields of various CPD substrates. (A) The relative repair quantum yields of T<>U, U<>T and U<>U to T<>T by photolyase by monitoring the formation of thymine/uracil bases using 266-nm absorption changes (inset) with 365-nm irradiation of the enzyme-substrate solution. With dissociation constants and known repair quantum yield of T<>T, the repair quantum yields of T<>U, U<>T and U<>U were obtained. (B) The dissociation constants of photolyase-CPD complexes were obtained by measuring the binding complexes relative to substrate concentrations and then fit using eq. 4.3.
Fig. 4.4 Femtosecond-resolved transient absorption dynamics of T<>U repair by photolyase at probe wavelengths from 710 to 266 nm. The transients probed at 266, 270, 300, 335 and 620 nm are deconvoluted in insets A-E, respectively. All transients were fitted by considering flavin-related species (dashed red in insets A-D and dashed pink and dark red in inset E), substrate intermediates of T -U (dashed blue) and U− (dashed dark green), and thymine/uracil products (dashed dark yellow).
Fig. 4.5 Femtosecond-resolved transient absorption dynamics of U<>U repair by photolyase at probe wavelengths from 710 to 266 nm. The transients probed at 266, 270, 300, 335 and 620 nm are deconvoluted in insets A-E, respectively. All transients were fitted by considering flavin-related species (dashed red in insets A-D and dashed pink and dark red in inset E), substrate intermediates of U–U (dashed blue) and U– (dashed dark green), and uracil products (dashed dark yellow).
Fig. 4.6 Femtosecond-resolved transient absorption dynamics of U<>T repair by photolyase at probe wavelengths from 710 to 266 nm. The transients probed at 266, 270, 300, 335 and 620 nm are deconvoluted in insets A-E, respectively. All transients were fitted by considering flavin-related species (dashed red in insets A-D and dashed pink and dark red in inset E), substrate intermediates of U–T (dashed blue) and T– (dashed dark green), and uracil/thymine products (dashed dark yellow).
Fig. 4.7 Reaction times and free energy diagrams of the elementary steps in splitting of CPD substrates. (A) Reaction times of each elementary step observed in repair of various substrates (T<>T, T<>U, U<>U and U<>T), including the forward ET, the C6-C6’ bond splitting, futile back ET and final electron return to complete the photocycle. Note the order in reaction times of three ET processes for four different substrates and also the faster bond splitting than the back ET and electron return. (B) The free energy profiles along the reaction coordinate after the forward ET in repair of CPD substrates with the time scales of the dynamics shown at the top. On the anionic surface, the solid curve represents the splitting of T<>T\(^-\) while the dashed curve for U<>U\(^-\). On the neutral surface, the bond-breaking activation barrier (dashed curve) is very high according to theoretical calculations. Note the different regions, normal or inverted, of three ET processes and the ring reclosure after the futile back ET.
Fig. 4.7 continued
Fig. 4.8 Active-site structures of enzyme-substrate complexes. (A) The relative positions of active cofactor FADH$^-$ and repaired substrate at CPD photolyase (*A. nidulans*). The red and golden arrows represent two electron tunneling pathways of the forward ET and electron return after repair, respectively. The gray arrows show the unfavorable through-space electron tunneling pathway. (B) The relative positions of FADH$^-$ and substrate 6-4 photoproduct of 6-4 photolyase (*A. thaliana*). Similarly, the red and dark-red arrows show the tunneling pathways of the forward and futile back ET between flavin and 6-4 photoproduct, similar to CPD photolyase. The through-space tunneling distance is 6.3 Å in gray arrows. (C) and (D), the space-filled representation of the structures in (A) and (B). The adenine-mediated tunneling pathways are completely filled with atom contacts while the through-space tunneling routes have gaps in the pathways, which decrease ET rates due to the weak electronic coupling.
CHAPTER 5

DYNAMICS AND MECHANISM OF REPAIR OF UV-INDUCED (6-4) PHOTOPRODUCT BY PHOTOLYASE
5.1 Introduction

The (6–4) photolyase contains a fully reduced flavin adenine dinucleotide (FADH⁻) as the catalytic cofactor (Figs 1.3). Upon excitation, the flavin cofactor donates an electron to the 6–4PP to generate a charge-separated radical pair (FADH⁺ + 6–4PP⁻).⁷,¹³,¹²⁰ However, if the C6–C4 bond in 6–4PP were split directly it would produce two damaged bases, because the hydroxyl group at the C5 of the 5’ pyrimidine cannot be transferred back to the 3’ pyrimidine (Fig. 1.3). In fact, (6–4) photolyase catalyzes this complex chemical reaction with no measurable error. Various hypothetical repair models,¹³,¹⁶,⁵⁶–⁶¹,¹⁴⁵ including formation of an oxetane intermediate before the photochemical reaction⁵⁶,⁵⁷ and, more recently, a model that includes formation of a water molecule following the primary photochemical reaction¹³,¹⁶, have been proposed to rationalize the bond breakages and group arrangements necessary for repair. Most models invoke proton transfer¹³,¹⁶,⁵⁶,¹⁴⁶ from a neighboring histidine residue, leading to a plausible scheme (Fig. 5.1).

5.2 Materials and methods

5.2.1 (6–4) photolyase and 6–4PP substrate

The purification of Arabidopsis thaliana (6–4) photolyase with an amino-terminal His tag has been described.¹⁴⁷,¹⁴⁸ We mutated His 364, a key amino acid for repair function, to polar Asn and Tyr, non-polar Met and Ala, acidic Asp and basic Lys for mutation studies. Mutant plasmids were constructed using QuikChange (Stratagene) based on the plasmid of wild-type enzyme. We sequenced the mutated DNA to ensure
that no additional mutation was introduced. In femtosecond-resolved studies, the protein concentrations were 300 μM for fluorescence up-conversion, 200 μM for transient absorption in the visible region and 50 μM in the ultraviolet region. The reaction buffer at pH 7.5 contains 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol and 50% (v/v) glycerol. The D₂O reaction buffer was prepared with 3 cycles of evaporating 1 ml reaction buffer to 0.5 ml and diluting it back to 1 ml using D₂O. Overall, more than 80% H₂O in the reaction buffer was exchanged to D₂O. We prepared the 6–4PP substrate as reported with some modifications. Briefly, we dissolved 5 mg d(GTATTATG) (synthesized by Integrated DNA Technologies) in 10 ml water, put it in a Petri dish at 4°C under argon in a sealed polyethylene pouch and irradiated it using two 254-nm germicidal lamps (General Electric, 15T8) at a 2-cm distance for 90 min. We purified the 6-4PP by high-performance liquid chromatography (HPLC), using a C18 reversed-phase column (Grace, 250 mm*10 mm) and a linear gradient of 8–9% (v/v) acetonitrile in 0.1 M triethylammonium acetate (pH 7.0) for 20 min at a flow rate of 4.5 ml/min. We selectively collected the 6–4PP by monitoring the absorbance at 260 nm and 325 nm. The final yield of 6–4PP is 0.5 mg, 10% of the starting material. The ratio of substrate to enzyme is 5:1 in all femtosecond resolved studies.

5.2.2 Enzyme activity

We measured enzyme activity as follows. We prepared a mixture of 1 μM enzyme with 50 μM 6–4PP in 200 μl reaction buffer at pH 7.5 (50 mM Tris-HCl, 100 mM NaCl, 10 mM dithiothreitol, 10% (v/v) glycerol) in a cuvette and then illuminated it
at room temperature (22 °C) using two white-light lamps (General Electric, F15T8) with a distance of 6 cm. We removed a 20-μl reaction sample every 30 min and heated it at 100 °C in darkness for 10 min. We removed the denatured protein by centrifuge at 14,000g for 10 min. We loaded the supernatant to an HPLC column and separated the repaired DNA using a C18 reversed-phase column (Alltech, 250 mm*4.6 mm) with the same solvent as in the purification of 6-4PP, except with an 8–12% (v/v) gradient. We integrated the curve of absorption at 260 nm against the HPLC retention time to determine the amount of 6-4PP that had been repaired. Plotted against the illumination time, the amount repaired increases linearly, and the slope is proportional to the repair quantum yield of the enzyme activity. We detected no repaired DNA in the substrate alone after white-light illumination. We measured the enzyme activity by monitoring the change in the absorption spectra (Fig. 5.2), following a reported method.\textsuperscript{148}

### 5.2.3 Femtosecond spectroscopy

We carried out all femtosecond-resolved measurements using the fluorescence up-conversion and transient absorption methods. The experimental layout has been reported.\textsuperscript{64} Briefly, for femtosecond-resolved fluorescence detection, we generated the pump wavelength at 400 nm by doubling 800 nm in a 0.2-mm-thick β-barium borate crystal (BBO, type I). We typically attenuated the pump pulse energy to 140–200 nJ before focusing it into the sample cell. We gated the resulting fluorescence from the sample using another 800-nm beam in a 0.2 mm BBO crystal to obtain fluorescence transients at desired wavelengths. For transient absorption measurements, we used optical
parametric amplifiers (OPA-800C and TOPAS, Spectra-Physics) to generate all desired probe wavelengths for different detection schemes. The spectral bandwidth is about 4 nm in the ultraviolet region. The instrument response time is about 500 fs for fluorescence detection and about 250 fs for transient absorption measurements. All experiments were done at the magic angle (54.7°). We kept samples stirring during irradiation to avoid heating and photobleaching. We carried out all enzyme reactions in the femtosecond-resolved measurements under anaerobic conditions.

5.3 Results and discussions

5.3.1 Electron injection into (6-4) photoproduct photolesion

To reveal the repair photocycle, we performed ultrafast spectroscopy to identify the reaction intermediates. We first characterized the initial ET by analyses of the excited FADH− dynamics using femtosecond fluorescence spectroscopy. By monitoring the weak FADH∗− emission at 550 nm (Fig. 5.3) we found that the fluorescence lifetime of FADH− in the absence of substrate is 3 ns but becomes drastically shorter in the enzyme-substrate complex (Fig. 5.4). The transient in the complex can be best represented by a stretched-single-exponential decay with τ=225 ps and β=0.8 (leading to an average ET time <τ_{FET}>=280 ps, see chapter 4), reflecting a heterogeneous ET dynamics7,94 which is continuously modulated by the active-site solvation as has been recently reported for (6-4) photolyase80 and also observed in photosynthesis96. These results were confirmed by transient absorption spectroscopy of the excited-state flavin dynamics (λ_{pr}=800 nm) as shown in Fig. 5.4. Importantly, with a series of replacement of the potential proton donor,
His364 in the active site, by charged (K and D), polar (N and Y) and hydrophobic (A and M) residues, we observed similar ET dynamics that follows a stretched-single-exponential decay with $\tau=147$-$281$ ps and $\beta=0.8$-$0.9$ (Table 5.1 and Fig. 5.5), leading to average forward ET time ($<\tau_{FET}>$) from 166 ps to 358 ps. This founding, along with the observation that the 64PP appears to be in its standard form in enzyme-complex x-ray structure$^{13}$, excludes the early repair model$^{56,57}$ that requires an oxetane precursor before the photochemical excitation. We conclude that cofactor FADH$^*$ in (6-4) photolyase is quenched by the 6-4PP substrate with an electron tunneling process in 280 ps (see chapter 4) and, after the electron injection, the 64PP repair takes place completely in the anionic ground state of 64PP$^*$. 

5.3.2 Proton transfer and back electron transfer

After the photoinduced charge separation (FADH$^*$+6-4PP$^*$), the reaction can evolve along two pathways: back ET (reaction rate constant $k_2$) or 6-4PP repair ($k_3$) (Fig. 5.1). Knowing the forward ET dynamics of FADH$^*$, we can map out the temporal evolution of FADH$^*$ by probing at wavelengths from 500 to 700 nm (Fig. 5.3) to follow 6-4PP repair. In Fig. 5.6A, the transient probed at 640 nm (red curve) shows drastically different behavior from that probed at 800 nm (blue curve), owing to the capture of the radical FADH$^*$ (green curve). We observed an apparent rise signal of FADH$^*$ in 51 ps (initial flat part of Fig. 5.6A inset) and a long plateau, indicating that complete 6-4PP repair takes longer than several nanoseconds. Because forward ET takes about 280 ps, the 51 ps is the overall decay time of the initially formed FADH$^*$ in Fig. 5.1 and dashed
purple curve in Fig. 5.6A) but appears as an apparent rise. Slower formation and faster decay result in apparent reverse kinetics and less FADH$^\bullet$ accumulation. The dynamics of the branched FADH$^\bullet$ in the repair channel (dashed light-blue curve in Fig. 5.6A) exhibit a complex formation (largely determined by the $k_1$ process) and a slow decay ($k_4$), but amplitude is mainly determined by the $k_3$ rate. By deconvolution (Appendix C), we measured average back ET in 57 ps ($k_2$) and repair in 481 ps ($k_3$), to form a 6–4PP$^\bullet$–anionic intermediate. From these rate constants we obtained a repair branching constant of 0.097, a value which is in excellent agreement with the reported steady-state-repair quantum yield of 0.1, suggesting that after the $k_3$ step, all subsequent reaction steps proceed to the final 6–4PP repair without any back ET that would lead to a futile cycle. These findings reveal that the underlying reason for the low repair quantum yield of (6–4) photolyase (0.1), compared to cyclobutane pyrimidine dimer photolyase (0.82), is the fast rate of back ET ($k_2 = 57$ ps) from 6–4PP$^\bullet$– to FADH$^\bullet$, relative to the parallel repair channel ($k_3 = 481$ ps), although the branchings of forward ET are quite high in both cases. For the series of mutants (His 364 Asn/Met/Tyr/Ala/Asp/Lys) used to test the proposed reaction mechanism, we observed that all transients probed in the 500–700 nm region show similar back-electron transfer dynamics in the range of 80–298 ps (Table 5.1 and Fig. 5.5), but decay to zero without any long plateaus (shown in Fig. 5.6B for the His 364 Asn mutant). This observation is critical to the proposed reaction scheme, as it indicates that in the mutants, even though ET from FADH$^\bullet$– to 6–4PP is essentially normal, the repair channel is completely shut off, and all FADH$^\bullet$ formed by initial charge separation follows a futile cycle back to FADH$^\bullet$– by charge recombination (Fig. 5.1).
These results are also consistent with our steady-state quantum yield measurements, which revealed a total lack of repair in any of the mutants (inset in Fig. 5.6B). Collectively, our data, in agreement with an earlier report\textsuperscript{57}, indicate that His 364 in the active site is a functional residue that is irreplaceable in the repair channel, and proton transfer from His 364 to 6–4PP is conceivably the rate-limiting step (\(k_3\)) in our reaction scheme (Fig. 5.1).

To confirm the proton transfer from H364, the repair reaction was carried out in D\(_2\)O. As shown in Fig. 5.6B for the wild-type photolyase, we observed a different transient with an obviously lower plateau, reflecting a slower repair process in 1240 ps (\(<\tau>\)) but with the similar forward (260 ps) and back (68 ps) ET dynamics as in H\(_2\)O. Thus, with the deuterated H364, the repair through the D\(^+\) transfer slows down by a factor of more than 2. Such a ratio of the rates (\(k_{H}/k_{D}\)) indicates no significant quantum tunneling effect\textsuperscript{150} for this PT process in photolyase. The x-ray structure shows a hydrogen-bond distance of 2.7 Å between the -NH of H364 and the -OH at the C5 position of the 5′ base (Fig. 1) and such a proton transfer is readily feasible. The lower plateau is about a half of that in H\(_2\)O and thus corresponds to a half decrease in the repair branching, in good agreement with the steady-state measured relative quantum yield of 1:2 in D\(_2\)O to H\(_2\)O (inset in Fig. 5.6B). We also studied both the repair dynamics and steady-state enzyme activity when the pH value was increased from 7 to 9 and did not observed any changes, consistent with the observation of H364 protonation even over a wide basic pH range.\textsuperscript{146} Thus, all these results reveal a key step of the proton transfer from H364 to 64PP\(^-\) between the enzyme and transient anionic substrate to generate a
protonated neutral radical 64PPH\(^\bullet\) in the repair pathway (Fig. 5.1). This critical proton transfer, induced by the initial forward electron transfer, completely eliminates the further futile back ET channel and more importantly, catalyzes subsequent repair reactions with high efficiency of nearly 100%.

We further examined the repair processes by detection of the 64PP-related species and the recovery of FADH\(^-\) in UV region. The typical transients are shown in Fig. 5.7 and are superposition of all species absorbing UV light. Because the H364N mutant exhibits no repair function and only follows a futile forward and back ET cycle, we were able to uniquely determine the absorption coefficient of the FADH\(^-\)^\(^*\) with wavelength (Fig. 5.3). By knowing all absorption coefficients of flavins (FADH\(^-\), FADH\(^-\)^\(^*\) and FADH\(^\bullet\)) and 64PP, and their related dynamics obtained by visible-light probing presented above, we obtained a transient dynamics of the 64PP intermediate (Figs. 5.7A and 5.7B) formed in about 481 ps and with the deuterated H364 in about 1240 ps and determined its absorption coefficient with a peak around 325 nm (Fig. 5.3). Thus, we detected the repair process and captured the protonated neutral radical 64PPH\(^\bullet\). After protonation, the intermediate radical decays in nanoseconds, corresponding to a series of atom arrangements with bond breaking and making for the complete 64PP repair on the time scale of tens of nanoseconds.

5.3.3 Repair efficiency and reaction photocycle

On the basis of these findings and previous data, including the crystal structure of the enzyme–substrate complex,\(^{13}\) a catalytic photocycle for the repair of thymine (6–4)
photoproduct was proposed (Fig. 5.8). In this scheme, the primary reactions are the initial electron transfer (I to II in Fig. 5.8) and the subsequent proton transfers (II to III in Fig. 5.8). The electron-transfer-induced transfer of a proton from a His residue in photolyase to the 6–4PP is a key step in the repair photocycle, like the ‘dividing line’ in the transition state, and makes the subsequent reactions ‘downhill’ without the possibility of back reaction. This critical step competes with the back electron transfer resulting in an overall repair quantum yield of about 0.1, which is probably the maximum value that could be achieved for such a structurally and chemically challenging reaction. The successive elementary steps naturally proceed to an intramolecular proton transfer from the hydroxyl group on the C5 of the 5′ base to the N3 at the 3′ base\textsuperscript{13,151} to form a transient zwitterion, and then an oxygen-atom attack of the C4 position at the 3′ base to form a transient oxetane-type structure (III in Fig. 5.8). The transient-water-molecule-formation model,\textsuperscript{13,16} which proposes direct breakage of the C–O bond at the 5′ base after the initial proton transfer, seems unlikely because it necessitates a series of proton transfer reactions, including the protonation of the carbonyl group at the 3′ base, and there are no potential proton donors in proximity to this carbonyl group. In addition, any interruption in such a complicated scheme proposed by this water model would be expected to give rise to damaged DNA at a significant rate; this is not observed in the repair reaction by (6–4) photolyase. In our scheme, in which a simple transient-oxetane formation facilitates the oxygen-atom transfer from the 5′ to 3′ base followed by C6–C4 bond split (IV in Fig. 5.8), would be less prone to mutagenic side reactions. This is because, after oxygen-atom transfer and C–C bond cleavage, the proton returns to the essential His 364 residue, and
the electron returns to FADH\(^*\), restoring the enzyme to its active form and the 6–4PP to two thymine bases (V in Fig. 5.8).
Table 5.1 The fitting time constants of all dynamic transients of 6-4PP repair.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>(\tau_{\text{lifetime}})</th>
<th>(&lt;\tau_{\text{FET}}&gt;)</th>
<th>(&lt;\tau_{\text{BET}}&gt;)</th>
<th>(&lt;\tau_{\text{PT}}&gt;)</th>
<th>(&lt;\tau_{\text{PR&amp;ER}}}&gt;)</th>
<th>(\beta^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT(H(_2)O)</td>
<td>3116</td>
<td>280</td>
<td>57</td>
<td>481</td>
<td>&gt;10000</td>
<td>0.8</td>
</tr>
<tr>
<td>WT(D(_2)O)</td>
<td>3159</td>
<td>260</td>
<td>68</td>
<td>1240</td>
<td>&gt;10000</td>
<td>0.8</td>
</tr>
<tr>
<td>H364N</td>
<td>2334</td>
<td>166</td>
<td>105</td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>H364M</td>
<td>2558</td>
<td>293</td>
<td>103</td>
<td></td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>H364Y</td>
<td>1885</td>
<td>332</td>
<td>138</td>
<td></td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>H364A</td>
<td>1699</td>
<td>348</td>
<td>113</td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td>H364D</td>
<td>4128</td>
<td>341</td>
<td>298</td>
<td></td>
<td></td>
<td>0.8</td>
</tr>
<tr>
<td>H364K</td>
<td>2846</td>
<td>358</td>
<td>80</td>
<td></td>
<td></td>
<td>0.8</td>
</tr>
</tbody>
</table>

\(^a\)All time constants are given in picoseconds. FET: forward electron transfer from FADH\(^{-}\) to 64PP; PT: proton transfer from H364 to 64PP; BET: back electron transfer from 64PP\(^{-}\) to FADH\(^{+}\); PR&ER: proton and electron returns after repair.

\(^b\)All the time constants except \(\tau_{\text{lifetime}}\) were obtained by fitting the transients with a stretched exponential model. The average time \(<\tau>\) used in text are calculated from \((\tau/\beta)\Gamma(1/\beta))^{-1}\) except that \(<\tau_{\text{FET}}>)=[((\tau_{\text{FET}}/\beta)\Gamma(1/\beta))^{-1}-(1/\tau_{\text{lifetime}})]^{-1}\).
Fig. 5.1 Proposed repair scheme of (6-4)PP. The proposed repair scheme includes forward electron transfer (FET, reaction rate $k_1$) after light excitation, back electron transfer (BET, reaction rate $k_2$) without repair, and the repair channel, comprising probable initial proton transfer (PT, reaction rate $k_3$) and late proton and electron return (PR and ER, reaction rate $k_4$) after repair.
Fig. 5.2 Changes of absorption spectra with time when the 64PP is repaired by (6–4) photolyase under white light. The thymine restoration was detected by the increase in absorption at 260 nm and by the decrease in absorption at 325 nm.
Fig. 5.3 Absorption spectra and coefficients of purified protein with FADH\textsuperscript{+}, converted active form FADH\textsuperscript{−}, damaged 64PP, and FADH\textsuperscript{−*}. Also determined is a 64PP-related reaction intermediate (Inter.) around 325 nm in Fig. 5.7. The fluorescence emission of FADH\textsuperscript{−*} is shown in inset with an arrow indicating the gated wavelength.
Fig. 5.4 Normalized signals detected by both fluorescence (gated around the emission peak of 550 nm) and absorption (probed at 800 nm) methods with and without the substrate in the active site show the same lifetime and forward ET decays. The ET dynamics is best represented by a stretched-single-exponential decay.
**Fig. 5.5** Femtosecond-resolved dynamics of FADH$^+$ in the (6-4) photoproduction repair by (6-4) photolyase. Transient absorption signals of H364N/M/Y/A/D/K were obtained at the probing wavelength of 800 nm.
Fig. 5.6 Femtosecond-resolved absorption dynamics of flavin species involved in the 6-4PP repair. (A) Transient absorption signal probed at 640 nm with both FADH$^+$ (blue curve) and FADH$^\cdot$ detection (green). The total FADH signal is from the two contributions of the initially formed one (dashed purple; $k_1$ formation and $k_2$+$k_3$ decay in Fig. 1) and the branched one in the repair channel (dashed cyan; $k_3$ formation and $k_4$ decay). Note the flat signal in tens of picosecond shown in inset, reflecting a fast apparent rise signal. (B) Transient absorption signals probed at 640 nm of the mutant H364N and the wild-type enzyme in D$_2$O compared with the wild type in H$_2$O, all showing the similar initial flat signals. But, for H364N the signal decays to zero and in D$_2$O the signal drops to about a half of the original plateau in H$_2$O. The corresponding relative steady-state quantum yield measurements are shown in inset.
Fig. 5.7 Femtosecond-resolved transient absorption dynamics of various species involved in the damaged DNA repair probed in UV region at 325 nm (a) and 315 nm (b) for the mutant H364N and the wild type in D₂O and H₂O. The mutant signals decay to zero, showing no repair with a futile ET cycle. The signals in the wild type include three contributions of overall flavin species (FADH⁻, FADH⁻* and FADH⁺), 64PP and a captured intermediate shown in insets. The determined absorption coefficients of FADH⁻* and the intermediate are shown in Fig. 5.2.
Fig. 5.8 Repair photocycle of (6-4) thymine photoproduct by (6-4) photolyase. The resolved elementary steps include a forward electron transfer in 225 ps upon excitation, a back electron transfer in 50 ps without any repair, and a parallel, catalytic proton transfer between the enzyme (H364) and the substrate, induced by the initial electron transfer, in 425 ps. This proton transfer is the determinant in the repair and determines the overall repair quantum yield. The subsequent repair reactions involve a series of atom arrangements with bond braking and making and final proton and electron returns (to H364 residue and flavin cofactor) to convert the 64PP to two thymine bases on the time scales of longer than ten nanoseconds.
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Appendix A: Kinetic data analyses of intramolecular ET within catalytic cofactor of photolyase

The proposed mechanism of intramolecular ET of FAD in various redox states is shown in Fig. A.1. In oxidized state (FAD_{ox}), if we assume the concentration of flavin excited is n_0, we can obtain the following equations according to the reaction scheme.

\[
[Lf^*]_{t=0} = n_0
\]  \hspace{1cm} [A.1]

\[
\frac{d[Lf^*(t)]}{dt} = -(k_{2,1} + k_{\text{lifetime}})[Lf^*(t)]
\]  \hspace{1cm} [A.2]

\[
\frac{d[Lf^{**}(t)]}{dt} = \frac{d[Ade^{**}(t)]}{dt} = k_{2,1}[Lf^*(t)] - k_{1,2}[Lf^{**}(t)]
\]  \hspace{1cm} [A.3]

\[
[Lf(t)] = n_0 - [Lf^*(t)] - [Lf^{**}(t)]
\]  \hspace{1cm} [A.4]

At 800, 630, and 580 nm, only Lf^* and Ade^{**} can be probed (Lf^* also has stimulated emission which cause negative signal at 580 nm). If assuming a concentration of n_0 oxidized lumiflavin is excited, we can unambiguously solve the dynamics of charge separation and recombination while considering:

\[
\Delta A(t) \propto (\varepsilon_{FAD^*_{abs}} - \varepsilon_{FAD^*_{emi}})[Lf^*(t)] + \varepsilon_{Ade^*}[Ade^*(t)]
\]  \hspace{1cm} [A.5]

Similarly, we can give out the kinetic equation of neutral semiquinoid FADH^* as follows:
\[ [\text{LfH}^*]_{t=0} = n_0 \]  

\[ [\text{LfH}^- (t)] = [\text{Ade}^{**} (t)] \]  

\[ \frac{d[\text{LfH}^* (t)]}{dt} = -(k_{4.1} + k_{\text{lifetime}})[\text{LfH}^* (t)] \]  

\[ \frac{d[\text{LfH}^- (t)]}{dt} = \frac{d[\text{Ade}^{**} (t)]}{dt} = k_{3.1}[\text{LfH}^* (t)] - k_{1.3}[\text{Ade}^{**} (t)] \]  

\[ [\text{LfH}^* (t)] = n_0 - [\text{LfH}^* (t)] - [\text{LfH}^- (t)] \]  

At 800 nm, only \( \text{LfH}^* \) and \( \text{Ade}^{**} \) have absorption signals, therefore this case is similar to the oxidized state.

\[ \Delta A(t) \propto \epsilon_{\text{LfH}^*}[\text{LfH}^* (t)] + \epsilon_{\text{Ade}^{**}}[\text{Ade}^{**} (t)] \]  

According to equation A.7 and A.10, we need to rewrite eq. A.11 as:

\[ \Delta A(t) \propto \epsilon_{\text{LfH}^*}[\text{LfH}^* (t)] + \epsilon_{\text{Ade}^{**}}[\text{Ade}^{**} (t)] + \epsilon_{\text{LfH}^-}([\text{LfH}^* (t)] - n_0) \]

\[ = \epsilon_{\text{LfH}^*}[\text{LfH}^* (t)] + \epsilon_{\text{Ade}^{**}}[\text{Ade}^{**} (t)] + \epsilon_{\text{LfH}^-}(n_0 - [\text{LfH}^* (t)] - [\text{LfH}^- (t)] - n_0) \]

\[ = (\epsilon_{\text{LfH}^*} - \epsilon_{\text{LfH}^-})[\text{LfH}^* (t)] + (\epsilon_{\text{Ade}^{**}} - \epsilon_{\text{LfH}^-})[\text{LfH}^- (t)] \]  

Therefore, the total absorbance signal can be sorted in two categories: one is related to the dynamics of LfH* but the absorption coefficient is proportional to the difference absorption coefficients between LfH* excited state and LfH* ground state. In addition, the second component is related to the dynamics of charge-separated intermediate Ade**(LfH−) but the intensity is proportional to the difference coefficient.
between Ade*+ and LfH*. By analyzing the kinetic data is this way, we can separate the relatively small signal of intermediate states from excited state and ground state.

If the FAD in at anionic semiquinone (FAD*) or at anionic hydroquinone state (FADH−), the intramolecular ET can also occur between lumiflavin moiety and adenine moiety. However, the adenine moiety acts as the electron acceptor in these cases. For FAD*− state, we can give out equations as follows:

\[
[Lf^{*-*}](t=0)=n_0
\]

\[
[Lf(t)]=[Ade^{*-} (t)]
\]

\[
\frac{d[Lf^{*-*} (t)]}{dt}=-(k_{3,1}+k_{\text{lifetime3}})[Lf^{*-*} (t)]
\]

\[
\frac{d[Lf(t)]}{dt} = \frac{d[Ade^{*-} (t)]}{dt} = k_{3,1}[Lf^{*-*} (t)] - k_{1,2}[Ade^{*-} (t)]
\]

\[
[Lf^{*-*} (t)]=n_0 - [Lf^{*-*} (t)] - [Lf(t)]
\]

Only Lf*− absorbs at 650 nm, we then can determined the dynamics of charge separation between Lf*− and Ade. At 350 nm and 348 nm, Lf and Lf*− have absorption and we also expect that adenine intermediate Ade*− possibly absorbs at these wavelengths. According to equation A.14 and A.17, we obtained:

\[
\Delta \lambda(t) \propto \epsilon_{L} [Lf^{*-*} (t)] + \epsilon_{Ade} [Ade^{*-} (t)] + \epsilon_{Lf} [Lf(t)] + \epsilon_{Lf^{*-*}} ([Lf^{*-*} (t)] - n_0)
\]

\[
= \epsilon_{L} [Lf^{*-*} (t)] + (\epsilon_{Ade} + \epsilon_{Lf}) [Lf(t)] + \epsilon_{Lf^{*-*}} (n_0 - [Lf(t)] - [Lf^{*-*} (t)] - n_0)
\]

\[
= (\epsilon_{L} - \epsilon_{L}) [Lf^{*-*} (t)] + (\epsilon_{Ade} + \epsilon_{Lf} - \epsilon_{Lf^{*-*}}) [Lf(t)]
\]
As in anionic semiquinoid state, the total absorbance signal can be sorted in two categories: one is related to the dynamics of Lf\(^{•-}\)\(^\ast\) but the absorption coefficient is proportional to the difference absorption coefficients between Lf\(^{•-}\)\(^\ast\) excited state and Lf\(^{•-}\) ground state. In addition, the second component is related to the dynamics of charge-separated intermediate Ade\(^{•-}\) (Lf) but the intensity is proportional to the difference coefficient of the sum of Ade\(^{•-}\) and Lf minus Lf\(^{•-}\).

Lastly, we may also obtain the dynamics of intramolecular ET at anionic hydroquinoid state (FADH\(^{−}\)) by fitting the following equations:

\[
[LfH^{•-}]_{t=0}=n_0
\]

\[
[LfH^{•}(t)]=[Ade^{•-}(t)]
\]

\[
[Ade(t)]=[LfH^{•-}(t)]+[LfH^{•-}(t)]
\]

\[
\frac{d[LfH^{•-}(t)]}{dt}=-(k_{5,1}+k_{\text{fittime2}})[LfH^{•-}(t)]
\]

\[
\frac{d[LfH^{•}(t)]}{dt}=\frac{d[Ade^{•-}(t)]}{dt}=k_{5,1}[LfH^{•-}(t)]-k_{1,5}[Ade^{•-}(t)]
\]

\[
[LfH^{−}(t)]=n_0-[LfH^{•−}(t)]-[LfH^{•}(t)]
\]

Only LfH\(^{−}\)\(^\ast\) absorbs at 800 nm, which provides information about forward ET. At 270 nm and 269 nm, LfH\(^\ast\), LfH\(^−\), and Ade have absorption and we also expect that adenine intermediate Ade\(^{•-}\) possibly absorbs at these wavelengths. According to equation A.20, A.21 and A.24, we obtained:
As anionic hydroquinoid state, the total absorbance signal can be sorted in two categories: one is related to the dynamics of LfH$^+$ but the absorption coefficient is proportional to the difference absorption coefficients between LfH$^{-}\ast$ excited state and LfH$^-$ ground state. In addition, the second component is related to the dynamics of charge-separated intermediate Ade$^\ast$ (LfH$^\ast$) but the intensity is proportional to the difference coefficient of the sum of Ade$^\ast$ and LfH$^\ast$ minus the sum of Ade and LfH$^-$. 

\[
\Delta A(t) \propto \varepsilon_{LfH^-}(t) + \varepsilon_{Ade^-}(t) + \varepsilon_{LfH^\ast}(t) + \varepsilon_{He^\ast}(t) + \varepsilon_{Ade^\ast}(t) + \varepsilon_{LfH^\ast}(t)
\]

\[
\Delta A(t) = \varepsilon_{LfH^-}(t) + \varepsilon_{Ade^-}(t) + \varepsilon_{LfH^\ast}(t) + \varepsilon_{He^\ast}(t) + \varepsilon_{Ade^\ast}(t) + \varepsilon_{LfH^\ast}(t)
\]

\[
\Delta A(t) = \varepsilon_{LfH^-}(t) + \varepsilon_{Ade^-}(t) + \varepsilon_{LfH^\ast}(t) + \varepsilon_{He^\ast}(t) + \varepsilon_{Ade^\ast}(t) + \varepsilon_{LfH^\ast}(t)
\]

\[
\Delta A(t) = \varepsilon_{LfH^-}(t) + \varepsilon_{Ade^-}(t) + \varepsilon_{LfH^\ast}(t) + \varepsilon_{He^\ast}(t) + \varepsilon_{Ade^\ast}(t) + \varepsilon_{LfH^\ast}(t)
\]

[\ref{A.25}]
Fig. A.1 The proposed mechanisms of intramolecular ET between the lumiflavin and adenine moieties of photolyase in the four different redox states.
Appendix B: Kinetic data analyses of CPD repair by photolyase

The scheme of CPD repair by photolyase, is shown in Fig. 3.1. Assuming that the enzyme-substrate complex concentration involved in reaction is \( n_0 \) upon excitation at time zero, the dynamics of each step involved in the entire reaction will be solved by fitting the following equations according to the reaction scheme:

\[
[FADH^-]_{t=0} = n_0 \quad [B.1]
\]

\[
\frac{d[FADH^-](t)}{dt} = -(k_{FET} + k_{LT})[FADH^-](t) \quad [B.2]
\]

\[
\frac{d[T<>T^-](t)}{dt} = k_{FET}[FADH^-](t) - k_{sp1}[T<>T^-](t) - k_{BET}[T<>T^-](t) \quad [B.3]
\]

\[
\frac{d[T-T^-](t)}{dt} = k_{sp1}[T<>T^-](t) - k_{sp2}[T-T^-](t) - k_{BET}[T-T^-](t) \quad [B.4]
\]

\[
\frac{d[T^-](t)}{dt} = k_{sp2}[T-T^-](t) - k_{ER}[T^-](t) \quad [B.5]
\]

\[
\frac{d[T](t)}{dt} = k_{sp2}[T-T^-](t) + k_{ER}[T^-](t) \quad [B.6]
\]

\[
[FADH^+(t)] = [T<>T^-](t) + [T-T^-](t) + [T^-](t) \quad [B.7]
\]

\[
[FADH^-](t) = n_0 - [FADH^+(t)] - [FADH^+(t)] \quad [B.8]
\]
Before pump excitation, the \([\text{FADH}^-]\) is \(n_0\) and the concentrations of \(\text{FADH}^*\), \(\text{FADH}'\), \(T<>T^-\), \(T^-T^-\), \(T^-\) and \(T\) are zero. According to the Beer-Lambert law, the signal of femtosecond transient absorption at any wavelength can be acquired as:

\[
\Delta A(t) \propto \varepsilon_{\text{FADH}^-} ([\text{FADH}^-^*(t)] - [\text{FADH}^-^*]_{t=0}) + \varepsilon_{\text{FADH}^*} ([\text{FADH}^*(t)] - [\text{FADH}^*]_{t=0}) \\
+ \varepsilon_{\text{FADH}^-} ([\text{FADH}^-^-(t)] - [\text{FADH}^-^-]_{t=0}) + \varepsilon_{T<>T^-} ([T<>T^-^-(t)] - [T<>T^-^-]_{t=0}) \\
+ \varepsilon_{T^-T^-} ([T^-T^-^-(t)] - [T^-T^-^-]_{t=0}) + \varepsilon_T ([T^-^-(t)] - [T^-^-]_{t=0}) \\
\propto \varepsilon_{\text{FADH}^-} [\text{FADH}^-^*(t)] + \varepsilon_{\text{FADH}^*} [\text{FADH}^*(t)] + \varepsilon_{\text{FADH}^-} ([\text{FADH}^-^-(t)] - n_0) \\
+ \varepsilon_{T<>T^-} [T<>T^-^-(t)] + \varepsilon_{T^-T^-} [T^-T^-^-(t)] + \varepsilon_T [T^-^-(t)] + \varepsilon_T [T^-^-] \\
\]  

[B.9]

We observed \(\text{FADH}^*\) at all wavelengths, while \(\text{FADH}'\) signal was probed from wavelengths of shorter than 620 nm. The \(\text{FADH}^-\) signal is captured from 500 to 260 nm. All thymine-related intermediates, which have absorption in UV region, were detected below 360 nm. The thymine product was detected clearly below 300 nm in absorption transients. There is no \(T<>T^-\) absorption, which is mainly shorter than 250 nm, at all wavelengths we probed, and thus no any signal contains the \(T<>T^-\) contribution.

In addition, the decay of \(\text{FADH}^*\) is controlled by both lifetime emission (\(k_{LT}\)) and forward ET, thus we observed a total rate of excited-state decay as \(k = k_{LT} + k_{FET}\) at all wavelengths. The forward ET rate was then deconvoluted from the fitting results. Also, according to equation B.7, the concentration of \(\text{FADH}^*\) is equal to the sum of three thymine-related intermediates. Since the \(T<>T^-\) has trace amount accumulation due to the ultrafast \(k_{sp1}\), the sum of signal of \(T^-T^-\) and \(T^-\) will be almost identical to that of \(\text{FADH}'\). Due to the fact that we do not have any absorption spectra of \(T^-T^-\) and \(T^-\) in protein environment, it is not easy to differentiate the total signal of \(T^-T^-\) and \(T^-\) intermediates.
from that of FADH'. Instead, only the species with the stronger absorption, T-T− or T−, can be usually obtained as shown in the inset of Fig. 3.3A. This is also the reason that only one intermediate, either T-T− or T−, shows the absorption spectrum in the UV region from 260 to 360 nm. Thus, the results of T-T− and T− are not the true absorption spectra and only represent the relative absorption between two intermediates. Their magnitudes were calibrated by comparison with the known flavin ground-state absorption.
Appendix C: Kinetic data analyses of 6–4PP repair by (6-4) photolyase

The scheme of 6–4PP repair by (6-4) photolyase shown below is slightly modified from Fig. 5.1 (Fig. C.1).

Assuming that the flavin concentration involved in reaction is \( n_0 \), after pump excitation at time zero the concentration at time \( t \) is solved as follows.

\[
\frac{d[FADH^+ (t)]}{dt} = -k_1 t, \quad [FADH^+]_{t=0} = n_0
\]

\[
[FADH^+ (t)] = n_0 e^{-k_1 t}
\]

However, as we observed in our previous studies of the CPD repair by CPD photolyase\(^7\), the kinetics of FADH\(^+\) is best represented by a stretched single-exponential decay, \( n_0 e^{-(k_1 t)^\beta} \). The observed heterogeneous dynamics result from the continuous modulation by the active-site motion. Thus, the dynamics of all the species in Fig C.1 can be modified to follow the stretched model by adding an exponential index \( \beta \) for all time constants \( t \).

\[
\frac{d[FADH^+ (t)]}{dt} = -k_1 t, \quad [FADH^+]_{t=0} = n_0
\]

\[
[FADH^+ (t)] = n_0 e^{-k_1 t}
\]

\[
\Rightarrow n_0 e^{-(k_1 t)^\beta}
\]

\[\text{[C.1]}\]
\[
\frac{d[FADH^*_{\text{initial}}(t)]}{dt} = k_1[FADH^*(t)] - (k_2 + k_3)[FADH^*_{\text{initial}}(t)],
\]

\[
[FADH^*_{\text{initial}}]_{t=0} = 0,
\]

\[
[FADH^*_{\text{initial}}(t)] = \frac{n_0 k_i}{k_2 + k_3 - k_1} \left[ e^{-k_i t} - e^{-(k_2 + k_3)t} \right]
\]

\[
\Rightarrow \frac{n_0 k_i^\beta}{k_2^\beta + k_3^\beta - k_1^\beta} \left[ e^{-(k_i^\beta + k_i^\beta)t} - e^{-(k_2^\beta + k_i^\beta)t} \right]
\]

\[
\frac{d[FADH^*_{\text{branched}}(t)]}{dt} = k_3[FADH^*_{\text{initial}}(t)] - k_4[FADH^*_{\text{branched}}(t)],
\]

\[
[FADH^*_{\text{branched}}]_{t=0} = 0,
\]

\[
[FADH^*_{\text{branched}}(t)] = \frac{n_0 k_i k_3}{(k_2 + k_3 - k_1)(k_4 - k_1)} e^{-k_i t}
\]

\[+ \frac{n_0 k_i k_3}{(k_2 + k_3 - k_1)(k_2 + k_3 - k_4)} e^{-(k_2 + k_3)t} \]

\[\quad - \frac{n_0 k_i k_3}{(k_4 - k_1)(k_2 + k_3 - k_4)} e^{-k_i t} \]

\[
\Rightarrow \frac{n_0 k_i^\beta k_3^\beta}{(k_2^\beta + k_3^\beta - k_1^\beta)(k_4^\beta - k_1^\beta)} e^{-(k_i^\beta + k_i^\beta)t}
\]

\[+ \frac{n_0 k_i^\beta k_3^\beta}{(k_2^\beta + k_3^\beta - k_1^\beta)(k_2^\beta + k_3^\beta - k_4^\beta)} e^{-(k_2^\beta + k_i^\beta)t} \]

\[\quad - \frac{n_0 k_i^\beta k_3^\beta}{(k_4^\beta - k_1^\beta)(k_2^\beta + k_3^\beta - k_4^\beta)} e^{-(k_i^\beta + k_i^\beta)t} \]

\[\text{[C.2]}\]

\[
The [FADH'(t)] was obtained by subtracting [FADH^*(t)] and [FADH^*'(t)] from the total flavin concentration of \(n_0\).
\]

\[
[FADH'(t)] = n_0 - [FADH^*(t)] - [FADH^*_{\text{initial}}(t)] - [FADH^*_{\text{branched}}(t)] \quad \text{[C.4]}
\]
The decay of 64PP follows the kinetics of FADH, and the formation of 64PP follows the kinetics of the initial part of FADH, which is formed by BET (See Fig. C.1).

\[
\frac{d[FADH_{\text{initial}}(t)]}{dt} = k_2[FADH_{\text{initial}}(t)], \quad [FADH_{\text{initial}}]_{t=0} = 0
\]

\[
[FADH_{\text{initial}}(t)] = \frac{n_0k_3}{(k_2 + k_3 - k_4)}(1 - e^{-k_4t})
\]

\[
- \frac{n_0k_4k_3}{(k_2 + k_3 - k_1)(k_2 + k_3)}(1 - e^{-(k_2 + k_3)t})
\]

\[
\Rightarrow \frac{n_0k_3^{\beta}}{(k_2^{\beta} + k_3^{\beta} - k_4^{\beta})}(1 - e^{-(k_2^{\beta} + k_3^{\beta})t})
\]

\[
- \frac{n_0k_4^{\beta}k_3^{\beta}}{(k_2^{\beta} + k_3^{\beta} - k_1^{\beta})(k_2^{\beta} + k_3^{\beta})}(1 - e^{-(k_2^{\beta} + k_3^{\beta})t})
\]

\[
[64PP(t)] = [64PP(t)_{\text{formation}}] + [64PP(t)_{\text{decay}}] = [FADH_{\text{initial}}(t)] + [FADH^{\prime}(t)] \tag{C.5}
\]

The 64PPH, the intermediate of 64PP after the PT, is formed with the same kinetics of FADH branched, but could has an independent decay rate, which is assumed to be \(k_3\) here (See the Fig. C.1).
\[
\frac{d[64PPH'(t)]}{dt} = k_3[FADH'\text{initial}(t)] - k_5[64PPH'(t)] , \quad [64PPH']_{t=0} = 0
\]

\[
[64PPH'(t)] = \frac{n_0k_1k_3}{(k_2 + k_3 - k_5^\beta)(k_5^\beta - k_1^\beta)} e^{-k_5^\beta t} + \frac{n_0k_1k_3}{(k_2 + k_3 - k_5^\beta)(k_2 + k_3 - k_5^\beta)} e^{-(k_2 + k_5^\beta)t} - \frac{n_0k_1k_3}{(k_5^\beta - k_1^\beta)(k_2 + k_3 - k_5^\beta)} e^{-k_1^\beta t}
\]

\[
\Rightarrow \frac{n_0k_1^\beta k_3^\beta}{(k_2^\beta + k_3^\beta - k_1^\beta)(k_5^\beta - k_1^\beta)} e^{-(k_2^\beta + k_3^\beta)t^\beta} + \frac{n_0k_1^\beta k_3^\beta}{(k_2^\beta + k_3^\beta - k_1^\beta)(k_5^\beta - k_3^\beta - k_5^\beta)} e^{-(k_2^\beta + k_3^\beta)t^\beta} \]

To obtain the time constants in the repair scheme, a series of experiments were carried out to probe different species with a wide range of probing wavelengths.

(a) FADH\textsuperscript{−} detection

At 800 nm, only the FADH\textsuperscript{−} has absorption. Before pump excitation the [FADH\textsuperscript{−}] is zero. Following the Beer-Lambert Law, the transient absorption signal is:

\[
\Delta A(t) \propto \varepsilon_{FADH^-}[\text{[FADH\textsuperscript{−}]\text{−}[FADH\textsuperscript{−}]\text{,}t=0}] \propto \varepsilon_{FADH^-}[\text{FADH\textsuperscript{−}}(t)]
\]

So, \(k_1\) and \(\beta\) were determined exclusively by fitting the transient using equation (1).

(b) FADH\textsuperscript{+} detection
In the 510-700 nm region, both the FADH\(^*\) and FADH\(^*\) have absorption. Before pump excitation, the [FADH\(^*\)] is zero. The total transient absorption signal becomes:

\[
\Delta A(t) \propto \varepsilon_{\text{FADH}^*} \left[ \text{FADH}^*(t) \right] + \varepsilon_{\text{FADH}^*} \left[ \text{FADH}^*(t) \right] - \varepsilon_{\text{FADH}^*} \left[ \text{FADH}^* \right]_{t < 0} \\
\propto \varepsilon_{\text{FADH}^*} \left[ \text{FADH}^*(t) \right] + \varepsilon_{\text{FADH}^*} \left[ \left( \text{FADH}^*(t)_{\text{initial}} \right) \right] + \varepsilon_{\text{FADH}^*} \left[ \text{FADH}^* \right]_{\text{branched}}(t) \right) \]

As shown in Fig. 5.6, the transients in this region are characterized by an apparent rise in 45 ps, which is dominated by the k\(_2\) rate, and a remarkable longtime plateau, whose amplitude is mainly proportional to the k\(_3\) rate. Therefore, such a set of rich features uniquely lets us determine the k\(_2\), k\(_3\), and k\(_4\) rates by fitting the transients. The results are consistent in all the transients probed in this region. The \( \varepsilon_{\text{FADH}^*} \) was also obtained because the \( \varepsilon_{\text{FADH}^*} \) is already known, and the resulting values were confirmed by fitting the transients of the H364 mutant, in which k\(_3\) and k\(_4\) are 0.

(c) Detection of FADH\(^-\), 64PP and possible intermediate(s)

In UV region, the additional species of FADH\(^-\), 64PP and 64PPH\(^*\) need to be considered. Before pump excitation, the [FADH\(^-\)] and [64PP] is \( n_0 \), and the [64PPH\(^-\)] is zero. So, the transient absorption signal is:

\[
\Delta A(t) \propto \varepsilon_{\text{FADH}^-} \left[ \text{FADH}^- (t) \right] + \varepsilon_{\text{FADH}^*} \left[ \left( \text{FADH}^*(t)_{\text{initial}} \right) \right] + \varepsilon_{\text{FADH}^-} \left[ \text{FADH}^- (t)_{\text{branched}} \right] \\
+ \varepsilon_{\text{64PPH}^*} \left[ \left( \text{64PPH}^* (t) \right) ight] - \varepsilon_{\text{64PPH}^-} \left[ \left( \text{64PPH}^- (t)_{\text{branched}} \right) \right] \]

\[
\propto \varepsilon_{\text{FADH}^*} \left[ \text{FADH}^*(t) \right] + \varepsilon_{\text{FADH}^*} \left[ \left( \text{FADH}^*(t)_{\text{initial}} \right) \right] + \varepsilon_{\text{FADH}^-} \left[ \left( \text{FADH}^* (t)_{\text{branched}} \right) \right] \\
+ \varepsilon_{\text{64PPH}^*} \left[ \left( \text{64PPH}^* (t) \right) \right] + \varepsilon_{\text{64PPH}^-} \left[ \left( \text{64PPH}^- (t) \right) \right] \]

\[
\propto \varepsilon_{\text{FADH}^-} \left[ \text{FADH}^- (t) \right] + \varepsilon_{\text{FADH}^*} \left[ \left( \text{FADH}^*(t)_{\text{initial}} \right) \right] + \varepsilon_{\text{FADH}^-} \left[ \left( \text{FADH}^* (t)_{\text{branched}} \right) \right] \\
+ \varepsilon_{\text{64PPH}^*} \left[ \left( \text{64PPH}^* (t) \right) \right] + \varepsilon_{\text{64PPH}^-} \left[ \left( \text{64PPH}^- (t) \right) \right] \]

\[
\propto \varepsilon_{\text{FADH}^-} \left[ \text{FADH}^- (t) \right] + \varepsilon_{\text{FADH}^*} \left[ \left( \text{FADH}^*(t)_{\text{initial}} \right) \right] + \varepsilon_{\text{FADH}^-} \left[ \left( \text{FADH}^* (t)_{\text{branched}} \right) \right] \\
+ \varepsilon_{\text{64PPH}^*} \left[ \left( \text{64PPH}^* (t) \right) \right] + \varepsilon_{\text{64PPH}^-} \left[ \left( \text{64PPH}^- (t) \right) \right] \]

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By fitting the transients of the H364 mutant, in which 64PPH is not formed, \( \varepsilon_{\text{FADH}^-} \) is uniquely determined knowing the \( \varepsilon_{\text{FADH}^+} \), \( \varepsilon_{\text{FADH}^-} \), and \( \varepsilon_{64\text{PP}} \). Then, the \( k_5 \) and \( \varepsilon_{64\text{PPH}^-} \) can be obtained from the transients of the wild type in this probing region.
Fig. C.1 A mechanism of 64PP repair by (6-4) photolyase