Investigation of Charge Recombination in Escherichia coli Photolyase and Vibrio cholera Cryptochrome DASH

Agnieszka Zieba
Abstract

Proton-coupled electron-transfer (PCET) is a mechanism of great importance in protein electron transfer and enzyme catalysis, and the involvement of aromatic amino acids in this process is of much interest. The DNA repair enzyme photolyase provides a natural system that allows for the study of PCET using a neutral radical tryptophan (Trp'). In *Escherichia coli* photolyase, photoreduction of the flavin adenine dinucleotide (FAD) cofactor in its neutral radical semiquinone form (FADH') results in the formation of FADH and Trp. Charge recombination between these two intermediates requires the uptake of a proton by Trp'. The rate constant of charge recombination has been measured as a function of temperature in the pH range from 5.5 to 10.0, and the data are analyzed with both classical Marcus and semi-classical Hopfield electron transfer theory. The reorganization energy associated with the charge recombination process shows a pH dependence ranging from 2.3 eV at pH ≤7 and 1.2 eV at pH(D) ≥ 10.0. These findings indicate that at least two mechanisms are involved in the charge recombination reaction. Global analysis of the data supports the hypothesis that PCET during charge recombination can follow two different mechanisms with an apparent switch around pH 6.5. At lower pH, concerted electron proton transfer (CEPT) is the favorable mechanism with a reorganization energy of 2.1 to 2.3 eV. At higher pH, a sequential mechanism becomes dominant with rate-limiting electron-transfer followed by proton uptake which has a reorganization energy of 1.0 to 1.3 eV. The observed 'inverse' deuterium isotope effect at pH < 8 can be explained by a solvent isotope effect that affects the free energy change of the reaction and masks the normal, mass-related kinetic isotope effect that is
expected for a CEPT mechanism. To the best of our knowledge, this is the first time that a switch in PCET mechanism has been observed in a protein.

The charge recombination reaction between FADH\(^-\) and Trp\(^*\) is also analyzed in *Vibrio cholera* cryptochrome 1 (VcCry-1), a flavoprotein similar to *E. coli* photolyase, but with limited DNA repair ability. Several methods for accumulating FADH\(^+\) are investigated due to the initially low radical concentration in VcCry-1. A significant effect of potassium ferricyanide on the rate of the charge recombination reaction in VcCry-1 and *E. coli* photolyase is observed. It is shown that the reaction rate constant increases significantly with an increase in potassium ferricyanide concentration. Transient absorption measurements reveal that the rate of the charge recombination reaction in VcCry-1 is slightly slower than in *E. coli* photolyase at pH 7.0.

Photoreduction of FAD and photodecomposition of 5,10-methenyltetrahydrofolate polyglutamate (MTHF) are examined in *E. coli* photolyase and VcCry-1 with comparison to the published results on *Arabidopsis thaliana* cryptochrome-3. Additional information on the MTHF cofactor in VcCry-1 is obtained. Alterations are made to the way the photoreduction and photodecomposition processes are carried out; such as the exposure time to different wavelengths of light, and chemicals used. The results demonstrate that the photoreduction and photodecomposition are much slower processes in VcCry-1 than in *E. coli* photolyase. The similarity in the behavior of AtCry3 and VcCry-1 compared to *E. coli* photolyase indicates similarity between these plant and bacterial members of the cryDASH family. Our study shows that the concentration of reducing agent matter, in the photoreduction and photodecomposition processes, and that FADH\(^-\) may play a crucial, intermediary role.
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and *Vibrio cholera* Cryptochrome DASH

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Introduction

Ultraviolet (UV) radiation has harmful effects on organisms such as mutagenesis and growth delay (1). Exposure to this light induces production of two major lesions on DNA: cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine-pyrimidone (PP) photoproduct (1) (Figure 1). CPD appearance is approximately 3-fold higher than the (6-4) PP (2). These unrepaired photolesions have several bad consequences such as apoptosis, mutation, and eventually carcinogenic events leading to skin cancer (2). Excessive tanning and depletion of ozone layer lead to an increase in skin cancer occurrence every year. Several mechanisms, such as photoreactivation, excision repair (base excision repair, BER, and nucleotide excision repair, NER), and mismatch repair are known to remove DNA lesions. In mammals, these lesions are removed from the DNA by the highly sophisticated NER mechanism (2). The structurally distinct lesions repaired with this mechanism involve CPDs, 6-4 PP, bulky chemical adducts, forms of oxidative damage, and DNA-intrastrand crosslinks (3). However, the repair of CPDs by NER is greatly diminished compared to (6-4) PP repair (3). NER-deficiency in humans results in appearance of syndromes such as, trichothiodystrophy (TTD), xeroderma pigmentosum (XP), or cockayne syndrome (CS) showing the importance of this repair mechanism (4).
The photoreactivation repair mechanism is used by photolyases. CPD photolyases and (6-4) photolyases are distinguished based on their repair ability. Photolyase can repair either CPDs or pyrimidine-pyrimidone (6-4) lesions but one subclass cannot repair both (1). The enzyme uses near UV/blue light as the energy source for splitting of the dimers (5). Approximately one dimer is thought to be monomerized after one blue-light photon is absorbed (5, 6) representing one of the most efficient DNA repair mechanisms that has existed from the early evolutionary ages (2).

Photolyase is a flavoenzyme that can be found across the three biological kingdoms (5). Photolyase demonstrating CPD repair has been identified in archaea, bacteria, fungi, viruses, plants, invertebrates, and other vertebrates including aplacental mammals. The 6-4 photolyases, however were found in organisms such as Drosophila, silkworm, Xenopus laevis, and rattle snakes (3). However, it is not found in placental mammals where it is thought that it disappeared through evolution. Several studies were undertaken where the use of photolyase in humans was analyzed. It was demonstrated that photolyase encapsulated into liposomes when applied to previously UV irradiated
skin with photoreactivating light provides efficient DNA repair and immunoprotection (7). Another study showed that transgenic mice that express CPD-photolyase, show enhanced DNA repair when CPDs are present as well as higher resistance to UV radiation (2).

Photolyases are 45-66 kDa monomeric proteins containing 420-616 amino acid residues (3). There are two non-covalently bound cofactors in DNA photolyase, flavin adenine dinucleotide (FAD) and 5,10-methenyltetrahydrofolate polyglutamate (MTHF) (Figure 2). *Escherichia coli* (*E. coli*) photolyase which is of our main interest consists of two domains showed in Figure 3: an N-terminal α/β domain including residues 1 to 131, and a C-terminal α-helical domain made up of residues 204 to 471. The MTHF cofactor is partially exposed to solvent whereas the FAD is buried within the α-helical domain with a U-shaped conformation (1). The distance from MTHF to FAD is 16.8 Å.

![Figure 2. Structures of 5,10-methenyltetrahydrofolate polyglutamate (MTHF) and flavin adenine dinucleotide (FAD).](image)
In the photolyase photoreactivation mechanism, MTHF serves as a light-harvesting cofactor where it absorbs a photon and transfers the energy to a fully reduced FAD (FADH\(^{+}\)). FADH\(^{+}\) is generated from which an electron is transferred to the pyrimidine dimer resulting in the neutral radical semiquinone form (FADH\(^{-}\)) and monomerized cyclobutane pyrimidines. The exact pathway of the electron transfer process is still debated (8, 9).

FADH\(^{-}\) is the active form of the enzyme needed for the photoreactivation. The isolated form of the enzyme, FADH\(^{+}\) can be activated to FADH\(^{-}\) by a second electron transfer in photolyase that is known as photoreduction (1). The pathway for this process is represented in Figure 4 and involves a tryptophan triad consisting of Trp\(_{382}\), Trp\(_{359}\), and Trp\(_{306}\) which is found in all photolyases and crytochromes (10-13) (Figure 5A). Despite
other proposed pathways, the electron hopping model through the tryptophan triad is the one that has been experimentally confirmed (1).

![Figure 4. Pathway for photoreduction (solid arrows) and charge recombination (dashed arrows) in photolyase (14).](image)

Interestingly, it is thought that the rate of the electron transfer is enhanced by each tryptophan being in the right position along the electron transfer pathway (13-16). Previously it was thought that Trp\textsubscript{382} is oxidized to Trp\textsubscript{382}\textsuperscript{+} by donating an electron to FADH\textsuperscript{−}, producing FADH\textsuperscript{−}. Trp\textsubscript{382}\textsuperscript{+} is reduced by Trp\textsubscript{359} which oxidizes to Trp\textsubscript{359}\textsuperscript{+} further being reduced by Trp\textsubscript{306} forming Trp\textsubscript{306}\textsuperscript{+} in 10 ns (13-14, 17) and in 300 ns Trp\textsubscript{306}\textsuperscript{+} being deprotonated to Trp\textsubscript{306}. Recently, a new rate was proposed where the photoreduction of FADH\textsuperscript{+} to FADH\textsuperscript{−} is done by Trp\textsubscript{382} which oxidizes to Trp\textsubscript{382}\textsuperscript{+} in 30 ps indicating that the other two inter-tryptophan electron transfer steps have to occur in less than 30 ps (13-14, 17). The approximate distance between the FAD cofactor and Trp\textsubscript{306} is 15 Å, and the individual steps range from 3.5 to 5.5 Å (18). The back electron transfer
mechanism which competes with retaining the active FADH\(^-\) is known as charge recombination. On a millisecond time scale, charge recombination occurs between FADH\(^-\) and Trp\(_{306}\)\(^*\) when no external electron donors are involved (14, 19-21). This process is showed to be pH dependent and affected by substrate binding (14, 19-20).

Protein radicals are important in nature because of their role in enzyme catalysis along with other significant reactions they undergo (15, 22). In enzymatic redox reactions, amino acid radicals serve as important intermediates (23). A recently postulated model for charge recombination in *E. coli* photolyase proposes that electron transfer from FADH\(^-\) to Trp\(_{306}\)\(^*\) is followed by the uptake of a proton by Trp\(_{306}\)\(^+\) at high pH. While a pure electron transfer from FADH\(^-\) to TrpH\(_{306}\)\(^+\) is suggested to persist at low pH (Figure 5B). It was proposed that the pK\(_a\) of TrpH\(_{306}\)\(^+\), which is at equilibrium with Trp\(_{306}\)\(^*\), causes the pH-dependent change in the charge recombination reaction (20). The substitution of hydrogen for deuterium affects the rate of enzyme-catalyzed redox processes resulting in either a small or a large kinetic isotope effect (KIE) (24). It is accepted that a significant deuterium isotope effect is a hallmark of concerted electron proton transfer (CEPT) and since it was not observed by Byrdin et al. CEPT was not taken into account as one of the possible mechanisms. The group only observed an ‘inverted’ deuterium isotope effect below pH 8.0, i.e., the rate of the reaction is faster with deuterium than with hydrogen (20).
The transfer of electrons serves as a key step in many important biological processes such as nitrogen fixation, aerobic respiration, and photosynthesis (25, 26). The reaction occurs when the reactants approach each other and the coupling of the electronic orbitals is enhanced, while other nuclear coordinates fluctuate (Figure 6).
Figure 6. Nuclear potential energy curves. Reactant and product vibrational wells are represented by R and P, respectively. \( \lambda \), \( \Delta G^0 \), \( \Delta G^* \), and \( H_{AB} \) are indicated as well as \( Q^* \) which is the coordinate of the transition state (27).

Undergoing the quantum mechanical transition, the configuration of the system evolves from the equilibrium state of the reactants into its product state (28). The electron transfer is described by Marcus theory of electron transfer:

\[
k_{ET} = \frac{2\pi}{\hbar} \frac{H_{AB}^2}{\sqrt{4\pi \lambda k_B T}} \exp \left( - \frac{(\Delta G^0 + \lambda)^2}{4 \lambda k_B T} \right)
\]

Equation 1

where \( \hbar \) is Planck’s constant, \( k_B \) is Boltzmann’s constant, \( \Delta G^0 \) is the change in standard free energy, \( T \) is the temperature, \( k_{ET} \) is the rate constant, \( \lambda \) is the reorganization energy, and \( H_{AB} \) is the electronic coupling matrix element. The reorganization energy is the energy required to distort the equilibrium configuration of the reactants and the surroundings leading to the equilibrium geometry of the products. \( H_{AB} \) represents the overlap of the electronic wavefunctions of the donor and the acceptor (28). When the
distance between the reactants is more than ~ 5 Å the electron transfer reactions are
treated nonadiabatically with weak electronic coupling present (H_{AB} small) (29).
However, when the reactants are in a close distance, the reaction is adiabatic and the
electronic coupling is large, signifying a high probability of the electron transfer. The
change in standard free energy is obtained as the difference in the reduction potential
(E_{m}^\circ) between the donor and the acceptor:

\[ \Delta G^0 = e \left[ E_m^0 (Donor) - E_m^0 (Accept) \right]. \] Equation 2

The quantum corrections have to be applied to the classical Marcus expression when \( \hbar \omega \),
which is associated with the characteristic frequency of the nuclear motion coupled to the
electron transfer, is larger than \( k_B T \) (30, 31). In the semi-classical Hopfield equation, \( k_B T \)
is replaced by \( \hbar \omega \coth(\hbar \omega / 2k_B T) \):

\[ k_{ET} = \frac{2\pi}{\hbar} \frac{H_{AB}^2}{2\pi^2 \hbar \omega \coth(\hbar \omega / 2k_B T)} \left[ \frac{\Delta G^0 + \lambda}{2\hbar \omega \coth(\hbar \omega / 2k_B T)} \right]. \] Equation 3

In the quantized version the electron is only allowed to transfer from discrete energy
levels without oscillating along the reactant potential energy surface before reaching the
intersection point for the transition into the product potential energy surface.

Various observations such as pH dependence and higher reorganization energy
suggested proton coupled electron transfer (PCET) (Figure 7) as one of the mechanisms
for the charge recombination reaction in \( E. coli \) photolyase. In amino acids, the transfer
of an electron is often coupled to its deprotonation (32-34). As an important biological
and chemical process PCET is a subject of great attention. For charge transport and
catalysis during primary metabolic steps enzymes often rely on this type of transfer (35).
It has also been observed in redox enzyme processes such as membrane proton pumping.
in cytochrome c oxidase, water catalysis by metallo-oxidases, and conversion of dioxyn to hydrogen peroxide. In processes involving radicals in an excited state as in green fluorescent protein photoreactions, photosynthesis, or simple steps in electrochemical dihydrogen evolution, PCET was also studied (36). PCET can follow a sequential or concerted mechanism or occur as hydrogen atom transfer. The sequential mechanism is described by an electron transfer (ET) followed by a proton transfer (PT) or vice versa. In the concerted electron-proton (CEP) transfer step, the proton and electron are transferred simultaneously. CEP transfer as a charge neutral reaction avoids the high-energy intermediate states associated with the pathway which are normally created by the sequential PTET or ETPT mechanisms. Due to the efficient reactions involving a low driving force, CEP transfer is called an energy-conservative mechanism.

\[ \text{FADH}^- , \text{TrpH}^+ \overset{e^-}{\rightarrow} \text{FADH}^+ , \text{TrpH} \]

\[ \text{H}^+ \overset{e^-}{\rightarrow} \text{H}^+ \]

**Figure 7.** Possible pathways for proton coupled electron transfer in charge recombination in photolyase.

In order to obtain a deeper understanding of PCET it is also studied using model compounds and by theory (35-44). PCET was studied on Ru-tyrosine and Ru-tryptophan model compounds by Hammarström and coworkers (32-34). They demonstrated that
PCET can switch from CEPT to ET followed by PT as a function of pH. A relatively high reorganization energy results from the CEP transfer mechanism. This idea served as one of the ways to discriminate between a CEPT and a sequential ET and PT mechanism. Besides the reorganization energy of up to 2.4 eV, other factors such as a pH-dependent rate constant, a large change in free energy, and a significant deuterium KIE were used for the discrimination between the PCET mechanisms. However, the reorganization energy has to be corrected for the pH-dependent entropy term which is approximated by the mixing term arising from the uptake of the proton from the bulk water disturbing the free-energy term (45):

\[ \Delta S_{\text{reaction}} = \Delta S_{\text{uptake}} \]

\[ \Delta S_{\text{uptake}} = -\Delta S_{\text{mixing}} = -R \ln(10) \cdot pH \]  

Equation 4

In this study, the model proposed by Byrdin et al. was tested analyzing the pH and temperature dependence of charge recombination. We have used Marcus and Hopfield theory of electron transfer as a way to determine the reorganization energy and the electronic coupling. Their values served as a way of distinguishing between concerted and sequential PCET mechanism in the charge recombination reaction in \textit{E. coli} photolyase. The charge recombination reaction was also examined in VcCry-1 with a full description of the project in PART II. Finally, photoreduction of MTHF was investigated in Part III.

Cryptochromes from the family of blue-light photoreceptors are also flavoproteins and related to photolyase. The enzyme has been found in bacteria, plants, and animals (5, 46). In plants, it is responsible for seedling development and regulating the stem growth. In animals, cryptochrome controls the circadian rhythm. It was also shown that fruit flies
achieve magnetosensitivity through use of this protein (47). Cryptochrome has also been proposed to act as a magnetoreceptor in migratory birds responsible for their orientation in Earth’s magnetic field (48, 49). It was thought that cryptochromes are proteins with a high photolyase sequence homology but without the ability for DNA repair. However, recently a great interest in cryptochrome-DASH (Cry-DASH) subfamily evolved because of its ability to repair CPDs on single stranded DNA (ssDNA) and its presence in humans.

*Vibrio cholera* cryptochrome 1 (VcCry-1) belongs to the Cry-DASH branch and it shows a high affinity for RNA (50). It was demonstrated that the CPD lesion repair efficiency on ssDNA and RNA by VcCry-1 is very similar to the efficiency of the CPD photolyase from *Vibrio cholera* (VcPhr) (50). Unfortunately, the repair efficiency of CPDs on dsDNA by VcCry-1 is negligible as compared to the repair by EcPhr. The two cofactors, FAD and MTHF which are bound to photolyase, also exist in Cry-DASH (51). When isolated, VcCry-1 contains the two cofactors in near stoichiometric amounts and the FAD is present as an anionic hydroquinone (FADH⁻) which then rapidly oxidizes to FADH⁺, and mainly FAD(ox) (50). In *E. coli* photolyase on the other hand MTHF is present in substoichiometric amounts, and FAD is oxidized to FADH' after isolation (50). Due to the possible structural differences in these two very similar proteins, the light driven electron transfers emerge as an interesting comparison. The analysis of the photoreduction and charge recombination reactions can provide additional information about these proteins. The charge recombination reaction, FADH⁻ + Trp₃₀₆⁺ + H⁺ → FADH' + TrpH₃₀₆ (14, 19), is of a special interest, and its exact mechanism in *E. coli*
photolyase being described in this thesis project. The difference in the oxidation states between \textit{E. coli} photolyase and VcCry-1 is shown in Figure 8.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{absorbance_spectra.png}
\caption{The absorbance spectra of \textit{E. coli} photolyase (mainly FAD\textsuperscript{H'}\textsubscript{ox}) in blue and VcCry-1 (mainly FAD\textsubscript{ox}).}
\end{figure}
Part I – Charge Recombination in DNA Photolyase

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma-Aldrich and Acros Organics and used without further purification. D2O was from Cambridge Isotope Laboratories, Inc.

Sample Preparation. Photolyase was overexpressed, isolated, and purified as described elsewhere (52-53). Purified photolyase was stored in 0.4 M K2SO4 and 20 mM potassium phosphate (pH 7.0) at -80 °C. Buffer exchange was done by two dilution and concentration cycles with the use of 30 kD NMWL centrifugal filter devices (Amicon Ultra, Millipore). The following buffers, each with 0.4 M K2SO4, were prepared: 20 mM citric acid for pH 5.5 and 6.0, 20 mM potassium phosphate for pH 7.0 and 8.0, 20 mM sodium pyrophosphate for pH 9.0, and 75 mM sodium bicarbonate for pH 9.5 and 10.0. A typical sample had a volume of 250 μL with photolyase at a concentration of 80 μM. The concentration of the flavin neutral radical in photolyase was determined from the absorbance at 580 nm with an extinction coefficient of \( \varepsilon_{580} = 4,800 \text{ M}^{-1}\text{cm}^{-1} \) (54). The samples for experiments in D2O solutions were prepared in the buffers described above. The pD of each solution was determined by adding 0.4 to pH electrode reading (55, 56).

Transient Absorption Measurements. Transient absorption spectroscopy was performed on a home-built system described below and shown in Figure 9. A 5 ns pulse at 532 nm from a Surelite Nd:YAG laser (I-20, Continuum) was used to excite the sample with a pulse energy of 50 to 60 mJ at 2-10 Hz repetition rate. The laser light was diffused through a mounted ground glass diffuser (220 GRIT, ThorLabs) to provide homogenous excitation of the sample. The probe light was generated with a 75 W Xe lamp (Optical
Building Blocks) and focused onto the sample with a lens. Infrared and ultraviolet light were removed from the probe beam with a heat filter (FSR-KG3, Newport), and a 50 nm bandwidth of light was selected by using a 550 nm cut-on filter and a 600 nm cut-off filter (FGL550S and FES0600, ThorLabs). Shutters and controllers (SH05 and SC10, ThorLabs, and VS25S2ZM1 and VCM-DI, Uniblitz) were used to block the laser pulses and the probe light to avoid unnecessary exposure of the sample to light. The shutter controllers were regulated by a pulse generator (9514 Plus Series, Quantum Composers) which was triggered on the preceding laser pulse detected with a photodiode (210 DET, ThorLabs). The probe light and the laser pulse made a 90° angle at the center of a quartz cuvette that was placed in a TLC 50™ temperature-controlled cuvette holder attached to the TC 125 temperature controller (Quantum North West) contained in a sample box (Model 2007, Optical Building Blocks). The cuvette holder was affixed to a circulating water bath (Little Giant Pump, Franklin Electric) and in-house dry air for cooling and purging purposes, respectively. The temperature was varied from -5 °C to +35 °C with 5 °C increments and monitored with a thermocouple (OMEGA) inside the sample. The transmitted probe light was focused into a monochromator (Model 2000, Optical Building Blocks), and the transmitted light intensity was detected with a photodiode (DET 36A, ThorLabs). The signal was attenuated with a variable terminator (VT1, Thorlabs) and recorded with a digital oscilloscope (TDS 2022, Tektronix). The output of the Xe lamp was directly monitored with a DET 210 photodiode connected to the oscilloscope through a variable attenuator to correct for fluctuations in the probe light.
At each temperature, two sets of 5 to 10 transient absorption traces were averaged. Three to five data sets were obtained for the temperature range at each pH. Samples were replaced upon the first signs of sample degradation (FAD oxidation) or trapping of FAD in its fully reduced state. Both cases led to loss of signal and poor signal-to-noise ratios. Neither oxidized nor fully reduced flavin contributed to the transient absorption signal under the excitation and detection conditions due to selective excitation of FADH* at 532 nm (52).

**Spectroscopic measurements of fully reduced and semiquinone FAD.** Fully reduced FAD in solution and in photolyase at various pH(D) values was prepared by purging samples with Nitrogen gas (N$_2$(g)) for 10 minutes. FAD in solution was photoreduced in the presence of 20 mM EDTA. FADH* in photolyase was photoreduced with visible light ($\lambda > 420$ nm) in the presence of 20 mM dithiothreitol, and the MTHF cofactor was removed by using UV-light ($\lambda = 300 – 400$ nm) for photodegradation.
(process explained in Part III). All irradiation was done with filtered light from a 75 W Xe lamp for 10 minutes at 0 °C. The progress of the photoreduction and photodegradation processes was monitored by checking the UV-vis absorption spectrum. The spectrum of the FAD semiquinone in photolyase was observed as a function of pH(D) using UV-vis absorption spectroscopy. Resonance Raman spectroscopy was done by Carlos Lucero and used to compare the spectra of the FAD semiquinone at various pH values (57).

**Determination of the FADH*/FADH\textsuperscript{−} reduction potential.** The FADH\textsuperscript{−}/FADH\textsuperscript{*} reduction potential in H\textsubscript{2}O solutions as a function of pH and in D\textsubscript{2}O buffer solution at pD 7.0 was measured by Dr. Yvonne Gindt’s research group at Lafayette College. For the measurements, a spectroelectrochemical method was used without a correction to the Ag/AgCl reference electrode for the D\textsubscript{2}O solvent (57).

**Data analysis.** For each pH and temperature, the rate constant of the charge recombination process was determined by fitting the transient absorption traces to a monoexponential decay function. The quality of the fit was not improved when a biexponential decay function was used. The averaged charge recombination rate constants, $k_{ET}$, at each pH as a function of temperature, $T$, were fit to the Marcus Equation 1 and semi-classical Hopfield Equation 3 for electron transfer to obtain the reorganization energy, $\lambda$, and the electronic coupling matrix element, $H_{AB}$. In the Hopfield equation the fitting was done using three common values for $\hbar\omega$: 200 meV, 70 meV, and 25 meV (30, 31). The change in standard free energy was obtained using Equation 2 with reduction potential of FADH\textsuperscript{−}/FADH\textsuperscript{*} and Trp\textsuperscript{*}/TrpH:

$$
\Delta G^0 = e\left[ E^0_m( FADH^- / FADH^\ast) - E^0_m(Trp^\ast / TrpH) \right].
$$

Equation 5
$E_m^0(\text{Trp}^*/\text{TrpH})$ is pH dependent due to the uptake of a proton during the charge recombination process. The entropy contribution was calculated using Equation 4 and applied as a correction to the $\Delta G^0$:

$$\Delta G^0 = \Delta G^0 + (T - 295) \cdot \Delta S_{\text{uptake}}$$  \hspace{1cm} \text{Equation 6}

Since the measurement of $E_m^0(\text{Trp}^*/\text{TrpH})$ was done at 295 K the entropy correction was 0 kJ/mol•K at that temperature (22). Data analysis was done using Origin 7.0 (Originlab) and Igor Pro 4.07 (Wavemetrics).

The experiments done in D$_2$O were analyzed the same way as the data from H$_2$O experiments, however, due to some uncertainty in the exact value for $\Delta S$ and unknown reduction potential for Trp$^*/$TrpH in D$_2$O, additional analyses were performed. One analysis included a 10% increase in the entropy term (58, 59). Another analysis resulted in obtaining $\lambda$ and $H_{AB}$ values using $\Delta S$ and reduction potential measurements done in H$_2$O. A third analysis used the measured FADH$^-$/$\text{FADH}^-$ reduction potential, Trp$^*/$TrpH reduction potential in H$_2$O, and the 10% correction on entropy. An additional modification to the analysis was done where the 10% correction was applied but $\Delta G^0$ was calculated from reduction potentials measured in H$_2$O. Since the samples in water solution were analyzed with and without the entropy correction in general, the same was done for the data derived from experiments that used deuterated water.
RESULTS

Temperature and pH-dependence of charge recombination in photolyase.

FADH$^-$ and $^{306}$Trp$^\prime$ are formed after the photoreduction of FADH which results in the disappearance of the FADH$^*$ absorbance between 450 and 650 nm (14, 60). Monitoring of the recovery of the FADH$^*$ allowed for measuring the rate constant of the charge recombination. The wavelength of the probe light does not affect the rate constant of the charge recombination (13, 14,19). The transient absorption traces generated by the reappearance of the FADH$^*$ absorbance at 580 nm due to the charge recombination process are shown in Figure 10.

![Figure 10](image)

**Figure 10.** Temperature and pH dependence of charge recombination in photolyase monitored at 580 nm following excitation at 532 nm with a 5 ns pulse. (A) Photolyase at pH 5.5 and at 30.0 °C (a) and at -5.0 °C (b). (B) Photolyase at -5.0 °C and at pH 5.5 (b) and pH 9.5 (c).

Although Trp$^\prime$ also absorbs at 580 nm, it disappears with the same kinetics that describe the reappearance of FADH$^*$ and will not complicate the analysis of the transient
absorption traces (14, 60). The temperature dependence of the reaction at pH 5.5 (Figure 10A) shows absorption changes at -5 °C and 30 °C with time constants for the charge recombination reaction of 2.6 ms and 1.0 ms, respectively.

The pH-dependence of the charge recombination reaction is illustrated in Figure 10B. At -5 °C, the reaction occurs with a time constant of 3.0 ms at pH 5.5 and 30.3 ms at pH 10. The rate constants were obtained after taking the reciprocal of the time constants. This pH-dependence of the rate constant for charge recombination is in agreement with earlier measurements (14, 19, 20). The averaged rate constants with the standard deviations of the charge recombination reaction as a function of pH and temperature are listed in Table 1. The larger standard deviations of the rate constant at lower pH values are due to the strong pH-dependence of the reaction in that pH range. However, the standard deviation between the data sets for the reorganization energy at low pH was very small. Therefore, the uncertainty of the rate constants at lower pH values does not affect the interpretation of our data. The transient absorption change does not return completely to zero when the experiments are done at low temperatures and especially higher pH values. Despite this complication, we determine the same rate constants as measured by others under these conditions (20). At higher pH values there may be heterogeneity in the availability of proton donors that interferes with charge recombination, resulting in two populations; one that can undergo charge recombination and one that cannot, trapping the FAD cofactor in the FADH⁻ state. The population that does not undergo charge recombination does not contribute to the observed rate constant and does not affect our analysis. This apparent incomplete charge recombination will be of interest for future studies and may elucidate the nature of the proton donor(s).
Table 1. Rate constant (s\(^{-1}\)) of charge recombination in DNA photolyase as a function of temperature and pH.

<table>
<thead>
<tr>
<th>T (K)</th>
<th>pH 5.5</th>
<th>pH 6.0</th>
<th>pH 7.0</th>
<th>pH 8.0</th>
<th>pH 9.0</th>
<th>pH 9.5</th>
<th>pH 10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>268.15</td>
<td>330 (±70)</td>
<td>270 (±50)</td>
<td>52.1 (±1.0)</td>
<td>32.86 (±0.03)</td>
<td>32.1 (±0.6)</td>
<td>32.95 (±0.17)</td>
<td>33.0 (±0.7)</td>
</tr>
<tr>
<td>273.15</td>
<td>360 (±60)</td>
<td>260 (±90)</td>
<td>58.9 (±1.2)</td>
<td>37.1 (±0.7)</td>
<td>36.7 (±1.6)</td>
<td>36.7 (±0.3)</td>
<td>37.1 (±0.5)</td>
</tr>
<tr>
<td>278.15</td>
<td>400 (±70)</td>
<td>290 (±100)</td>
<td>68 (±5)</td>
<td>42.8 (±0.7)</td>
<td>42.0 (±1.6)</td>
<td>41.8 (±0.5)</td>
<td>42.0 (±1.5)</td>
</tr>
<tr>
<td>283.15</td>
<td>450 (±75)</td>
<td>320 (±110)</td>
<td>79 (±4)</td>
<td>48.9 (±0.4)</td>
<td>47.6 (±1.9)</td>
<td>47.7 (±0.7)</td>
<td>47.1 (±1.0)</td>
</tr>
<tr>
<td>288.15</td>
<td>520 (±90)</td>
<td>370 (±120)</td>
<td>93 (±8)</td>
<td>56.8 (±0.8)</td>
<td>53.9 (±0.3)</td>
<td>54.3 (±1.0)</td>
<td>54.3 (±0.9)</td>
</tr>
<tr>
<td>293.15</td>
<td>580 (±110)</td>
<td>450 (±180)</td>
<td>115 (±16)</td>
<td>64 (±4)</td>
<td>62.5 (±0.7)</td>
<td>67.2 (±1.0)</td>
<td>68 (±7)</td>
</tr>
<tr>
<td>298.15</td>
<td>720 (±140)</td>
<td>500 (±190)</td>
<td>133 (±16)</td>
<td>76.6 (±1.8)</td>
<td>73.6 (±0.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>303.15</td>
<td>870 (±150)</td>
<td>590 (±220)</td>
<td>160 (±20)</td>
<td>89 (±4)</td>
<td>88.9 (±1.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>308.15</td>
<td>1070 (±80)</td>
<td>750 (±370)</td>
<td>178 (±7)</td>
<td>109 (±14)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Determination of reorganization energy and electronic coupling matrix element.**

Measuring the temperature dependence or the free energy dependence of the electron transfer rate constant allows for determining the reorganization energy value through experiments (61). We chose the temperature dependence approach because modifying the cofactors to modify reduction potentials in DNA photolyase would not be trivial. We used the approximation that the reduction potentials of the species involved are constant over the temperature range of the experiments. Figure 11 shows the temperature dependence of the rate constant of charge recombination at pH 7.0. The values of $\lambda$ and $H_{AB}$ were obtained with and without the entropy correction (Eq. 6) by fitting the temperature dependence of the rate constant with the classical Marcus theory of electron transfer (Eq. 1). The thermodynamic parameters that were used for the fitting procedure are listed in Table 2. Dr. Yvonne Gindt from Lafayette College provided experimentally determined $E_m^0(FADH^-/FADH^+)$ in *E. coli* photolyase, and no obvious pH dependence of
$E_m^0(\text{FADH}^\cdot /\text{FADH}^\cdot)$ was observed (Table 2). The value of $E_m^0(\text{Trp}^\cdot /\text{TrpH})$ in mV was determined by using an empirical formula: (22, 62)

$$E_m^0(\text{Trp}^\cdot /\text{TrpH}) = 1070 - (pH - pK_a) \cdot 53,$$

Equation 7

with $pK_a = 3.7$ for $\text{TrpH}^\cdot$. The study by Tommos et al. involved $\text{TrpH}$ in a protein, reflecting our system more closely and for that reason their data was used in our analysis (22).

**Table 2.** Thermodynamic parameters used for fits with classical Marcus (Eq. 1) and semi-classical Hopfield (Eq. 2) theory to the temperature dependence of the charge recombination rate constant.

<table>
<thead>
<tr>
<th>pH(D)</th>
<th>$E_m^0(\text{FADH}^\cdot /\text{FADH}^\cdot)$ (mV)</th>
<th>$E_m^0(\text{Trp}^\cdot /\text{TrpH})$ (mV)</th>
<th>$\Delta G^0$ (eV)</th>
<th>$\Delta S_{\text{uptake}}$ ($10^{-3}$ eV/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>16$^a$ (45)$^b$</td>
<td>975 (1004)</td>
<td>-0.951 (-0.959)</td>
<td>-1.09</td>
</tr>
<tr>
<td>6.0</td>
<td>24 ± 10 (45)$^b$</td>
<td>948 (978)</td>
<td>-0.924 (-0.933)</td>
<td>-1.19</td>
</tr>
<tr>
<td>7.0</td>
<td>0 ± 6 (45 ± 6)</td>
<td>895 (925)</td>
<td>-0.895 (-0.880)</td>
<td>-1.39</td>
</tr>
<tr>
<td>8.0</td>
<td>11 ± 7 (45)$^b$</td>
<td>842 (872)</td>
<td>-0.831 (-0.827)</td>
<td>-1.59</td>
</tr>
<tr>
<td>9.0</td>
<td>14 ± 4 (45)$^b$</td>
<td>789 (819)</td>
<td>-0.775 (-0.774)</td>
<td>-1.79</td>
</tr>
<tr>
<td>9.5</td>
<td>8.5$^a$ (45)$^b$</td>
<td>763 (792)</td>
<td>-0.754 (-0.747)</td>
<td>-1.89</td>
</tr>
<tr>
<td>10.0</td>
<td>7.5$^a$ (45)$^b$</td>
<td>736 (766)</td>
<td>-0.728 (-0.721)</td>
<td>-1.98</td>
</tr>
</tbody>
</table>

$^a$Values extrapolated from measurements at pH 6.0 through pH 9.0; $^b$Values for D$_2$O experiments in parentheses; $^c$Data from Ref. 22 estimated with empirical formulas Eq. 7 and Eq. 8 for H$_2$O and D$_2$O, respectively; $^d\Delta S_{\text{uptake}}$ is assumed the same in H$_2$O and D$_2$O solutions (see text).

For the data in Figure 11, values of $\lambda = 1.88$ eV and $H_{\text{AB}} = 7.1 \cdot 10^{-7}$ eV were obtained when the entropy correction was included; the results are listed in Table 3. The small standard deviations of $\lambda$ shown in the table indicate a good reproducibility at each pH value. The inset in Figure 11 shows the variation of $\lambda$ with pH when analyzed with or
without the entropy correction. It is clear that the inclusion of the entropy correction affects the magnitude of the reorganization energy but not its pH-dependence. At pH 7.0, the average reorganization energy is $1.98 \pm 0.09 \text{ eV}$ with entropy correction and increases to $2.53 \pm 0.10 \text{ eV}$ when the entropy term is omitted.

**Figure 11.** Temperature dependence of the rate constant of charge recombination in *E. coli* photolyase at pH 7.0 fitted to the classical Marcus (solid line) and semi-classical Hopfield (dashed line, $h\omega = 25 \text{ meV}$) theory of electron transfer (Eq. 1 and Eq. 2) with correction for entropy (Eq. 5). Inset: The reorganization energy as a function of pH with (▲) and without (■) the entropy correction from classical Marcus theory and with entropy correction (▼) from semi-classical Hopfield ($h\omega = 25 \text{ meV}$) theory. The solid lines are added to guide the eye.
Table 3. pH-dependence of the reorganization energy ($\lambda$) and electronic coupling matrix element (H_{AB}) obtained from a fit with classical Marcus (Eq. 1) and semi-classical Hopfield (Eq. 2) theory to the charge recombination rate constants as a function of temperature in H$_2$O and D$_2$O solutions.

<table>
<thead>
<tr>
<th>pH</th>
<th>$\lambda$ (eV)</th>
<th>H_{AB} (10^{-6} eV)</th>
<th>$\lambda$ (eV)</th>
<th>H_{AB} (10^{-6} eV)</th>
<th>$\lambda$ (eV)</th>
<th>H_{AB} (10^{-6} eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>2.54 (±0.14)</td>
<td>21.5</td>
<td>2.08 (±0.15)</td>
<td>3.33</td>
<td>2.30 (±0.17)</td>
<td>11.3</td>
</tr>
<tr>
<td>6.0</td>
<td>2.42 (±0.16)</td>
<td>13.3</td>
<td>1.95 (±0.15)</td>
<td>2.00</td>
<td>2.15 (±0.17)</td>
<td>5.22</td>
</tr>
<tr>
<td>7.0</td>
<td>2.53 (±0.10)</td>
<td>11.5</td>
<td>1.98 (±0.09)</td>
<td>1.15</td>
<td>2.19 (±0.11)</td>
<td>3.75</td>
</tr>
<tr>
<td>8.0</td>
<td>2.31 (±0.13)</td>
<td>5.56</td>
<td>1.72 (±0.11)</td>
<td>0.40</td>
<td>1.89 (±0.14)</td>
<td>1.26</td>
</tr>
<tr>
<td>9.0</td>
<td>2.17 (±0.01)</td>
<td>3.64</td>
<td>1.52 (±0.01)</td>
<td>0.24</td>
<td>1.66 (±0.01)</td>
<td>0.64</td>
</tr>
<tr>
<td>9.5</td>
<td>2.03 (±0.06)</td>
<td>2.34</td>
<td>1.37 (±0.05)</td>
<td>0.16</td>
<td>1.49 (±0.06)</td>
<td>0.38</td>
</tr>
<tr>
<td>10.0</td>
<td>2.03 (±0.10)</td>
<td>2.91</td>
<td>1.33 (±0.07)</td>
<td>0.16</td>
<td>1.46 (±0.10)</td>
<td>0.40</td>
</tr>
</tbody>
</table>

Charge recombination in D$_2$O buffer solutions. The charge recombination rate constants measured at 10 °C are shown in Figure 12 as a function of pH(D). A small isotope effect of 1.1 – 1.4 is observed above pH(D) 8.0. Below pH(D) 8.0, an ‘inverse’ isotope effect is seen. These results are shown in the inset of Figure 12 and are in agreement with an earlier report (20). Obtaining the thermodynamic parameters in D$_2$O allowed analyzing the charge recombination reaction in D$_2$O resulting in $\lambda$ and H_{AB} values.
Figure 12. The pH-dependence of the rate constant of charge recombination at 10 °C in H₂O (■) and in D₂O (●). The pD values of the D₂O buffer solutions were determined by reading the pH electrode and adding 0.4. Inset: The pH-dependence of the reorganization energy on pH (■) and pD (●), and the KIE as a function of pH(D) (○).

The FADH/FADH⁺ reduction potential was measured by Dr. Gindt in *E. coli* photolyase at pD 7.0 and determined to be +45 (±6) mV vs NHE. It is a small increase of 45 mV compared to the potential at pH 7.0. Based on the fact that we did not find any evidence for the pH-dependence of $E_m^0(FADH/FADH^+)$, it was assumed that there was no pD-dependence of the reduction potential either. A slightly more positive reduction potential is in agreement with other studies of FAD-containing proteins (63-64). For the general analysis, the entropy change associated with the proton uptake in D₂O solutions was assumed to have the same value as in H₂O solutions with pH replaced by pD (Equation 4). Finally, the value of the Trp⁺/TrpH reduction potential in D₂O solution is
not available; however, it has been well-documented that weak acids experience an increase in $pK_a$ in $D_2O$, represented by $\Delta pK_a = pK_a^D - pK_a^H$, in solution and proteins (65-69). It has been proposed that the Trp cationic radical has $\Delta pK_a = 0.56$ in $D_2O$ (20). To estimate $E_m^0(Trp^*/TrpD)$ in $D_2O$ solutions, we modified Eq. 7 by shifting the pH-dependence by 0.56:

$$E_m^0(Trp^*/TrpD) = 1070 - (pD - pK_{aD}^D) \cdot 53,$$

Equation 8

with $pK_{aD}^D = 3.7 + \Delta pK_a = 4.26$ for $TrpD^*$ in $D_2O$ solutions. All the thermodynamic parameters used to fit the experimental data are shown in Table 2 and the results for $\lambda$ and $H_{AB}$ with the thermodynamic parameters for the $D_2O$ data are shown in Table 3. The dependence of $\lambda$ on $pD$ is shown in the inset of Figure 12. The value of $\lambda$ obtained is always slightly smaller in $D_2O$ experiments than in $H_2O$ experiments. We want to emphasize that there is some uncertainty in the thermodynamic fit parameters for $D_2O$ experiments which may give rise to this discrepancy. The results from the additional analysis which included the 10% entropy correction and reduction potential measurements in $H_2O$ or $D_2O$ are shown in Table 4. The highest $\lambda$ is acquired from the combination of 10% correction on entropy and $\Delta G$ with reduction potentials measured in $H_2O$. Figure 13 compares the different ways of analyzing studies in deuterated water as well as the study in water solution where trivial difference between all measurements is distinguished. The main analysis where only a correction on $FADH^+/FADH$ reduction potential was applied demonstrated that $\lambda$ does show the same dependence on $pD$ as on $pH$. Also a significant increase in $\lambda$ arises from removing the entropy term from the analysis for both $H_2O$ and $D_2O$ experiments.
Table 4. Reorganization energy and electronic coupling in D$_2$O obtained from using different combinations of entropy and $\Delta G$.

<table>
<thead>
<tr>
<th>pH</th>
<th>$\lambda$ (eV)$^a$</th>
<th>$\lambda$ (eV)$^b$</th>
<th>$\lambda$ (eV)$^c$</th>
<th>$\lambda$ (eV)$^d$</th>
<th>$\lambda$ (eV)$^e$</th>
<th>$H_{AB}$ (eV)$^a$</th>
<th>$H_{AB}$ (eV)$^b$</th>
<th>$H_{AB}$ (eV)$^c$</th>
<th>$H_{AB}$ (eV)$^d$</th>
<th>$H_{AB}$ (eV)$^e$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.0</td>
<td>1.90</td>
<td>1.93</td>
<td>1.89</td>
<td>2.34</td>
<td>2.31</td>
<td>2.37x10$^{-6}$</td>
<td>2.41x10$^{-6}$</td>
<td>2.06x10$^{-6}$</td>
<td>1.00x10$^{-5}$</td>
<td>1.00x10$^{-5}$</td>
</tr>
<tr>
<td>7.0</td>
<td>1.86</td>
<td>1.93</td>
<td>1.89</td>
<td>2.42</td>
<td>2.35</td>
<td>1.07x10$^{-6}$</td>
<td>1.10x10$^{-6}$</td>
<td>9.22x10$^{-7}$</td>
<td>8.45x10$^{-6}$</td>
<td>8.40x10$^{-6}$</td>
</tr>
<tr>
<td>8.0</td>
<td>1.65</td>
<td>1.70</td>
<td>1.65</td>
<td>2.24</td>
<td>2.19</td>
<td>3.55x10$^{-7}$</td>
<td>3.58x10$^{-7}$</td>
<td>3.04x10$^{-7}$</td>
<td>3.41x10$^{-6}$</td>
<td>3.39x10$^{-6}$</td>
</tr>
<tr>
<td>9.0</td>
<td>1.40</td>
<td>1.44</td>
<td>1.40</td>
<td>2.01</td>
<td>1.96</td>
<td>1.48x10$^{-7}$</td>
<td>1.52x10$^{-7}$</td>
<td>1.29x10$^{-7}$</td>
<td>1.52x10$^{-6}$</td>
<td>1.51x10$^{-6}$</td>
</tr>
</tbody>
</table>

$^a$ Using 10% correction on entropy and $\Delta G$ with $E_m$ (FADH$^-$/FADH$^+$) measured in D$_2$O.

$^b$ Using 10% correction on entropy and $\Delta G$ with reduction potentials measured in H$_2$O.

$^c$ Using entropy and $\Delta G$ as in H$_2$O.

$^d$ Without entropy term and $\Delta G$ with reduction potentials measured in H$_2$O.

$^e$ Without entropy term and $\Delta G$ with $E_m$ (FADH$^-$/FADH$^+$) measured in D$_2$O.

Figure 13. pH-dependency of reorganization energy resulting from various analyses on D$_2$O data measurements.
Effect of quantum correction on reorganization energy and electronic coupling matrix element. The results shown in Table 3 and Figure 11 represent the analysis of the data using the semi-classical Hopfield expression including the entropy correction where the value of 25 meV was used for $h\omega$. We were not able to fit the data with $h\omega = 200$ meV. When a value of $h\omega = 70$ meV was used, $\lambda$ and $H_{AB}$ were unrealistically high (e.g., 4 eV $\leq \lambda \leq$ 7 eV). An excellent fit to the data (indistinguishable from the classical fit) was obtained using $h\omega = 25$ meV with values of $\lambda$ and $H_{AB}$ that are slightly higher compared to those from the classical Marcus expression. However, the trends that are observed for $\lambda$ and $H_{AB}$ versus pH are the same as those observed with the classical Marcus expression. Even smaller values of $h\omega$ such as 20 meV, 15 meV, 10 meV, and 1 meV were tested where $\lambda$ and $H_{AB}$ get closer to the values obtained with the classical Marcus expression. When a fit with $h\omega$ as a free parameter was performed the value for it was extremely small ($10^{-3} - 10^{-4}$ meV). The analysis of the data obtained in D$_2$O with the semi-classical Hopfield expression ($h\omega = 25$ meV) shows the same trend observed with the classical Marcus expression; $\lambda$ is slightly lower in D$_2$O than in H$_2$O and decreases as a function of pD. However, the values of $\lambda$ and $H_{AB}$ in D$_2$O are slightly higher for the semi-classical Hopfield case compared to the classical Marcus case (Table 3).

Stability of FADH' and FADH- as a function of pH(D). It was assumed that FADH' and FADH- are stable over the entire pH(D) range of our study. However, the FAD-cofactor could change the protonation state within the pH(D) range of our study due to flavin hydroquinone pK$_a$ of 6.7 and the flavin radical semiquinone pK$_a$ around 8.5 (70, 71). For that reason the stability of FADH' and FADH- in photolyase was tested as a function of pH and the possible change of reaction equilibrium in flavoproteins is shown
is Figure 14. Figure 15 shows absorption spectra of *E. coli* photolyase at pH 5.5 and pH 9.0; FADH* is observed throughout the entire pH- and pD- (data not shown) range. A small amount of oxidized FAD at higher pH values we noticed, however no evidence for the anionic radical semiquinone (FAD ̈) was observed. If any FAD ̈ is formed, it may rapidly decay to oxidized FAD. The resonance Raman spectra of FADH* in *E. coli* photolyase obtained by Carlos Lucero in the pH range from 5.5 to 9.0 support this finding and are identical to those reported before (Figure 15) (53).

![Diagram](attachment:image.png)

**Figure 14.** Possible change of reaction equilibrium in flavoproteins.
**Figure 15.** Absorption spectra of the FADH* in DNA photolyase at pH 5.5 (dashed line) and pH 9.0 (solid line). Inset: Resonance Raman spectra of FADH* in DNA photolyase at pH 5.5, 6.0, 7.0, 8.0, and 9.0.

The difference in absorbance between FADH$_2$ at pH 5.5 and FADH* at pH 8.0 (Figure 16, inset) was demonstrated by taking the absorption spectrum of reduced FAD in solution, which is also in agreement with the spectra presented in a literature (71). The reduced photolyase with photodecomposed MTHF, presents no divergence between the spectra of pH 5.5 and 9.0 (Figure 16). The pK$_a$ occurrence around 6.5 has an effect on the equilibrium of FADH$_2$⇌FADH*+H$^+$ for other flavoproteins but not photolyase. The emission spectrum also confirms no change occurring in photolyase with variation in pH. The protein environment in photolyase keeps the neutral radical and the reduced flavin the same even through the transition states near the two pK$_a$'s indicated above.
The analysis of the experiments showed that the reorganization energy of the charge recombination reaction between FADH$^{-}$ and Trp$_{306}^ -$ in photolyase is pH dependent. At lower pH, the reorganization energy is around 2.0 (2.3) eV, and it decreases to about 1.3 (1.5) eV at higher pH (Table 3). The values in the parentheses represent the result of analysis where semi-classical Hopfield equation was used and this
notation will be used for the remainder of Part I. Either a change in the structure of the reactants and/or the products or a change in the mechanism of charge recombination could give rise to the pH-dependence. No change in protonation state of $^{306}\text{TrpH}$ and $^{306}\text{Trp}^*$ is expected because the pK$_a$ values of TrpH and TrpH$^{++}$ of about 17 and 3.7, respectively, (22, 34) lie outside the pH range of our study. Also the stability of the protonation states of FADH$^+$ and FADH$^-$ in photolyase over the pH range of this study was confirmed by the absorption and resonance Raman spectra. Therefore, we rule out that changes to the structures of the reactants and products of charge recombination reaction are responsible for the pH-dependence of the reorganization energy.

Global analysis was used to test the Byrdin et al. and our model, to ascertain that the pH-dependence of the reorganization energy of the charge recombination is due to a change in mechanism. In the model that was proposed by Byrdin et al. $^{306}\text{TrpH}^{++}$ and $^{306}\text{Trp}^*$ are explicitly included as electron acceptors (Figure 5B) (20). The charge recombination in the Byrdin model which we will refer to as the ET-model occurs from FADH$^-$ to $^{306}\text{TrpH}^{++}/^{306}\text{Trp}^*$ depending on the pH and with a pK$_a$ of 4. Equation 9a describes the pure electron transfer between FADH$^-$ and $^{306}\text{TrpH}^{++}$ and is labeled ET(I). The charge recombination between FADH$^-$ and $^{306}\text{Trp}^*$ follows ETPT mechanism in which rate-limiting electron transfer is followed by a proton transfer step at high pH. This mechanism is referred to as ET(II) and described by Equation (9b). The second model is labeled as the CEPT-model, where based on the high reorganization energy value a CEPT mechanism is proposed by us to dominate at low pH (Figure 5C) (34) and is described by Equation 9c. At the higher pH values, ETPT takes place where a rate-
limiting electron transfer between \( \text{FADH}^- \) and \( ^{306}\text{Trp}^* \) is followed by a proton transfer, which is the same as ET(II).

\[
\text{ET(I)}: \quad \text{FADH}^- + ^{306}\text{TrpH}^{+*} \rightarrow \text{FADH}^* + ^{306}\text{TrpH} \quad \text{Equation 9a}
\]

\[
\text{ET(II)}: \quad \text{FADH}^- + ^{306}\text{Trp}^* \rightarrow \text{FADH}^* + ^{306}\text{Trp}^- \quad \text{Equation 9b}
\]

\[
\text{CEPT}: \quad \text{FADH}^- + ^{306}\text{Trp}^* + \text{H}^+ \rightarrow \text{FADH}^* + ^{306}\text{TrpH} \quad \text{Equation 9c}
\]

The two models differ in the mechanism at the lower pH (ET vs. CEPT) but also in the origin of the pH-dependence of the charge recombination rate constant. In the ET-model, this pH-dependence is explained by the equilibrium between \( ^{306}\text{TrpH}^{+*} \) and \( ^{306}\text{Trp}^* \) with a \( pK_a \) of about 4 (20). In our CEPT-model, it originates from the pH-dependence of the thermodynamic parameters that affect the CEPT rate constant.

The data from the experiments done in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \) in the pH(D) range of 5.5 to 10.0 was used to test the two proposed models for charge recombination in DNA photolyase. The thermodynamic parameters that were used for each model are listed in Table 5.

**Table 5.** Thermodynamic parameters used for the global analysis fit to the two models in H\(_2\)O and D\(_2\)O solution.

<table>
<thead>
<tr>
<th></th>
<th>( E_m^0(\text{FADH}^-/\text{FADH}^*) ) (mV)</th>
<th>( E_m^0(\text{Trp}^*/\text{TrpH}) ) (mV)</th>
<th>( \Delta S_{\text{uptake}} ) (eV/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEPT</td>
<td>12.25 (45)(^a)</td>
<td>1070 (-[\text{pH}(D) - pK_a] \cdot 53)^b</td>
<td>(-R\ln(10)pH(D))</td>
</tr>
<tr>
<td>ET(I)</td>
<td>12.25 (45)(^a)</td>
<td>1070 (1070)(^a)</td>
<td>0</td>
</tr>
<tr>
<td>ET(II)</td>
<td>12.25 (45)(^a)</td>
<td>365 (395)(^a)</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\)Values for \( \text{D}_2\text{O} \) are given between parentheses; \(^b\)\( pK_a = 3.7 \) and 4.26 in \( \text{H}_2\text{O} \) and \( \text{D}_2\text{O} \) solutions, respectively.

The pH(D) and temperature dependence of the free energy change of the reaction is only required by the CEPT mechanism (Eq. 9c). Because no proton uptake is involved in the
pure ET mechanisms, ET(I) and ET(II), the pH(D)-dependence of reduction potential of
the tryptophan, and the temperature dependence can be eliminated causing no change in
the entropy. \( H_{AB} \) and \( \lambda \) are global fit parameters for each mechanism as the reactants and
products stay the same for each specific mechanism within the pH(D)-range. The
fraction of charge recombination following the low pH(D) mechanism, CEPT, \( x \), is a
local fit parameter, while \( x = \left[ 1 + 10^{\left( pD - pK_a \right)} \right]^{-1} \) is used to describe the fraction following
ET(I) in the ET-model with the pK\(_a\) as a global fit parameter (20). All other
thermodynamic parameters are determined from experimental and literature values and
are kept constant with the exception of the \( E_m^0(\text{Trp}^*/\text{TrpH}) \) and \( \Delta S_{\text{uptake}} \) pH(D)-dependent
values for the CEPT mechanism. \( E_m^0(\text{TrpH}^*/\text{TrpH}) = 1070 \) mV is used for ET(I), (22)
and, by using Eq. 7, \( E_m^0(\text{Trp}^*/\text{Trp}^-) = 365 \) mV for the ET(II) mechanism with pH = 17
(the pK\(_a\) of TrpH has been estimated to be 17) (34). The rate constant of charge
recombination, \( k_{CR} \), for the ET-model and the CEPT-model at a given pH is described in
the global analysis using the following two expressions:

**ET-model:** \[ k_{CR} = x \cdot k_{ET(I)} + (1 - x) \cdot k_{ET(II)} \] \hspace{1cm} \text{Equation 10a}

**CEPT-model:** \[ k_{CR} = x \cdot k_{CEPT} + (1 - x) \cdot k_{ET(II)} \] \hspace{1cm} \text{Equation 10b}

The results of the global analysis of the data in H\(_2\)O and D\(_2\)O solutions with both the
classical Marcus expression (Eq. 1) and the semi-classical Hopfield expression (Eq. 3) for
electron transfer are shown in Tables (6) and (7), and the fits to both models are shown in
Figure 16. Both models fit well to the data; however the CEPT model with the semi-
classical Hopfield expression provides the best overall result. For the ET-model, a pK\(_a\) of
5.8 and 5.0 is found for H\(_2\)O and D\(_2\)O, respectively, while the CEPT-model shows a
transition between mechanisms (\( x = 0.5 \)) around pH(D) 6.5 and 6.0 for classical Marcus
and semi-classical expressions, respectively. All these values deviate from the one proposed by Byrdin et al., who used a $pK_a = 4.0$, the value for TrpH in solution, for the ET-model (20).

Table 6. Results of the global analysis fit of the two models to the pH/D-dependent charge recombination rate constants, and calculated charge recombination rate constants and deuterium isotope effects (KIE). Results are shown for both classical Marcus and semi-classical Hopfield electron transfer theory.\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>H(_2)O</th>
<th></th>
<th></th>
<th></th>
<th>D(_2)O</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\lambda$ (eV)</td>
<td>$H_{AB}$ (10(^{-6}) eV)</td>
<td>$\Delta G^\pm$ (eV)</td>
<td>$k$ (s(^{-1}))</td>
<td>$\lambda$ (eV)</td>
<td>$H_{AB}$ (10(^{-6}) eV)</td>
<td>$\Delta G^\pm$ (eV)</td>
<td>$k$ (s(^{-1}))</td>
</tr>
<tr>
<td><strong>CEPT-model</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEPT</td>
<td>2.09</td>
<td>5.15</td>
<td>$c$</td>
<td>$c$</td>
<td>1.96</td>
<td>7.01</td>
<td>$c$</td>
<td>$c$</td>
</tr>
<tr>
<td></td>
<td>(2.31)</td>
<td>(10.5)</td>
<td></td>
<td></td>
<td>(2.12)</td>
<td>(11.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET(II)</td>
<td>1.05</td>
<td>0.51</td>
<td>0.115</td>
<td>39.1</td>
<td>1.02</td>
<td>0.41</td>
<td>0.110</td>
<td>31.0</td>
</tr>
<tr>
<td></td>
<td>(1.25)</td>
<td>(1.06)</td>
<td>(0.162)</td>
<td>(37.3)</td>
<td>(1.19)</td>
<td>(0.74)</td>
<td>(0.149)</td>
<td>(30.2)</td>
</tr>
<tr>
<td><strong>ET-model (pK(_a) = 5.8)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ET(I)</td>
<td>2.68</td>
<td>41.1</td>
<td>0.246</td>
<td>752</td>
<td>2.47</td>
<td>71.1</td>
<td>0.212</td>
<td>9520</td>
</tr>
<tr>
<td></td>
<td>(2.98)</td>
<td>(96.5)</td>
<td>(0.310)</td>
<td>(751)</td>
<td>(2.72)</td>
<td>(145)</td>
<td>(0.265)</td>
<td>(9480)</td>
</tr>
<tr>
<td>ET(II)</td>
<td>1.53</td>
<td>5.89</td>
<td>0.226</td>
<td>43.6</td>
<td>1.60</td>
<td>7.32</td>
<td>0.243</td>
<td>34.0</td>
</tr>
<tr>
<td></td>
<td>(1.78)</td>
<td>(13.3)</td>
<td>(0.286)</td>
<td>(45.4)</td>
<td>(1.70)</td>
<td>(8.36)</td>
<td>(0.268)</td>
<td>(35.6)</td>
</tr>
</tbody>
</table>

\(^a\)Results for semi-classical expression with $\hbar \omega = 25$ meV are given between parentheses; \(^b\) $\Delta G^\pm = (\Delta G^{0h} + \lambda)^2/4$; \(^c\) These values are pH/D-dependent for the CEPT mechanism and are listed in Table 7.
Figure 17. Results of global analysis fits of the CEPT- (A, B) and ET- (C, D) models to the pH- and temperature-dependence of the charge recombination rate constants. Either classical Marcus (A, C) or semi-classical Hopfield (B, D) electron transfer theory was used. For the analysis with the semi-classical electron transfer theory, $h\Omega_o$ was set to 25 meV. For clarity, only the data and fits (solid lines) for pH 5.5 – 9.0 are shown.
Table 7. Results for the pH-dependent parameters of the global analysis fit for the CEPT-mechanism. Results are shown for analysis with classical Marcus and the semi-classical Hopfield electron transfer theory.\textsuperscript{a}

<table>
<thead>
<tr>
<th>pH/D</th>
<th>$x$</th>
<th>$\Delta G^\ddagger$ (eV)\textsuperscript{b}</th>
<th>$k$ (s\textsuperscript{-1})</th>
<th>$x$</th>
<th>$\Delta G^\ddagger$ (eV)\textsuperscript{b}</th>
<th>$k$ (s\textsuperscript{-1})</th>
<th>KIE</th>
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<tbody>
<tr>
<td>5.5</td>
<td>1.0</td>
<td>0.156</td>
<td>533</td>
<td>0.80</td>
<td>0.130</td>
<td>2900</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>(0.81)</td>
<td>(0.199)</td>
<td>(641)</td>
<td>(0.94)</td>
<td>(0.163)</td>
<td>(3220)</td>
<td>(0.20)</td>
</tr>
<tr>
<td>6.0</td>
<td>0.82</td>
<td>0.163</td>
<td>389</td>
<td>0.40</td>
<td>0.138</td>
<td>2150</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>(0.66)</td>
<td>(0.208)</td>
<td>(0.225)</td>
<td>(0.47)</td>
<td>(0.171)</td>
<td>(2400)</td>
<td>(0.20)</td>
</tr>
<tr>
<td>7.0</td>
<td>0.26</td>
<td>0.179</td>
<td>203</td>
<td>0.09</td>
<td>0.153</td>
<td>1160</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td>(0.21)</td>
<td>(0.225)</td>
<td>(248)</td>
<td>(0.10)</td>
<td>(0.187)</td>
<td>(1310)</td>
<td>(0.19)</td>
</tr>
<tr>
<td>8.0</td>
<td>0.20</td>
<td>0.196</td>
<td>103</td>
<td>0.03</td>
<td>0.169</td>
<td>606</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>(0.16)</td>
<td>(0.242)</td>
<td>(128)</td>
<td>(0.03)</td>
<td>(0.204)</td>
<td>(694)</td>
<td>(0.18)</td>
</tr>
<tr>
<td>9.0</td>
<td>0.45</td>
<td>0.213</td>
<td>50.7</td>
<td>0.02</td>
<td>0.185</td>
<td>306</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>(0.33)</td>
<td>(0.261)</td>
<td>(64.0)</td>
<td>(0.01)</td>
<td>(0.221)</td>
<td>(359)</td>
<td>(0.18)</td>
</tr>
<tr>
<td>9.5</td>
<td>0.29</td>
<td>0.222</td>
<td>35.1</td>
<td>$c$</td>
<td>$c$</td>
<td>$c$</td>
<td>$c$</td>
</tr>
<tr>
<td></td>
<td>(0.08)</td>
<td>(0.270)</td>
<td>(44.9)</td>
<td>$c$</td>
<td>$c$</td>
<td>$c$</td>
<td>$c$</td>
</tr>
<tr>
<td>10.0</td>
<td>0.0</td>
<td>0.231</td>
<td>24.2</td>
<td>0.03</td>
<td>0.203</td>
<td>150</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>(0.13)</td>
<td>(0.279)</td>
<td>(31.3)</td>
<td>(0.0)</td>
<td>(0.239)</td>
<td>(180)</td>
<td>(0.17)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Results for semi-classical expression with $\hbar \omega = 25$ meV are given between parentheses; $\Delta G^\ddagger = (\Delta G^0 + \lambda)^2/4\lambda$; \textsuperscript{b}No data recorded at pH = 9.5.

For the analysis with the classical Marcus expression, the CEPT-model gives rate constants for ET(II) of 39 s\textsuperscript{-1} and 31 s\textsuperscript{-1} in H\textsubscript{2}O and D\textsubscript{2}O, respectively. These values are in good agreement with those predicted by Byrdin et al.; $k_2 = 43$ s\textsuperscript{-1} and 30 s\textsuperscript{-1} in H\textsubscript{2}O and D\textsubscript{2}O, respectively, for ET(II) (20). The ET-model also predicts consistent rate constants for ET(II); 44 s\textsuperscript{-1} and 34 s\textsuperscript{-1} in H\textsubscript{2}O and D\textsubscript{2}O, respectively. Byrdin et al. proposed a rate constant of $k_1 = 40,000$ s\textsuperscript{-1} for ET(I), (20) while both the ET-model and the CEPT-model give a much lower rate constant of 750 and 530 s\textsuperscript{-1} for ET(I) and CEPT, respectively, though the CEPT rate constant could reach 1,500 s\textsuperscript{-1} at pH = 4. Finally, the value of $H_{AB}$
for ET(I) in the ET-model is 8-times larger than the one for CEPT, and $H_{AB} = 5.2 \cdot 10^{-6} \text{eV}$ for CEPT is very close to a calculated value of $H_{AB} = 6.2 \cdot 10^{-6} \text{eV}$ for ET from $^{306}\text{TrpH}$ to $\text{FADH}^\cdot$ in $E. \text{coli}$ photolyase (72).

When using the semi-classical Hopfield expression with $h\omega = 25 \text{meV}$ for analysis, $\lambda$ resulted in a 0.2 to 0.3 eV increase, and $H_{AB}$ increased by a factor of 2 as compared to the analysis with the classical Marcus expression (Table 6 and 7). A similar trend between the values of $h\omega$, $\lambda$, and $H_{AB}$ was observed for ET between cytochrome $c_2$ and the $\text{Rhodobacter sphaeroides}$ reaction center (RC) (29). The semi-classical analysis produced slightly lower rate constants for ET(II) in H$_2$O and D$_2$O for the CEPT-model and a little higher for the ET-model. The value of the CEPT rate constant increases to $641 \text{s}^{-1}$ ($1,860 \text{s}^{-1}$ at pH 4.0), and the rate constant for ET(I) stays the same as for the analysis with the classical Marcus expression. These values are still well below the $40,000 \text{s}^{-1}$ that was proposed by Byrdin et al. (20) The value of $H_{AB}$ for ET(I) is 9-times larger than for CEPT. In the latter case, the value of $H_{AB} = 10.5 \cdot 10^{-6} \text{eV}$ in H$_2$O and is still very close to the calculated value of $6.2 \cdot 10^{-6} \text{eV}$ (72). CEPT-model describing charge recombination in $E. \text{coli}$ photolyase is strongly supported by our results obtained either by the classical or semi-classical expression.

The rate constant of ET(II) at high pH is in an excellent agreement between the two models presented. However, the rate constant found at low pH is much higher in the Byrdin et al. model than in the CEPT-model presented by our group. They estimate $k_1 = 40,000 \text{s}^{-1}$ for ET(I), (20) and we find $750 \text{s}^{-1}$ for ET(I) with $pK_a = 5.8$ and $1,500 \text{s}^{-1}$ ($1,860 \text{s}^{-1}$) for CEPT at pH 4.0. This large discrepancy is most likely due to the fact that ET(I) in the earlier work only accounts for 3% or less of the observed rate over the pH-
range (20). Thus, the rate constant \( k_1 = 40,000 \text{ s}^{-1} \) is based on a very small part of the data set and represents a miniscule contribution to the observed rate at \( \text{pH} \geq 5.5 \). Because of that the rate constant presented by Byrdin et al. carries a very large uncertainty with it. On the other hand, our analysis shows the results where CEPT (Table 7) and ET(I) contribute up to 80\% and 66\% to the observed rate (pH 5.5), respectively. In order to determine the values a much larger data set was analyzed based on the pH and temperature dependence of the rate constants. Classical and semi-classical expressions for electron transfer theory were used with literature data to determine \( \Delta G^0 \) in order to obtain \( \lambda \) and \( H_{AB} \). Based on all the restrictions applied, our approach results in a more accurate value of the rate constant. The significantly overestimated value presented by Byrdin et al. could easily come from the less restricted analysis. When the pK\(_a\) was kept fixed at 4 in the global analysis of the ET-model, we did find a much larger ET(I) rate constant of 15,000 s\(^{-1}\). However, the resulting fit to the data gave a \( \chi^2 \) value that is 15-times larger than any of the other fits, and this result was rejected.

**CEPT-model vs. ET-model.** From the work by Hammarström and coworkers we learned that reorganization energy is an important parameter in distinguishing between different electron transfer mechanisms (34). Knowledge of this allowed us to test for a CEPT mechanism in the charge recombination reaction in DNA photolyse. The analysis of the ET-model gave unusually high \( \lambda \) of 2.7 (3.0) eV for the ET(I) mechanism. Pure ET reactions that occur in water or other polar solvents did result in such large reorganization energies; however that was never a case for ET in a protein (28, 29, 31, 61, 73). Generally, pure ET reactions have reorganization energies between 0.7 and 1.3 eV (31, 61). Also the reorganization energy of 1.5 (1.8) eV that was predicted for ET(II)
in the ET-model is high compared to other reported values. In addition, when the ET-model is evaluated with a fixed pKₐ of 4, which is favored by Byrdin et al., or with the fraction x as a free parameter as for the CEPT-model, the reorganization energies remain high (data not shown); values of 2.7 to 2.8 eV and 1.2 to 1.3 eV are found for ET(I) and ET(II), respectively.

The CEPT model, provides reorganization energies of \( \lambda = 2.1 \ (2.3) \text{ eV} \) and 1.1 (1.3) eV for the CEPT and ET(II) mechanisms, respectively. The reorganization energy value of 2.4 eV observed for CEPT in Ru-Tyr model compounds and in hydrogen-bounded phenols where \( \lambda = 1.0 - 2.4 \text{ eV} \) show a little higher value than the one observed for CEPT in our model (34, 74). However, the lower \( \lambda \) value for CEPT in charge recombination in photolyase is expected and appears reasonable because of the smaller solvent-contributions to the outer sphere reorganization energy by CEPT and ET reactions in proteins.

In the CEPT model the reorganization energy for the ET(II) mechanism has a value of 1.1 (1.3) eV and is in the expected range for pure protein electron transfer (31, 61). The literature values obtained by experiments and calculations for a pure electron transfer with either tryptophan or flavin as one of the reactants involve \( \lambda \) of 1.1 – 1.2 eV (75-77) and 1.0 – 1.4 eV (78-82), respectively. In ET with flavin as one of the reactants reorganization energies as high as 1.8 and 2.2 eV were measured where they were assigned to electrostatics in the active site and to required conformational changes, respectively (83, 84). Calculations and experiments on photoinduced ET between a flavin and tryptophan predict a \( \lambda \) of 0.7 – 2.1 eV (85, 86). In these studies \( \lambda \) values larger than 1.3 eV are mainly a result of solvent contributions to the outer sphere reorganization...
energy and less likely to involvement of the flavin excited state. In ET that involves flavin, tryptophan, or both, average reorganization energy of 1.2 eV is expected. CEPT model predicts a very close $\lambda$ value for the ET(II) mechanism.

The CEPT-model is strongly supported by the analysis with either the classical Marcus theory or with the semi-classical Hopfield theory where the value of reorganization energy allowed discriminating between the PCET mechanisms. Therefore, our analysis provides a far more realistic description of charge recombination reaction in *E. coli* photolyase and gives the favored model. The global analysis was only used as a convenient tool where the two possible models could be evaluated simultaneously. As a result we obtained well defined reorganization energy values, whereas the values of $H_{AB}$ and $x$ were sensitive to variations. $H_{AB}$ and $\lambda$ varied significantly in the analysis of the ET(II) mechanism for ET-model in D$_2$O experiment resulting in large, unjustified KIE values (~10). However, we did not encounter this problem with the analysis of the CEPT-model. Although the $H_{AB}$ and $x$ values varied for the global analysis, reorganization energy values did not deviate more than 0.05 eV.

For the CEPT-model a slightly improved fit compared to the classical Marcus theory was obtained with semi-classical Hopfield analysis where a value of 25 meV was used for $h\omega$ (Figure 17). An increase by 0.2 eV in the values of $\lambda$ and a 2-fold increase in $H_{AB}$ evolved from the semi-classical analysis. Although a value of $h\omega = 70$ meV is more common, (87) values of 25 meV or less have been reported before, e.g., in electron transfer between cytochrome c$_2$ and the *Rb. sphaeroides* photosynthetic RC in and within that RC (29, 88). The value of $h\omega = 25$ meV corresponds to a temperature of 290 K (17 °C), which falls in the middle of the temperature range of our study. Smaller values of
hω resulted in minimal or no improvement in the quality of the fits, but they did bring the values of λ and H_{AB} closer to those obtained with the classical Marcus theory. This suggests that the charge recombination process in *E. coli* photolyase in the temperature range of this study is described almost equally well by the classical Marcus theory and the semi-classical Hopfield theory and that electron transfer may only be coupled to low-frequency nuclear motions. A similar conclusion was reached for electron transfer between cytochrome *c*₂ and the *R. sphaeroides* RC (29).

*The deuterium isotope effect on charge recombination.* Based on the large reorganization energy, the CEPT-model is strongly favored for the charge recombination reaction in H₂O and D₂O for pH(D) < 7.0. However, occurrence of KIE is also an important indicator of PCET. In our model, the ET(II) mechanism demonstrates a rather small KIE of 1.26, which in fact is in agreement with values reported for pure ET and rate-limiting ET followed by PT (89, 90). However, the expected KIE > 1 is not observed for the CEPT mechanism (38, 42, 36, 34, 74, 91-93). Instead, an 'inverse' KIE is observed below pH(D) 8.0. The lack of large KIE can be caused by the solvent isotope effects on the thermodynamic parameters that may be masking the KIE that is associated with the mass of the deuteron. The earlier study by Byrdin et al. also reported the 'inverse' KIE and it has been observed in pure electron-transfer in proteins (20, 94).

The physical and chemical properties of D₂O are well documented to be different from those of H₂O; these differences can affect the physicochemical properties of molecules that are dissolved in these solvents (95, 96). Each term that is affecting the rate constant was carefully examined for its contribution to the observed 'inverse' KIE. The pre-exponential factor in both expressions, classical and semi-classical is mainly
dependent on $H_{\text{AB}}$, where the exponential factor is determined by the activation energy, $\Delta G^\ddagger$. Theoretical descriptions of PCET include a separate term for the proton transfer that factors in the mass of the proton (36, 38, 39). Such a factor is larger in H$_2$O than in D$_2$O and does not explain the observed 'inverse' KIE. Therefore, we will focus on $H_{\text{AB}}$ and $\Delta G^\ddagger$.

$H_{\text{AB}}$ contains the strength of the electronic coupling which decays with donor-acceptor distance (28, 61). The strength of the electronic coupling depends upon the electronic/molecular orbitals that are involved in the electron transfer and the energy differences between them. Since the absorption spectrum of the FAD cofactor is the same in H$_2$O and D$_2$O solutions, the (differences between) energy levels of the FAD cofactor are not sensitive to the isotope effect. Although such data are not available for $^{30}$TrpH and $^{30}$Trp', and we do not expect any significant change in their energy levels either. Although the solvent cavity may be slightly smaller in D$_2$O, we do not expect that this has any appreciable effect on the distance between the electron donor and acceptor in photolyase. A smaller ET distance could slightly increase $H_{\text{AB}}$ in D$_2$O solutions (31, 61, 94) but this effect is not observed for $H_{\text{AB}}$ of the ET(II) mechanism for which the global analysis predicts $H_{\text{AB}}$(D$_2$O) < $H_{\text{AB}}$(H$_2$O). Therefore, we expect the effect of D$_2$O on the electronic coupling matrix element to be insignificantly small with $H_{\text{AB}}$(D$_2$O) $\approx$ $H_{\text{AB}}$(H$_2$O). This is in agreement with the results from the global analysis for the CEPT-model which has $H_{\text{AB}}$(D$_2$O) $\approx$ 1.1 to 1.4 times $H_{\text{AB}}$(H$_2$O) (Table 6).

The activation energy, $\Delta G^\ddagger$, is the most likely source of the observed 'inverse' KIE and is given by (28):

$$\Delta G^\ddagger = (\Delta G^{0\ddagger} + \lambda)^2 / 4\lambda.$$  

Equation 11
Following Eqs. 5 and 6, it has contributions from \( \lambda \), \( \Delta S_{\text{uptake}} \), \( E_m^0(\text{FADH}^+/\text{FADH}^-) \), and \( E_m^0(\text{Trp}^+/\text{TrpH}) \). The reorganization energy, \( \lambda \), consists of an inner sphere contribution, \( \lambda_i \), and an outer sphere contribution (solvent reorganization), \( \lambda_o \) (28). The reactants and products are the same in H\(_2\)O and D\(_2\)O except for the exchangeable protons; thus, \( \lambda_i \) is likely unchanged. From dielectric continuum theory, the outer sphere contribution is given by (28):

\[
\lambda_o = (\Delta e)^2 \left[ \frac{1}{2a_1} + \frac{1}{2a_2} - \frac{1}{r} \right] \left[ \frac{1}{D_{op}} - \frac{1}{D_s} \right],
\]

Equation 12

with \( \Delta e \) the charge that is transferred, \( a_1 \) and \( a_2 \) the radii of the two (spherical) reactants, \( r \) the center-to-center distance between reactants, and \( D_s \) and \( D_{op} \), the relative dielectric constant (\( \varepsilon_r \)) and the optical dielectric constant (square of the refractive index, \( n \)) of the solvent, respectively.

At 20 °C, the values of \( \varepsilon_r \) and \( n \) are 80.21 and 1.333 and 79.89 and 1.328 for H\(_2\)O and D\(_2\)O, respectively, (97-99) to give a \( \lambda_o \) that is 0.7% larger in D\(_2\)O than in H\(_2\)O. A potentially smaller solvent cavity in D\(_2\)O may slightly reduce the center-to-center distance between FAD and \( ^{306}\text{Trp} \), and the first term of \( \lambda_o \) could be somewhat smaller in D\(_2\)O with \( a_1 \) and \( a_2 \) assumed constant. The likely net result would be a slightly larger reorganization energy in D\(_2\)O than in H\(_2\)O. However, the global analysis finds a smaller \( \lambda \) in D\(_2\)O than in H\(_2\)O, with the \( \lambda \) in D\(_2\)O appearing to be underestimated by the global analysis fit. This result is most likely due to an underestimation of the effect of D\(_2\)O on \( \Delta G^0' \).

\( \Delta G^0' \) depends on \( \Delta S_{\text{uptake}} \) and the reduction potentials of FADH\(^+\) and TrpH. The entropy term included in our analysis only takes into account the cratic (mixing) contribution of the change in entropy due to proton uptake (\( \Delta S_{\text{uptake}} \)), which is the same in
H₂O and D₂O solutions for properly adjusted pH and pD values (Eq. 4). The much smaller unitary contribution (solute-solvent interactions) to $\Delta S_{\text{uptake}}$ is not considered. Since hydrogen bonding interactions are thought to increase by 10% in D₂O, the unitary term may be up to 10% larger in D₂O (58, 59). This would cause $\Delta S_{\text{uptake}}$ to be slightly smaller (less negative) in D₂O than in H₂O and would give rise to a small increase in both $\Delta G^{0'}$ and $\Delta G^\ddagger$, and, therefore, to a small decrease in $k_{\text{CEPT}}$ in D₂O. The increase of $E_m^0(\text{FADH}^\gamma/\text{FADH}^\gamma)$ in photolyase by 45 ± 6 mV in D₂O solutions also leads to an increase in $\Delta G^\ddagger$ and a decrease of $k_{\text{CEPT}}$ in D₂O. Therefore, the solvent isotope effects on $\Delta S_{\text{uptake}}$ and $E_m^0(\text{FADH}^\gamma/\text{FADH}^\gamma)$ cannot explain the observed 'inverse' KIE.

Finally, we consider the effect of D₂O on $E_m^0(\text{Trp}^\gamma/\text{TrpH})$. In our analysis, we have taken into account the fact that the pKₐ of a weak acid increases between 0.4 and 0.8 in D₂O (65-67) and the pKₐ of $^{306}\text{TrpH}^{\text{**}}$ has been proposed to increase by 0.56 in D₂O (20). Such an increase introduces a ‘horizontal’ shift in the pH(D)-dependence of $E_m^0(\text{Trp}^\gamma/\text{TrpH})$ in D₂O (Eq. 8) and causes an increase in $E_m^0(\text{Trp}^\gamma/\text{TrpH})$ of about 30 mV at pD values above its pKₐ. This would result in a more negative $\Delta G^{0'}$ and in a decrease in $\Delta G^\ddagger$ which, in turn, leads to an increase in $k_{\text{CEPT}}$. The effect is canceled out by the +45 mV increase measured in $E_m^0(\text{FADH}^\gamma/\text{FADH}^\gamma)$. Therefore, $E_m^0(\text{Trp}^\gamma/\text{TrpH})$ must increase by more than 45 mV to cause a more negative $\Delta G^{0'}$ and a decrease in $\Delta G^\ddagger$ that would explain the increase in $k_{\text{CEPT}}$ in D₂O. Either the pKₐ of $^{306}\text{TrpH}^{\text{**}}$ increases by more than 0.56 in D₂O or $E_m^0(\text{Trp}^\gamma/\text{TrpH})$ is at least 45 mV more positive in D₂O. If some combination of these changes occur, it would result in a more negative $\Delta G^{0'}$, a decrease in $\Delta G^\ddagger$, and an increase in $k_{\text{CEPT}}$ in D₂O.
Although we cannot rule out that small, seemingly insignificant changes to $H_{AB}$, $\lambda$, and $\Delta S_{\text{uptake}}$ in D$_2$O may add up to a more substantial solvent isotope effect, the most likely cause of the 'inverse' KIE is an increase in $E_m^0(\text{Trp'}/\text{TrpH})$ in D$_2$O. This conclusion is not merely a product of the global analysis, because global analysis of the CEPT-model does properly predict the observed KIE of ET(II). An increase in $E_m^0(\text{Trp'}/\text{TrpH})$ or a larger $\Delta pK_a$ of TrpH$^{+}$ in D$_2$O would decrease the activation energy and increase the rate constant of the CEPT. On the basis of theoretical considerations and experimental observations, a KIE of 1 to 2 is expected for CEPT that involves short-range, adiabatic proton transfer (38, 42, 74, 91, 100-102). Our analysis indicates that it is possible that such a small but real KIE is masked by a solvent isotope effect on the thermodynamic parameters of the ET cofactors, resulting in the observed inverse KIE of 0.18. Therefore, the observed 'inverse' KIE of the CEPT-mechanism can be explained by the solvent isotope effect on mainly the reduction potentials of the donor and acceptor molecules, while other factors ($\lambda$, $H_{AB}$, and $\Delta S_{\text{uptake}}$) may make minor contributions. This further supports the presence of a CEPT mechanism at low pH during charge recombination in *E. coli* photolyase.

**Switching between CEPT and ET in photolyase.** The following analysis is the same whether the classical Marcus theory or the semi-classical Hopfield theory of electron transfer is used. Although $H_{AB}$ for the CEPT mechanism is constant over the pH-range, the rate constant of charge recombination for this mechanism varies with pH because of the pH-dependence of $E_m^0(\text{Trp'}/\text{TrpH})$ and the entropy correction for the proton uptake. The rate constant at 10 °C varies from 530 (640) s$^{-1}$ at pH 5.5 to 24 (31) s$^{-1}$ at pH 10 (Table 7). The pH-dependence of the CEPT rate constant has the interesting consequence
in that it becomes smaller than the pH-independent rate constant of ET(II) at pH 9.5. Although the pH-dependence of fraction $x$ (Table 7) indicates that the ET(II) mechanism becomes dominant above pH 6.5, the global analysis of the CEPT model suggests that the CEPT mechanism still contributes at higher pH values. This may be due to the limited temperature range of the experiments at higher pH values in H$_2$O experiments. The FADH* state of the enzyme was much more stable in D$_2$O experiments, and a larger temperature range could be studied. At higher pD values, the global analysis of these data shows a much smaller contribution of CEPT. Since our analysis shows that $k_{\text{CEPT}} \approx k_{\text{ET(II)}}$ around pH 9.5 and that the switch between CEPT and ET(II) occurs between pH 6 and pH 7, it is not likely that the CEPT and ET(II) mechanisms compete on a kinetic basis. Either a real switch occurs on the basis of favorable thermodynamics for one mechanism or the availability of a suitable proton donor with a pK$_a$ around 6.5 modulates the two mechanisms.

Analysis of the thermodynamic and other parameters of the CEPT and ET(II) mechanisms provides insight into the switch between the two mechanisms around pH 7. The driving force, $\Delta G^{0'}$, of the CEPT mechanism becomes less negative with increasing pH but is more favorable than $\Delta G^{0'}$ of the ET(II) mechanism which is unchanged over the pH range (Tables 6 and 7). The activation energy, $\Delta G^\circ$, of the CEPT mechanism increases with pH and is larger than that of the ET(II) mechanism over the entire pH range. The apparent switch between CEPT and ET occurs below pH 7. In that pH range, $\Delta G^{0'}$ is about 8 times larger for CEPT than for ET, and $\Delta G^\circ$ is larger for CEPT than for ET. This suggests that a delicate balance between a favorable driving force and an unfavorable activation energy may determine the switch between CEPT and ET.
mechanisms, similar to what has been proposed for the model compounds studied by Hammarström and coworkers (33, 34). Unlike the model compounds, no normal KIE is observed, and the CEPT rate constant is larger than the ET(PT) rate constant in photolyase.

The 'inverse' KIE is most likely due to modification of the thermodynamic parameters in D₂O solutions, especially, \( E_m^0(\text{Trp'}/\text{TrpH}) \) as discussed above. Since the observed 'inverse' KIE of the CEPT mechanism indicates that there may only be a small isotope effect (KIE ≈ 1 – 1.5) due to the difference in mass, there is likely only a low-barrier adiabatic proton-transfer over a short distance, and the proton donor may be hydrogen-bonded to \(^{306}\text{Trp'}\) (42, 74, 91, 100-103). This suggests that a balance between \( \Delta G^0 \) and \( \Delta G^\dagger \) may not be the only contribution to the switch and that the availability of the proton must also be considered. The crystal structure shows that there are no amino acids sufficiently close to \(^{306}\text{TrpH}\) to act as proton-donor during CEPT but a water molecule with its oxygen atom at 2.8 Å from the \(^{306}\text{TrpH}\) indole nitrogen could fulfill that role (18). The presence of a water molecule hydrogen bonded to \(^{306}\text{Trp'}\) could be tested as a function of pH by using resonance Raman spectroscopy. Resonance Raman spectra of \(^{306}\text{Trp'}\) in photolyase at pH(D) 7.4 show no evidence of a hydrogen bond to the indole nitrogen, in agreement with a switch to the ET(II) mechanism at that pH (104).

The global analysis predicts that \( H_{\text{AB}} \) is about 10 times larger for CEPT than for ET(II). Since the FAD cofactor is the same in the reactant and product states for both mechanisms, this difference would be due to tryptophan in the product state. In this state, tryptophan is TrpH and Trp\(^-\) for the CEPT and ET mechanism, respectively. This would suggest that the presence of Trp\(^-\) results in poorer electronic coupling between reactant
and product states assuming the distance between the FAD cofactor and $^{306}$TrpH does not change with pH.

Finally, $-\Delta G^0$ is significantly smaller than the reorganization energy for either mechanism. This indicates that either mechanism occurs in the Marcus normal region and will display a temperature dependent rate constant (28). A small temperature dependence of the rate constant is already observed within the temperature range of our study, and extension of our study to lower temperatures could uncover additional details about either mechanism.

**CONCLUSION**

The charge recombination between FADH$^-$ and $^{306}$-Trp in *E. coli* photolyase is characterized by a pH-dependent reorganization energy. Global analysis of the data shows that this pH-dependence is best explained by a model that predicts that charge recombination occurs through concerted electron proton transfer (CEPT) with a large reorganization energy ($\lambda = 2.1$ to 2.3 eV) below pH 7 and through rate limiting electron transfer followed by proton transfer (ETPT) with a lower reorganization energy ($\lambda = 1.1$ to 1.3 eV). The switch from the CEPT to the ET mechanism occurs at a pH of about 6.5 and is not due to a straightforward kinetic competition on the basis of the rate constants. It is most likely due to either a delicate balance between driving force ($\Delta G^0$) and activation energy ($\Delta G^+$) or the availability of a nearby proton donor. As judged from the quality of the global analysis fit, the semi-classical Hopfield theory of electron transfer provides a slightly better analysis of our results than the classical Marcus theory.

The charge recombination displays an unexpected 'inverse' isotope effect below pH 8 for the proposed CEPT mechanism. Our analysis indicated that a small, normal
KIE of about 1.4 can easily be masked by solvent isotope effects on $E_m^0(\text{FADH}^-/\text{FADH}^*)$ and $E_m^0(\text{Trp}^*/\text{TrpH})$ that lower $\Delta G^\pm$ and increase $k_{\text{CEPT}}$ in D$_2$O solutions. Since $E_m^0(\text{FADH}^-/\text{FADH}^*)$ in photolyase increases by $45 \pm 6$ mV in D$_2$O solutions, we predict that $E_m^0(\text{Trp}^*/\text{TrpH})$ increases by at least that amount. The $E_m^0(\text{Trp}^*/\text{TrpH})$ itself may increase in D$_2$O or can appear to increase as a result of an increase in the $pK_a$ of TrpH$^{+^+}$. 
Part II – Charge Recombination in VcCry-1

MATERIALS AND METHODS

**Materials.** Chemicals were purchased from Sigma-Aldrich and Acros Organics and used without further purification.

**Sample Preparation.** VcCry-1 was overexpressed, isolated, and purified as described elsewhere (57). VcCry-1 was stored in 0.4 M K₂SO₄ and 20 mM potassium phosphate (pH 7.0) at -80 °C. Samples were diluted with 0.4 M K₂SO₄, 20 mM potassium phosphate buffer at pH 7.0 for the experiments. The concentration of the flavin neutral radical and oxidized flavin with an extinction coefficient of $\varepsilon_{450} = 11,000$ M⁻¹cm⁻¹ in VcCry-1 was determined by recording the absorption spectrum on UV/Vis spectrophotometer (described on Materials and Methods section Part I) and adding the flavin concentration at 580 nm and 450 nm (5, 54, 57).

**Accumulating FADH⁺ with a use of chemicals.** 20 mM sodium dithionite (Na₂S₂O₄) was titrated into VcCry-1 samples (20 – 80 μM) with 5 μL increments for a total of 10 – 50 μL. VcCry-1 sample was incubated at a room temperature with Na₂S₂O₄ for about 30 minutes, and the absorbance spectrum was obtained to confirm the full reduction of FAD to FADH⁺. Next, 10 mM potassium ferricyanide (K₃Fe(CN)₆) was titrated into the VcCry-1 sample in order to oxidize FADH⁺ to FADH⁺.

A second method to produce FADH⁺ in VcCry-1 by using chemicals involved photoreduction of FAD with EDTA. Full oxidation of the flavin was confirmed by taking the absorption spectrum, and the sample was illuminated with white light for photoreduction of FAD to FADH⁺. Illumination of the sample was performed by the use of the Xenon lamp and in the sample box at $t = 5$ °C.
**Accumulating FADH⁺ with a use of chemicals followed by column purification.**

VcCry-1 samples where FADH⁺ was accumulated by the use of reducing/oxidizing reagents were run through silica columns (Bio-Spin 30 Tris Columns) in order to remove the excess of these chemicals.

**Accumulating FADH⁺ using UV light illumination.** VcCry-1 samples of 20-80 µM were illuminated with UV light for a duration of 2 to 5 minutes in order to obtain the highest possible concentration of FADH⁺. For the UV light illumination a UV filter was placed in the sample box in front of the cuvette holder and the light was generated by the Xenon lamp.

**Transient Absorption Measurements.** The same transient absorption spectroscopy system was used as described in Materials and Methods section in Part I. During the experiments the samples were kept at either 5 or 10 °C.

**Data Analysis.** Determining the charge recombination rate constants followed the procedure described in the Data Analysis section Part I.

**RESULTS.**

**FADH⁺ Accumulation.** In order to investigate the charge recombination reaction in VcCry-1 the way it was examined in *E. coli* photolyase, the accumulation of a higher concentration of FADH⁺ in VcCry-1 was necessary. The highest concentration of FADH⁺ was accumulated with the use of Na₂S₂O₄ and K₃Fe(CN)₆. The use of EDTA and illumination led to only a low concentration of FADH⁺ which quickly oxidized to FAD. When the VcCry-1 sample with accumulated FADH⁺ was run through a column for removing the chemicals a lot of the FADH⁺ was lost and led to its fast oxidation to FAD. The use of chemicals was not necessary with the UV light illumination method. After a
short illumination of the VcCry-1 sample the concentration of FADH' increased. However, the concentration was too low for successful transient absorption measurements. Because the highest concentration of FADH' was accumulated with the use of Na₂S₂O₄ and K₃Fe(CN)₆, that method was used for preparing the samples for the transient absorption measurements.

**Charge Recombination Rate Constants in VcCry-1.** The investigation of the charge recombination in VcCry-1 was examined the same way as in *E. coli* photolyase. Unfortunately, the pH and temperature dependence could not be observed in VcCry-1 because of the unstable FADH' at pH values other than 7.0 and at temperatures other than 5 and 10 °C.

The rate constants that were obtained from the transient absorption spectroscopy measurements with VcCry-1 sample at 5 °C and at pH 7.0 are shown in Table 8. The rate of the charge recombination reaction highly oscillates between each measurement on the same sample. However, with an increasing concentration of K₃Fe(CN)₆ in the sample, the rate constant clearly increases. The data shows that the rate constant is highly affected by the concentration of K₃Fe(CN)₆ in the VcCry-1 sample. The same observation was made for the rate of charge recombination in *E. coli* photolyase, where the rate was faster with an addition of K₃Fe(CN)₆ (data not shown).
Table 8. Rate constants for charge recombination in VcCry-1 at 5 °C and pH 7.0.

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Besides the oscillation of the rate constant caused by K₃Fe(CN)₆, the signal in the transient absorption measurements showed unusual behavior. Several transient absorption traces are shown in Figure 18 where the first trace was acquired for VcCry-1 at pH 7.0 and with FADH⁺ accumulated by the Na₂S₂O₄/K₃Fe(CN)₆ method. However, after taking the 11th measurement of the same sample, the rate constant became slower and the signal did not decay to zero. The 21st measurement of still the same sample shows a signal that decays almost to 0 but with a slower rate constant. When 5 µL of K₃Fe(CN)₆ was added to that sample, the signal showed a complete decay to zero and also a rate constant as fast as in the first measurement.
**DISCUSSION AND CONCLUSION**

The accumulation of sufficient FADH$^\cdot$ for the transient absorption experiments is the biggest obstacle in investigating the charge recombination reaction in VcCry-1. Our experiments show that the chemicals used in producing FADH$^\cdot$ highly affect the rate of the charge recombination mechanism in VcCry-1 and *E. coli* photolyase. Because of the rate oscillations, accurate analysis of the charge recombination rate constants becomes impossible at this time. From our preliminary observations, the rate of the charge recombination reaction in VcCry-1 is slower than in *E. coli* photolyase. However, a lack of the pH and temperature dependent studies on VcCry-1 hinders the examination of the mechanism that the charge recombination reaction follows.
Future experiments where higher concentrations of FADH\(^+\) are accumulated through a different method with chemicals that do not cause side reactions will be necessary for the investigation of the charge recombination reaction in VcCry-1. The similarities between *E. coli* photolyase and VcCry-1 as well as their different functions serve as an interesting point of study. Since *E. coli* photolyase provides an ideal system to study PCET, that could also be true for VcCry-1, if only an adequate way of accumulating FADH\(^+\) can be determined.
PART III – Photoreduction of FAD and Photodecomposition of MTHF in *E. coli* Photolyase, and VcCry-1.

**INTRODUCTION**

The process of photodecomposition was first described by Heelis et al. with experiments performed on *Escherichia coli* photolyase (EcPhr) (21). Their studies showed that irradiation of EcPhr with white light leads to photodecomposition of only the second cofactor, MTHF, without altering FAD. The experiments demonstrated that the photoreduction process rate is dependent on the concentration of the reducing agent; they proposed that the rate of MTHF photodecomposition by white light illumination is almost the same with and without the use of dithiothreitol (DTT). It was also noted that MTHF photodecomposition is not directly connected to changes in the FAD cofactor. However, studies of Jorns et al. on the function and interaction of MTHF in EcPhr showed that during the photobleaching of the second cofactor with yellow light the formation of FADH⁺ occurs (107). Langenbacher et al. demonstrated the difference in photoresponse between plant and animal cryptochromes as well as photolyase (51). They observed proton transfer in the photoreduction of FAD in cryptochromes but not in photolyases (51).

There are still uncertainties regarding the differences between EcPhr and *Vibrio cholerae* cryptochrome 1 (VcCry-1). Studies by Sokolova et al. on EcPhr and VcCry-1 showed that the pteridine ring of MTHF in EcPhr interacts with the protein matrix differently than VcCry-1 (50). In EcPhr the carbonyl of its pteridine ring has a stronger hydrogen bonding along with a more polar environment comparing to VcCry-1. On the other hand, in VcCry-1 the hydrogen bonding to the pteridine ring amine hydrogens is
stronger. The lack of photodecomposition of MTHF was observed in VcCry-1 and explained to be a result of the differences in hydrogen bonding of MTHF suggesting a higher stability of the cofactor in VcCry-1 compared to EcPhr. Further work on cryDASH can also reveal important information on the mechanism of the single stranded DNA repair, and how diverse it is from the DNA photolyase repair mechanism of CPD lesions on double stranded DNA. It is known that MTHF is not involved in DNA repair (21, 108), and Sokolova et al. also demonstrated that loss of MTHF does not perturb the interactions between the protein and FAD in EcPhr.

Understanding the differences and similarities between DNA photolyase and various types of cryptochrome DASH are essential for expanding the overall knowledge of their specific roles and mechanisms of action. Arabidopsis thaliana cry3 (AtCry3) is a type of cryDASH isolated from a plant. Recently, a new electron transfer pathway was proposed to exist in AtCry3 that results in photodecomposition of MTHF (109). The discovered electron transfer requires a whole tryptophan triad consisting of Trp-356, Trp-409, and Trp-432, which is also involved in FADH* photoreduction. Side-directed mutagenesis of Trp-356 to phenylalanine allowed for testing of the tryptophan triad’s role in photodecomposition of MTHF. The results demonstrated that the intact tryptophan triad is essential for efficient photobleaching of the second cofactor as well as FAD photoreduction in both DNA photolyase and AtCry3 (109). In AtCry3, it was determined that the photodecomposition of MTHF is much slower than in EcPhr. Also, the photoreduction of the flavin radical in AtCry 3 is slower than in DNA photolyase (109). The findings on MTHF photodecomposition in VcCry-1 by Sokolova et al. are different compared to studies done on AtCry3 by Moldt et al (50). Due to the fact that both
AtCry3 and VcCry-1 belong to the cryDASH family it is important to investigate the potential differences in the photodecomposition process. Other studies involving these proteins were conducted, and it is now known that accumulation of a fully reduced flavin results from blue light irradiation in AtCry3 and VcCry-1 (51).

The crystal structures of DNA photolyase and AtCry3 give a lot of information about the protein. Since the crystal structure of VcCry-1 is still not known, comparing its behavior to AtCry3 can provide significant supplemental information regarding its role and mechanism. The structures of cryptochromes such as, Arabidopsis thaliana Cry1, Arabidopsis thaliana Cry3, and Synechocystis Cry-DASH provide structural similarities to DNA photolyase. Their protein fold contains FAD in a U-shaped conformation, and the conserved tryptophans placed at the proper distances allow for electron transfer leading to photoreduction of FADH* similar to the one occurring in DNA photolyase (110).

The procedure for photodecomposition of MTHF in AtCry3 undertaken by Moldt et al. was also taken into account in order to compare the MTHF removal from VcCry-1, AtCry3, and EcPhr. Their approach also involved mutation of Trp-356 where phenylalanine was introduced which is thought to be inactive in the FAD photoreduction process in DNA photolyase; such a mutation was not pursued in the current work (109). The photoreduction and photodecomposition experiments on AtCry-3 wild type and Trp-356 mutant were conducted using blue light and ultraviolet light illuminations. The research however did not involve examining the role of reducing agents in the processes of reducing FAD and removing MTHF. Based on the experiments performed on EcPhr
and AtCry-3, it is suggested that 5,10-methenylTHF is converted to 5,10-methyleneTHF by a transfer of two electrons and one proton under UV light irradiation (109).

In this work, investigation of photoreduction of FADH' and photodecomposition of MTHF in VcCry-1 and EcPhr was undertaken. Photoreduction of the flavin radical is done in the presence of a reducing agent and by illumination with long wavelength light which FADH' absorbs (1). The MTHF cofactor can also be removed with the use of sodium borohydride (50). For the photodecomposition of the MTHF cofactor UV light is used, where FADH' does not absorb much but MTHF does (21). However, the photobleaching at 380 nm does not signify an elimination of the second chromophore but its chemical conversion (109).

In order to analyze possible effects due to temperature of the sample, chemicals added, time exposure to different wavelengths of light, and air contact; various approaches towards photoreduction of FADH'/FAD(ox) and photodecomposition of MTHF in VcCry-1 are examined.

**MATERIALS AND METHODS**

**Materials.** Chemicals were purchased from Sigma-Aldrich and Acros Organics and used without further purification.

**Sample Preparation and Experiments.** *E. coli* photolyase and VcCry-1 were overexpressed, isolated, and purified as described elsewhere (52-53, 57). Purified *E. coli* photolyase and VcCry-1 were stored in 0.4 M K$_2$SO$_4$ and 20 mM potassium phosphate (pH 7.0) at -80 °C. Samples were diluted with 0.4 M K$_2$SO$_4$, 20 mM potassium phosphate buffer at pH 7.0 for every experiment and stored in a quartz cuvette at 10 °C while being irradiated. Various methods described below were used for preparing the
samples for experiments. The sample box and lamp that were used are described in Materials and Methods of Part I. The set up used is shown in Figure 19.

**Temperature Controlled Cuvette Holder**

![Temperature Controlled Cuvette Holder](image)

**Figure 19.** The lamp and Sample Box set up used for irradiation of the samples.

**Samples exposed to oxygen, without any chemicals added.** EcPhr and Vc Cry-1 samples were diluted to 43 μM and 72 μM, respectively. Two filters were used interchangeably for photoreducing the flavin cofactor followed by photodecomposition of the MTHF cofactor from both photolyase and cryptochrome. A filter with a cut-on of 455 nm let through light above 455 nm to photoreduce the flavin radical and oxidized flavin. Next, the ultra-violet (UV) filter was used to photodecompose the MTHF cofactor. The redox changes occurring inside the protein were monitored by taking absorbance spectra at short time intervals at the beginning and then with slightly longer ones.

**Samples without oxygen and no chemicals added.** *E. coli* photolyase and VcCry-1 samples were prepared with the concentration of 45 μM in pH 7.0 buffer. The samples were sealed and purged with N₂ for about 10 minutes in order to remove any oxygen left in the sample to prevent unnecessary oxidation of the protein. The same
procedure for photoreduction of FADH* and photodecomposition of MTHF is used as with the sample that was exposed to oxygen.

**Samples without oxygen, with reducing agents.** There are different chemicals that can be used for reducing FAD/FADH* to FADH~, such as DTT, β-mercaptoethanol, or sodium dithionite. The reducing agents: dithiothreitol and β-mercaptoethanol serve as the electron donor, indirectly reducing FADH* with the use of illumination. The light initiates the following process: 

\[
\text{FADH}^* + \text{TrpH} \rightarrow \text{FADH}^~ + \text{Trp}^* + \text{H}^+ 
\]

where an agent such as DTT or β-mercaptoethanol reduces Trp* to TrpH, while trapping FADH~. The 45 μM *E. coli* photolyase and VcCry-1 samples including dithiothreitol with a final concentration of 10 mM were sealed and purged with N₂ for about 10 minutes. The photoreduction occurs much faster with the reducing agent and for that reason the illumination time has to be shortened and the absorption spectra of the sample have to be taken more often, especially at the beginning of the irradiation. After FADH* was reduced, the MTHF was photodecomposed using UV light. Due to the fact that there should not be any oxidation in the sample, the removal of the second cofactor occurs much faster as well. Unfortunately, use of any chemicals can cause side reactions that can perturb the comparison between the samples. For that reason it is important to check for any differences occurring between the samples prepared with or without oxygen and chemicals.

Sodium dithionite, which is another chemical donating an electron to FADH* directly, was utilized as a comparison to the sample reduced with dithiothreitol. As pointed out earlier, the chemicals can cause unwanted reactions to occur, disturbing the overall outcome of the experiment. Sodium dithionite reduces FADH* without irradiation
after which MTHF can be removed with UV light as in the other samples. Any differences in photodecomposition of MTHF were analyzed where reduction of the flavin radical using various chemicals was carried out first. Different sodium dithionite concentrations were used to reduce $\text{FADH}^*/\text{FAD}_{(ox)}$ in VcCry-1 and compared to the use of DTT for the photoreduction process. 446 $\mu$M sodium dithionite was titrated into the cryptochrome sample with 10 $\mu$L increments. Complete $\text{FADH}^*/\text{FAD}_{(ox)}$ reduction occurred after about 30 minute incubation of the cryptochrome sample with dithionite in room temperature. Also in VcCry-1 the behavior of FAD and MTHF was examined where 20 mM DTT was used with only UV illumination without prior reduction of $\text{FADH}^*/\text{FAD}_{(ox)}$.

$\beta$-mercaptoethanol was used as another type of a reducing agent which is about half as efficient as DTT for photoreduction of FAD$^*$ (21). 10 mM $\beta$-mercaptoethanol in the final 45 $\mu$M *E. coli* photolyase or 45 $\mu$M VcCry-1 solution was used at first. The second trial involved using 40 mM $\beta$-mercaptoethanol. After purging the sample for about 10 minutes with N$_2$, it was illuminated with blue light in order to reduce flavin radical. UV light was used afterwards in order to photodecompose the MTHF cofactor.

**RESULTS**

*Flavin radical photoreduction and photodecomposition of MTHF in E. coli photolyase.* In Figure 8, the original absorption spectra for *E. coli* photolyase and VcCry-1 demonstrate the differences in flavin oxidation states and where MTHF absorbs with their wavelength maximum absorbencies represented in Table 9.
Table 9. Wavelength of maximum absorbance for MTHF and FAD oxidation states.

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{\text{max}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHF</td>
<td>380 nm</td>
</tr>
<tr>
<td>FAD$_{\text{(ox)}}$</td>
<td>443 nm</td>
</tr>
<tr>
<td>FADH$^+$</td>
<td>580 nm</td>
</tr>
<tr>
<td>FADH$^-$</td>
<td>$\approx 350-380$ nm</td>
</tr>
</tbody>
</table>

Figure 16 illustrates the photoreduced FAD and photodecomposed MTHF in *E. coli* photolyase and VcCry-1. The results from experiments on *E. coli* photolyase are presented in Table 10.

Table 10. Various conditions for photoreduction of FAD and photodecomposition of MTHF in *E. coli* photolyase.

<table>
<thead>
<tr>
<th></th>
<th>$E. \text{coli Photolyase}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7</td>
</tr>
<tr>
<td>Air Exposure</td>
<td>Full</td>
</tr>
<tr>
<td>Sample Concentration</td>
<td>43 $\mu$M</td>
</tr>
<tr>
<td>Reducing Agent Used</td>
<td>None</td>
</tr>
<tr>
<td>Chemical Concentration</td>
<td>N/A</td>
</tr>
<tr>
<td>Light Used to reduce FAD</td>
<td>Blue</td>
</tr>
<tr>
<td>Time to reduce FAD (min)</td>
<td>154</td>
</tr>
<tr>
<td>Light used to reduce MTHF</td>
<td>UV</td>
</tr>
<tr>
<td>Time of photodecomposing MTHF (min)</td>
<td>65</td>
</tr>
<tr>
<td>MTHF completely reduced</td>
<td>No</td>
</tr>
<tr>
<td>FAD Oxidation</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Variations to the experimental conditions such as air exposure or chemical addition revealed important information regarding the sample behavior. With full air exposure and no reducing agent, it took 154 minutes of irradiation with blue light in order to photoreduce the flavin radical. However, the MTHF was still not bleached after 65
minutes of irradiation with UV light. On the other hand, when the sample was sealed off and degassed using N₂, the irradiation with blue light took 74 minutes and the flavin radical was completely reduced. Unfortunately, 15 minutes of illumination with UV light caused the sample to degrade. In both experiments oxidation of the flavin occurred despite the initial reduction of FADH⁺/FAD(ox).

In experiments with anaerobic samples where DTT was used as a reducing agent, FAD(ox) was not observed. In an 11uM EcPhr sample containing 10 mM DTT, FAD⁺ was reduced with blue light within 80 seconds, and the MTHF photodecomposed within 8 minutes of irradiation with UV light. In 45 uM EcPhr samples with 20 mM DTT, FADH⁺ was reduced with blue light in 3 to 7 minutes (Figure 19) and MTHF peak was bleached with UV light in 9 to 13 minutes (Figure 20). Use of 10 mM β-mercaptoethanol solution in the sample instead of DTT was not as efficient as the other reducing agents (Table 10 and 11). Switching to the UV filter caused FADH⁺ and FAD(ox) to appear in the spectra while the MTHF peak was decreasing suggesting the involvement of FADH⁺ as an electron donor. It is observed that the flavin radical reappears after it is photoreduced, however, not in such amount as it is observed in the study with β-mercaptoethanol (111) (Figure 21, Table 12).

A higher concentration of 40 mM β-mercaptoethanol was used to maintain more of the flavin in its reduced form. Still, it was not possible to produce the desired effect, most probably due to a less favorable reduction potential of β-mercaptoethanol in comparison to the -0.33 V reduction potential of DTT (112).
Figure 19. Absorbance changes as a function of time for the photoreduction of FAD in *E. coli* photolyase (circles) and VcCry-1 (squares) under blue light illumination with 20mM DTT.

Figure 20. Absorbance changes as a function of time for the photodecomposition of MTHF in *E. coli* photolyase (circles) and VcCry-1 (squares) under blue light illumination with 20mM DTT.
Table 11. Photoreduction of FAD and photodecomposition of MTHF in VcCry-1 and EcPhr.

<table>
<thead>
<tr>
<th></th>
<th>VcCry-1</th>
<th>EcPhr</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Air Exposure</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Sample Concentration</td>
<td>45 μM</td>
<td>45 μM</td>
</tr>
<tr>
<td>Reducing Agent Used</td>
<td>β-mercaptoethanol</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>Chemical Concentration</td>
<td>10 mM</td>
<td>40 mM</td>
</tr>
<tr>
<td>Light Used to reduce FAD</td>
<td>Blue Light</td>
<td>Blue Light</td>
</tr>
<tr>
<td>Time to reduce FAD (min)</td>
<td>56 min</td>
<td>52 min</td>
</tr>
<tr>
<td>Light used to reduce MTHF</td>
<td>UV</td>
<td>UV</td>
</tr>
<tr>
<td>Time of photodecomposing MTHF (min)</td>
<td>133 min</td>
<td>109 min</td>
</tr>
<tr>
<td>MTHF completely reduced</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>FAD Oxidation</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Figure 21. 45 μM EcPhr with 20 mM DTT UV light illumination with absorbance changes. The absorbance increases in the region below 320 nm due to accumulation of FADH⁺. Peak at 380 nm decreases due to photodecomposition of MTHF. At 580 nm, small reappearance of FADH⁺ is seen with the maximum observed after 1 min irradiation of the sample.
Table 12. FADH* absorbance change in EcPhr under UV light illumination after complete photoreduction.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Abs. at 580 nm</th>
<th>FADH* change in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.0132</td>
<td>54.7</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0240</td>
<td>100.0</td>
</tr>
<tr>
<td>2.0</td>
<td>0.0225</td>
<td>93.4</td>
</tr>
<tr>
<td>3.0</td>
<td>0.0208</td>
<td>86.5</td>
</tr>
<tr>
<td>4.0</td>
<td>0.0180</td>
<td>74.8</td>
</tr>
<tr>
<td>5.0</td>
<td>0.0164</td>
<td>68.0</td>
</tr>
<tr>
<td>6.0</td>
<td>0.0157</td>
<td>65.4</td>
</tr>
<tr>
<td>7.0</td>
<td>0.0148</td>
<td>61.6</td>
</tr>
<tr>
<td>8.0</td>
<td>0.0150</td>
<td>62.2</td>
</tr>
<tr>
<td>9.0</td>
<td>0.0142</td>
<td>59.2</td>
</tr>
</tbody>
</table>

Flavin radical reduction and photodecomposition of MTHF in VcCry-1. The different conditions under which the experiments were done are presented in Table 11, 13, and 14.

Table 13. Photoreduction of FAD and photodecomposition of MTHF in VcCry-1 under various conditions.

<table>
<thead>
<tr>
<th>VcCry-1</th>
<th>7</th>
<th>7</th>
<th>7</th>
<th>7</th>
<th>7</th>
<th>7</th>
<th>7</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Air Exposure</td>
<td>Full</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Sample Concentration</td>
<td>72</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
<td>45</td>
</tr>
<tr>
<td>Reducing Agent Used</td>
<td>None</td>
<td>DTT</td>
<td>DTT</td>
<td>DTT</td>
<td>DTT</td>
<td>DTT</td>
<td>DTT</td>
<td>DTT</td>
</tr>
<tr>
<td>Chemical Concentration</td>
<td>None</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Light Used to reduce FAD</td>
<td>Blue</td>
<td>Blue</td>
<td>Blue</td>
<td>Blue</td>
<td>Blue</td>
<td>Blue</td>
<td>Blue</td>
<td>None</td>
</tr>
<tr>
<td>Time to reduce FAD (min)</td>
<td>146</td>
<td>24</td>
<td>25</td>
<td>25</td>
<td>29.5</td>
<td>51</td>
<td>17</td>
<td>N/A</td>
</tr>
<tr>
<td>Light used to reduce MTHF</td>
<td>UV</td>
<td>UV</td>
<td>UV</td>
<td>UV</td>
<td>UV</td>
<td>UV</td>
<td>UV</td>
<td>UV</td>
</tr>
<tr>
<td>Time of photodecomposing MTHF (min)</td>
<td>125</td>
<td>52</td>
<td>38</td>
<td>42</td>
<td>39</td>
<td>48</td>
<td>130</td>
<td>27</td>
</tr>
<tr>
<td>MTHF completely reduced</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>FAD Oxidation</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

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Table 14. Photoreduction of FAD and photodecomposition of MTHF in VcCry-1 with various sodium dithionite concentrations.

<table>
<thead>
<tr>
<th>pH</th>
<th>VcCry-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air Exposure</td>
<td>None</td>
</tr>
<tr>
<td>Sample Concentration</td>
<td>45 µM</td>
</tr>
<tr>
<td>Reducing Agent Used</td>
<td>Sodium dithionite</td>
</tr>
<tr>
<td>Chemical Concentration</td>
<td>~446 µM</td>
</tr>
<tr>
<td>Light Used to reduce FAD</td>
<td>None</td>
</tr>
<tr>
<td>Time to reduce FAD (min)</td>
<td>~55 min RT</td>
</tr>
<tr>
<td>Light used to reduce MTHF</td>
<td>UV</td>
</tr>
<tr>
<td>Time of photodecomposing MTHF (min)</td>
<td>115 min</td>
</tr>
<tr>
<td>MTHF completely reduced</td>
<td>No</td>
</tr>
<tr>
<td>FAD Oxidation</td>
<td>Yes</td>
</tr>
</tbody>
</table>

In a 72 uM VcCry-1 sample with full air exposure, the flavin in radical and oxidized state was reduced in 146 minutes using only blue light. After illuminating this sample with UV light for 125 minutes, the MTHF did not decrease completely. In a 45 uM sample with 20 mM DTT, the reduction of FADH /FAD(ox) took place in 24 to 29.5 minutes of irradiation with blue light (Figure 19). UV light illumination photodecomposed MTHF in 38 to 52 minutes (Figure 20). In few cases some FAD(ox) and FADH appeared while using UV light (Figure 22, Table 15). Sodium dithionite, DTT, and β-mercaptoethanol concentration dependent studies were also carried out. We observed that the photoreduction and photodecomposition reactions are dependent on the concentration of the reducing agent. With higher concentration of the reducing agent, it took less time to reduce FADH /FAD(ox) and photodecompose the MTHF. The investigation of the behavior of the sample only under UV light was also completed. It turned out that for a
45 μM VcCry-1 sample with 20 mM DTT, the flavin radical and MTHF peak decreased completely in 37 minutes with no oxidation present (Figure 23).

![Graph](image-url)

**Figure 22.** 45 μM VcCry-1 with 20 mM DTT UV light illumination with absorbance changes. The absorbance increases in the region below 320 nm due to accumulation of FADH*. The peak at 380 nm decreases due to photodecomposition of MTHF. At 580 nm reappearance of FADH* is seen with the maxima observed after 6 minute irradiation of the sample. FAD(ox) increase is observed at around 443 nm.
Table 15. FADH• absorbance change in VcCry-1 under UV light illumination after complete photoreduction.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Abs. at 580 nm</th>
<th>FADH• change in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.0063</td>
<td>38.9</td>
</tr>
<tr>
<td>3</td>
<td>0.0121</td>
<td>75.3</td>
</tr>
<tr>
<td>6</td>
<td>0.0161</td>
<td>100.0</td>
</tr>
<tr>
<td>9</td>
<td>0.0065</td>
<td>40.6</td>
</tr>
<tr>
<td>12</td>
<td>0.0122</td>
<td>76.2</td>
</tr>
<tr>
<td>15</td>
<td>0.0109</td>
<td>67.6</td>
</tr>
<tr>
<td>18</td>
<td>0.0122</td>
<td>76.2</td>
</tr>
<tr>
<td>21</td>
<td>0.0101</td>
<td>63.0</td>
</tr>
<tr>
<td>24</td>
<td>0.0106</td>
<td>65.8</td>
</tr>
<tr>
<td>27</td>
<td>0.0103</td>
<td>63.9</td>
</tr>
<tr>
<td>30</td>
<td>0.0098</td>
<td>61.1</td>
</tr>
<tr>
<td>33</td>
<td>0.0122</td>
<td>75.7</td>
</tr>
<tr>
<td>36</td>
<td>0.0092</td>
<td>57.5</td>
</tr>
<tr>
<td>39</td>
<td>0.0084</td>
<td>52.6</td>
</tr>
</tbody>
</table>

Figure 23. A. 45 μM VcCry-1 sample containing 20 mM DTT (1) was illuminated with UV light (2). Here the photoreduction of FADH'/FAD_{(ox)} and photodecomposition of MTHF took 37 minutes. B. 45 μM VcCry-1 sample containing 20 mM DTT (1) was first illuminated with blue light which photoreduced FADH'/FAD_{(ox)} in 25 minutes. Afterwards UV light was used for MTHF photodecomposition for 39 minutes (2).
DISCUSSION

In order to compare the photoreduction of the FAD cofactor and the photodecomposition of the MTHF cofactor we have to realize that the starting points in each enzyme are already different due to the different stoichiometry of FADH\textsuperscript{*} and FAD\textsubscript{(ox)} in the protein. When the sample is exposed to oxygen the photoreduction has to compete with oxidation. In the aerobic experiments the continuous air supply causes the photoreduction of FAD to occur much slower where most of the FADH\textsuperscript{*} oxidizes to FAD\textsubscript{(ox)} which interferes with the photodecomposition of MTHF. Photolyase samples with a mixture of FADH\textsuperscript{*} and FAD\textsubscript{(ox)} degrade faster and monitoring the photodecomposition of MTHF becomes inaccurate. This also suggests the importance of FADH\textsuperscript{*}. The difference in oxidation states, especially the amount of FAD\textsubscript{(ox)} in VcCry-1 and EcPhr causes an inconvenience in comparison of the two proteins.

The absorption spectra obtained for the different samples at various stages during illumination can be compared by looking at the change in absorbance at 380 nm where MTHF absorbs. The observed phenomenon that the photodecomposition of MTHF in DNA photolyase occurs much faster than in CryDASH (AtCry3) was reaffirmed by the experiments on photolyase and VcCry-1 described above. However, it is more interesting to see whether VcCry-1 behaves the same as the cryptochrome DASH isolated from a plant. By comparing it to AtCry-3, we are able to see whether cryDASH from different organisms behave similarly while undergoing the photoreduction and photodecomposition processes.

For AtCry3, it was determined that the intact tryptophan triad is needed in photodecomposing MTHF. The studies on AtCry-3 and AtCry-3 mutant showed that the
photoreduction of FADH' and photodecomposition of MTHF is absent when there is a mutation of one of the tryptophans (109). However, other mechanisms can exist through which the second cofactor can be photodecomposed from AtCry-3 and VcCry-1 and other conditions may be required. Unfortunately, at this time it is impossible to distinguish what can serve as an electron donor for reducing MTHF in VcCry-1. However, based on our results we acknowledge that the reducing agents make a strong contribution to the photoreduction and photodecomposition processes. These are the most likely mechanisms:

1) $\text{MTHF} \overset{e^-}{\leftarrow} \text{Trp}^{356}, \text{Trp}^{409}, \text{Trp}^{432} \overset{\text{reducing agent}}{\leftarrow}$

2) $\text{MTHF} \overset{e^-}{\leftarrow} \text{FADH}^- \overset{e^-}{\leftarrow} \text{Trp}^{356}, \text{Trp}^{409}, \text{Trp}^{432} \overset{\text{reducing agent}}{\leftarrow}$

3) $\text{MTHF} \overset{e^-}{\leftarrow} \text{reducing agent}$

4) $\text{MTHF} \overset{e^-}{\leftarrow} \text{FADH}^- \overset{e^-}{\leftarrow} \text{reducing agent}$

The use of the reducing agent greatly decreases the necessary illumination time, and the change in concentration of the reducing agent affects the rate at which the photoreduction process occurs. Moreover, the results reveal that DTT concentration not only affects the FADH'/FAD$_{(ox)}$ photoreduction but also MTHF photodecomposition rate. That could indicate a rapid rereduction of Trp$^{432}$ accelerating the reduction and photodecomposition processes. However, that could also be an indication of a direct reaction occurring between MTHF and DTT.

After the FADH'/FAD$_{(ox)}$ was photoreduced and the samples were illuminated with UV light, the flavin in the radical and oxidized redox state reappeared (Figure 24, 25).
**Figure 24.** The change in EcPhr absorbance after blue and UV light illumination. 45 μM EcPhr sample with 40 mM β-mercaptoethanol was illuminated with blue light for 11 minutes for photoreduction of FADH'/FAD(ox) and with UV light for 31 minutes for MTHF photoreduction.
Figure 25. The change in VcCry-1 absorbance after blue and UV light illumination. 45 µM VcCry-1 sample with 40 mM β-mercaptoethanol was illuminated with blue light for 52 minutes for photoreduction of FADH\(^{\text{red}}/\text{FAD(ox)}\) and with UV light for 109 minutes for MTHF photoreduction.

The increase in FADH\(^{\text{red}}\) absorbance during MTHF photodecomposition was seen by Jorns et al. as well as in our experiments (107). Jorns group proposed that FADH\(^{-}\) is the electron donor to MTHF, resulting in some buildup of FADH\(^{\cdot}\). When β-mercaptoethanol was used as a reducing agent the illumination with UV light photodecomposed MTHF while the FADH\(^{\cdot}/\text{FAD(ox)}\) absorbance increased dramatically in both EcPhr and VcCry-1 which was completely photoreduced before UV light irradiation. This would make a strong case for FADH\(^{-}\) as an intermediate. In AtCry3, it was seen that in the presence of β-mercaptoethanol the blue light illumination leads to the fully reduced flavin state (112), however nothing is said about further UV light irradiation and appearance of FADH\(^{\cdot}\) or
FAD_{(ox)}. The oxidation state of flavin can play an important role in photodecomposition of MTHF. There are two possible mechanisms where the oxidation state of flavin would matter:

1\textsuperscript{st} Possibility: $\text{Trp}^{356} \rightarrow \text{FAD} \rightarrow \text{MTHF}$

2\textsuperscript{nd} Possibility: $\text{Trp}^{356} \rightarrow \text{MTHF (reaction 1)}$ \quad $\text{FAD (reaction 2)}$.

If the reaction follows the first possibility the oxidation state would matter because the reduction of FAD would cause the reduction of MTHF. In the second possibility where the electron comes directly from the tryptophan reducing the MTHF (reaction 1) the oxidation state of FAD does not matter. In reaction 2 FAD gets reduced without affecting MTHF, unless the reduction of FAD is more favorable than the reduction of MTHF where the oxidation state of FAD would matter.

The lack of MTHF photodecomposition from VcCry-1 previously observed by Sokolova et al. was most probably caused by the different experimental conditions. The samples were under 350.7 nm excitation from a Kr⁺ - laser with 10mW power for about 30 min (50). Our current data indicate that the higher sample concentrations used without reducing agent and weak light intensity along with short illumination time will not result in MTHF photodecomposition in VcCry-1. However, the much slower photodecomposition rate in VcCry-1 compared to EcPhr can be explained by the higher stability of MTHF in VcCry-1 potentially based on their reduction potentials that are regulated by interactions with the protein (50).

Further investigation of this matter could reveal other aspects that may explain the exact mechanism for photodecomposition of MTHF in VcCry-1. In AtCry-3 the FAD can be reduced with β-mercaptoethanol and blue light irradiation, and MTHF is
photodecomposed with UV light in the presence of FADH$. Time of FAD reduction and photodecomposition of MTHF cannot be directly compared between AtCry-3, EcPhr, and VcCry-1 since the concentrations of the samples, and light intensity slightly differ. The mutation experiment on AtCry-3 strongly suggests that the tryptophan triad is the starting point. It is likely that the dependence on DTT concentration shows the reduction of Trp* trapping FADH$^\cdot$/MTHF$^\cdot$. The importance of the oxidation state of FAD is of a future interest.

**CONCLUSION**

Various responses to reducing FAD and photodecomposing MTHF under diverse experimental conditions were shown. Based on the measurements obtained it is well confirmed that the photoreduction and photodecomposition in VcCry-1 occurs much slower than in *E. coli* photolyase. At this point the findings signify a similarity between AtCry-3 and VcCry-1 since the same correlation exists between the plant subfamily of cryDASH with photolyase, as the bacteria type cryDASH has with photolyase. Using different conditions for photoreduction FADH$^\cdot$/FAD$_{ox}$ and MTHF photodecomposition revealed that there are more possibilities for the mechanism which these processes can follow in VcCry-1. The experiments revealed an important role that reducing agents and oxidation state of FAD play in photodecomposition of MTHF.
References