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Hydrogen Bond Switching among Flavin and Amino Acids Determines the Nature of Proton-Coupled Electron Transfer in BLUF Photoreceptors

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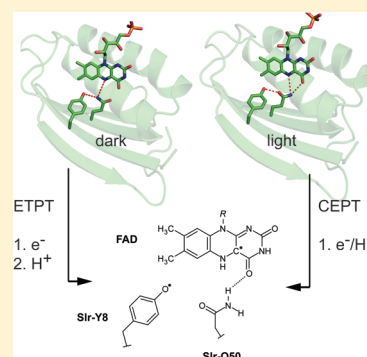
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S Supporting Information

ABSTRACT: BLUF domains are flavin-binding photoreceptors that can be reversibly switched from a dark-adapted state to a light-adapted state. Proton-coupled electron transfer (PCET) from a conserved tyrosine to the flavin that results in a neutral flavin semiquinone/tyrosyl radical pair constitutes the photoactivation mechanism of BLUF domains. Whereas in the dark-adapted state PCET occurs in a sequential fashion where electron transfer precedes proton transfer, in the light-adapted state the same radical pair is formed by a concerted mechanism. We propose that the altered nature of the PCET process results from a hydrogen bond switch between the flavin and its surrounding amino acids that preconfigures the system for proton transfer. Hence, BLUF domains represent an attractive biological model system to investigate and understand PCET in great detail.

SECTION: Biophysical Chemistry



Biological photoreceptors consist of a pigment to absorb light of a certain wavelength region bound to a protein that reacts upon photoinduced chemical or structural changes of the pigment to subsequently modulate its interaction with a signaling partner. Most photoreceptors reversibly switch from a dark state to a light-adapted or signaling state upon light absorption. The light-adapted state, a molecular ground state that activates downstream effectors and hence causes a biological reaction, is metastable and recovers thermally to the dark-adapted state.

BLUF photoreceptors are small flavin containing proteins¹ involved in phototaxis,² photosynthetic gene regulation,³ and virulence⁴ and have also been found coupled to various enzyme domains.^{2,5–9} The latter have gained immense attention as so-called optogenetic tools.^{9–13} The essential components of the photosensory apparatus consist of the isoalloxazine moiety of the flavin, a conserved tyrosine and glutamine side-chain. Figure 1 shows the X-ray structure of the Slr1694 BLUF domain from *Synechocystis* sp. PCC 6803¹⁴ (also known as SyPixD) with the flavin binding pocket highlighted. In the BLUF dark state, after excitation with blue light an electron is transferred within a few tens of picoseconds from the tyrosine to the flavin, resulting in a spectroscopically observable FAD^{•−} anion semiquinone radical.^{15,16} Subsequently FAD^{•−} is protonated, most likely by the same tyrosine, to form a neutral flavin semiquinone (FADH[•])/tyrosyl (TyrO[•]) radical pair. Thus, proton-coupled electron transfer (PCET) underlies

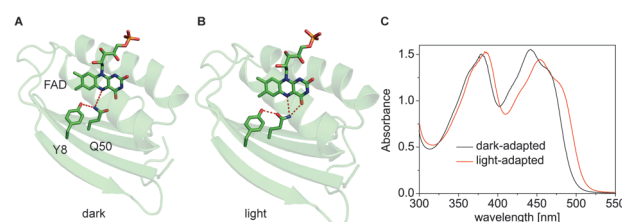


Figure 1. Dark- and light-adapted states of Slr1694. A and B show the presumed respective tyrosine/glutamine/flavin hydrogen bond network. The absorbance of both states is presented in C.

BLUF photoactivation, whereby electron transfer precedes proton transfer by picoseconds. During radical recombination a rearrangement of the hydrogen bond network around the flavin takes place leading to a 10–15 nm red shift in the visible absorbance of the flavin as shown in Figure 1C,³ as well as to a downshift of the carbonyl vibrations of the flavin.¹⁷ The resulting hydrogen bond-switched state is formed within less than 100 ps and remains stable for about 10 decades of time and is therefore considered as the light-adapted or signaling state of the BLUF domain.¹⁵ The details of this hydrogen bond switch are still under considerable discussion since crystal and

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NMR structures gave contradicting findings about dark and light-adapted states with respect to glutamine orientation.^{6,14,18–24} A likely mechanism involves rotation of the glutamine amide.^{15,18} However, recent theoretical calculations introduced new models for the hydrogen bond switch, involving tautomerization with or without rotation of the glutamine side-chain.^{25,26}

Because of their unique primary photochemistry and their small size, BLUF domains represent an ideal naturally occurring model system to investigate PCET in great molecular detail. PCET is a key step in solar energy conversion that still requires mechanistic elucidation, especially in photosystem II where a key tyrosine mediates redox processes between P680 and the oxygen-evolving complex.^{27–29} With BLUF domains we have an easily accessible protein-chromophore complex whose photoreactivity is determined by well-defined structural constraints and controlled redox partner interactions through hydrogen bonding. Deeper understanding of these evolutionary optimized environments is of major importance to design efficient artificial systems for solar energy conversion.³⁰

In this study we investigated the photochemistry of the light-adapted ground state of Slr1694 from *Synechocystis* sp. PCC 6803. We chose this protein because it is available in milligram amounts of high purity and its light-driven PCET processes are kinetically well-resolved in the dark-adapted state to signaling-state forward reaction.^{15,16} To keep Slr1694 in the light-adapted state conveniently, we used the slow-photocycling W91F mutant of Slr1694, which exhibits a 50-fold slower recovery to the dark state as compared to wild type (223 s vs 4 s, for W91F and wild type, respectively).³¹ Background illumination with a blue LED then induces formation of the 12 nm red-shifted light-adapted state at near 100% population. It is important to note that the ultrafast light-induced dynamics of the Slr1694-W91F dark state remain essentially identical to those of the wild type.³¹ With the information on the ultrafast electronic and vibrational dynamics obtained on this system here we show that in the light-adapted state the same neutral flavin semiquinone tyrosyl radical pair is formed as in the dark-adapted state reaction. Most importantly we show that its formation by proton coupled electron transfer is transformed from a sequential fashion in the dark-adapted state into a concerted fashion in the light-adapted state, hence making it an ideal model system to study PCET. These changes in photodynamics furthermore strongly support one of the highly debated molecular models of both states as postulated earlier¹⁶ and will be discussed below.

Ultrafast visible absorption dynamics were recorded by transient pump/probe absorption spectroscopy on light-adapted Slr1694-W91F in H₂O and D₂O buffer with excitation at 400 nm on an experimental setup described previously.³² Data treatment and analysis is described in detail in the Supporting Information. The obtained data was sufficiently described by global analysis³³ using a sequential model with four lifetimes.

The evolutionary-associated difference spectra (EADS) are displayed in Figure S2, whereas kinetics at selected wavelengths are shown in Figure 2A. Formation of a flavin semiquinone intermediate is clearly observed by the rising absorption between 550 and 650 nm. The signal is overlaid with the excited state absorption (ESA) at 520 nm and above 630 nm and partly diminished by stimulated emission (SE). This is best demonstrated by the change in absorption at 556 nm (Figure 2A). The initial negative absorption by SE is quickly recovered

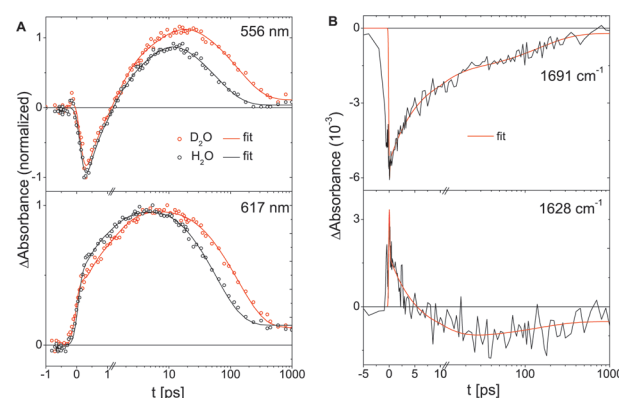


Figure 2. Time traces of the light-adapted state absorption changes after 400 nm excitation at selected wavelengths together with their fit. The traces at 556 nm (A) represent SE of the flavin, which is compensated within the first 2 ps by semiquinone absorption. Characteristic absorption of the flavin neutral semiquinone is observed at 617 nm, which is biased by overlap with SE within the first 10 ps. This leads to an apparent slowed down formation of the semiquinone in D₂O due to a longer lived excited state lifetime in D₂O (Figure 3A). At delays greater than 10 ps the absorbance change is significantly slowed down in D₂O at both wavelengths. The IR traces (B) recorded in D₂O buffer at 1691 and 1628 cm⁻¹ display the decay of the carbonyl stretch of the flavin and a tyrosine species, respectively. The time axis is linear until 1 (A) and 10 ps (B) and logarithmic after indicated by the skewed lines.

in an H/D isotope independent manner. After reaching a maximum, the signal, now fully belonging to the FAD neutral semiquinone, decays again. This decay is slowed down in the deuterated species. The corresponding EADS (Figure S2A/B) represent mixtures of FAD excited state, FAD neutral semiquinone, and a nondecaying rather featureless spectrum with slightly rising absorbance toward the red edge of the spectral window and a bleach in the flavin ground state absorption region. This spectrum contributes very little to the overall spectral evolution and is therefore difficult to interpret. It might be attributed to solvated electron generation by two-photon ionization³⁴ observed previously in PYP³⁵ or a minor fraction of triplet species.³⁶

To obtain the pure spectra from this mixture we performed target analysis³³ using a simple compartmental model (Figure 3A). A parallel reaction (green) is included to attribute for the putative photoionization product, which is instantly formed and remains constant on the time scale of the experiment. The mixture of excited state and semiquinone spectra is addressed by introducing a minor heterogeneity of the excited state. Only one of the two spectrally identical excited state species forms the neutral semiquinone. The resulting species-associated difference spectra (SADS) are displayed in Figure 3B. The excited state spectra are as a result of the imposed constraint identical and decay with 1 and 5 ps in H₂O. The FAD excited-state lifetime in dark-adapted Slr1694 has multiphasic lifetimes of 7, 40, and 180 ps.¹⁵ Thus, the FAD excited-state lifetime is significantly shortened with respect to that observed in the dark-adapted state,^{15,16} as previously observed for the light-adapted states of the Tll0078 and AppA BLUF domains.^{37,38} The spectra are highly similar to the EADS and show the ground-state bleach (GSB) of the light-adapted state at 452 nm as well as ESA at 510 nm and from 600 to 725 nm. Stimulated emission (SE) is observed at 560 nm. The fast disappearance corresponds to the formation of the FADH[•] neutral semi-

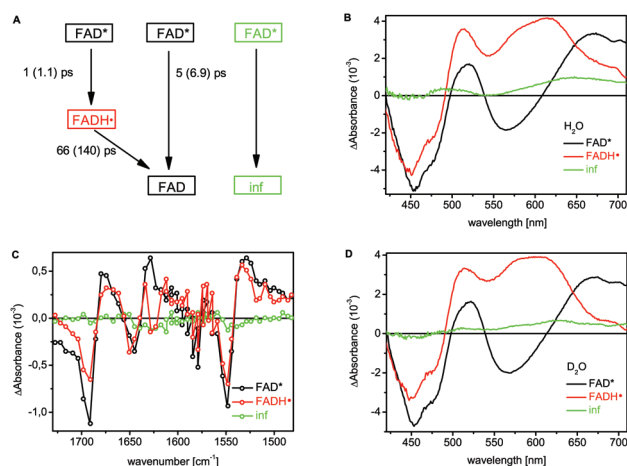


Figure 3. Target analysis of vis and IR absorption data using the model in A with the corresponding lifetimes in H₂O (D₂O). B and D show the SADS of H₂O and D₂O visible absorption data sets. The black and red spectra ideally represent flavin excited state and neutral flavin semiquinone, respectively. The green, nondecaying spectrum contributes little to the data sets and is tentatively assigned to a two-photon product or a minor amount of flavin triplet species. C shows the SADS of the IR data set in D₂O, obtained by simultaneous analysis of both vis and IR data. The black spectrum is identified as a flavin excited state whereas the red spectrum features a new bleach at around 1628 cm⁻¹, which is attributed to the tyrosine radical pair partner.

quinone (red), which lives for 66 ps. The species is formed with an efficiency of about 40% as judged by the loss of GSB. The FADH* spectrum shows the same GSB and additional absorptive features between 550 and 625 nm, representing the neutral radical absorption.¹⁵ The nondecaying spectrum (green) is identical to the spectrum from the sequential analysis. In D₂O we observe similar spectra with different lifetimes. The excited states decay with 1.1 and 6.9 ps, which represents a KIE of 1.1 and 1.4, respectively. Thus, we observe a significant kinetic isotope effect for the slow fraction of FAD* decay (5 vs 6.9 ps). The red spectrum is formed with an efficiency of about 50% and represents the absorption of a neutral flavin semiquinone FADH*. The spectrum decays with 140 ps and shows a KIE of about 2.1.

Strikingly, the spectra and the lifetimes of the FADH* neutral semiquinone are practically identical to the dark state intermediate of this particular Slr1694 mutant, in H₂O as well as D₂O.³¹ Therefore the process of radical recombination, which leads to the red-shifted, oxidized BLUF state, must be highly similar to that of the dark state reaction. This observation strongly suggests that, in the light adapted state of BLUF domains, the same FADH* intermediate with regard to hydrogen-bond pattern and glutamine orientation is formed as in the dark state reaction.

To confirm the identity of the dark- and light-adapted state intermediates as suggested above, we identified the corresponding radical pair partner of the neutral flavin semiquinone by femtosecond infrared spectroscopy. To prevent the large underlying absorption of the OH bending mode of water we measured the spectral dynamics between 1460 and 1740 cm⁻¹ in D₂O. The most prominent signals are observed at 1691 cm⁻¹ (Figure 2B) and 1549 cm⁻¹ (Figure S3), which represent the carbonyl stretch and the C=N out-of-phase vibration of the flavin, respectively. Both signals decay completely with the same kinetics on the observed time scale. The induced

absorption of the carbonyl stretch at 1674 cm⁻¹ and the bleach of the xylene ring vibration of the flavin at 1647 cm⁻¹ decay in parallel (Figure S3). The corresponding induced absorption of the latter at 1628 cm⁻¹ (Figure 2B), however, rises initially but then decays quickly within 5 ps and becomes negative, indicating a ground-state bleach of a molecular group other than the flavin. The negative absorption then decays within the observed time scale. A bleach at around 1620 cm⁻¹ was previously assigned to a tyrosine ring vibration and interpreted as a loss of an electron and a proton in formation of a tyrosyl radical.¹⁶ The corresponding EADS of the IR measurements (Figure S2C) are highly similar to the SADS (Figure 3C). Both show a prominent carbonyl bleach of the flavin cofactor at around 1690 cm⁻¹ and the C=N vibration at 1550 cm⁻¹ as previously observed.¹⁶ The former is downshifted relative to the dark state by ~10 cm⁻¹ and indicates stronger hydrogen bonding as previously observed for the light-adapted state by steady state FTIR.¹⁷ The occurrence of a previously identified tyrosine signature at 1628 cm⁻¹ clearly demonstrates that the radical pair consists of the flavin and the close-by tyrosine, identical to the dark state intermediate.¹⁶ The IR absorbance difference spectra of FAD* and FADH reported in Figure 3C may be compared to the corresponding spectra in dark-adapted BLUF domains.¹⁶

Upon dark-state excitation, PCET in Slr1694 and most likely in BLUF domains in general occurs in a sequential fashion where proton transfer lags electron transfer by picoseconds.^{15,16,39} Here, we have shown that a FADH* neutral semiquinone similar or identical to that formed in the dark-state reaction, is very rapidly produced by electron and proton transfer in 1 ps without any observable anionic FAD*- radical intermediate. Hence, PCET occurs in a rather concerted fashion after light-adapted state excitation in BLUF domains. In general, a concerted mechanism features a lower activation barrier since it does not involve the charged high-energy intermediates that result from separated proton and electron transfer.⁴⁰ The reconfiguration of the hydrogen-bond network around the FAD chromophore in the BLUF domain very likely is responsible for the altered nature of the PCET process. To facilitate the obviously more efficient proton tunnelling in the light-adapted state of BLUF, the molecular interactions need to be preconfigured for proton transfer either by shortening the proton transfer distance or by establishing an indirect proton conducting wire. Similar to the AppA and Tll0078 BLUF domains, the heterogeneity in the excited-state decay in Slr1694-W91F is significantly reduced in the light-adapted state.^{37,38} This supports the general assumption that the coordination of the flavin becomes tighter, more rigid and less heterogeneous in the light-adapted state. After femtosecond excitation the light-adapted excited state decays rapidly with an H/D isotope independent lifetime of 1 ps to form the neutral flavin radical. Because we do not observe any electron transfer intermediate like an anionic semiquinone here, the proton transfer clearly must occur in less than 1 ps, which can be explained by the increased basicity of the nearby N5 atom in the excited state of flavin.⁴¹ It should be noted that the time scale of this reaction is in the range of skeletal motions (~300 fs) that finally mediate excited state proton transfer as previously observed in GFP.⁴² Hence, the proton transfer rate observed here has been pushed close to the physical limit by the reconfiguration of the hydrogen bond network around the flavin in the light adapted state.

The exact configuration of the hydrogen bond network and orientation of the glutamine side chain in the light-adapted state, which determine the changed reactivity we observe here, are still under heavy debate.^{16,18,19,25,26,43–45} Recent NMR and FTIR studies showed that an unusually strong hydrogen bond from the tyrosine hydroxyl group to the glutamine is formed in the BLUF light-adapted state.^{45,46} The molecular model of the light-adapted state that we proposed earlier (Figure 4A,

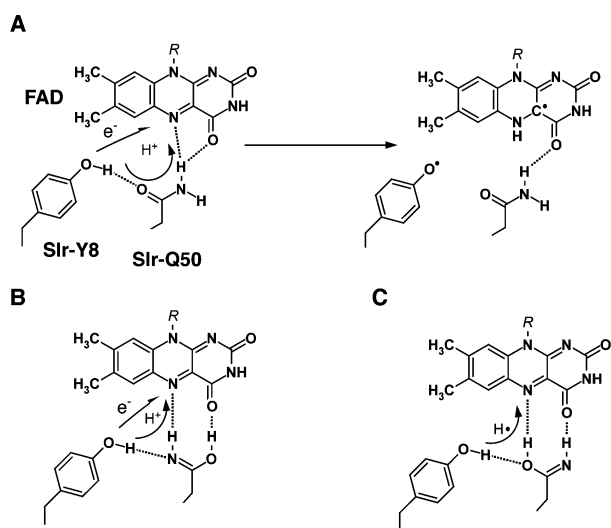


Figure 4. Models for PCET in the light state of Slr1694. The reaction involving concerted electron/proton transfer (CEPT) as proposed here is displayed in (A), with CEPT from Y8 to FAD taking place with a time constant of 1 ps after photoexcitation, see the main text for details. Alternative starting geometries (B and C) might involve the tautomeric forms observed in computational models and might involve direct H-atom transfer (C).

identical to the light state configuration in ref 15) provides the basis for such a strong hydrogen bond between tyrosine and glutamine. On the basis of the kinetic information on PCET in the light state of BLUF provided here, we propose that the formal hydrogen atom transfer is occurring by a concerted electron and proton transfer with a time constant of 1 ps, with direct electron transfer from the tyrosine and proton transfer by a Grotthuss-like mechanism along the amide group of the glutamine to FAD N5, to result in formation of a FADH•-Y8• radical pair. Due to the presumably tighter coordination of the flavin in the light-adapted state, the proton is instantly transferred. An intermediary imidic glutamine side chain, which might form upon transfer of the tyrosine hydroxyl proton to the amide carbonyl and release of an amide nitrogen proton to N5, instantly reautomerizes to the energetically more favorable amide form (Figure 4A, right). Note that the resulting molecular configuration between Y8•, Q50, and FADH• (Figure 4A, right) matches that proposed for the FADH•-Y8• radical pair intermediate of the dark-state reaction of the same protein,¹⁵ which is consistent with our observation that these two radical pairs have similar spectra, the same lifetime of 66 ps and the same kinetic isotope effect of 2.1 upon H/D exchange. Yet, the details of the binding pocket with respect to side chain configuration remain to be further characterized and cannot be assigned here completely. A direct hydrogen atom transfer from the tyrosine seems unlikely concerning the already hydrogen bonded N5 of the flavin and the rather large distance of 4–5 Å to the flavin.

In QM/MM studies of Domratcheva et al.²⁶ and Sadeghian et al.^{25,47} and spectroscopic studies by Stelling and co-workers⁴³ alternative models for the BLUF light-adapted state were proposed that involve tautomeric forms of Q50, as indicated in Figure 4, parts B and C. Although a tautomeric form may energetically be unfavorable for the long-lived signaling state in BLUF⁴⁸ we will discuss these models with respect to strong hydrogen bonding as observed by FTIR⁴⁶ and fast PCET observed in this study. In Domratcheva's model (Figure 4B), the imidic nitrogen of the glutamine side chain receives a hydrogen bond from Q50 and donates a hydrogen bond to flavin N5.²⁶ In such a molecular configuration, a concerted mechanism with a very short proton relay involving only the nitrogen atom of the amide group of Q50 would present a very efficient proton transfer path consistent with the experimental results presented here. However, this molecular configuration for the light-adapted state is difficult to reconcile with the presence of an unusually strong hydrogen bond between Y8 and Q50:⁴⁶ in general stronger H-bonds are formed to the oxygen rather than to the nitrogen of an amide or an imidic acid due to the higher electronegativity of oxygen. Hence, we consider this model less likely.

In Sadeghian's model the imidic acid oxygen of the glutamine side chain receives a hydrogen bond from Q50 and donates a hydrogen bond to flavin N5 (Figure 4C).²⁵ In this molecular configuration, a direct H-atom transfer from Y8 to FAD may readily occur because the H-bond of the imidic Q50 side chain to N5 is easily removed by tautomerization to the energetically favorable amide form. However, this molecular model is not consistent with formation of an unusually strong hydrogen bond between Y8 and the imidic oxygen of Q50: strong hydrogen bonds acquire significant covalent bond character,⁴⁹ which would destabilize the tautomeric form of the glutamine side chain even further, rendering this model for the BLUF light-adapted state less likely.

An interesting result from our experiments is the H/D-isotope dependent decay of a fraction of FAD excited-states, in 5 ps in H₂O and 6.9 ps in D₂O (Figure 3A). This fraction does not form an obvious proton associated product such as a flavin neutral semiquinone, but rather returns to the FAD ground state directly. The isotope-dependent excited-state decay might be due to a reconfiguration of the H-bond network in the excited state. Most likely FAD N5 attracts an H-bond stronger due to its increased basicity in the singlet excited state.⁴¹ The observation of this process in only a fraction of the proteins shows that a certain degree of ground-state heterogeneity exists in the light-adapted state, with a protein configuration in which the electron transfer is inefficient, probably due to an unfavorable tyrosine/flavin orientation.

In summary, our study demonstrates that an intricate biological hydrogen bond network tunes the photoinduced electron/proton transfer of a flavin/protein complex from a sequential to a concerted reaction. Since the BLUF domain can be easily switched between both reactivities it clearly represents a most powerful model system for further studies on proton-coupled electron transfer in biological systems.

■ ASSOCIATED CONTENT

● Supporting Information

Experimental procedures for sample preparation, spectroscopy and data analysis as well as supplementary data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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