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Communication

Enhancing Proton-Coupled Electron Transfer in Blue Light Using FAD Photoreceptor $AppA_{BLUF}$

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ABSTRACT: The Blue Light Using FAD (BLUF) photoreceptor utilizes a noncovalently bound FAD to absorb light and trigger the initial ultrafast events in receptor activation. FAD undergoes 1 and 2 electron reduction as an enzyme redox cofactor, and studies on the BLUF photoreceptor PixD revealed the formation of flavin radicals ($FAD^{•-}$ and $FADH^{•}$) during the photocycle, supporting a general mechanism for BLUF operation that involves PCET from a conserved Tyr to the oxidized FAD. However, no radical intermediates are observed in the closely related BLUF proteins $AppA_{BLUF}$ and BlsA, and replacing the conserved Tyr with fluoro-Tyr analogs that increase the acidity of the phenol OH has a minor effect on $AppA_{BLUF}$ photoactivation in contrast to PixD where the photocycle is halted at $FAD^{•-}$. The hydrogen bonding network in BLUF proteins contains several strictly conserved residues but differs in the identity of amino acids that interact with the flavin C2═O. In PixD there are two hydrogen bonds to the C2═O, whereas there is only one in $AppA_{BLUF}$. Using TRIR we show that the introduction of a second hydrogen bond to the C2═O in $AppA_{BLUF}$ results in the formation of flavin radicals ($FAD^{•-}$ and $FADH^{•}$) during the photocycle. Subsequent replacement of the conserved Tyr (Y21) in the double mutant with 2,3,5-trifluoroTyr prevents radical formation and generation of the light state, indicating that the $AppA_{BLUF}$ photocycle is now similar to that of PixD. The ability to trigger PCET provides fundamental insight into the role of electron transfer in the mechanism of BLUF photoactivation.

Proton-coupled electron transfer (PCET) is a fundamental mechanism in biological systems, involving simultaneous electron transfer and proton transfer, often forming stable radical species.[1,2] This process is integral to many biochemical reactions including energy conversion, enzyme catalysis and redox regulation.[3−5] In addition, PCET has also been discovered in blue light using a flavin (BLUF) domain photoreceptors which control a wide-range of biological functions by modulating the activity of covalently or noncovalently bound output domains.[6−8] Understanding the mechanism of BLUF photoreceptor operation remains a central challenge to their use in optogenetic applications, and provides a unique opportunity to observe and modulate PCET in real time.[9]

BLUF domain photoreceptors are primarily found in prokaryotes but are also present in other domains of life including eukaryotes, such as *Euglenozoa* and fungi.[10,11] The BLUF domain has a ferredoxin-like fold comprised of five parallel and antiparallel β-sheets and two α-helices, which surround the isoalloxazine ring of the FAD chromophore (Figure 1).[12−15] Light absorption results in the ultrafast perturbation of a conserved hydrogen bond network that surrounds the isoalloxazine ring leading to receptor activation on the μs-ms time scale. Seminal studies on PixD (Slr1694) revealed the formation of flavin radicals during the photocycle,[23] leading to a general model for BLUF photoactivation in which concerted or stepwise transfer of an electron and proton from a strictly conserved Tyr (Y8) to the oxidized flavin results in the formation of $FAD^{•-}$ and $FADH^{•}$ radical intermediates. The Tyr-flavin radical pair then recombines

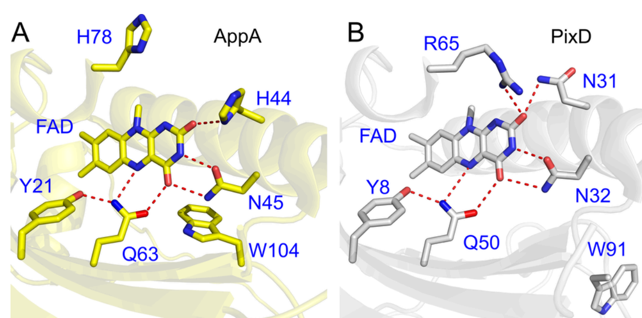

Figure 1. Flavin binding pocket in AppA and PixD. (A) AppA (PDB: 1YRX).[12] (B) PixD (PDB: 2HFN).[16] The conserved Gln (Q63/Q50) is assumed to be in the enol tautomer that forms during light state formation.[17−22]

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resulting in formation of the light state. In agreement with this mechanism, an increase in acidity of the PixD Tyr pK_a by replacement with fluoro-Tyr residues halts the photocycle at $FAD^{\bullet-}$.[24] However, in contrast to PixD, no radical intermediates are observed in the ultrafast IR spectra of the homologous BLUF proteins AppA and BlsA,[25−27] while replacement of the conserved Tyr in AppA with fluoro-Tyr residues does not affect light state formation although the rate of dark state recovery is increased.[28] The data on AppA calls into question the relevance of PCET as a universal mechanism for BLUF operation. Inspection of the BLUF flavin binding pocket reveals that there is variation in the residues that interact with the flavin C2═O group (Figure 1) which has been proposed to account for differences in BLUF mechanism.[24,29−31] To evaluate this hypothesis, we first replaced H44 in the AppA BLUF domain ($AppA_{BLUF}$) with Phe, Asn, Arg, and Ala, the residues found in that position in other BLUF proteins.[13,16,32] All the H44 single mutants underwent the characteristic red shift in FAD absorbance at 450 nm which signifies formation of the light state in BLUF photoreceptors (Figure S1, Table S1).[33,34] In addition, the rate of dark state recovery for the H44 single mutants differed by only ∼2-fold, in contrast to the 57-fold difference in the rate of recovery between wild-type $AppA_{BLUF}$ and PixD (Table 1).

Table 1. Kinetics of Dark State Recovery[a]

	k_{H2O} (min^{-1})	k_{D2O} (min^{-1})	kie
$AppA_{BLUF}$			
Wild-type	0.040 ± 0.001	0.0078 ± 0.0001	5.1
H44N	0.094 ± 0.002	0.013 ± 0.001	7.5
H78R	0.054 ± 0.001	0.016 ± 0.001	3.4
H44N,H78R	0.068 ± 0.001	0.024 ± 0.001	2.8
$3FY_{21}$ H44N,H78R	2.31 ± 0.02	0.24 ± 0.01	9.6
$3FY_{21}$	2.04 ± 0.12	0.23 ± 0.12	8.8
PixD			
Wild-type	2.28 ± 0.24	0.60 ± 0.018	3.8
$3FY_6$	13.2 ± 0.72	1.38 ± 0.048	9.5

[a]Dark state recovery was monitored by following the change in 450 nm flavin absorbance as a function of time.

We then assessed the effect of the mutations by ultrafast time-resolved multiple probe infrared spectroscopy (TRMPS) which probes the IR difference spectrum of the sample as a function of time after a 450 nm 100 fs pump pulse.[35,36] Evolution-associated difference spectra (EADS) were then generated by global analysis of the TRMPS data using Glotaran with a sequential model (Figure S2).[37]

The ultrafast IR spectroscopy measurements provide information on the ground state structure of the flavin in the dark state and the kinetics of excited state decay and ground state recovery, probed by monitoring time-dependent changes in the intensity of the ∼1380 cm^{-1} transient and ∼1547 cm^{-1} bleach bands, respectively. Specific changes in the frequency of the band assigned to the C2═O vibrational mode were observed which shifted from 1650 cm^{-1} in wild-type $AppA_{BLUF}$ to 1642 cm^{-1} in H44N $AppA_{BLUF}$ (Figure S2).[24,28] In addition, the C2═O band was observed at 1650 cm^{-1} in H44F and 1649 in H44R $AppA_{BLUF}$ while the intensity of the 1650 cm^{-1} was suppressed in H44A $AppA_{BLUF}$. The C2═O band is found at 1638 cm^{-1} in PixD where the analogous residue is Asn, and thus the shift in C2═O from 1652 to 1642 cm^{-1} suggests that the environment around C2═O in $AppA_{BLUF}$ has become more "PixD-like". However, the kinetics of the excited state decay and the ground state recovery, which probe the formation of an intermediate state during photoactivation, exhibit only minor differences (∼2−3-fold) for each H44 variant (Figure S3 and Table S2). In addition, the vibrational marker at ∼1520 cm^{-1} assigned to the flavin radical is not detected in the early time scale of the TRMPS experiment, suggesting other factors in addition to the specific residue at position 44 also modulate the electron transfer process during photoactivation.

The PixD crystal structure reveals that R65 is also hydrogen bonded to the FAD C2═O, and we speculated that this residue might play a role in stabilizing the charge separation of the isoalloxazine ring. We therefore replaced H78 with Arg in both the wild-type $AppA_{BLUF}$ and the H44N variant (i.e., H78R and H44N,H78R). TRIR data for H78R and H44N,H78R $AppA_{BLUF}$ (Figure 2 and Figure S4) shows the instantaneous formation of excited state absorption (bands at 1383 and 1420 cm^{-1}) and ground state bleaches (at 1545, 1634, and 1690 cm^{-1}) as observed for wild-type $AppA_{BLUF}$.[28,38,39] The 1634 cm^{-1} band is assigned to the C2═O of FAD that is further red-shifted compared to H44N $AppA_{BLUF}$ (1642 cm^{-1} Figure S2) supporting the presence of a second hydrogen bond to C2═O as observed in PixD where the C2═O frequency is at 1638 cm^{-1}. However, transients are now observed at 1507 and 1521 cm^{-1} within 1 ps in the TRIR spectra of H44N,H78R $AppA_{BLUF}$ (Figure 2), supporting the light-induced formation of tyrosine and FAD radical species, $Tyr^{\bullet+}$, and $FAD^{\bullet-}$, respectively.[24,40,41] The second EADS forms in 8 ps, showing the decay of FAD* and $Tyr^{\bullet+}$, the formation of a band at 1532 cm^{-1} which is assigned to the $FADH^{\bullet}$ radical, and recovery of the ground state.[38,40,42] The third EADS forms in 92 ps and includes the decay of a transient at 1532 cm^{-1} assigned to $FADH^{\bullet}$, followed by the decay of other vibrational modes in the amide region (Figure 2A and B). The kinetics for the formation and decay of the 1521 and 1532 cm^{-1} bands indicates that the electron and proton transfer is stepwise, since decay of the 1521 cm^{-1} band precedes that of the 1532 cm^{-1} band (Figure S5). In addition, the final TRIR spectrum closely matches the steady-state FTIR light minus dark difference spectrum of H44N,H78R $AppA_{BLUF}$ (Figure S6), as well as wild-type $AppA_{BLUF}$,[17,28] indicating that the double mutation has not affected the ability of the photoreceptor to adopt the final signaling state.

The observation of flavin radicals in the TRIR spectra of the H44N,H78R $AppA_{BLUF}$ mutant is supported by transient absorption (TA) measurements that report on the oxidation state of the FAD (Figure S7). Specifically, global fitting of the TA data demonstrate that the excited state of FAD (FAD*-FAD) forms instantaneously and decays to the semiquinone state ($FADH^{\bullet}$-FAD) in 4 ps. However, although formation of the excited state was also observed for the H78R variant, there was no evidence of a radical intermediate. To further investigate the radical formation mechanism in the H44N,H78R variant, we modulated the electron transfer by replacing the conserved residue Y21 with 3FY21 and $2,3,5F_3Y21$, which have the structures shown in Figure 3.

Figure 2C and E show the temporal evolution of the TRIR spectrum for $2,3,5F_3Y21$ H44N,H78R and 3FY21 H44N,H78R variants, respectively. At the first EADS, the fluoro-Tyr variants contain the same transients and bleaches as the Y21 H44N,H78R variant. However, the vibrational marker of the radical species at ∼1510 to 1530 cm^{-1} only shows a

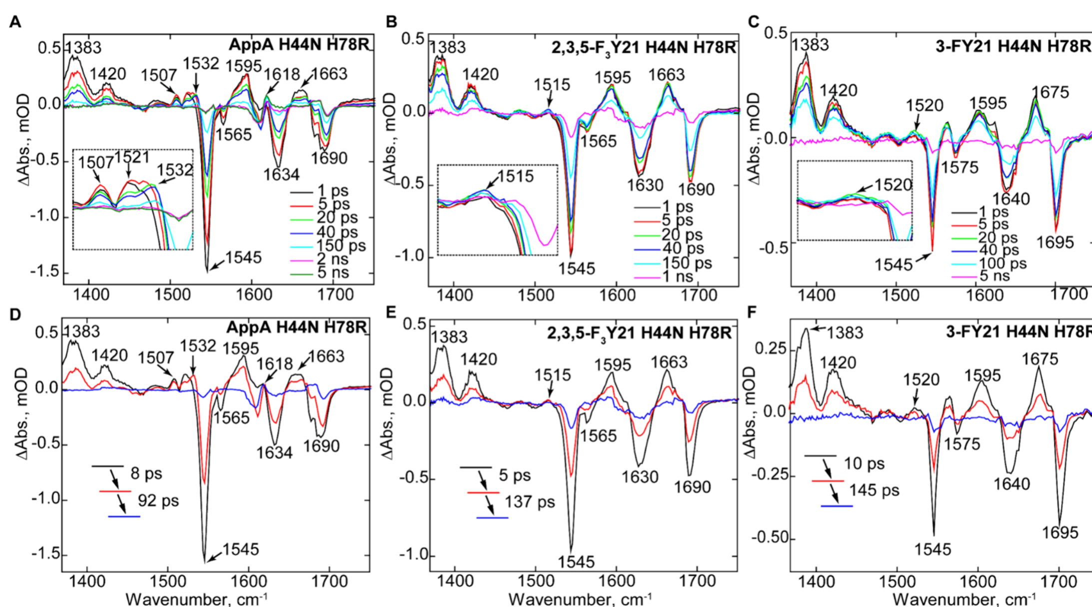

Figure 2. TRIR spectra of the H44N,H78R $AppA_{BLUF}$ variants. Temporal evolution of the H44N,H78R (A), the $2,3,5F_3Y21$ H44N,H78R (C), and 3FY21 H44N,H78R (E) spectra was recorded between 100 fs and 5 ns after 450 nm excitation. Transients assigned to $FADH^{\bullet}$ (1532 cm^{-1}) and $FAD^{\bullet-}$ (1515 cm^{-1}) are shown in the inset. (B) EADS of H44N,H78R were obtained from a global fit of the TRIR data in A. (D) EADS of $2,3,5F_3Y21$ H44N,H78R were obtained from a global fit of the TRIR data in C. (F) EADS of 3FY21 H44N,H78R were obtained from a global fit of the TRIR data in E.

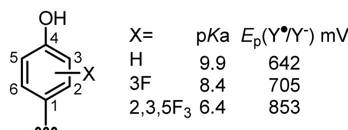

Figure 3. Structure, pK_a, and reduction potential of the fluoro-Tyr residues incorporated into $AppA_{BLUF}$.

weak amplitude at 1520 and 1515 cm^{-1} for 3FY21 and $2,3,5F_3Y21$, respectively; as discussed below, the kinetics are heterogeneous and not all FAD* excitations will lead to radical intermediates. The weak amplitude of the 1520 cm^{-1} band in the 3FY21 variant suggests that only a small population of $FADH^{\bullet}$ and $FAD^{\bullet-}$ is formed during photoactivation, while the lack of a transient at 1532 cm^{-1} in $2,3,5F_3Y21$ H44N,H78R suggests that the increase in acidity of Y21 prevents the formation of the $FADH^{\bullet}$ state. In addition, the 1383 cm^{-1} transient decays more rapidly than the 1545 cm^{-1} band, indicating that decay of the excited state involves intermediate species that precede formation of the ground state in $2,3,5F_3Y21$ H44N,H78R. However, the presence of a band at 1515 cm^{-1} indicates that $FAD^{\bullet-}$ still forms in this variant, suggesting that photoactivation is halted at this state (Figure S8, Figure 2C).

The results are similar to studies on PixD, where replacing the conserved Tyr with 2,3,5F3 Tyr prevents the formation of the $FADH^{\bullet}$ state, and only a small population of the $FAD^{\bullet-}$ state is observed.[8,24] In addition, the 1618 cm^{-1} transient observed in H44N,H78R $AppA_{BLUF}$ is red-shifted from the position found in wild-type $AppA_{BLUF}$ (1630 cm^{-1}) and disappears in the 3FY21 and $2,3,5F_3Y21$ H44N,H78R spectra (Figure 2A and C). The 1618 cm^{-1} band is assigned to the protein modes perturbed upon formation of the final signaling state,[28] and the absence of this mode in 3FY and $2,3,5F_3Y21$ H44N,H78R $AppA_{BLUF}$ is consistent with the photocycle halting at $FAD^{\bullet-}$.

Previously we proposed a mechanism for dark state recovery for both $AppA_{BLUF}$ and PixD that involves proton transfer from the conserved Tyr (Y21 or Y8) to the conserved Gln (Q63 or Q50).[24,28] While broadly similar, key differences between the two proteins included the absolute rate of recovery, which was 57-fold faster in PixD, and the extent of proton transfer in the rate-limiting transition state which was essentially complete in $AppA_{BLUF}$ but only ∼40% complete in PixD. By monitoring the change in FAD absorbance at ∼450 nm as a function of time, we found that the rate of dark state recovery increased only ∼2-fold in H44N, H78R $AppA_{BLUF}$ (Table 1). In addition, we found that the recovery rate of 3FY21 H44N,H78R $AppA_{BLUF}$ increased 34-fold, which is similar to the effect in wild-type $AppA_{BLUF}$ (51-fold increase in 3FY21 $AppA_{BLUF}$), while the recovery rate in 3FY6 PixD increases only 5.8-fold. In other words, like wild-type $AppA_{BLUF}$, proton transfer during dark state recovery in H44N,H78R $AppA_{BLUF}$ has a strong dependence on the pK_a of Y21 indicating that the recovery mechanism has not been significantly affected by the double mutation and remains "AppA-like". This conclusion is supported by the observation of large normal isotope effects on the rate of recovery (Table 1).

In summary, although interactions with the C2═O do not affect dark state recovery, replacement of H44 and H78 in $AppA_{BLUF}$ with Arg and Asn, the residues in PixD that hydrogen bond to the FAD C2═O, alters the photoactivation mechanism so that radical intermediates are now observed. In addition, further modulation of the photocycle by altering the acidity of Y21 in H44N,H78R $AppA_{BLUF}$ indicates that $FAD^{\bullet-}$ and $FADH^{\bullet}$ lie on the pathway to light state formation. These observations support a model for BLUF photoreceptors in which two hydrogen bonds to the C2═O are needed to facilitate charge separation and PCET from the conserved Tyr to the isoalloxazine ring. The present results thus reveal that two mutations switch the dark to light state reaction of BLUF domains from a pathway with no observable radical

intermediates to one where they are readily observed, yet leaving the dark state recovery unchanged.

Both the lack of evidence for electron transfer (ET) in AppABLUF using time-resolved spectroscopy and the failure of the dark state decay rate to follow expectations of a simple ET model when the energetics are tuned using fluoro-Tyr analogs led us to propose a chemically plausible neutral pathway. In that pathway population of FAD* is sufficient in itself to initiate structure change in the highly conserved Gln. More recent QM/MM calculations support a much more complex mechanism,[43−45] than either that neutral pathway or a simple initiation in a single step ET reaction. In the calculated mechanism ET coupled to proton transfer, both of which rates are tuned by modulating the acidity of the conserved Tyr, is energetically required for light state formation, but the mechanism intimately involves dynamics of the protein around the flavin, including a solvation step to stabilize a Tyr-flavin charge transfer (CT) state and the presence of successful/unsuccessful ET pathways depending on fluctuations in the orientation of the Trp residue.[21,46−48] The absence of observable metastable radical intermediates in $AppA_{BLUF}$ and other BLUF domains could therefore arise from this complexity, the nature of which has been addressed here. Specifically, the interactions at C2═O characterized by mutagenesis must perturb the secondary protein dynamics and CT state stabilization, altering population and decay rate of radical intermediates to be above or below an observability threshold. This departure from simple kinetic schemes and the existence of multiple relaxation pathways is consistent with the observed inhomogeneity in BLUF photophysics,[49] and will have implications for the overall yield of light state formation and thus light driven processes. These could be quantified by appropriate assays.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.4c11817.

Supplementary figures including dark state recovery kinetics, TRIR, TRMPS, and TA spectra (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BLUF, Blue light utilized flavin; FAD, Flavin adenine dinucleotide; PCET, Proton-coupled electron transfer; TRMPS, Time-resolved multiple probe spectroscopy; TRIR, Time-resolved infrared spectroscopy; QM/MM, quantum mechanics/molecular mechanic; EADS, evolution associated difference spectra

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