Photophysics of the Blue Light Using Flavin Domain

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Conspectus

Light activated proteins are at the heart of photobiology and optogenetics, so there is wide interest in understanding the mechanisms coupling optical excitation to protein function. In addition, such light activated proteins provide unique insights into the real-time dynamics of protein function. Using pump-probe spectroscopy, the function of a photoactive protein can be initiated by a sub-100 fs pulse of light, allowing subsequent protein dynamics to be probed from femtoseconds to milliseconds and beyond. Among the most interesting photoactive proteins are the Blue Light Using Flavin (BLUF) domain proteins, which regulate the response to light of a wide range of bacterial and some euglenoid processes. The photosensing mechanism of BLUF domains has long been a subject of debate. In contrast to other photoactive proteins, the electronic and nuclear structure of the chromophore (flavin) is the same in dark- and light-adapted states. Thus, the driving force for photoactivity is unclear.

To address this question requires real-time observation of both chromophore excited state processes and their effect on the structure and dynamics of the surrounding protein matrix. In this Account we describe how time resolved infra-red (IR) experiments, coupled with chemical biology, provides important new insights into the signalling mechanism of BLUF domains. IR measurements are sensitive to changes in both chromophore electronic structure and protein hydrogen bonding interactions. These

contributions are resolved by isotope labelling chromophore and protein separately. Further, a degree of control over BLUF photochemistry is achieved through mutagenesis, while unnatural amino acid substitution allows us to both fine tune the photochemistry and time resolve protein dynamics with spatial resolution.

Ultrafast studies of BLUF domains reveal non-single exponential relaxation of the flavin excited state. That relaxation leads within one nanosecond to the original flavin ground state bound in a modified hydrogen-bonding network, as seen in transient and steady-state IR spectroscopy. The change in H-bond configuration arises from formation of an unusual enol (imine) form of a critical glutamine residue. The dynamics observed, complemented by quantum mechanical calculations, suggest a unique sequential electron then double proton transfer reaction as the driving force, followed by rapid reorganization in the binding site and charge recombination. Importantly, studies of several BLUF domains reveal an unexpected diversity in their dynamics, although the underlying structure appears highly conserved. It is suggested that this diversity reflects structural dynamics in the ground state at standard temperature, leading to a distribution of structures and photochemical outcomes. Time resolved IR measurements were extended to the millisecond regime for one BLUF domain, revealing signalling state formation on the microsecond timescale. The mechanism involves reorganization of a β-sheet connected to the chromophore binding pocket via a tryptophan residue. The potential of site-specific labelling amino acids with IR labels as a tool for probing protein structural dynamics was demonstrated.

In summary, time-resolved IR studies of BLUF domains (along with related studies at visible wavelengths and quantum and molecular dynamics calculations) have resolved the photoactivation mechanism and real-time dynamics of signalling state formation. These measurements provide new insights into protein structural dynamics, and will be important in optimising the potential of BLUF domains in optobiology.

Key References

Hall, C.R., Tolentino, J., Iuliano, J.N., Adamczyk, K., Lukacs, A., Greetham, G. M., Sazanovich, I., Tonge, P. J., Meech, S. R., 'Site Specific Protein Dynamics Probed by Ultrafast Infrared Spectroscopy of a Noncanonical Amino Acid' J. Phys. Chem. B, 2019, 123, 9592 – 9597[1] DOI: 10.1021/acs.jpcb.9b09425

Through the introduction of unnatural amino acids with strong IR absorption the spatial and temporal evolution of BLUF domain structure can be followed.

Gil, A., Laptenok, S. P., Iuliano, J. N., Lukacs, A., Verma, A., Hall, C.R., Yoon, E., Brust, R., Greetham, G. M., Towrie, M., French, J. B., Meech, S. R., Tonge, P. J., *'Photoactivation of the BLUF Protein PixD Probed by Site-Specific Incorporation of Fluorotyrosine Residues'* J. Amer. Chem. Soc., **2017**, *139*, 14638–14648[2] D**OI**: 10.1021/jacs.7b07849

Exchange of a critical tyrosine residue for a series of fluorotyrosines allows fine control over pK_a for proton transfer and reaction free energy for electron transfer, and therefore acts as a probe of the light sensing mechanism.

Brust, R., Lukacs, A., Haigney, A.L., Addison, K., Gil, A., Towrie, M., Clark, I. P., Greetham, G. M., Tonge P. J., Meech S. R., 'Proteins in Action: Femtosecond to Millisecond Dynamics of the Blue Light Sensing Protein AppA' J. Amer. Chem. Soc. 2013, 135, 16168-16174[3] DOI: 10.1021/ja407265p

Light absorption can act as the 'effector' for 'optical allostery'. By tracking the evolution in protein structure over multiple timescales the allosteric dynamics and pathway can be inferred.

Lukacs, A., Brust, R., Haigney, A. L., Laptenok, S. P., Addison, K., Gil, A., Greetham, G. M., Towrie, M., Tonge, P. J., Meech, S. R. , 'BLUF Domain Function Does Not Require a Metastable Radical Intermediate State' J. Amer. Chem. Soc., 2014, 136, 4605-4615[4] DOI: 10.1021/ja4121082

There is considerable diversity in the mechanism of different BLUF domains. At least two categories are identified. In one, electron transfer intermediates are obscured. The origin of the obscurity is not trivial kinetics, but arises from distinct ground state dynamics.

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1. Background and motivation Blue Light Using Flavin (BLUF) domain proteins are a family of photoactive flavoproteins found mainly in bacteria but also in eukaryotes.[5-8] They act as sensing modules for a range of light-controlled processes, including bacteriochlorophyll biosynthesis, biofilm formation, phototaxis and controlling levels of cyclic-AMP. Consistent with their diverse function, BLUF domains are found in a range of protein structures, either as elements of larger proteins that change structure upon irradiation or in protein complexes that dissociate under illumination.[9] Despite this diversity, the BLUF domain itself is modular and can be swapped between BLUF containing proteins without loss of function.[10] Modularity makes the BLUF domain an attractive element for optogenetics, in which light-sensing proteins are recruited to lend light-activation to specific protein functions.[11] Consequently those functions can be stimulated on demand with spatial and temporal control. Optogenetics is a powerful tool with demonstrated therapeutic potential,[11,12] which has added urgency to research aiming to understand and optimise operation of BLUF domains.

Photoactive flavoproteins are widespread in nature, and three types have been identified: light-oxygen-voltage (LOV) domains; photolyase/cryptochrome family; BLUF domains.[7,13] A fourth type, a fatty acid photodecarboxylase, has very recently been identified.[14] In each, the chromophore is the isoalloxazine moiety of either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), absorbing at around 450 nm (oxidized form, Figure 1A). The light-sensing mechanism of the BLUF domains is the least understood. In LOV domains triplet FMN reacts with an adjacent cysteine to form a meta-stable adduct, initiating a change in protein structure.[15] In cryptochromes excited FAD (FAD*) is photoreduced by electron transfer from a conserved Trp triad, leading to charge separation, proton transfer in plant cryptochromes,[16] and subsequent chemical reaction.[17] In both cases the flavin changes chemical state between dark and light-adapted forms. In contrast, in BLUF domains FAD is fully oxidized in both dark and light-adapted states. The only difference is a 15 nm red-shift in the electronic spectrum on formation of the light-adapted state, suggesting a change in chromophore environment. This raises the

question: *what is the driving force for structure change in BLUF proteins*? Here we describe experiments that probe the BLUF light-sensing mechanism using time-resolved infrared spectroscopy (TRIR), isotope editing and unnatural amino acid (UAA) substitution. These studies are placed in the context of time-resolved electronic spectroscopy and quantum chemical calculations.

2. BLUF structure and photochemistry The structure of the PixD BLUF domain (a light-sensor for the photophobic response of cyanobacterium *Synechocystis sp.PCC6803*) is shown in Figure 1B.[18] The isoalloxazine moiety is bound between two α-helices, which are aligned parallel to a five-strand β-sheet, such that the isoalloxazine plane is at right angles to it. The flavin is held in place by multiple H-bonds with residues in the β-sheet and α-helices. These H-bonding residues are highly conserved, suggesting that the intricate H-bonding network (Figure 1C) is of functional significance. Mutagenesis showed that residues Q50 and Y8 are essential for observation of the characteristic light-activated red-shift. Both are integral to the H-bonding network.[19] Direct evidence for light-driven change in this network was provided by vibrational spectroscopy, which showed a red-shift in an FAD mode, assigned to a stronger H-bond with the C4=O carbonyl in the light-adapted state.[20] Residues capable of forming H-bonds at C4=O include N32, Q50, M93 and W91.

Configuration of the Met and Trp residues in dark adapted structures has been the topic of debate (Figure 1C,D). The earliest crystal structures, of the BLUF domain protein AppA ($AppA_{BLUF}$, involved in control of bacteriochlorophyll biosynthesis) from *Rhodobacter sphaeroides*, showed Trp adjacent to the isoalloxazine ring and involved in the H-bonding network; the W_{in} configuration (Figure 1D).[21] Later structures suggested Trp is switched out of the H-bonding network (W_{out}), replaced by Met, yielding a distortion in the β-sheet structure (Figure 1C).[22] It was suggested that Trp/Met exchange might be the switch between light and dark-adapted states. Most recent structures show a W_{out} dark-state configuration in AppA, consistent with studies of fluorescence polarisation.[23]

Two crystal structures of light-adapted BLUF domains have been reported. Neither showed evidence for extensive structure change, such as a W/M switch, but both indicated light-induced changes in the β4-β5 strands of the β-sheet, suggesting their involvement in signalling state formation.[22,24] Residues in this region were also perturbed in NMR studies of light and dark-adapted states.[25] The most recent study showed light-adaptation was associated with only modest structure changes,[26] returning the focus to understanding light modulation of the H-bonded network (Figure 1C,D).

3. TRIR of BLUF domains.

Our high sensitivity ultrafast TRIR pump-probe experiments are described elsewhere.[27,28] In TRIR the 450 nm pump−IR probe delay of up to 6 ns is controlled by varying the optical pathlength. In the time-resolved multiple-probe spectroscopy (TRMPS) configuration a separate synchronised 1 kHz source provides the 450 nm pump, and optical and electronic delays combine to yield 10 kHz multiplexed time-delayed IR probe pulses from 100 fs to 1 ms. TRIR is a difference method, so only vibrations modified by photoexcitation and subsequent structure changes appear. Vibrational modes shifted due to excitation of FAD yield negative signals in difference spectra, while modes of FAD* and any photoproducts give positive signals. The frequency and/or intensity of vibrational modes of many amino acids are sensitive to their H-bonding environment, so TRIR/TRMPS experiments track protein structure evolution in real-time from 100 fs to 1 ms.[29,30]

The TRIR spectrum of PixD is shown in Figure 2A.[2] There are negative signals (bleaches) at 1700, 1638, 1580 and 1547 cm^{-1}. These were assigned to FAD based on TRIR of FAD or FMN in solution, complemented by density functional theory (DFT) calculations and isotope labelling.[31,32] The two highest wavenumber bleaches are C4=O and C2=O stretch modes, coupled to the N3H wag. DFT showed these are not exclusively localised on specific carbonyls. Instead, depending on H-bonding environment and N3H/D exchange, their character evolves between two localized modes (with C4=O at higher

wavenumber) and a delocalised O=C4−(N3H)−C2=O symmetric/anti-symmetric pair. This feature is important in understanding the sensitivity of these modes to their H-bonding environment.[31] The 1580 and 1547 cm^{-1} bleaches were assigned to flavin ring modes.

FAD* contributes positive features appearing instantaneously (*i.e.* during the <100 fs pump pulse). Calculation and assignment of excited state vibrations is less straightforward than for ground states, but there are recent assignments for FAD*.[33-36] The transient pair at 1380 and 1420 cm^{-1} arise from C=C/C=N ring modes of FAD*. The complex set of transients below 1700 cm^{-1} are calculated to have contributions from FAD* carbonyl modes, but comparison with FAD* in solution[37] suggests additional contributions from surrounding protein residues (e.g. at 1623 cm^{-1}).

Turning to temporal evolution (Figure 2A), the FAD* transient at 1380 cm^{-1} decays with non-single-exponential kinetics, and a mean lifetime of 87 ps. Since the lifetime of FMN* in aqueous solution is 4.5 ns,[38] picosecond decay indicates efficient quenching in PixD (the lifetime of FAD in aqueous solution is much shorter, but that reflects intermolecular quenching by the adenine moiety, which plays no role in BLUF domains, as it is usually anchored at the protein solution interface, Figure 1B). The intense bleach at 1547 cm^{-1} is a good indicator of ground state recovery, which occurs with a mean time of 137 ps, slower than FAD* decay. This is characteristic of intermediate state kinetics, and TRIR resolves the intermediates as a pair of transients at 1515 and 1535 cm^{-1}, which initially rise and then decay. One plausible FAD* quenching mechanism is electron transfer, which is often observed in flavoproteins, independent of whether they are photoactive or not.[39] The mechanism is charge separation (CS) between adjacent Tyr (or Trp)-FAD* pairs to form $Y^{\bullet+}$ and $FAD^{\bullet-}$ (although subsequent proton transfer may occur). The BLUF structure (Figure 1B-D) shows Tyr (and possibly Trp) residues near the chromophore. To assign the 1515/1535 cm^{-1} modes we synthesised a model compound for the neutral radical $FADH^{\bullet}$ and studied its TRIR, observing a strong ground state bleach at 1528 cm^{-1},[40] which supports electron transfer as the quenching step in PixD, with $FADH^{\bullet}$ as the 1535 cm^{-1} intermediate.[41]

The same conclusion was reached in transient optical absorption measurements,[42,43] but overlap of the broad FAD* and radical spectra, necessitated sophisticated kinetic analysis.

Given the obvious formation and decay of intermediates, the TRIR data were globally analysed assuming sequential kinetics. Global analysis fits the spectral evolution to a sum of first order kinetic steps and recovers a set of Evolution Associated Difference Spectra (EADS).[44] Three intermediates and one final component were required for an adequate fit (Figure 2B). The fastest step (2.5 ps) showed a rise of the 1515 cm^{-1} transient, followed by a 20 ps rise at 1535cm^{-1}, with both decaying with time constants of 110 ps and 525 ps. The existence of two radical intermediates in the PixD TRIR suggests sequential electron then proton transfer steps, TyrOH-FAD*→$TyrOH^{\bullet+}$-$FAD^{\bullet-}$→$TyrO^{\bullet}$-$FADH^{\bullet}$; this mechanism features prominently in theoretical calculations of the BLUF mechanism (below) and is represented schematically in Figure 3. Note that multi-exponential kinetics point to inhomogeneity in ground-state population.

The final EADS (Figure 2B) is significant as it reveals a persistent change in structure associated with formation of the light-adapted state. The most prominent feature is a positive/negative pair at 1690 cm^{-1} indicating a shift to lower wavenumber of a carbonyl mode of FAD. This occurs within 500 ps, but after the radical species recombine. The shift suggests stronger H-bonding to a flavin C4=O in the light-adapted state arising within 1 ns consistent with transient electronic spectroscopy and steady state IR.[43,45]

4. Diversity in BLUF photodynamics. While it is established that BLUF domains are modular, their photocycles exhibit variation; light to dark recovery of PixD takes seconds, but in $AppA_{BLUF}$ it takes fifteen minutes.[5] Similar diversity was found in primary process. In contrast to PixD, early studies of $AppA_{BLUF}$ failed to identify radical intermediates, although FAD* decay was non-exponential and quenched (mean decay time was 255 ps).[46,47] In contrast, the photoinactive Y21W mutant of $AppA_{BLUF}$ (equivalent to Y8 in PixD) showed typical radical intermediate features associated with $FAD^{\bullet-}$ (at 1521 cm^{-1}), presumably

arising because CS in Trp/FAD* is more energetically favourable than for Tyr.[4,48] Thus, radical intermediates are readily observable in $AppA_{BLUF}$, but are not observed during formation of its light-adapted state.

The only indication of light-activated structure change in $AppA_{BLUF}$ was a transient at ca 1666 cm^{-1} formed during the excitation pulse (Figure 4A).[46] Although an FAD* carbonyl might contribute here, no such mode was observed for FAD* in solution,[37] or in the light-adapted state of $AppA_{BLUF}$ or a photoinactive mutant Q63L. Thus the 1666 cm^{-1} transient was assigned to a mode of an amino acid residue instantaneously perturbed by electronic excitation. This assignment was confirmed by ^{13}C labelling the apoprotein. One candidate is Q63 (equivalent to Q50 in PixD), which is essential for light-activation, and forms an H-bond with N5 in the dark-state (Figure1D). To test this the Q63E mutant was studied.[49] Replacing Gln with Glu allows the H-bond network of $AppA_{BLUF}$ to be retained, but the C=O mode of the Glu sidechain will be shifted relative to Gln. Electronic excitation of Q63E indeed resulted in a new instantaneous bleach at 1724 cm^{-1}, with a corresponding transient at 1704 cm^{-1}. The 1724 cm^{-1} bleach was assigned to the carbonyl of the Glu RCOOH side chain (confirmed by ^{13}C labelling). A stable protonated Glu carboxylate sidechain suggests a change in pK_a in the flavin binding pocket, compared to aqueous solution. That the Glu C=O mode shifts to 1704 cm^{-1} on FAD* formation points to weakening of the C=O bond, indicating increased H-bond strength with N5 on FAD* formation. The instantaneous transient appearance shows that changes in the H-bonding network can be induced by changes in electronic state alone, without major reorganisation of nuclear structure.

There are two plausible explanations for the failure to observe radical intermediates in $AppA_{BLUF}$. First, observation is kinetically restricted, because charge recombination (CR) is much faster than CS.[43] A consequence would be a very short-lived CS state, restricting the extent of nuclear reorganisation possible during the intermediate lifetime. Second, electron transfer in PixD is incidental to mechanism

(as it is in many photo-inactive flavoproteins[39,50]) and a neutral pathway, driven by changes in the H-bond network alone, is sufficient for BLUF light-state formation.

To test the hypothesis that electron transfer in $AppA_{BLUF}$ occurs with rate limiting CS, UAA substitution was employed. The putative donor, Y21, was exchanged for a series of fluorotyrosines (FY), modifying the redox potential and pK_a.[4] Under the assumption of CS being the primary step, and assuming applicability of a simple Marcus model in the normal region, the prediction was that the CS rate would increase when Y21 was exchanged for 2-or 3-FY due to the change in redox potential. In contrast, a threefold decrease in FAD* decay rate was observed. The same experiment in PixD led to slower CS, consistent with the faster PixD reaction being in the Marcus inverted region.[2] Thus modulation of electron transfer by FY substitution in PixD was consistent with its obvious CS reaction, but inconsistent with CS in $AppA_{BLUF}$.[4] Ultrafast data on six BLUF domains were reviewed.[2] In three examples PixD, OaPAC (a light-controlled adenylyl cyclase from *Oscillatoria acuminata*) and PapB[51] (from purple bacterium *Rhodopseudomonas palustris*, which controls biofilm formation) radical intermediates were observed, while in three others, AppA, BlrB (from *Rhodobacter sphaeroides*, a small BLUF domain of unknown function) and BlsA (from Acinetobacter baumannii, a regulator influencing a number of light sensitive processes) none were detected.[52,53] This suggests the existence of at least two classes of BLUF domain with distinct kinetics. Consideration of protein structure and/or sequence suggests radical intermediates are observed when two amino acids can H-bond to C2=O, but absent when there is only one H-bonding partner (see Figs 1C,D). It would be interesting to investigate this hypothesis in other BLUF domains, and seek a role for C2=O H-bonding on CS.

There is ambiguity in modulation of reduction potential by FYs, as Tyr fluorination modifies both redox potential and pK_a (hence H-bond interactions).[54] FY exchange effects on TRIR were extended to three different BLUF domains ($AppA_{BLUF}$, PixD and OaPAC) (Figure 4). The exchange does not perturb the main features of the BLUF domain ground state *i.e.* the bleach modes associated with the isoalloxazine ring

are unchanged (Figure 4). However, as the pK_a of YF reduces formation of the light-adapted state (indicated by a red-shift in the C=O mode) is suppressed. It is likely that as Tyr pK_a reduces the critical H-bond network is disrupted and the Tyr may be deprotonated at the lowest pK_a.

Figure 4 also provides further evidence of BLUF domain diversity. In $AppA_{BLUF}$ the effect of FY exchange on FAD* is an increase in mean lifetime as pK_a decreases, with subnanosecond (quenched) components lengthening and decreasing in weight.[4] In PixD, the red-shifted C4=O is only formed with 2-FY, which has the smallest pK_a change. When the red-shifted C4=O state is absent there is no radical intermediate at 1520-35 cm^{-1}, but FAD* is still quenched (Figure 4B). In OaPAC (Figure 4C) radical and product formation are suppressed for larger pK_a shifts, as for PixD, but FAD* quenching is also suppressed, to the extent that the triplet state of FAD* is formed. These data highlight the key roles of the H-bond network and proton transfer in formation of the light adapted state. At lower pK_a formation of tyrosinate could occur and promote electron transfer (consistent with assignment of the 1515 cm^{-1} transient to $FAD^{\bullet -}$[55]) but stable light-adapted states cannot form.

5. A role for Gln tautomerization The absence of radical intermediates in $AppA_{BLUF}$ led us to consider possible neutral pathways to the light-adapted state (Figure 3).[46,49] The 1666 cm^{-1} transient assigned to Q63 was proposed to result from side chain keto-enol (or equivalently amide-imide) tautomerization, leading to ultrafast H-bond reorganization around C4=O, initially without nuclear reorganisation. Stabilising the light-adapted state after FAD* decay requires nuclear structure change, proposed to be rotation of the Q63 tautomer. The eventual decay of the 1666 cm^{-1} mode as the red-shifted state formed was assigned to relaxation of the unstable enol back to the keto form; the mechanism is illustrated in Figure 5. A role for Q63 rotation in stabilising the light-adapted state was proposed earlier, where electron transfer was the driving force and Gln retained the keto form.[21,42] The pathway in Figure 5 is consistent with absence of a CS state in $AppA_{BLUF}$, although the origin of enol tautomer stability is unclear.

Domratcheva et al[56] and Saghdegian et al[57] proposed a role for keto-enol tautomerization on the basis of theoretical calculations. They calculated that red-shifts in electronic and IR absorption were reproduced by formation of the Q63 enol, and that keto-enol tautomerization was accompanied by restructuring of the H-bond network. The driving force was electron transfer from Y21 to flavin, followed by proton transfer from the Y21 phenol to Q63, as seen in PixD TRIR (but not $AppA_{BLUF}$). Since these original observations Gln tautomerization has become an important feature of theoretical and experimental studies of BLUF domains.

Two recent studies investigated Gln tautomerization, combining IR difference spectroscopy, isotope labelling and computation. Domratcheva et al computed light-state spectra for unlabelled and ^{15}N labelled Gln in BlrB and compared the result with IR difference measurements.[58] Data were consistent with W_{out} and Gln oriented with its carbonyl H-bonded to Y9. Electronic excitation results in electron and proton transfers such that the Gln enol forms with OH and NH both H-bonded to the C4=O. Iwata et al studied ^{15}N and ^{13}C labelled $AppA_{BLUF}$, and concluded that light-state formation requires tautomerization and rotation of Gln, but that this state was stable with W_{in}.[59] They also reported a downshifted frequency of a phenolic OH, consistent with a Tyr-enol H-bond, which was reproduced in a computational study.[60] While there are differences in detail between these studies (which investigated different BLUF domains) evidence of a role for Gln tautomerization is compelling. Both require the usually unstable enol tautomer to persist for the lifetime of the light-state (tens of minutes in $AppA_{BLUF}$), which is remarkable, requiring the light-adapted state to be rigid (which further suggests relaxation of the 1666 cm^{-1} transient (Figure 5) reflects enol rotation rather than relaxation to keto[46]).

6. Comparison to calculations. Further mechanistic detail followed from quantum chemical and molecular dynamics simulations of experimental data.[61-67] Most calculations accurately reproduce electronic and IR spectral shifts on light-state formation by assuming Gln enol formation leading to a new H-bond network, and usually including Gln rotation. Calculation of excited state relaxation yields

data comparable to ultrafast experiments. Initially the locally excited (LE) state of FAD* is stable, with a $Tyr^{\bullet+}$–$FAD^{\bullet-}$ CS state at higher energy. Calculations suggest that structural evolution in the LE state and nuclear reorganisation in the protein stabilises the CT state, such that it crosses the LE state and electron transfer occurs. Details vary and a number of relaxation pathways and structures have been considered, but key parameters are nuclear reorganization in the isoalloxazine ring, electrostatic interactions with the FAD* charge distribution and Tyr–FAD distance.[61-67] Importantly, for CS to be rapid (as suggested by experiment) most pathways require a specific geometry of the conserved Gln. The Gln geometry which stabilises the CT state is also well aligned for protonation of its carbonyl by the very acidic $Tyr^{\bullet+}$ proton. This proton transfer is followed by a second to $FAD^{\bullet-}$, to yield the enol and a neutral radical pair. Most recent calculations suggest rotation of the enol to make an H-bond at C4=O. CR recovers the fully oxidized form of FAD but now in the metastable light-adapted state. The final reorganisation requires at least a several picosecond lifetime for the diradical state. This mechanism (Figure 6A) is consistent with TRIR measurement in PixD (Figure 2) and OaPAC (Figure 4) and a recent light-adapted crystal structure.[26]

An explanation for the absence of detectable radical intermediates in several BLUF domains is required. Calculation has not yet found an energetically accessible route for the mechanistically feasible neutral pathway to the Gln tautomer.[46,49] The explanation of rate determining CS and fast CR was contradicted by FY data and the requirement for a several picosecond lifetime in the CS state.[4] A possible explanation lies in the inhomogeneous FAD* kinetics (a feature of all ultrafast studies of BLUF domains)[42,46] and the dynamic FAD binding site suggested by calculations.[61,67] Domratcheva established that the key Gln residue rotates freely over a low barrier even in the dark-state, so different conformers exist at room temperature.[61] Hammes-Schiffer et al showed that (i) Gln reorients in the dark-state of $AppA_{BLUF}$ on a nanosecond timescale and is coupled to location of the conserved Tyr and (ii) that the Trp/Met exchange can be thermally activated over a barrier of ca 50 $kJmol^{-1}$, suggesting both conformers exist; W_{in} was slightly more stable than W_{out}. Further, W_{in} can adopt two distinct orientations, only one of

which supports the electron and proton transfer.[66,67] Thus, residues around the flavin adopt a number of structures that may quench the excited state with a range of rates and mechanisms, but not lead to photactivation, consistent with non-single exponential FAD* decay. Formation of the $Y^{\bullet+}$-$FAD^{\bullet-}$ (hence the light-adapted state) requires a specific geometry which must also support proton transfer. Thus, the rate-determining step may be fluctuations in the FAD environment to generate structures competent for CS and proton relay. These ideas are incorporated into the kinetic scheme shown in Figure 6, where fluctuations 'gate' the reaction in either ground or excited state (Figure 6B). Such a scheme allows photoexcitation of 'activated' ground state geometries, and subsequent CS, enol formation (consistent with instantaneous appearance of the 1666 cm^{-1} transient) and rotation while maintaining overall low concentrations of the radical intermediates compared to bleached flavin.[4,46] Thus, the distinction between BLUF domains that do and do not show radical intermediates may be population of ground states competent to undergo CS and proton transfer. BLUF domains with prominent radical intermediates have more stable (or frequently accessed) geometries for light-state formation, and possible higher light-adapted state yields. The BLUF domain dependent effects of FY exchange (Figure 4) and mutagenesis may be to modify *both* photochemistry and ground state dynamics. An important test of this hypothesis will be measurements of the relative quantum efficiency of different BLUF domains; such measurements are planned.

7. Dark-adapted to signalling state: optical allostery. The red-shifted light-adapted state formed in <1 ns is not the final signalling state. Local changes in the flavin H-bonding network must translate into structure changes in the β-sheet, modulating, for example, the affinity of the BLUF domain for output partner proteins. It is of general interest to follow such allosteric structure changes in real time, as they are a key feature of many protein functions.

Structural evolution in $AppA_{BLUF}$ was measured from <1 ps to >100 μs by TRMPS.[3] Most spectral changes are complete in 10 ns, but evolution in a 1622/1631 cm^{-1} bleach/transient pair and a transient/bleach pair associated with C4=O continues for microseconds (Figure 7A). Assignment of the former to protein amide I modes was confirmed by ^{13}C labelling, and the wavenumber implies the change is in β-sheet residues. Lineshape evolution for the 1622/1631 cm^{-1} pair suggests a time dependent spectral shift to higher wavenumber (e.g. loosening of H-bonds). However, kinetic analysis (Figure 7B) reveals a more complex picture; the transient evolves faster than the bleach, suggesting complex non-single exponential kinetics and the involvement of more than one residue. Further, although evolution in the C4=O pair was of low amplitude, it had a separate timescale to the 1622 cm^{-1} bleach. Thus, after formation of the red-shifted state there is structural evolution on distinct microsecond timescales modifying the β-sheet (some 10Å from Q63) and the H-bond network around the flavin.

These measurements were extended to W104A and M106A mutants of $AppA_{BLUF}$.[3] Both show formation of red-shifted C4=O, but reversion to the dark-state is accelerated in W104A.[68] In vivo W104A acts as if locked in a light-adapted structure. Both factors suggest involvement of W104 in signalling state formation.[69,70] In line with this, W104A mutation has a large effect on TRMPS data (Figure 7C). The 1631 cm^{-1} transient appears within 10 ns, but the 1622 cm^{-1} bleach appears only weakly. Thus in W104A perturbation to the β-sheet does not develop, confirming W104's role in signal propagation. In contrast the transient/bleach associated with C4=O develops more strongly than in $AppA_{BLUF}$ and within 10 ns. Thus, formation of the red-shifted state is enhanced and accelerated by W104 mutation, but communication of photoexcitation to the β-sheet is suppressed. M106A, was qualitatively similar to $AppA_{BLUF}$, presenting no evidence for its role in allostery.

TRIR reveals allosteric dynamics, but the level of detail is limited; Figure 7A shows that β-sheet is perturbed, but not which residues. Ideally, one would like single residue resolution to track allosteric

dynamics. Residue selective isotope labelling is possible, but usually all occurrences of the residue are labelled, and the down-shifted amide modes are in a spectroscopically crowded region. An alternative is to employ UAA substitution to exchange one occurrence of a residue for an 'IR beacon' such as cyano- or azidophenylalanine (AzPhe). These probes are site specific and absorb strongly in blank regions of the IR spectrum; they are widely applied in steady state protein IR.[71-73]

We incorporated AzPhe in $AppA_{BLUF}$/PixD in place of W104/91 and M106/93.[1] Results were complicated by the complex lineshape of AzPhe, but revealed a number of new features (Figure 8). On replacing W104/91 the azido mode responds instantaneously to electronic excitation, suggesting direct involvement of this residue in the H-bonding network (e.g. via Q63/50). The azido mode subsequently evolves on a bi-exponential timescale, with kinetics similar to FAD* decay. In PixD M93AzPhe showed a weaker response than W91 but a similar timescale, while in $AppA_{BLUF}$ M106AzPhe response was very weak. These preliminary studies highlight the potential of 'IR beacons' for tracking allosteric dynamics.

8. Dark-state recovery. The BLUF photocycle is completed by recovery from signalling to dark-state, which takes seconds to tens of minutes, depending on the BLUF domain. This recovery phase has been less investigated, although Masuda and co-workers showed it was slower in D_2O than H_2O, indicating a role for proton tunnelling in the rate determining step, and that W104A mutation accelerated recovery.[5]

We probed the recovery mechanism through exchange of Y21/Y8 in $AppA_{BLUF}$/PixD for a series of FY derivatives, modifying the Tyr pK_a.[2,74] In contrast to the forward reaction, the recovery is well represented by a single exponential (Figure 9A). The kinetic isotope effect is >5 in all cases, consistent with proton tunnelling. Even in cases where the characteristic red-shift of the C4=O mode was absent from EAS (Figure 4) the absorption red-shift can be observed (Figure 9B). However, as the p*K*a of Y21/8 is decreased the stability (or yield) of the red-shifted state is greatly reduced, favouring a

photostationary state closer to the dark-state. This may reflect an effect of Tyr pK_a on the population of ground state orientations competent to undergo CS (Figure 6B).

Figure 9C shows a Brønsted plot for the recovery rate constant, *k*. The effect of Y21 pK_a on AppA_{BLUF} is remarkable, a ca 900 fold acceleration over the pKa range 9.0-7.2, in sharp contrast to the forward photoreaction. Further, the slope is close to 1 suggesting a transition state in which the H-bond is essentially broken. It is difficult to ascertain which H-bond is involved, as it depends in part on the structure of the red-shifted state, which is unresolved. Interestingly the larger rate constant for recovery in PixD was less sensitive to Y8 pK_a, increasing by a factor of 15 across the range. Further the slope of the Brønsted plot for PixD was between 0.43 (H_2O) and 0.28 (D_2O) suggesting less proton transfer in the transition state, consistent with mechanistic diversity among BLUF domains.

8. Conclusions Photoactivated protein function combined with TRIR allows the study of real time protein dynamics. The complete photocycle of BLUF domains has been observed, from <1 ps to >1 ks. Application of a battery of spectroscopic and theoretical methods clarified the mechanism of BLUF domain photoactivation. The most likely route is electron and double proton transfer in the FAD-Gln-Tyr triad leading to Gln in its enol form, generating the red-shifted state and modified H-bond network. Most data point to Gln being rotated in the light-adapted state, but there are experimental data and calculations to the contrary, so this is still to be unambiguously resolved. Significant diversity among BLUF domains exists, and accounting for it suggests further levels of complexity. In particular, protein dynamics in the ground state feed into photochemical dynamics. This complexity presents new challenges, but warrants investigation. It is likely that the complexity resolved in BLUF domains is the rule rather than the exception in functional protein dynamics. It is evident in BLUF domains because of the high signal-to-noise and time resolution attained. Thus, BLUF domain photodynamics will remain an excellent model system for observation and calculation of protein dynamics.

Formation of the red-shifted state is not the end of the story. In $AppA_{BLUF}$ protein dynamics are observed on a microsecond timescale, assigned to allostery leading to the signalling state. This observation yields new mechanistic detail, which was refined using mutagenesis and UAA exchange for IR beacons. The combination of light-activated proteins, TRIR, UAA exchange and QMMM calculations is a powerful probe of allostery.

Figure Legends

Figure 1. A. Numbering convention for isoalloxazine. B Structure of the PixD BLUF domain (PDB:2HFO). The flavin ring is blue, the β-sheet yellow and the α-helices red. C. Detail of the H-bond network and key residues in PixD. D. As C but for $AppA_{BLUF}$ (PDB:1YRX).

Figure 2. A. TRIR of PixD excited at 450 nm. B. EADS arising from global analysis (insets highlight the intermediate kinetics). Adapted with permission from ref. [2] (2017) ACS.

Figure 3. Schematic potential energy surfaces highlighting primary processes in BLUF domains following electronic exciation. Solid curved arrows indicate the ultrafast electron and proton transfer pathways, the dashed arrow shows a possible 'neutral' route. The final recovery (black curved arrow) occurs in seconds to minutes.

Figure 4. Dynamic diversity among three BLUF domains illustrated through the dependence on pK_a of the putative Tyr electron donor by Y/FY exchange. TRIR were data excited at 450 nm for A. $AppA_{BLUF}$ (the absence of $FAD^{\bullet -}$/ $FADH^{\bullet}$at 1510 - 1530 cm^{-1} is obvious) B. PixD (the $FADH^{\bullet}$ is not observed for lower pK_a). C. OaPAC, where radical intermediates are also not observed at reduced pK_a but the ground state recovery is slow (nanoseconds).

Figure 5. Proposed mechanism for a neutral pathway to the light-adapted state via Gln tautomerization (Adapted with permission from ref. [49] (2011) ACS).

Figure 6. A. Schematic mechanism of the most likely reaction pathway – the prominence of the radical intermediate is obvious. B. Extension to scheme A to include ground and excited state equilibria as suggested by calculations and which may suppress radical population. {i/j} implies dynamics equilibria for W and Q orientations. Arrow indicates Q orientation. (k) or (e) indicated keto or enol forms of Q respectively.

Figure 7. Allosteric Dynamics in AppA. A. TRIR showing β-sheet dynamics at 1622/31 cm-1 (* indicates triplet FAD) as well as formation of the red shifted C4=O in the light adapted state. B. Illustrating the distinct kinetics of the components of the β-sheet relaxation. C. Effect of W104A mutation on allosteric relaxation, where formation of the red-shifted C4=O is fast and β-sheet dynamics suppressed. Adapted with permission from ref. 3 (2013) ACS.

Figure 8. A. Effect of AzPhe W104AzPhe (which may be W_{in} or W_{out}) replacement on TRIR. B. TRIR response of the azido mode in W104AzPhe $AppA_{BLUF}$. Adapted with permission from ref. [1] (2019) ACS.

Figure 9. Light- to dark-state recovery modulated by Y21FY. A. Single exponential recovery dynamics for $AppA_{BLUF}$ and five FY mutants measured at 293 K. B. Extent of red-shift in PixD and its mutants after constant 450 nm irradiation. The red-shift develops, but the photostationary state increasingly favours the dark state at lower FY pK_a. C. Brønsted plots for PixD and $AppA_{BLUF}$ as a function FY pK_a. Adapted with permission from refs. [2,74] (2017, 2016) ACS.

Biographies

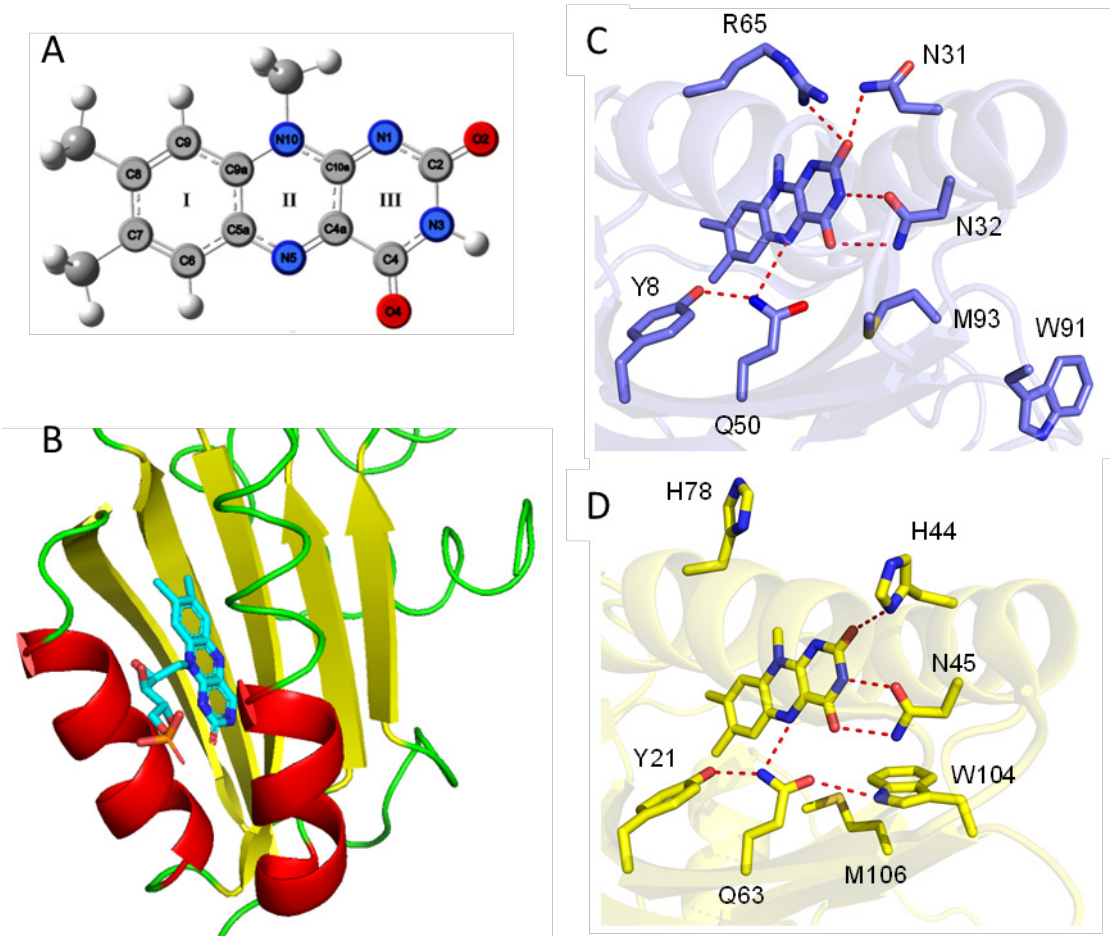
Andras Lukacs is a biophysicist with interest in ultrafast spectroscopy and photochemistry (particularly photochemistry of flavoproteins including BLUF domain proteins, photolyases, and cryptochromes). He graduated as an engineer-physicist in 1997 at the Technical University of Budapest (Hungary). He is currently Associate Professor of Biophysics at the Medical School of University of Pécs (Hungary) where he obtained his Ph.D. degree. He was Marie Curie student and postdoctoral fellow at the Laboratory of Optics and Biology at Ecole Polytechnique (France) and senior research associate at the School of Chemistry at The University of East Anglia.

Peter Tonge is a chemical biologist with interests from drug development to photosensor proteins. He studies the role of drug− target kinetics in drug discovery and the structural dynamics of photosensor proteins and develops positron emission tomography radiotracers to diagnose infections. Peter is currently a Distinguished Professor of Chemistry and of Radiology (by courtesy) at Stony Brook University, NY, where he is the Chair of the Department of Chemistry and Director of the Center for Advanced Study of Drug Action. He is also an Associate Editor for ACS Infectious Diseases. Peter earned his B.Sc. and Ph.D. degrees in Biochemistry from Birmingham University, U.K., and was a SERC/NATO postdoctoral fellow at the National Research Council of Canada (NRCC). After positions as a Research Associate and Research Officer at NRCC, he was a Staff Investigator at the Picower Medical Research Institute before joining Stony Brook University.

Steve Meech is a physical chemist with interests in ultrafast photochemistry (particularly molecular motors, coupled dimers and protein chromophores), liquid state dynamics and photobiology (including fluorescent protein photophysics and light sensing flavoproteins). He is currently professor of Physical Chemistry at The University of East Anglia, from where he graduated BSc in 1978. He studied for his PhD at Southampton University and the Royal Institution, and held postdoctoral appointments and

fellowships at The Royal Institution, UK, Wayne State University, USA, Groningen University, The Netherlands and The Institute for Molecular Science, Japan.

Figure 1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O2
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I
II
III
C7
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C6
N5
C4
O4
B
C
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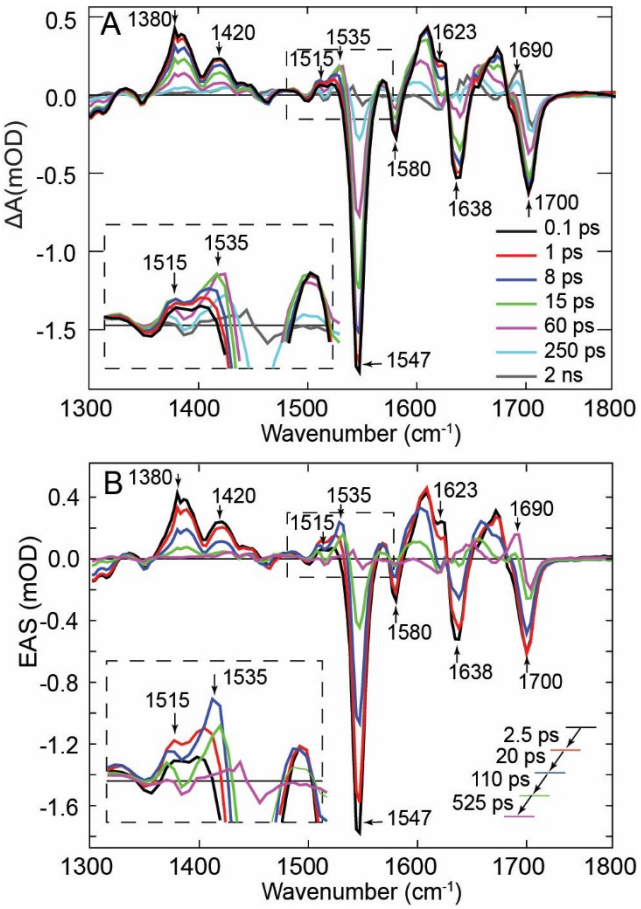
M106
Q63

Figure 2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1380
1420
1515
1535
1623
1690
1580
1638
1700
1547
0.1 ps
1 ps
8 ps
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60 ps
250 ps
2 ns
ΔA(mOD)
Wavenumber (cm-1)
B
1380
1420
1515
1535
1623
1690
1580
1638
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1547
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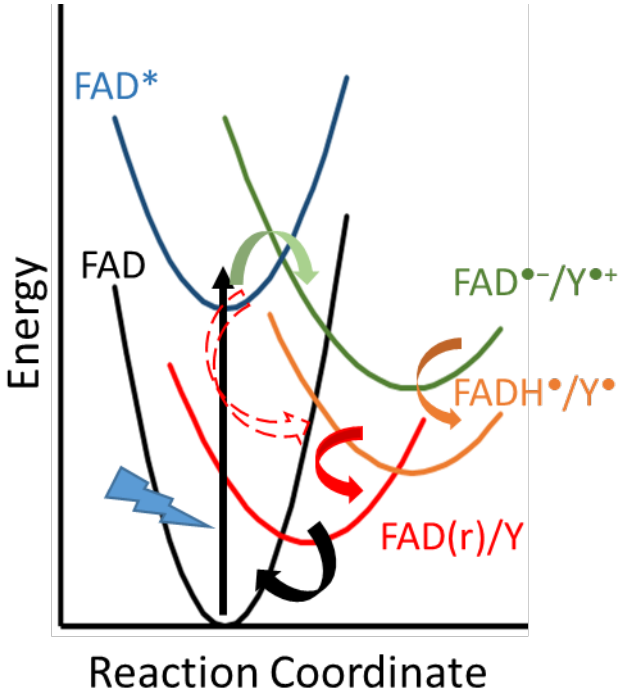
110 ps
525 ps
EAS (mOD)
Wavenumber (cm-1)

Figure 3



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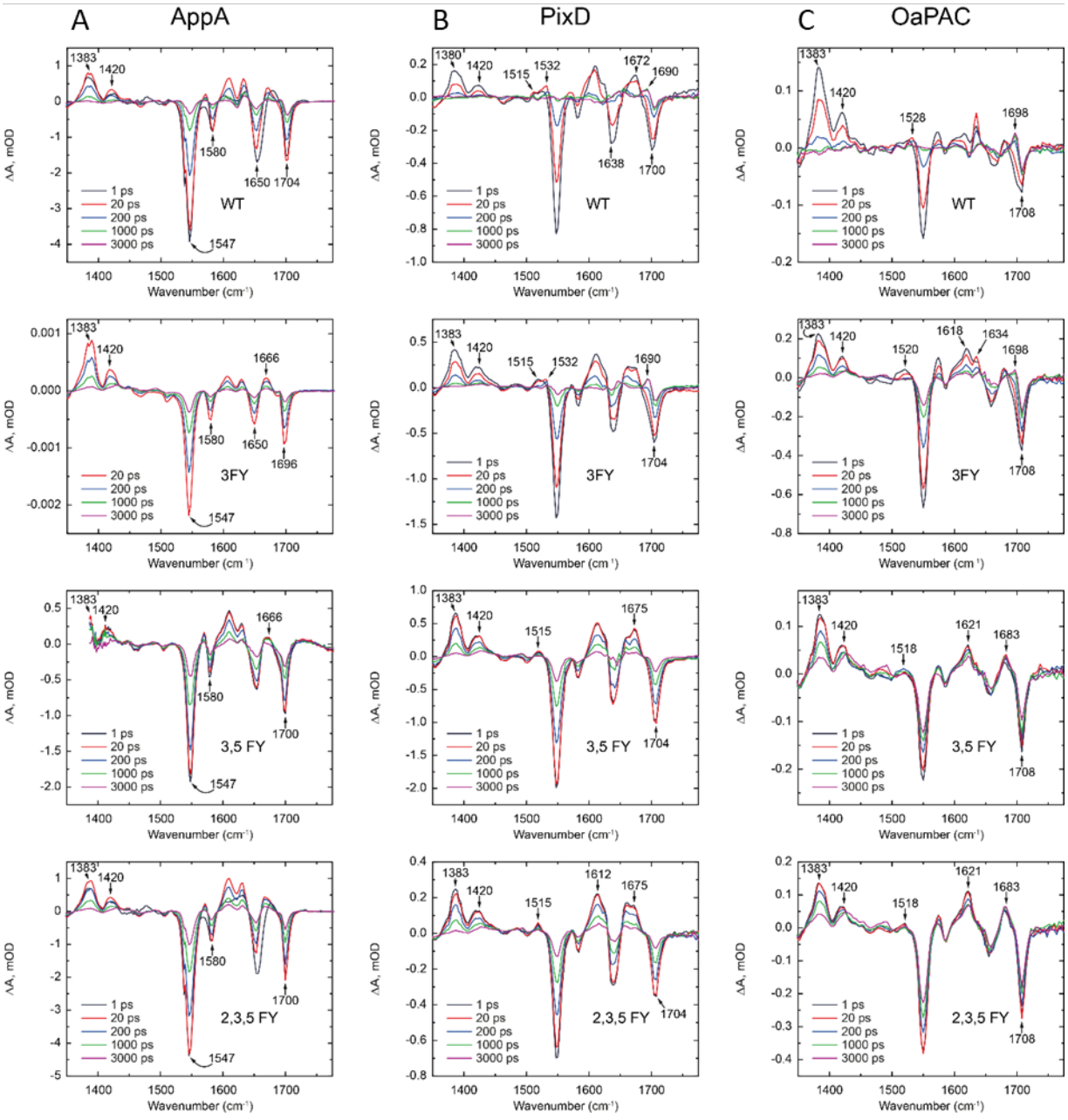
A
AppA
B
PixD
C
OaPAC
ΔA, mOD
Wavenumber (cm⁻¹)
1 ps
20 ps
200 ps
1000 ps
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3FY
3,5 FY
2,3,5 FY
1383
1420
1515
1532
1672
1690
1638
1700
1528
1698
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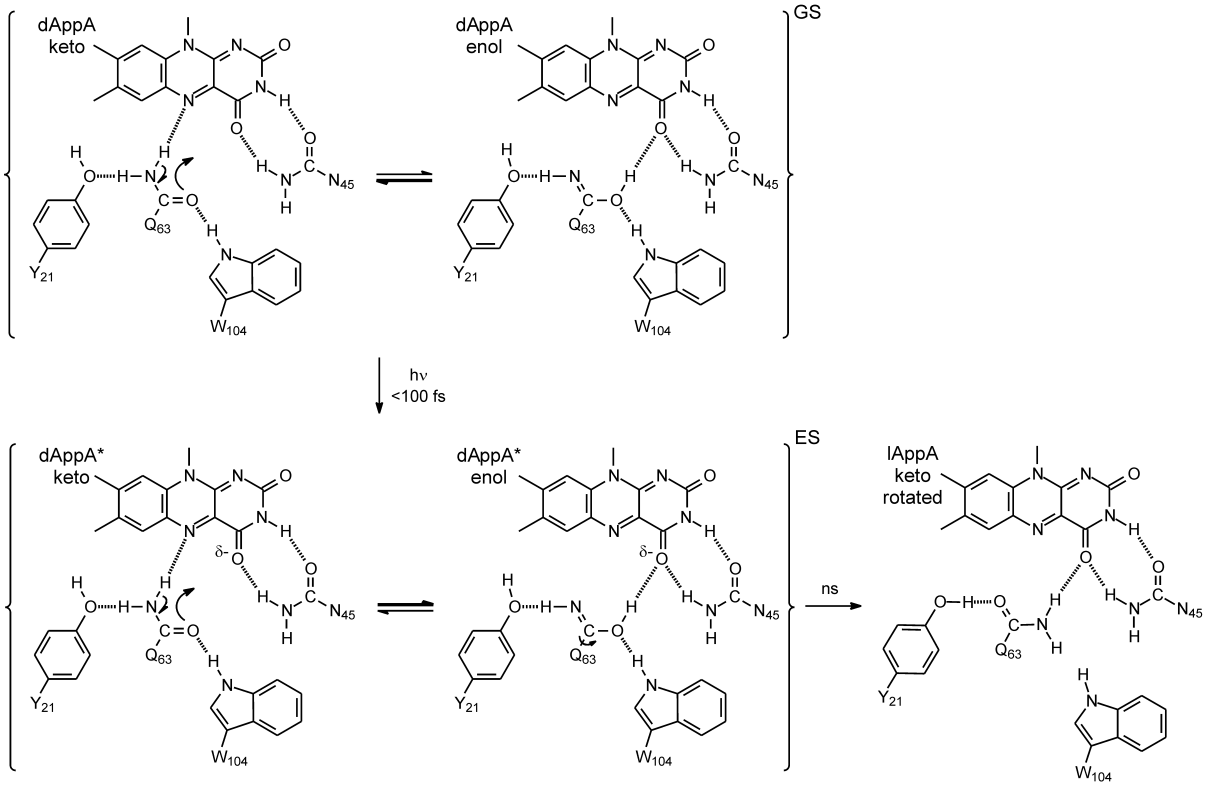
1612

Figure 5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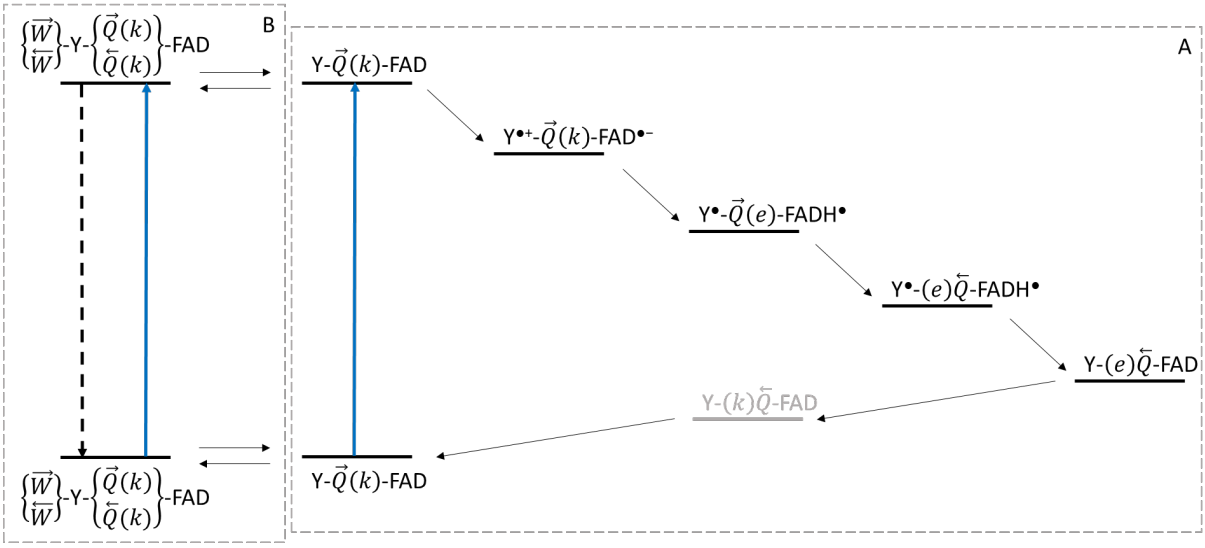
W104
N45

Figure 6


B
$\{\vec{W}\}$-Y-$\{\vec{Q}(k)\}$-FAD
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A
Y-$\vec{Q}(k)$-FAD
Y$^{\bullet+}$-$\vec{Q}(k)$-FAD$^{\bullet-}$
Y$^{\bullet}$-$\vec{Q}(e)$-FADH$^{\bullet}$
Y$^{\bullet}$-$(e)\overleftarrow{Q}$-FADH$^{\bullet}$
Y-$(e)\overleftarrow{Q}$-FAD
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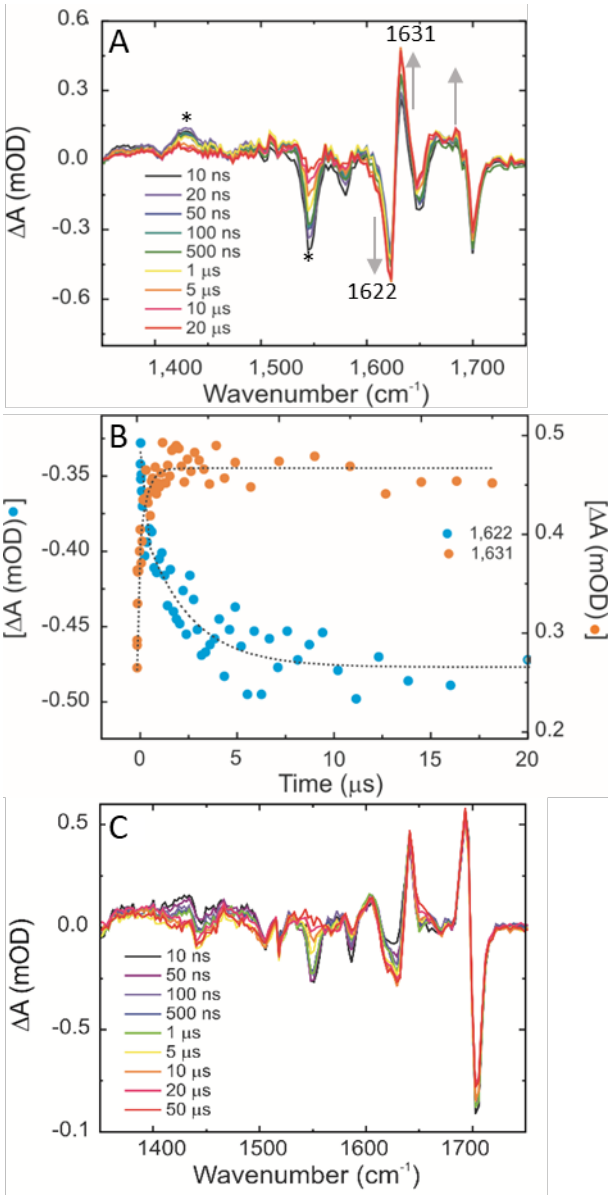
Y-$\vec{Q}(k)$-FAD

Figure 7


A
1631
1622
*
*
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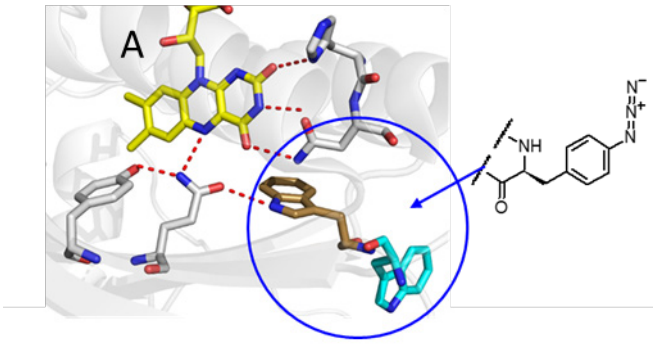
20 ns
50 ns
100 ns
500 ns
1 µs
5 µs
10 µs
20 µs
ΔA (mOD)
Wavenumber (cm-1)
B
1,622
1,631
[ΔA (mOD)•]
[ΔA (mOD)•]
Time (µs)
C
10 ns
50 ns
100 ns
500 ns
1 µs
5 µs
10 µs
20 µs
50 µs
ΔA (mOD)
Wavenumber (cm-1)

Figure 8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NH
O
N
N
N

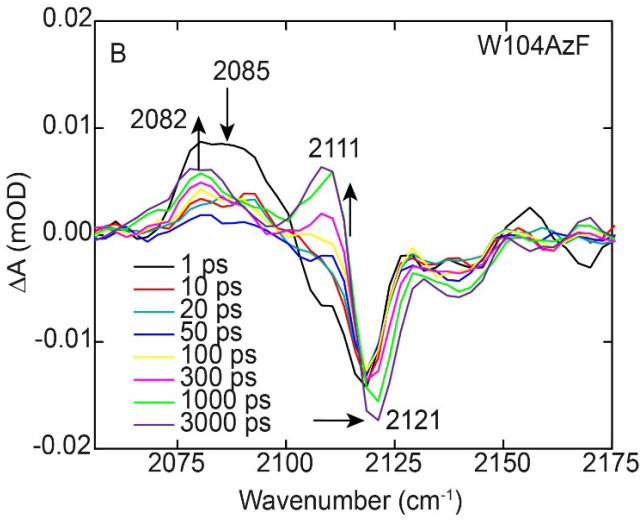
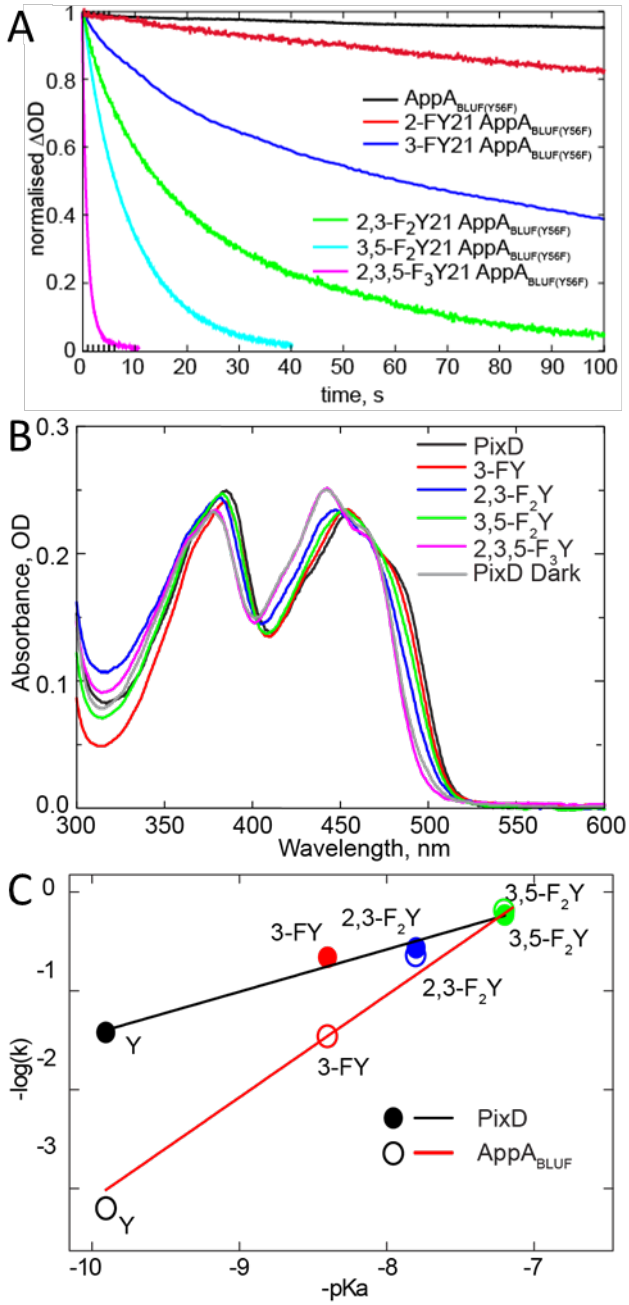

B
W104AzF
2085
2082
2111
2121
0.02
0.01
0.00
-0.01
-0.02
ΔA (mOD)
1 ps
10 ps
20 ps
50 ps
100 ps
300 ps
1000 ps
3000 ps
2075
2100
2125
2150
2175
Wavenumber (cm-1)

Figure 9


A
normalised ΔOD
time, s
AppA BLUF(Y56F)
2-FY21 AppA BLUF(Y56F)
3-FY21 AppA BLUF(Y56F)
2,3-F2Y21 AppA BLUF(Y56F)
3,5-F2Y21 AppA BLUF(Y56F)
2,3,5-F3Y21 AppA BLUF(Y56F)
B
Absorbance, OD
Wavelength, nm
PixD
3-FY
2,3-F2Y
3,5-F2Y
2,3,5-F3Y
PixD Dark
C
-log(k)
-pKa
3-FY
2,3-F2Y
3,5-F2Y
Y
PixD
AppABLUF

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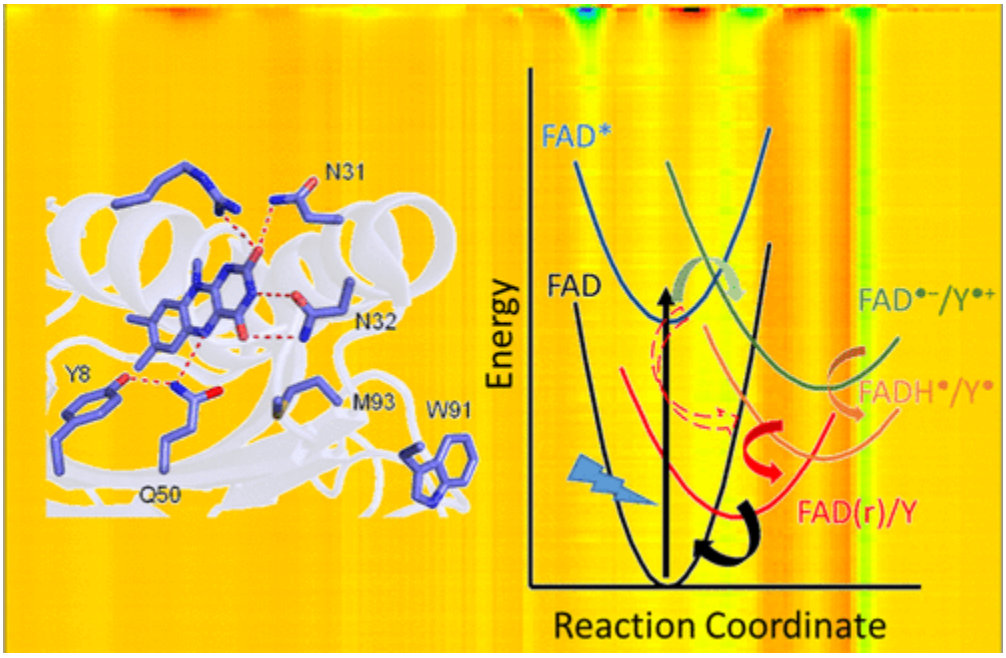
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