Site Specific Protein Dynamics Probed by Ultrafast Infrared Spectroscopy of a Noncanonical Amino Acid

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Abstract

Real-time observation of structure change associated with protein function remains a major challenge. Ultrafast pump-probe methods record dynamics in light activated proteins, but the assignment of spectroscopic observables to specific structure changes can be difficult. The BLUF (blue light using flavin) domain proteins are an important class of light sensing flavoprotein. Here we incorporate the unnatural amino acid (UAA) azidophenylalanine (AzPhe) at key positions in the H-bonding environment of the isoalloxazine chromophore of two BLUF domains, PixD and $AppA_{BLUF}$; both proteins retain the red shift on irradiation characteristic of photoactivity. Steady state and ultrafast time resolved infrared (TRIR) difference measurements of the azido mode reveal site-specific information on the nature and dynamics of light driven structure change. AzPhe dynamics are thus shown to be an effective probe of BLUF domain photoactivation, revealing significant differences between the two proteins, and a differential response of the two sites to chromophore excitation.

1. Introduction

Blue light sensing proteins play key roles in both mediating the response of a range of organisms to light and in optobiology.[1-3] Of the three photoactive flavoprotein families,[4] the BLUF domain is least understood. Uniquely in BLUF domains the oxidation state of the isoalloxazine chromophore is unchanged between dark and light adapted states.[2, 5] Only a *ca* 15 nm red shift in the electronic spectrum of the fully oxidized flavin co-factor is observed, which develops in < 1 ns and is associated with a change in H-bonding interactions between the isoalloxazine and its protein matrix.[6-16] This local structure change results in reorganization of the secondary structure leading to signalling state formation on the microsecond timescale.[17]

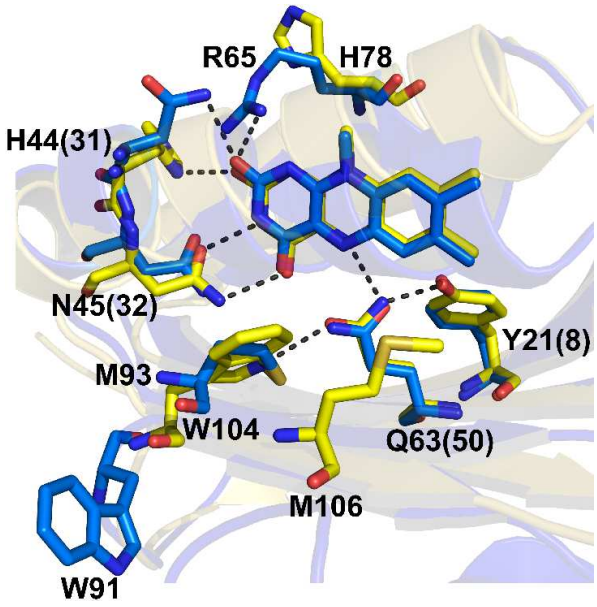

Figure 1. O*verlay of dark state structures of the isoalloxazine ring and the key residues in the binding region for AppA$_{BLUF}$ (Yellow, PDB 1YRX) and PixD (Blue, PDB 2HFO). The relevant residue numbers are given as AppA (PixD).*

Structures of the flavin binding region for two BLUF domains proteins, PixD and AppA, are shown in Figure 1 (the latter, isolated by truncation of AppA, is hereafter labelled AppA$_{BLUF}$); secondary structures

are shown in Supporting Information (S1).[18-20] The Tyr and Gln, residues are highly conserved and essential for formation of the red-shifted flavin spectrum.[11, 20-21] Trp and Met residues are semi-conserved and proposed to be involved in signalling state formation;[13, 17-18, 22-23] they occupy different positions in PixD and AppA, with the Trp in either 'in' ($AppA_{BLUF}$) or 'out' (PixD) conformations, with the vacancy in Trp 'out' occupied by Met.[18, 24-25]

Ultrafast spectroscopy of the PixD BLUF domain showed that the red shift arises following ultrafast electron and proton transfer between the conserved Tyr and excited isoalloxazine.[8, 26-27] It was proposed that this leads to rotation of the Gln residue, which is the switch for changes in protein secondary structure, possibly communicated through Trp and or Met (Figure S1). However, evidence of electron transfer proved elusive in some other BLUF domains,[9, 16, 28] and alternative mechanisms were proposed, including H-bond reorganization and tautomerization of the conserved Gln, a mechanism supported by experiment and calculation.[10, 16, 29-30] Recent TRIR measurements where Tyr was exchanged for fluorotyrosine suggested further diversity in PixD and $AppA_{BLUF}$ photoactivation mechanisms.[26, 31]

X-ray structures for light- and dark-adapted BLUF domains do not unambiguously resolve the photoactivation mechanism. Studies of $AppA_{BLUF}$ suggested a significant structural perturbation on photoactivation, including a change from Trp 'in' to 'out' structures.[18, 32] In contrast, a recent study of the OaPAC BLUF domain in dark and light adapted forms did not find evidence for large structure changes.[33-34] Recent free energy simulations coupled with electronic structure calculations suggest a dynamic picture of the flavin binding fold, with Trp_{in}/Met_{out} and Trp_{out}/Met_{in} in thermal equilibrium at physiological temperatures, modulating the probability of Tyr to flavin electron transfer and H-bond reorganization.[35-37] Other calculations suggest a dynamic binding pocket, with spontaneous glutamine rotation occurring on the time scale of the photoreaction.[38]

Here we site specifically probe the dynamic response of $AppA_{BLUF}$ and PixD to electronic excitation, combining μOD sensitivity ultrafast TRIR with UAA substitution at the critical Met and Trp positions. AzPhe is chosen as the site selective IR probe since the N_3 mode near 2100 cm^{-1} is a strong absorber, well isolated from protein vibrational modes and sensitive to its H-bonding environment.[39]

2. Experimental

Procedures for expression and purification of $AppA_{BLUF}$, PixD and the AzPhe labelled BLUF domains are presented in Supporting information S6a. Protein solutions (ca 2 mM) were placed in a Harrick cell with a 6 micron pathlength for steady state FTIR difference measurements. Irradiation was with a 455 nm LED (S6b). TRIR measurements were performed as previously described[26] and detailed in S6c.

3. Results and Discussion

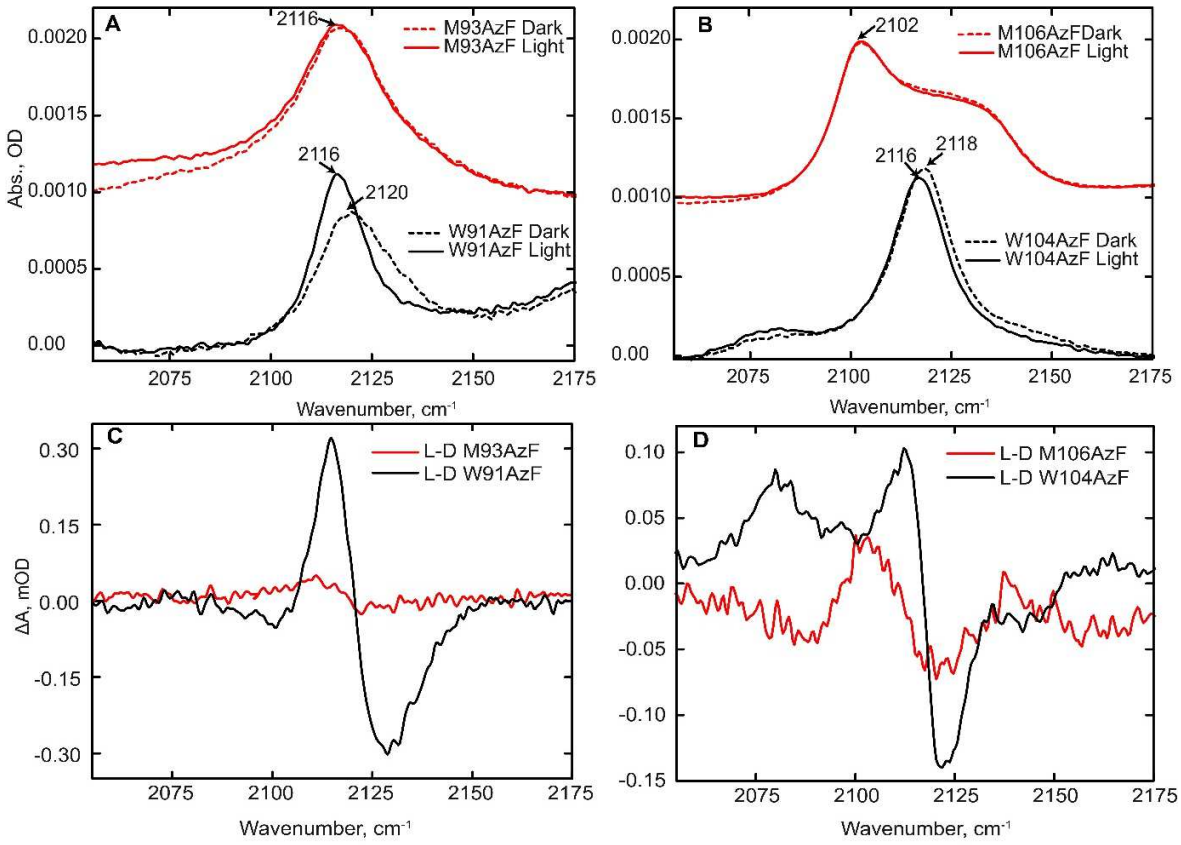

Figure 2. Steady state IR and IR difference data for the azido mode. A. PixD: Black W91AzPhe, Red M93AzPhe (offset for clarity). Dash line is for the dark-adapted proteins and the solid for light-adapted. Spectra were recorded separately and buffer signal has been subtracted. B. As A for $AppA_{BLUF}$: W104azPhe and M106AzPhe. C. The light minus dark IR difference spectra for PixD W91AzPhe (black) and M93AzPhe (red) obtained from a direct subtraction of unirradiated and irradiated samples (not a subtraction of the separate spectra in A). D. $AppA_{BLUF}$ as for C.

Figures 2A, B display steady state FTIR spectra of the azido asymmetric stretch, where the semi-conserved Met (93/106) and Trp (91/104) residues in the flavin binding pocket of PixD/$AppA_{BLUF}$ respectively, were exchanged for AzPhe. Spectra were measured for both dark and light- adapted states. In PixD the azido mode maximum is at 2116 cm^{-1} for three samples but slightly blue shifted to 2120 cm^{-1} for dark adapted W91AzPhe. In each case a single somewhat asymmetric lineshape is observed, with

only minor differences between them (peak wavenumber and widths are tabulated in Table S1). In sharp contrast to PixD, $AppA_{BLUF}$ presents a multimode lineshape. The AzPhe104 and 106 FTIR spectra both show at least two contributions, none of which have a common frequency. Conversion from dark- to light-adapted states gives rise to small changes across the lineshape (Table S1).

Such variable and complex lineshapes for AzPhe are consistent with model studies of this probe.[39] When the azido moiety is substituted on an aromatic ring (as in AzPhe) a multimode lineshape similar to that in Figure 2B was assigned to a Fermi resonance; recent data suggests resonance between the azido stretch and two combination bands as the origin of the multimode lineshape.[40] The model studies also showed that the N_3 mode is insensitive to polarity but blue shifts in H-bonding, and especially aqueous, environments and that the Fermi resonance is less pronounced when azido is substituted on an aliphatic group.[39] Comparing Figures 2A and 2B it is evident that the protein environment itself tunes the azido mode between a multimode ($AppA_{BLUF}$) and single (albeit asymmetric) mode profile (PixD). This tuning must arise from a differential influence of the environment on the combination and N_3 asymmetric stretch modes contributing to the resonance. Thus, while the complexity of the lineshape creates challenges for modelling, it also provides new information on site interactions, in this case revealing differences on the two BLUF domains.

The distinction between the two BLUF domains extends to the light minus dark difference spectra (Figures 2C, D). Crucially, the AzPhe mutants of both $AppA_{BLUF}$ and PixD retain the ability to form the red-shifted light adapted state on irradiation; data are shown in S3, where changes in the light to dark recovery rate due to AzPhe are described. For PixD the asymmetric differential lineshape for W91AzPhe (Figure 2C) is consistent with a shift to a less strongly H-bonded environment on irradiation. The M93AzPhe shows a weaker difference spectrum indicating a smaller change in its environment on irradiation. For $AppA_{BLUF}$ the spectra are complicated by the multimode lineshape, but both W104AzPhe and M106AzPhe are sensitive to photoactivation, with light induced changes occurring across the

lineshape. Thus, while both positions 104 and 106 are coupled to light induced structure change on electronic excitation, there is no gross change in the azido spectrum, such as would be expected if the residues exchanged positions, and thus H-bond environment; this is consistent with OaPAC BLUF domain structure data, where only small changes are seen.[33]

The data in Figure 2 show that electronic excitation of isoalloxazine perturbs the environment of the AzPhe. A separate question is whether the azido group perturbs the electronic structure or photophysics of the isoalloxazine ring, introducing for example an additional source for electron transfer quenching. To address such potentially misleading effects we performed control experiments probing the TRIR response of $AppA_{BLUF}$, W104F and W104AzPhe in the fingerprint region (1300 – 1800 cm^{-1}); measurements were made in D_2O due to the intense IR absorption of the H_2O bending mode. These three samples separately address the effect of mutation alone (the W104 mutations were previously shown to modify the photocycle[17]) and any additional effect of the N_3 group. The data are discussed in detail in S7 (Figures S6 and S7). From these data we conclude that in addition to supporting the BLUF photocycle, W104AzPhe is a site selective probe which does not introduce any new quenching channel, and does not perturb the TRIR spectra and kinetics of $AppA_{BLUF}$ any more than the mutation W104F.

Figure 3 presents the ultrafast temporal evolution of the azido mode in response to isoalloxazine electronic excitation. These data allow site-specific insights into the real time response of the protein to optical excitation. Figure 3A shows that W104AzPhe responds instantaneously (within 1 ps) to excitation of the chromophore, with a prompt decrease in absorbance (bleach) at the peak (2118 cm^{-1}) and an increase (transient) at 2110 cm^{-1} (see S4 for time domain data at specific wavenumbers). The instantaneous response in AzPhe104 could reflect long-range electrostatic interactions, as the isoalloxazine ring undergoes a 2.3 D increase in dipole moment on excitation.[41] However, Stark and solvent shift experiments show that the azido mode is only weakly dependent on electric field.[39, 42] Thus,

we assign the instantaneous perturbation of the azido mode in AzPhe104 on flavin excitation to intermolecular interaction through an extended H-bonded network.[43] The

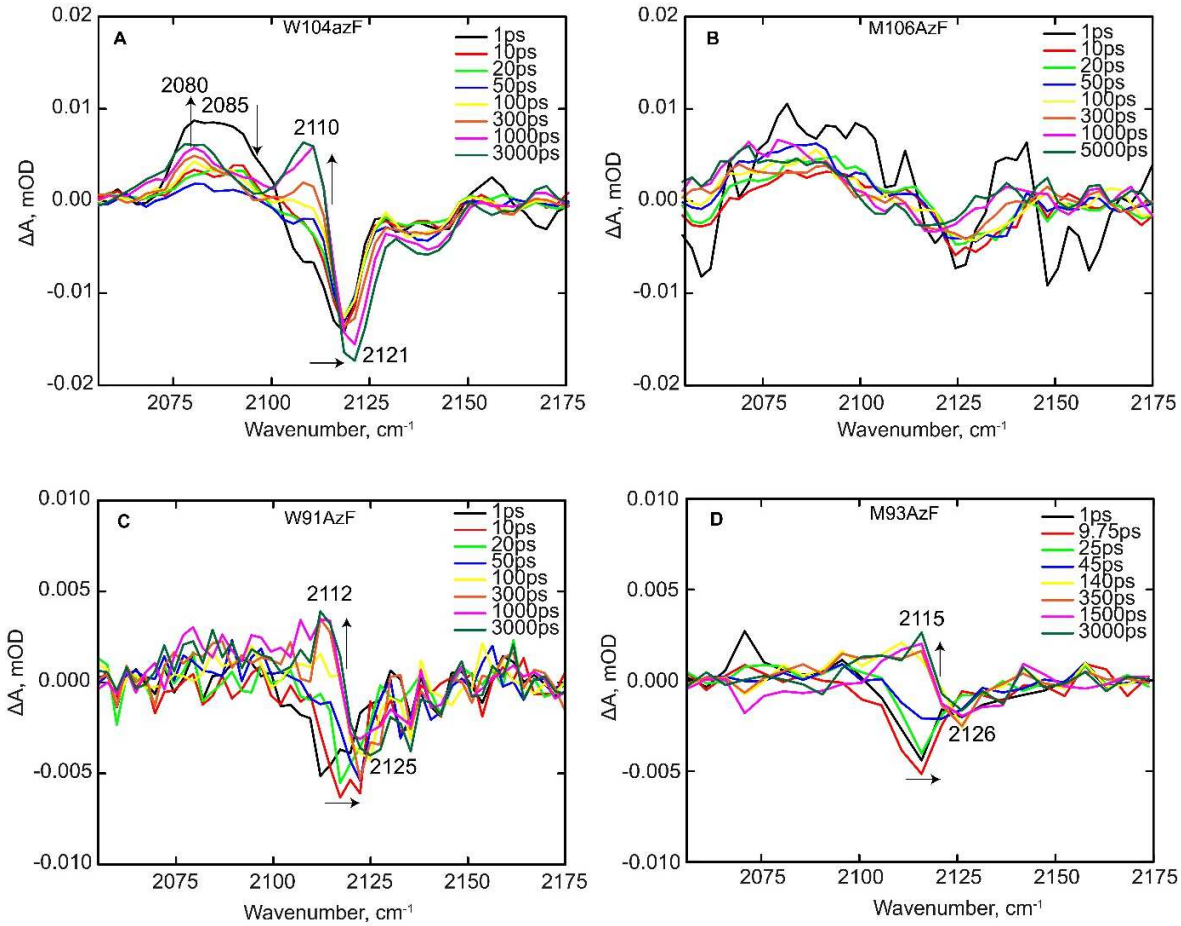

Figure 3. Time resolved IR difference response of the azido mode following ultrafast excitation of isoalloxazine cofactor. A. $AppA_{BLUF}$ W104 AzPhe; B. $AppA_{BLUF}$ M106AzPhe; C. PixD W91AzPHE; D. PixD M93AzPhe.

distance of closest approach in the crystal structure is 5 Å (Figure 1) precluding formation of a direct chromophore – AzPhe H-bond, so an indirect pathway, probably via Q63 (Figure 1), must operate.

The W104AzPhe TRIR spectra (Figure 3A) show complex evolution as a function of time, with the broad transient absorption at 2085 cm^{-1} decaying in a few picoseconds before growing in again (at 2080 cm^{-1}), accompanied by formation of a new transient at 2110 cm^{-1}. The bleach amplitude at 2118 cm^{-1} initially decreases but then increases and shifts to higher wavenumber (see also single wavenumber time profiles in S4). After 3 ns the azido spectrum is constant, and has a profile similar to the IR difference spectrum (Figure 2D).

It is interesting to compare the transient response of W104AzPhe for dark- (Figure 3A) and light- (Figure S5) adapted forms of $AppA_{BLUF}$. Although the FTIR spectra are very similar (Figure 2B), excitation of the light adapted (red-shifted) isoalloxazine results in a weaker response in the azido mode. There is an instantaneously formed transient at 2085 cm^{-1} which decays in ca 3 ps forming a bleach at 2118 cm^{-1}, which then decays in ca 15 ps to the baseline. This is as expected from earlier TRIR studies of $AppA_{BLUF}$ in the fingerprint region, where relaxation is fast and no final spectrum is observed.[16] Thus, the AzPhe response in $AppA_{BLUF}$ indeed probes protein structural evolution following photoexcitation, and does not simply reflect chromophore excitation (which is common to both light and dark adapted states).

In contrast to dark-adapted W104AzPhe, dark-adapted M106AzPhe responds only weakly to electronic excitation. The only measureable evolution occurs after 300 ps at around 2125 cm^{-1} (Figure 3B). There is also an 'S' shaped baseline, indicating a weak instantaneous perturbation of the broad (Figure 2B) azido spectrum, which appears promptly and persists for nanoseconds; unlike W104AzPhe, no picosecond kinetics were resolved. Thus, formation of the final difference spectrum (Figure 2D) must occur on a timescale longer than 5 ns.

The time resolved PixD spectra broadly reflect changes in steady state difference spectra (Figures 3 C,D). In W91AzPhe there is an instantaneous bleach, indicating communication between site 91 and the chromophore, which deepens within 10 ps and forms the differential lineshape observed in the final

spectrum in <300 ps (Figure 2C). This is faster than in $AppA_{BLUF}$, which correlates with the faster excited state decay and overall faster photocycle of PixD.[28] The timescale of the azido IR response of M93AzPhe (Figure 3C) is similar to W91AzPhe, although the amplitude is smaller. The later spectra have a larger amplitude than expected from the very weak steady state difference spectrum (Figure 2C), suggesting a decrease occurs on a longer (> 3 ns) timescale.

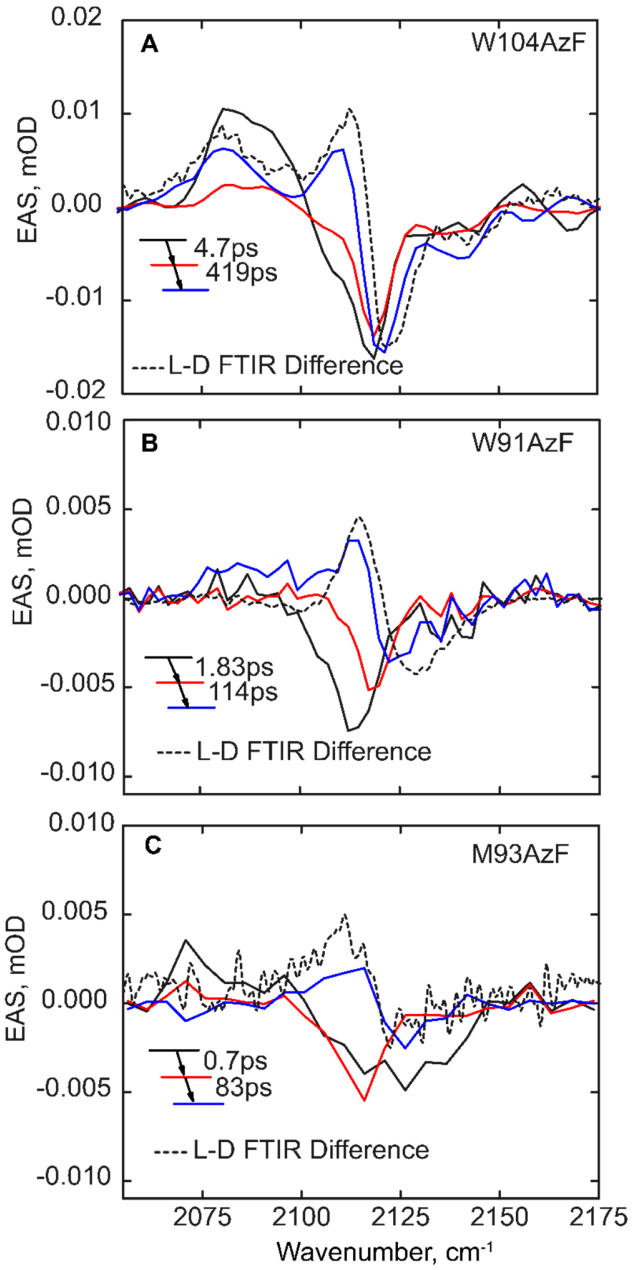

Figure 4. Global analysis EADS. A. AppA_{BLUF} W104AzPhe; B. PixD W91AzPHE; C. PixD M93AzPhe. In each case the data were globally fit to a sequential first order kinetics model with an initial state (black) one intermediate state (red) which forms on a sub 10 ps timescale and subsequently relaxes to a final state (blue). The final state is compared with the FTIR difference spectra (grey dash line). All analyses used the glotaran software package (see also S6).[44]

The three cases where the data revealed a significant spectral evolution were fit by global analysis, which required a two-step model (Figure 4, S6). W104AzPhe has the slowest response and a complex evolution in its spectral profile. This is significant as earlier studies identified W104 as important in forming the $AppA_{BLUF}$ light adapted signalling state;[17] in contrast, M106AzPhe had only a small amplitude response to electronic excitation. For both W91AzPhe and W104AzPhe a final spectrum similar to the steady state IR difference spectrum evolves within several hundred picoseconds. However, in all three cases there are minor differences in peak position and amplitude between the final spectrum recovered from global analysis and steady state IR difference spectra. This suggests further spectral evolution on timescales longer than 5 ns. Comparing the analysis for $AppA_{BLUF}$ and PixD, the AzPhe response in the latter is faster, consistent with faster dynamics in PixD.[26] Further, given the larger amplitude of the early time response in $AppA_{BLUF}$ (where a stable charge separated state is not observed) than PixD, we conclude that the dominant contribution to the time dependent azido response is evolution of H-bonded interactions rather than a Stark effect. This accords with analysis of solvent dependent FTIR spectra.[39]

4. Conclusion

A combination of TRIR with UAA substitution has resolved site-specific real time structural dynamics in a protein. This opens up the possibility of mapping allosteric pathways in real time.[17, 45] In PixD and $AppA_{BLUF}$ the Trp and Met sites implicated in formation of the signalling state reveal distinct spectra and different amplitudes in response to photoactivation. Azido modes in the two Trp sites both respond instantaneously to flavin excitation, which is assigned to interaction through the extended H-bonding network. This in-turn requires that the AzPhe substituted in the Trp position occupies an 'in' configuration at room temperature, consistent with recent calculations.[36-37] The effect is larger in $AppA_{BLUF}$ than PixD suggesting either stronger interaction with the H-bond network, or a different position of the dynamic equilibrium, leading to a larger population of the 'in' configuration. The azido

response evolves in a biphasic fashion on a sub nanosecond timescale, with a fast phase perhaps indicative of structural reorganization of the H-bonded interactions, followed by a slower frequency shift to yield a final differential lineshape on a sub-nanosecond timescale. This final lineshape reveal some differences to the IR difference spectra suggesting the presence of some nanosecond time scale structural relaxation.

Supporting Information

Additional details of proteins structure; tabulated steady state IR data; details on light-dark photocycle; single wavenumber transient data; AzPhe dynamics in the light adapted state; experimental details including protein preparation, sample preparation, ultrafast spectroscopy; control experiments on $AppA_{BLUF}$ and W104F in the fingerprint region to assess effect of AzPhe substitution on the flavin binding site.

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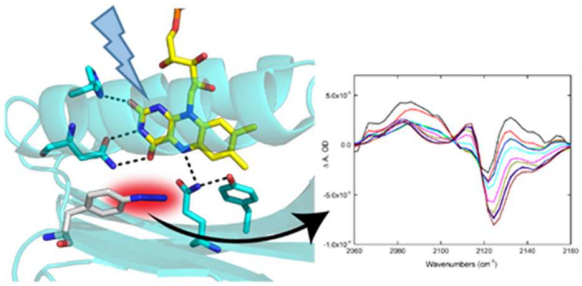
ToC Graphic


5.0x10⁻⁴
0.0
-5.0x10⁻⁴
-1.0x10⁻³
Δ A, OD
2060
2080
2100
2120
2140
2160
Wavenumbers (cm⁻¹)