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Proteins in Action: Femtosecond to Millisecond Structural Dynamics of a Photoactive Flavoprotein

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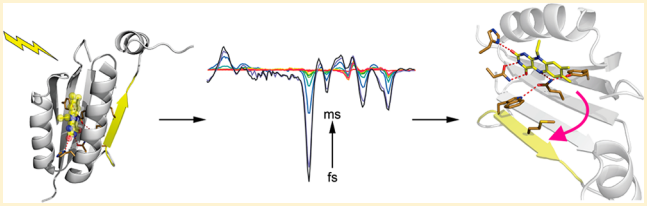
Supporting Information

ABSTRACT: Living systems are fundamentally dependent on the ability of proteins to respond to external stimuli. The mechanism, the underlying structural dynamics, and the time scales for regulation of this response are central questions in biochemistry. Here we probe the structural dynamics of the BLUF domain found in several photoactive flavoproteins, which is responsible for light activated functions as diverse as phototaxis and gene regulation. Measurements have been made over 10 decades of time (from 100 fs to 1 ms) using transient vibrational spectroscopy. Chromophore (flavin ring) localized dynamics occur on the pico- to nanosecond time scale, while subsequent protein structural reorganization is observed over microseconds. Multiple time scales are observed for the dynamics associated with different vibrations of the protein, suggesting an underlying hierarchical relaxation pathway. Structural evolution in residues directly H-bonded to the chromophore takes place more slowly than changes in more remote residues. However, a point mutation which suppresses biological function is shown to 'short circuit' this structural relaxation pathway, suppressing the changes which occur further away from the chromophore while accelerating dynamics close to it.



INTRODUCTION

The underlying mechanism of protein function involves time dependent changes in structure occurring on multiple time scales, from subpicosecond to seconds.[2−4] Recording and modeling the full range of protein dynamics is critical to the understanding and manipulation of protein function. Consequently, the real time measurement and analysis of protein dynamics is a major objective of modern biophysics. In many cases, protein activity is modulated through interaction with external stimuli such as allosteric effectors that bind to regions of the protein remote from the effector site and result in long-range structural changes. Although allosteric effectors are normally considered to be small organic molecules, photons of light that trigger photoreceptor activation may be considered analogous to allosteric modulators. The application of pulsed lasers to such photoactive proteins thus provides a natural starting time, from which real time structural dynamics can be measured. For example, time-resolved X-ray diffraction has provided detailed insights into photoinduced structural dynamics in a number of proteins.[6−13] The formation of the signaling state of the photoactive yellow protein (PYP) has recently been recorded on a one hundred picosecond to millisecond time scale. However, X-ray diffraction requires the protein to be studied in a crystalline environment,[6,8,13] which may perturb or even suppress large scale structural changes. Solution phase X-ray scattering has also been applied to study PYP dynamics.[11,12] Important insights into the shape changes, which occur following optical excitation, are obtained, but scattering data yield less microscopic structural detail than diffraction experiments. In this work we describe time-resolved measurements of light induced structural dynamics in a photoactive flavoprotein in solution over a very wide time range, from hundreds of femtoseconds to hundreds of microseconds. The protein dynamics are recovered from measurements of the time-resolved infrared difference (TRIR) spectra, which are sensitive to structural changes in both the chromophore and the surrounding protein. To achieve this, we exploit the recently developed method of ultrafast time-resolved multiple probe spectroscopy (TRMPS).[14,15]

A number of blue light sensing photoreceptors utilize flavins as the chromophore, where light absorption is localized in the flavin (isoalloxazine) ring. The photoactive protein, AppA (activation of photopigment and PUC A protein), is a flavoprotein photoreceptor from *Rhodobacter sphaeroides* that regulates photosystem biosynthesis in response to both light

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and oxygen levels.[16,17] The protein comprises two domains: an N-terminal blue-light utilizing flavin (BLUF) domain, which binds the flavin adenine dinucleotide (FAD) chromophore (Figure 1A), and a C-terminal domain that is the binding site

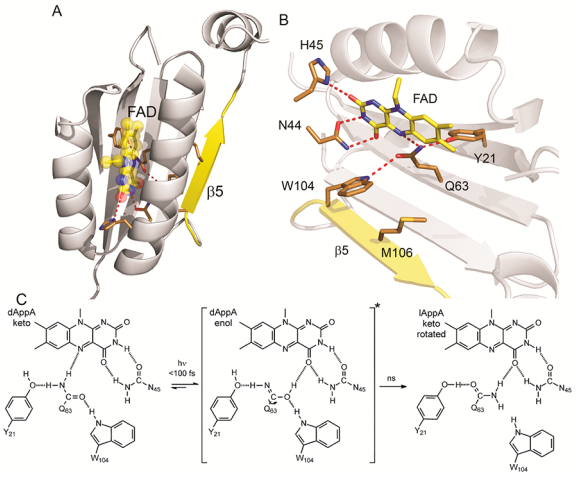

Figure 1. Structure and H-bonding of FAD in $AppA_{BLUF}$. (A) Crystal structure of $AppA_{BLUF}$ showing flavin binding between helices 1 and 2. (B) The H-bonding network around the flavin that includes the key residues Y21, Q63, W104, and M106. The figure was made using Pymol,[1] and the structure 1YRX.pdb.[5] (C) Details of the proposed H-bonding network changes in $dAppA_{BLUF}$ around the chromophore following photoexcitation.

for the transcription factor, PpsR. In low light, low oxygen environments AppA sequesters PpsR, but under blue light illumination, it undergoes a conformational change resulting in the release of the transcription factor, which then binds to DNA to inhibit photosystem biosynthesis.[18,19] The BLUF domain is of particular interest since it is a modular unit found in a number of blue light sensing proteins where it controls functions as diverse as phototaxis, the photophobic response and gene expression.[17,20−22] Recently, it was proposed that the modular nature of the BLUF domain lends itself to applications in the emerging field of optogenetics.[23]

BLUF domain proteins exhibit a two state, reversible photocycle characterized by a *ca.* 10 nm red shift in the absorption spectrum of the flavin ring of FAD, which itself remains intact and in its oxidized state in both dark and signaling states.[21,22] The red shift occurs within 1 ns and the photocycle is completed by recovery of the dark adapted (dAppA) ground state in 30 min.[24] A number of crystal structures have been solved for the N-terminal BLUF domain of AppA ($AppA_{BLUF}$), revealing an intricate H-bonding network surrounding the chromophore (Figure 1B).[5,25] Studies of the structure of $dAppA_{BLUF}$ and its light adapted signaling state ($lAppA_{BLUF}$) suggest that a key step in forming the signaling state is rotation of the conserved glutamine (Q63 in AppA) adjacent to the flavin ring; in-line with this, Q63 is found to be an essential residue for photoactivity.[5,26] On the basis of previous work and our study of the photoinactive mutant Q63E, we proposed a refinement to this model in which tautomerization of Q63 precedes rotation, leading to the formation of a new H-bond to the flavin C_4═O carbonyl (Figure 1C).[27,28] This is consistent with stationary state vibrational spectra, where a red shift in the C_4═O stretch mode (indicative of stronger H-bonding) is observed between dark and light adapted states.[5,19] Other conserved residues critical to photoactivation include Y21 and W104.[29] In the following, we report femtosecond to millisecond TRIR of $dAppA_{BLUF}$ and two of its mutants (W104A and M106A) as they evolve from the dark to the signaling state. We probe both the evolution in protein structure and the pathway of the structure change as it propagates away from the chromophore.

EXPERIMENTAL METHODS

Materials. FAD (disodium salt) was from Sigma Aldrich. D_2O (99.9 atom %) and [U-$^{13}C_6$]-D-glucose (99 atom %) were from Cambridge Isotope Laboratories. Ampicillin (disodium salt), 100x MEM vitamins, and Minimal media were from Fisher.

Protein Expression and Purification. Mutants were prepared by site directed mutagenesis using pfu Turbo (Agilent). For W104A, the primers used were 5′ TTT GCG GGA GCG CAC ATG CAG CTC TCC TGC TCG 3′ (forward) and 5′ CGA GCA GGA GAG CTG CAT GTG CGC TCC CGC AAA 3′ (reverse). For M106A, the primers used were 5′ TTT GCG GGA TGG CAC GCG CAG CTC TCC TGC TCG 3′ (forward) and 5′ CGA GCA GGA GAG CTG CGC GTG CCA TCC CGC AAA 3′ (reverse). $AppA_{BLUF}$, its mutants and [U-^{13}C]-$AppA_{BLUF}$ were expressed in BL21(DE3) *Escherichia coli* cells and purified as described previously.[28]

Steady State Fourier Transform Infrared (FTIR) Spectroscopy. Light minus dark FTIR spectra were obtained on a Vertex 80 (Bruker) FTIR spectrometer. Here, 80 μL of 2 mM protein was placed between two CaF_2 plates equipped with a 50 μm spacer. 128 scans were accumulated at a 3 cm^{-1} resolution. The light state was generated by 3 min irradiation using a 460 nm high power mounted LED (Prizmatix). The LED used had a mounted objective providing a focused blue beam on the surface of the infrared cell.

TRMPS. The TRMPS method exploits the high signal-to-noise and stable pulse-to-pulse timing of a 10 kHz amplified titanium sapphire laser pumping OPAs for the generation of ∼50 fs IR probe pulses, described elsewhere.[14] A second 1 kHz amplified titanium sapphire laser provides the visible pump pulses (450 nm, ∼100 fs, 1 μJ, ∼120 μm diameter spot size at the sample). The two amplifiers are synchronized with the 65 MHz repetition rate of the common titanium sapphire seed laser, with the seed laser optically delayed before the 1 kHz amplifier to achieve 100 fs to 15 ns relative pump−probe delays. For delay times between 15 ns and 100 μs, the oscillator seed pulse train is used to add steps of 15 ns to the pump laser delay. For times between 100 μs and 1 ms, the 10 kHz probe pulses provide a data point every 0.1 ms, until the following pump pulse starts the experiment again every 1 ms (at the 1 kHz repetition rate of the pump laser). Pump polarization was set to 54.7° relative to the IR beam to eliminate contributions from orientational relaxation.

RESULTS AND DISCUSSION

Chromophore Dynamics. Figure 2A shows the time dependent TRIR spectra for $dAppA_{BLUF}$ between 2 ps and 10 ns after 450 nm excitation of the flavin ring of FAD, the chromophore responsible for blue light absorption in photoactive flavoproteins. These difference spectra comprise the following: negative bands (bleaches) associated with depletion of the flavin ground state population, or with photoinduced changes in the vibrational spectrum of the protein, occurring either directly through electronic excitation at t = 0 or as a result of subsequent structural dynamics; positive bands associated with vibrations of the electronically excited state of the flavin, or with modes of the protein which shift as a result of electronic excitation, or of products formed subsequently. The dominant subnanosecond relaxation is well fit by a biexponential function with components of tens and hundreds of picoseconds consistent with an inhomogeneous distribution of ground state structures leading to a distribution of decay

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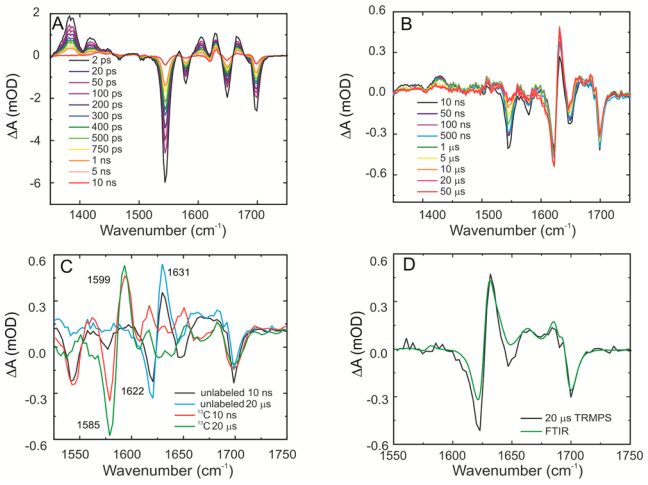

Figure 2. Time resolved IR difference spectra for $dAppA_{BLUF}$. (A) TRIR spectra recorded between 2 ps and 10 ns after excitation of $dAppA_{BLUF}$ at 450 nm. The fast and complete decay of the singlet excited state is evident in the transient flavin modes at 1380 cm^{-1}. However, the ground state recovery is incomplete, e.g., at 1547 cm^{-1} and some transient (probably triplet) state is formed. (B) Relaxation in the $dAppA_{BLUF}$ TRIR spectrum between 10 ns and 50 μs after excitation. The electronic ground state recovers fully (1547 cm^{-1}) but formation of a new environment is indicated by the shift and incomplete recovery in the carbonyl mode at 1703 cm^{-1}. The temporal evolution in the 1622/1631 cm^{-1} pair of protein modes is also evident. .(C) Effect of ^{13}C isotope exchange in $dAppA_{BLUF}$ measured 10 ns and 20 μs after excitation. (D) Comparison of the TRIR spectra recorded 20 μs after excitation with the stationary state IR difference spectrum for the light minus dark states.

rates (Supporting Information Table S1).[27,28,30] The two highest frequency bleach modes at 1700 and 1650 cm^{-1} are associated with two carbonyl stretches of the FAD ground state, and are sensitive to the H-bond environment.[31−34] The intense bleach at 1547 cm^{-1} and the weaker one at 1580 cm^{-1} are FAD ring modes. The two positive peaks formed immediately on excitation at 1408 and 1383 cm^{-1} are not assigned to specific vibrational modes, but are associated with the excited state of the chromophore rather than with frequency shifted protein modes, as proven by comparison with the TRIR of the flavin ring in free solution (Supporting Information Figure S1).

The mechanism of the primary photochemical step in AppA underlying the data of Figure 2A is controversial. Ultrafast optical spectroscopy assigned the primary step to electron transfer between excited FAD and an adjacent conserved tyrosine residue, Y21 (Figure 1B) followed by proton transfer.[24,35−37] The resultant changes in electronic structure were proposed to lead to changes in the H-bonding network prior to back electron transfer. This assignment was based on analysis of the complex multiphase kinetics and observations of a radical-like spectrum in the transient visible absorption of a related BLUF domain protein, PixD.[30] We proposed an alternative mechanism for AppA photoactivity where excitation of FAD itself is sufficient to induce a change in the H-bonding network, giving rise to keto−enol tautomerization in Q63, which then leads on to the required structure change. This assignment was based on the absence of radical states of FAD in the subnanosecond TRIR spectra of $dAppA_{BLUF}$,[27] the observed prompt perturbation of the protein network on excitation and the relative quenching and recovery kinetics of FAD.[27,28] There is theoretical support for both mechanisms,[38−40] and importantly, both agree that light induced structure change in the network of amino acids surrounding FAD occurs within 1 ns, which ultimately leads to formation of the signaling state.

The focus of this paper is on the previously hidden evolution in protein structure, which propagates from the N-terminal FAD binding site to ultimately release PpsR at the C-terminal, thus controlling photosystem biosynthesis. This requires analysis of the TRIR spectra beyond 10 ns.

Protein Dynamics. Although it is evident from Figure 2A that most population has returned to the ground state within 10 ns, a weak spectrum remains; it is the evolution of this spectrum which reveals the subsequent protein dynamics. Exploiting an apparatus with high signal-to-noise, a 10 kHz data acquisition rate and the ability to measure TRIR from 100 fs to 1 ms,[15] we have time-resolved data beyond 10 ns (Figure 2B). It was established (Figure 2A) that by 10 ns the initially excited singlet state of FAD has completely relaxed (e.g., from the disappearance of the 1383 cm^{-1} transient mode of the flavin S_1 state). However, the ground state has not been completely repopulated as can be seen from the persistence of flavin localized bleach modes at 1547 and 1703 cm^{-1} in the TRIR. In addition, a weak transient, possibly a triplet state (see Supporting Information, Figure S1), is formed, giving rise to the weak absorption near 1440 cm^{-1}. The observation of residual ground state bleaches after the excited state decay is complete proves the existence of long-lived intermediate protein structures in the photocycle. Figure 2B probes the relaxation of this structure on a longer time scale, and shows that complete recovery of the 1547 cm^{-1} chromophore ground state mode occurs on the microsecond time scale, while the mode associated with the C_4=O carbonyl of the flavin ring at 1700 cm^{-1} recovers with the same rate (Table 1), but to a nonzero bleach level, i.e., this chromophore mode has not fully recovered its initial state within 50 μs. Data beyond 50 μs (out to 1 ms) showed no further change in the TRIR spectrum. These data thus point to microsecond dynamics refilling the original ground state, while the latter feature indicates that the spectrum associated with the signaling state, a shifted flavin C_4=O mode due to altered H-bond interactions,[19] has formed within tens of microseconds.

Table 1. Kinetic Analysis of $AppA_{BLUF}$, W104A and M106A

peak/cm^{-1}	$dAppA_{BLUF}$/μs	W104A/μs	M106A/μs
1547	5.4 ± 0.5	5.2 ± 0.6	4.5 ± 0.5
1622	2.1 ± 0.3	2.6 ± 0.6	2.2 ± 0.4
1631	1.5 ± 0.3	N.A.	1.2 ± 0.4
1688	5.6 ± 0.8	N.A.	6.3 ± 1.1
1703	5.3 ± 0.7	N.A.	5.8 ± 0.8

Most significantly, microsecond dynamics associated specifically with the protein are evident in the complex dispersive band profile between 1600 and 1640 cm^{-1}, which also continues to evolve after the excited state decay of FAD (Figure 2B). Since there are no strong flavin chromophore modes in this region (Supporting Information, Figure S1), these changes must be assigned to structural evolution in the protein. This result demonstrates the sensitivity of vibrational spectroscopy to protein dynamics; in the electronic spectrum no evolution was detected between 10 ns and 15 μs.[41] The assignment of the 1622/1631 cm^{-1} dispersive profile to protein modes was confirmed by repeating the experiment in uniformly

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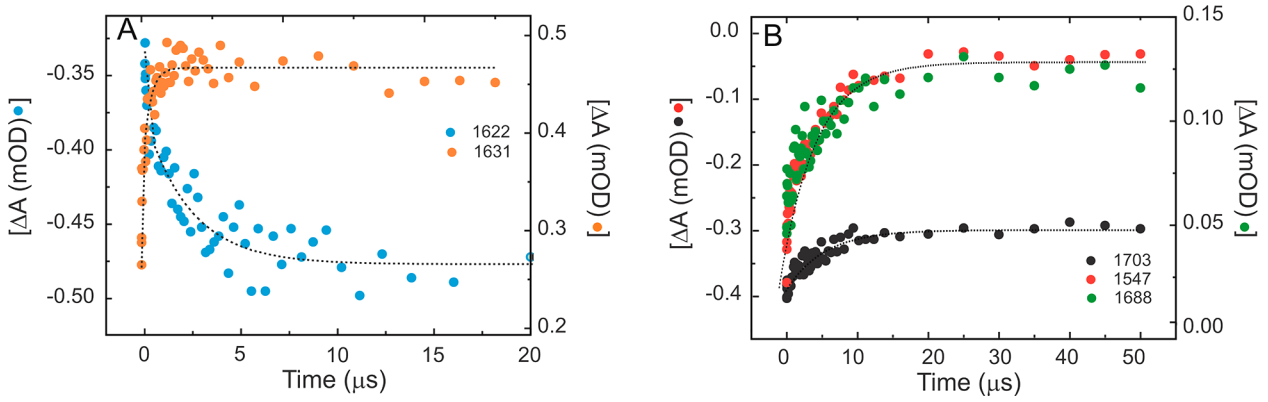

Figure 3. Comparison of protein and chromophore mode kinetics. (A) Kinetics of protein modes, showing that the linked pair at 1622/1631 cm^{-1} exhibit distinct kinetics. The growth of the transient occurs more rapidly than the evolution of the bleach. (B) Kinetics associated with the recovery of the chromophore modes at 1547 cm^{-1} (complete recovery) and 1703 cm^{-1} (partial recovery (Figure 2B)) and the growth of the 1688 cm^{-1} transient. The slower dynamics associated with the chromophore recovery and growth of the light adapted state compared to the protein modes in (A) is apparent. The relevant optical density axes are indicated by the symbol color.

^{13}C labeled protein, U-^{13}C $dAppA_{BLUF}$; TRIR data recorded after 10 ns and 20 μs are shown in Figure 2C. Both parts of the dispersive line shape are red-shifted by 36 ± 2 cm^{-1} from the unlabeled spectrum, consistent with an isotope shift. In contrast, modes assigned to the FAD chromophore are unshifted.[28] The assignment of the dispersive band to protein is in good agreement with the stationary state IR difference spectra of Masuda and co-workers, who proposed on the basis of the observed frequencies that these changes arose from a C═O (amide) mode of the β-sheet structure, which is linked to FAD bound in the α-helix region by the key residues W104, Q63 and Y21 (Figure 1B).[19]

In figure 2D the TRIR spectrum recorded at 20 μs is compared with a steady state IR difference spectrum recorded with the same (approximately 3 cm^{-1}) resolution. These spectra show that the structural dynamics associated with these protein modes are essentially complete within 20 μs, with no further changes in TRIR being observed out to 1 ms. The formation of the signaling state within 20 μs is however longer than the nanosecond time scale associated with the red shift of the FAD chromophore observed by ultrafast electronic spectroscopy.[41] The microsecond time scale is significant in the light of an NMR study of light and dark adapted forms of the BLUF domain, which suggested that the structural changes which occur are of small scale but take place in residues relatively remote from the FAD chromophore, including in the β-sheet.[42] The present data thus show that structural changes taking place at distances in excess of 10 Å from the chromophore can occur on the microsecond time scale.

Further detail can be recovered from analysis of the kinetics between 10 ns and 10 μs after excitation. For the 1622/1631 cm^{-1} dispersive pair (associated with protein bleach and absorption respectively), the kinetics are presented in Figure 3A. A striking result is that these two bands are kinetically distinct and not therefore linked by a simple first order shift to lower frequency of a single protein mode. The transient absorption at 1631 cm^{-1} rises in *ca.* 1.5 μs, which is reproducibly faster than the 2.1 μs development of the 1622 cm^{-1} bleach (Table 1). This result is not unexpected, as the structural changes between the light and dark states are spread over a number of residues,[42] each of which may have a slightly different vibrational frequency associated with its amide backbone. The kinetics associated with changes occurring on more than one residue will be both more complex than a simple first order process and hierarchical in nature. This is likely to result in stretched exponential or dispersive kinetics rather than single exponential relaxation,[43] although the present signal-to-noise does not permit the extraction of anything more than a characteristic time scale for the dynamics associated with each mode.

Consideration of the relaxation times of other modes is consistent with this more complex picture (Figure 3B). The weak transient feature at 1688 cm^{-1} develops on a longer time scale than the protein modes (Table 1), but on the same time scale as the partial bleach recovery at 1703 cm^{-1}. The 1688 cm^{-1} feature is well resolved in the light minus dark difference spectrum (Figure 2D) and was assigned previously by Masuda and co-workers to an increase in H-bonding between the flavin carbonyl mode and protein in the light adapted state. The present results thus suggest a phase in protein structural reorganization slower than seen in the protein modes in Figure 3A. Significantly, the slower structural reorganization occurs in protein residues sufficiently close to the flavin chromophore to be involved in H-bonding with it. This contrasts with the faster changes assigned to the more remote residues in the β-sheet (Figure 3A). This result shows that there is no simple relationship between the time scale of the protein response to electronic excitation and distance from the chromophore.

The chromophore bleach modes (e.g., at 1547 cm^{-1}) also recover on this longer time scale, as does the very weak transient absorption around 1440 cm^{-1}. These might both be assigned to relaxation of a triplet state, since a similar (but faster) evolution is observed for FMN in aqueous solution (Figure S1). In an effort to resolve the triplet state in $AppA_{BLUF}$, we studied the photoinactive mutant Q63E on the picosecond to microsecond time scale (Figure S2).[28] In this case, no 1440 cm^{-1} transient was observed. However, the recovery of the flavin ground state was markedly faster in Q63E than in $dAppA_{BLUF}$ (Figure S2), so this result cannot rule out a contribution from the triplet state. Thus, although the developing transient absorption at 1688 cm^{-1} can be reliably assigned to a change in the protein environment around the chromophore which is slower than the changes seen at 1622/1631 cm^{-1}, the similarities in time scales mean that we cannot rule out a role for the triplet state in the microsecond recovery kinetics of the BLUF domain ground electronic state at 1547

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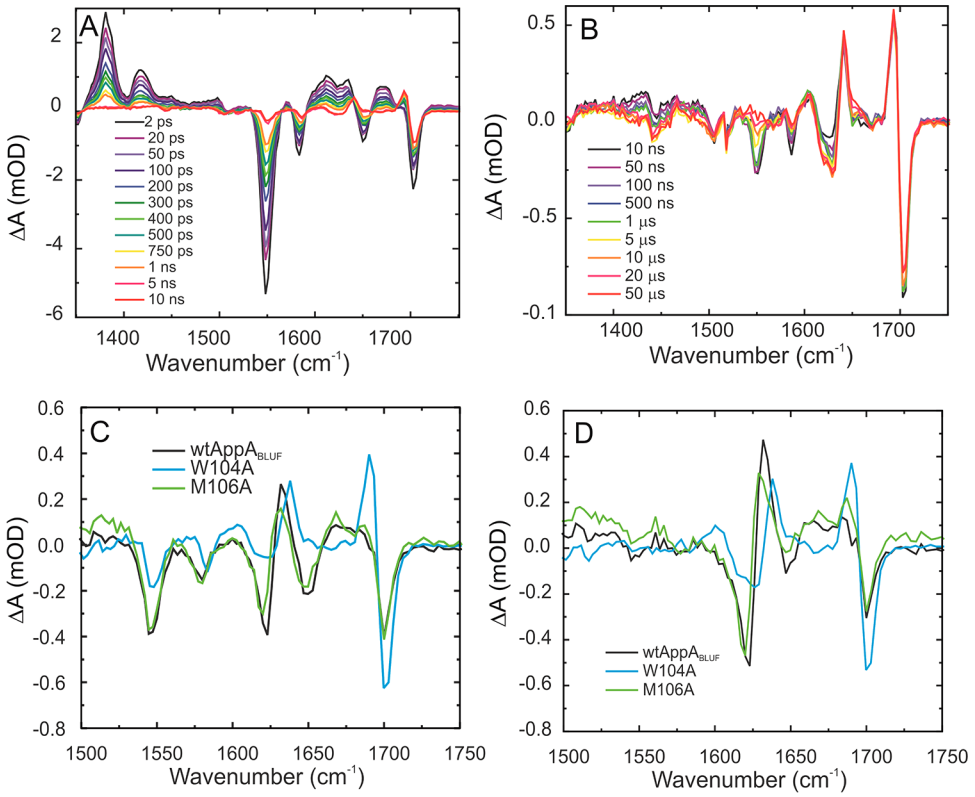

Figure 4. Transient IR spectra for dAppA$_{BLUF}$ and two mutants. (A) Femtosecond to nanosecond TRIR of W104A. (B) Microsecond dynamics of W104A. (C) Comparison of the TRIR spectra of AppABLUF and the two mutants 10 ns after excitation. (D) As for (C) but 20 μs after excitation.

cm^{-1}. We complemented the above analysis of individual assigned modes with a global analysis of the ps $-\mu$s spectrum (Supporting Information Figure S3), assuming a sequence of first order kinetics, which reproduced the behavior described here.

Modification of Protein Dynamics in W104A AppA$_{BLUF}$. To further characterize the relaxation pathway in AppA$_{BLUF}$, we measured the 100 fs to 1 ms dynamics associated with two mutants which both link the flavin binding pocket to the β-sheet (Figure 1A) and show the red-shift in FAD absorption between dark and light adapted states characteristic of photoactivity: W104A and M106A. Compared to dAppA$_{BLUF}$, W104A is known to dramatically accelerate the recovery of the dark state, by a factor of 80, while M106A gives rise to a more modest enhancement of 1.5.[44] There are distinct differences between the TRIR data for W104A and dAppA$_{BLUF}$ between 100 fs to 10 ns (Figures 2A and 4A). Most strikingly the flavin ground state (1547 cm^{-1}) recovers and a red-shifted C$_4$=O transient species (1688 cm^{-1}) develops simultaneously in W104A, both on the nanosecond time scale (Figure 4A and Figure S3). The 1688 cm^{-1} mode is assigned to a rearrangement in H-bonding between the protein and the flavin ring[19] and only appears on the microsecond time scale in dAppA$_{BLUF}$ (Figure 3); evidently this rearrangement has very different dynamics in W104A. A second striking difference is in the kinetics associated with the protein mode line shape on the nanosecond to millisecond time scale (Figure 4B). In W104A the positive feature (1631 cm^{-1}) appears immediately and shows no further evolution, while the negative feature (1622 cm^{-1}) does grow over time but to a level which is much weaker than in dAppA$_{BLUF}$. This is clearly illustrated by the comparison of the transient spectra for W104A, M106A and dAppA$_{BLUF}$ at 10 ns and 10 μs (Figure 4, panels C and D, respectively). In Figure 4C, the fast appearance of the 1688 and 1631 cm^{-1} features is apparent, while Figure 4D shows that there is much weaker development of the transient bleach (1622 cm^{-1}) in W104A, while dAppA$_{BLUF}$ and M106A are very similar, and in particular both show the development of the 1688 cm^{-1} transient and the protein modes occur on the microsecond time scale (Table 1, Figure S5). Inspection of the kinetics associated with each mode (Figure S5) confirms the lack of development beyond 10 ns for most modes in W104A and shows that the 1622 cm^{-1} bleach mode develops more rapidly than in M106A and dAppA$_{BLUF}$, which are in every respect similar.

These data confirm that W104 is a key residue in communicating the electronic excitation of the flavin ring to the protein backbone.[45] It was established in steady state IR difference measurements that mutations in W104 suppress the appearance of protein modes.[46] The present data shows that this is a mechanistic change rather than a kinetic one, i.e., for W104A, the photoinduced change in protein structure observed in dAppA$_{BLUF}$ never occurs, rather than occurs and rapidly relaxes. It is significant that W104A forms the red-shifted flavin carbonyl associated with the signaling state (Figure 4), and that this mode forms on the nanosecond time scale (Figure 4A, Figure S3). We suggest that in W104A the structural evolution revealed in the microsecond TRIR of dAppA$_{BLUF}$ is 'short circuited', i.e., there is a light induced change in the local H-bonding environment of the FAD chromophore, which leads to the nanosecond spectral shift in the C$_4$=O mode, but that the longer range structural changes observed in dAppA$_{BLUF}$, and critical for protein function, do not develop. Instead, the

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ground state structure recovers on a time scale similar to that of $dAppA_{BLUF}$ (Table 1).

Such a short-range change in structure is consistent with the eighty-fold increase in the rate of dark state recovery in W104A, compared to $dAppA_{BLUF}$ and with biochemical measurements of AppA antirepressor activity, which showed much lower activity for W104A than for wild type.[29] Evidently, a spectral shift alone is not sufficient to indicate a photoactive state of the BLUF domain.

CONCLUSIONS

Time resolved infrared spectroscopy has revealed the time scale and pathway of structural dynamics in a BLUF domain. Structural dynamics were probed on the 100 fs to 1 ms time scale. Following electronic excitation, the primary events are associated with relaxation of the flavin excited electronic state. This occurs on a subnanosecond time scale, and mainly results in recovery of the initial ground state, with a minor fraction of excited states leading to perturbation of the local structure of the protein and possibly some triplet state formation. The secondary steps involve protein structural dynamics which convert these local perturbations into the changes which eventually form the signaling state, releasing the repressor molecule. The time scale for these protein structural dynamics is 1−5 μs. The fastest steps communicate optical excitation to the protein via the W104 residue in 1−2 μs, and more than one residue is involved, such that different protein modes present different response times. There are also slower dynamics which reflect relaxation in the vicinity of the chromophore, showing that the rate of structural change is not simply related to distance from the chromophore. In the W104A mutant, communication to the protein is suppressed, but fast H-bond rearrangements still occur around the chromophore, suggesting changes in the chromophore spectrum alone are not a good measure of photoactivity.

ASSOCIATED CONTENT

Supporting Information

Transient IR spectra of FMN in solution and Q63E; some further kinetic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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