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

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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,⁶⁻⁸¹³ 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 (isalloxazine) ring. The photoprotein, 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. 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 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. 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.

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. Recently, it was proposed that the modular nature of the BLUF domain lends itself to applications in the emerging field of optogenetics. 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. Recently, it was proposed that the modular nature of the BLUF domain lends itself to applications in the emerging field of optogenetics.

**EXPERIMENTAL METHODS**

**Materials.** FAD (disodium salt) was from Sigma Aldrich. D$_2$O (99.9 atom %) and [U-13C$_6$]-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’ TTG GCC GGA GCG CAT ATG 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’ TTG GCC GGA TGG CAC GCG CAG CTC TCC TGC TCG 3’ (forward) and 5’ CGA GCA GGA GAG CTG CAT GTG CGC TCC CGC AAA 3’ (reverse). AppA$_{ADH}$, its mutants and [U-13C]-AppA$_{BLUF}$ were expressed in BL21(DE3) E. coli cells and purified as described previously.

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

Table 1. Kinetic Analysis of AppA\textsubscript{BLUF} W104A and M106A

| peak/cm\textsuperscript{-1} | dAppA\textsubscript{BLUF}/\(\mu\)s | W104A/\(\mu\)s | M106A/\(\mu\)s |
|---------------------------|-----------------|----------------|----------------|
| 1547                      | 5.4 \(\pm\) 0.5 | 5.2 \(\pm\) 0.6 | 4.5 \(\pm\) 0.5 |
| 1622                      | 2.1 \(\pm\) 0.3 | 2.6 \(\pm\) 0.6 | 2.2 \(\pm\) 0.4 |
| 1631                      | 1.5 \(\pm\) 0.3 | N.A.           | 1.2 \(\pm\) 0.4 |
| 1688                      | 5.6 \(\pm\) 0.8 | N.A.           | 6.3 \(\pm\) 1.1 |
| 1703                      | 5.3 \(\pm\) 0.7 | N.A.           | 5.8 \(\pm\) 0.8 |

nonzero bleach level, i.e., this chromophore mode has not fully recovered its initial state within 50 \(\mu\)s. Data beyond 50 \(\mu\)s (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 \(C_s=O\) mode due to altered H-bond interactions,\textsuperscript{19} has formed within tens of microseconds.

Most significantly, microsecond dynamics associated specifically with the protein are evident in the complex dispersive band profile between 1600 and 1640 cm\textsuperscript{-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 \(\mu\)s.\textsuperscript{41} The assignment of the 1622/1631 cm\textsuperscript{-1} dispersive profile to protein modes was confirmed by repeating the experiment in uniformly
The assignment of the dispersive band to protein contrast, modes assigned to the FAD chromophore are reproducibly faster than the backbone. The kinetics associated with changes occurring on the microsecond time scale is significant. The microsecond time scale is distinct from the nanosecond time scale associated with the red shift of the FAD chromophore observed by ultrafast electronic spectroscopy. 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 \(\beta\)-sheet. 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 \(\mu\)s 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 unshifted. The assignment of the dispersive band to protein contrast, modes assigned to the FAD chromophore are reproducibly faster than the backbone. The kinetics associated with changes occurring on the microsecond time scale is significant. The microsecond time scale is distinct from the nanosecond time scale associated with the red shift of the FAD chromophore observed by ultrafast electronic spectroscopy. 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 \(\beta\)-sheet. 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.

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We complemented the above analysis of individual assigned modes with a global analysis of the ps $\sim$ μs spectrum (Supporting Information Figure S3), assuming a sequence of first order kinetics, which reproduced the behavior described here.

Modification of Protein Dynamics in W104A AppABLUF.

To further characterize the relaxation pathway in AppABLUF, we measured the 100 fs to 1 ms dynamics associated with two mutants which both link the flavin binding pocket to the $\beta$-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 dAppABLUF, 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 dAppABLUF 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=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 and only appears on the microsecond time scale in dAppABLUF (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 dAppABLUF. This is clearly illustrated by the comparison of the transient spectra for W104A, M106A and dAppABLUF 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 dAppABLUF 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 dAppABLUF 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 dAppABLUF 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 dAppABLUF 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=O mode, but that the longer range structural changes observed in dAppABLUF and critical for protein function, do not develop. Instead, the
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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