Proteins in Action: Functional Dynamics of Blue Light Sensing Proteins from Femtoseconds to Milliseconds

Contact *s.meech@uea.ac.uk;ptonge@notes.cc.sunysb.edu*

Kiri Addison
University of East Anglia
School of Chemistry, Norwich NR4 7TJ, UK

Richard Brust
Stony Brook University
Dept. of Chemistry, Stony Brook University, New York 11994-3410 USA

Agnieszka Gil
Stony Brook University
Dept. of Chemistry, Stony Brook University, New York 11994-3410 USA

Gregory Greetham
Central Laser Facility
Research Complex at Harwell, Harwell Campus, Oxon OX110QX, UK

Allison Haigney
Stony Brook University
Dept. of Chemistry, Stony Brook University, New York 11994-3410 USA

Andras Lukacs
University of Pec
Department of Biophysics, Szigeti ut 12, 7624 Pec, Hungary

Sergei Laptenok
University of East Anglia
School of Chemistry, Norwich NR4 7TJ, UK

Stephen Meech
University of East Anglia
School of Chemistry, Norwich NR4 7TJ, UK

Igor Sazanovich
Central Laser Facility
Research Complex at Harwell, Harwell Campus, Oxon OX110QX, UK

Peter Tonge
Stony Brook University
Dept. of Chemistry, Stony Brook University, New York 11994-3410 USA

Introduction

The underlying mechanism of protein function involves time dependent changes in structure occurring on multiple time scales, from subpicosecond to seconds. Understanding the full range of protein dynamics will critical to the interpretation, analysis and ultimately 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. Such triggers are normally small organic molecules. However, 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.

In this article we report on our recent investigations of one such family of proteins, the BLUF (blue light using FAD) domain proteins. We will focus on the photoactive flavoprotein AppA (activation of photopigment and PUC A protein), which is a blue light photoreceptor from *Rhodobacter sphaeroides* that regulates photosystem biosynthesis in response to both light and oxygen levels. The protein comprises two domains: an N-terminal BLUF domain, which binds the flavin adenine dinucleotide (FAD) chromophore (Figure 1), and a C-terminal domain that is the binding site for the transcription factor, PpsR. In low light, low oxygen environments AppA binds two molecules of PpsR, but under blue light illumination, it undergoes a conformational change resulting in the release of the transcription factor, which then binds to DNA, inhibiting 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. Recently, it was proposed that the modular nature of the BLUF domain lends itself to applications in the emerging field of optogenetics.

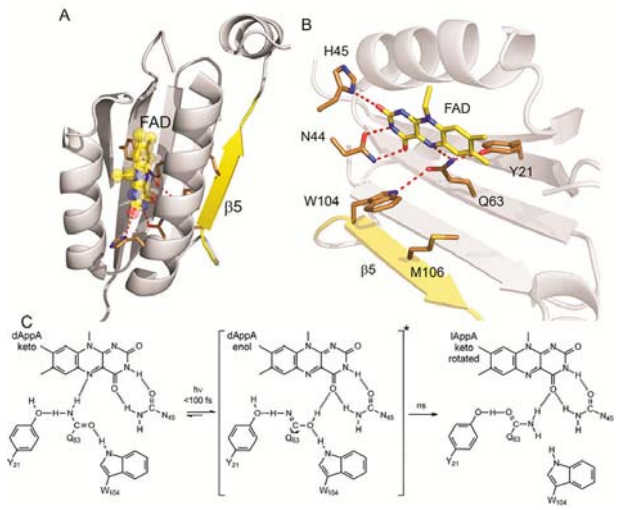

Figure 1 A Structure of AppA BLUF domain showing binding of FAD. The H-bonding network is illustrated in more detail in B. In C a potential change in the H-bonding structure as a result of electronic excitation is shown.

In this report we will describe our recent studies of the AppA BLUF domain ($AppA_{BLUF}$) utilising both the ultrasensitive transient infrared (TRIR) measurements afforded by the ULTRA system and the sensitive measurement of transient IR on timescales from 100 fs to 1 ms, which exploited the capabilities of the recently commissioned TRMPS spectrometer.[1,2] The former method is used to probe the primary mechanism of BLUF domain photochemistry.

Preliminary measurements and careful calibration studies suggested that the previously proposed radical intermediate mechanism was not operating, a result which is here supported by extensive studies of AppA mutants, other BLUF domains and by using artificial amino acid substitution to modify the predicted rate. The TRMPS method was used to follow structural dynamics in the protein following the initial photochemical step. The microsecond protein dynamics observed reveal the pathway of propagation of structure change through the protein, which, we show, is short circuited by mutations known to suppress photoactivity. The data and description presented here are based on two published papers, which should be consulted for complete details[1,2].

Primary Mechanism in BLUF Domain Function

The originally proposed and most widely accepted model for the primary process in BLUF domains is electron transfer from a highly conserved tyrosine residue (Y21 in AppA) to the photoexcited flavin ring, $Y21{-}FAD^* \rightarrow Y21^{\bullet+}{-}FAD^{\bullet-}$.[3] This assignment is based on two important observations: the formation of a radical like spectrum in ultrafast transient electronic spectroscopy of PixD and the observation of complex multiexponential kinetics in the decay of the transient electronic spectrum. Such multiexponential kinetics could be consistent with sequential formation of $FAD^{\bullet-}$ and $FADH^{\bullet}$ on a subnanosecond time scale.

In our earlier TRIR studies and in some transient absorption experiments considerable efforts were made to observe the radical intermediate states.[4] In AppA none were observed, although in another BLUF domain (PixD) a radical intermediate was characterised.[3] These data led us to propose a non-radical pathway to the required light induced H-bond reorganization.[4]

The increased sensitivity and spectral range of the ULTRA TRIR spectrometer allowed us to make simultaneous measurements of excited state decay (1380 cm^{-1} transient absorption) and ground state recovery (1548 cm^{-1}).[1] These modes were characterised by a comparison of the transient spectrum of AppA with that of the chromophore flavin ring system (Figure 2A,B). In addition we had previously characterised the spectroscopy of the radical intermediate, which has a strong feature at *ca* 1528 cm^{-1}.[5] Thus a TRIR experiment permits the complete characterisation of the electron transfer kinetics. The results are shown in Figure 2. The first thing to note in Figure 2A is that there is no new state formed at 1528 cm^{-1}. Thus, there is no spectroscopic evidence for formation of any appreciable population of a radical intermediate state in AppA. Further the high signal-to-noise TRIR allows detailed kinetic analysis. Although the kinetics are non-single exponential the decay of the excited state exactly mirrors the recovery of the ground state, a result which precludes the formation of any intermediate state with a lifetime longer than the excited state decay.

To ensure that a radical intermediate could be observed we exchanged Y21 for a tryptophan (Trp), which has a greater driving force for the electron transfer reaction. In this case the flavin radical intermediate was easily observed at the expected wavenumber, and the excited state decay was faster than the ground state recovery.[1] In addition the spectrum of the Trp radical cation was detected. Thus, radical reactions in BLUF domains are readily observed and characterised by TRIR.

To investigate the observation of radical states in the PixD BLUF domain we investigated its TRIR. In this case, in agreement with [3], we indeed detected the electron transfer intermediate, which shows that BLUF domains are not uniform in their behaviour despite having similar structures. This raises the question of which is the more common case. Two further BLUF domains were studied, and in neither case was there any evidence for a radical intermediate state.[1]

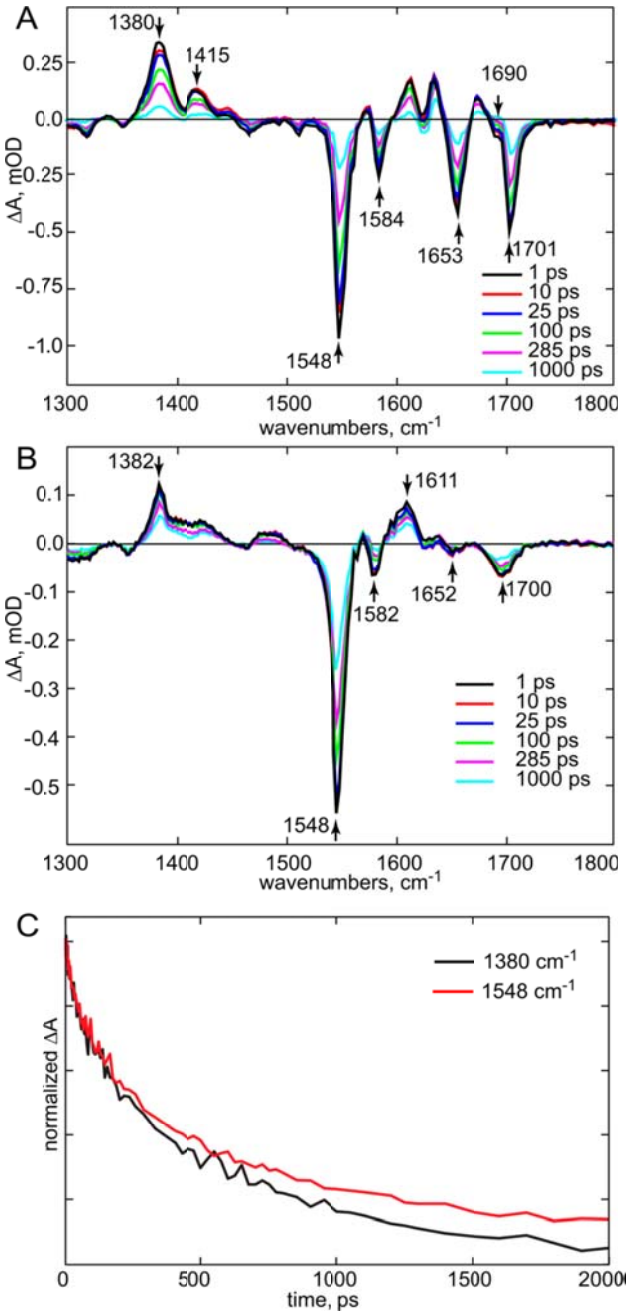

Figure 2. TRIR data for A. $AppA_{BLUF}$ and B. The flavin FMN in free solution – note the corresponding bands in the protein. C. Comparison of excited state decay (black) and ground state recovery kinetics (red, inverted for comparison). The kinetics are the same within experimental error, although the recovery is to a constant long lived offset.

The remaining explanation for the absence of a radical intermediate state is that the forward reaction is slow compared to the back electron transfer, so no intermediate population is formed. To analyse this possibility we assume that the electron transfer can be described by Marcus theory. We then modified the driving force (and therefore also the reorganization energy) for the electron transfer utilising unnatural amino acid replacements (fluorotyrosines in place of the Y21 residue). For the known redox potentials the faster recombination than charge separation requires that the separation be in the normal region of the Marcus curve. In that case the rate was expected to be acceleratred by fluorotyrosine substitution, whereas a retardation was observed.[1] Thus, the primary mechanism of BLUF domain cannot be a simple electron transfer pathway. We have proposed an alternative neutral pathway involving light stimulated keto-enol tautomerization in the Q63 residue.

Real Time Study of Functional Dynamics

The BLUF domain is an anti-repressor, which binds the repressor PpsR in the dark. Its function requires the release of PpsR following light absorption. Since PpsR is bound at the C terminal, well removed from the N terminal flavin binding domain this is a nice example of allostery. Because the process can be initiated by light absorption the TRMPS method affords us the opportunity to observe this in real time.[2]

The TRIR and TRMPS data for $AppA_{BLUF}$ are shown in figure 3A and B. Figure 3A shows the first 10 ns of the relaxation, and it is evident that ground state recovery dominates. However, after 10 ns a small fraction (*ca*. 10%) of the ground state has not recovered. The high sensitivity and very wide time range of TRMPS allows us to follow the evolution of this minor population. Most significantly the sigmoidal lineshape around 1622/1631 cm^{-1} continues to evolve on the 1-5 µs time scale. In figure 3C the measurements are repeated for fully ^{13}C exchanged $AppA_{BLUF}$. The flavin peaks are unshifted while the peaks evolving on the microsecond time scale undergo a shift typical of $^{12}C/^{13}C$ exchange, confirming their assignment to protein modes. Finally in 3D the TRMPS spectrum recorded after evolution is complete is compared with the steady state IR difference measurement. The similarity between the two sets of data established the timescale for the light induced structural change as a few microseconds. Detailed studies of the kinetics at each mode were made and revealed a range of time scales for structural dynamics. Interestingly the kinetic relaxation associated with the 1700 band system, unambiguously assigned to the chromophore ground state carbonyl, was slower than the main protein modes, suggesting the speed of response to photoexcitation is not determined solely by distance from the absorber.[2]

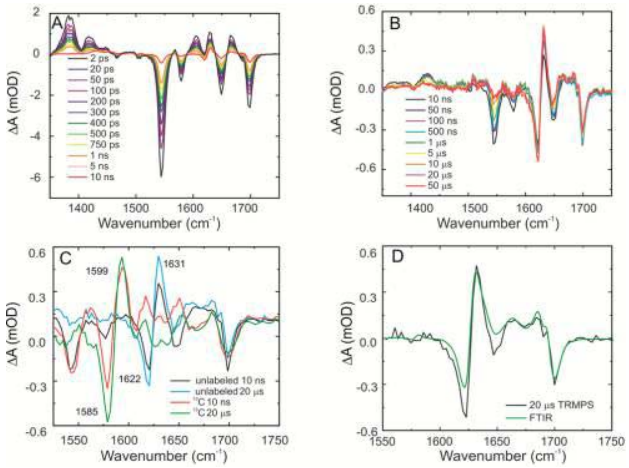

Figure 3. A. 0 – 10 ns dynamics of $AppA_{BLUF}$. B TRMPS data on the evolution between 10 ns and 50 µs after excitation of the flavin. Note the continuing evolution of the 1622/1631cm^{-1} pair. C. The effect of $U^{13}C$ substitution. D. Comparison of 20 µs and steady state FTIR difference spectra.

To investigate further the pathway for the propagation of the structure change we measured TRMPS for the W104A mutant of AppABLUF.[2] This mutant has been shown to be photoactive in the sense that the characteristic 15 nm spectral shift in the flavin absorption is observed on light activation. However, this mutant has greatly reduced antirepressor activity compared to wild type AppA.[6] The TRMPS measurements show that the effect of flavin excitation on the protein modes is greatly attenuated in W104A. However, the dynamics associated with structure change around the carbonyl mode (1700 cm^{-1}) are accelerated from microseconds to nanoseconds. We ascribe this to a short circuiting of the structure change, such that changes in the vicinity of the chromophore occur rapidly, but are not communicated further. This confirms the key role of W104A in signaling state formation.

Conclusions

Ultrasensitive transient IR spectroscopy has provided new insights into the mechanism of action of BLUF domain proteins from femtoseconds to milliseconds.

A series of TRIR studies have been conducted to probe the primary photochemical event, including: $AppA_{BLUF}$; a series of mutants of $AppA_{BLUF}$; unnatural amino acid substituted $AppA_{BLUF}$; three different BLUF domains.[1] These experiments do not support a key role for a metastable radical intermediate, which featured in many proposed mechanisms. Rather we proposed an alternative non-radical pathway.

The evolution of the vibrational spectrum of $AppA_{BLUF}$ has been followed over eight decades in time to map out the pathway of structure change following flavin photoexcitation.[2] The timescale for the structure change was 1 – 10 microseconds, and the role of some key residues was demonstrated.

Acknowledgements

This work was funded by and EPSRC-NSF Joint Research Grant to to SRM (EP/K000764/1), and PJT (CHE-1223819). We are grateful to STFC for access to the Central laser facilities (program 101005 to SRM and PJT)

References

1. R. Brust, A. Lukacs, A. Haigney, K. Addison, A. Gil, M. Towrie, I. P. Clark, G. M. Greetham, P. J. Tonge and S. R. Meech, J. Amer. Chem. Soc., **2013**, *135*, 16168
2. A. Lukacs , R. Brust, A. Haigney, S. Laptenok, K. Addison, A. Gil, M. Towrie, G. M. Greetham, P. J. Tonge and S. R. Meech, J. Amer. Chem. Soc., **2014**, *136*, 4605
3. C. Bonetti, M. Stierl, T. Mathes, I. H. M. van Stokkum, K. M. Mullen,T. A. Cohen-Stuart, R. van Grondelle, P. Hegemann, J. T. M. Kennis, Biochemistry **2009**, *48*, 11458
4. A. L. Stelling, K. L. Ronayne, J. Nappa, P. J. Tonge and S. R. Meech, J. Amer. Chem. Soc., **2007**, *129*, 15556
5. A. Lukacs, R. K. Zhao, A. Haigney, R. Brust, M. Towrie, G. M. Greetham, P. J. Tonge and S. R. Meech, J. Phys. Chem. B, **2012**, *116*, 5810
6. S. Masuda, Y Tomida, H Ohta, K I. Takamiya, J. Mol. Biol. **2007**, *368*, 1223