Photodynamics in light sensing proteins: A time resolved infra-red study of the BLUF (blue light sensing protein using FAD) domain of AppA

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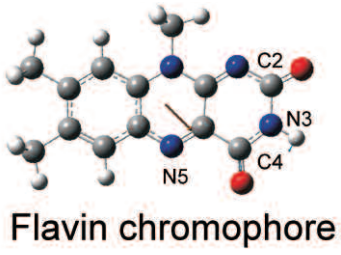
Introduction

Photoreceptors regulate the response of organisms to light, and are involved in controlling processes such as circadian rhythms, phototropism and photosystem biosynthesis[1]. The currently known photoreceptors include the rhodopsins,[2] the xanthopsins such as photoactive yellow protein,[3] and the bilin-containing phytochromes[4] together with three flavoprotein families: light-oxygen-voltage (LOV) domain-containing proteins such as the phototropins;[5,6] photolyase-like cryptochromes;[7] blue light using FAD (BLUF) proteins[2]. In each case there is intense interest in understanding how light absorption results in formation of the signaling state of the protein. For the rhodopsins, xanthopsins and phytochromes, major structural changes accompany light absorption, driven by photoisomerization of the chromophore. Light absorption by the LOV domain proteins leads to formation of a cysteinyl-flavin adduct, while in the cryptochromes blue-light absorption is accompanied by the one electron reduction of the oxidized flavin leading to the formation of a neutral flavin semiquinone signaling intermediate.

In contrast to the LOV domain proteins and cryptochromes, photoexcitation of the BLUF domain proteins does not result in either covalent modification or reduction of the flavin chromophore. Instead, light absorption causes a more subtle change in the chromophore and the major spectroscopic signature of signaling state formation is a small (10 nm) red shift in the visible absorption spectrum of the flavin. The structural changes which accompany signaling state formation have been best characterized for the N-terminal BLUF domain of AppA, a transcriptional antirepressor from the photosynthetic bacterium *Rhodobacter sphaeroides*.[8] In *R. sphaeroides*, photosystem biosynthesis is inhibited under aerobic conditions and by high intensity blue (450 nm) light, effects which are mediated by the PpsR transcription factor. Under low light conditions, the dark state of AppA binds to PpsR forming an AppA-$PpsR_2$ complex that is unable to bind DNA. Upon blue light photoexcitation AppA dissociates from PpsR allowing formation of the repression-competent PpsR tetramer which binds to DNA



Flavin chromophore

and inhibits gene transcription[8]. The molecular details of how photoexcitation of the AppA N-terminal BLUF domain alters the ability of the cysteine-rich C-terminal domain to bind PpsR remain to be determined. The primary events in this mechanism are the subject of the present study, in which ultrafast time resolved infra red (TRIR) spectroscopy is used to record both kinetic data and provide structural information at the level of vibrational spectroscopy.

The BLUF domain of AppA was prepared and purified as previously described[9]. To investigate the key primary processes we also prepared photoactive (W104F) and inactive (Q63L) mutants. Further we have exchanged the FAD chromophore for lumiflavin, FMN and riboflavin, to investigate the role of the binding of the chromophore in controlling protein dynamics. This also allowed us to study isotopically labeled riboflavin chromophore, which aids the assignment of transient vibrational modes. The isotopes used in the current study were ^{13}C substituted at either the C2 position or doubly substituted at C4 and C10. The numbering convention used for the isoalloxazine ring of the flavin chromophore is shown in the scheme above.

Results and discussion

One of the curiosities of AppA is that the Flavin chromophore is retained throughout the photocycle, and the principle manifestation of the change from dark (dAppA) to light adapted (lAppA) or signaling state is a 10-15 nm red shift in the electronic spectrum. We (among others[10]) have investigated whether or not the photocycle operates when the FAD chromophore has been replaced

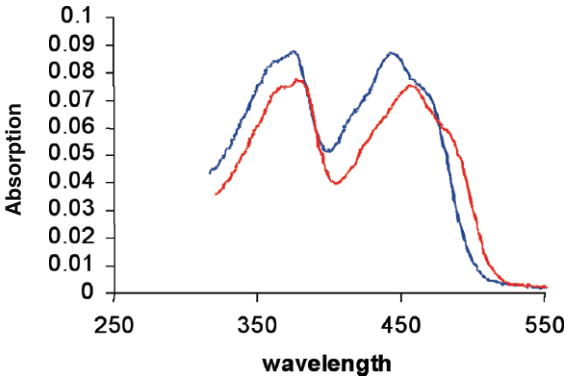


Figure 1. Linear absorption spectra of dark adapted (blue) and light adapted (red) AppA when the chromophore is riboflavin. For TRIR measurements the excitation wavelength was at 400 nm.

by other naturally occurring flavins. In figure 1 we show that photoactivity is retained when the chromophore is exchanged to riboflavin which differs from FAD in having no adenine moiety and in the structure of the chain. Similar behaviour was observed when flavin mononucleotide and lumiflavin chromophores were exchanged for FAD. The observation of photoactivity in the riboflavin substituted AppA is significant because we can use the isotopically substituted riboflavin synthesized in Munich as an aid to assigning the complex TRIR spectra of the protein in both light and dark adapted state.

In figure 2 the TRIR spectra for riboflavin bound to AppA recorded 2 ps after excitation are shown, and contrasted with the same result for riboflavin with ^{13}C substituted at either the C2 or the C4 carbon atom. In figure 2 a negative delta OD corresponds to a bleach following excitation at 400 nm, with a positive value indicating formation of a new transient state. Bleaches forming on the time scale of a few picoseconds can be ascribed to the ground state and promptly formed transient the excited state absorption.

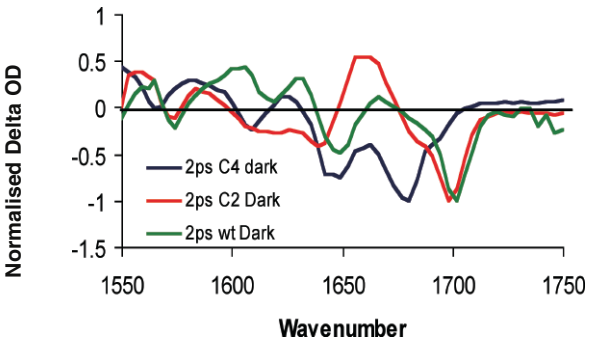


Figure 2. TRIR spectra at 2 ps after excitation of wild type (green) C2 (red) and C4 (blue) ^{13}C substituted riboflavin.

These data immediately allow us to confirm our earlier assignment of the highest wavenumber (ca 1700 cm^{-1}) feature to a ground state mode which is dominated by the C4=O4 carbonyl stretch and the next highest wavenumber (1650 cm^{-1}) to the C2=O2 localised mode[11]. The only other modes influenced by substitution are at 1610 and 1570 wavenumbers in the C4/C10 substitution, suggesting these may both be ring modes of the carbonyl containing ring.

Equally interesting is the excited state absorption. In wt Rf a weak promptly formed transient absorption at 1666 cm^{-1} is observed. In the C2 isotope this is much more strongly developed, presumably because the competing C2=O2 bleach has now been removed. If this transient absorption were simply due to the excited state of C4=O4 the expectation is that in the C4 isotope the excited state would shift down in parallel with the ground state bleach. There is little evidence that this is the case. For example the C2=O2 mode seems at least as strong in the C4 isotope as in wild type. Possible explanations include (a) that the transient absorption is not associated with the chromophore, but is a perturbed protein mode (b) that the electronic structure in the excited state is sufficiently different to the ground state that the vibrational frequencies in ground and excited states are not coupled, such that the excited state mode is less sensitive to isotopic substitution.

We have also studied TRIR spectra for AppA/riboflavin in both the light and dark adapted forms. The data measured 2 ps after excitation are shown in figure 3. The changes between the dark and signaling states are quite pronounced, and analogous to those reported by us in AppA/FAD[9]. In particular the changes around the C4=O4 are interesting, with the single band in dAppA becoming essentially a doublet in lAppA. This suggests that in the signaling state of AppA the chromophore occupies at least two sites. The second interesting observation is that the weak absorbance around 1666 cm^{-1} in dAppA becomes a bleach in lAppA. This is further evidence that this transient is not associated with the chromophore, but may reflect a mode of the protein strongly coupled to it.

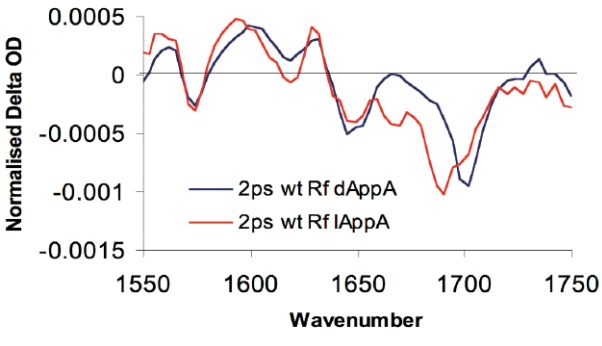


Figure 3. The TRIR spectra at 2 ps after excitation for dAppA (blue) and lAppA (red).

Conclusions

We have observed transient infra-red spectra for the photosensor protein AppA. It has been established that the flavin ring is the critical element for the photocycle, and that photoactivity is retained for flavin molecules as different as FAD and lumiflavin. We have used isotope substitution to assign the vibrational modes of the chromophore in the protein. Some evidence has been found for modes of the protein which are coupled to chromophore excitation. Finally the TRIR method has been used to investigate differences between the light and dark adapted forms of the protein. Photoinduced disorder was observed, indicating the formation of new sites or H-bonding environments for the chromophore under irradiation.

Acknowledgements

We are grateful to STFC for access to the facilities. SRM is grateful to EPSRC for financial support, and MK thanks the JSPS for a studentship. This work was supported by NIH grant GM66818 to PJT. We are also grateful to Prof Marcus Fischer University Hamburg for his collaboration with the riboflavin isotope study.

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