Ultrafast infrared spectroscopy of an isotope-labelled photoactivatable flavoprotein

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Introduction

Light regulates many biological processes including visual perception, circadian rhythms, phototropism, photoperiodism, phototaxis and photosystem biosynthesis. These events are mediated by a series of photoregulated proteins that includes the rhodopsins, xanthopsins and phytochromes as well as the flavin-containing cryptochromes, phototropins and BLUF photosensors.[1] BLUF domain photosensors differ fundamentally from the rhodopsins, xanthopsins and phytochromes since the flavin chromophore cannot undergo large scale reorganization upon excitation. Consequently the protein matrix must have evolved to sense subtler changes in chromophore structure resulting from light absorption.

The transcriptional antirepressor AppA from the photosynthetic bacterium *Rhodobacter sphaeroides* regulates gene transcription in response to both light and oxygen. AppA consists of two domains: the N-terminal BLUF domain and a C-terminal domain that is responsible for the oxygen sensitivity of the protein. Formation of the light-induced signaling state in AppA is characterized by a 10 nm red shift in the 445 nm electronic transition of the isoalloxazine chromophore and is accompanied by a strengthening of hydrogen bond(s) to the C4=O group of the chromophore from the protein.[2] X-ray crystallographic studies[3] have shown that the isoalloxazine C4=O group participates in a hydrogen bond network that also includes a conserved glutamine (Q63), tyrosine (Y21) and tryptophan (W104) (Figure 1), and, together with NMR spectroscopy, support a model for light activation that involves rotation of the Q63 side chain and an alteration in the hydrogen bonding environment of Y21 and W104.[2] As a consequence of this light-induced change in hydrogen bonding, W104 may move leading to formation of the signaling state of the protein. The AppA signaling state has a half-life of 15 min, it is formed within 1 ns of photoexcitation, an event thought to involve electron transfer from Y21 to the neighboring chromophore.[4] To provide further information on the early structural changes that result from light absorption, we undertook ultrafast time-resolved infrared (TRIR) studies of the AppA BLUF domain ($AppA_{BLUF}$) which led to the proposal, now supported by computational studies, that Q63 tautomerizes, rather than rotates, on the ultrafast time scale.[5] A critical experimental observation in our model was the identification of a transient absorption (TA) at 1666 cm^{-1} in proteins capable of forming the signaling state that was tentatively assigned to Q63. However, although the vibrational spectra contain a wealth of structural detail, attempts to fully assign them have been hindered by the difficultly in site-specifically incorporating isotopes into the flavin chromophore. Here we report TRIR spectra of $AppA_{BLUF}$ reconstituted with [2-$^{13}C_1$]- and [4,10a-$^{13}C_2$]riboflavin (Rf). These data allow the first unambiguous assignment of bands in the TRIR spectrum to vibrational modes arising from the C2=O and C4=O groups in the isoalloxazine ring.

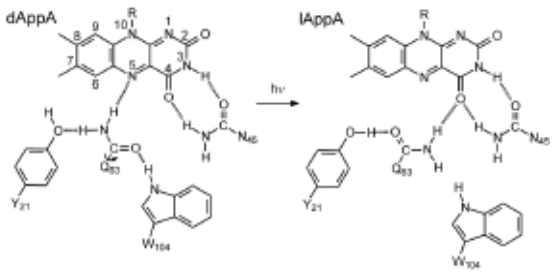

Figure 1. Environment of the isoalloxazine ring in AppA. In Rf R is ribose while in FAD, R is ADP-ribose. Hydrogen bonding interactions are shown by dashed lines. Photoexcitation leads to changes in the hydrogen bond network, one model for which involves a rotation of Q63[1].

Results and discussion

Previous work indicated that the AppABLUF photocycle is largely unperturbed by replacement of FAD with Rf. Consequently, we reconstituted wild-type $AppA_{BLUF}$ with the two labeled Rf isotopologues as well as unlabeled Rf and obtained TRIR spectra with 400 fs time resolution following 400 nm excitation

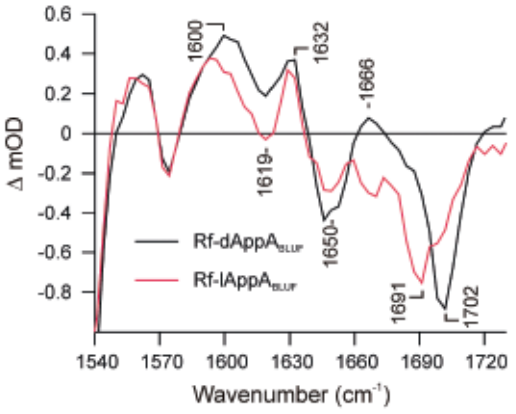

Figure 2. TRIR spectra of riboflavin (Rf) bound to dAppA$_{BLUF}$ and lAppA$_{BLUF}$. Difference spectra 2ps after photoexcitation. ΔmOD, change in absorbance expressed as milliOD.

using PIRATE facility at the CLF. In Figures 2 and 3 transient IR spectra are shown for Rf in solution or bound to AppA$_{BLUF}$. The amplitude for each TRIR spectrum has been normalised to the intense bleach of the flavin mode at 1540 cm^{-1}. These are difference spectra generated from the excited minus unexcited transmission of the IR probe pulse. Thus a negative peak (bleach) formed within the time resolution can be ascribed to the ground state modes of the flavin chromophore, while positive bands (TA) are associated with vibrations of the newly created excited state. For Rf bound to dark AppA$_{BLUF}$ (Rf-dAppA$_{BLUF}$, Figure 2) intense bleach bands are observed at 1702, 1650, 1575 and 1540 cm^{-1}, while there is a broad TA with an onset at 1630 cm^{-1} that extends to lower wavenumber (not shown). These data for Rf-dAppA$_{BLUF}$ are very similar to previous spectra of FAD-dAppA$_{BLUF}$ and include the 1666 cm^{-1} TA marker mode characteristic of the photoactivateable state of the protein. The transient IR spectra were followed for up to 100 ps and no further changes in spectral shape were detected.

The transient IR data for light activated AppA$_{BLUF}$ reconstituted with Rf (Rf-lAppA$_{BLUF}$) 2 ps after excitation are also shown in Figure 2. In comparison to the Rf-dAppA$_{BLUF}$ spectrum the highest frequency bleach splits into a doublet and shifts to lower wavenumber. In addition, there is a new bleach around 1670 cm^{-1}, coupled with a weaker bleach at 1650 cm^{-1}. The formation of a doublet structure at 1700 and 1690 cm^{-1} in what is proved below to be the C4=O transient bleach indicates that the Rf C4=O in Rf-lAppA$_{BLUF}$ experiences at least two hydrogen bonding environments, one of which is more strongly hydrogen bonded than in Rf-dAppA$_{BLUF}$. The formation of a red shifted doublet in the light activated state of FAD-lAppA$_{BLUF}$ was reported in our earlier paper. Thus, the first conclusion to be drawn from the present work is that the similar behaviour of Rf-AppA$_{BLUF}$ and FAD-AppA$_{BLUF}$ on irradiation observed in steady state spectroscopy, persists in transient IR spectroscopy. Crucially this shows that Rf is a suitable model for FAD in AppA.

Figure 3A shows the transient IR spectra recorded 3 ps after excitation for unlabeled and both ^{13}C-labeled Rf in pD 8 D_2O buffer. Importantly, the spectra of

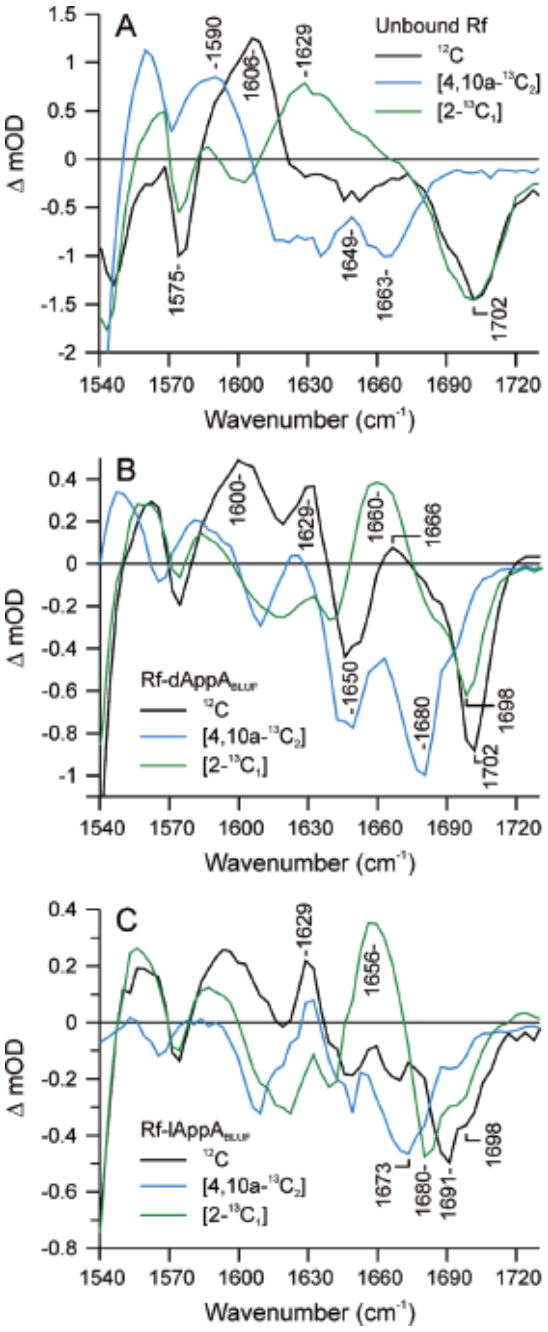

Figure 3. TRIR spectra of the Rf isotopologues in solution and bound to dAppA$_{BLUF}$ and lAppA$_{BLUF}$.
A: 3ps TRIR spectra of Rf in buffer.
B: 2ps TRIR spectra of Rf bound to dAppA$_{BLUF}$.
C: 2ps TRIR spectra of Rf bound to lAppA$_{BLUF}$.

isotopically-labelled Rf confirm the assignment of the highest wavenumber transient bleach (1702 cm^{-1}) to a mode dominated by the C4=O stretch. This mode shifts by -39 cm^{-1} in [4,10a-^{13}C$_2$]Rf. The mode with the next highest wavenumber for Rf in buffer (1650 cm^{-1}) is rather broad and of low intensity. This mode contains a major contribution from C2=O stretch on the basis of a large (-45 cm^{-1}) shift to lower wavenumber in [2-^{13}C$_1$]Rf, accompanied by only a small (< 5 cm^{-1}) shift in the mainly C4=O localised mode.

The shift of the C2=O bleach to lower frequency in [2-^{13}C$_1$]Rf reveals a broad intense TA with a peak around 1630 cm^{-1}. We assign this to the C4=O mode in the excited state. In unlabeled Rf this mode is

presumably hidden due to overlap with the bleach associated with C2=O. In [4,10a-$^{13}C_2$]Rf in buffer the shift of the C4=O mode to 1663 cm^{-1} is accompanied by an increase in the TA intensity around 1590 cm^{-1}, which can be assigned to a shift in the associated excited state mode. These data suggest that the carbonyl modes of Rf in buffer are shifted to lower frequency and broadened in the excited electronic state, compared to the ground state.

Qualitatively the same spectra and isotope shifts are observed for the carbonyl bleaches in Rf-dAppA$_{BLUF}$ (Figure 3B) but the two high wavenumber bleaches at 1702 and 1650 cm^{-1} are narrower and better resolved, with additional intensity in C2=O mode. Again, for [4,10a-$^{13}C_2$]Rf bound to dAppA$_{BLUF}$ the C4=O mode is now observed at 1680 cm^{-1}, with little change in the C2=O mode. Conversely, labelling C2 has little effect on the 1700 cm^{-1} mode but a dramatic effect on the 1650 cm^{-1} band. This important observation now confirms the assignment of the two bands to C4=O and C2=O as proposed previously for FAD-dAppA$_{BLUF}$.

A large transient is observed at 1660 cm^{-1} in [2-$^{13}C_1$]Rf-dAppA$_{BLUF}$ (Figure 3B). This is likely a combination of the 1666 cm^{-1} mode observed in unlabeled Rf-dAppA$_{BLUF}$ and an excited state mode revealed by the shift of C2=O bleach to lower frequency. For Rf in solution the TA seen at 1630 cm^{-1} was assigned to C4=O in the excited state. It is possible that this mode also contributes to the intensity at 1660 cm^{-1} in [2-$^{13}C_1$]Rf-dAppA$_{BLUF}$. If so, then this suggests a significant difference in the interaction between excited Rf in the specific hydrogen bonding environment of dAppA$_{BLUF}$ compared with the distribution of hydrogen bonding geometries in the solvent. However, such a localised mode assignment predicts that the TA should shift in [4,10a-$^{13}C_2$]Rf-AppA$_{BLUF}$, at least attenuating the bleach around 1645 cm^{-1}; no such attenuation is observed.

A similarly complex picture emerges from the study of labelled Rf-lAppA$_{BLUF}$ (Figure 3C). Labelling of C4 results in a significant shift to lower frequency in the doublet observed at 1690/1700 cm^{-1}, consistent with the C4=O assignment. In addition, as seen for dAppA$_{BLUF}$, the TA at 1660 cm^{-1} is clearly resolved in the spectrum of [2-$^{13}C_1$]Rf-lAppA$_{BLUF}$. However the position of this TA is the same in Rf-dAppA$_{BLUF}$ and Rf-lAppA$_{BLUF}$, in contrast to the major component of the C4=O peak which shifts by 10 cm^{-1} upon formation of the signalling state of the protein. The shift in the C4=O bleach causes the high wavenumber edge of the TA to shift somewhat, but the low wavenumber edge overlaps precisely with that in dAppA. Thus, it is not clear that this TA correlates with the C4=O bleach. Once again, there is no striking evidence for the strong 1660 cm^{-1} TA in either ^{12}C Rf-lAppA$_{BLUF}$ or [4,10a-$^{13}C_2$]Rf-lAppA$_{BLUF}$ (Figure 3C).

The apparent dependence of the intensity of the 1660 cm^{-1} TA on the position of the ^{13}C label is surprising, and not readily reconciled with a straightforward assignment to the C4=O mode in the excited electronic state. One interpretation is that the excited state modes are no longer localised in the same way as they are in the ground state, but delocalised over (among others) both carbonyl stretches. The coupling leading to delocalisation may then be particularly sensitive to frequency shifts in one of the component modes, giving rise, for example, to the unexpected effects of [2-$^{13}C_1$] substitution. Indeed it may be that in Rf-AppA$_{BLUF}$ mode coupling extends beyond the Rf chromophore to include nearly resonant modes in the surrounding residues (which may themselves be delocalised, as has been observed in antiparallel β-sheet). Coupling between flavin and protein modes was reported in the DFT calculations of Unno *et al*. Previously we assigned changes in the transient IR spectra in the 1660-1670 cm^{-1} region of FAD-AppA$_{BLUF}$ to a marker mode for photoactivity. The data presented here raise the possibility that the TA corresponding to that marker mode may reflect photoinduced modifications of flavin excited state – protein coupling. This proposal can be tested by recording TRIR spectra, of isotope edited AppA. Such measurements are in progress.

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