BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2011,** *50,* 1321–1328 1321
DOI: 10.1021/bi101589a

Ultrafast Infrared Spectroscopy of an Isotope-Labeled Photoactivatable Flavoprotein†

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Received September 30, 2010; Revised Manuscript Received January 8, 2011

ABSTRACT: The blue light using flavin (BLUF) domain photosensors, such as the transcriptional antirepressor AppA, utilize a noncovalently bound flavin as the chromophore for photoreception. Since the isoalloxazine ring of the chromophore is unable to undergo large-scale structural change upon light absorption, there is intense interest in understanding how the BLUF protein matrix senses and responds to flavin photoexcitation. Light absorption is proposed to result in alterations in the hydrogen-bonding network that surrounds the flavin chromophore on an ultrafast time scale, and the structural changes caused by photoexcitation are being probed by vibrational spectroscopy. Here we report ultrafast time-resolved infrared spectra of the AppA BLUF domain ($AppA_{BLUF}$) reconstituted with isotopically labeled riboflavin (Rf) and flavin adenine dinucleotide (FAD), which permit the first unambiguous assignment of ground and excited state modes arising directly from the flavin carbonyl groups. Studies of model compounds and DFT calculations of the ground state vibrational spectra reveal the sensitivity of these modes to their environment, indicating that they can be used as probes of structural dynamics.

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 blue light using flavin (BLUF)[1] photosensors (*1*). BLUF domain photosensors differ fundamentally from the rhodopsins, xanthopsins, and phytochromes since the flavin chromophore does not undergo large-scale reorganization upon excitation (*2*). 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 (*2*). 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 (*2*) and is accompanied by a strengthening of hydrogen bond(s) to the C4═O group of the chromophore from the protein (*3*−*5*). X-ray crystallographic studies 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). This structure, together with NMR spectroscopy, supports 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 (*6*−*9*). As a consequence of this light-induced change in hydrogen bonding it has been proposed on structural grounds that W104 may move, leading to formation of the signaling state of the protein. Recent spectroscopic evidence suggests that W104 may move, closer to the flavin in the signaling state (*10*), possibly contributing to acceleration of the ground state recovery time in the light-adapted form (*11*, *12*).

Although the AppA signaling state has a half-life of 15 min (*2*), it is formed within 1 ns of photoexcitation (*13*), an event thought to involve electron transfer from Y21 to the neighboring chromophore (*14*). 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 (*11*) that Q63 tautomerizes, rather than rotates, on the ultrafast time scale. Such a tautomerization is a key feature in some recent computational studies of the primary steps in BLUF photoactivation (*15*, *16*). 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 perturbed by flavin excitation. However, although the vibrational spectra contain a wealth of structural

†Funded by NSF (P.J.T.; CHE-0822587), EPSRC (S.R.M.; EP/G002916), and the Hans Fischer Gesellschaft (A.B.).

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[1]Abbreviations: FAD, flavin adenine dinucleotide; Rf, riboflavin; BLUF, blue light using flavin; $AppA_{BLUF}$, AppA BLUF domain; $dAppA_{BLUF}$, dark-adapted $AppA_{BLUF}$; $lAppA_{BLUF}$, light-adapted $AppA_{BLUF}$; TRIR, time resolved infrared.

Published on Web 01/10/2011

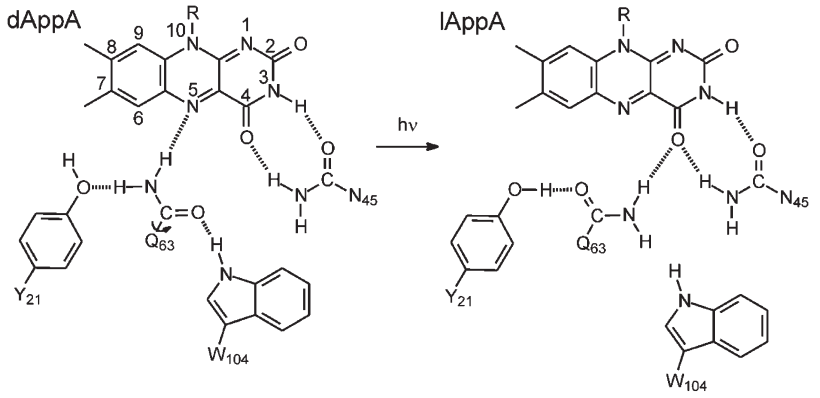

FIGURE 1: Environment of the isoalloxazine chromophore in AppA. Putative hydrogen-bonding interactions are shown by dashed lines. Photoexcitation may lead to changes in the hydrogen bond network, one model for which involves a rotation of Q63 (*6*).

detail, attempts to fully assign the spectra have been hindered by the difficulty in site-specifically incorporating isotopes into the flavin chromophore, which has been shown to be a powerful tool in assigning transitions observed in TRIR (*17*). As a result of the limited spectroscopic data available on AppA, alternative interpretations of the TRIR data are plausible (*18*). Here we report TRIR spectra of $AppA_{BLUF}$ reconstituted with [4,10a-$^{13}C_2$] riboflavin and [2-$^{13}C_1$]-FAD. These data allow the assignment of bands in the TRIR spectrum to vibrational modes arising mainly from the carbonyl groups in the isoalloxazine ring. We complement these $AppA_{BLUF}$ measurements with an experimental and theoretical study of the carbonyl modes of FAD, riboflavin, and their isotopes in aqueous solution, which reveals their sensitivity to isotopic substitution and to their H-bonding environment.

MATERIALS AND METHODS

Materials. Riboflavin and FAD (disodium salt) were from Sigma Aldrich. D_2O (99.9 atom %) was from Cambridge Isotope Laboratories. Ampicillin (disodium salt) was from Fisher Scientific. Ni-NTA resin was from Novagen. ITPG was from Gold-ChemBio.

Synthesis of Riboflavin and FAD Isotopes. [2-$^{13}C_1$]-FAD and [4,10a-$^{13}C_2$]riboflavin were prepared according to Tishler et al. (*19*). Riboflavin isotopologues were converted enzymatically to the cognate FAD isotopologues.

Protein Expression and Purification. The BLUF domain of AppA ($AppA_{BLUF}$; residues 5−125) was expressed in BL21-(DE3) *Escherichia coli* cells using a construct in which the $AppA_{BLUF}$ coding sequence had been inserted into a pET15b vector (Novagen) so that protein was produced with a N-terminal His tag. Protein expression and purification were performed in the dark essentially as described previously (*11*) with the following modifications. After growth at 30 °C for 5 h followed by induction using 0.8 mM IPTG at 18 °C overnight, the cell pellet resulting from a 1 L culture was resuspended in 40 mL of buffer A (50 mM NaH_2PO_4 buffer, pH 8, containing 10 mM NaCl) to which were added 200 μL of the protease inhibitor phenylmethanesulfonyl fluoride (50 mM stock solution in ethanol) and 14 μL of β-mercaptoethanol. The cells were than lysed using sonication, cell debris was removed by centrifugation (33000 rpm for 90 min), and the soluble fraction was incubated with a 10-fold molar excess of either FAD or riboflavin for 45 min on ice in the dark to ensure a homogeneous population of protein-bound chromophore (*20*). Following incubation, the solution was loaded onto a Ni-NTA column (1 × 10 cm) that had been preequilibriated with pH 8 phosphate buffer and then washed with 50 mL of buffer A. The column was then washed with buffer A containing increasing concentrations of imidazole until $AppA_{BLUF}$ eluted at 250 mM imidazole. The fractions containing protein were pooled, dialyzed against buffer A overnight, and concentrated to 1.5 mM. Protein purity was assessed by SDS−PAGE and UV−vis spectroscopy (protein, ε_{270} = 35800 M^{-1} cm^{-1}; FAD, ε_{446} = 8500 M^{-1} cm^{-1}). Chromophore content was determined by the ratio of protein to FAD absorbance (4.2 for wild-type $AppA_{BLUF}$ with FAD bound (*21*)). To exchange the protein into D_2O, samples of AppA were frozen in liquid N_2, lyophilized overnight, redissolved in D_2O, and allowed to incubate for 5 h after which this process was repeated three to four times. Both exchanged and unexchanged proteins were stored as lyophilized powders at −80 °C until needed.

Binding of Riboflavin and FAD Isotopologues. Approximately 5 mg of riboflavin isotopologue was solubilized in 50 μL of DMSO. The solution was added to 15 mL of D_2O buffer (1 mM NaCl, 20 mM Na_2HPO_4, pD 8) and allowed to incubate at 60 °C for at least 1.5 h. The solution was cooled to 4 °C and combined with 0.5−1 mL of 1.5 mM $AppA_{BLUF}$ bound to unlabeled riboflavin. The mixture was kept in the dark at 4 °C for 1.5 h. The protein was then concentrated to between 1.5 and 2 mM by centrifugation using Amicon filters with a MW cutoff of 3000 Da. To remove unbound ligand, the protein was diluted with buffer and then reconcentrated using an Amicon filter.

The [2-$^{13}C_1$]-FAD isotopologue was incorporated into $AppA_{BLUF}$ by incubating a 0.54 mM solution of the purified protein with 1.4 mM [2-$^{13}C_1$]-FAD. This protein flavin mixture was then washed by repeated cycles of concentration and dilution with buffer A using a 3000 Da Amicon filter until free flavin could not be detected in the eluate. The protein sample (∼0.5 mM) was then incubated a second time with an excess of [2-$^{13}C_1$]-FAD (1.42 mM), followed by repeated washing until no free flavin could be detected in the ultrafiltrate. Using this method the final percent isotope incorporation was estimated to be 92%.

Time-Resolved Infrared Spectroscopy. Ultrafast time-resolved IR (TRIR) spectra were measured at the STFC Central Laser Facility using two different TRIR spectrometers. The first provided an ∼300 fs temporal resolution at a 1 kHz repetition rate; the system and data collection methods have been reported previously (*11*, *22*, *23*). In these experiments the spot size and power of the laser pump pulse were controlled so as to prevent photobleaching of the sample (which was rastered in the beam) while retaining good signal-to-noise ratio in the spectra. Typical

settings were a 100 μm radius spot size and excitation pulse energies less than 2.0 μJ per pulse, so that ~10% of the sample within the irradiation volume was excited by each pulse. Measurements typically consisted of probe vibrational spectra in the region 1550−1750 cm^{-1} recorded at three to five randomly ordered time delays, with a 10 s collection period for each delay. Typically eight samples, each around 1.5−2.5 mM, were measured for each spectrum in a 50 μm path length cell, and the results were averaged. Electronic absorption spectra were taken after each experiment to check for photobleaching, as previously described (*11*). All protein and most aqueous flavin solution measurements were made in deuterated water due to its superior IR transmission characteristics, but some flavin solution measurements were made in H_2O, in which case the path length was reduced to 6 μm.

The second TRIR system exploited a recently developed high-sensitivity 10 kHz repetition rate source with ~100 fs time resolution. The apparatus has been described in detail elsewhere (*24*). The key differences compared to the 1 kHz source were an improved signal-to-noise ratio resulting from the faster repetition rate and more stable source and a wider spectral bandwidth permitting the 1400−1800 cm^{-1} wavenumber range to be measured in one experiment. For this source the excitation spot size was ~100 μm radius, and the pulse energy was kept below 400 nJ. The possible effect of the higher repetition rate on the photokinetics of the photoactive samples was considered. For this 10 kHz source photoactive proteins were studied in a flow cell which was used in addition to rastering of the sample holder in the beam path, thus minimizing photobleaching and degradation of the protein and the photoconversion of dark-adapted $AppA_{BLUF}$. Even under these conditions if the pump pulse energy exceeded 600 nJ, the transient spectrum measured at 3 ps was observed to be a mixture of dark- and light-adapted $AppA_{BLUF}$. Below 400 nJ the dark-adapted $AppA_{BLUF}$ spectrum was independent of pump intensity. Under these conditions the spectra and the kinetics (ground state recovery) were compared with those measured on the 1 kHz system and were seen to be the same within the signal-to-noise ratio. The IR probe again recorded transient difference spectra (pump on−pump off) at time delays between 1 ps and 2 ns. After the measurements were recorded, the extent of photoconversion was shown to be negligible using absorbance spectroscopy. To retain the same spectral resolution as for the 1 kHz system, the probe was measured by two carefully matched 128 pixel detectors, yielding a resolution of 3 cm^{-1} per pixel. Spectra were calibrated relative to the IR transmission of a pure *cis*-stilbene standard sample placed at the sample position.

Light-adapted $AppA_{BLUF}$ was prepared by irradiating $dAppA_{BLUF}$ at 365 nm using a hand-held UV illuminator. Photoconversion was monitored using UV−vis spectroscopy and was found to be complete within 3 min.

Density Functional Theory Calculations. Density functional theory (DFT) calculations were performed to support the assignments based on isotopic substitution. So as to avoid complications associated with possible multiple conformations of the side chain and to allow a greater number of calculations, all of the frequencies given refer to lumiflavin rather than FAD. The DFT calculations were made for gas phase using the Gaussian 03 software package, B3LYP method, and 6-31G basis set. This procedure can obviously lead to discrepancies between the experimental data and the calculations, so for the purpose of assignment the relative spectral shifts are more relevant than the exact wavenumber. H-bonding effects of the solvent were investigated by adding specific water molecules. For the H_2O/D_2O comparison we exchanged the H atom at the N3 position with D. The resulting frequencies were in every case multiplied by 0.9614, the accepted scaling factor for the B3LYP/6-31G calculations (*25*). Although the theory predicts only a maximum ±34 cm^{-1} difference between the theoretical and the experimental data, this rule had to be relaxed for the high frequencies (1650, 1700 cm^{-1}) where there was strong evidence for a matching vibration that was more than 34 cm^{-1} away.

RESULTS AND DISCUSSION

The TRIR spectra of dark- ($dAppA_{BLUF}$) and light-adapted ($lAppA_{BLUF}$) $AppA_{BLUF}$ contain a wealth of information on the early structural changes that accompany protein photoexcitation (*11*). However, in order to fully interpret the TRIR spectra and thereby enhance our understanding of the mechanism of AppA photoactivation, isotope labels must be incorporated site specifically into both the chromophore and the surrounding amino acids in order to assign vibrational modes associated with changes upon photoexcitation. Obvious targets for site-specific labeling include groups that are involved in direct interactions between chromophore and protein, including the carbonyl groups in the FAD isoalloxazine ring that are hydrogen bonded to amino acids surrounding the chromophore. The isoalloxazine C4═O groups is of particular interest since models for AppA activation propose a strengthening of hydrogen bond(s) to this group in the light-activated protein (Figure 1), and we and others have assigned bands in the vibrational spectra to both the C4═O and the adjacent C2═O groups (*5*, *11*, *26*−*30*). The frequency of these bands alter upon photoconversion, supporting the importance of protein interactions with the carbonyl groups in the AppA photocycle. While DFT calculations have been used to calculate the normal mode composition of each band (*31*), true band assignments require selective isotope labeling in order, for example, to delineate the degree of vibrational coupling between the two carbonyl groups. In this work we first probe through TRIR measurements and DFT calculations the sensitivity of the carbonyl modes of FAD or riboflavin to isotopic substitution at C2 and C4 and also to H/D exchange at the N3 atom (known to be coupled to the carbonyl stretches) (*29*, *32*). We further probe the environment sensitivity of these modes by calculating the effect of specific H-bonding interactions. With these data it becomes possible to investigate the same modes in AppA in both its dark- and light-adapted states.

In Figure 2 the transient vibrational spectra at a 3 ps time delay after excitation for FAD and [2-$^{13}C_1$]-FAD are shown and compared with DFT calculations of the ground state IR transmission spectrum. Experimental data were recorded in H_2O, and calculations were made with a proton at N3. The experimental spectra in Figure 2 are difference spectra of the excited state minus unexcited (ground) state transmission where the negative modes are associated with loss of the ground state and referred to as bleaches, and the positive modes are associated with the newly generated excited state. The main observed effect of isotopic substitution is a shift in the band with the second highest frequency from 1663 to 1625 cm^{-1}. This compares nicely with the calculated downshift of 38 cm^{-1} in the DFT data as a result of the same substitution and is thus consistent with the assignment of this band to a mode that is principally associated with motions of the C2═O group. Although the DFT calculations reveal that this mode is strongly coupled to the N3H wag, there is no

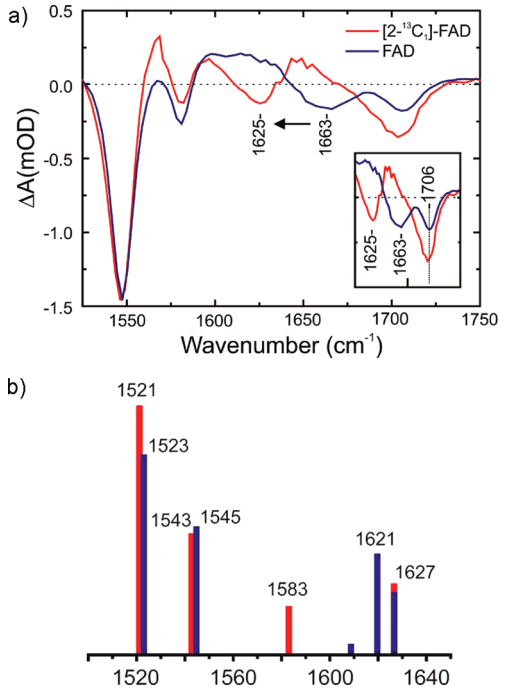

FIGURE 2: TRIR and calculated spectra of unlabeled FAD and [2-$^{13}C_1$]-FAD in H_2O. FAD concentration was 6 mM in phosphate buffer, pH 8, and the TRIR spectra were recorded with a time delay of 3 ps.

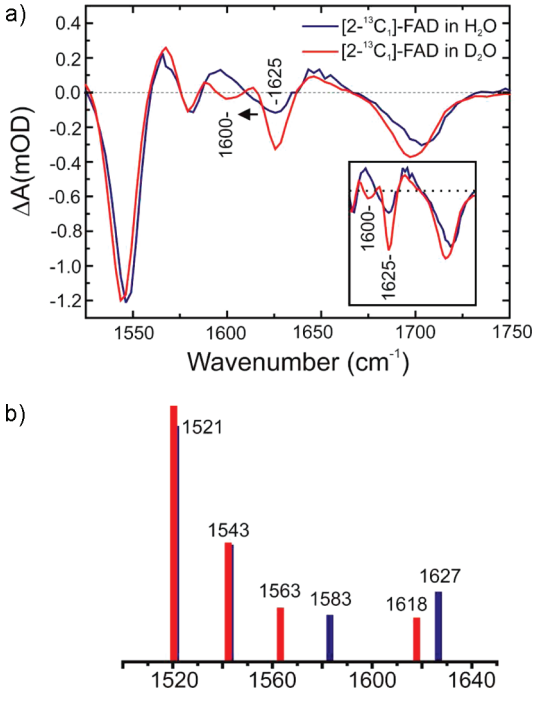

FIGURE 3: TRIR and calculated spectra of [2-$^{13}C_1$]-FAD in H_2O and D_2O. FAD concentration was 6 mM in pH or pD 8 phosphate buffer, and the TRIR spectra were recorded with a time delay of 3 ps.

significant movement of C4=O upon isotopic substitution at C2, indicating that the two carbonyl modes are not strongly coupled.

In Figure 3 the effect of exchange of N3H for N3D is shown both experimentally for [2-$^{13}C_1$]-FAD in water and D_2O and in DFT calculations. Again, the agreement between calculated and experimental data is good. Both of the carbonyl localized modes shift down in frequency in D_2O, with the larger effect being seen

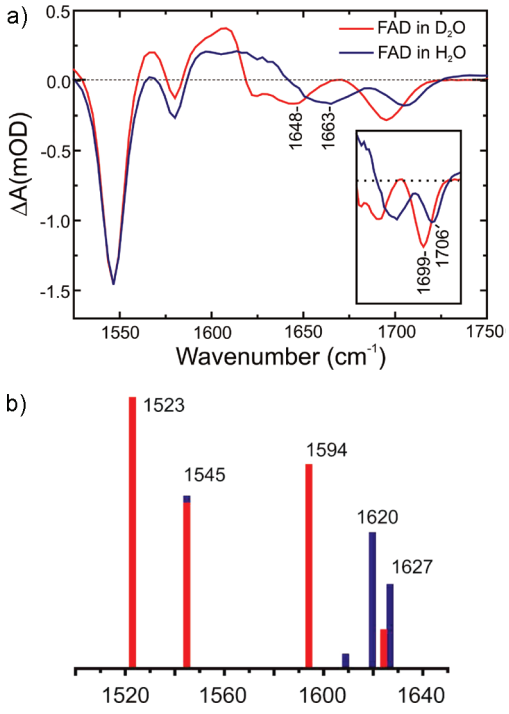

FIGURE 4: TRIR and calculated spectra of unlabeled FAD in H_2O and D_2O. FAD concentration was 6 mM in pH or pD 8 phosphate buffer, and the TRIR spectra were recorded with a time delay of 3 ps.

for the C2=O mode in this case (1625−1600 cm^{-1}). This result indicates the importance of H/D exchange at N3 and is consistent with the DFT calculations which indicate coupling between C2=O and N3H. The effect of deuteration on the vibrational data will be significant when comparing theory and experiment since the vast majority of IR difference spectra are made in D_2O. A consequence of the downshift of C2=O is the appearance of a bleach at 1625 cm^{-1}. We suggest that this mode can be assigned to the adenine of the FAD (*33*) which was partially obscured by the excited state absorption of C2=O. Support for this assignment comes from the observation (not shown) that no such mode appears for FMN when D_2O is exchanged for H_2O, although all other shifts are very similar; clearly, the adenine mode lends some uncertainty to the location of the C2=O mode in H_2O, but the importance of the H/D exchange is clear.

The effect of H/D exchange is even more marked in the case of unlabeled FAD in H_2O and D_2O (Figure 4). Previous Raman studies on riboflavin demonstrated that H/D exchange of FAD resulted in a 13 cm^{-1} decrease in the frequency of a band at 1714 cm^{-1} (*34*). This is consistent with the current data where a 7 cm^{-1} decrease in the band at 1706 cm^{-1} is observed (Table 1). In the DFT calculation for the chromophore in H_2O (N3H) the two highest frequency modes are close in wavenumber with the stretch largely localized on the C4=O (1627 cm^{-1}) and C2=O (1620 cm^{-1}) modes, respectively, and coupled to the N3H wag. In contrast, the DFT calculation of the chromophore in D_2O (N3D) shows that the two frequencies separate and the character changes to an asymmetric (1594 cm^{-1}) and symmetric (1624 cm^{-1}) C2/C4=O character pair (again coupled with the N3D wag). The effect seen experimentally is consistent with this result, but the modes are already well separated in H_2O although they do move further apart in D_2O, as predicted by calculation (Figure 4). The origin of the apparent discrepancy between N3H experiment and DFT calculations was investigated by including a specific water molecule in the calculations, H-bonded to the C=O oxygen

Table 1: Observed and Calculated Vibrational Modes (cm^{-1}) of FAD and Riboflavin in H_2O and D_2O[a]

	unlabeled FAD		[2-$^{13}C_1$]-FAD		unlabeled Rf		[4,10a-$^{13}C_2$]-Rf	
	obsd	calcd	obsd	calcd	obsd	calcd	obsd	calcd
H_2O	1547	1523, $C10_aN_1$	1547	1521, $C10_aN_1$				
	1581	1545, $C4_aN_5$	1581	1543, $C4_aN_5$				
	1663	1619, C_2=O + N_3 wag	1625	1583, C_2=O + N_3 wag				
	1706	1627, C_4=O + N_3 wag	1703	1627, C_4=O + N_3 wag				
D_2O	1547	1523, $C10_aN_1$	1545	1521, $C10_aN_1$	1547	1523, $C10_aN_1$	NA	1515, $C10_aN_1$
	1581	1545, $C4_aN_5$	1581	1543, $C4_aN_5$	1575	1545, $C4_aN_5$	1565	1536, $C4_aN_5$
	1648	1594, C_2=O, C_4=O asym + N_3 wag	1600	1563, C_2=O, C_4=O asym + N_3 wag	1648	1594, C_2=O, C_4=O asym + N_3 wag	1620	1570, C_4=O, C_2=O asym + N_3 wag
	1699	1624, C_4=O, C_2=O sym + N_3 wag	1697	1618, C_4=O, C_2=O sym + N_3 wag	1700	1624, C_4=O, C_2=O sym + N_3 wag	1663	1608, C_2=O, C_4=O sym + N_3 wag

[a]Observed frequencies are from the experimental TRIR measurements. Calculated frequencies are for lumiflavin and were generated using Gaussian 03 (B3LYP/6-31G). Both unlabeled FAD and [2-$^{13}C_1$]-FAD were measured in H_2O and D_2O. Unlabeled riboflavin (Rf) and [4,10a-$^{13}C_2$]riboflavin were measured in D_2O only.

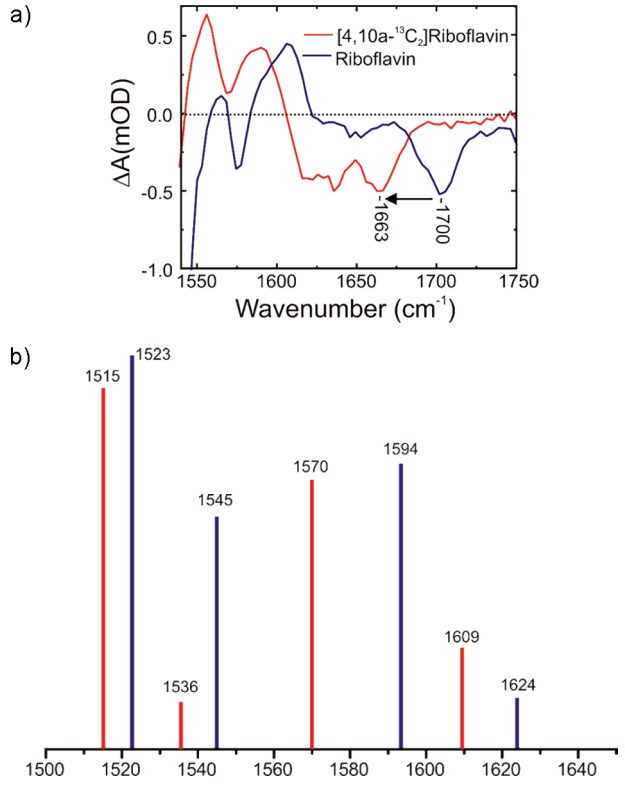

FIGURE 5: TRIR and calculated spectra of unlabeled riboflavin and [4,10a-$^{13}C_2$]riboflavin in D_2O. TRIR spectra of 1 mM riboflavin (black) and [4,10a-$^{13}C_2$]riboflavin (red) in pD 8 phosphate buffer recorded with a time delay of 3 ps.

atoms or to N3H. The largest effect was on H-bonding to N3H, causing the separation of the two carbonyl frequencies in FAD to increase by 30 cm^{-1}. From this it may be concluded that the pattern of H-bonding modes is susceptible not only to H/D exchange but also to the local H-bonding environment. Such measurements will be useful in probing H-bonded interactions in the protein.

The transient spectra in Figure 4 are in good agreement with our previous study in D_2O (*31*). It is interesting to compare these measurements with the data of Wolf et al. (*29*), who studied riboflavin in the non-H-bonding solvent DMSO. The loss in the hydrogen bonding gives rise to a shift of the carbonyl peaks to higher frequencies. The DMSO data have the highest frequency mode at 1710 cm^{-1} compared to ~1706 cm^{-1} in aqueous FAD and 1700 cm^{-1} in riboflavin solution. Hydrogen bonding causes a larger shift in the C2=O localized mode of more than 20 cm^{-1} from 1676 cm^{-1} in DMSO to ~1650 cm^{-1} in both riboflavin and FAD in aqueous solution. The solvent-induced shifts in the ring modes at 1581 and 1546 cm^{-1} are much smaller, confirming the sensitivity of the carbonyl modes to H-bonding.

In addition to labeling at C2, the effect of ^{13}C-labeling at C4 has been probed using [4,10a-$^{13}C_2$]riboflavin. Specific labeling of only C4 is challenging, and the synthetic route to the isotopomer studied here resulted in labeling at both C4 and C10a of the isoalloxazine ring. From the TRIR data in Figure 5, the most obvious effect of labeling in the experimental data is a 37 cm^{-1} decrease in the highest observed bleach mode, indicating that this mode has a substantial fraction of C4=O character. This is in agreement with previous steady-state Raman spectra on FAD where a 34 cm^{-1} shift in this mode was observed upon ^{13}C-labeling of C4=O (*26*). DFT calculations for isotopically labeled lumiflavin show the same magnitude shift of the peaks at 1523 and 1545 cm^{-1} as the respective measured peaks at 1547 and 1575 cm^{-1}; i.e., both of the higher frequency modes shift down in frequency. The shift of the lower frequency mode of the pair is less evident experimentally, perhaps because it is poorly resolved (Figure 5). It may also be that these modes for riboflavin in D_2O are more localized than suggested by the calculation because of the effect of H-bonding discussed above.

Equipped with the above spectroscopic characterization of isotopically substituted flavins, a more detailed discussion of the data for the protein reconstituted with its ligand can be made. We measured TRIR spectra of $AppA_{BLUF}$ reconstituted with [2-$^{13}C_1$]-FAD and [4,10a-$^{13}C_2$]riboflavin. While FAD is more soluble in aqueous solution than riboflavin, and thus more convenient to work with, previous studies based on electronic spectroscopy suggest that the photocycle of AppA is not affected by replacing FAD with riboflavin (*3*, *32*). To confirm this proposal, we first compared the TRIR spectra of $dAppA_{BLUF}$ and $lAppA_{BLUF}$ reconstituted with either FAD or riboflavin (Figure 6). Based on peak position, relative intensity, and the kinetic information shown, $AppA_{BLUF}$ is not affected by replacing FAD with riboflavin as the flavin chromophore. The $dAppA_{BLUF}$ spectra show intense bleaches observed at 1695, 1650, 1580, and 1547 cm^{-1} for $AppA_{BLUF}$ reconstituted with both riboflavin and FAD. Transient absorptions are observed at 1630, 1595, and 1565 cm^{-1} for both FAD and riboflavin in addition to a broad transient at ~1670 cm^{-1}.

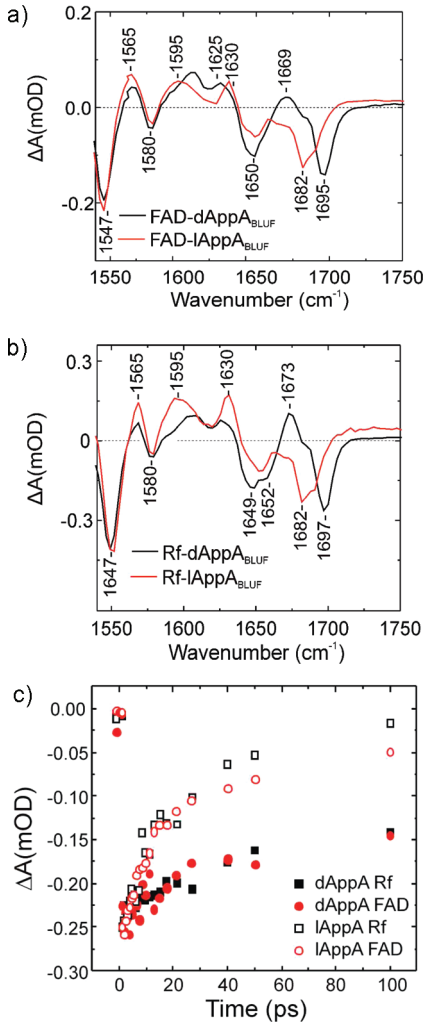

FIGURE 6: TRIR spectra and kinetic data of dAppA$_{BLUF}$ and lAppA$_{BLUF}$ bound to riboflavin or FAD in D_2O. (a) TRIR spectra of dAppA$_{BLUF}$ (black) and lAppA$_{BLUF}$ (red) bound to riboflavin. (b) TRIR spectra of dAppA$_{BLUF}$ (black) and lAppA$_{BLUF}$ (red) bound to FAD. Protein concentration was 2 mM in pD 8 phosphate buffer, and the TRIR spectra were recorded with a time delay of 3 ps. (c) Comparison of kinetic measurements of AppA$_{BLUF}$ bound to riboflavin and FAD in D_2O measured at 1547 cm^{-1}

The latter transient has been previously reported by us to be associated with a photoactivatable state of the protein through studies of both light- and dark-adapted states and photoactive and inactive mutants (*11*).

The TRIR spectra for lAppA$_{BLUF}$ bound to FAD and riboflavin are also shown in Figure 6. In comparison to the dark state, the high-frequency bleach at ~1695 cm^{-1} shifts to lower frequency and splits into a doublet at 1691 and 1682 cm^{-1}. The bleach at 1650 cm^{-1} found in both the dark and light state spectra is lower in intensity in the light state when compared to the dark state. In addition, the transient at ~1670 cm^{-1} is no longer observed in the light state, which is in agreement with previous measurements (*11*). The first conclusion that can be drawn from this work is that the similarities reported in the steady-state data for AppA$_{BLUF}$ bound to either FAD or riboflavin are also observed in TRIR spectroscopy, demonstrating that riboflavin is a suitable model for FAD in AppA$_{BLUF}$ and that the H-bonding pattern is similar for both chromophores.

In Figure 6c the ground state recovery kinetics for dAppA$_{BLUF}$ and lAppA$_{BLUF}$ are compared bound to both FAD and riboflavin.

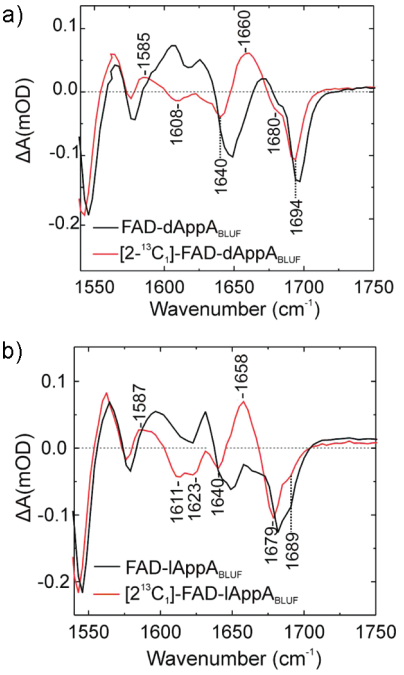

FIGURE 7: TRIR spectra of FAD and [2-$^{13}C_1$]-FAD bound to dAppA$_{BLUF}$ and lAppA$_{BLUF}$. (a) TRIR spectra of dAppA$_{BLUF}$ bound to unlabeled FAD (black) and [2-$^{13}C_1$]-FAD (red). (b) TRIR spectra of lAppA$_{BLUF}$ bound to unlabeled FAD (black) and [2-$^{13}C_1$]-FAD (red). The protein concentration was 2 mM, and the samples were prepared in 50 mM phosphate D_2O buffer, pD 8. The time delay was 3 ps.

The kinetics for riboflavin and FAD are indistinguishable within experimental error, consistent with the conclusion above. Also immediately noticeable for both chromophores is that the ground state recovery is much faster in lAppA$_{BLUF}$, suggesting faster excited state quenching, as discussed elsewhere (*11*). A discussion of the ultrafast kinetics is beyond the scope of this paper, but the nonexponential recovery is evident. For lAppA$_{BLUF}$ the fast components dominate. A substantial fraction of the dAppA$_{BLUF}$ recovery occurs on a sub-100 ps time scale, as was also observed in earlier studies (*11*, *35*). Measurements of transient electronic spectroscopy however report a dominant 600 ps component (e.g., in fluorescence decay), accompanied by faster components (*13*). The ground state recovery appears to be faster than the fluorescence decay suggests inhomogeneous decay kinetics with the faster components being connected to ground state recovery.

The TRIR spectra of dAppA$_{BLUF}$ and lAppA$_{BLUF}$ bound to [4,10a-$^{13}C_2$]riboflavin confirm assignments of the high-frequency band as involving mainly the C4═O localized stretch and the lower frequency band as mainly arising from the C2═O stretch. First we consider dAppA$_{BLUF}$ bound to [2-$^{13}C_1$]-FAD (Figure 7a) which shows only a small shift in the 1695 cm^{-1} bleach. In addition, there is a shift of the 1650 cm^{-1} mode to lower frequency (1640 cm^{-1}). This bleach is much less intense in comparison to the unlabeled spectra; however, we suggest that the broad positive band spanning from 1630 to 1585 cm^{-1} overlaps with the 1640 cm^{-1} feature, reducing its intensity. Similar changes are observed in the TRIR spectrum of lAppA$_{BLUF}$ upon ^{13}C-labeling of C2═O (Figure 7b), and thus these data confirm the assignment of mainly C4═O localized character to the high-frequency mode and the C2═O vibration as the main contributor to the low-frequency mode, as proposed previously for FAD bound to dAppA$_{BLUF}$ (*11*). These assignments align with our earlier study and are similar to another

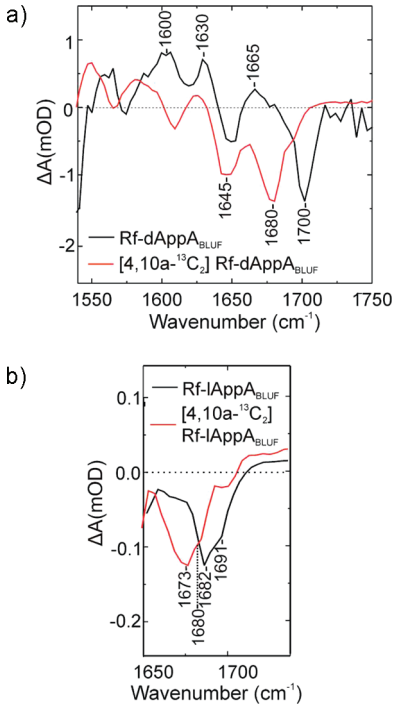

FIGURE 8: TRIR spectra of unlabeled riboflavin and [4,10a-$^{13}C_2$] riboflavin bound to dAppA and lAppA. (a) TRIR spectra of dAppA$_{BLUF}$ bound to unlabeled riboflavin (black) and [4,10a-$^{13}C_2$] riboflavin (red). (b) TRIR spectra of lAppA$_{BLUF}$ bound to unlabeled riboflavin (black) and [4,10a-$^{13}C_2$]riboflavin (red). The protein concentration was 2 mM, and the samples were prepared in 50 mM phosphate D_2O buffer, pD 8. The time delay was 3 ps.

TRIR study of a BLUF domain, Slr1694 BLUF, although in that case a high wavenumber mode of low amplitude was assigned as the highest frequency carbonyl stretch.

Figure 8 shows the TRIR spectra of AppA$_{BLUF}$ bound to [4,10a-$^{13}C_2$]riboflavin and allows assignment of the high-frequency carbonyl mode as largely localized on the C4=O stretch. The 1700 cm^{-1} peak in dAppA$_{BLUF}$ shifts by 20 cm^{-1} to 1680 cm^{-1} (Figure 8a), and this isotope shift also appears in lAppA where the 1682 cm^{-1} mode shifts by roughly 10 cm^{-1} to 1673 cm^{-1} (Figure 8b). These isotope shifts seen upon ^{13}C-labeling of C4=O further validate assignment of the 1700 cm^{-1} mode in dAppA$_{BLUF}$ and the 1682 cm^{-1} mode in lAppA$_{BLUF}$ to a mainly C4=O mode of the flavin chromophore.

Another important aspect of both the light and dark AppA$_{BLUF}$ spectra is the appearance of an intense transient absorption at 1660−1670 cm^{-1}. This mode is seen in the unlabeled spectra at 1669 cm^{-1} in dAppA$_{BLUF}$ and was found earlier (*11*) to be characteristic of AppA mutants capable of undergoing a photocycle, being absent in lAppA$_{BLUF}$ and the photoinactive Q63L mutant. On the basis of those measurements the transient absorption was assigned to a protein mode, most likely Q63, perturbed by flavin excitation. The shift in the 1650 cm^{-1} bleach to lower frequency upon labeling removes overlap with the 1669 cm^{-1} mode increasing intensity of the transient, making the mode appear slightly red shifted. The same experiment in lAppA$_{BLUF}$ reveals for the first time a transient absorption. These observations suggest the transient may be associated with an excited state mode of the flavin (localized on C4=O, for example). However, such a simple assignment is not consistent with observations on AppA mutants. Further, the spectra in Figure 8a show that a shift in the C4=O bleach from 1700 to 1680 cm^{-1} in dAppA$_{BLUF}$ bound to riboflavin is not accompanied by an increase in bleach around 1650 cm^{-1}; indeed, the opposite is the case. In fact, attempts to model the transient absorption in Figure 7 and 8 solely on the basis of the isotope shifts seen in the bleach modes were not successful. The data presented here thus raise the possibility that the transient corresponding to the marker mode may reflect photoinduced modifications of flavin excited state−protein coupling. This proposal will be tested by recording TRIR spectra of the AppA protein that has been isotopically edited; such measurements are planned.

CONCLUSIONS

The carbonyl modes of the isoalloxazine ring can be used as tools for probing structural dynamics of the primary photoprocesses of AppA. The frequency and character of these modes are a sensitive function of their environment. The time-resolved IR data for FAD and riboflavin in solution and bound to AppA$_{BLUF}$ are similar in peak position and intensity. This result confirms the assumptions made from steady-state experiments that riboflavin is a good model for FAD in the BLUF domain of AppA. On the basis of this information, we are confident that both riboflavin and FAD can be used interchangeably as models to study flavin excited state dynamics of AppA. Isotope labeling of FAD and riboflavin has enabled the assignment of various ground and excited state modes observed in the TRIR spectra of AppA$_{BLUF}$. Specifically, the high-frequency and low-frequency carbonyl modes are confidently assigned as mainly C4=O and C2=O localized modes, respectively. However, isotopic exchange and DFT calculations showed that these modes have significant mixed character notably N3H wag which is medium dependent in agreement with earlier studies. In addition, the 1660−1670 cm^{-1} transient absorption previously reported as a photoactive marker has been designated a protein−chromophore band based on the changes seen upon isotope labeling. Currently, methods to isotopically label the protein in order to ensure greater confidence in the assignment of this marker vibrational mode are under development, while new mutants are being prepared to modify the H-bond configuration around the chromophore.

ACKNOWLEDGMENT

We are grateful to STFC for access to the ULTRA laser facility.

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