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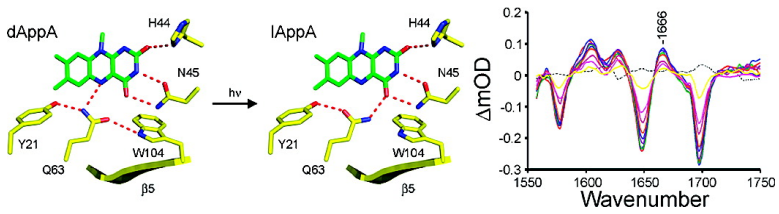

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Ultrafast Structural Dynamics in BLUF Domains: Transient Infrared Spectroscopy of AppA and Its Mutants

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Abstract: The structural dynamics following photoexcitation of a photosensing BLUF (blue light sensing using FAD) domain protein have been investigated by ultrafast transient infrared spectroscopy. Specifically, the transcriptional antirepressor AppA from *Rhodobacter sphaeroides* has been studied in the light and dark adapted forms and in photoactive and inactive mutants W104F and Q63L. A transient absorption has been observed at 1666 cm^{-1} which is a marker mode for the photoactive state of the protein. This instantaneously formed transient is tentatively assigned to a vibrational mode of a protein residue modified through its interaction with the excited state of the chromophore. A plausible candidate consistent with the mutant studies is the carbonyl stretch of the Q63 amide side chain. These results suggest that modification of the strength of protein chromophore H-bonded interactions is the primary step in the BLUF domain photocycle. No new species were observed to be formed during the first nanosecond. Measurement of the ultrafast ground state recovery showed that the excited state of light adapted AppA is strongly quenched compared to the dark adapted state. It is proposed that the reorganization which occurs to form the signaling state is favorable to electron-transfer quenching.

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 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

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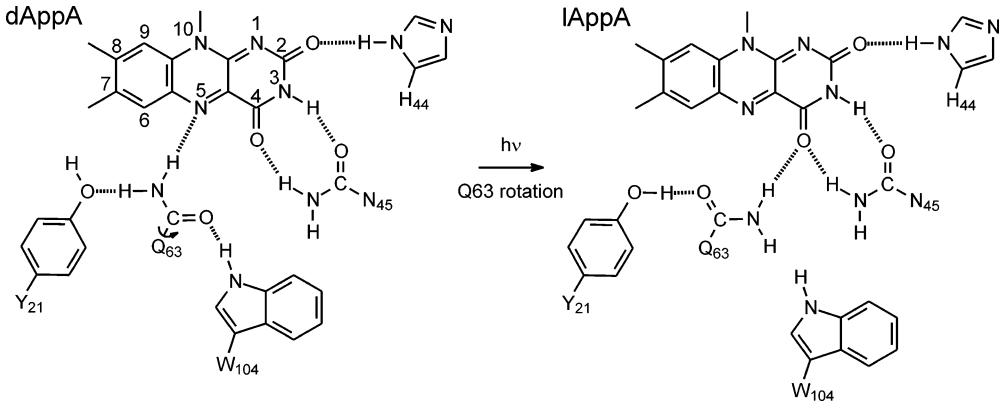

Figure 1. Flavin AppA Interactions for dAppA and lAppA. The structure of the active site of the AppA, for both the dark (dAppA) and proposed light (lAppA) adapted forms. Interactions shown in the figure are based on the crystal structure determined by Anderson et al. (1YRX.pdb)[10]. For clarity, only the FAD isoalloxazine ring is shown. The atom numbering scheme used throughout is also shown.

are the subject of the present study, in which ultrafast time-resolved infrared (TRIR) spectroscopy is used to both record kinetic data and provide structural information at the level of vibrational spectroscopy.

The structural changes accompanying photoexcitation of the AppA BLUF domain have previously been probed by X-ray crystallography,[9−11] NMR spectroscopy,[12,13] and vibrational spectroscopy.[14−16] These studies resulted in a model for the light induced structural transformation of AppA involving rearrangement of amino acid side chains in the vicinity of the flavin chromophore particularly Y21, Q63, and W104 (Figure 1). Photoexcitation of dark adapted AppA (dAppA), which has absorption bands at 377 and 445 nm, results in formation of the signaling form of AppA (light adapted AppA, lAppA) characterized by a red-shifted flavin spectrum with absorption bands at 384 and 462 nm.[8] Ultrafast electronic spectroscopy has shown that the red-shifted form of the protein is generated from the FAD singlet excited state within 1 ns with a quantum yield of 0.24[17] and thermally relaxes back to dAppA over a period of about 30 min.[8]

Photoexcitation must result in a conformational change in AppA, and a model has been proposed in which Q63 rotates and forms a hydrogen bond with the flavin C4═O carbonyl.[10] The X-ray structure of dAppA indicates that the flavin interacts with the protein through several contacts including hydrogen bond interactions involving C2═O, C4═O, N3, and N5 (Figure 1).[10] The flavin C2═O is hydrogen bonded to the imidazole side chain of H44, while the C4═O is hydrogen bonded to the side chain of N45 which also interacts with the flavin N3. Finally, the flavin N5 accepts a hydrogen bond from the amide side chain of Q63 which also donates a hydrogen bond to Y21. The hydrogen-bonding network involving Q63 is completed by an interaction between the amide carbonyl of Q63 and the indole nitrogen of W104.[10] Photoexcitation from this ground state is proposed to result ultimately in rotation of the Q63 side chain so that the amide NH_2 group is hydrogen bonded to the flavin C4═O and the amide C═O accepts a hydrogen bond from Y21 (Figure 1). Loss of the hydrogen bond with W104 triggers the conformational change in the protein leading to the signaling state.

This structural model is broadly in agreement with existing steady-state spectroscopic data. NMR studies show (i) appearance of a downfield resonance in lAppA assigned to the Y21 OH proton[13] and (ii) loss of the resonance assigned to the Q63 amide NH_2 group in dAppA, consistent with rotation of Q63.[12] Formation of a second hydrogen bond to the C4═O in lAppA is in agreement with the difference IR[14,16] and Raman studies[15] that show an ∼12 cm^{-1} decrease in the position of a band at 1710 cm^{-1} in dAppA assigned to the C4═O stretching vibration.

X-ray structural studies on the C20S mutant of AppA have resulted in an alternative model in which M106 interacts with Q63 in place of W104.[9] While broadly similar to the dAppA structure solved by Anderson et al.,[10] the subtle differences observed between the two crystallographic dark states support the importance of β-strand 5 (residues W104-L108) in transmitting the initial photochemical event to the remainder of the protein. The importance of W104, as well as Q63 and Y21, is clearly shown by site-directed mutagenesis. While mutagenesis of Q63 or Y21 results in proteins that are photoinactive,[14,18,19] replacement of W104 with Ala results in a protein that relaxes back to dAppA ∼150-fold faster than the wild-type AppA while also eliminating bands in the difference IR ascribed to changes in β-sheet structure caused by photoexcitation.[20]

The dynamics of the phototransformation in AppA can be addressed by ultrafast spectroscopy. Transient electronic spectroscopy has already shown that the signaling state is formed within 1 ns[17] and has recently led to the proposal that the structural changes are mediated by an electron-transfer reaction

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involving formation of the FAD radical anion.[21] In the present study, we have used ultrafast time-resolved infrared (TRIR) spectroscopy to probe the initial structural events following light absorption in AppA in more detail. Ultrafast time-resolved vibrational spectra have been obtained in dark (dAppA) and light adapted (lAppA) protein and in photoactive (W104F) and inactive (Q63L) mutants. Distinct differences related to the potential for photoactivity have been uncovered in both the spectra and the kinetics. In particular, a promptly formed transient marker mode for the photoactivatable state has been identified, and a tentative assignment to a prompt modification of the FAD−protein H-bonding has been made. No additional states were resolved within the 1 ns time window investigated.

Experimental Section

The BLUF domain (residues 5−125) of AppA was amplified by PCR using the upstream primer AppaNdeIF: 5′ GCG *CAT ATG* CTC GAG GCG GAC GTC ACG 3′ and the downstream primer AppaBamHIR 5′-CGC *GGA TCC* CTA CTG CCG GCT CTC GGC-3′ (restriction sites in italics). The amplified PCR product was digested with NdeI and BamHI (Invitrogen) and ligated into the Nde1/BamHI restriction sites of the plasmid pET15b so that a His_6-tag was encoded at the C terminus of the protein. Site directed mutagenesis was performed using Herculase (Stratagene). Primer sequences for W104F were 5′-CGC CGC TTT GCG GGA TTT CAC ATG CAG CTC TCC-3′ (forward) and 5′-GGA GAG CTG CAT GTG AAA TCC CGC AAA GCG GCG-3′ (reverse); primer sequences for Q63L were 5′-GGC GTC TTC TTC CTC TGG CTC GAA GGC-3′(forward) and 5′-GCC TTC GAG CCA GAG GAA GAC GCC-3′ (reverse). All constructs were verified by DNA sequencing.

Wild-type and mutant AppA proteins were expressed and purified according to standard procedures.[9, 16] BL21DE3 *E. coli* cells transformed with plasmids containing the wild-type BLUF domain (residues 5 − 125), or the mutants Q63L and W104F, were grown in 1 L of LB medium (4 L flask) containing 0.5 mM ampicillin at 30 °C for 4 h until an OD600 was reached. Protein expression was then induced by addition of 0.8 mM IPTG followed by incubation at 18 °C overnight in the dark. Cells were harvested by centrifugation and resuspended in 30 mL of lysis buffer (50 mM sodium phosphate, 150 mM NaCl, pH 8.0) to which 15 μL of the protease inhibitor PMSF (50 mM stock solution in ethanol) had been added. After lysing the cells by sonication, cell debris was removed by centrifugation (33 K, 1.5 h) and the supernatant was allowed to incubate on ice in the dark with a molar excess of FAD (Sigma Aldrich) for 1 h to ensure a homogeneous population of protein bound chromophore.[22] Subsequently, AppA was purified by Ni-NTA chromatography (Qiagen), using 30 to 40 mM imidazole buffer (50 mM sodium phosphate, 200 mM NaCl, pH 8) as the wash buffer and 250 mM imidazole in the same buffer to elute the protein from the column. Column fractions containing protein were combined and dialyzed overnight in the dark against 10 mM Tris-HCl pH 8 buffer containing 150 mM NaCl. Protein purity was assessed by SDS-PAGE electrophoresis and UV−vis spectroscopy (protein, $\epsilon_{270} = 35\,800\ M^{-1}\ cm^{-1}$; FAD, $\epsilon_{446} = 8500\ M^{-1}\ cm^{-1}$). Chromophore content was determined by the ratio of protein to FAD absorbance (∼4.2 for wild-type AppA with FAD bound).[23] Wild-type AppA and the mutants were concentrated to ∼1 mM, frozen in liquid N_2, and lyophilized overnight. Proteins were then reconstituted with deuterated buffer (10 mM Tris-HCl, 1 mM NaCl, pD 8), allowed to exchange for ∼5 h in the dark at 4 °C, frozen in liquid N_2, and lyophilized overnight. The protein samples were subjected to at least 4 to 5 rounds of exchange into the deuterated buffer and were then stored at −20 °C.

Steady-state absorption spectra were recorded at 25 °C on a Varian Cary 300 UV−vis spectrometer. Photoconversion experiments were performed with a 500 W Oriel XeHg lamp using a 405 nm band-pass filter. Typically, 40 μM Appa in 10 mM Tris-HCl, 1 mM NaCl, pH 8 buffer was exposed to up to 40 mW of 400 nm light in a 1 cm cuvette for 3 min. Steady-state IR spectra were recorded at 25 °C using a BioRAD FTS-40A FTIR spectrometer. For the steady-state IR difference experiments, AppA was exchanged into pD 8 D_2O buffer containing 10 mM Tris-HCl and 1 mM NaCl and then concentrated using a centricon 10. Subsequently, 50 μL of 2.5 mM protein were placed in a CaF_2 cell equipped with a 50 μm spacer, and 32 scans were accumulated at a resolution of 4 cm^{-1}. To generate the IR difference spectrum of light minus dark AppA, the protein sample was photoconverted in situ with 3 min of irradiation from the lamp described above, and the spectrum obtained before irradiation was subtracted from that obtained following photoconversion.

Ultrafast time-resolved IR spectra were measured at the STFC Central Laser Facility. A description of the system and data collection methods has been published.[24, 25] The laser system comprises an amplified titanium sapphire laser operating at a 1 kHz repetition rate, pumping a pair of optical parametric amplifiers. The linearly polarized pump beam was at 400 nm (second harmonic of Ti: sapphire). The 150 fs pulse width allowed for roughly 300 fs time resolution. The spot size and power of the pulse were adjusted so as to prevent photobleaching of the sample while retaining good signal-to-noise in the spectra (see below). The pump beam was chopped at 500 Hz and delayed with respect to the IR probe pulse by means of an optical delay line. Typical settings included a 200 μm spot size and energies invariably less than 1 μJ per pulse. Probe radiation in the IR region was generated by difference frequency mixing of the OPA output in a $AgGaS_2$ crystal. Transmitted probe and reference spectra were collected on matched MCT detectors (each 2 × 32 element arrays) in spectral regions approximately 150 cm^{-1} in width. Samples were contained in cells with CaF_2 windows separated by spacers of 25 μm. Measurements typically consisted of spectra recorded at 3−5 randomly ordered time delays, with a 10 s collection period for each delay. Typically eight samples were measured for each spectrum presented, and the results were averaged.

In most measurements the relative angle between the pump and probe beams was set to the magic angle (54.7°) to suppress the effect of orientational relaxation. However, additional data may be obtained from polarization resolved measurements. The angle between the electronic and vibrational transition moments (θ) was determined by exciting the samples with 400 nm radiation polarized parallel or perpendicular to the linearly polarized IR probe. In either case the measured pump-on minus pump-off IR data were combined to generate the transient IR difference spectra at each delay time. For parallel and perpendicular polarization resolved data θ is obtained from the anisotropy, D, defined as the ratio of parallel to perpendicular intensity in the TRIR difference spectra

$$D = \Delta A_{||}/\Delta A_{\perp}$$ [26]

via the relation

$$\cos\theta = \left(\frac{2D-1}{D+2}\right)^{1/2}$$

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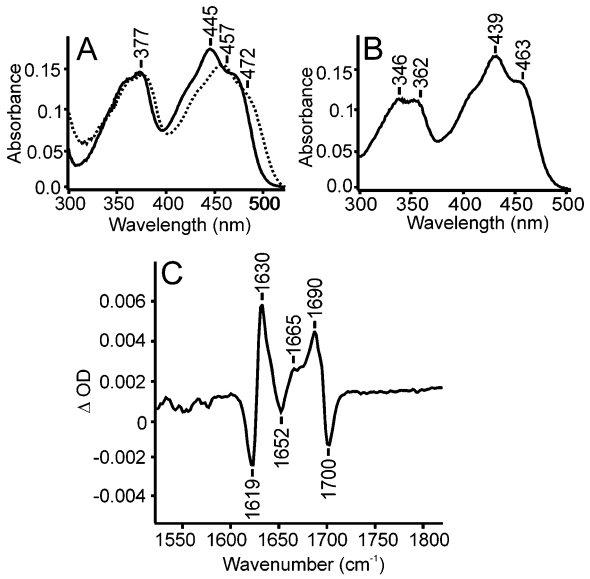

Figure 2. UV−visible absorption and FTIR difference spectra of wild-type and mutant AppA. Steady-state absorption spectra for (A) wild-type AppA (dark, solid; irradiated, dashed) and (B) AppaQ63L. (C) The steady-state IR difference spectrum of wild-type AppA (light minus dark) in pD 8 buffer. IR spectra were obtained using 2.5 mM AppA in a 50 μm CaF_2 cell. The difference spectrum was obtained by subtracting the IR spectrum of AppA acquired before irradiation from that acquired after 3 min of irradiation with a 500 W XeHg lamp.

The assumption is that the protein itself does not rotate significantly between excitation and probing (which is reasonable on the picosecond time scale of the measurements reported here). Thus, for angles θ varying from 0 to 90° D may take values between 0.5 and 3. The calculation cannot distinguish between θ and $\theta + 90°$ so two solutions exist. The electronic transition dipole moment of the isoalloxazine ring has been shown to lie approximately along a line connecting C4 and N9. The application of the polarization method has been discussed in more detail elsewhere.[27]

The pixel to wavenumber conversion was based on the IR spectrum of water vapor. The reproducibility of spectral position was $\pm$ 3 cm^{-1} and the absolute accuracy is estimated as $\pm$5 cm^{-1}.

Considerable care was taken to avoid photobleaching of the samples; all samples were raster scanned over an ~1 cm^2 area during data accumulation. For dAppA the sample with a concentration of ca. 1 mM was additionally placed in a low volume flow cell. This sample could be irradiated with the intensity described above for extended periods without any measurable change in the transmission spectrum. For the W104F and Q63L mutants, which were available only in lower volumes, the sample cell was rastered and kept under irradiation for only several tens of seconds. In that case it was established that much less than 5% of the original sample had been photoconverted in any one measurement. After this brief period of irradiation the sample was replaced with an identical fresh cell, and the measurement continued while the original sample recovered in the dark. In this way sufficient data for the high signal-to-noise spectra shown below were accumulated.

Results and Discussion

Figure 2 shows the steady-state absorption spectrum of wild-type AppA obtained before and after irradiation with 400 nm light (15 to 25 mW, 3 min), as well as the spectrum of the photoinactive mutant Q63L. The wild type protein exhibits a 12 nm red shift upon blue light absorption (445 to 457 nm), and relaxes back to the 445 nm form in ~30 min in agreement

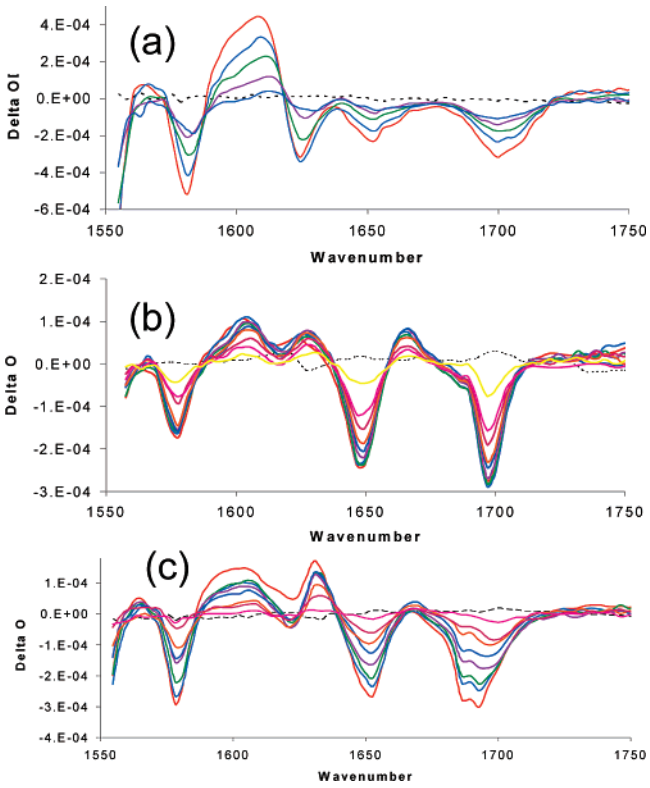

Figure 3. TRIR spectra of dAppA, lAppA, and FAD. (a) TRIR spectra of FAD in D_2O measured with delay times of 1 (red), 5 (blue), 10 (green), 20 (violet), and 50 ps (indigo) after excitation. (b) TRIR for dAppA recorded 1 (red), 5 (blue), 10 (green), 20 (violet), 50 (indigo), 100 (orange), 300 (plum), 500 (pink), and 2000 ps (yellow) after excitation. Note that data for 1, 5, 10 ps are overlapped indicating the slow initial decay. (c) TRIR in lAppA 1 (red), 5 (blue), 8 (green), 15 (violet), 20 (indigo), 50 (orange), 200 (plum), and 1000 ps (pink) after excitation.

with previous observations.[8] In contrast, the absorption spectrum of Q63L (Figure 2B) shows no red shift upon illumination and is blue-shifted relative to wild-type AppA (439 nm compared to 445 nm). Again these data are in accord with existing literature.[19] Finally, the spectra of the photoactive mutant W104F, obtained before and after irradiation, were observed to be very similar to the corresponding spectra of the wild-type protein (data not shown). Thus, the characteristic red shift associated with formation of the signaling state is apparent for wild-type AppA but is absent for Q63L. The W104 mutant, while photoactive, has an increased rate of recovery to its dark state, again in agreement with earlier studies.[20] Also shown (Figure 2c) is the steady-state FTIR difference spectrum of wild-type AppA (light minus dark). Two prominent bands are observed with the characteristic shape associated with a spectral shift between light and dark states. The higher wavenumber mode around 1695 cm^{-1} is ascribed to a shift of C4═O, consistent with increased hydrogen bonding, as previously observed by Masuda.[16] Shifts at lower wavenumber (around 1625 and 1658 cm^{-1}) were assigned to modifications of protein rather than chromophore modes,[16] although chromophore modes may also contribute in this region (see below).

In Figure 3 the TRIR spectra for dark and light adapted AppA are compared with the corresponding TRIR spectra for FAD in D_2O.[27] The TRIR spectra are difference spectra generated from sequential pump-on minus pump-off IR transmission measurements at a given time delay. Thus negative OD features indicate loss of absorption (bleaching) after excitation by the pump pulse,

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while positive features indicate the generation of states not present in the ground state. The kinetics of each state can then be determined from the dependence of the magnitude of the change in OD on the delay time between pump and probe pulses. In the spectral region investigated four main bleaches are observed, all of which appear promptly on excitation. Hence these transitions can be assigned to ground state modes of the FAD chromophore in its local environment. The assignment of these bands for FAD in D_2O was discussed in our earlier paper, on the basis of TRIR polarization spectroscopy and DFT calculations.[27] The highest wavenumber mode (1703 cm^{-1}) corresponds to the normal mode involving the C4═O carbonyl stretch. The next band at 1650 cm^{-1}, which is somewhat asymmetric, is assigned to the C2═O carbonyl. Both of these modes also involve a substantial contribution from the N3H-(D) bend.[27,28] The next highest mode (1624 cm^{-1}), which is somewhat overlapped by a transient bleach, was tentatively assigned to a ring I mode and a C4aN5 stretch. The remaining mode in Figure 3a at 1580 cm^{-1} is mainly due to the C4aN5 stretch, which is again overlapped with the transient absorption. There is in addition a much more intense mode at 1546 cm^{-1} which includes a contribution from C10aN1 stretch.[27] These assignments are in broad agreement with earlier Raman and IR data.[28,29] In addition to these bleaches, a broad transient absorption is observed in the 1580 cm^{-1} to 1640 cm^{-1} region of FAD. This absorption, which is also formed promptly on excitation, is most likely due to carbonyl modes in the excited electronic state, an assignment consistent with the observed polarization dependence.[27]

There are striking differences between the TRIR spectra of FAD in D_2O and FAD bound in dAppA (Figure 3b). First, the ground state bleach transitions which develop promptly (within the excitation pulse) are appreciably sharper in dAppA, although they are not shifted from the aqueous solution frequency by more than 3 cm^{-1} (approximately the spectral resolution of our instrument). This spectral narrowing is ascribed to the existence of a range of static (inhomogeneous) and dynamic broadening processes for FAD in solution, arising from multiple H-bond structures in D_2O which may exchange on an ultrafast time scale. In contrast, in AppA the chromophore interacts with the protein through a number of specific H-bond interactions, most notably between O2 and H44, O4 and D45, and N5 and Q63 (Figure 1).[10] It is suggested that this reduced range of H-bond geometries leads to the spectral narrowing observed. As a consequence of these narrower lines, a shoulder on the low-energy side of the C4═O bleach at about 1690 cm^{-1} is observable in dAppA which is not resolved for FAD in D_2O, or predicted by the DFT calculations. This structure will be discussed further below. Second, a new transient absorption is observed in dAppA at 1666 cm^{-1}, which is absent in FAD. This new transient reaches its maximum amplitude within 10 ps and is therefore characteristic of electronically excited FAD in the dAppA protein environment. The spectral shape in the present energy range does not evolve as a function of time, as evidenced by the observation that early time (5 ps) minus late time (1000 ps) difference spectra recover the baseline over the entire spectral range when the late time spectrum is multiplied by a normalization factor (data not shown). Thus the most distinct difference between FAD and dAppA is the appearance of the 1666 cm^{-1} transient absorption. No new species are observed to evolve from the excited dAppA in the present 1 ns time frame.

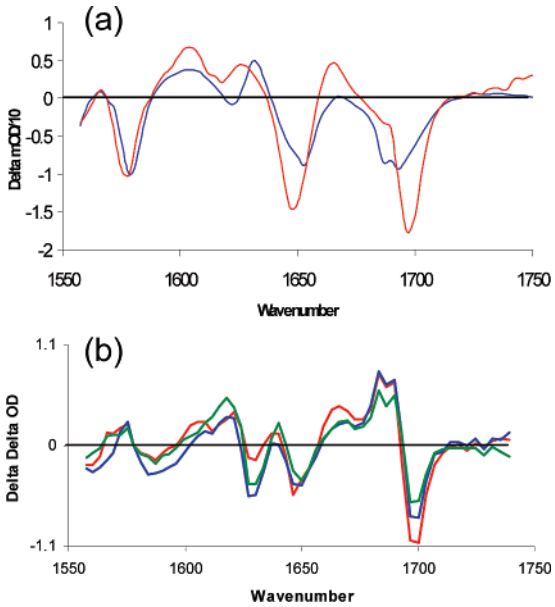

Figure 4. TRIR spectra of dAppA and lAppA after 5 ps. (a) A comparison of TRIR spectra of dAppA (red) and lAppA (blue) recorded 5 ps after excitation. (b) Difference TRIR spectra ((dAppA − lAppA) 5 (red), 20 (blue), and 50 ps (green) after excitation.

The TRIR spectra for lAppA are themselves significantly different to those of dAppA (Figure 3c). This difference is highlighted in Figure 4, where the TRIR spectra recorded for both samples measured 5 ps after excitation are compared, along with a time-resolved lAppA - dAppA difference TRIR spectrum. The latter spectrum is comparable to the steady-state IR difference spectrum (Figure 2c) but will not reflect spectral evolution in dAppA for long times after excitation. It is interesting to note the absence in Figure 4 of the intense difference band around 1625 cm^{-1} (Figure 2c) suggesting the assignment of this mode to a slow (nanosecond or longer) evolution in the protein spectra.

In Figures 3c and 4 it can be seen that the C4═O bleach has shifted to lower frequency in lAppA and developed a broader more complex profile. The weak shoulder noted for this mode at 1690 cm^{-1} in dAppA has increased in intensity relative to C4═O, so that the highest frequency band has developed into a doublet in lAppA. A doublet was previously reported for this mode in the IR spectrum of solid lumiflavin by Abe et al.[28,29] and was proposed to arise from a Fermi resonance of C4═O with a combination band involving the N3H deformation mode. This proposal was based on the effect of deuteration or methylation of N3, thus confirmation of the importance of Fermi resonance in lAppA requires TRIR studies with appropriately substituted FAD (an experiment which is planned). Exchange of the flavin N3─H with deuterium is base catalyzed, and the extent to which this position has exchanged with solvent in our AppA samples prepared in D_2O is unknown. The overall shift to lower wavenumber for the C4═O mode on irradiation is consistent with steady-state IR difference spectra (Figure 2c).[14−16] Such a downshift in wavenumber corresponds to a weakening of the C4═O bond on formation of lAppA, which can be ascribed to stronger hydrogen bonding with the protein. The data in Figure 3b and c and Figure 4 are thus consistent

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Table 1. Polarization Resolved Data for d and lAppA Compared to FAD[a]

	D (dAppA)	θ/deg (dAppA)	*D* (lAppA)	θ/deg (lAppA)	*D* (FAD)	θ/deg (FAD)
C4═O	1.0	$57^{-7}{}_{+10}$	1.1	$51^{-8}{}_{+10}$	0.6	$74^{-9}{}_{+10}$
C4═O (sh)	0.7	$67^{-8}{}_{+12}$	0.8	$62^{-7}{}_{+12}$		
C2═O	1.8	$34^{-9}{}_{+10}$	1.7	$36^{-10}{}_{-10}$	1.25	$47^{-5}{}_{+8}$
TA (1600)	0.9	$58^{-7}{}_{+10}$	1.1	$51^{-8}{}_{+10}$	1.25	$47^{-5}{}_{+8}$
C4aN5	3.2	0 ± 20	3.5	0 ± 20	3.2	0 ± 20

[a] The ratios *D* and the associated angles θ were calculated as described in the Experimental Section. The modes concerned follow the numbering scheme in Figure 1, but TA (1600) stands for the strong transient absorption at 1600 cm^{-1}. (sh) = shoulder. The errors reported represent an assumed 25% error in the determination of *D*. Repeated measurements fell within this range for all modes investigated.

with both previous assignments of steady-state IR data[14,16] and the structural data which suggest the formation of a new H-bond between O4 and Q63 in lAppA.[10] The third major difference apparent in Figure 3 is that the transient absorption seen at 1666 cm^{-1} in dAppA but not in FAD is also absent in lAppA. This is a particularly significant finding, because it suggests that this excited-state mode is a signature for AppA in its dark adapted photoactivatable form. This mode will be discussed below in connection with the anisotropy data and the TRIR spectra of the AppA mutants.

Further changes are apparent in the light−dark ΔTRIR spectrum (Figure 4b). The intensity of the C2═O bleach near 1650 cm^{-1} is somewhat attenuated in lAppA, which also appears to influence the intensity of the transient absorption around 1600 cm^{-1}, leading to a complex ΔTRIR profile in this region. Thus, this FAD localized mode may contribute to the changes observed in the steady-state IR difference spectrum (Figure 2c) around 1650 cm^{-1}, although these are usually ascribed to protein modes. In contrast, the bleach associated with the C4aN5 stretch at 1581 cm^{-1} is barely affected by irradiation, with at most a small shift to lower frequency being observed.

These assignments have been further investigated with polarization resolved TRIR spectroscopy (Table 1, Figure 5). The measured anisotropy, *D*, yields the angle between the transition dipole moments of the electronic transition moment excited by the visible pump radiation and that of the mode probed by the IR. The two components of the C4═O doublet in dAppA and lAppA have quite distinct polarization behaviors, with the "main" band showing $D > 1$ and the shoulder $D < 1$ in all samples studied (to the extent that this "shoulder" becomes the major band in Figure 5b). Thus although the absolute error in *D* may reach 25%, the variation in the ratio *D*(main)/*D*(shoulder) was much smaller. This result suggests that the doublet reflects the existence of two distinct modes, rather than a Fermi doublet associated with a single transition.

The differences between θ for FAD and l or d AppA deserve comment (Table 1). For the carbonyl localized modes a significant and reproducible difference in the mean θ value is observed (on the order of a 10° to 25° decrease in the protein, most markedly for the C4═O mode). In principle this could reflect a reorientation of the electronic transition moment of FAD on binding. However, no such shift is observed for the C4aN5 mode, which should be as sensitive as the carbonyl modes to such a reorientation. Thus it seems likely that the strong specific H-bonds formed between the isoalloxazine ring and the binding site lead to a reorientation of the vibrational

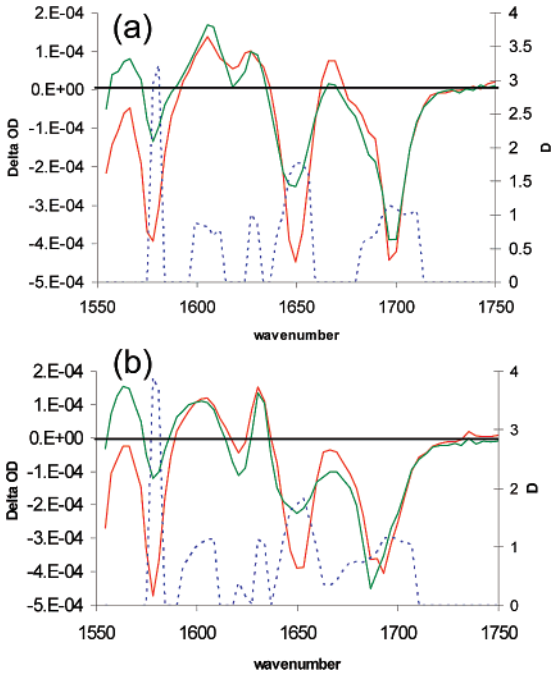


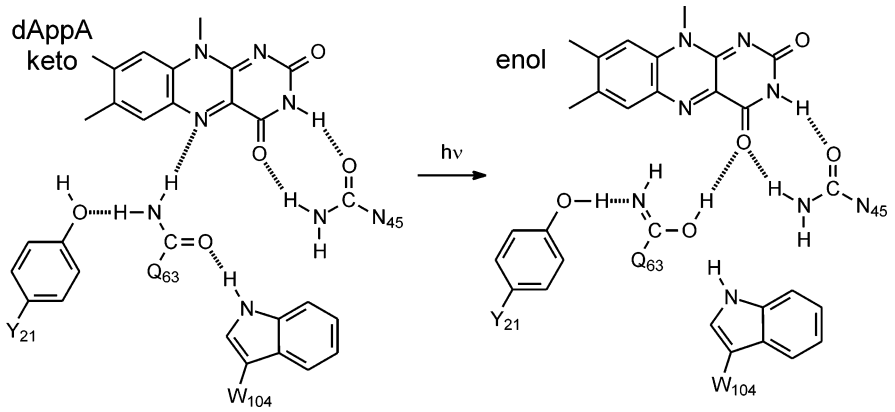
Figure 5. Polarization resolved TRIR spectra. Parallel (red) and perpendicular (green) TRIR for (a) dAppA and (b) lAppA are shown. Also shown (blue) is the anisotropy (*D*).

transition moments of the carbonyl localized mode. The shoulder measured on the C4═O transition has a mean angle 10° larger that that for the main band. It is suggested that this shoulder arises from sites with different H-bonding environments (and thus different θ) to the main band, the population in such sites being increased on irradiation (Figure 3 and 5).

Finally, the transient absorption observed at 1666 cm^{-1} for dAppA but not for FAD in solution or lAppA is a function of polarization, with the transient being most intense for perpendicular pump and probe polarizations (Figure 5). (Unfortunately a meaningful value of *D* cannot be obtained because the ΔOD in parallel polarization is close to zero.) However, this polarization sensitivity does serve to distinguish the 1666 cm^{-1} transient from the C4═O bleach and 1600 cm^{-1} transient, both of which have $D \approx 1$. If the 1666 cm^{-1} transient arose from an excited-state mode of FAD it would have to be assigned to C2═O, which is also strong in perpendicular polarization. This mode would then, unusually, have an increased frequency in the excited electronic state, due to, for example, due to weaker H-bonding. Such a weakening of the H-bonding to C2═O upon excitation may be consistent with the recent structural data which find an increase in the FAD to H44 separation on formation of lAppA. However NMR data lead to the opposite conclusion, with a downfield shift reported for the H44 NH proton, consistent with its involvement in stronger H-bonding to C2═O. Thus this transient, which is characteristic of the photoactivatable form of AppA, cannot easily be assigned to an FAD mode.

An alternative assignment for the 1666 cm^{-1} transient absorption is to a frequency shift in a mode of the protein, caused by electronic excitation of FAD. For example, a change in the proton affinity of N5 or N3 could lead to a shift in frequency of the amide modes of Q63 or N45, respectively. The fact that the transient absorption is only observed for dAppA suggests the assignment to Q63. Such protein modes can certainly be observed in TRIR;[24] however, a corresponding

Scheme 1. Keto−enol Tautomersim of Q63



bleach is then expected but has not been observed in dAppA. One possibility is that the transient absorption in dAppA is indeed associated with a bleach hidden under C4═O, while in lAppA this protein mode is shifted down in frequency to form the new peak at 1686 cm^{-1} and the transient absorption is now obscured by C2═O. However, the different anisotropies of the bleach and transient absorption (Figure 5) argue against this assignment. These spectra might be further resolved by single value decomposition analysis to permit a more detailed assignment, but that will certainly require data of better signal-to-noise than is presently available. The significance of this mode as a marker mode for the photoactivateable state of AppA will be highlighted in consideration of the mutant forms of AppA considered below. Here we discuss possible assignments of the new transient.

Recourse to the extensive literature on IR modes associated with amino acid side chains reveals that the primary amide of glutamine gives rise to a band at 1668 cm^{-1} in H_2O with an extinction coefficient of 377 M^{-1} cm^{-1}.[30] Thus the 1666 cm^{-1} mode that appears in the TRIR of dAppA following photoexcitation is nominally in the correct position for the amide mode of glutamine in H_2O. Using the value 377 M^{-1} cm^{-1}, the intensity of the 1666 cm^{-1} band (0.08 mOD) is equivalent to a Gln concentration of 40 μM. Given the protein concentration of 2 mM and using the upper limit for the quantum efficiency of 25%, the magnitude of the band is similar to that expected assuming that 10% of the molecules are excited per shot ($\approx$50 μM). This level of excitation is consistent with the molar extinction coefficient of flavin (ca. 10^4 M^{-1} cm^{-1}) and the excitation intensity of <1 μJ cm^{-2} focused to a 200 μm spot. Similarly, the intensity of the bleach at 1703 cm^{-1} is 0.28 mOD, about 3.5-fold more intense than the 1666 cm^{-1} band. Assuming that the 1703 cm^{-1} bleach arises solely from the flavin C4═O mode, the difference in intensities of the 1666 and 1703 cm^{-1} bands matches the known difference in extinction coefficients for glutamine (377 M^{-1} cm^{-1}) and the flavin C4═O (1210 M^{-1} cm^{-1}, using uracil as a model for the isoalloxazine ring).[31] These simple calculations suggest that alterations in the ground state population of the flavin are directly coupled to a change in environment of the residue (presumed in this analysis to be Q63) that gives rise to the band at 1666 cm^{-1}.

Two other matters should be addressed when considering the assignment of this band to the primary amide side chain of glutamine. First, this vibrational mode is sensitive to N-deuteration. The amide mode of acetamide in the solid is at 1675 cm^{-1} for $CH_3C(=O)-NH_2$ and 1650 cm^{-1} for $CH_3C(=O)-ND_2$.[32] In addition, the amide mode of *N*-acetyl-glutamine-methyl ester is around 1635 cm^{-1} in D_2O. Since the TRIR experiments are performed on protein samples in D_2O, it is likely that the Q63 side chain has already exchanged with solvent, and thus the 1666 cm^{-1} band in dAppA would have to be assigned to the N-deuterated form of this residue. In turn this would require that the glutamine side chain is less strongly H-bonded in the protein than in solution, and we note that the amide band of N-deuterated acetamide $CH_3C(=O)-ND_2$ is at 1668 cm^{-1} in $CHCl_3$ while the corresponding band for $CH_3C(=O)-NH_2$ is at 1680 cm^{-1}.[32] It should be noted that we do not know the deuteration state of the Q63 side chain. However we can be confident that the extent of deuteration of any exchangeable protons in the protein that would influence the positions of bands in the TRIR spectrum does not change during photocycling, since it was demonstrated that the TRIR spectra themselves do not change during repeated photocycling. It is well-known that a fraction of protons in proteins are protected from solvent exchange by their environment; however the flavin chromophore binds only relatively weakly to the protein so it is unlikely that the Q63 amide group is sequestered from solvent.

A second mechanism that should be considered when discussing light induced structural changes associated with primary amides concerns the possibility for keto−enol tautomerism of the amide group. For acetamide in an argon matrix this results in replacement of the keto C═O at 1727 cm^{-1} with bands at 1681 and 1676 cm^{-1} arising from the C═N stretch of two enol isomers of acetimidic acid ($CH_3(C-OH)=NH$).[33] Although the most stable enol tautomer of acetamide is 50 kJ mol^{-1} less stable than the keto form,[33] it is plausible that the enol could be stabilized by interactions with the AppA and the isoalloxazine ring. Keto−enol tautomerism raises the intriguing possibility that changes in H-bonding associated with the Q63 side chain could arise without formal rotation of the group, since tautomerization will alter the H-bond donor and acceptor ability of the C═O and NH_2 groups (Scheme 1).

Returning to the TRIR data further assignment of the 1666 cm^{-1} band will have to await experiments utilizing isotopically labeled protein. Such experiments, which have been shown to

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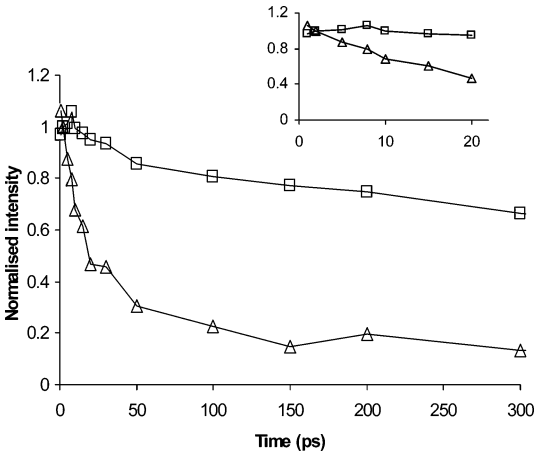

Figure 6. Ground state recovery kinetics for wild-type AppA. Ground state recovery kinetics measured at the C4═O mode of dAppA (□) and lAppA (△). Inset shows early time data.

be useful in unravelling the complex TRIR of the green fluorescent protein, are planned for AppA.

Further differences between lAppA and dAppA are apparent in the kinetic data (Figure 6). The temporal recovery of the bleach modes are a direct measure of the ground state recovery kinetics following electronic excitation. These data show that lAppA recovers its ground state very much more rapidly than dAppA. The dAppA bleach is instantaneous and remains approximately constant for the first 5 ps (see inset to Figure 6) and then the ground state recovers with a nonexponential time profile on the time scale of >1ns, which is consistent with the time-resolved fluorescence and transient absorption measurements of Gauden et al.[17] The lack of relaxation over the first few picoseconds may reflect vibrational cooling in the S_1 state occurring in a few picoseconds prior to the onset of ground state recovery (there is an excess energy of >2500 cm^{-1} following excitation at 400 nm). This assignment is supported by the observation of a small narrowing and shift to higher wavenumber of the transient at 1600 cm^{-1}. This takes place during the first 5−10 ps, along with the absence of ground state recovery. This behavior is characteristic of vibrational cooling in the S_1 state and also consistent with the report of rapid (1−2 ps) internal conversion by Gauden et al.[17] The quantum yield of signaling state (lAppA) formation on excitation of dAppA has been measured as 24% with an additional 9% populating the triplet state.[17] The data in Figure 6 suggest that approximately 80% of the ground state has been repopulated in 2 ns, the longest time accessible in the present experiment. This is qualitatively consistent with the quantum yield data but quantitatively suggests that the product yields determined represent upper limits.

The origin of the rapid ground state recovery in lAppA is not clear, although a similar result has been reported previously in time-resolved fluorescence experiments.[17,34,35] In aqueous solution FAD itself exhibits a rapid ground state recovery which is due to a quenching interaction between the excited isoalloxazine ring and the adenine. If the adenine ring is absent (as in flavin mononucleotide and riboflavin) the lifetime is in the nanosecond range. Thus the long lifetime in AppA shows that the isoalloxazine ring of FAD in AppA is bound in such a way as to restrict access to the adenine ring. The structural changes reported between dAppA and lAppa are not so large as to allow access to the adenine group leading to the quenching observed. In addition the spectral shift between dAppA and lAppA is not so large as to suggest a major perturbation of the electronic or nuclear structure of FAD sufficient to promote rapid internal conversion. On the other hand flavins are known to be susceptible to electron-transfer quenching by tyrosine and tryptophan. In AppA, Tyr21 has been suggested to be capable of charge-transfer quenching of the excited state.[18,35] The observation of quenching in lAppA suggests that accompanying the H-bond and structural rearrangement which occur after photoexcitation there is also a translational or orientational rearrangement of the chromophore−Tyr21/Trp104 geometry such that FAD is more accessible to charge-transfer quenching by these residues. Such a reorganization may be sufficient to promote the charge-transfer quenching. Although the reorganization is not large, the mechanism is plausible because the rate constant for electron-transfer reactions exhibits a very strong (exponential) dependence on distance and is sensitive to donor acceptor orientation.[36] Consideration of the published structure of d and lAppA suggests that Trp104 has moved away from the FAD, whle Tyr 21 may be closer. In addition the lAppA state may be more accessible to the aqueous environment, which can be an important factor in promoting the rate of Tyr−flavin electron-transfer reactions.[37]

Electron-transfer quenching is however expected to lead to ionized products, which will have distinct vibrational spectra; no such product modes have been observed. The absence of product modes does not rule out the charge-transfer mechanism. One possibility is that the charge separated state is not stabilized in the protein and that reverse charge transfer to reform the ground state occurs on such a fast time scale that the intermediate population is insignificant. Alternatively, it is possible that the modes associated with the products are absent because they are too weak or at frequencies outside the window studied.

Finally we note that the kinetic and spectral data contain evidence of an inhomogeneous site distribution for FAD in lAppA (Figure 3c). The rapid ground state recovery reveals a minor slowly recovering population with a C4═O mode shifted to higher wavenumber, close to that of C4═O in dAppA. This is assigned to a fraction of the initial dAppA population which could not be photoconverted. This could either arise from a photoinactive initial population in dAppA or from an inhomogeneous distribution of FAD sites following photobleaching. In the latter case the high wavenumber C4═O mode correlates with an FAD site inaccessible to the quenching mechanism which dominates the lAppA kinetics.

Next we consider TRIR spectra for the photoinactive (Q63L) and photoactivatable (W104F) mutants which are shown in Figure 7. The TRIR spectra for Q63L are remarkably similar to those for FAD in D_2O (Figure 3), although the ground state recovery is slower. Significantly, this photoinactive mutant does not have the transient absorption around 1666 cm^{-1}, which is further evidence that this mode is characteristic of the photo-

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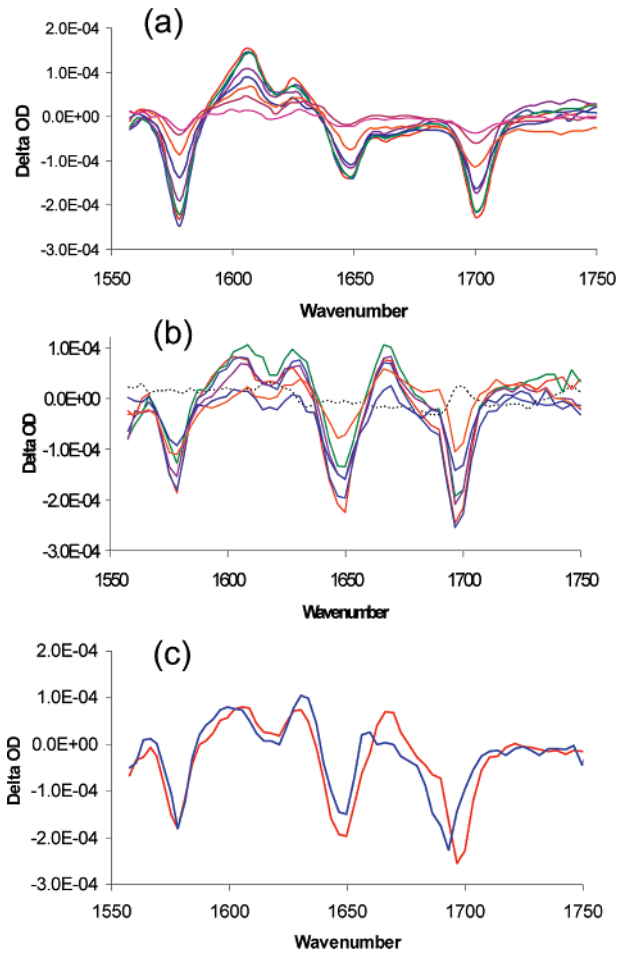

Figure 7. TRIR Spectra of Q63L and W104F. (a) TRIR spectra of the photoinactive Q63L mutant recorded 5 (red), 10 (blue), 20 (green), 50 (violet), 100 (indigo), 200 (orange), 500 (plum), and 1000 ps (pink) after excitation. (b) TRIR for the W104F mutant 2 (red), 5 (blue), 20 (green), 100 (violet), 500 (indigo), and 1000 ps (orange) after excitation. (c) A comparison of the TRIR spectra 5 ps after excitation (red dark adapted, blue light adapted).

activatable state, and reflects a modification of the Q63 C═O stretch on photoexcitation of the FAD. This is further supported by the TRIR measurements on the dark adapted W104F mutant, which is capable of forming the lAppA state, and in which the 1666 cm^{-1} transient is observed (Figure 7). The TRIR spectra for the dark and light adapted W104F mutant 5 ps after excitation are compared in Figure 7c. The TRIR associated with these two spectra are quite similar to those which result from dAppA to lAppA photoconversion (Figure 4a). The C4═O mode is shifted to lower wavenumber, consistent with increased involvement in H-bonding with the protein. The spectral profile again becomes more complex, with the weak shoulder in the dark adapted form becoming markedly more significant in the light adapted state. Crucially, in the photoconverted state the transient absorption at 1666 cm^{-1} is absent. Thus the changes observed in the FAD to dAppA to lAppA spectra are reproduced by the Q63L, dW104F, lW104F series, confirming in particular the correlation of the transient absorption at 1666 cm^{-1} with the photoactive state of the protein. Since the changes to the FAD chromophore throughout this series are minor (leading only to the 10 nm red shift reported in Figure 2), we have assigned this marker mode to a perturbation of the protein (probably a glutamine) induced by excitation of FAD. It is proposed to arise because of changes in the H-bonding network following immediately upon excitation, causing a change in the frequency of a C═O mode of an adjacent residue Q63 (or possibly N45). In line with the earlier discussion the changes observed between dAppA and lAppA may reflect a change in the H-bond strength between Q63 and the flavin.

Conclusion

Structural dynamics in the AppA protein have been investigated on an ultrafast time scale through TRIR spectroscopy. Light and dark adapted forms and photoactive and inactive mutants have been investigated. A transient absorption marker mode for proteins capable of forming the signaling state on photon absorption has been identified. This transient, which is formed promptly on excitation, is suggested to arise from an H-bond reorganization in the protein rather than a chromophore mode. It was proposed that electronic excitation of FAD leads to changes in the proton affinity of atoms involved in H-bonding (N5, N3, C2═O). This in turn results in the breaking or disruption of the network of residues involving Q63, N45, and H44. The mutant data confirm the significance of this 1666 cm^{-1} mode and suggest a role for Q63. A more definite assignment will be possible by ^{13}C labeling of the Q in AppA. We have exploited such methods in studies of green fluorescent protein dynamics,[38] and similar experiments for AppA are planned.

A number of other changes in the vibrational spectroscopy between FAD in solution and bound in AppA were observed. Broadly these measurements are consistent with existing steady-state experiments. In addition the TRIR spectra suggest that irradiation leads to a degree of inhomogeneity in the AppA structure, particularly around the C4═O bond. Interestingly none of the spectra revealed the growth of new vibrational modes, suggesting that further changes associated with signaling state formation take place outside the temporal or energy window of these experiments. We plan to extend these ranges in future experiments.

Ground state recovery kinetics were also recorded. Delayed ground state recovery for dAppA in the first few picoseconds was tentatively assigned to relaxation in the upper state. The very rapid quenching of lAppA compared to dAppA was suggested to arise from enhanced electron-transfer quenching of the isoalloxazine ring by an adjacent amino acid, induced through structural changes in the light adapted form.

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