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Light-Induced Flipping of a Conserved Glutamine Sidechain and Its Orientation in the AppA BLUF Domain

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AppA is a transcriptional antirepressor of photosynthesis gene expression in *Rhodobacter sphaeroides* that regulates cellular responses to light and oxygen.[1] It is a blue-light photoreceptor that mediates blue light induced photosynthesis gene repression.[2] The N-terminus of AppA contains a BLUF (blue light photoreceptor utilizing flavin adenine dinucleotide (FAD)) domain (residues 16−108) and binds to flavins.[3,4] Absorption of blue light results in a ∼10 nm red shift of the UV/vis absorbance spectrum.[4] The lifetime of the red-shifted absorbance spectrum for the light-induced signaling state of AppA is among the longest of all known flavin-based photoreceptors (∼1800 s), yet is formed within 1 ns from the singlet-state flavin.[5,6] Emerging evidence from spectroscopic and mutational studies[7−9] suggests that light catalyzes flipping of the conserved Q63 side chain ∼180 degrees to form a strong hydrogen bond between the Q63 side chain amide and the flavin C4═O, altering the electronic configuration of the flavin isoalloxazine ring and giving rise to the observed ∼10 nm red-shift of its UV/vis absorbance spectrum, as well as ∼16 cm^{-1} downshift of the C4═O stretching mode in FTIR spectra. In contradiction to this, the recently reported crystal structure of AppA mutant C20S[10] has been interpreted that in the dark the Q63 side chain is oriented with its carbonyl oxygen near the Y21OH group and its side chain amide close to the flavin O4 (opposite the dark state orientation proposed by Anderson et al.[11]) and that in response to light it flips by ∼180 degrees.[10]

In light of this discrepancy, it is very important to determine which model represents the dark state and light-induced signaling state orientation of Q63 side chain for AppA in solution. Here we present NMR data describing changes in the core of the BLUF domain of AppA in response to blue light illumination supporting the model that, in solution, AppA WT undergoes a flip of the Q63 side chain in response to light to form strong hydrogen bonds between the Q63 side chain amide and the flavin C4═O and between the Q63 side chain carbonyl oxygen and Y21 side chain hydroxyl, as proposed by Anderson et al.[11]

The chemical shift position of the FAD H3 proton was previously unknown and prevented a description of the light-induced changes in the FAD isoalloxazine ring and its immediate environment. We report the assignment of the flavin H3 proton NMR frequency in the dark (11.16 ppm, 290 K) and in the light-induced signaling state of AppA (11.80 ppm, 290 K). Figure 1 shows the downfield shifted region of NMR spectra of the dark state and light-induced signaling state of ^{15}N-labeled AppA. The FAD H3 resonance was previously assigned to the Hδ1 resonance of H44 because it appeared in the HSQC spectrum of AppA (Figure 1, inset). Exchange of the ^{15}N-labeled FAD for unlabeled FAD leaves an identical 1D NMR spectrum (Figure 1a), but because the FAD H3 is attached to ^{14}N, it is not split into a doublet in 1D ^{1}H NMR spectra recorded without decoupling of ^{15}N (Figure 1b), demonstrating the correct assignment.

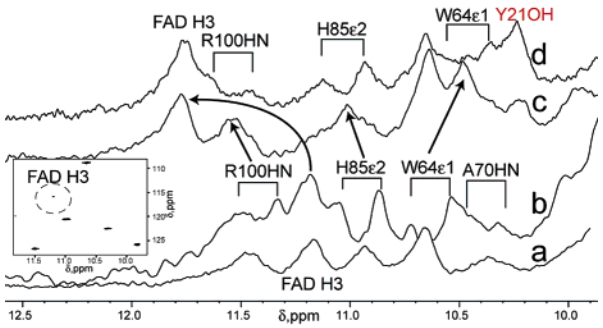

Figure 1. Region of ^{1}H NMR spectra of ^{15}N-labeled AppA5−125 with unlabeled FAD (a) in the dark using ^{15}N decoupling, (b) in the dark and ^{15}N-coupled, (c) illuminated (150 mW, 200 ms per 15 s) and ^{15}N-decoupled, and (d) illuminated and ^{15}N-coupled. All protein NH resonances are split by the $^{1}J_{NH}$ coupling, while the unlabeled FAD H3 resonance is not. The Y21OH resonance at 10.22 ppm is not split and does not appear in HSQC spectra of the light-induced signaling state. Assignments of the resonances in the light-induced signaling state are indicated by arrows. The inset shows the HSQC spectrum of the same protein with ^{15}N-labeled FAD and a visible H3 resonance.

The difference in chemical shift of the H3 proton between the dark state and the light-induced signaling state is the largest observed change for all resonances in the protein ($\Delta\delta$ ∼0.6 ppm). The observed change in chemical shift for the FAD H3 proton resonance can be caused either by a change in the electronic structure of the flavin isoalloxazine ring or by a change in the local chemical environment due to a structural change of the protein. Other photoreceptor proteins such as PYP or LOV domains are known to undergo large structural unfolding transitions in their signaling states,[12,13] but it is thought that AppA undergoes only a small structural perturbation in response to light.[4,14,15] From NOESY spectra in Figure 2, it can be seen that the chemical shifts of dark state NOEs to the flavin H3 proton (S41β, H44ϵ1, and V75γ2) are virtually unchanged in the light. In addition, other resonances in this region of the NOESY spectrum show small chemical shift changes and almost identical NOE patterns. The high degree of similarity of these NOE patterns is consistent with very little overall structural perturbation of the flavin binding core. Therefore, the light-induced change in chemical shift of the FAD H3 proton resonance is most likely caused by changes in the electronic structure of the flavin isoalloxazine ring. Formation of a strong hydrogen bond to the C4═O position in the light-induced signaling state withdraws electron density from the ring structure and deshields the H3 proton and is the most likely cause of the downfield shift observed for the FAD

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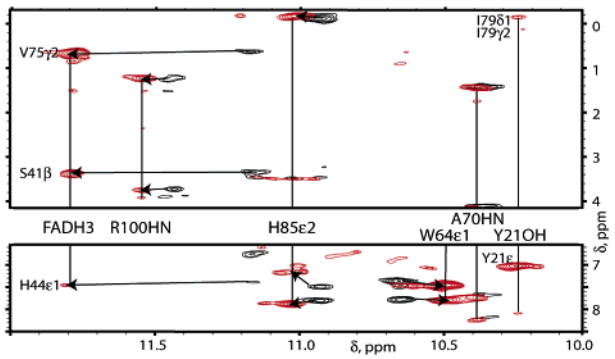

Figure 2. NOESY spectrum of unlabeled AppA5−125 in the dark (black) and light (red). Vertical bars highlight the light state resonances. Additional weak unassigned peaks appear in the light state.

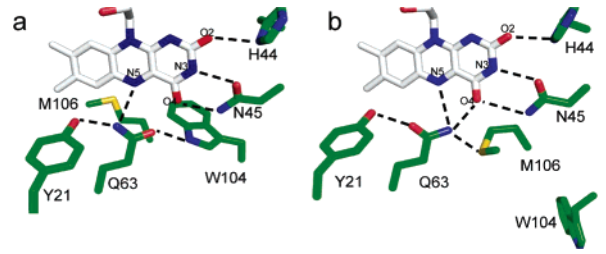

Figure 3. Dark state crystal structures of AppA (a) WT and (b) mutant C20S. The orientation of the Q63 side chain in (b) corresponds to the light-induced state of AppA proposed by Anderson et al.[11] except for the hydrogen bond to M106 (hydrogen bond distances shown as dotted lines).

H3 proton resonance. Our observation supports FTIR data on the light-induced signaling state of AppA[7] and other BLUF domains.[16,17]

The ultrafast kinetics reported for FAD signaling state formation,[5] combined with these NMR observations, are consistent with a small, local change in protein structure such as reorientation of the Q63 side chain. From the dark state crystal structure of AppA WT (Figure 3a),[11] it can be seen that rotation of the side chain of Q63 by ∼180 degrees in the light-induced state would place its side chain carbonyl group in perfect position for making a hydrogen bond with Y21OH (see Figure 3b). In support of this light-induced flip of Q63, a new resonance at 10.22 ppm can be seen in the NOESY spectrum of the light-induced signaling state that arises from the Y21OH proton (Figure 2). Observation of this tyrosine hydroxyl resonance indicates that the hydroxyl proton exists in a very stable hydrogen bonding arrangement that prevents fast exchange of the proton with solvent in the light (see Supporting Information),[18] which is in agreement with the orientation of the Q63 side chain in the light state as proposed by Anderson (see Figure 3b).[11] We do not observe this Y21OH resonance in spectra of the dark state, consistent with the structure in Figure 3a, in which the Y21OH proton exists in a very hydrophobic pocket, and does not have a hydrogen bonding partner available.

Examination of the available structural information on AppA and other BLUF domains also supports our conclusion on the dark state orientation of Q63. The side chain orientation of Q63 in AppA crystal structures was determined by analyzing nearby hydrogen bond donors and acceptors to model the most likely orientation of the amide and carbonyl moieties.[10,11] The structure of AppA mutant C20S in the dark[10] shows the conserved Q63 side chain amide in a hydrogen bonding arrangement with conserved M106 Sδ (Figure 3b). However, the orientation of the Q63 side chain in this structure may be influenced by the C20S mutation and a DTT molecule (present in the crystallization buffer) covalently linked to C19, very near the Y21 and Q63 sidechains. The orientation of Q63 in the C20S mutant structure is opposite that found in the dark state crystal structure of AppA WT,[11] which shows the conserved Q63 side chain carbonyl oxygen in hydrogen bonding arrangement with the indole NH of conserved W104 (Figure 3a) and does not show any covalent adducts. The solution structure of AppA WT in the dark[15] shows that W104 is in the core of the protein, with its indole NH able to hydrogen bond with the Q63 side chain carbonyl oxygen (as in Figure 3a). This orientation is supported by more than 20 separate NOEs (see Supporting Information), demonstrating that, in the dark and in solution, W104 is buried in the core of AppA WT. This dark state orientation of Q63 agrees with available spectroscopic data[8] and is independent experimental confirmation of the orientation of the Q63 side chain in the crystal structure of AppA WT in the dark.[11]

We conclude here that, in the light-induced signaling state of AppA, the structure of the flavin binding core in general is only minimally altered. The observed FAD H3 chemical shift change and NOE patterns provide evidence that the strongest local effect of light-induced signaling state formation is alteration of the electronic structure of the flavin isoalloxazine ring upon formation of a strong hydrogen bond between the flavin C4═O and Q63 side chain amide. Observation of the Y21OH resonance in the light confirms formation of a strong hydrogen bond between the Y21OH and the Q63 side chain carbonyl group. These conclusions are supported by inspection of available crystal and solution structural data, are consistent with the model proposed by Anderson et al. for orientation of the Q63 side chain,[11] and confirm flipping of the Q63 side chain as the likely mechanism of signaling state formation. How this light-induced side chain flip affects the rest of the BLUF domain is currently under study.

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Supporting Information Available: Materials and methods, supplemental results, and discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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