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The Solution Structure of the AppA BLUF Domain: Insight into the Mechanism of Light-Induced Signaling

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The transcriptional antirepressor AppA from the photosynthetic bacterium Rhodobacter sphaeroides *senses both the light climate and the intracellular redox state. Under aerobic conditions in the dark, AppA binds to and thereby blocks the function of PpsR, a transcriptional repressor. Absorption of a blue photon dissociates AppA from PpsR and allows the latter to repress photosynthesis gene expression. The N terminus of AppA contains sequence homology to flavin-containing photoreceptors that belong to the BLUF family. Structural and chemical aspects of signal transduction mediated by AppA are still largely unknown. Here we present NMR studies of the N-terminal flavin-binding BLUF domain of AppA. Its solution structure adopts an α/β-sandwich fold with a $\beta\alpha\beta\beta\alpha\beta\beta$ topology, which represents a new flavin-binding fold. Considerable disorder is observed for residues near the chromophore due to conformational exchange. This disorder is observed both in the dark and in the light-induced signaling state of AppA. Furthermore, we detect light-induced structural changes in a patch of surface residues that provide a structural link between light absorption and signal-transduction events.*

Introduction

Photosynthetic organisms regulate their photosynthesis-related gene expression in response to the ambient light and redox conditions. AppA is a transcriptional antirepressor found in *Rhodobacter sphaeroides* that senses these two signals in the cell and alters the expression of photosynthesis genes accordingly.[1] AppA is a 450-residue blue-light photoreceptor protein that was identified by its ability to mediate blue-light repression of bacteriochlorophyll synthesis.[2] The N terminus of AppA shows sequence homology to other flavin-containing photoreceptors of the BLUF-domain family[3] and binds flavin.[4–6] The C-terminal domain of AppA contains a cysteine motif reminiscent of disulfide-isomerizing proteins.[7] Full-length AppA acts as an antirepressor of photosynthesis gene expression under low-light and anaerobic conditions by binding through its C-terminal domain to the PpsR transcriptional repressor.[8] Once bound to AppA, PpsR can no longer bind to its target DNA sequences.[4] Under high-light conditions, the process is reversed: absorption of blue light by its chromophore causes AppA to dissociate from PpsR, freeing PpsR to bind to DNA. The C-terminal domain of AppA as such can relay redox signals to PpsR; information about the ambient light climate is presumably sequentially relayed from the N-terminal BLUF domain to the C-terminal domain.[8]

The photoactivated (signaling) state of AppA is long-lived ($\tau \sim 30$ min) and has a red-shifted absorption spectrum.[4,9,10] This signaling state, which has one of the longest lifetimes of all known flavin-based photoreceptors, is formed directly from the singlet excited state of flavin adenine dinucleotide (FAD) on an ultrafast (< 1 ns) timescale and without any apparent intermediate being involved.[11] The ultrafast kinetics of signaling-state formation suggest that differences in structure between the dark-adapted state and the light-induced signaling state of AppA are small and closely associated with the flavin, without global unfolding of the protein. Other photoreceptors characterized in the last few years, such as PYP, the LOV domains, and the rhodopsins, display very different photochemistries, with several spectroscopically distinguishable intermediates that gradually reduce the energy of the system until a stable, long-lived signaling state is formed.[12] It is known that all these proteins undergo large structural changes or unfolding events to reach the signaling state.[13–17]

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Secondary-structure prediction based on the primary sequence of AppA reveals no similarity to known flavin-binding proteins or other photoreceptors.[3] Two recent crystal structures, one of AppA [18] and the other of the BLUF domain Tll0078 from *Thermosynechococcus elongatus* BP1,[19] give important clues regarding the structural basis of FAD binding and photochemistry, and demonstrate a novel flavin-binding fold for the BLUF domains. Important questions remain regarding both the structure and dynamics of the dark-adapted state and light-induced changes in the structure and dynamics of AppA. Therefore, we have expressed the N-terminal BLUF domain of AppA (residues 5–125) heterologously, solved its solution structure using NMR spectroscopy, and studied its light-induced signaling state. Our results contribute to the understanding of how light absorption is transmitted into structural changes in this photoreceptor protein and subsequently into a signal-transduction event.

Experimental Section

Plasmid construction: The expression plasmid was constructed by using a ligation-independent cloning procedure based on both the PCR and the vector having cohesive ends.[20] The DNA fragment coding for amino acids 5 to 125 of AppA was amplified by PCR with the sense primer $AppA_5F$: 5′-GCCGCGCGGCAGCCTGCTCGAGGCGGACGTC-3′ and the antisense primer $AppA_{125}R$: 5′-CAAGAAGAACCCCTTACTGCCGGCTCTCGG-3′ by using *Rhodobacter sphaeroides* strain 2.4.1 RK1[21] genomic DNA as a template. Agarose gel-purified DNA was treated with T4 DNA polymerase (0.5 units in a 15 μL volume (33 mM Tris acetate pH 9.0, 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol, and 100 μg mL^{-1} bovine serum albumin)) in the presence of dATP (2.5 mM). After incubation for 30 min at room temperature, the mixture was heated for 15 min at 65 °C and cooled on ice.

The T4 DNA polymerase-treated PCR product was hybridized with vector pLICHIS (linearized vector pLICHIS was a kind gift from Dr. R. N. de Jong), and *Escherichia coli* JM101 was transformed with the DNA. Transformants were checked for the presence of the recombinant plasmids by single colony PCR by using the T7 forward and reverse primers. This cloning procedure resulted in the construct pLIC-$AppA_{5-125}$ expressing amino acids 5–125 of AppA with an N-terminal His-tag followed by a thrombin-cleavage site. The construct was verified by commercial sequencing (BaseClear, Leiden, the Netherlands).

Protein production and purification: Uniformly ^{15}N or ^{13}C,^{15}N-enriched AppA was produced from *Escherichia coli* BL21(DE3) transformed with the pLIC-$AppA_{5-125}$, grown at 37 °C in M9 medium[22] by using $^{15}NH_4Cl$ (0.5 g L^{-1}) and glucose (5 g L^{-1}) or $^{15}NH_4Cl$ (0.5 g L^{-1}) and [^{13}C]-glucose (2 g L^{-1}; Cambridge Isotope Laboratories, Maryland, USA). Protein expression and purification were performed as previously reported.[23] Reconstitution with unlabeled FAD was performed as reported.[6] The purity of the protein was checked by SDS-PAGE by using the PHAST system (Pharmacia) and UV/Vis spectroscopy, and the flavin content of the samples was determined by TLC.[6,24] Protein concentrations were determined by using an extinction coefficient of 8.5 mM^{-1} cm^{-1} at 446 nm for protein-bound FAD.[9]

NMR assignment: Backbone ^1H, ^{15}N and ^{13}C NMR assignments of AppA 5–125 were obtained by using triple-resonance experiments HNCO, HN(CA)CO, HNCA, HNCACB, CBCA(CO)NH, HBHA(CO)NH, HN(CA)HA (for a review see ref. [25]) on a Bruker Avance 700 MHz spectrometer. Side-chain assignments were obtained from 3D (H)CCH COSY and 3D H(C)CH TOCSY experiments[26] on a Bruker Avance 600 MHz spectrometer equipped with a triple-resonance cryoprobe. In H/D-exchange experiments, AppA was lyophilized from an aqueous solution and resuspended in 99.9% D_2O. ^1H,^{15}N HSQC spectra were acquired at 750 MHz and 310 K for 72 h after the exchange. Backbone amide protons that were visible after the first HSQC spectrum in D_2O were allowed for use in hydrogen-bond restraints for the structure calculation. NOESY spectra were recorded on a Bruker Avance 900 MHz spectrometer equipped with a triple-resonance probe. Two 3D heteronuclear-edited NOESY spectra (^1H,^{15}N NOESY-HSQC and 3D ^1H,^{13}C NOESY-HSQC) in H_2O (mixing times 75 and 100 ms, respectively) and three 2D ^1H,^1H NOESY experiments in D_2O (mixing times 50, 100, and 150 ms) were recorded at 900 MHz. Of the three 2D NOESY spectra, only the experiment at 50 ms mixing time was used, because of significant spin diffusion with the longer mixing times. FAD resonances were assigned by using 2D and 3D NOESY and 2D TOCSY spectra. FAD resonances do not appear in the HSQC spectra; this indicates that the chromophore had been completely replaced by unlabeled FAD added during the reconstitution stage.

Light excitation of AppA was achieved by using continuous light input (240 mW) from an argon laser, with a setup previously described.[27] Monitoring the chemical shift of the water resonance confirmed that heating of the NMR sample was negligible under the conditions used. Mainly small chemical-shift changes in the light-induced state of AppA allowed assignment of most of the light-state resonances.

Pulse field gradient NMR experiments: In order to determine the translational diffusion coefficient of AppA, a series of one-dimensional pulse field gradient NMR experiments was recorded on a Bruker Avance 750 MHz spectrometer with a three-axis gradient probe (*x*-axis for the bipolar gradient pulse pair and *y*- and *z*-axes for residual-water-signal crushing) by using modified Pulse Gradient-Stimulated Echo Longitudinal Encode–Decode sequences as described previously.[28] In addition to AppA 5–125, the diffusion coefficients of several proteins with a known three-dimensional structures were measured at 310 K. These were used as references to determine the apparent hydrodynamic radius of AppA 5–125. These include chymotrypsin inhibitor-2 (M_W = 7.3 kDa), hen-egg-white lysozyme (M_W = 14.3 kDa), and lipase from *Pseudomonas mendocina* (M_W = 27.6 kDa). Theoretical translational diffusion coefficients of these proteins at 310 K were calculated by using HYDROPRO.[29]

NMR structure calculations: Initial NMR structures were calculated for AppA 5–125 by using distance restraints generated from four NOESY spectra: 2D ^1H,^1H NOESY in H_2O, 2D ^1H,^1H NOESY in D_2O, 3D ^1H,^{15}N NOESY-HSQC, and 3D ^1H,^{13}C NOESY-HSQC. Dihedral-angle restraints were generated by using Cα, Cβ, CO, $^1H\alpha$, and ^{15}N chemical shifts to search a database of protein structures with the program TALOS.[30] Hydrogen-bond restraints were introduced for amino acids in stable secondary structure elements showing extensive protection from exchange with solvent D_2O. Automated NOESY assignment and NMR structure calculations were performed iteratively by using ARIA 1.2.[31,32] 50 structures were calculated in iterations 1–7, and the lowest in energy were used for automated NOE assignment. A set of manually assigned NOEs was used to facilitate the automated assignment procedure. The resulting set of 2041 unambiguous NOEs, in combination with the dihedral-angle restraints was then used to calculate the final ensemble of structures following the RECOORD structure-calculation protocol.[33] 200

structures were generated, and the 50 lowest NOE energy structures were refined in water. The lowest energy structure was then used as a starting point for a new round of calculations. The resulting 20 lowest energy structures were then analyzed by using PROCHECK and WHATCHECK.[34,35]

Results and Discussion

AppA assignment

All backbone ^{1}H, ^{15}N, and ^{13}C frequencies in AppA 5–125 were assigned by using triple-resonance experiments, with the exception of the six amino acids from M106 to S111. Assignment of backbone proton resonances for residues M106, Q107, L108, and S109 was made by using NOESY spectra. Backbone amide proton resonances for these residues were very weak due to chemical exchange line broadening. More than 94% of the observable side-chain protons were assigned. ^{1}H, ^{15}N, and ^{13}C chemical shift values have been deposited in the BioMagResBank (accession number 6692). An assigned ^{1}H,^{15}N HSQC spectrum is presented in the Supporting Information.

AppA secondary structure

The secondary structure of AppA 5–125 is summarized in Figure 1. AppA contains 5 β-strands for residues 17–24, 51–56, 61–67, 88–95, and 103–108, and 2 α-helices for residues 31–47 and 70–81. The stability of the secondary-structure elements is evidenced by the H/D-exchange data. Strands 1–4 show stable hydrogen bonding. In contrast, strand 5 lacks protection from exchange with D_2O; this indicates that it might undergo conformational fluctuations that allow exchange of its HN protons. Many of the residues in the two α-helices show protection from solvent exchange; this indicates that they are not unfolding transiently.

AppA exists as a dimer in solution

NMR diffusion measurements for AppA 5–125 show a linear relationship between the experimentally derived translational diffusion coefficients and the theoretical ones. When these were compared to the translational diffusion coefficients of standard proteins, a dimeric state for AppA 5–125 in solution was found. The diffusion coefficient was the same for protein concentrations ranging from 0.1 to 1.0 mM; this indicates that AppA 5–125 exists as a dimer under the solution conditions studied. This finding is in agreement with gel-filtration studies of the purified BLUF domain of AppA[9] and our purification scheme, in which AppA has a retention time that is consistent with a molecular weight of 32 kDa and close to the expected value for a dimer.

AppA solution structure

Semiautomated NOE assignments and structure calculations were performed by using a dimer version of ARIA 1.2. Since the resulting set of NOEs could be interpreted solely on the basis of a monomer structure, the final structure calculations and refinement were only performed for the monomer (see Experimental Section). Coordinates for the solution-structure ensemble of AppA 5–125 have been deposited in the PDB under accession code (2bun). The solution structure of AppA 5–125 adopts an α/β-sandwich fold with a βαββαββ topology. In Figure 2 the structure shows a five-stranded β-sheet (four antiparallel strands and one parallel strand), with two antiparallel helices running in the same direction as the strands on top of the sheet. The FAD chromophore binds on top of the β-sheet and between the two α helices, with the ribose, phosphate, and adenine moieties extending out into solution. The flavin-binding pocket is very hydrophobic, with a few polar or charged residues in positions where they make specific contacts with the flavin N5, N3, O4, and O2 atoms. Of the residues making contact with the flavin, almost all are completely conserved within the BLUF domain sequence family. The methyl groups of the flavin ring are packed against the aromatic rings of Y21 and F61 and side chains of L54 and Q63 from the sheet, and against nearby hydrophobic side chains from both helices (L34, I37, V38, I79, R84, and H85). The flavin isoalloxazine

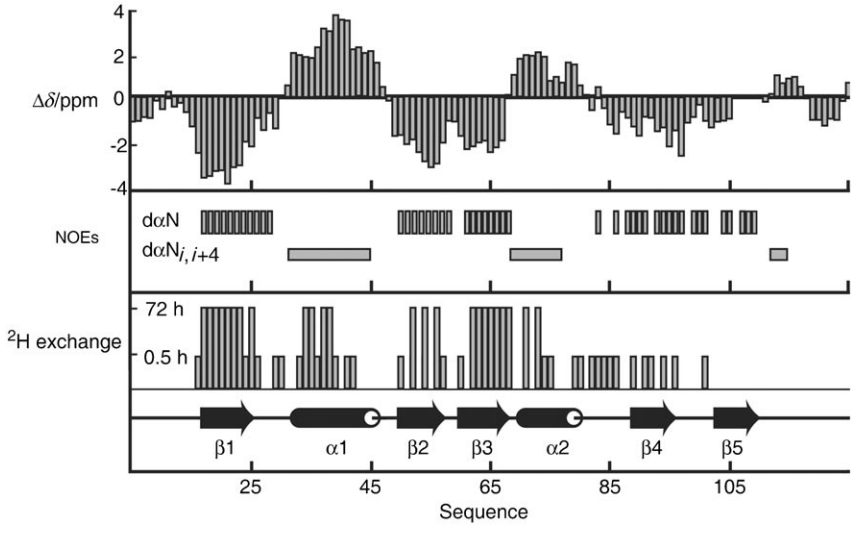

Figure 1. Secondary structure of AppA 5-125. The top panel shows deviation of $^{13}C\alpha$ resonances from random-coil chemical-shift values. The deviation was calculated as a running average of three consecutive residues. The second panel shows NOEs that are diagnostic of β-sheet (dαN) or α-helical (dαN$_{i,i+4}$, supported by dαN$_{i,i+2}$ and dNN$_{i,i+2}$ NOEs) structures. The third panel shows H/D-exchange data, with short bars for resonances detected 30 min after exchange and tall bars for resonances present after three days at 310 K in the dark. A summary of secondary structural elements is depicted at the bottom.

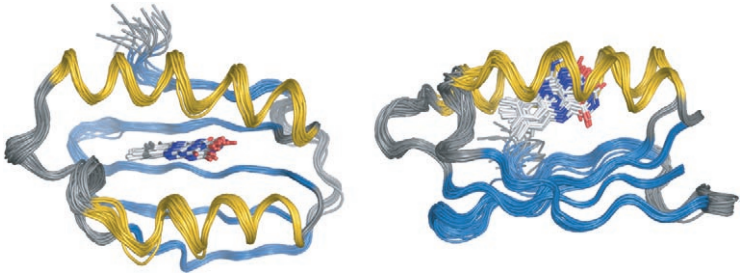

Figure 2. Ensemble of solution structures of AppA, with the flavin chromophore carbons in white, oxygen in red, and nitrogen in blue. α-helices are indicated in yellow, β-sheet in blue and loops in gray.

ring extends along the helices, with the O2 and H3 groups making hydrogen-bond contacts with H44 Hδ1 and N45 Hδ in the solution ensemble.

Structural statistics are presented in Table 1. Heterogeneity of the flavin orientation is reflected in elevated RMSD values for the structure ensemble if FAD is included, although the orientation of the flavin isoalloxazine ring within the core of AppA is generally consistent. Figure 2 shows the ensemble of structures fitted onto the stable structured core of the protein (residues 15–108). The RMSD of the NMR ensemble from the mean structure is 0.45 ± 0.10 Å for backbone atoms and 0.9 ± 0.12 Å for all heavy atoms. If FAD is included in the calculation, the average RMSD increases to 0.58 ± 0.10/1.35 ± 0.13 Å. The variation in the flavin can be seen in the structure ensemble in Figure 2. Ramachandran statistics for the ensemble show that more than 93% of the residues have most favorable or allowed dihedral angles, 5% have generously allowed angles, and less than 2% have disallowed angles. The two residues that consistently have disallowed angles are Q58 and L91. Q58 lies in a β-hairpin between strands 2 and 3, and it is in position 2 in a slightly modified type I′ β-turn conformation.[36] L91 lies in a bulge region of β-strand 4 where the side chains of L91 and A92 point in the same direction. All distance restraints for L91 are well satisfied, NOE data for this residue are very good, and the side-chain orientation matches that in the crystal structure of AppA.[18] The number of NOE violations is small; this indicates that the structural ensemble satisfies our NOE data very well.

During review of this manuscript, the coordinates for the crystal structure of the BLUF domain of AppA were released, and the structure of a second BLUF domain BlrB from *Rhodobacter sphaeroides* was published and released.[18,37] Comparison of the NMR solution structure of the AppA BLUF domain with the three crystal structures of BLUF domains reveals identical folds for the proteins and identical hydrogen-bonding arrangements for the side chains that contact the flavin chromophore. The pair-wise backbone RMSD between the solution structure of AppA and the other three BLUF domain structures is 1.43 Å for the crystal structure of AppA (1YRX), 1.92 Å for the BLUF domain Tll0078 (1X0P), and 2.01 Å for the BLUF domain BlrB (2BYC).

Table 1. Structural statistics for the solution ensemble of AppA 5–125

RMSD [Å] with respect to the mean:	Backbone/heavy
AppA 15–108, FAD	0.58 ± 0.10/1.35 ± 0.13
AppA 15–108	0.45 ± 0.10/0.95 ± 0.12
Number of experimental restraints	
intraresidue NOEs	834
sequential NOEs ($\|i-j\|=1$)	515
medium range NOEs ($1<\|i-j\|\leq 4$)	247
long range NOEs ($\|i-j\|>4$)	445
total NOEs	2041
Dihedral angle restraints	50
NOE restraint violations	
number of NOE violations > 0.3 Å	1.20
max violation [Å]	0.42
number of dihedrals with violations > 5°	1.00
RMSD [Å] for NOE restraints	0.038 ± 0.001
Ramachandran analysis	
residues in most favored region [%]	80.5
residues in additionally allowed regions [%]	13
residues in generously allowed regions [%]	5.2
residues in disallowed regions [%]	1.3

Conformational exchange in dark-state AppA

The dynamic disorder in the flavin within AppA causes line broadening of resonances near the flavin, fewer observed NOEs for these residues, and disorder in the structural ensemble. Variation in the orientation of the flavin chromophore in the solution structure of AppA is due to a lack of distance restraints to some parts of the flavin isoalloxazine ring. The H3 and H6 protons from the flavin chromophore are not observed in NMR spectra due to exchange line broadening. In addition to the H3 and H6 protons, broad line widths are seen for all resonances of amino acids near the flavin ring, while the line width of resonances in the core of the protein, farther away from the flavin or on the surface of AppA, do not exhibit such line broadening. The proximity of all broadened residues to the flavin ring suggests that the flavin isoalloxazine ring exchanges between different conformational states on the μs–ms timescale, and is thus responsible for the line broadening observed in dark-state AppA.

Resonances exhibiting line broadening in NMR spectra are mapped onto the solution structure of AppA 5–125 in Figure 3. Side-chain resonances for H44, N45, L54, F61, Q63, L65, and W104 all have weak intensity and very few NOEs due to line broadening, and all of these amino acids come into close contact with the flavin ring. Backbone HN resonances from these residues also have broad line widths; this suggests that dynamics in the flavin chromophore are coupled through side-chain contacts to fluctuations in the protein backbone. The presence of these μs–ms timescale motions in AppA in the dark indicates that the chromophore samples different states even in the absence of light. UV-visible characterization of the AppA photocycle showed multiexponential decay of the singlet-excited state of the flavin chromophore to reach the signaling state,[11] which could reflect the presence of multiple

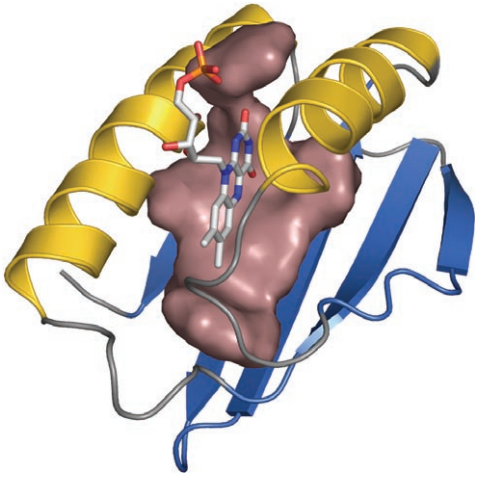

Figure 3. Fold of AppA, with line-broadened side chains shown as a brown surface. All broadened side chains make contact with the flavin chromophore.

conformations for the AppA-bound flavin. Since the lines of all of the side chains that hydrogen bond to the flavin isoalloxazine ring are broadened, it is possible that the source of the μs–ms timescale motions of the flavin ring involves rearrangement or fluctuations in strength of hydrogen bonds to the protein.

Exchange between multiple conformations in a protein often appears in a crystal structure as weak electron density or large B factors. Indeed, weak electron density is observed in the crystal structure of AppA,[18] especially for residues near the flavin ring. The authors speculated that the disorder observed in the crystals stemmed either from the low ambient-light conditions during data acquisition causing partial population of the light-induced state, or from the electrons in the X-ray source causing reduction of the flavin ring to create radical species that damage the protein. While both causes might have affected the quality of the crystal data, our NMR line-broadening data show that the residues near the flavin are dynamic in the dark.

Light-dependent changes in AppA

Figure 4A shows overlaid HSQC spectra for dark-adapted and light-induced states of AppA. The spectra are almost superimposable, with few and mostly small chemical-shift changes upon light excitation. The similarity of the two HSQC spectra demonstrates that no large structural unfolding event occurs in the light-induced state. This similarity is consistent with analysis of the AppA photocycle by using UV-visible spectroscopy,[11] FTIR difference spectroscopy,[38] and 1D ^{1}H NMR spectroscopy.[9] In spectra of AppA completely converted to the light-induced state, some resonances disappear with a concomitant appearance of a new resonance at a slightly different chemical-shift value; this is typical of slow exchange between the two states on the NMR timescale, and is consistent with the observed long lifetime of the light-induced state of AppA.

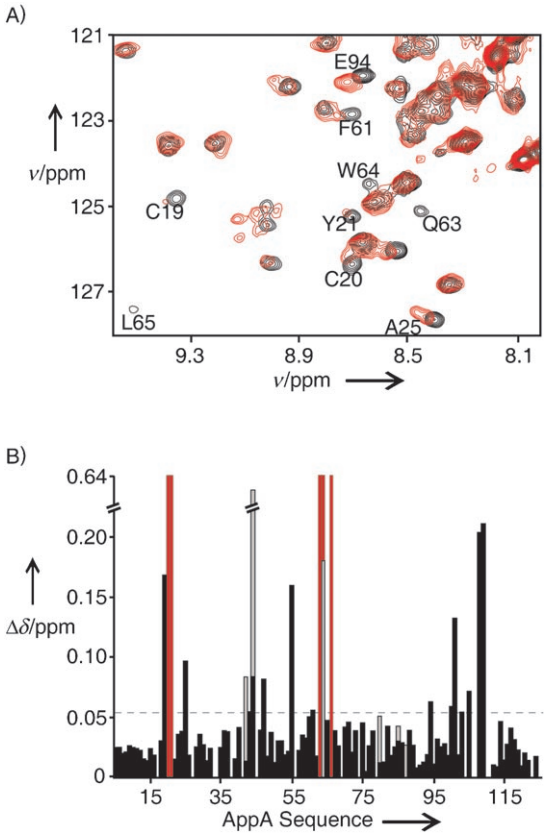

Figure 4. Light-induced changes in AppA. A) Dark- (black) and light-adapted (red) HSQC spectra for AppA. B) Combined ^{1}H and ^{15}N chemical-shift changes between the dark and light HSQC spectra of AppA. Chemical-shift difference values were calculated as $(|\delta H_{light}-\delta H_{dark}|+0.145*|\delta N_{light}-\delta N_{dark}|)$. The red boxes represent peaks that disappeared from the HSQC spectrum of the light-excited state, but for which a light-state resonance could not be assigned, and the gray boxes represent chemical-shift changes for side-chain HN resonances added to the top of the value for the backbone resonance. The dashed line at 0.055 represents the mean chemical-shift change.

Most of the light-induced chemical shifts are small enough to allow assignment from the position of the dark-state resonance and are shown in Figure 4B. Of 131 assigned backbone and side-chain resonances in the ^{1}H,^{15}N HSQC spectrum, 109 were assigned in the light state, no conclusion could be drawn about light-induced changes for 16 resonances due to signal overlap, and six resonances disappeared in the light-induced state. For five HN backbone resonances in the HSQC spectrum of AppA (C20, Y21, Q63, W64, E66) and the side-chain NH_2 of Q63, the intensity of the dark-state resonance decreases as the protein is excited into the light state, but no new peak appears. This could be due to spectral overlap, large differences in chemical shift between the dark and light states, or chemical-exchange line broadening in the light state. These resonances correspond to parts of the sequence with large changes in chemical shift upon illumination.

Notably from this group of resonances, the Q63 side-chain NH_2 disappears in the light-induced state; this indicates that the environment and/or structure of this side chain have

changed in the light state. This observation is consistent with the model for photoactivation of AppA proposed from FTIR measurements by Masuda et al.,[38] in which Q63 reorients to form a strong hydrogen bond with flavin C(4)=O, which is catalyzed by increased basicity at the N5 position in the singlet-excited state of the flavin. Additional evidence in support of this model is revealed by comparison of the structure of AppA with Tll0078. The strength of the hydrogen bond between C(4)=O and the protein is in part determined by the electron density on the flavin isoalloxazine ring. Placement of a positive charge near C(2)=O withdraws electron density from the flavin ring and weakens the C(4)=O hydrogen bond to the protein. In Tll0078, the positively charged side chain of R65 is very close to C(2)=O (2.7 Å), while no positively charged side chains are near C(2)=O in the solution structure of AppA. Tll0078 recovers much more quickly ($\tau \sim 2$ s) from the light-induced state than does AppA ($\tau \sim 30$ min), presumably because of a weaker hydrogen bond between C(4)=O and Q50 in Tll0078 (corresponding to Q63 in AppA).

Evidence for specific light-induced changes in the AppA BLUF domain is seen in the chemical shifts of AppA. The side-chain Hδ1 resonance of H44 has the largest light-induced chemical-shift change that we assigned in AppA (the ^{1}H chemical shift moves from 11.3 ppm in the dark to 11.8 ppm in the light). This downfield change in chemical shift indicates stronger hydrogen bonding of the H44 Hδ1 in the light-induced signaling state. H44 Hδ1 is hydrogen bonded to C(2)=O in most of the dark-state solution structures in our ensemble. It is likely that H44 Hδ1 is also bonded to C(2)=O in the light-excited state, but a light-induced increase in the hydrogen-bonding character at position C(2)=O of the flavin was not identified in a previous FTIR study of AppA.[38] In studies of cyanobacterial BLUF domain Slr1694, the light-induced state is characterized by stronger hydrogen bonding to both C(4)=O and C(2)=O.[39] The importance of hydrogen-bonding interactions in stabilizing the light-induced signaling state is emphasized by our observation that the rate of recovery to the ground state is accelerated by more than one order of magnitude when imidazole is added to a solution of AppA (Laan et al., unpublished results).

If the residues with light-induced changes in chemical shift (such as H44 Hδ1) are mapped onto the solution structure of AppA, the structural changes that are important for signaling with the C-terminal domain of AppA are highlighted (Figure 5).

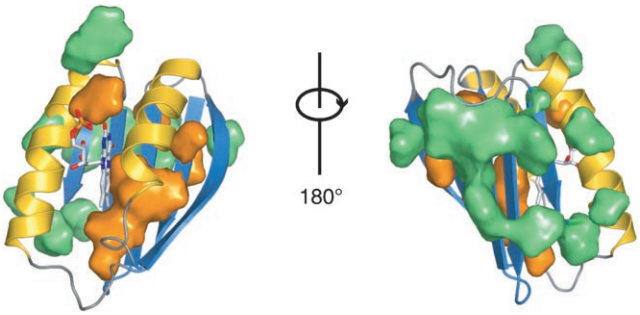

Figure 5. Residues that demonstrate chemical-shift changes in AppA upon illumination are shown in orange (<40% solvent exposure) and green (>40% solvent exposure).

The affected residues can be loosely organized into two groups based on their level of exposure to solvent. The buried residues include side chains that make contact with the flavin (C19, Y21, F61, Q63, H44), as well as adjacent residues in the β-sheet (C20 and E66) that connect the first group of buried residues to the second group of surface-exposed residues (which includes F55, W64, E94, R100, F101, G103, H105, Q107, L108, and S109). The only residues outside the flavin-binding pocket that are highly conserved in the BLUF domain sequence family (T51, E66, R99, and F101) are part of this group of residues that connects the flavin-binding pocket to the surface that displays light-induced changes.

It has been proposed that the BLUF domain of AppA interacts with the C terminus of AppA to disrupt the AppA–PpsR complex in the presence of light.[8,38] The pattern of chemical-shift changes that we observe with NMR strongly suggests that the surface of the BLUF domain of AppA pictured in Figure 5 is responsible for that function, and also gives clues as to how changes in side chains that make contact with the flavin propagate the light signal into a surface change. In the structure of the Tll0078 BLUF domain,[19] the homologous surface interacts with a C-terminal protein sequence on this surface. The same surface is buried in a dimer interface in the crystal structure of the BLUF domain of AppA, although both the presence of a detergent molecule and gaps in the electron density at the interface suggest that the interface might not be specific. As mentioned above, during calculation of the solution structure, no intermolecular NOEs were identified; this suggests that the dimer interface might be dynamic or non-specific. Full-length AppA was demonstrated to be a monomer in solution,[4] but truncated forms of the protein (AppA 1–156 and AppA 5–125) form dimers in solution; this is consistent with our diffusion measurements and gel-filtration chromatography. In full-length AppA, this hydrophobic surface most likely associates with the C-terminal domain of AppA, where alteration of this interaction disrupts the AppA-PpsR complex to allow repression of photosynthesis gene expression.

Conclusion

Line broadening of protein side-chain resonances involved in hydrogen-bonding interactions with the flavin chromophore demonstrates that, even in the dark, these residues sample different conformations. The conformation and/or dynamics of these residues are altered in the light-induced signaling state of AppA to form a stable hydrogen-bonding arrangement with the flavin chromophore. This long-lived signaling state of AppA is propagated through the β-sheet to cause changes in the hydrophobic surface of AppA. We conclude that light-induced changes in the structure and/or dynamics of this surface of AppA are responsible for disruption of the interaction between the C-terminal domain of AppA and PpsR, either directly or indirectly.

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