Redox and Light Control the Heme-Sensing Activity of AppA

Liang Yin, Vladimira Dragnea, George Feldman, Loubna A. Hammad, Jonathan A. Karty, Charles E. Dann III, Carl E. Bauer

Department of Molecular and Cellular Biochemistry, and Department of Chemistry, Indiana University, Bloomington, Indiana, USA

ABSTRACT The DNA binding activity of the photosystem-specific repressor PpsR is known to be repressed by the antirepressor AppA. AppA contains a blue-light-absorbing BLUF domain and a heme-binding SCHIC domain that controls the interaction of AppA with PpsR in response to light and heme availability. In this study, we have solved the structure of the SCHIC domain and identified the histidine residue that is critical for heme binding. We also demonstrate that dark-adapted AppA binds heme better than light-excited AppA does and that heme bound to the SCHIC domain significantly reduces the length of the BLUF photocycle. We further show that heme binding to the SCHIC domain is affected by the redox state of a disulfide bridge located in the Cys-rich carboxyl-terminal region. These results demonstrate that light, redox, and heme are integrated inputs that control AppA’s ability to disrupt the DNA binding activity of PpsR.

IMPORTANCE Photosynthetic bacteria must coordinate synthesis of the tetrapyrroles cobalamin, heme, and bacteriochlorophyll, as overproduction of the latter two is toxic to cells. A key regulator controlling tetrapyrrole biosynthesis is PpsR, and the activity of PpsR is controlled by the heme-binding and light-regulated antirepressor AppA. We show that AppA binds heme only under dark conditions and that heme binding significantly affects the length of the AppA photocycle. Since AppA interacts with PpsR only in the dark, bound heme thus stimulates the antirepressor activity of PpsR. This causes the redirection of tetrapyrrole biosynthesis away from heme into the bacteriochlorophyll branch.

A
AppA from Rhodobacter sphaeroides (1) is a blue-light photoreceptor discovered a decade ago that utilizes flavin as a chromophore. It is among the first identified members of a broadly disseminated BLUF (sensors of blue light using FAD) family of photoreceptors (2). Since their identification, there has been extensive research on the BLUF photocycle providing significant insight on how light absorption by a bound flavin leads to a conformational change in the BLUF domain (3). In many cases, it remains unclear how light-driven alterations in the BLUF input domain result in changes in the downstream output module. Among BLUF proteins with a linked output domain, only two examples of downstream signal transduction events have been described at a molecular level. One example is YcgF from Escherichia coli (4, 5) and its homolog BlrP1 in Klebsiella pneumoniae (6) where light excitation of the BLUF domain stimulates phosphodiesterase activity of the EAL output domain that converts c-di-GMP to linear diguanylate (6). The other example is AppA where light excitation of the BLUF domain inhibits an output domain to function as an antirepressor of the photosystem-specific repressor PpsR (1).

PpsR in R. sphaeroides controls heme, bacteriochlorophyll, and photosynthesis gene expression with the activity of the PpsR-AppA system highly regulated by numerous input signals such as light, redox, and heme (1, 7, 8). Several input signals directly affect the activity of PpsR, while others affect the ability of AppA to interact with PpsR. For example, under aerobic growth conditions, two cysteine residues in PpsR are in an oxidized state which stimulates its DNA binding activity (1). This is contrasted by anaerobic conditions where these cysteines are in a reduced state which impedes DNA binding. PpsR also binds heme via a conserved Cys residue present in the DNA-binding domain and a histidine present in a Per-Arnt-Sim (PAS) domain (7). Bound heme affects the interaction between PpsR and its target DNA (7).

The antirepressor AppA consists of an N-terminal BLUF domain that utilizes flavin to sense light, a middle SCHIC (sensor containing heme instead of cobalamin) domain (9) that binds heme and a redox-responding C-terminal Cys-rich domain (Fig. 1A). The Cys-rich carboxyl domain is capable of reducing the redox-active Cys in PpsR under dark anaerobic conditions to lower the DNA binding affinity of PpsR (1, 8). Additionally, dark-adapted AppA is able to form a stable PpsR−AppA complex that cannot bind to target DNA sequences (1). Interestingly, the interaction of AppA with PpsR is inhibited by blue-light absorption of the BLUF domain, a regulatory feature that imparts light control of photosynthesis gene expression (1). A final aspect of AppA regulation involves binding of heme to the SCHIC domain of AppA that is thought to promote interaction of AppA with PpsR (10). The SCHIC domain has sequence similarity to the well-characterized vitamin B12-binding domain of methyltransferases (9, 10), but unlike methyltransferases, the SCHIC domain differentially binds heme over B12 (9, 10).

Several research groups have reported that the SCHIC domain
binds heme, but the details of this interaction have not been clarified. One study used an AppA variant that had the C-terminal Cys-rich motif replaced with a maltose-binding protein (MBP). Studies with this construct indicated that heme binds to AppA-MBP only in the absence of oxygen and that heme is gradually discoordinated from AppA as the oxygen level increases (9). A second heme binding study used a His6-tagged AppA variant that lacked the BLUF domain ([H9004]/N-AppA-His6) (10). Since neither study tested full-length untagged AppA, it has remained unclear whether oxygen or light has an effect on heme binding to AppA.

In this study, we demonstrate that dark-adapted AppA binds heme better than light-excited AppA does and that heme bound to the SCHIC domain significantly reduces the length of the BLUF photocycle. We also solved the structure of the SCHIC domain and identified the histidine residue that is critical for heme binding to AppA. In this study, we demonstrate that dark-adapted AppA binds heme better than light-excited AppA does and that heme bound to the SCHIC domain significantly reduces the length of the BLUF photocycle. We also solved the structure of the SCHIC domain and identified the histidine residue that is critical for heme binding to AppA. In this study, we demonstrate that dark-adapted AppA binds heme better than light-excited AppA does and that heme bound to the SCHIC domain significantly reduces the length of the BLUF photocycle. We also solved the structure of the SCHIC domain and identified the histidine residue that is critical for heme binding to AppA.

RESULTS

AppA binds heme under both anaerobic and aerobic conditions. We examined the interaction of AppA with heme by purifying tagless full-length AppA from an E. coli overexpression system. As previously reported by our group, there were no significant amounts of heme observed when full-length AppA was purified from the E. coli overexpression system (1). However, it does contain flavin adenine dinucleotide (FAD) as the BLUF domain cofactor. To ensure that our experimental conditions covered a wide range of oxygen concentrations, we prepared AppA either in degassed buffer (oxygen level of <1 mg/liter) or in air-saturated buffer (oxygen level of >6 mg/liter) for use in interaction studies with heme. Both samples exhibited almost identical absorption spectra, indicating no oxygen-dependent effect on heme binding by full-length tagless AppA (see Fig. S1 in the supplemental material). This is in contrast to a previous report that AppA binds to heme only when oxygen is absent (9). Calculating the amount of heme binding in replicate reconstitution experiments showed that AppA binds to heme only when oxygen is absent (9). Calculating the amount of heme binding in replicate reconstitution experiments showed that AppA binds to heme only when oxygen is absent (9).
could be described by a single exponential equation (Fig. 1C). The heme-binding rate under dark aerobic conditions was fitted with the concentration of AppA, yielding a $K_d$ (dissociation constant) of $1.25 \pm 0.44 \mu M$ (Fig. 1D). This rather weak binding affinity is similar to values reported for other heme-sensing proteins such as PpsR ($1.9 \mu M$) (7) and R-transferase ($0.15 \mu M$) (11).

Light excitation of the BLUF domain impedes heme binding by the SCHIC domain. To test whether the light-regulated BLUF domain has a role in regulating SCHIC domain interaction with heme, we monitored the kinetics of heme binding in dark- and light-adapted AppA using stopped-flow analysis. As shown in Fig. 1C, there is no significant binding of heme to lit AppA observed over the course of the experiment. This is in contrast to dark-adapted AppA that exhibits exponential binding of heme as monitored by amplification of $A_{412}$ (Fig. 1C). This result indicates that AppA binds heme effectively only under dark conditions.

In previous studies, we described several mutations in the BLUF domain (AppA$_{Y21F}$ [AppA with a Y-to-F change at position 21] and AppA$_{Q63E}$) that remain in a lit signaling state irrespective of whether these proteins are under dark or illuminated conditions (12, 13). We assayed heme binding with AppA$_{Y21F}$ and AppA$_{Q63E}$ in the dark to confirm whether there is indeed a connection between light sensing and heme binding. As shown in Fig. 2A, incubation of AppA$_{Y21F}$ and AppA$_{Q63E}$ with heme for 30 min shows that both proteins bind heme very poorly in the dark as evidenced by large amounts of free unbound heme with a peak at 370 nm and only a slight shoulder of bound heme with a peak at 412 nm. This is in contrast to wild-type AppA, which shows excellent binding under similar dark conditions (Fig. 2A).

We also observed that light excitation of the BLUF domain affected the spectrum of prebound heme. In this analysis, light excitation of AppA-heme complex resulted in a slight but reproducible reduction of the Soret peak at 412 nm (Fig. 2B), which indicates a weakening of AppA-heme interaction. A dark-minus-light differential spectrum of the AppA-heme sample clearly shows this reduction at 412 nm and an alteration of flavin absorbance at the 500-nm region attributed to the BLUF photocycle (Fig. 2C). Note that similar analysis with BLUF mutants AppA$_{Y21F}$ and AppA$_{Q63E}$ did not show alterations in the Soret peak at 412 nm upon exposure to light, as these mutants are in locked in an illuminated state (Fig. 2C).

Bound heme pushes the BLUF domain toward the dark state conformation. Given that light excitation of the BLUF domain affects heme binding to the SCHIC domain, we asked whether heme bound to the SCHIC domain inversely affects the state of the BLUF domain. To test this possibility, we measured the BLUF photocycle by monitoring spectral changes in the flavin absorption region. Dark-adapted AppA has a broad flavin absorption peak with a maximum at 450 nm that upon light excitation red-shifts $\sim 10$ nm as a result of a conformational change in the BLUF domain. This excitation spectral shift subsequently falls back to the ground state with a half-life of $\sim 15$ min when the protein returns from a lit to a dark conformation (1). The dark and lit state flavin absorption spectra show the same spectral features in both

FIG 2 Integration of light sensing and heme binding in AppA. (A) Absorption spectrum of heme and different AppA constructs under aerobic conditions. AppA mutants and heme were mixed at a 2:1 ratio and incubated for at least 20 min before the spectrum was taken. The spectrum of the protein with flavin is subtracted. Normalized absorbance is shown on the right and top axes. (B) Absorption spectrum of AppA and AppA-heme complex in dark (D) and lit (L) states. The spectrum of protein was not subtracted. (C) Differential spectrum of AppA mutants and heme (dark state minus light state). The samples in Fig. 2A were used here. (D) Recovery kinetics of AppA/AppA-heme light cycle. AppA and AppA-heme complex were light excited and put back in dark conditions. Absorbance at 493 nm was measured to monitor the photocycle of the BLUF domain, and the data were fitted to a single-exponential model.
AppA and AppA with bound heme indicating that the BLUF domain has the same general conformation whether or not heme is bound (Fig. 2B). However, the length of the AppA-heme photocycle is considerably shorter, with a decay half-life of ~4 min compared to AppA itself that has a half-life of ~15 min (Fig. 2D). This indicates that the BLUF domain prefers a dark conformation when heme is bound to the SCHIC domain.

The SCHIC domain uses His^{284} to interact with heme. We obtained crystals of the SCHIC domain (AppA amino acids 186 to 398) (Fig. 3A; see Table S1 in the supplemental material) without bound heme using hanging drop vapor diffusion. Unfortunately, the SCHIC domain alone does not incorporate heme in stoichiometric amounts, so we were not successful in growing SCHIC crystals containing heme. Due to the inherent problems of heme solubility, we were also unsuccessful in loading heme by soaking protein crystals with heme containing mother liquor. Nevertheless, the solved SCHIC domain apo-structure at 2.04 Å does provide valuable information on a putative heme-binding cleft.

The crystal lattice contains two copies of a homodimeric SCHIC domain that have similar conformations with a root mean square deviation (RMSD) of 0.40 Å between the dimers. Individual subunits are comprised of an N-terminal helix bundle followed by a C-terminal α/β Rossmann fold (Fig. 3A). As shown in Fig. 3B, the SCHIC structure superposes very closely with the vitamin B_{12}-binding domain from methionine synthase (MetH, 1BMT.pdb) (Fig. 3B) (14). The MetH-B_{12} structure contains a well-defined B_{12}-binding cleft, with a loop where a highly conserved histidine residue directly coordinates with the cobalt in B_{12} (Fig. 3C) (14). The SCHIC domain also contains a similar loop with a potential iron-coordinating histidine (His^{284}) located 2 amino acids further down this loop than for the cobalt-coordinating histidine present in MetH-B_{12} (Fig. 3C). The imidazole ring in SCHIC His^{284} is also rotated and moved with an average distance of 6.4 Å from the His^{759} present in MetH (Fig. 3C).

To test whether heme forms a ligand to His^{284}, we mutated His^{284} to alanine within full-length AppA and then spectrally analyzed the ability of the mutant peptide to bind heme. Incubation of AppA_{H284A} with heme showed a peak at 370 nm, which is the spectral characteristic of free unbound heme compared to the 412-nm Soret peak of heme bound to wild-type AppA (Fig. 3D). This observation indicates that the Ala substitution disrupted heme and that His^{284} indeed coordinates with the heme iron.

A disulfide bond in the Cys-rich motif affects the SCHIC-heme interaction. Even though full-length AppA is capable of binding heme in the presence of molecular oxygen, this result does not rule out that changes in the redox state of the Cys-rich C-terminal domain can affect heme binding. Previous studies from our laboratory have shown that AppA contains a single re-
Heme Sensing by AppA

Numerous studies show that *Rhodobacter sphaeroides* uses the PpsR-AppA regulatory system to coordinate biosynthesis of heme and bacteriochlorophyll with synthesis of photosystem apoproteins (1, 15, 16). In this study, we demonstrate that tagless full-length AppA can bind heme under both anaerobic and aerobic conditions (Fig. 1B). This finding is in contrast to a report by Moskvin et al. (9), which indicated that oxygen can discoordinate heme from AppA in a concentration-dependent manner. This apparent difference likely stems from the fact that our study utilized full-length tagless AppA, while the study by Moskvin et al. utilized an MBP-tagged AppA that also contained a truncation of the Cys-rich motif.

If heme is not an oxygen sensor, then what is its function when excited? The heme Soret peak in reduced AppA exhibits a 6-nm redshift along with a decrease in intensity (Fig. 4C). This is distinctly different than that of oxidized wild-type AppA where the heme Soret peak decreases without a redshift upon light excitation (Fig. 2B). To confirm that this effect is a result of the reduced disulfide bridge, we constructed a Cys<sup>399</sup>-to-Ala point mutation. Purified AppA<sub>C399A</sub> also exhibited the same 6-nm redshift along with a decrease in intensity as described above for reduced AppA (Fig. 4D). Interestingly, AppA with a Cys<sup>406</sup> Ala mutation (the other residue of the disulfide pair) exhibited a dark/light spectrum that is similar to the spectrum of oxidized wild-type AppA (Fig. 5A). Further analysis showed that a disulfide bond can form between Cys<sup>399</sup> and a neighboring Cys (Cys<sup>405</sup>) in this construct (Fig. 5B), which appears to function like the original Cys<sup>399</sup>–Cys<sup>406</sup> disulfide bond present in wild-type AppA. Taken together, these results indicate that the presence of a disulfide bridge in the Cys-rich motif has an effect on the heme binding properties of the SCHIC domain.

The effect of this reduced disulfide on heme binding can also be demonstrated by assaying absorbance changes using stopped-flow experiments. As discussed above, heme binding with wild-type AppA can be analyzed by stopped-flow analysis by measuring the rise in absorbance at 412 nm that can be described in a single exponential equation (Fig. 1C). Loss of the Cys<sup>399</sup>–Cys<sup>406</sup> disulfide bridge in AppA<sub>C399A</sub> results in a similar rapid rise at 412 nm, followed first by a short plateau and then by a slow decrease in absorbance. As discussed above, the presence of a disulfide bridge in the heme Soret spectral species followed by slow decay to an alternate state that is capable of undergoing the 6-nm redshift upon light excitation of the BLUF domain.

Finally, as discussed earlier, the presence of heme in AppA that has Cys<sup>399</sup>–Cys<sup>406</sup> in its oxidized disulfide state shortens the BLUF photocycle half-life to ~4 min, which is significantly faster than the 15-min half-life observed with oxidized AppA that does not contain heme (Fig. 2D). Interestingly, heme-induced reduction of the BLUF photocycle is absent when the disulfide in AppA is reduced with DTT. Under this condition, the photocycle half-life is ~11 min with or without bound heme. The latter result suggests that the redox state of this disulfide affects signal communication between the BLUF domain and the heme-binding SCHIC domain.

DISCUSSION

doxy active pair of cysteines that function as a dithiol/disulfide couple with a midpoint potential (\(E_m\)) of ~325 mV (8). This potential is very similar to the \(E_m = 320\) mV potential of a disulfide pair in PpsR that AppA is capable of reducing (1, 8). To determine which cysteines are redox active, we performed matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis of trypsin-digested iodoacetamide (IAM)-labeled AppA. The tryptic peptide AppA<sub>397-409</sub> was further analyzed using liquid chromatography coupled to electrospray ionization tandem mass spectrometric analysis (LC-ESI-MS-MS) which shows the presence of a Cys<sup>399</sup>–Cys<sup>406</sup> disulfide bridge (Fig. 4; see Fig. S2 in the supplemental material).

To address whether the Cys<sup>399</sup>–Cys<sup>406</sup> disulfide bond affects the AppA-heme interaction, we reduced the disulfide bond with dithiothreitol (DTT) and then analyzed the heme spectrum. The heme spectrum of reduced AppA exhibits a normal Soret peak at 412 nm in the dark state. However, when the BLUF domain is light excited, the Soret peak of AppA<sub>C399A</sub>-heme is redshifted from 412 nm to 418 nm. (D) When light excited, the Soret peak of AppA<sub>C399A</sub>-heme is redshifted from 412 nm to 418 nm.

FIG 4 The disulfide bridge in the Cys-rich motif affects the SCHIC-heme interaction. (A) A disulfide bridge was identified within peptide AppA<sub>397-409</sub>. Under oxidized conditions, neither Cys<sup>399</sup> nor Cys<sup>406</sup> can be modified by iodoacetamide (IAM). Under reduced conditions, the disulfide bridge formed between Cys<sup>399</sup> and Cys<sup>406</sup> is reduced, and both residues can be modified by iodoacetamide (DTT-IAM). Under oxidized conditions, the disulfide bridge formed between Cys<sup>399</sup> and Cys<sup>406</sup>, which forms the alternative Cys<sup>399</sup>–Cys<sup>405</sup> disulfide bond, does not exhibit a slow decrease in the Soret peak after binding heme (Fig. 5D). We interpret this result as evidence that the binding of heme in reduced AppA first involves the formation of the 412-nm Soret spectral species followed by slow decay to an alternate state that is capable of undergoing the 6-nm redshift upon light excitation of the BLUF domain.

The effect of this reduced disulfide on heme binding can also be demonstrated by assaying absorbance changes using stopped-flow experiments. As discussed above, heme binding with wild-type AppA can be analyzed by stopped-flow analysis by measuring the rise in absorbance at 412 nm that can be described in a single exponential equation (Fig. 1C). Loss of the Cys<sup>399</sup>–Cys<sup>406</sup> disulfide bridge in AppA<sub>C399A</sub> results in a similar rapid rise at 412 nm, followed first by a short plateau and then by a slow decrease in absorbance. As discussed above, the presence of a disulfide bridge in the heme Soret spectral species followed by slow decay to an alternate state that is capable of undergoing the 6-nm redshift upon light excitation of the BLUF domain.
it is bound to the SCHIC domain of AppA? The absorption characteristics of the flavin region in both dark and light states are identical with or without bound heme, indicating that the BLUF domain undergoes a similar hydrogen bond rearrangement in the presence or absence of heme (Fig. 2B). However, the length of the AppA photocycle is dramatically reduced when oxidized AppA contains bound heme relative to that observed with AppA alone. The consequence of a quicker photocycle is that it would increase the population of AppA that is present in the dark state. Given that only AppA in the dark state is capable of interacting with PpsR, the binding of heme would therefore increase the antirepressor activity of AppA (Fig. 6A). This would result in increased bacteriochlorophyll and light-harvesting gene expression to promote maximal synthesis of the photosystem (Fig. 6B). The involvement of heme in controlling the AppA photocycle appears to be a means to ensure that increased synthesis of the photosystem does not occur unless there is sufficient heme available to handle the essential function of electron transfer. In purple bacteria, cytochrome biosynthesis is essential, while synthesis of the photosystem is not. Furthermore, electrons derived from photosynthesis need to be shuttled back to the photosystem via heme-containing cytochromes. Consequently, it is not surprising that heme has a role in controlling the amount of photosystem synthesis via the regulation of AppA activity.

The solved crystal structure of the SCHIC domain shows remarkable sequence similarity with the vitamin B12-binding domain in MetH (9, 10). Heme and vitamin B12 have significant differences and central metals. For example, heme has methyl and formyl side groups with the bound iron free to form axial ligands. This is in contrast to vitamin B12, where the corrin ring has 7 propionamide side groups and 2 alcohol side groups as well as a bulky attached dimethylbenzimidazol ribonucleotide group that forms a lower axial ligand to the bound cobalt. B12 also contains a second upper axial cobalt (Co) ligand typically comprised of a methyl or 5'-deoxyadenosine group. Thus, it is surprising that the crystal structure of the SCHIC domain is so similar to the structure of the B12-binding domain from MetH (Fig. 2B).

Despite these similarities, there are several subtle changes that likely contribute to tetrapyrrole selectivity (Fig. 7A). For example, the loop in MetH that forms a lower axial ligand of His to Co is altered in the SCHIC structure such that the His is displaced by two residues (Fig. 3C). In addition, MetH-B12 has Phe708 and Leu715 located at one end of the second helix in the helix bundle that serves as a cap to cover the reactive methyl ligand of cobalamin (met cap) (14). Located in a similar position of this helix in the SCHIC structure is Leu239 and a positive charged Arg246 that are in contact with a loop extended from the Rossmann fold (extension) (Fig. 7B). These changes would block the binding of Met- or Adeno-B12 to the SCHIC domain (Fig. 7C). This extension also forms a hydrogen bond network with the His loop in the SCHIC domain that locks the orientation of Fe-binding His284 in a position (see Fig. S4 in the supplemental material) that is distinct from the Co-coordinating His289 in MetH-B12 (14). Finally, there is additional space in the core of the MetH-B12 Rossmann fold that anchors the bulky attached nucleotide tail of vitamin B12 (14). In the SCHIC domain, the access to this docking space is blocked by Glu289 which resides on the His loop (dock lock [Fig. 7B and D]). These changes likely constitute selectivity of these similar tetrapyrrole binding domains for heme versus B12.

Interestingly, the described regions critical for heme/B12 differentiation map to an extension in the SCHIC domain Rossmann fold that exhibit different conformations in the two subunits of
the SCHIC crystal structure (Fig. 7B and E). In one subunit, part of this extension is moved ~7 Å, forming different interactions with the methyl cap and the His loop (see Fig. S4 in the supplemental material). This could represent the “empty” state versus an “en route” position toward heme-bound conformation. As a heme-sensing module, SCHIC is probably not at its most stable state and would not bind heme, since it does not bind heme tightly. This might explain our failure to grow crystals of SCHIC-heme complex or to reconstitute SCHIC crystals with heme.

This might explain our failure to grow crystals of SCHIC-heme complex or to reconstitute SCHIC crystals with heme.

Protein-heme interactions. Heme reconstitution with AppA and AppA mutants was performed as described previously (7). The sample was either degassed or air bubbled when needed with dissolved oxygen concentration measured using an oxygen CHEMets kit (CHEMetrics).

UV-visible absorption spectrum was recorded using a Beckman DU 640 spectrophotometer as described previously (7). To convert AppA into a light-excited state, dark-adapted samples were illuminated with strong white light for 30 s immediately before spectral analysis unless noted otherwise. Note that the heme spectrum itself stays identical in dark and illuminated conditions (see Fig. S4 in the supplemental material).

Purification of SCHIC for crystallization. To construct an SCHIC domain expression vector, AppA with amino acids 186 to 398 (AppA186-398) was cloned into pTYB12 plasmid (New England Biolabs) with NotI-HindIII restriction sites of pSUMO. The pTY-AppA plasmid was subjected to site-directed mutagenesis (Herculase II; Stratagene) to create point mutations in the BLUF domain (AppA221F and AppA398E). All point mutations were created by the standard QuickChange (Agilent Technologies) protocol.

Protein purification for biochemical experiments. SUMO-tagged full-length AppA was overexpressed and purified as described previously (7). Proteins cloned into the pT7 plasmid (AppA221F and AppA398E) were purified using chitin beads and the manufacturer’s protocol (New England Biolabs) followed by gel filtration chromatography using Sephadryl S-200 HP resin (GE Healthcare). All purified proteins were purified in a buffer of 20 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 5% glycerol, and all experiments were performed in the same buffer unless otherwise noted.

Materials and Methods

Strains, media, and growth conditions. Strain BL21(DE3) (Novagen) was used for protein overexpression in E. coli. Luria broth (LB) was used for agar-solidated plates and liquid cultures for E. coli. The antibiotics used and their final concentrations were as follows: ampicillin, 100 µg ml⁻¹; kanamycin, 50 µg ml⁻¹ for agar-solidated plates and 25 µg ml⁻¹ for liquid cultures.

Plasmid construction. Wild-type AppA, AppA CYS mutants (AppA C399A and AppA C406A) and AppA His mutants (AppA H284A, AppA H128A, and AppA H356A) were expressed in E. coli using modified small ubiquitin-like modifier SUMO-I (LifeSensors Inc.) overexpression system. The coding region was cloned from pTY-AppA (24) and inserted into the NdeI-NotI restriction sites of pSUMO. The pT7-AppA plasmid was subjected to site-directed mutagenesis (Herculase II; Stratagene) to create point mutations in the BLUF domain (AppA221F and AppA398E). All point mutations were created by the standard QuickChange (Agilent Technologies) protocol.
FIG 7 Architectural basis of heme/B12 differentiation. (A) Sequence alignment of AppA SCHIC and selected MetH vitamin B12-binding domains. Three sequences, the R. sphaeroides, Rhodobacter capsulatus, and E. coli sequences, are shown. The MetH B12-binding domains were aligned first. Residues that are identical, highly similar, or less similar were shown with black, medium gray, or light gray background, respectively. The alignment was then compared to the SCHIC sequence based on sequence and structure homology. The regions in AppA SCHIC domain that are critical for heme/B12 differentiation are shown on a red background. (B) Crystal structure of SCHIC domain (subunit A). Regions critical for heme/B12 differentiation other than the His loop are highlighted as followed: extension (blue), met cap (red), and dock lock (yellow). (C) Structure of the extension and met cap regions in SCHIC (orange) and MetH (green). Note that these two regions are in contact in SCHIC (orange) (hydrogen bond between R246 and L334) but far away from each other in MetH (green). The contact in SCHIC would block the B12 binding cleft in MetH (broken green line). (D) Structure of the dock lock in SCHIC (orange) and MetH (green). Note that E289 occupies more space in SCHIC (orange) than G762 does in MetH (green). (E) The extension loop in SCHIC is flexible and interacts differently with the met cap and His244 in molecule A (orange) and molecule B (blue) in the crystallographic dimer. The detailed hydrogen networks are shown in Fig. S3 in the supplemental material.

Data collection and processing. Diffraction data were collected at the Berkeley beamline Advanced Light Source (ALS) 4.2.2. using remote data collection. The best anomalous data set was collected using selenium peak (4 selenium atoms in the SCHIC molecule) at 2.2-Å resolution. Data collection proceeded for 210° with a step of 1°. The crystal mosaicity was ~0.8. The best diffraction native data set was collected at 2.05-Å resolution. Initial indexing by HKL2000 provided primitive monoclinic space group P21. The initial phase estimates using SeMet data set were computed using Phaser (26). Autobuild software of Phenix (27) then provided the beginning model. Coot (28) and Phenix were used for model building and final refinement. Final refinement statistics are shown in Table S1 in the supplemental material.

Stopped-flow spectroscopy. Dynamics of the AppA-heme reaction was measured using a KinTek stopped-flow SF-300 instrument. Various concentrations of AppA and heme were mixed in the reaction chamber with absorbance at 412 nm monitored. For analysis of the reaction of heme with dark state AppA, AppA was kept in the dark for at least 20 min before the reaction was initiated. For the reaction of heme with lit state AppA, dark state AppA was loaded into a clear glass injection syringe and then illuminated for at least 20 s before and during the time course of the monitored reaction. The $K_d$ of AppA-heme interaction was calculated using the standard model as reported before (29).

Mapping cysteine residues in AppA. The presence of reduced Cys sulhydryl residues in AppA was determined by incubating ~10 μM AppA (either oxidized or 10 mM DTT-reduced) with 10 or 20 mM iodoacetamide at 22°C for 10 min. Excess iodoacetamide was then removed by passing through a MidiTrap G-25 (GE Healthcare) desalting column or a Zeba spin desalting column with a molecular weight cutoff of 7,000 (7K MWCO) (Thermo Scientific), depending on the sample volume. The samples were then treated with trypsin (protein/trypsin ratio is 10:1) at 37°C overnight. The digestion was stopped by the addition of trifluoroacetic acid (TFA) to a final concentration of 0.1% and desalted using ZipTip pipettes with C18 resin (Millipore). The samples eluted from ZipTip pipettes by 60% acetonitrile and 0.1% TFA were used for MALDI mass spectrometry (LC-MS-MS) (30). An Eksigent two-dimensional (2D) Ultra liquid chromatograph (AB Sciex, Foster City, CA) injected 5 μl
of extract onto a trapping column (100 by 0.1 mm) packed with 5-µm Michrom Magic C18 particles and desalted for 8 min at a flow rate of 4 µL/min using LC buffer A (0.1% [vol/vol] formic acid [Sigma-Aldrich], 3% [vol/vol] acetonitrile [EM Science] in high-performance liquid chromatography [HPLC]-grade water [EM Science]). The trapping column was then placed in line with a Picofrict column (150 by 0.075 mm) with an integrated ESI tip (New Objective, Woburn, MA) packed with the same material as in the trap. A 35-min gradient from 10 to 85% LC buffer B (0.1% [vol/vol] formic acid, 3% [vol/vol] water in acetonitrile) separated the peptide mixture prior to tandem mass spectrometry in an LTQ-Orbitrap XL mass spectrometer (Thermo, Waltham, MA). The mass/ charge ratios of the intact peptide ions were measured at a resolving power of 15,000 in the Orbitrap; the fragment ions were generated, and their charge ratios of the intact peptide ions were measured at a resolving power of 40,000 in the orbitrap; the fragment ions were measured in the LTQ ion trap. The resulting mass spectra were manually interpreted; the MS-Product module of ProteinProspector (http://prospector.ucsf.edu) (31) was used to predict the fragment ion masses.

Protein structure accession number. The final structure has been deposited in the Protein Data Bank (PDB) under accession number 4HEI.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org /lookup/suppl;doi:10.1128/mBio.00563-13/-/DCSupplemental.

Figure S1, TIF file, 10.1 MB.
Figure S2, TIF file, 3.3 MB.
Figure S3, TIF file, 6.9 MB.
Figure S4, TIF file, 3.3 MB.
Table S1, DOCX file, 0.1 MB.

ACKNOWLEDGMENTS
We thank Jay Nix at the ALS, Berkeley, CA, for help during crystallographic data collection. We also thank Spencer Anderson and Hua Yuan for advice and help with data processing of various SCHIC crystal forms and Jared Cochran for help with stopped-flow kinetic studies.

This work was supported by NIH grant R37 GM040941 to C.E.B.

REFERENCES
1. Masuda S, Bauer CE. 2002. AppA is a blue light photoreceptor that antirepresses photosynthesis gene expression in Rhodobacter sphaeroides. Cell 110:613–623.
2. Gomelsky M, Klug G. 2002. BLUF: a novel FAD-binding domain involved in sensory transduction in microorganisms. Trends Biochem. Sci. 27:497–500.
3. Losi A, Gärtnert W. 2012. The evolution of flavin-binding photoreceptors: an ancient chromophore serving trendy blue-light sensors. Annu. Rev. Plant Biol. 63:49–72.
4. Hasegawa K, Masuda S, Ono TA. 2006. Light induced structural changes of a full-length protein and its BLUF domain in YcgF(Blrp), a blue-light sensing protein that uses FAD (BLUF). Biochemistry 45:3785–3793.
5. Rajagopal S, Key JM, Purcell EB, Boereoma DJ, Moffat K. 2004. Purification and initial characterization of a putative blue light-regulated phosphodiesterase from Escherichia coli. Photochem. Photobiol. 80:542–547.
6. Barends TR, Hartmann E, Griese JJ, Beitlich T, Kirienko NV, Ryjenkov DA, Reinstein J, Shoeman RL, Gomelsky M, Schlichting I. 2009. Structure and mechanism of a bacterial light-regulated cyclic nucleotide phosphodiesterase. Nature 459:1015–1018.
7. Yin L, Dragnea V, Bauer CE. 2012. PpsR, a regulator of heme and bacteriochlorophyll biosynthesis, is a heme-sensing protein. J. Biol. Chem. 287:13850–13858.
8. Kim SK, Mason JT, Knaff DB, Bauer CE, Settardahl AT. 2006. Redox properties of the Rhodobacter sphaeroides transcriptional regulatory proteins PpsR and AppA. Photosynth. Res. 89:89–98.
9. Moskvin OV, Kaplan S, Gilles-Gonzalez MA, Gomelsky M. 2007. Nomenclature of heme-based oxygen odoxidase with a revealing evolutionary history. J. Biol. Chem. 282:28740–28748.
10. Han Y, Meyer MH, Keusgen M, Klug G. 2007. A haem cofactor is required for redox and light signalling by the AppA protein of Rhodobacter sphaeroides. Mol. Microbiol. 64:1090–1104.
11. Hu RG, Wang H, Xie Z, Varshavsky A. 2008. The N-end rule pathway is a sensor of heme. Proc. Natl. Acad. Sci. U. S. A. 105:76–81.
12. Dragnea V, Arunkumar AI, Lee CW, Giedroc DP, Bauer CE. 2010. A Q63E Rhodobacter sphaeroides AppA BLUF domain mutant is locked in a pseudo-light-excited signaling state. Biochemistry 49:10682–10690.
13. Dragnea V, Arunkumar AI, Yuan H, Giedroc DP, Bauer CE. 2009. Spectroscopic studies of the AppA BLUF domain from Rhodobacter sphaeroides: addressing movement of tryptophan 104 in the signaling state. Biochemistry 48:9969–9979.
14. Drennan CL, Huang S, Drummond JT, Matthews RG, Lidwig ML. 1994. How a protein binds B12: a 3.0 Å X-ray structure of B12-binding domains of methionine synthase. Science 266:1669–1674.
15. Olsen S, Ponnampalam SN, Bauer CE. 1998. CtrI bound to distant binding sites interacts cooperatively to aerobically repress photopigment biosynthesis and light harvesting II gene expression in Rhodobacter capsulatus. J. Biol. Chem. 273:18391–18396.
16. Ponnampalam SN, Bauer CE. 1997. DNA binding characteristics of CtrI. A redox-responsive repressor of bacteriochlorophyll, carotenoid, and light harvesting II gene expression in Rhodobacter capsulatus. J. Biol. Chem. 272:18391–18396.
17. Winkler A, Heintz U, Lindner R, Reinstein J, Soehman RL, Schlötter I. 2013. A ternary AppA-PpsR-DNA complex mediates light regulation of photosynthesis-related gene expression. Nat. Struct. Mol. Biol. 20: 859–867.
18. Eriksen JS, Mackenzie IR. 2008. Substrate promiscuity: normal function and role in neurodegeneration. J. Neurochem. 104:287–297.
19. Harbal R, Chen Z, James S, Bennett HP, Ni F. 1996. The hairpin stack fold, a novel protein architecture for a new family of protein growth factors. Nat. Struct. Biol. 3:747–752.
20. Schaffer MA, Fischer RL. 1988. Analysis of mRNAs that accumulate in response to low temperature identifies a thiolo protein gene in tomato. Plant Physiol. 87:431–436.
21. Watanabe H, Abe K, Emori Y, Hosoyama H, Arai S. 1991. Molecular cloning and gibberellic-induced expression of multiple cysteine proteinases of rice seeds (oryzains). J. Biol. Chem. 266:16897–16902.
22. Granell A, Harris N, Pisabarro AG, Carbonell J. 1992. Temporal and spatial expression of a thiolo protein gene during pea ovary senescence, and its regulation by gibberellin. Plant J. 2:907–915.
23. Berti PJ, Storer AC. 1995. Alignment/phyllogy of the papain superfamily of cysteine proteases. J. Mol. Biol. 246:273–283.
24. Kraft BJ, Masuda S, Kikuchi J, Dragnea V, Tollin G, Zaleski JM, Bauer CE. 2003. Spectroscopic and mutational analysis of the blue-light-photoreceptor AppA: a novel photocycle involving flavin stacking with an aromatic amino acid. Biochemistry 42:6726–6734.
25. Studier FW. 2005. Protein production by auto-induction in high density shake cultures. Protein Expr. Purif. 41:207–234.
26. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Terwilliger TC, Zwart PH, Headd JJ, Weyand G, Bricogne G, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. 2009. PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D 66:213–221.
27. Emsley P, Lohkamp B, Scott WG, Cowtan K. 2010. Features and development of Coot. Acta Crystallogr. D 66:486–501.
28. Liu M, Tanaka WN, Zhu H, Xie G, Dooley DM, Lei B. 2008. Direct hemin transfer from IsdA to IsdB in the iron-regulated surface determinant (Isd) heme acquisition system of Staphylococcus aureus. J. Biol. Chem. 283:6668–6676.
29. Tang XJ, Thibault P, Boyd RK. 1993. Fragmentation reactions of multiply-protonated peptides and implications for sequencing by tandem mass spectrometry with low-energy collision-induced dissociation. Anal. Chem. 65:2824–2834.
30. Clausen BR, Baker P, Burlingame AL. 1999. Role of accurate mass measurement (± 10 ppm) in protein identification strategies employing MS or MS/MS and database searching. Anal. Chem. 71:2871–2882.
31. Martin SA, Biemann K. 1987. A comparison of KeV atom bombardment mass spectra of peptides obtained with a two-sector mass spectrometer with those obtained from a four sector mass spectrometer. Int. J. Mass Spectrom. Ion Processes 78:213–228.