A ternary AppA–PpsR–DNA complex mediates light regulation of photosynthesis-related gene expression

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The anoxygenic phototrophic bacterium *Rhodobacter sphaeroides* uses different energy sources, depending on environmental conditions including aerobic respiration or, in the absence of oxygen, photosynthesis. Photosynthetic genes are repressed at high oxygen tension, but at intermediate levels their partial expression prepares the bacterium for using light energy. Illumination, however, enhances repression under semiaerobic conditions. Here, we describe molecular details of two proteins mediating oxygen and light control of photosynthesis–gene expression: the light-sensing antirepressor AppA and the transcriptional repressor PpsR. Our crystal structures of both proteins and their complex and hydrogen/deuterium-exchange data show that light activation of AppA–PpsR₂ affects the PpsR effector region within the complex. DNA binding studies demonstrate the formation of a light-sensitive ternary AppA–PpsR–DNA complex. We discuss implications of these results for regulation by light and oxygen, highlighting new insights into blue light–mediated signal transduction.

Organisms from all kingdoms of life are able to perceive environmental stimuli required for adaptation to their habitats. The facultatively phototrophic alphaproteobacterium *R. sphaeroides* is remarkably versatile in adjusting its energy generation to environmental cues. Aerobic respiration is its preferred mode of deriving energy. A decrease in oxygen tension activates the expression of genes that encode components of the photosynthetic apparatus. This prepares the bacterium for using photosynthesis as an alternative energy source upon oxygen depletion. At intermediate oxygen levels, however, when photosynthesis genes are partially expressed in the dark, light inhibits the formation of the photosynthetic apparatus because the combination of oxygen and photosynthesis results in photo-oxidative stress.

Light and oxygen are perceived and integrated by the AppA–PpsR regulatory system, wherein PpsR is a master repressor of photosynthesis genes and AppA a light- and oxygen-sensitive antirepressor and this has provided limited mechanistic insights into light-induced conformational changes. Therefore, we set out to obtain structural insights into the light dependence of AppA–PpsR₂ interactions and their consequences on DNA binding. Here, we present crystal structures of AppA, PpsR and an AppA–PpsR₂ core complex. On the basis of functional studies and structural analysis of light-induced changes, using hydrogen/deuterium exchange coupled to MS (HDMS), we propose a direct light-signaling mechanism through a ternary AppA–PpsR–DNA complex. We show that AppA–PpsR₂ stability is only mildly affected by blue light. Rather, this complex interacts with PpsR-binding sites on DNA to prevent formation of the PpsR–DNA repressor complex in a light-dependent manner. Together, these results highlight molecular details of the molecular mechanism and provide a new model of ligand-modulated regulation of photosynthesis genes in *R. sphaeroides*.

**RESULTS**

**Characterization of AppA, PpsR and their complex**

To understand how blue-light sensing in AppA affects DNA binding of PpsR, we generated protein constructs encompassing the domains required for protein complex formation and light-signal transduction (Fig. 1a). We addressed the oligomeric state of full-length PpsR, which is described as either a dimer or tetramer, by multianalyte light scattering (MALS) analysis coupled to size-exclusion chromatography. Quantification of the average molar mass yielded ~150 kDa (Fig. 1b), suggesting a trimer with 51 kDa per monomer. However, pronounced peak tailing and a continuous decrease of the molar-mass signal...
or octamer binding, respectively. (Fig. 1b) suggested a dynamic equilibrium of PpsR tetratomers and dimers. We quantified this transition by using microscale thermophoresis (MST; Supplementary Note) and obtained a $K_d$ of 0.9 µM for the 2 PpsR $\rightleftharpoons$ PpsR tetratomer equilibrium on the basis of PpsR concentration (Fig. 1b, inset). If not indicated otherwise, the AppA construct used throughout this study was AppAΔ C399 C20S (Supplementary Note and Supplementary Fig. 1a–d), which is subsequently referred to as AppAΔC. We quantified the AppAΔC interaction with dimeric PpsR (AppA–PpsR stoichiometry4) by using MST and determined a $K_d$ of 1.3 µM for AppAΔC–PpsR2 (Fig. 1c).

Notably, the higher kinetic stability of AppAΔC–PpsR2 (Supplementary Fig. 1c) compared to PpsR1 (Fig. 1b) that we observed upon gel filtration also suggests that association and dissociation kinetics influence the available concentration of molecular species, thereby affecting light sensing and DNA binding.

**Illumination of AppAΔC–PpsR2**

Unexpectedly, we observed no light-induced dissociation of AppAΔC–PpsR2, as described previously4, in our gel-filteration experiments with continuous illumination at AppAΔC concentrations above the $K_d$. Instead, illumination induced changes in elution volumes for AppAΔC and AppAΔC–PpsR2, indicating conformational changes of both species (Supplementary Fig. 2a). To circumvent the problem of limited light penetration into the column, we performed native PAGE under dark and light conditions (Supplementary Fig. 2b,c). These experiments confirmed the light stability of the binary complex, and we observed a retardation of AppAΔC–PpsR2 migration similar to that observed in the size-exclusion experiments (Supplementary Fig. 2a). Semiquantitative evaluation of the AppAΔC–PpsR2 affinity (Fig. 2a) showed that complex stability is only mildly affected by illumination. The effective concentration for 50% response (EC50) increased only 1.6-fold from dark (1.4 µM) to light (2.3 µM). Additional experiments...
Table 1 Data collection and refinement statistics

|                     | AppA Δ399 C20S Se-SAD* | AppA Δ399 C20S | AppA Δ399 wild type | PpsR ΔHTH Se-SAD* | PpsR ΔHTH | AppA–PpsR<sub>2</sub> core complex |
|---------------------|-------------------------|----------------|---------------------|------------------|-----------|----------------------------------|
| **Data collection** |                         |                |                     |                  |           |                                  |
| Space group         | P<sub>3</sub>12         | P<sub>3</sub>12 | P<sub>3</sub>12     | P<sub>3</sub>21     | P<sub>3</sub>21  | P<sub>2</sub>1<sub>2</sub>1<sub>2</sub> |
| a, b, c (Å)         | 70.5, 70.5, 381.5       | 70.3, 70.3, 382.1 | 70.5, 70.5, 383.8   | 117.8, 117.8, 211.0 | 117.5, 117.5, 211.8 | 89.8, 188.3, 51.9 |
| α, β, γ (°)         | 90.0, 90.0, 120.0       | 90.0, 90.0, 120.0 | 90.0, 90.0, 120.0   | 90.0, 90.0, 120.0  | 90.0, 90.0, 120.0   | 90.0, 90.0, 90.0 |
| **Refinement**      |                         |                |                     |                  |           |                                  |
| Resolution (Å)      | 0.9786                  | 1.0000         | 1.0000              | 0.9790           | 0.9483    | 0.9998                          |
| Wavelength          | 0.9786                  | 1.0000         | 1.0000              | 0.9790           | 0.9483    | 0.9998                          |
| I / σ               | 48–2.6 (2.7–2.6)        | 58–3.5 (3.6–3.5) | 60–5.5 (5.6–5.5)    | 50–2.8 (2.9–2.8)  | 50–1.75 (1.85–1.75) |                 |
| Complete (%)        | 99.7 (99.3)             | 99.7 (99.8)    | 99.6 (99.9)         | 99.9 (100)       | 99.9 (99.6) | 99.9 (99.9)         |
| Redundancy          | 20.0 (20.1)             | 5.5 (5.7)      | 4.4 (4.6)           | 21.8 (22.9)      | 5.2 (5.2)  | 8.6 (8.5)         |
| **Data collection and refinement statistics**

performed with an AppA variant (Q63E) locked in the light state further confirmed that light-activated AppA interacts with PpsR (Supplementary Fig. 2d.e).

**AppA modulates DNA binding of PpsR in a ternary complex**

Because light activation of AppA affects PpsR-mediated transcriptional regulation, we characterized the influence of AppA on the PpsR-DNA interaction. Using electrophoretic mobility shift assays (EMSAs; Supplementary Fig. 3a) we measured PpsR binding to a 70–base pair (bp) (named puc I) or 250-bp (puc II) DNA fragment, both containing the two palindromes of the <i>R. sphaeroides</i> puc gene cluster (GenBank X68796), and differing only in their extensions. Semiquantitative data evaluation showed the characteristic cooperative binding (Fig. 2b) described previously<sup>4,6</sup>. Using the Hill equation, we obtained an EC<sub>50</sub> of 1 µM AppA<sub>Δ</sub> and increasing concentrations of PpsR<sub>Δ</sub> compared to ~0.25–2.5 µM PpsR<sub>Δ</sub> and AppA<sub>Δ</sub>–PpsR<sub>Δ</sub>, respectively; Supplementary Fig. 3a compared to Fig. 2c demonstrates that the ternary complex is formed at lower PpsR concentrations than those for PpsR<sub>Δ</sub> and that AppA<sub>Δ</sub>–PpsR<sub>Δ</sub> binding to DNA shows reduced cooperativity.

Illumination reduces the affinity of AppA<sub>Δ</sub>–PpsR<sub>Δ</sub> for DNA

Whereas previous studies propose light-induced AppA–PpsR<sub>Δ</sub> dissociation<sup>4</sup>, our new results indicating only a subtle light response of the binary complex. A comparison of the transitions from free to bound DNA (~0.5–1.5 µM compared to ~0.25–2.5 µM PpsR for PpsR<sub>Δ</sub> and AppA<sub>Δ</sub>–PpsR<sub>Δ</sub>, respectively; Supplementary Fig. 3a compared to Fig. 2c) demonstrated that the ternary complex is formed at lower PpsR concentrations than those for PpsR<sub>Δ</sub> and that AppA<sub>Δ</sub>–PpsR<sub>Δ</sub> binding to DNA shows reduced cooperativity.

To characterize the influence of AppA on DNA-binding properties of PpsR, we tested different concentrations of AppA<sub>Δ</sub>. Below 1 µM AppA<sub>Δ</sub>, the apparent affinity of PpsR for puc I was slightly reduced compared to that of PpsR in the absence of AppA<sub>Δ</sub>, as reported previously<sup>4,6</sup>. Of note, we observed an additional faint band, and increasing the AppA<sub>Δ</sub> concentration to 1.5 µM resulted in an increase in intensity of this newly observed species that indicated the formation of a ternary AppA<sub>Δ</sub>–PpsR–puc I complex (Fig. 2c and Supplementary Fig. 3d). Even an excess of PpsR was not able to compete with AppA<sub>Δ</sub>–PpsR<sub>Δ</sub> for DNA, which suggests a higher affinity or higher kinetic stability of the ternary complex. A comparison of the transitions from free to bound DNA (~0.5–1.5 µM compared to ~0.25–2.5 µM PpsR for PpsR<sub>Δ</sub> and AppA<sub>Δ</sub>–PpsR<sub>Δ</sub>, respectively; Supplementary Fig. 3a compared to Fig. 2c) demonstrated that the ternary complex is formed at lower PpsR concentrations than those for PpsR<sub>Δ</sub> and that AppA<sub>Δ</sub>–PpsR<sub>Δ</sub> binding to DNA shows reduced cooperativity.
Figure 3 Domain organization of AppA supports the dual sensor function. (a) Secondary structure representation of AppA with individual domains color-coded according to Figure 1a. (b) Overall AppAΔC structure with domains colored according to panel a. (c) Stereo view of conserved BLUF residues around the flavin cofactor (yellow) showing the positioning of Trp104. Residues contacting Trp104 from the core β-sheet and the capping helix are shown as stick models. (d) AppAΔC structure colored with respect to deuterium incorporation in the dark after 15-s labeling. Implications of the color-coding and details for the normalization procedure (Dnorm) are described in Supplementary Note. (e) Changes in relative deuterium incorporation between dark- and blue-light conditions after 15 s labeling. The overall structure of AppAΔC is colored according to the observed changes in deuterium uptake between light- and dark-states. ΔDrel, absolute difference in relative deuterium incorporation. Shades of red correspond to elements with an increased deuterium uptake in the light state, representing a destabilization of secondary structure or deprotection due to loss of interaction. Shades of blue indicate stabilization upon complex formation or an increase in secondary-structure stability. Full details of all comparative experiments are shown in Supplementary Movies 1–6.

PpsR (5 µM AppAΔC and 13 µM PpsR) for binding (Fig. 2e). This again highlights the importance of the kinetic stability of different species involved in the light response. A detailed analysis of ternary-complex illumination at AppAΔC concentrations (20 µM) enabling formation of AppAΔC–PpsR2 throughout the PpsR titration range (0–30 µM; Fig. 2f) showed that stable ternary-complex formation occurs only at one order of magnitude higher PpsR concentrations compared to those under dark conditions (Fig. 2c). However, a quantitative comparison is complicated because the cooperativity of DNA binding and the kinetics of ternary complex disassembly are affected by illumination.

The AppAΔC structure supports the dual-sensor model

To identify elements affected by illumination, we solved the crystal structure of AppAΔC to 2.6-Å resolution (Table 1), revealing molecular details beyond the BLUF domain. (Schematic and cartoon representation of the overall structure is shown in Fig. 3a, b and Supplementary Fig. 4.) The core BLUF domain closely resembles the structure of the isolated domain (r.m.s. deviation 0.3 Å to AppA BLUF 1–124 (ref. 16)). In particular, strand βB_{AppA} (in notation in which subscripts denote the domain and the protein: B, BLUF; H, 4HB; S, SCHIC; A, AppA; N, N domain; I, PAS1; 2, PAS2; P, PpsR) displays a kink corresponding to the tryptophan-out conformation16. Notably, Trp104 is sandwiched between the core β-sheet of the BLUF domain and an amphipathic helix (amino acid (aa) 141–162) capping this β-sheet (Fig. 3c). To exclude that the C20S substitution causes the tryptophan-out orientation, we solved the AppAΔC99 wild-type structure (PDB 4HH1) showing an identical Trp104 conformation. The linker between the BLUF and SCHIC domains consists of the BLUF capping helix, some stretches without secondary-structure elements and a four-helix bundle (4HB). The SCHIC domain has a flavodoxin-like fold, as expected from its relationship with the cobalamin-binding superfamily7. Structural details of the SCHIC domain are described (Supplementary Note and Supplementary Fig. 4b). A notable feature of the AppAΔC structure is the weak interaction between the BLUF and SCHIC domains. A surface representation (Supplementary Fig. 4c) suggests that both domains use the linker region and the 4HB as ‘binding platforms’ without strong interactions at the BLUF-SCHIC interface. This is in line with the current view that AppA integrates two stimuli and communicates them to PpsR.

Illumination affects central elements of the BLUF domain

Comparative HDX experiments performed under identical experimental conditions for two states of a protein provide information on structural differences. Therefore, HDX is ideally suited for complementing crystallographic studies in terms of AppA–PpsR2 complex formation and illumination. In addition, differences in deuterium exchange kinetics correlate with secondary-structure stability (Supplementary Note and Fig. 3d).

In the overall structure of AppAΔC (Fig. 3e), changes in deuterium uptake at initial time points are restricted to the BLUF domain and the capping helix. A substantial stabilization upon illumination is observed for the (α1–β2)β_{AppA} element, involving Ser41 and Asn45, which interact with the flavin cofactor, and the βB_{AppA} strand containing Met106. Notably, the central part of the capping helix becomes slightly destabilized upon illumination, which suggests its involvement in light-signal integration.
The importance of PAS domains for oligomerization of PpsR

To obtain a better understanding of PpsR, we set out to determine its structure. We crystallized a construct lacking the HTH motif (PpsR\text{\textsubscript{\text{AMTH}}}; Fig. 4a) and determined the structure to 2.8-Å resolution (Table 1). This revealed a tetrameric assembly of the triple PAS protein in the asymmetric unit (Fig. 4b), which is composed of two antiparallel dimers, each with a parallel dimerization interface ranging from the N domain through the α-helical Q linker (αQ) to the PAS1 domain. Although the PAS2 domains also form a homodimer, the overall dimer symmetry is broken, owing to interaction of the PAS2 domains with αQ of the other dimer. On the basis of the C termini of the PAS2 domains, the position of the HTH motif is expected to be close to αQ of the second dimer (Fig. 4c and Supplementary Fig. 5), and this might contribute to the strong evolutionary conservation of this region (Supplementary Fig. 6a). Although structural rearrangements upon DNA binding cannot be excluded, the distant positioning of two HTH dimers in the tetramer is unlikely to cause efficient binding of puc palindromes that are separated by a half turn of the DNA double helix. Notably, αQ provides another antiparallel oligomerization interface with a symmetry-related tetramer, thereby forming an octameric assembly (Supplementary Fig. 5a,b). In this case, the proposed location of the HTH motifs would be ideally positioned for DNA binding (Fig. 4c), and this explains the cooperativity and the 1:8 stoichiometry of puc-PpsR binding, as derived from our active site titration.

AppA\textsubscript{AC}–PpsR\textsubscript{2} formation affects elements of light signaling

We addressed structural changes of AppA\textsubscript{AC}–PpsR\textsubscript{2} formation and illumination by HDX (Fig. 4d–g). The complex formation–induced changes in deuterium exchange, as mapped onto the structures (Fig. 4d,f), show a pronounced stabilization of the 4HB and the BLUF capping helix in AppA\textsubscript{AC}. In addition, the N-terminal region of SCHIC, extending from the linker to the 4HB through (β1, α1, β2 to α2)\textsubscript{β\_A}, experiences a reduction in deuterium exchange upon complex formation. Several of these elements show above-average deuterium incorporation in free AppA\textsubscript{AC} (Fig. 3d). Notably, not only the capping helix but also additional light-responsive BLUF elements show changes upon complex formation. Whereas the (α1–β2)\textsubscript{B\_A} region is destabilized in the presence of PpsR, the (β3–capping helix)\textsubscript{B\_A} strand shows a similar stabilization to that observed upon illumination. In addition, the (β1–α1)\textsubscript{B\_A} and (β5–capping helix)\textsubscript{B\_A} loop regions become stabilized upon complex formation.

Deuterium-incorporation characteristics of PpsR alone (Supplementary Fig. 6b) demonstrated that, similarly to AppA, regions involved in complex formation (Fig. 4f) belong to elements with above-average deuterium incorporation. This is most pronounced for parts
of the Q-linker region that exhibit bimodal deuterium-incorporation characteristics (EX1 kinetics\(^2\)) reflecting complex dissociation during labeling (Supplementary Fig. 6c). This extreme form of deuterium exchange is caused by a faster chemical exchange rate of free PpsR compared to the reassociation kinetics of the protected AppA\(_{AC}\)-PpsR\(_2\) species. This is supported by HDX experiments performed with different AppA concentrations (Supplementary Fig. 6d–f). Analysis of the titration curve, using the law of mass action, provided a \(k_2\) estimate of \(-1.5\ \mu\text{M}\), in agreement with our MST data. We observed additional elements with pronounced stabilization in the N domain, including loops around \(\alpha_{1N_p}\) and \(\alpha_{3N_p}\). Similarly, \(\beta_{41_p}\) and \(\beta_{51_p}\) and their connecting loop become stabilized upon complex formation. Notably, the PAS2 domain behaves oppositely, and complex formation leads to desaturation of the \(\alpha_{32_p}\), \(\beta_{42_p}\), and \(\beta_{52_p}\) elements. Given the AppA–PpsR\(_2\) stoichiometry, this supports a role of this region in PpsR homotetramerization as seen in the crystal structure (Fig. 4b). In addition, this suggests that an interaction interface of AppA on \(\alpha Q\) prevents PpsR oligomerization by blocking the PAS2-binding site. The HTH region (Fig. 5a–e) shows merely subthreshold stabilization upon complex formation, and this indicates that AppA\(_{AC}\) does not prevent DNA binding of PpsR by interacting with the HTH motif.

HDX measurements of light-adapted AppA\(_{AC}\)-PpsR\(_2\) additionally support the notion that illumination does not lead to complex dissociation (Fig. 4e,g), owing to sustained stabilization of \(\alpha Q\) and the N and PAS1 elements described above. \(\alpha Q\) shows only \(-10\%\) of the stabilization upon complex formation, and this can be explained with a small, light-induced reduction in AppA–PpsR\(_2\) concentration due to experimental conditions limiting light-independent saturation of the binary system (Fig. 2a). Therefore, the N–Q–PAS1 domains of PpsR form a light-independent core-binding interface with AppA. The PAS2 domain, in contrast, shows partial reversibility of the changes observed upon complex formation. Moreover, the C-terminal HTH motif is pronouncedly stabilized and shows EX1-like kinetics for peptides from this region (Fig. 5b). The observation of a bimodal distribution points to the presence of an AppA\(_{AC}\)-PpsR\(_2\) species with the HTH motif in a conformation different to dark-adapted complex or free PpsR. This might originate either from a light-state AppA-induced HTH dimerization or from interaction with light-responsive AppA elements.

HDX changes induced by illumination of PpsR-complexed AppA\(_{AC}\) are clustered around the BLUF domain and partially correspond to observations of isolated AppA\(_{AC}\). The \((\alpha_1\beta_2)_{\alpha_1}\) region shows substantial stabilization, whereas the central part of the capping helix partially reverts to a more flexible state (Fig. 5f–j). Apart from these changes, only the region between the pairs of helices in the 4HB (aa 213–222) is destabilized. The remaining elements in the 4HB and the SCHIC domain are unaffected by illumination and therefore belong to the core interface defined for PpsR above. Notably, the observation of light-responsive elements from the BLUF core through the capping helix to PAS2 and the DNA-binding HTH motif provides a basis for direct light regulation in the binary complex.

The complex structure suggests BLUF and PAS2–HTH proximity

On the basis of core-complex regions identified by HDX, we generated corresponding constructs of AppA (4HB–SCHIC) and PpsR
Figure 6 Molecular details of the AppA–PpsR complex. (a) Overall structure of the core complex, color-coded according to domains of AppA (Fig. 3b) and PpsR (Fig. 4c). (b) Superposition of HDX data with the observed complex interface. Data of the 15-s time points (as in Fig. 4d,f) are mapped on the core complex with N–Q–PAS1 shown in transparent surface and the AppA domains in cartoon representation. The full time course can be seen in Supplementary Movie 6. (c) Model of the AppA–PpsR complex, based on the individual structures, with alignment of the PAS1 dimers of PpsRHTH and the 4HB and SCHIC of AppA, respectively. The HTH domains are placed in analogy to Figure 4c.

We crystallized their complex and determined its structure to 1.75-Å resolution (Table 1). The observed assembly matches the AppA–PpsR stoichiometry and confirms the binding of AppA to αQ (Fig. 6a). Additional interactions of AppA with the N and PAS1 domains of different PpsR protomers lead to a pronounced rotation of ~70° of the PAS1-domain dimer along the αQ axis and explain the observed asymmetry of the complex. Details of the complex interface accord well with our HDX results (Fig. 6b). The pronounced stabilization in the (α3–β3)N–P region can be explained by the observed contact with (α3–β4)A. Similarly, αQ interacts with αH3–A and the linker region to the SCHIC domain including (α1 and β2)H–A. In addition, (β4–β5)L–P is sandwiched between the connecting loop of the 4HB to the SCHIC domain and (α1–β2–α2)H–A. The interactions observed in the core complex fully explain the stabilization of all PpsR elements observed by HDX, suggesting that the BLUF domain does not interact extensively with PpsR. Rather, we propose that AppA elements that are stabilized upon complex formation but do not contact PpsR, that is, (α1–α2)H–A and the BLUF capping helix, are stabilized internally. Notably, these elements are also in proximity in the AppA crystal structure. Combined with the observation that the arrangement of the 4HB and the SCHIC domain is almost identical in the isolated and complex structures (r.m.s. deviation 0.6 Å), it is likely that the AppA structure resembles that of AppA stabilized upon PpsR binding. We therefore combined the complex structure with the PpsRHTH and AppA crystal structures (Fig. 6c).

In this model, the BLUF domain approaches the PAS2–HTH region of PpsR, which can explain the direct transmission of light-induced changes from the photoreceptor to the PAS2–HTH region that we observed by HDX (Supplementary Figs. 7 and 8). In addition, the close positioning of the BLUF domain and the DNA-binding element is probably responsible for the light-induced destabilization of the ternary complex observed in EMSAs.

DISCUSSION

The AppA–PpsR system serves as a master regulator of R. sphaeroides photosynthesis genes in response to oxygen and light. Our studies on AppA, PpsR and their cognate DNA provide detailed functional and structural insights for both proteins and their complexes, with implications for their biological function. The crystal structure of the transcriptional repressor PpsR reveals an intricate tetrameric assembly composed of two head-to-tail PpsR dimers. Both the N and the PAS1 domains form homodimers between which αQ forms a coiled-coil-like structure that serves as binding site for either PAS2 domains of another PpsR dimer, which would lead to tetramer formation, or the AppA light sensor, which would result in formation of an AppA–PpsR complex. The relevance of the PpsR tetramer is supported by the evolutionary conservation of the interaction between α3–P and αQ. However, the tetramer architecture cannot explain the highly cooperative DNA-binding mode observed for PpsR, because of the large distance between HTH dimers. In addition, active site titration data support an octameric PpsR species for DNA binding. Such an assembly is mediated by αQ of symmetry-related tetramers, and it brings two HTH dimers into proximity, thus allowing them to cooperatively bind target promoter sequences. The DNA binding of PpsR octamers was also proposed for homologs from Bradyrhizobium [21]. The importance of these oligomeric states is further supported by the requirement of the N domain for DNA binding of PpsR in vivo [9] and in vitro [11]. On the basis of the PpsR structure, this requirement can be rationalized by the impaired dimer and tetramer formation of constructs lacking the N domain, as suggested previously [11], a consequence that would also affect octamer formation.

In R. sphaeroides, the action of PpsR is modulated by the antirepressor AppA. The two proteins form a complex in vitro and in vivo [4] to enable light- and oxygen-dependent regulation of gene expression. Previously, it was suggested that photon absorption by the AppA BLUF domain triggers dissociation of AppA–PpsR2 (ref. 4). This model is based on gel-filtration data in which AppA, PpsR and their complex are observed in comparable quantities, a result indicating experimental conditions close to the Kd of the complex. In this case, even subtle changes in affinity would lead to pronounced differences in the relative amounts of involved species. This interpretation is supported by the reduced, but not eliminated, fraction of complex upon illumination [4]. Analogous experiments using AppA concentrations above the Kd of AppA–PpsR2 resulted in no dissociation; moreover, native PAGE and HDX data also did not support substantial light-induced dissociation of the complex. Rather, our data indicate a small light-dependent decrease of AppA–PpsR affinity. By combining structural and HDX data, we identified a light-independent core-binding interface consisting of the 4HB and the SCHIC domain of PpsR.
AppA and the N–Q–PAS1 region of PpsR. Illumination influences the BLUF domain and capping helix of AppA and the HTH motif of PpsR, which supports a light-signaling pathway through allosteric structural changes. This hypothesis is supported by the fact that the AppA–PpsR2 complex also binds the PpsR-binding sites on DNA. This binding indicates structural ‘preorganization’ of AppA and PpsR in their complex, which would promote ternary complex formation and thereby reflect a form of configurational cooperativity. In contrast to the original binary description of PpsR binding to DNA as a repressor, the introduction of a third component enables different properties of potential DNA complexes (PpsR2–DNA compared to AppA–PpsR2–DNA), and this probably reduces repressive strength and thereby promotes photosynthesis-gene expression. Furthermore, light modulation of AppA–PpsR2–DNA affinity in addition to the subtle light-induced dissociation of AppA–PpsR2 potentially enhances in vivo control. In particular, the concentration-dependent competition of the ternary complex with DNA binding of PpsR provides a fine-tunable control system responding to illumination over a wider range of protein concentrations. Notably, AppA and PpsR expression levels are inversely regulated in response to changing oxygen levels. This results from the interplay between the PrrB–A and AppA–PpsR regulatory cascades in which the transcriptional regulator PrrA was shown to positively affect AppA expression upon transition from aerobic to anaerobic conditions. These oxygen-induced changes in concentration may be sufficient to explain in vivo regulation in response to changing light and oxygen levels by the ternary system. Because the cellular concentrations of AppA and PpsR are not known, we cannot exclude a regulatory mechanism based on complex dissociation at concentrations close to the Kd under in vivo conditions. However, ternary-complex formation occurs at lower concentrations of PpsR and therefore increases the potential for transcriptional regulation as described above. In the new model, the light-dependent repressive effect of AppA under semiaerobic conditions is explained by the interference of BLUF elements with the HTH motif of PpsR. This reduces AppA–PpsR2 affinity for DNA and enables excess PpsR to bind promotor sequences (Fig. 7). This mechanism is consistent with observations under anaerobic conditions in which light-induced repression was demonstrated for a strain lacking the PrrB activator.

Our study describes the first detailed structural characterization, to our knowledge, of a BLUF protein in complex with its noncovalent effector. We revealed molecular details of their interaction interface and also provided structural details of the linker region C-terminal to the BLUF domain and its importance for light-regulated modulation of DNA binding. Our study contributes to a better understanding of the modularity and details of the signaling process of the BLUF photoreceptor family. Previously published crystal structures of the AppA BLUF domain show two different conformations of strand β5 that result in either Trp104 (ref. 15) or Met106 (ref. 16) being positioned in the vicinity of the flavin chromophore, a result that might originate from the use of different protein constructs. These observations suggested different mechanisms for the photoreaction and spurred a controversy over the dark-state structure of AppA and other BLUF proteins (reviewed in ref. 27). Our AppAAC structure confirmed that the dark-state conformation features Met106 close to the flavin cofactor and Trp104 in the ‘out’ conformation in which it contacts residues of the newly observed BLUF capping helix, which interacts with residues of β5 and the BLUF core β-sheet. HDX experiments showed that these structural elements are affected by illumination, which is in line with previous NMR experiments and theoretical and spectroscopic studies on the AppA BLUF domain.

Combined with information from other BLUF proteins, common aspects of light-induced changes in the vicinity of the flavin cofactor are emerging that suggest that the β-sheet (especially β5) and the C-terminal extensions of BLUFs are important for signal transduction (reviewed in ref. 27). Because HDX is not limited by the size of the system, we also addressed the structural changes in AppA–PpsR. Our results showed that complex formation is a prerequisite for structuring the C-terminal extension of BLUF, which experiences a pronounced destabilization upon illumination. This increased flexibility enables the BLUF domain to interfere with DNA binding of the complex, which is in line with in vivo data demonstrating restoration of light signaling upon complementation of a system containing BLUF-free AppA with BLUF provided in trans.

The requirement of a preformed complex for light signaling is of general interest not only for BLUF proteins but also for other photoreceptor families. So far, one system comprising a BLUF and an effector domain was characterized structurally and functionally: the light-regulated cyclic di-GMP phosphodiesterase BlrP1 (ref. 30). Although some elements involved in transmitting the signal to the effector domain are conserved between BlrP1 and AppA, the arrangement of the C-terminal extensions of the BLUF domains differ substantially. BlrP1 represents a BLUF system that is covalently tethered to its effector; however, the majority of BLUF domains signal through noncovalent interaction. One such system, the BLUF protein PixD and its partner PixE, involved in phototaxis control, has been the...
subject of detailed molecular and physiological characterization. Common aspects of light signaling in the family have, so far, not been described, and details of the noncovalent interactions are not known for the majority of BLUF proteins. However, knowledge of the complex interface appears to be critical for addressing structural changes and elucidating how the light signal is transmitted to the effector. The structural characterization of AppA–PpsR2 not only provides new information in this direction but also highlights an important caveat. Crystal structures obtained from isolated domains need to be interpreted with caution because effector-binding regions may be unstructured without an interaction partner.

Combining these results with other currently investigated BLUF systems will allow a separation of system-specific aspects of signal transduction from common features involved in BLUF signaling, and this will be useful in the rational design of artificial BLUF-based photosensors with application in the growing field of optogenetics.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Coordinates and structure factors have been deposited in the Protein Data Bank, with accession codes 4H1H for AppA<sub>4A</sub>, 4HH1 for wild-type AppA<sub>4A</sub> A399, 4H2H for PpsR<sub>4H2</sub> and 4H3H for the AppA–PpsR<sub>2</sub> core complex.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

We acknowledge K.-A. Seifert for excellent technical support. We thank the Dortmund and Heidelberg team for data collection at beamline X10SA (Swiss Light Source, Villingen, Switzerland). We are grateful to A. Meinhart for help with structural data analysis and EMSAs and C. Roome for IT support. We thank M. Müller and M. Gradl for assistance in MS. We are grateful to M. Gomelsky and M. Cury for stimulating discussions and comments on the manuscript and to P. Hegemann (Humboldt University, Berlin, Germany) for providing the pET28 AppA<sub>4A</sub> plasmid. We thank G. Stier (Biochemistry Center, Heidelberg University, Heidelberg, Germany) for providing the pET_M11 plasmid and the TEV protease clone. We acknowledge financial support by the European Molecular Biology Organization (EMBO) (ALTF 1309–2009 to A.W.), the Austrian Science Fund (FWF) (J 3242–B09 to A.W.), the German Research Foundation (FOR526 to I.S.) and the Max Planck Society.

AUTHOR CONTRIBUTIONS

A.W. and I.S. designed the project. A.W., U.H. and R.L. cloned, expressed and purified AppA variants. U.H. cloned, expressed and purified the PpsR constructs. A.W., U.H. and J.R. performed and interpreted photocycle, MST and MAL5 experiments. A.W. carried out EMSAs as well as HDX-MS, crystallized the AppA variants and solved their structure. U.H. crystallized PpsR and the AppA–PpsR<sub>2</sub> core complex, and A.W. and U.H. determined the corresponding structures. A.W., R.L., J.R. and R.S. analyzed and interpreted HDX data. A.W. and I.S. wrote the manuscript. All authors discussed the results and the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Protein expression and purification. We generated AppA constructs by PCR amplification from wild-type pET28_AppA (provided by P. Hegemann). For Ni-NTA-based affinity purification, a C-terminal hexahistidine tag was included in reverse primers for AppA constructs. Primers for amplifying the full-length, Δ399 and ΔHβ–SCHIC domain AppA variants are described in Supplementary Table 1. The C20S mutation was introduced in pET21_AppA_full-length and pET28_AppA Δ399; the Q63E mutation was generated in pET28_AppA Δ399. In both cases, site-directed mutagenesis was performed according to the QuickChange method (Agilent; primers in Supplementary Table 1), and correct clones were identified by DNA sequencing.

An E. coli codon-optimized gene (GeneArt) served as the template for amplification of different PpsR constructs. We generated PCR products representing full-length PpsR constructs, the ΔH7M and the N–Q–PAS1 construct (primers in Supplementary Table 1). The PCR products were cloned into pET_M111 (provided by G. Stier) to allow for use of the TEV-cleavable N-terminal histidine tag during purification.

Protein expression was performed in E. coli BL21 (DE3). Cultures were incubated with IPTG at an optical density of 0.6–0.9. All constructs except full-length AppA were expressed at 18 °C overnight in the presence of 0.2 or 0.5 mM IPTG for AppA or PpsR, respectively. Full-length AppA was expressed at 24 °C for 6 h with 0.2 mM IPTG. All AppA experiments from the point of induction onward were carried out under safe-light conditions, if not stated otherwise. Cells were harvested by centrifugation and the pellets resuspended in buffer A (20 mM CHES, pH 9, 10 mM imidazole, 300 mM NaCl and 5% (w/v) glycerol) including EDTA-free protease inhibitor (Roche). We performed cell lysis by using a microfluidizer (Microfluidics) and clarified lysates by ultracentrifugation at 180,000g before Ni-NTA chromatography. The column was washed with buffer A containing 50 mM imidazole and the protein eluted in buffer B with 200 mM imidazole.

AppA fractions incubated with excess FMN were concentrated with Amicon centrifugal filter units and loaded onto a Superdex 200 10/300 GL column equilibrated in buffer B (10 mM CHES, pH 9, 150 mM NaCl and 5% (w/v) glycerol). After elution from the Ni-NTA column, PpsR constructs were dialyzed overnight against buffer C (10 mM CHES, pH 9, 100 mM NaCl, 2 mM EDTA and 5% (w/v) glycerol) with parallel TEV cleavage with a ratio of ~1:25 for TEV/substrate. For removal of histidine-tagged TEV and the cleaved histidine tag, the dialyzed sample was reloaded onto the Ni-NTA column and the flow through used for further purification. Full-length PpsR was loaded onto a HiTrap heparin column (GE Healthcare) and eluted with a NaCl gradient in the buffer system used for dialysis. All proteins were finally purified by size-exclusion chromatography in analogy to AppA.

Selenomethionine-substituted proteins were expressed in E. coli BL21 (DE3) with modified growth medium according to ref. 35 before Ni-NTA chromatography. The column was washed with buffer A containing 50 mM imidazole and the protein eluted in buffer B with 200 mM imidazole. AppA fractions incubated with excess FMN were concentrated with Amicon centrifugal filter units and loaded onto a Superdex 200 10/300 GL column equilibrated in buffer B (10 mM CHES, pH 9, 150 mM NaCl and 5% (w/v) glycerol). After elution from the Ni-NTA column, PpsR constructs were dialyzed overnight against buffer C (10 mM CHES, pH 9, 100 mM NaCl, 2 mM EDTA and 5% (w/v) glycerol) with parallel TEV cleavage with a ratio of ~1:25 for TEV/substrate. For removal of histidine-tagged TEV and the cleaved histidine tag, the dialyzed sample was reloaded onto the Ni-NTA column and the flow through used for further purification. Full-length PpsR was loaded onto a HiTrap heparin column (GE Healthcare) and eluted with a NaCl gradient in the buffer system used for dialysis. All proteins were finally purified by size-exclusion chromatography in analogy to AppA.

Static light scattering. AppA and PpsR (25 µM) were preincubated at 4 °C for 30 min and subjected to size-exclusion chromatography at RT on a Superdex 200 10/300 GL column (GE Healthcare) equilibrated in buffer B. The chromatography system was connected to an 18-angle light-scattering detector (Dawn Heleos, Wyatt Technology) combined with a refractive-index detector (Waters). We included parallel illumination from a royal-blue (455 nm) collimated LED lamp (Thorlabs). For the latter purpose, samples were preirradiated for 2 min at 600 µW cm⁻² blue light in the slot, and during electrophoresis (10 V cm⁻¹ for 160 min) constant illumination of the gel was kept at 30 µW cm⁻². Gels were stained with GelRed (Biotium) for DNA visualization, then proteins were stained with InstantBlue (Biozol). For the active site titration, we titrated PpsR (2.5 µM) with 100 to 700 nM puc II. The gel was run at RT for 70 min at 10 V cm⁻¹.

Crystallization and structure elucidation. Crystallization was performed at 20 °C. Trigonal crystals of AppA Δ399 wild type and C20S were grown in hanging-drop geometry with 15 mg ml⁻¹ protein and 0.3 M Tris–Cl, pH 7.4, 1.3 M NaCl and 0.3 M MgCl₂ as reservoir. Growth of selenomethionine-labeled AppA Δ399 included 50 mM DTE in the reservoir. Crystals were harvested after a 2-h soak in reservoir solution saturated with NaCl and cryocooled in liquid nitrogen.

Trigonal PpsR AHTH crystals were grown in hanging-drop vapor-diffusion setups with 0.1 M Tris–Cl, pH 8.5, 1 M ammonium sulfate, 12% (w/v) glycerol, 6% (w/v) xylitol and 1% dioxane as reservoir and protein at 30 mg ml⁻¹. Crystals were harvested after 2 weeks and cryocooled after cryoprotection in 50 mM Na-P₆ buffer, pH 6.8, 12% (w/v) glycerol and 3 M Na malonate. Selenomethionine-labeled protein was crystallized accordingly.

The complex of PpsR N–Q–PAS1 and the AppA ΔHβ–SCHIC was crystallized in sitting-drop vapor-diffusion geometry with a solution of 0.1 M HEPES, pH 7, 64 mM trisodium citrate, 10% (w/v) PEG 5000 monomethyl ether was mixed with the preformed complex at a concentration of 8.3 mg ml⁻¹ (1:5 stoichiometry of AppA/PpsR). For cryoprotection, reservoir solution containing 20% glycerol was added to the drop. After 1-min incubation, crystals were harvested and cryocooled in liquid nitrogen.

Diffraction data were acquired at 100 K at beamline X10SA at the SLS (PSI, Villingen, Switzerland). Data were processed with XDS56. Phasing and refinement were performed with PHENIX7. Refinement included an initial simulated annealing (torsion) step followed by several rounds of maximum-likelihood least-squares refinement of models modified with Coot38 using σα-weighted 2mFo–DFo and Fc–Fo electron density maps. Details of the refinement procedures are presented in Supplementary Note. Data collection, processing and refinement statistics are summarized in Table 1.

Hydrogen/deuterium-exchange mass spectrometry. We incubated separately purified AppA ΔM and PpsR at 4 °C for 30 min, using a 1:1-fold excess of AppA on the basis of the AppA–PpsR stoichiometry. Purification of AppA ΔM–PpsR was performed on a Superdex 200 10/300 GL column, and the pooled complex fractions were concentrated to 200 µM AppA ΔM–PpsR. We prepared AppA ΔM in analogy at a concentration of 200 µM, and PpsR samples were set up at a final concentration of 400 µM. We performed deuterium labeling in triplicate after a 90-s preincubation at 20 °C, then diluted the reactions by 20-fold in D₂O with 10 mM CHES, 150 mM NaCl, 5% glycerol (d3), pH 9.5. For complex measurements, this resulted in ~75% of AppA ΔM bound to PpsR in equilibrium. For blue-light measurements, we included parallel illumination from a royal-blue (455 nm) collimated LED lamp (Thorlabs) for the preincubation step and during labeling to provide a light intensity of 600 µW cm⁻² at 450 nm. Aliquots of 20 pmol (complex and AppA ΔM) or 40 pmol for PpsR were removed after 15 s, 1, 5 and 20 min and quenched in ice-cold 200 mM ammonium formate acid buffer, pH 2.6. We then injected the samples into a cooled HPLC setup (0.5 °C). Deuterated samples were digested on a pepzin column (Applied Biosystems) at 10 °C. Resulting peptides were desalted on a 2-cm C18 guard column (Discovery Bio C18, Supelco) and separated in the presence of 0.6% formic acid with a 20-min acetonitrile gradient (15 to 50%) on a reversed-phase column (Discovery Bio Wide Pore C18 10 × 0.1 cm, 3 µm) and injected into a maxISI ESI-UVH-TOF (Bruker) for measurement of deuterium incorporation. Details of data evaluation are in Supplementary Note.35

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