The structure of a complete phytochrome sensory module in the Pr ground state

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Phytochromes are red/far-red photochromic biliprotein photoreceptors, which in plants regulate seed germination, stem extension, flowering time, and many other light effects. However, the structure/functional basis of the phytochrome photoswitch is still unclear. Here, we report the ground state structure of the complete sensory module of Cph1 phytochrome from the cyanobacterium *Synechocystis* 6803. Although the phycocyanobilin (PCB) chromophore is attached to Cys-259 as expected, paralleling the situation in plant phytochromes but contrasting to that in bacteriophytochromes, the ZZZssa conformation does not correspond to that expected from Raman spectroscopy. We show that the PHY domain, previously considered unique to phytochromes, is structurally a member of the GAF (cGMP phosphodiesterase/adenylyl cyclase/FhlA) family. Indeed, the tandem-GAF dumbbell revealed for phytochrome sensory modules is remarkably similar to the regulatory domains of cyclic nucleotide (cNMP) phosphodiesterases and adenylyl cyclases. A unique feature of the phytochrome structure is a long, tongue-like protrusion from the PHY domain that seals the chromophore pocket and stabilizes the photoactivated far-red-absorbing state (Pfr). The tongue carries a conserved PRxSF motif, from which an arginine finger points into the chromophore pocket close to ring D forming a salt bridge with a conserved aspartate residue. The structure that we present provides a framework for light-driven signal transmission in phytochromes.

biliprotein | photochromicity | photoreceptor | protein structure | sensory histidine protein kinase

Phytochromes are a family of red/far-red photochromic biliprotein photoreceptors known in plants (1), cyanobacteria (2, 3), fungi (4), and nonphotosynthetic bacteria (5). In plants, phytochromes are the principal photoreceptors regulating light-dependent seed germination, seedling deetiolation and flowering, thus mediating the most radical environmental effects on development known in nature. Photochemical activation of the module in the red-absorbing ground state (Pr) begins with a Z→E isomerisation (photoflip) of the D ring of the bilin chromophore within picoseconds of photon absorption (6, 7). However, the mechanism underlying the subsequent intramolecular signal transduction is unclear.

The discovery of prokaryotic phytochromes (2, 3, 5) was important for two reasons. First, the domain map they provided for phytochromes as a whole revealed their origins as sensory histidine protein kinases (SHPKs), molecules extensively used in “two-component” perception systems in prokaryotes but also known in fungi and plants. Second, they could be produced at high purity in large amounts by means of overproduction in *Escherichia coli*, enormously facilitating biophysical studies in the context of molecular genetics. Whereas in bacteriophytochromes (Bphs) the chromophore is attached to a Cys residue close to the N terminus (8), plant phytochromes and cyanobacterial phytochromes like Cph1 from *Synechocystis* 6803 attach their chromophores within the central GAF domain of the sensory module (9, 10) (Fig. 1A). Consequently, Cph1 represents a valuable evolutionary link between Bphs and plant phytochromes.

Pioneering x-ray crystallographic studies of the ≈35-kDa PAS (Period/Arnt/Single-minded)–GAF bidomain of BphP from *Deinococcus radiodurans* provided an important insight into phytochrome 3D molecular structure and function (11). In particular, unexpected aspects of the chromophore conformation and microenvironment were revealed in addition to an unusual knot formed by the N terminus and a loop extending from the GAF domain. However, functional interpretation of this and subsequent PAS–GAF structures (12, 13) is compromised by the fact that these molecules are dysfunctional: whereas the Pr ground state is spectrally similar to the native molecule, a stable Pfr state does not arise after photon absorption. It seems that Pfr absolutely requires a functional PHY domain, as the complete sensory module (PAS–GAF–PHY tridomain) of Cph1 is photochemically identical to that of the native molecule, whereas mutations affecting the PHY domain often lead to ≈10-nm hypsochromic shifts and/or compromise photochromicity (10, 14–17).

Here, we report the 3D structure of the Cph1 sensory module in the Pr ground state, revealing details of the PHY domain and its likely important role in signal transduction. This domain shows clear structural similarity to the GAF superfamily; thus, phytochromes are “tandem GAF” proteins resembling phosphodiesterases and adenylyl cyclases. Also, the new structure shows a chromophore conformation similar to that described for bacteriophytochromes, quite different from that predicted from Raman spectroscopy (18). We discuss the implications of the structure for the changes likely to be associated with photoactivation.

Results and Discussion

We purified the holoprotein in its Pr ground state by affinity and size-exclusion chromatography and identified appropriate crystallization conditions. UV-Vis absorbance spectra of Cph1 in the crystalline state resemble those of solutions (Fig. 1D), implying that the structure indeed represents that of the free molecule. The structure was solved at 2.45 Å resolution by multiwavelength anomalous diffraction (MAD)-phasing (*R* factor, 24.4%; *R*_{free}, 27%, [see supporting information (SI) Table S1]).

Crystallizing as an antiparallel dimer [Fig. S1], the sensor is bilobal, the N-terminal PAS domain and the central, chromophore-binding GAF domain forming the larger lobe, the overall architecture of which, including a figure-of-eight knot made by the N terminus, is similar to that of the PAS–GAF bidomains of bacteriophytochromes (Figs. 1B and 2B) (12). The second, smaller lobe comprises the C-terminal PHY domain

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The authors declare no conflict of interest.

Data deposition: The atomic coordinates and structure factors for the Cph1 sensory module have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2VEA).

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Fig. 1. Structure and spectral characteristics of the Cph1 phytochrome sensory module from *Synechocystis* 6803. (A) Domain boundaries of Cph1 phytochrome. In the recombinant Cph1 sensory module described, the C-terminal histidine kinase transmitter (Leu-515–Asn-748) is replaced by a (His)₆ tag. (B) Ribbon representation of the sensory module structure showing the N-terminal α -helix (green) and PAS (blue), GAF (orange) and PHY (red) domains. The PCB chromophore (cyan) is covalently attached to Cys-259. Disordered loop regions (Gln-73–Arg-80, Gly-100–Asp-101, Arg-148–Gln-150, and Glu-463–Gly-465) are indicated as dotted lines. The molecular surface calculated by PYMOL (probe radius, 1.4 Å) is shown in gray. (C) Omit electron density of the adduct between the PCB chromophore and Cys-259 contoured at 2 σ . (D) UV/Vis spectra of the Cph1 sensory module in solution at room temperature (red line) and in crystalline form at 100 K (■) in the Pr state (Upper) and after red light irradiation (Lower). Whereas in solution a photoequilibrium at 70% Pfr is reached, the mole fraction is $\approx$ 50% in the crystal. Spectra from crystals were recorded at the Cryobench of the ESRF, Grenoble. Photoconversion was done by irradiating for 10 s at room temperature with a 635 nm argon laser focused to 100 μ m.

(Thr-324–Glu-514) of the sensor. DALI comparisons of this structure show clearly that it belongs to the GAF domain family (Fig. 2C). A structure-based alignment with related domains and a summary of the secondary structure is shown in Fig. S2. The weak but significant sequence similarity (<15% identity at the amino acid level) lead to an earlier proposal that the PHY and GAF domains are orthologous and structurally related (19, 20). Although, like other members of the PAS superfamily, GAF domains commonly bind small, hydrophilic cofactors, no electron density potentially representing such a ligand was seen in our PHY structure.

The PAS-GAF and PHY lobes form an intricate structural unit connected at two points. First, a 66-Å and almost perfectly linear α -helix (α_9 , Phe-299–Ala-345) connects the two domains covalently. Tandem GAF arrangements of this kind are typical of the regulatory modules of cNMP phosphodiesterases (21, 22) and eubacterial adenylyl cyclases [phosphodiesterase (PDE) and adenylyl cyclase (AC), respectively] (23, 24). In both of these enzyme classes, the tandem-GAF domains act as allosteric, cNMP-dependent switches regulating cNMP synthesis or hydrolysis. Both show two GAF domains separated by a 50- to 60-Å connecting α -helix, astonishingly similar to the situation in the Cph1 sensor (Fig. 2A) (22, 24). Interestingly, cGMP has been

implicated in plant phytochrome signaling (25, 26), but neither the structure we present nor the equivalents in plant phytochromes seem likely to provide a binding site for cGMP. Second, in Cph1 an additional connection between the two lobes is provided by an unusual 49 residue (Pro-442–Gln-490) tongue-like protrusion between β_{16} and α_{15} of the PHY domain. This tongue extends back as a long, kinked β -hairpin from the PHY lobe toward the GAF domain, making intimate contact not only with the GAF surface but also with α_1 (Thr-4–Leu-18) protruding through the knot, interactions corroborated by cross-linking data for the bacteriophytochrome Agp1 (27). The tongue is present in all phytochrome classes and includes several highly conserved residues, which contribute either to its interaction with the GAF domain (PRxSF motif, see below) or to its unusual conformation along the GAF-PHY surfaces (WGG motif: Trp-450–Gly-452, Glu-480) (Fig. S3). Length variations within the phytochrome tongues are restricted to their tips; that the tips probably interact only weakly with the GAF domain is suggested by the fact that the tongue tip is partly disordered in our structure (Glu-463–Gly-465). Interestingly, the shortest tongues are found in noncanonical phytochromes of the Cph2 type, which are not only shortened by nine residues but also miss the N-terminal PAS domain.

Whereas earlier structural data from bacteriophytochrome PAS-GAF bidomains implied partial solvent access to the

Fig. 4. Comparisons of the chromophore pocket in Cph1 (orange) and bacteriophytochrome (yellow) (12) with their respective chromophores PCB (cyan) and biliverdin (green). Waters are shown as red spheres. (A) The subsite for interactions between the bilin propionate groups and the GAF domain, showing the different conformations adopted by Arg-222 and Phe/Tyr-216 in Cph1 and bacteriophytochrome. (B and C) The ring D microenvironment in Cph1 and bacteriophytochrome, respectively. The molecular surfaces of the proteins (gray) show similar cavities with a triangular cross section providing space for the Z→E photoflip. The chromophore is sealed off from the solvent in the case of the Cph1 complete sensory module, whereas the bacteriophytochrome bidomain pocket is open to the solvent (note the numerous waters).

Cph1/plant-type phytochromes attach their chromophores to the GAF domain (9, 10), our structure revealing the single carbon–thioether link between chromophore ring A and the sulfur of Cys-259 (Figs. 1C, 3B, and 3C). In bacteriophytochromes, two carbon atoms link ring A to a cysteine near the N terminus, whereas in Cph1 the corresponding residue, Leu-18, together with Leu-15 and Ile-20 forms part of the hydrophobic walling around rings A and B of the chromophore. Also, the thioether linkage is shielded from the solvent by Tyr-458 and Leu-469–Pro-471 of the tongue. The Cph1 structure clearly shows that the chromophore adopts a *ZZZssa* conformation (Figs. 1C and 3C) as seen in bacteriophytochromes, contrasting with predictions based on vibrational spectroscopy (28), which favored a more linear *ZZZasa* conformer. However, there is little doubt that *ZZZssa* is correct. First, the electron density only fits this conformer (Fig. 1C) (the datasets used exhibited minimal x-ray damage). Second, the *R* stereochemistry of the PCB chromophore (and of phytochromobilin used in plant phytochromes) precludes a thioether linkage to Cys-259 in *ZZZasa* because this residue and the vinyl of ring A would then be on opposite sides rather than adjacent to each other as required (29).

The detailed structure of the chromophore binding cleft of the complete Cph1 sensory module shows important similarities to and differences from those of bacteriophytochrome PAS–GAF bidomains. Pro-471 and Arg-472 form a conserved PRxSF motif with the R472A mutant showing a Pr-specific hypsochromic shift (Fig. S4A) as some other mutations within the tongue (16). Whereas the side chain of Pro-471 shields ring A of the chromophore, Arg-472 acts as an arginine finger pointing into the PCB-binding cleft to form a salt bridge to the conserved acidic residue Asp-207 of the GAF domain. This salt bridge is poised between rings A and D of the chromophore and connected to it by a hydrogen-bonding network (Fig. 3B and C) similar to what is seen in earlier structures. His-260, water 3 above the three chromophore ring nitrogens and the hydrogen bonds connecting them are also structurally conserved. Similarly, the side chains of residues Tyr-176, Val-186, Tyr-203 (Phe in most bacteriophytochromes), Pro-204, and Tyr-263 form a conserved hydrophobic subpocket around ring D, resembling the situation in bacteriophytochromes. However, the structure of the complete Cph1 sensory module additionally shows that Phe-475 of the tongue

closes the pocket, thereby shielding the chromophore from the solvent. Also, the chromophore in Cph1 is less twisted than in earlier bacteriophytochrome structures. Our model shows tilts of 9.8°, 1.4°, and 26.3° between rings A–B, B–C, and C–D, respectively, the latter in particular being much less than the ≈40–50° reported earlier for bacteriophytochromes (12, 13) (Figs. 4B and C). This moderate tilt would afford complete π -orbital connectivity through the ring system and, thus, strong absorption of red light. Mutational data support the notion that stronger aplanarity might result from the missing PHY domain (16). In the Cph1 structure, the ring D methyl carbon is closer to the Tyr-263 hydroxyl, the steric interaction preventing a more coplanar conformation. The relative chromophore planarity in Cph1 apparently arises from a slightly different orientation of Tyr-263 caused by its interaction with the Asp-207–Arg-472 salt bridge and Phe-475 of the tongue (Fig. 4B and C). As a consequence, ring D is no longer coplanar with the imidazole moiety of His-290, with which it forms a conserved hydrogen bond via its carbonyl group. Another difference is seen for the cleft accommodating the propionate side chains of ring B and C (Fig. 4A). In Cph1 Arg-222 is bridged via water 10 to both propionates and comes within 4 Å of the PAS–GAF domain interface. Also, Phe-216, conserved in plant and cyanobacterial phytochromes, is replaced by a tyrosine in the biliverdin-dependent fungal and bacteriophytochromes. Instead of Arg-222, this tyrosine hydrogen bonds to the ring B propionate, thus allowing the side chain of Arg-222 to point further into the GAF domain.

The structure we report would allow the chromophore to adopt the expected *ZZEssa* Pfr conformation (6, 30) and helps explain how light-induced conformational changes in the chromophore might be transmitted to the protein. Whereas the weak NMR signals obtained for Cph1 have been interpreted as reflecting considerable chromophore mobility inconsistent with strong interactions with the protein (31, 32), both the Cph1 and the earlier bacteriophytochrome structures indicate close packing around chromophore rings A–C. Such tight packing would rule out major conformational changes in that region of the chromophore without associated dramatic changes in the protein. The tight packing especially around the C10 atom connecting rings B and C might rather allow the protein to perceive small changes in chromophore position. In contrast, as in earlier

structures, there is ample space for ring D to rotate on $Za \rightarrow Ea$ isomerisation (Fig. 4B and C), the primary photochemical event (7). Of the two conserved tyrosine residues required for Pfr production in plant phytochromes (33), one is probably Tyr-176, immediately below ring D: the Y176H mutant fails to photoconvert, rather losing its excitation energy by fluorescence (16). The second might be Tyr-263 above the ring or Tyr-203 (Phe in bacteriophytochromes) at the side (Fig. 4B). According to our structure, the ring D photoflip would place the carbonyl group next to Asp-207 and Tyr-263, stabilising the Pfr conformation, and by forming a hydrogen-bonding network with these residues, disturb the salt bridge between Asp-207 and Arg-472. The resulting changes transmitted to the protein surface would explain the differential accessibility of the tongue to proteases in the Pr and Pfr states (27, 34). Interestingly, D207A mutants bleach in red light without generating Pfr (10), whereas R472A mutants have only minor effects on the absorbance properties. However, unlike the WT sensory module, R472A fails to dimerise in the Pr state, whereas Pfr dimerisation is unaffected (Fig. S4). Thus, the tongue is probably important in transmission of the light signal to the surface of the molecule, disruption of the salt bridge to Arg-472 being a likely first step. Arg-472 might couple changes on ring D photoisomerization to the tongue conformation, because it hydrogen bonds to the main chain oxygen between Gly-451 and Gly-452 at the kink of the tongue (Fig. S3).

On the other side of the chromophore, the salt bridge between the ring B propionate and Arg-254, conserved even in some nonphytochrome biliproteins, might be an alternative route for signal transmission within the sensory module (Fig. 4A). Arg-254 and Arg-472 mutants show similar changes in their state-dependent dimerisation (Fig. S4), implying that they are both involved in mediating intramolecular signal transduction to the surface of the molecule. Indeed, Arg-254 is essential in plant phytochrome A signaling (A. Nagatani, personal communication). The ring D photoflip could change the position of the bilin rings B and C, affecting the salt bridges and thus the PAS/GAF-domain interface, for example by an outward/inward movement of conserved Arg-222. The B propionate might even reassociate to Arg-222; an analogous signaling system involving arginine-heme propionate salt bridges has been proposed for the FixL oxygen sensor (35). The C-ring propionate is positioned differently in Cph1 relative to bacteriophytochrome structures (Fig. 4A), hydrogen-bonding to Thr-274 (and via two waters to Ser-272). These interactions too might be important in signaling. Last, the loss of the ring D-His-290 interaction could easily be transmitted to the protein surface by means of His-291.

Besides the conformation, protonation of the bilin is an important factor in optimizing light absorption (36, 37). Indeed, recent liquid and solid-state NMR data clearly show that all four chromophore nitrogens are protonated in both Pr and Pfr states (31, 32). However, none of the known phytochrome structures shows acidic side chains near the chromophore that could act as counterions for the positively charged tetrapyrrole system. Asp-207 points its side chain away from the chromophore to form the salt bridge to Arg-472. Also, the D207A mutation seems not to affect Pr protonation (10, 38). However, the pK of the bound chromophore might be much higher than in free solution, so that histidine or even tyrosine and cysteine could act as proton donors. Conserved His-260 probably acts as a proton source/sink by hydrogen-bonding via water 3 to the nitrogens of rings A–C, as in the H260Q mutant the chromophore deprotonates above pH 8 (10). The partial negative charges of the Asp-207 backbone oxygen and the His-260 $\delta 1$ nitrogen might stabilize the cationic chromophore (20). The His-290–ring D interaction mentioned above might have a similar role. The ZZZssa Pr conformation together with complete protonation in both Pr and Pfr constrains models for the mechanism of interconversion by red and far-red

light. Despite measurable transient proton release and uptake from phytochromes during photoconversion (37), there is no obvious proton conductance channel leading from the chromophore. Because proton exchange occurs in the millisecond time range, it is likely that conformational changes of the protein are involved. The loss of the ring D-His-290 interaction on photoisomerisation could trigger chromophore deprotonation (37), whereas later reprotonation might occur by means of His-260 and/or the now freed side chain of Asp-207. It would seem essential for effective long-wavelength light absorption that an extensive π -electron system is present, requiring the protonation of all of the ring nitrogens in Pr and Pfr states, as is indeed the case (31, 32).

Characteristically, apophytochromes bind and ligate their chromophores autocatalytically (39). However, in contrast to earlier structures, the chromophore-binding pocket seen in our structure is closed (Fig. 3A). Also, the PAS and GAF domains are knotted together, and the tongue binds both the GAF domain and the N terminus protruding through the knot. Thus, in the context of the discrete kinetic steps observed during autoassembly (40), how the chromophore enters the pocket becomes an intriguing question.

Contradicting earlier assumptions, plant phytochrome signaling seems to arise from the N-terminal sensory module, the C-terminal half of the molecule being responsible for dimerisation and Pfr-dependent nuclear localization (41, 42). Thus, our structure of the complete Cph1 sensory module is relevant to the primary molecular mechanism underlying light-regulated plant development. The state-dependent surface changes we observe (Fig. S4), probably reflecting changes in the tongue and perhaps other regions of the protein, might be progenitors of the cryptic nuclear targeting signal (43) and partner binding (44) specific to Pfr. In contrast to the bacteriophytochrome bidomain molecules whose structures have been reported to date, the complete Cph1 sensory module is photochemically similar to the full-length holoprotein (6). The crystals too have similar UV-Vis absorbance properties to Cph1 in solution; indeed, it is possible to photoconvert the Pfr crystals to Pr under appropriate conditions (Fig. 1D and Fig. S5). However, photoconversion in the crystalline state coincided with a severe loss of diffraction, so that other approaches will be needed to derive the structure of Pfr.

Sensory histidine protein kinases have a central role in the biology of most prokaryotic organisms, domain swapping of Cph1 and EnvZ (45) implying a common regulatory mechanism. As Cph1 can be activated and deactivated noninvasively by picosecond light pulses any number of times, it might represent a particularly useful system for investigating this mechanism. In all, structure/functional studies of phytochromes have widespread significance in biology.

Materials and Methods

The complete sensory module (residues 1–514) of Cph1 phytochrome from *Synechocystis* 6803 was expressed as a histidine-tagged holoprotein in *E. coli* by overproducing the recombinant apoprotein together with PCB generated from host heme by means of coexpressed hemeoxygenase and biliverdin reductase (46, 47). Mutants R254A and R472A [kindly provided by Hortensia Faus (Free University, Berlin) and Georgios Psakis (Justus Liebig University, Giessen), respectively] were generated by site-directed mutagenesis and sequenced. All extracts were purified by Ni^{2+} -affinity and size-exclusion chromatography by using infrared (940 nm $\pm$ 45 nm) visualization equipment.

Tetragonal crystals were grown in drops comprising 500 nl of 10 mg/ml Pr in 2.5 mM Tris-HCl, pH 7.8/15 mM NaCl, plus 500 nl of reservoir solution (2 M sodium acetate/0.1 M magnesium acetate, pH 6.7) at 18°C in darkness and visualized under infrared or blue-green light (490 $\pm$ 20 nm). SeMet-labeled crystals were grown under essentially the same conditions. Crystals were frozen in reservoir buffer supplemented with 25% (wt/vol) magnesium acetate.

Native datasets were recorded at beamline X13 [European Molecular Biology Laboratory (EMBL), Hamburg, Germany] and ID14–3 [European Syn-

chrotron Radiation Facility (ESRF), Grenoble, France]. A 2.8-Å MAD dataset was recorded at BW7A (EMBL, Hamburg, Germany) from a SeMet-labeled crystal. The crystals (space group $P4_32_12$) were strongly anisotropic, necessitating that the diffraction data be rescaled for successful structure determination by using the Diffraction Anisotropy Server (UCLA-DOE Structure Evaluation Server, University of California, Los Angeles). The effective resolution for the anisotropic native dataset corresponds to 2.45 Å. Initial MAD-phasing succeeded with the AUTO-RICKSHAW suite (48) and was followed by refinement of the selenium sites by using the SHARP package (49). Further automated and manual refinement with REFMAC5 (50) and COOT (51) finally yielded a model of the sensory module defined for residues Leu-4-His-520 omitting disordered loop regions Gln-73–Arg-80, Gly-100–Asp-101, Arg-148–Gln-150, and Glu-463–Gly-465.

Radiation damage was estimated by examination of difference Fourier syntheses of datasets collected from the same crystal after different doses of

X-ray radiation. Stereochemical restraints (9) were used for the PCB–Cys-259 adduct as the available data were not sufficient to resolve the stereochemistry at the C3' atom. Figures were made by using the program PYMOL 1.0 (52). Please see *SI Materials and Methods* for more details.

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