Phytochrome three-dimensional structures and functions

Jon Hughes1
Plant Physiology, Justus Liebig University, Giessen, Germany

Abstract
The complete three-dimensional sensory module structures of the Pr ground state of Synechocystis 6803 Cph1 and the unusual Pfr ground state of the bacteriophytochrome PaBphP (PDB codes 2VEA and 3C2W respectively) have now been solved, revealing an asymmetrical dumbbell form made up of a PAS (Period/ARNT/Singluminded)-GAF (cGMP phosphodiesterase/adenylate cyclase/FhlA) bidomain carrying the chromophore and the smaller PHY (phytochrome-specific) domain. The PHY domain is structurally related to the GAF family, but carries an unusual tongue-like structure which contacts the larger lobe to seal the chromophore pocket. In 2VEA, the tongue makes intimate contact with the helical N-terminus; both the N-terminus and the tongue structures are quite different in 3C2W. As expected, the structures reveal ZZsssa and ZZEsssa chromophore conformations in 2VEA and 3C2W respectively, associated with tautomeric differences in several nearby tyrosine residues. Two salt bridges on opposite sides of the chromophore, as well as the associations of the C-ring propionates also differ. It is still unclear, however, which of these structural differences are associated with bacteriophytochromes compared with Cph1 and plant-type phytochromes, the unusual 3C2W Pfr ground state functionality compared with the Pr ground state or the Pr compared with Pfr photoisomerism. To access the latter unambiguously, both Pr and Pfr structures of the same molecule are required. New solid-phase NMR data for Cph1 in the Pr, Pfr and freeze-trapped intermediate states reveal unexpected changes in the chromophore during Pfr → Pr photoconversion. These, together with our efforts to solve the three-dimensional structure of a complete phytochrome molecule are also described.

Introduction
Phytochromes are dimeric, red/far-red photochromic, bili-protein photoreceptors ubiquitous in plants, widespread in bacteria and known in fungi. Generally speaking, the Pr ground state absorbs predominantly in the red region (λ_{max} 650–670 nm), leading to photoconversion to the Pfr state which absorbs predominantly in the far-red region (λ_{max} 705–730 nm). Pfr is more or less thermally stable, with conversion back into Pr requiring a second photon. In plants, phytochromes play a central role in mediating light-signalling, three–dimensional molecular structure.

Key words: linear tetrapyrrole, photoreceptor, phytochrome, sensory histidine protein kinase, signalling, three-dimensional molecular structure

Phytochrome three-dimensional structures

Prokaryotic phytochromes provided not only the ’missing link’ between plant phytochromes and their SHPK ancestors (and thereby a clear domain map for the phytochrome family), but also access to powerful biophysical methods such as X-ray crystallography and NMR on account of the relative ease with which they can be prepared. The first 3D (three-dimensional) structural data were obtained for the 35 kDa cases, they are easily overproduced as apoproteins in Escherichia coli and autoassembled to form functional holoproteins when provided with appropriate bilin chromophore cofactors either in vitro or in vivo [3,4]. Essentially, canonical phytochromes consist of an N-terminal sensory module comprising PAS (Period/ARNT/Singluminded), GAF (cGMP phosphodiesterase/adenylate cyclase/FhlA) and PHY (phytochrome-specific) domains and a C-terminal transmitter module comprising a likely helix–loop–helix dimerization/phosphoacceptor domain and an ATPase catalytic domain. Plant phytochromes bear an additional ’Quail module’ between these two. Like plant phytochromes [5,6], Cph1 (cyanobacterial phytochrome 1) from the cyanobacterium Synechocystis 6803 attaches its chromophore to a GAF-domain cysteine residue via a thioether link [7], whereas the more primitive bacteriophytochromes [8] use a cysteine residue close to the N-terminus [9]. A domain map is shown in Figure 1.

Abbreviations used: BV, biliverdin; Cph1, cyanobacterial phytochrome 1; Cph1ΔΔ2, Cph1 sensory module; DHP, dimerization and histidine phosphoacceptor; GAF, cGMP phosphodiesterase/adenylate cyclase/FhlA; MAS, magic angle spinning; PAS, Period/ARNT/Singluminded; PCB, phycocyanobilin; Pfr, phytochrome photochrome state absorbing maximally in the far-red region; PHY, phytochrome-specific; Pr, phytochrome chromophore state absorbing maximally in the red region; SHPK, sensory histidine protein kinase; 3D, three-dimensional.

1email jon.hughes@uni-giessen.de
Figure 1 | Cph1 domain relationships

Pfam domains (to scale) of phytochrome and SHPK families. CAT, catalytic ATPase domain; X, missing phosphoacceptor; ♦, chromophore-binding site; 2VEA, 2.45 Å structure of Cph1Δ2.

PAS–GAF bidomain fragment of a bacteriophytochrome from *Deinococcus radiodurans*, DrBph1 (PDB code 1ZTU) [10]. This groundbreaking structure showed, among other things, that the polypeptide is knotted around the PAS domain. Moreover, the chromophore is in a periplanar ZZZssa configuration, rather different from that which had been predicted from Raman data. Similar structures followed [11,12]. However, the chromophore pocket was clearly incomplete in these fragments, probably because of the omission of the PHY domain. Indeed, the role of the latter is clearly important in light sensory function because changes to this part of any phytochrome generally prevent the appearance of *bona fide* Pfr. Producing X-ray crystallographic data for the complete PAS–GAF–PHY sensory module is difficult, however, because its red/far-red photochromicity requires that all operations, including crystal screening and picking, are carried out in total darkness with the help of IR visualization systems. A collaboration between the Giessen and Marburg laboratories was nevertheless successful in crystallizing and solving the structure of Cph1Δ2, the Cph1 sensory module, at 2.5 Å (1 Å = 0.1 nm) resolution [13]. This 2VEA structure was published alongside 3C2W [14] for the equivalent module of a ‘bathy bacteriophytochrome’ from *Pseudomonas aeruginosa*, PaBph. ‘Bathy’ phytochromes are unusual in that, although they autoassemble as Pr, this rapidly converts into a Pfr-like state by an unknown thermal mechanism. Whereas 3C2W thus provides the first Pfr 3D structure, the non-photochemical Pr → Pfr ‘bathy’ function is encrypted within it. This, together with the different chromophore attachment site and other fundamental differences, makes it risky to extrapolate from a 2VEA/3C2W comparison with the Pr → Pfr photoconversion mechanism.

The PHY domain

2VEA revealed the PHY domain, hitherto considered unique to phytochromes, to be a GAF superfamily member and thus phytochromes to be ‘tandem-GAF’ proteins. Several nucleotide cyclases and phosphodiesterases carry tandem GAF modules as allosteric regulators of cyclic nucleotide synthesis and hydrolysis respectively. As microinjection studies implied that plant phytochromes signal via cGMP [15–17], this correlation is intriguing. Structurally, however, cyclic nucleotide binding to the PHY domain is unlikely. Analogously to other tandem-GAF proteins, the globular PHY domain is kept at a distance from the larger PAS–GAF lobe by the long α9 connecting helix. Uniquely in the GAF superfamily, however, it carries a long, tongue-like, hairpin loop which stretches back to and makes contact with the PAS–GAF lobe. In 2VEA, it seals the chromophore pocket and makes intimate contact with the N-terminal helix, α1. This arrangement is interesting because α1 and α7 are colinear at the surface of the molecule (Figure 2 background), perhaps providing a conditional binding surface for a partner.

The chromophore and its pocket

Consistent with biochemical studies [5–7,9], 2VEA shows ring A of the PCB (phycocyanobilin) chromophore attached

Figure 2 | Stereo pair of the chromophore and surrounding pocket in the 2VEA Pr structure

Turquoise, PCB chromophore; green, N-terminus; gold, GAF domain; red, tongue; blue, nitrogen; pink, oxygen; yellow, sulphur; —, hydrogen bonds; A, B, C, D, chromophore pyrrole rings.
Figure 3 | Stereo pair of the chromophore and nearby groups in the 2VEA (Pr) and 3W2C (Pfr) structures (overlay following GAF 3D alignment)

Blue, nitrogen; pink, oxygen; —, hydrogen bonds differing between 2VEA and 3W2C. 2VEA: turquoise, PCB chromophore ZZssa; green, carbon. 3W2C: pale turquoise, BV chromophore ZZEssa; grey, carbon.

by a single-carbon thioether link to Cys259 (Cph1 numbering) in the GAF domain, whereas the bacteriophytochrome structures show the less reduced BV (biliverdin) attached by a two-carbon link to a cysteine residue near the N-terminus. The Pr structures show a ZZZssa conformation with the D ring tilted as a result of hydrogen-bonding of the carbonyl group to His290 and its homologues (Figures 2 and 3).

Pr is characterized by a sharp intense absorbance band near 660 nm. Under acidic conditions, a similar band also appears in free bilins [7,18], suggesting that the phytochrome chromophore is kept protonated in an ‘acid pocket’ within the protein. Indeed, NMR work has confirmed that all four chromophore nitrogens are protonated [19,20]. One of the big surprises of the 3D structures is therefore that not a single acidic side chain lies anywhere near the chromophore. How protonation occurs is still unclear, but two perfectly conserved residues above and below the chromophore are likely to be important (Figure 2). In 2VEA, whereas the backbone oxygen of Asp 207 below the chromophore is hydrogen-bonded to the chromophore nitrogens, the His260 δ nitrogen above is connected to them via a strategically placed water molecule. His260 seems to act as a proton buffer for the chromophore [7] (see below).

Two prominent salt bridges associated with the chromophore are probably also important in phytochrome function (Figure 2). In 2VEA, Arg472 of the tongue points into the chromophore pocket to form a salt bridge with Asp207 whose main-chain oxygen is hydrogen-bonded to the protonated nitrogens of chromophore rings A, B and C. Apparently, this construction is important in Pfr function: mutations which disturb the salt bridge bleach in red light without forming the typical Pfr peak [7]. A second salt bridge links the ring B propionate carboxy group to Arg254 in 2VEA. Interestingly, although this residue is conserved in all phytochromes and even in some non-phytochrome biliproteins, mutants at this site are spectrally almost normal.

Photoconversion

In canonical phytochromes and bacteriophytochromes, the Pr → Pfr transition is initiated by a photon-driven S0 → S1 excitation leading to a Z → E isomerization of the chromophore D ring. This can also occur in free bilins, but, in phytochromes, both Z and E states are thermally stable, generally requiring a photon for state conversion. 2VEA and, to some extent, 3C2W explain the stability of the respective states, but we need to discover the structural changes associated with the rest of the photocycle and to associate these with the causal mechanism of intramolecular signal transduction before we can claim to understand how phytochromes work. 2VEA and 3WC2 represent a major advance towards this goal, but it has yet to be achieved.

Whereas the Pr structures all show a periplanar ZZZssa conformation, the 3C2W Pfr structure implies ZZEssa, consistent with the expected primary photochemistry of Pr → Pfr. 3C2W shows associated differences in the positions of several tyrosine side chains around ring D; in particular, the nitrogen is hydrogen-bonded to the Tyr176 hydroxy group (Figure 3). It has long been known that tyrosine residues are important in photoconversion [21], indeed not only does the Cph1 Y176H mutant fluoresce brightly rather than photoconverting [22], but also the equivalent mutation in plant phytochrome A behaves constitutively as Pfr [23]. It is thus clearly tempting to deduce a photoconversion mechanism from conformational differences between 2VEA and 3C2W. In the latter, the D ring is tilted 50° anticlockwise in relation to rings B and C as a result of the stearic clash between the methyl side chains, thus it would seem obvious that the D ring in Pr must break its association with His290 and rotate clockwise to generate Pfr, while the latter would have an energetically easier task to flip anticlockwise back to Pr, explaining the lower-energy Pfr absorbance band. CD data imply, however, that the D ring has a clockwise tilt in Cph1 Pfr, implying that the Pr → Pfr isomerization is anticlockwise.
Whereas all current Pr structures show the His260 bands when the pH of the medium is varied between 7 and 9. It is characteristic of tongue mutants that Pr photobleaches without the geometrical appearance of the typical Prf peak, implying that the tongue is important in the formation or stabilization of the Pfr state. Indeed, the Asp207–Arg472 salt bridge in 2VEA is not seen in the 3C2W Pfr structure: the Arg472 homologue is almost 10 Å distant, while the Asp207 homologue is associated instead with the chromophore ring D nitrogen. Here too, however, an interpretation in terms of photoconversion is unwise as the 3C2W sequence is very different from that of 2VEA in many regions. For example, the Asp207 homologue in 3C2W is also hydrogen-bonded to an arginine residue absent from Cph1 and many other phytochromes. Similarly, the N-terminal α1 helix of 2VEA is not seen in 3C2W (neither is there any contact to the tongue), a difference more likely to be functionally associated with chromophore attachment to that portion of the bacteriophytochrome molecule and the radical function of the other salt bridge between the B ring propionate and the Arg222-homologous residue.

Protonation and deprotonation

All four nitrogens of the bilin chromophore are fully protonated in both Pr and Pfr states [19,20], probably central to the very efficient light capture typical of phytochromes ($\epsilon_{\text{Pr}}$ at $\lambda_{\text{max}} = 85 \text{mM}^{-1} \cdot \text{cm}^{-1}$). Surprisingly, how protonation occurs is not clear from the structures: the protein places no acidic side chains near the chromophore. His260 seems to play a critical role in protonation, however, the H260Q mutant losing and regaining its Pr- and Pfr-typical absorbance bands when the pH of the medium is varied between 7 and 9 [7]. Whereas all current Pr structures show the His260 side chain bridging the C ring propionate to the chromophore nitrogens via a conserved water molecule, in 3C2A the propionate is quite differently oriented, its carboxy group being shared between the His260 and Tyr275 homologues and the poorly conserved Ser275 (homologous with Ala288 in Cph1). It might be that the ring C propionate itself is the counterion for the protonated nitrogen. Interestingly, a bleached intermediate (Pbl or metaRb) in the 10–100 ms time range following photon absorption by Pr has been detected in plant and Cph1 phytochromes, interpreted as transient chromophore deprotonation [27–29]. Indeed, transient proton release/recapture in this time range has been demonstrated [29,30]. Propionate reorientation might be induced by photoconversion and associated with transient deprotonation. New MAS (magic angle spinning)–NMR data for Cph1 Pfr→Pr [30a] provide no evidence for a deprotonated intermediate, however. Ironically, equivalent Pr→Pfr data could not be obtained.

Dimerization and signalling

A technical problem in studying the properties of Pfr is that the absorbance spectral overlap with Pr limits photoinduced Pfr occupancy to 70–85%. Our Cph1∆2 (sensory module) preparations form dimers at concentrations above ~1 mg/ml; remarkably the Pfr affinity is ~20-fold higher than that of Pr [31]. The biological significance of this effect is unclear. As the complete phytochrome molecule is stably dimerized by interactions between the helix–loop–helix DHP (dimerization and histidine phosphoacceptor) domains, the orientation of the sensory modules is constrained. On the other hand, the dimerization behaviour of Cph1∆2 clearly results from state-dependent changes at the surface of the molecule which might be the basis of interdomain or intermolecular signalling. Interestingly, several mutants showing wild-type absorbance properties and Pfr dimerization no longer dimerize in the Pr state (J. Maillet and J. Hughes, unpublished work). Serendipitously, state-dependent dimerization allows us to prepare Pfr at 100% occupancy via size-exclusion chromatography [31,32]. As the Pfr state of Cph1 is quite stable in darkness at room temperature, this greatly simplifies studies of Pfr and the Pfr→Pr side of the photocycle.

Outlook

In the last 4 years, X-ray crystallography has provided a totally new insight into phytochrome molecular function. Much remains to be done, however, and, as always, generating appropriate crystals is the crucial bottleneck. Fortunately, experience has shown functional phytochromes to be particularly difficult objects of study for liquid NMR [19,33,34]: protein fragments below 30 kDa might yield structural information, but, unfortunately, at least in the case of canonical phytochromes, such fragments are generally non-functional. Solid-state NMR has great potential [20,30a,35] and would avoid the latter problem, but is also currently limited to domains of <30 kDa, which would have to be labelled separately from the rest of the protein; no easy task.

The difficulty in interpreting 2VEA and 3C2W in terms of photoconversion underlines the fact that 3D structures of a canonical phytochrome in both Pr and Pfr states would...
We are thus trying to crystallize Cph1/Δ1 from Pfr preparations at 100% occupancy. We also want to solve the crystal structure of full-length Cph1. This would be of significance not only for phytochrome research, but also for studies of the SHPK superfamily. These molecules lie at the heart of the ‘two-component’ signalling systems which bacteria generally rely upon for environmental perception and acclimation. As they are absent from the animal kingdom, they potentially represent excellent targets for therapeutic drugs, whose intelligent design is currently thwarted by the lack of 3D structural data. As Cph1 can be switched on and off non-invasively within milliseconds any number of times, it represents an ideal molecule for basic studies of SHPK function. Conversely, 3D structural data for a plant phytochrome would be of great interest to plant biologists.

Note added in proof (received 12 February 2010)

Subsequently to the submission of the present review, Ulijasz et al. [36] have published liquid NMR-based structural models for both the ground state and photoactivated state of a GAF-domain fragment of ‘SyB-Cph1’ phytochrome from the thermotolerant cyanobacterium Synechococcus OSB. Interestingly, this phytochrome is able to bind PCB and shows 630 nm/704 nm photochromicity even though the N-terminal PAS domain typical of phytochromes is absent. Indeed, the GAF fragment alone binds PCB and can photoconvert into a Pfr-like state, albeit with a blue-shifted λ_max at 689 nm. An earlier report by the same authors described ground state structures essentially similar to those of the known crystal structures, but noted that the chromophore itself appeared to be highly mobile [37]. The recent liquid NMR-based structure of the Cph1 chromophore in situ is entirely consistent with these data, confirming the periplanar ZZZssa conformation [33]. The Ulijasz main-chain ensembles are in accord with other published structures, implying modest state-related changes in comparison with the apparent high mobility of various regions of the peptide. Much more problematical are the detailed predictions of the side-chain positions and, particularly, the possible chromophore conformations in the 20 lowest-energy structural models for each state. The ground state ensemble shows a wide range of conformers, with rings A and D generally nearly perpendicular to the plane of rings B and C, whereas the photoactivated chromophore ensemble shows four of the 20 models with the C-ring flipped so that the propionate points towards ring A (Figure 4). Another feature of the models is that many of the individual rings are themselves non-planar (puckered). This is probably wrong as only pyrrolidinones (non-aromatic pyrroles) like ring A are significantly flexible [38]. Failing to apply this constraint would probably affect the rest of the structural prediction. Most surprisingly, however, the photoflip itself seems to

Figure 4 | Stereo pair of the chromophore ensembles from the 2KOI ground state and 2KLI photoactivated state of the ‘SyB-Cph1’ GAF domain as deduced from liquid NMR.
Blue, nitrogen; red, oxygen; turquoise, carbon (ground state Pr); green, carbon (photoactivated Pfr-like state). A, B, C and D are chromophore pyrrole rings. The hydrogens have been removed for clarity.
involves ring A rather than ring D. This is, to say the least, controversial. A similar variety of conformations is seen for several amino acid side chains including the His^{290} and Arg^{257} homologues. All earlier crystallographic, liquid NMR and MAS–NMR studies on bacteriophytochrome and canonical plant/Cph1-like phytochromes paint a fairly consistent, if not complete, picture in harmony with spectral data. Although crystal contact forces do affect protein structure and X-ray crystallography is always associated with radiation damage, there is little reason to expect either to have had a major effect on the published phytochrome structures. The Ulijasz models, on the other hand, present a very different view which is hard to correlate with other work. How, for example, is a bilin able to absorb at such long wavelengths when rings A and D are so twisted as to be electronically decoupled from rings B and C? Clearly, solving the structure of a 20 kDa GAP domain via liquid NMR is no easy task, while achieving it for a light-activated state at ~80% occupancy in a population of photocycling molecules would be a daunting challenge by any standards. A single incorrect assignment would generate quite incorrect structural models. Time will tell whether the new data indeed represent the reality of phytochrome structure in its two states.

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