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PHYTOCHOME STRUCTURE AND SIGNALING MECHANISMS

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Abstract

Phytochromes are a widespread family of red/far-red responsive photoreceptors first discovered in plants, where they constitute one of the three main classes of photomorphogenesis regulators. All phytochromes utilize covalently attached bilin chromophores that enable photoconversion between red-absorbing (P_r) and far-red-absorbing (P_{fr}) forms. Phytochromes are thus photoswitchable photosensors; canonical phytochromes have a conserved N-terminal photosensory core and a C-terminal regulatory region which typically includes a histidine-kinase-related domain. The discovery of new bacterial and cyanobacterial members of the phytochrome family within the last decade has greatly aided biochemical and structural characterization of this family, with the first crystal structure of a bacteriophytochrome photosensory core appearing in 2005. This structure and other recent biochemical studies have provided exciting new insights into the structure of phytochrome, the photoconversion process that is central to light sensing, and the mechanism of signal transfer by this important family of photoreceptors.

Keywords

phytochrome; biochemistry; biliprotein; photoreceptor; light signaling; photochemistry

GENERAL INTRODUCTION

Phytochrome was first discovered in plants in 1959 as the photoreceptor that mediates plant growth and development in response to long-wavelength visible light (9). Phytochrome measures the ratio of red light (R) to far-red light (FR), thereby allowing the plant to assess the quantity of photosynthetically active light and trigger shade avoidance responses (89). Phytochromes are found in all flowering plants and cryptophytes, and this important family of developmental regulators constitutes one of the three major classes of photoreceptors in higher plants, with the others being cryptochromes and phototropins (3,8,91).

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SIDE BAR

Phytochromes as Sensors of Oxygen-Dependent Heme Catabolism. The bilin chromophores incorporated by all phytochromes are synthesized from heme in two steps. First, a heme oxygenase converts heme into BV, which is directly incorporated as the chromophore of BphP and Fph phytochromes. In plants and cyanobacteria, however, BV is further reduced to yield PΦB in higher plants and PCB in cyanobacteria and green algae. Conversion of BV to PΦB is carried out by HY-2 in the chloroplast, while reduction of BV to yield PCB is instead carried out by PcyA. Both HY-2 and PcyA belong to a conserved family of ferredoxin-dependent bilin reductases.

The kinase activity and regulatory signaling state of many phytochromes are regulated not only by light but by the presence or absence of chromophore. The synthesis of chromophore is itself dependent on the heme metabolism of the cell, because chromophore will only be produced sparingly if cells are starved for heme or oxygen. Hence, phytochrome signaling is sensitive to heme metabolism and oxygen levels. Phytochromes therefore integrate both the light environment and the metabolic state of the cell to affect a single signaling readout. Bilin metabolism has recently been reviewed (26).

RELATED CHAPTERS (Vol. 57, 2006)

Complementary Chromatic Adaptation (David Kehoe)

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Tetrapyrrole Synthesis (Ayumi Tanaka)

More recently, phytochrome-related proteins have been isolated from other taxa. The first such protein to be discovered was the cyanobacterial chromatic adaptation sensor RcaE (51). Since this initial discovery, R/FR-sensing phytochromes have been discovered in cyanobacteria (Cph1/CphA, Cph2, and CphB/BphP), non-oxygenic bacteria (bacteriophytochromes or BphPs), and even fungi (Fphs), demonstrating that this class of photosensors is not limited to photosynthetic organisms (49). The true extent of the phytochrome family is only now becoming apparent with the advent of genome sequencing. Supplemental Table 1 lists currently known (or suspected) phytochromes and phytochrome-related proteins, and a sequence alignment of more than 120 of these proteins is shown in Supplemental Figure 1.

While microbial phytochromes have proven amenable systems for biochemical and spectroscopic analyses, much remains to be determined about the function of many of these molecules *in vivo*. The BphPs from *Rhodopseudomonas palustris* have been shown to regulate the biosynthesis of the photosynthetic apparatus in this organism (32,33), while BphPs are known to regulate pigment biosynthesis in *Deinococcus radiodurans* and *Rhodospirillum centenum* (14,45). The phytochrome from the filamentous fungus *Aspergillus nidulans* has recently been implicated in sexual development (5). These functions are thus conceptually analogous to functions of plant Phys: phytochromes regulate the metabolic response of the organism to its light environment.

PHYTOCHROMES ARE BILIPROTEIN PHOTOSWITCHES

Photoconversion: the “Central Dogma” of Phytochromes

An early key in defining the action of phytochrome in plant biology was the observation that both the spectrum of phytochrome preparations and the action spectrum of many plant responses were reversibly altered by illumination (89). Illuminating dark-grown tissues with red light converts phytochrome from the R-absorbing P_r form to the FR-absorbing P_{fr} form which triggers photomorphogenesis (Figure 1A). This change is reversible, with far-red illumination restoring P_r, and involves both primary photochemistry and subsequent thermal steps. For detailed review of the microscopic steps involved in the phytochrome photocycle, we refer the reader to detailed reviews on this topic (36,93).

R/FR photoreversibility, the hallmark of plant Phys, is rarely observed in other organisms, because higher fluences or continuous light are typically required to maintain a threshold P_r/P_{fr} ratio for light responsiveness (91). This reflects a thermal process known as dark reversion, in which P_{fr} phytochrome is slowly converted to P_r phytochrome in the absence of light. While dark reversion is an intrinsic property of all phytochromes, plant phytochromes have apparently evolved to exhibit slower dark reversion (31). Interestingly, BphPs that possess far-red absorbance maxima in the thermal ground state with photoconversion to P_r-like species have recently been described (32,48,100). Whether this spectral inversion proceeds via a reverse of the normal dark reversion pathway or via some other mechanism is not yet clear for any of these bathyBphPs. However, despite these spectral variations, it is clear that the biological outputs from phytochromes reflect the ratio of the P_r and P_{fr} forms, and that this ratio is determined by the light environment, by the forward and reverse rates of photoconversion, and by the rates for thermal interconversion between these forms.

The Modular Domain Architecture of Phytochromes

The large and steadily growing number of phytochrome sequences now known permit classification into subfamilies (Supplemental Table 1; (50, 69)) and delineation of conserved sequences and domains that are either ubiquitous among phytochromes or conserved in different subfamilies (Figure 1B; Supplemental Figure 1). Plant Phys, Cph1s, and most BphPs share a common architecture consisting of an N-terminal photosensory region with 3 conserved

domains (termed P2 or PAS domain, P3 or GAF domain, and P4 or PHY domain) and a C-terminal regulatory histidine kinase or histidine kinase-related domain (HKRD). Plant Phys have an additional N-terminal extension termed P1 known to inhibit dark reversion (102) and two additional regulatory PAS domains recently shown to be important for nuclear localization (13). Fungal Fphs have distinct N-terminal extensions and additional C-terminal response regulator domains (RR/REC) (5, 29). The other class of cyanobacterial phytochromes, the Cph2s, lack the N-terminal P2/PAS domain altogether but have other GAF domains duplicated C-terminal to the P4/PHY domain.

Canonical phytochromes thus consist of a PAS-GAF-PHY N-terminal photosensory module typically combined with a C-terminal HKRD module. PAS and GAF domains are present in other signaling molecules; for example, the photosensory Lov domains of the phototropins are PAS domains (19,37), while GAF domains have been implicated as regulators of cyclic nucleotide metabolism in organisms as diverse as cyanobacteria and mammals (62,63). Although there is not yet experimental structural information about the PHY domain, P4/PHY domains typically exhibit low similarity to GAF domains. It has therefore been proposed that this domain also assumes a GAF fold (69). The concatenation of PAS, GAF, and PHY domains attached to HKRD modules typify all classes of phytochromes and phytochrome-related proteins.

Phytochrome Chromophore Structure

The characteristic absorbance spectra and photoconversion of phytochromes reflect their association with a linear tetrapyrrole bilin chromophore which is normally covalently attached via a thioether linkage (Fig 2A). Photoconversion is known to involve a *Z–E* isomerization about the C15–C16 double bond of the bilin, as apophytochrome neither photoconverts nor exhibits a typical phytochrome absorbance spectrum (36,94). The exact nature of this chromophore varies for different subfamilies of phytochromes: plant Phys use phytochromobilin (PΦB, Fig 2A), while Cph1s and Cph2s instead utilize phycocyanobilin (PCB). Both bilins are covalently attached at $C3^1$ to a conserved Cys in the P3/GAF domain of the photosensory core (56,59,80,105). In contrast, BphPs and Fphs incorporate biliverdin IXα (BV) chromophores (Fig 2A). In these proteins, the more oxidized BV is attached to a conserved Cys upstream of the P2/PAS domain, apparently via a $C3^2$ linkage (58,103). This linkage appears less stable in BphPs than the $C3^1$ linkage to PCB, based on recent evidence for its reversibility (86). Covalent attachment does not appear to be a prerequisite for photoconversion; indeed, a mutant BphP lacking the nucleophilic Cys residue has been shown to function as an enzyme for production of C15–C16 *E* bilins (60). Covalent attachment likely provides a more stable holoprotein that is better suited to reversible photoswitching. Phytochromes are thus photoswitchable photosensors that utilize bilin chromophores.

THE STRUCTURE AND FUNCTION OF THE PHYTOCHROME PHOTOSENSORY CORE

Phytochromes are Bilin Lyases

Because the bilin precursor of the Cph1 and Cph2 phytochrome chromophores are identical to those used in assembly of the cyanobacterial phycobiliprotein antennae complexes, it was expected that phytochromes would also require bilin lyases for assembly of holoprotein. Surprisingly, plant, bacterial, and fungal apophytochromes are all able to self-ligate to appropriate bilins *in vitro* in the absence of other proteins or cofactors. This intrinsic bilin lyase activity has been mapped to the P3/GAF domain by truncation analysis, with the P2/PAS and P4/PHY domains important for tuning the spectroscopic porperties of the bound bilin (105). While removal of the P4/PHY domain permits bilin assembly for Phys, Cphs and BphPs, such truncated phytochromes typically exhibit reduced efficiency of photoconversion and enhanced

dark reversion (50,77,81,105). The P4/PHY domain thus seems to play an accessory role in reducing both unproductive modes of de-excitation and dark reversion. In contrast, deletion of either the P2/PAS or P3/GAF domains typically yielded unstable or misfolded protein; indeed, AphA, a Cph1 from *Anabaena sp.* PCC7120, is known to absolutely require a small extension N-terminal to the P2/PAS domain for bilin assembly (108). However, because the cyanobacterial Cph2 phytochromes lack a P2/PAS domain, it was possible to express a Cph2 P3/GAF domain alone and demonstrate formation of a covalent PCB adduct (105). The bilin lyase activity of phytochromes thus wholly resides in the P3/GAF domain.

The assembly reaction of Cph1 with PCB was recently examined by stopped-flow absorbance spectroscopy (6). PCB is shown to rapidly bind noncovalently in a cyclic, porphyrin-like conformation with *Z,syn* geometry at C5, C10, and C15, which is the most stable configuration in solution (18;Figure 2B). A second intermediate with red-shifted long-wavelength absorbance maximum and enhanced long-wavelength absorbance intensity appears soon after, followed by a blue-shift of the long-wavelength absorbance maximum concomitant with thioether bond formation. The second intermediate exhibits the spectral signature of a bilin in a more extended conformation with all four nitrogens protonated (18,34,35). ^{15}N-NMR characterization of the P_r state in Cph1 corroborates this interpretation (97). Recent studies have isolated point mutations in the P3/GAF domain of Cph1 that form covalent PCB or PΦB adducts exhibiting a porphyrin-like conformation or even form covalent adducts with an endogenous porphyrin (21). The extended chromophore configuration is thus not necessary for covalent attachment, and indeed this work raises the intriguing possibility that porphyrins or metalloporphyrins may also modulate phytochrome function.

The Crystal Structure of the DrBphP Photosensory Core

A major breakthrough in understanding the photochemistry and structure of phytochrome was the unveiling of a 2.5 Å crystal structure for the BV-bound P2/PAS and P3/GAF domains of DrBphP, the BphP from *Deinococcus radiodurans*, in the P_r state (103). This structure confirms that the P2 and P3 domains adopt PAS and GAF folds, as expected (Fig. 3A). Gaps and insertions within these domains in the extended phytochrome family largely fall outside of secondary structure elements, as would be expected for a conserved fold, and both domains exhibit conserved cores (Figure 3A). Consistent with biochemical studies, the BV chromophore is covalently attached to Cys24, apparently by linkage to $C3^2$ rather than $C3^1$ as in Phys or Cph1 (Figure 2A), and is deeply buried within a conserved pocket in the P3/GAF domain.

Unexpectedly, the interface between the PAS and GAF domains is formed by a deep trefoil knot (Figure 3B). Such knots have only been recognized relatively recently, so known examples are relatively few (76, 101). Phytochrome biosynthesis thus holds the promise of providing new insight into knot formation. The phytochrome knot is formed from sequence lying between Cys24 and the P2/PAS domain proper, which passes through a “lasso” formed by P3/GAF sequence between the fourth and fifth strands of the central GAF β sheet (103). The trefoil knot is centered on the conserved Ile35, which lies within the N-terminal sequence element required for bilin assembly of AphA (108), and also contains Arg254, a conserved residue directly interacting with the B-ring carboxylate of the biliverdin chromophore. It is therefore highly likely that this architecture will be conserved among other phytochromes. Additional N-terminal extensions such as the P1 region in plant phytochrome or the large N-terminal extensions of Fphs must either be largely unstructured or else could be proteolytically removed to facilitate this folding process. The potential regulatory role of such extensions is an interesting topic for future investigation.

Mutations in the PAS and GAF domains of plant phytochromes that result in altered function *in vivo* (Supplemental Table 2) have been mapped onto the DrBphP structure (Figure 3A). While several loss-of-function mutations cluster about the chromophore-binding pocket as

expected, others occur in residues at the interface between the PAS domain and the trefoil knot, such as Gly118, Ser134, and Ile208 in Arabidopsis PhyB (Supplemental Table 2). Such mutations might well affect the proper folding of these domains. Both loss-of-function and gain-of-function alleles have been isolated in the "back-side" helices of the GAF domain, which lie on the other side of the central β sheet from the chromophore-binding pocket (e.g., mutation of Arabidopsis PhyA Glu229 and pea PhyB Val238). It is thus conceivable that these helices play a role in signal transduction via light-mediated regulation of either intramolecular interactions (with the P4/PHY domain, C-terminal regulatory domains, or plant Phy P1 sequence) or direct intermolecular interactions with downstream signaling components.

The BV chromophore of DrBphP is unequivocally bound in the C5–*Z,syn* C10–*Z,syn* C15–*Z,anti* configuration in the P_r state (Figure 4), ending a controversy which has lasted for some time. The chromophore is deeply buried in the GAF domain, and both the carboxylate side chains and the tetrapyrrole ring system are excluded from bulk solvent. The B-ring carboxylate forms a tight, bidentate association with Arg254, while the C ring carboxylate is associated with His260, Ser272, and Ser274 (Figure 4A).

The chromophore ring system is on one side packed onto the highly conserved motif formed by Asp207, Ile208, and Pro209 and on the other closely apposed to His260. Assuming that this structure reflects a protonated BV species, as seems likely based on spectral characterization of the crystals, the positive charge that is delocalized across the B- and C-ring NH moieties is sandwiched between the backbone carbonyl oxygen of Asp207 and the side chain of His260. The charge of the ring system is thus closely associated with the partial negative charges of the His260 δ1 nitrogen and the backbone carbonyl oxygen of Asp207, which together should suffice to stabilize the charged, protonated BV ring system.

Although DrBphP was crystallized without the PHY domain, this crystal structure nevertheless provides unique insight into the photoconversion process. The BV A-ring is sandwiched between secondary structure elements of the GAF domain and is covalently attached to Cys24. The C10 methine bridge is tightly packed by the B- and C-rings of chromophore as well as by the conserved Asp207, Ile208, Pro209, Ala212, Tyr216, and His260 (Figure 4B). C10 is thus held tightly in place, so photochemistry cannot occur about this position, explaining the lack of photoconversion and the intense fluorescence observed upon assembly of apophytochrome with a bilin containing a saturated C15 bridge (71). In contrast, the D-ring is in a looser environment that would sterically permit more ready rotation. The D-ring pocket is also lined with highly conserved residues (Figure 4C), at least one of which is known to be critical for photochemistry (20, 21). This structure thus provides strong evidence that the conversion of P_r to P_{fr} proceeds with rotation of the D-ring and only the D-ring of the chromophore.

THE PHYTOCHROME PHOTOCYCLE AND DARK REVERSION

The "Forward" Reaction: Pr to P_{fr} Phototransformation

The DrBphP crystal structure is in the P_r state, so the exact structure of P_{fr} must await future investigation. However, in combination with other data, this structure provides a new basis for more directed speculation about the photochemical pathway than was possible without any experimental structure. Several Resonance Raman studies have led to proposal of a C15–*E,anti* geometry for the P_{fr} chromophore, although without any consensus as to the structure of the P_r chromophore (1,22,54,66,67,70). Examination of both Phys and Cph1 by FT-IR spectroscopy provides evidence that the primary photoproduct formed upon irradiation of P_r, lumi-R, has a P_{fr}-like configuration (23–25). The subsequent dark reactions leading to P_{fr} have been proposed to involve a further rotation about C15 to generate a C15–*E,syn* conformation (2), but the crystal structure of DrBphP indicates that this conformer would be sterically disfavored (103). Moreover, a recent study utilizing synthetic bilins that are unable to rotate

about C15 (44) demonstrated that only the C15–*E,anti* BV analog yielded a BphP adduct with properties similar to those of P_{fr} (Figure 5A). This approach also correctly identified the C15–*Z,anti* conformation of the P_r chromophore (44). Taken together with the crystal structure, these data provide good evidence that conversion of P_r to P_{fr} is best described by a single photochemical isomerization of the chromophore about the C15–C16 double bond, with both the lumi-R primary photoroduct and P_{fr} adopting a C15–*E,anti* conformation (Figure 5B).

Unlike P_r, the P_{fr} state is not stable in solution and can only be observed within its native protein matrix (88). The substantial red-shift of P_r relative to P_{fr} (Figure 5A) indicates either a much more extended conformation, which is not the case for the proposed C15–*E,anti* configuration, or greater electron density on the D-ring that extends the effective length of the conjugated system and red-shifts the resulting spectrum. Such electron density could readily result from the hypothetical P_{fr} structure shown in Figure 5B, which would arise from two proton transfers between the chromophore and the protein. The greater electron density on the D ring is consistent with the observed red shift of P_{fr}. The O-protonated P_{fr} species proposed in Figure 5B would explain the instability of the P_{fr} chromophore upon denaturation (87), because it is not significantly populated in solution (18). A recent NMR study of Cph1 presented evidence that all four nitrogens were protonated in the P_{fr} state (97), but it is unclear whether these technically challenging experiments would have been able to distinguish between such a model and one in which one proton is shared between the B- and C-ring nitrogens, particularly in light of the apparently weak NMR intensities seen for deprotonated bilin nitrogens (18).

The proposed P_{fr} structure in Figure 5B would be stabilized in the chromophore-binding pocket through the action of a proton acceptor (taking a proton from the B/C-ring NH moieties) and a proton donor (transferring a proton to the D-ring carbonyl oxygen). Recently, the pK_a of the chromophore ring system in holoprotein has been estimated at ~9.5, suggesting that conserved Tyr or Cys residues could be viable proton donors in addition to conserved His, Asp, or Glu residues. It should now be possible to test this model and others by mutagenizing candidate proton donors and acceptors based on the DrBphP crystal structure.

In this proposed model, the photoconversion of P_r to P_{fr} proceeds via initial photoisomerization of the C15–C16 double bond followed by proton transfers and conformational changes of the protein matrix. The P_{fr} state is known to be much less fluorescent than the P_r state, and the proposed structure in Figure 5B provides a possible explanation for this: excited chromophore molecules that do not undergo photochemistry could readily undergo proton transfer either via tunneling between the B- and C-rings or at the D-ring carbonyl, leading to spectrally silent de-excitation.

The "Reverse" Reaction: P_{fr} to P_r Phototransformation

The conversion of P_{fr} to P_r is known to proceed via a distinct pathway from that of conversion from P_r to P_{fr} (36). For at least one phytochrome, recent FT-IR data provide evidence that the lumi-F primary photoproduct adopts a P_r-like configuration, which would imply a C15–*Z,anti* configuration, but this may not be universal (24). While the P_{fr} structure proposed in Figure 5B is hypothetical and is presented here as a conceptual aid, one can see that the reverse reaction from this species would indeed proceed via a different pathway with a different primary photoproduct. Subsequent thermal relaxation of lumi-F to P_r would entail a different pathway of proton transfers and protein conformational changes, with the residue donating a proton to the D-ring in the P_{fr} state again becoming protonated and the proton acceptor returning the proton to the B/C-ring system. Additional structural information about the P_{fr} state will be needed before a more informed description of the photochemical reverse reaction can be attained.

Dark Reversion

The P_{fr} state is also thermally unstable in most phytochromes, with restoration of the P_r state over time in a process known as dark reversion. It has long been known that multiple factors are capable of modulating the rate of dark reversion, such as changes in pH, ionic strength, reducing agents or metal ion concentration (27). By definition, this process cannot be triggered by spectral techniques, so it is much less amenable to study than photoconversion. The proposed P_{fr} structure suggests a mechanism for dark reversion via an alternate resonance form with single bond character about C15–C16 that could therefore thermally rotate to the C15–*Z,anti* configuration of P_r (Figure 5B, center). Reversion of this intermediate to P_r via a series of steps reminiscent of the photochemical process can then be envisaged. Although dark reversion is not yet well characterized, it makes an important contribution to the balance between P_r and P_{fr} and hence to determining the output state of a given phytochrome. Indeed, evidence that dark reversion of plant Phys is fluence rate dependent (39), can be reduced by interaction with other proteins (99) or enhanced by missense mutations (16) suggests that regulation of dark reversion may play a significant role in Phy signal output.

PHYTOCHROME AFTER DARK: FROM PHOTOCHEMISTRY TO SIGNALING

In view of the diversity of regulatory domains associated with a conserved bilin-binding GAF domain of phytochromes, the molecular mechanisms of signal output are expected to vary widely. Phytochromes with histidine kinase(-related) regulatory domains are the most widespread - an observation that strongly suggests that plant phytochromes evolved from a two component sensor precursor with a tetrapyrrole binding pocket (69). Indeed, prokaryotic phytochromes of the Cph1 and BphP families are predominantly ATP-dependent histidine kinases that mediate phosphotransfer to aspartate residues of their cognate response regulators, which are often encoded within the same operon (50). Despite the nature of the output domain, it is well accepted that phytochrome signaling involves light-mediated changes in interactions between photosensory and regulatory domains that are best understood for plant phytochromes (79).

Molecular mechanisms of prokaryotic phytochrome signaling

Signal transfer by prokaryotic phytochromes most frequently utilizes the two component signaling paradigm, i.e. ligand-dependent histidine kinase activation and phosphotransfer to a response regulator that directly regulates transcription or motility (82). Phosphotransfer is both bilin- and light-modulated for Cph1s and BphPs, a result consistent with the regulation of photoreceptor homodimerization and substrate interaction dynamics by these input signals (69,78). Bilin binding stimulates kinase activity for Cph1s, while red light inhibits both autophosphorylation and response regulator phosphorylation in a mechanistic interpretation depicted in Figure 6A (17,43,59,107). Although some BphPs show photoregulation similar to Cph1 (33,43,61), other BphPs exhibit a reversal in polarity with P_{fr} being more active than P_r (4,48), while others show no effect of light on autophosphorylation (100). Unfortunately, the structural basis of this diversity in biochemical signal output is not readily revealed by comparison of the protein sequences; we expect that compensatory changes in both the photosensory and regulatory domains will be responsible.

Molecular mechanisms of plant phytochrome signaling

Our understanding of plant phytochrome signaling has benefited from extensive genetic, biochemical and cell biological investigations going back many years (7,10,11,30,42,68,73, 83,84,90,95). For this reason, the following discussion is limited to recent data that most directly impinge upon the molecular basis of phytochrome signaling. As depicted in Figure 1B, the structure of plant phytochromes (Phys) has been remarkedly preserved throughout evolution (69). In contrast with Cph1s and BphPs, plant phytochromes are obligate dimers

consisting of two ~120 kDa subunits with both regulatory PAS and HKRD subdomains contributing to the high affinity subunit-subunit interaction (47). Small x-ray scattering and EM analysis indicates that the phytochrome holoprotein has similar overall dimensions to mammalian immunoglobulin Gs (46,74,75). Encoded by small gene families in angiosperms (64), phytochromes fall into two classes - those that are light-labile (phyAs) and those that are light-stable (phyB-F). While phyAs are mostly homodimeric, recent studies reveal that light-stable phytochromes are also found as tightly bound heterodimers (92). Based on this structural property, it is clear that light-regulated subunit-subunit dissociation cannot be the signaling mechanism used by plant phytochromes.

The recent discovery that the P1-P3 photosensory core domains of plant phytochrome are fully sufficient for phytochrome signaling as long as the truncated polypeptide is targeted to the plant cell nucleus as a homodimeric holoprotein was another paradigm-shifting observation in the field of phytochrome research (65,77). This work, along with a plethora of other studies using GFP-labeled phytochromes (reviewed in (68,72,73)) and cytoplasm-anchored phytochrome (41), indicate that phytochrome signaling requires dynamic cytoplasm-to-nuclear relocalization following its photoactivation. Since the nuclear localization signal (NLS) has been localized to the C-terminal PAS domains (13), the regulatory domains must play a dual role in phytochrome signaling - to maintain the homodimer and to target the photoreceptor to the nucleus. The evidence that plant phytochromes are serine/threonine kinases suggests that ATP-binding and/or protein phosphorylation mediated by the regulatory domains also play a role in light signaling (106).

Phytochrome phosphorylation has been shown to contribute to desensitization of the light signal (52,53). However, the hypothesis that light-regulated protein phosphorylation is the trigger enabling the photoreceptor to 'uncouple' itself from a cytoplasmic anchor thereby exposing the NLS, remains a viable one (90). In this signaling model depicted in Figure 6B, photoconversion of P_r to P_{fr} with red light effects a conformational change that facilitates phosphotransfer to a bound anchoring molecule (X). The P_{fr}(ADP):X-P complex dissociates upon ATP-ADP exchange, enabling exposure of the PAS-localized NLS and P_{fr} migration to the nucleus where the N-terminal photosensory domain can interact with regulatory transcription factors (42). The altered activity of the putative anchoring molecule(s) X-P is envisaged to initiate a cytoplasmic output signal. Little is presently known about the cytoplasmic signaling pathway, candidate phytochrome-interacting cytoplasmic substrates have been identified (90). Once in the nucleus, P_{fr} accumulates in sub-nuclear foci or speckles whose appearance are correlated with the output signal (12). Speckles are thought to represent sites of transcription factor degradation, although other hypotheses have been proposed (11). Speckle formation requires the intact C-terminus, i.e. both PAS and HKRD domains, suggesting this region plays an additional signaling role in the nucleus (77). P_{fr} autophosphorylation and/or subsequent dark reversion are envisaged to complete the phytochrome signaling cycle, whereupon free phosphorylated forms of phytochrome are degraded (phyA) or recycled (PhyB-E).

Through isolation of missense alleles of phytochromes, genetic approaches have provided valuable insight into the molecular basis of phytochrome signaling. Such mutant alleles can be categorized into two classes: hyposensitive (loss-of-function) and hypersensitive (gain-of-function) alleles (Supplemental Table 2). While a large majority of the loss-of-function mutations fall within the regulatory PAS domains (85), loss-of-function mutations also occur throughout the photosensory region. Where tested, the molecular bases for loss-of-function phenotypes include increased dark reversion, reduced nuclear targeting and altered sub-nuclear localization. Known gain-of-function mutations are rare (55)(15) (104), with some falling in the photosensory core (Figure 3A). These mutations could enhance the translocation of P_{fr} to the nucleus or inhibit nuclear turnover of phytochrome (104). The accumulation of additional

mutant alleles, together with x-ray crystallographic analysis, will be a powerful combination to assess the molecular basis of phytochrome signaling in the future.

Phytochrome signaling mechanisms are still evolving

There are a number of BphPs that lack HKRDs altogether, and other catalytic/regulatory domains have been inserted in their place during evolution (32,50). This type of exchange appears to have occurred many times in the past, but the probability that the new phytochrome chimera remained functional is small since few such phytochromes exist (outside the cyanobacteria). Domain exchange is likely responsible for the emergence of the plant phytochrome lineage since their regulatory PAS and HKRD modules appear evolutionarily distinct from HKRDs found on the extant prokaryotic phytochromes (64). Domain exchange has occurred more recently in primitive plants to yield the neochromes, which are functional chimeras of a plant phytochrome photosensory P1-P4 domain and a blue-light sensing phototropin (98). It is clear that the most extensive phytochrome evolution has taken place in the cyanobacteria, which probably reflects the abundance of multiple bilin ligands and the need of these photosynthetic bacteria to adapt to light environments that are enriched in blue, green or red wavelengths (57).

SUMMARY LIST

- Phytochromes photoconvert between P_r and P_{fr} states, and the ratio of these states determines the signaling state of phytochrome.
- Phytochromes have a modular domain architecture with a conserved N-terminal photosensory core and a C-terminal regulatory region.
- Phytochromes utilize bilin chromophores that photoisomerize during the conversion between P_r and P_{fr}.
- The conserved N-terminal photosensor of phytochromes can be fused to a variety of regulatory domains which can act in bacterial two-component pathways or in more complex pathways in plants.
- Recent structural breakthroughs and biochemical results have defined the P_r state of the chromophore and provide new insight into the structure of the P_{fr} state.

FUTURE DIRECTIONs

- Understanding the structural changes associated with photoisomerization.
- Understanding how those changes alter the function of the regulatory domains to trigger signaling.
- Understanding the biological functions of phytochromes in nonphotosynthetic microbes and of divergent, phytochrome-related molecules in plants and cyanobacteria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

GLOSSARY

bilin
: a linear tetrapyrrole, metabolically derived from heme.

dark reversion
: conversion of P_{fr} to P_r in a light-independent thermal process; also can be used to describe the P_r to P_{fr} thermal conversion for the bathyBphPs.

P2/PAS
: the PAS domain in the phytochrome photosensory core.

P3/GAF
: the GAF domain in the phytochrome photosensory core.

P4/PHY
: the PHY domain in the phytochrome photosensory core, thought to be a phytochrome-specific GAF domain.

photoconversion
: photochemical conversion of P_r phytochrome to P_{fr} and back.

P_r
: red-light-absorbing phytochrome state.

P_{fr}
: far-red-light-absorbing phytochrome state.

ACRONYMS LIST

bathyBphP
: atypical BphP that exhibits P_{fr}-like spectrum in the thermal ground state, with photoconversion to a species with a P_r-like spectrum.

BphP
: member of the bacteriophoytochrome subfamily.

BV
: biliverdin IXα

Cph1, Cph2
: phytochrome subfamilies named after cyanobacterial phytochromes 1 and 2, exemplars of these subfamilies from *Synechocystis* sp. PCC 6803.

EM
: electron microscopy.

Fph
: member of the fungal phytochrome subfamily.

FR
: far-red light.

FT-IR
: Fourier transform infrared spectroscopy.

GAF

domain acronym derived from vertebrate cGMP-specific phosphodiesterases, cyanobacterial adenylate cyclases, and formate hydrogen lyase transcription activator FhlA.

HKRD

histidine kinase related domain.

PAS

domain acronym derived from period clock (PER) protein, aromatic hydrocarbon receptor nuclear translocator (ARNT), and single minded (SIM).

PCB

phycocyanobilin.

PΦB

phytochromobilin.

Phy

member of the plant phytochrome subfamily.

R

red light.

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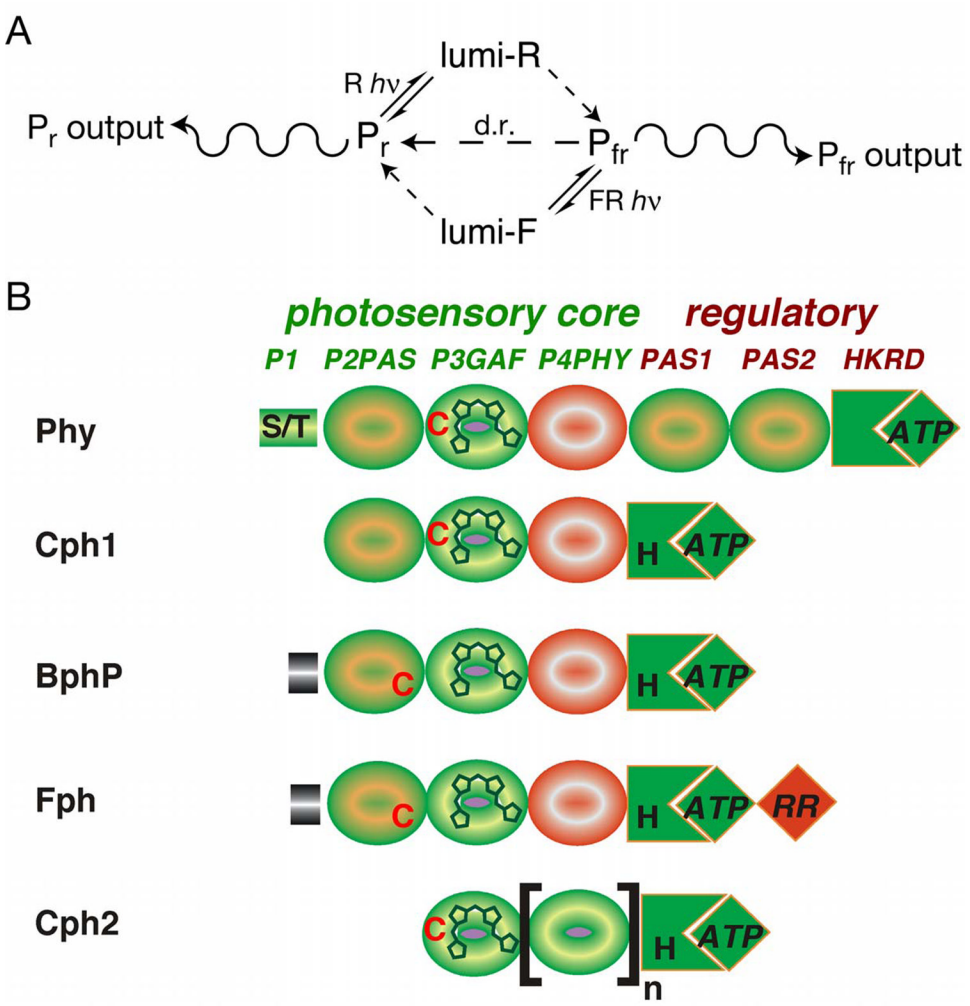

Figure 1. Domain structure and chromophores of phytochromes

(a) The phytochrome photocycle. Illumination of P_r phytochrome with red light (R) produces lumi-R as the primary photoproduct. This is subsequently converted to P_{fr} via multiple light-independent steps. P_{fr} can be converted into P_r either by illumination with far-red light (FR), producing lumi-F and then P_r via subsequent thermal steps, or by an entirely thermal process known as dark reversion (d.r., center). The ratio between P_r and P_{fr} (and hence between the two physiological outputs) is thus determined by the light environment and by the rate of dark reversion. (b) Domain architecture of the extended phytochrome family. The five classes of phytochromes possess an N-terminal photosensory core region and typically share regulatory output domains related to those found on two component histidine kinases (HKRD). The P3/GAF domain is associated with the bilin chromophore and is highly conserved. All phytochromes except those found in the Cph2 subfamily share P2/PAS domains, while P4/PHY photosensory domains are specific to phytochromes and are thought to have folds similar to GAF domains (69). Plant phytochromes (Phys) possess two additional PAS domains within the regulatory region. Fungal phytochromes (Fphs) have a domain structure similar to those of the cyanobacterial phytochrome 1 (Cph1) and bacteriophytochrome (BphP) families, except for an additional C-terminal response regulator receiver domain (RR) extension and variable N-terminal extensions.

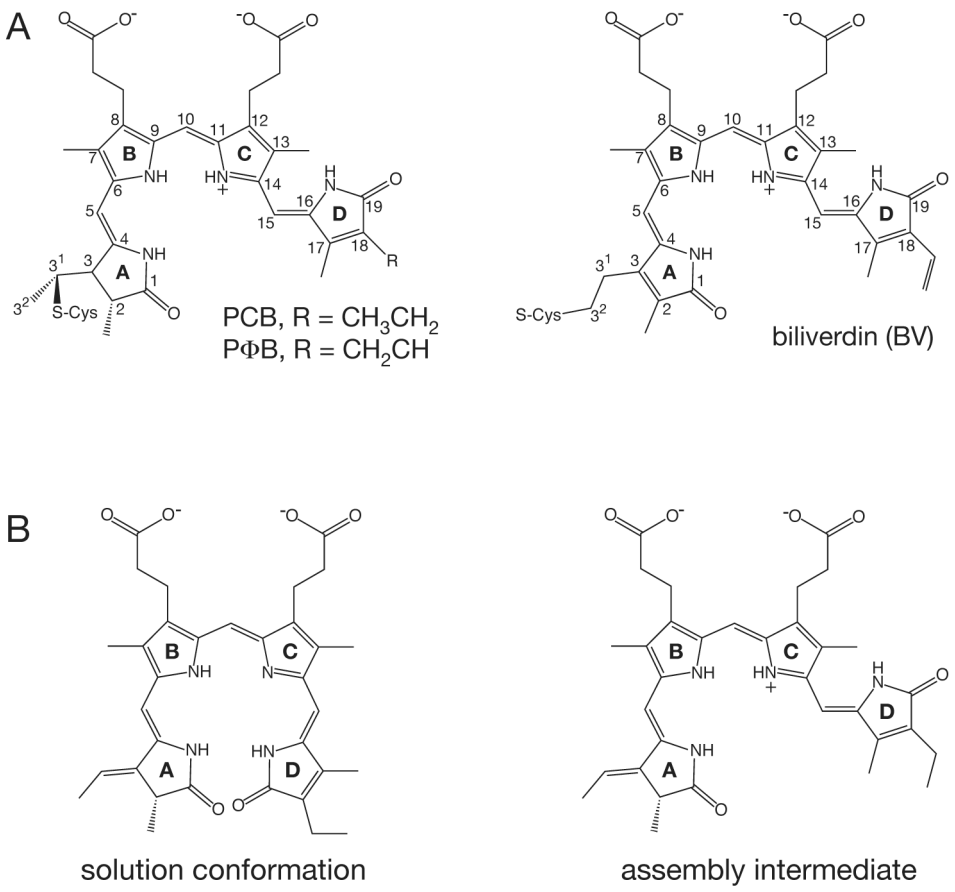

Figure 2. Chromophore structure and sssembly

(a) The structures of the bilin chromophores utilized by known phytochromes are shown. Left, phycocyanobiliin (PCB) and phytochromobilin (PΦB) chromophores share a reduced A ring and differ only at the C18 side chain. These chromophores are utilized by plant and algal Phys and cyanobacterial Cph1s and Cph2s. Right, the BphPs and Fphs instead utilize biliverdin (BV) as chromophore. All chromophores are shown in the C5–*Z,syn* C10–*Z,syn* C15–*Z,anti* configuration adopted in the P_r state (103). (b) Conformations of PCB thought to be present during the assembly reaction with Cph1 are shown (6). The cyclic, porphyrin-like C15–*Z,syn* species (left) is the most stable in solution at neutral pH and initially binds to apoCph1. After binding, the B/C ring system becomes protonated, driving adoption of a C15–*Z,anti* conformation (right) which is characterized by enhanced, red-shifted long wavelength absorbance. This species then becomes covalently attached to Cys259 of Cph1 to give the P_r structure shown in (a). BV is bound to a different Cys upstream of the P2/PAS domain of BphPs (58,103).

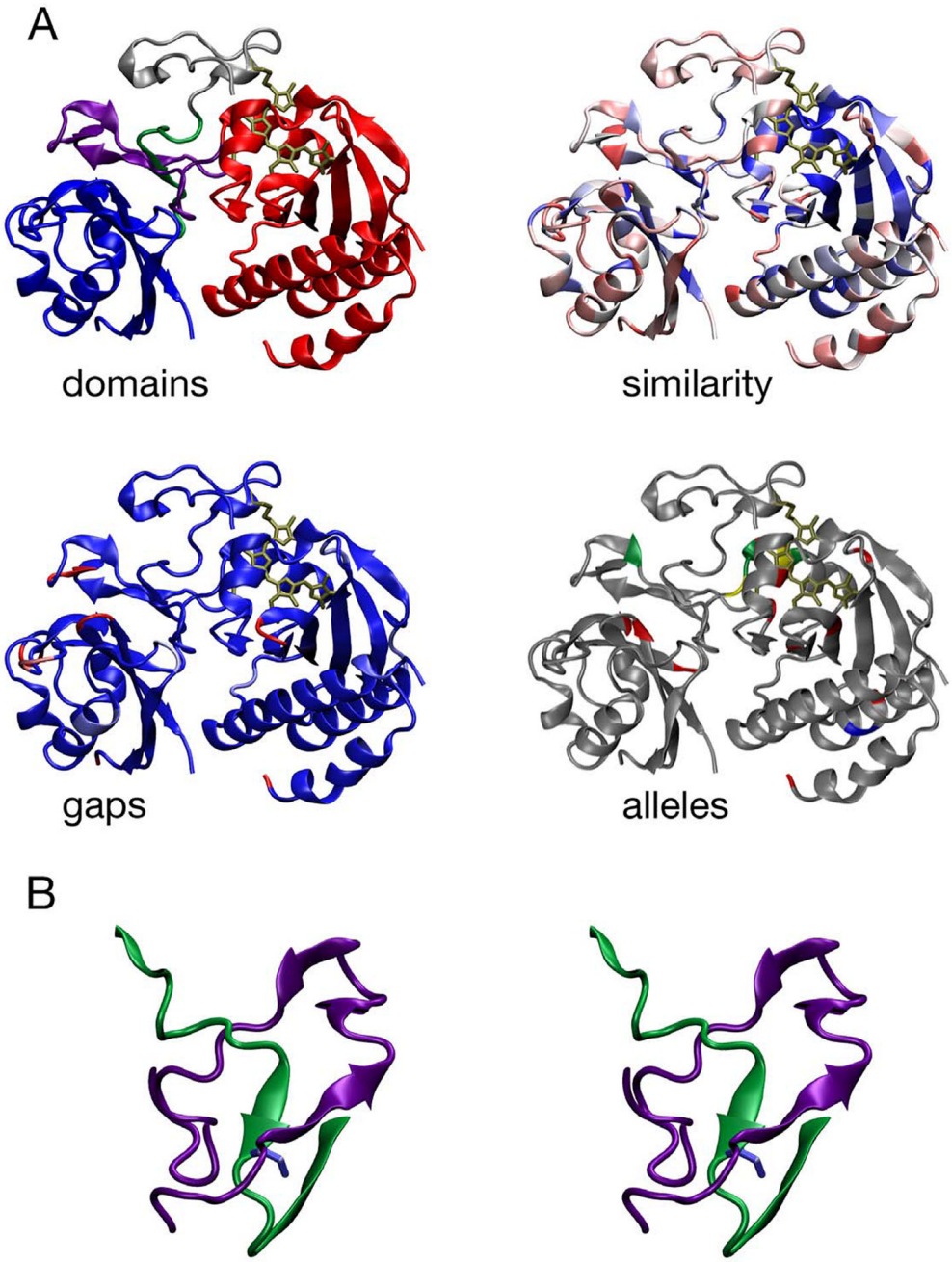

Figure 3. Conservation of the PAS and GAF domains of phytochromes

The 2.5 Å crystal structure of DrBphP P2/PAS and P3/GAF domains (PDB code 1ZTU: (103)) is shown with bound BV chromophore covalently attached to Cys_{24} (bronze) colored by domains (top left), similarity (top right), gaps (bottom left), and known alleles of plant Phys (bottom right). The DrBphP structure colored by domains (top left) uses the following color scheme: PAS, blue; GAF, red; N-terminal knot interface, green; GAF insert knot interface, purple; N-terminus, grey. The DrBphP structure colored by similarity (top right) uses a normalized BLOSUM62 matrix (38) and the alignment of 122 phytochromes presented in Supplemental Figure 1. A continuous color scale is used, ranging from dark blue (100% conserved) to bright red (variable). The DrBphP structure colored by length of gaps (bottom

left) uses the alignment in Supplemental Figure 1. A continuous color scale ranges from light blue (no gaps) to bright red (gaps ≥ 5 amino acids long), with a gap defined as a position where any phytochrome has insertions relative to DrBphP. The DrBphP structure colored by the location of alleles in plant phytochromes (bottom right) shows alleles that have been reported within the PAS/GAF domains of DrBphP against a grey background (see Supplemental Table 2). Loss-of-function alleles are colored red, gain-of-function alleles are colored blue, positions with multiple phenotypes are colored yellow, and silent alleles are colored green. Figure 3 and Figure 4 were prepared using VMD (40), Tachyon (96), STRIDE (28) and homolmapper (N. C. R. and J. C. L., unpublished results). (b) Stereoview of the conserved trefoil knot at the interface between the PAS and GAF domains by residues 27–43 (green, upstream of the PAS domain and the first beta strand of the PAS domain) and 227–257 (purple). Ile35 (blue) is at the center of the knot.

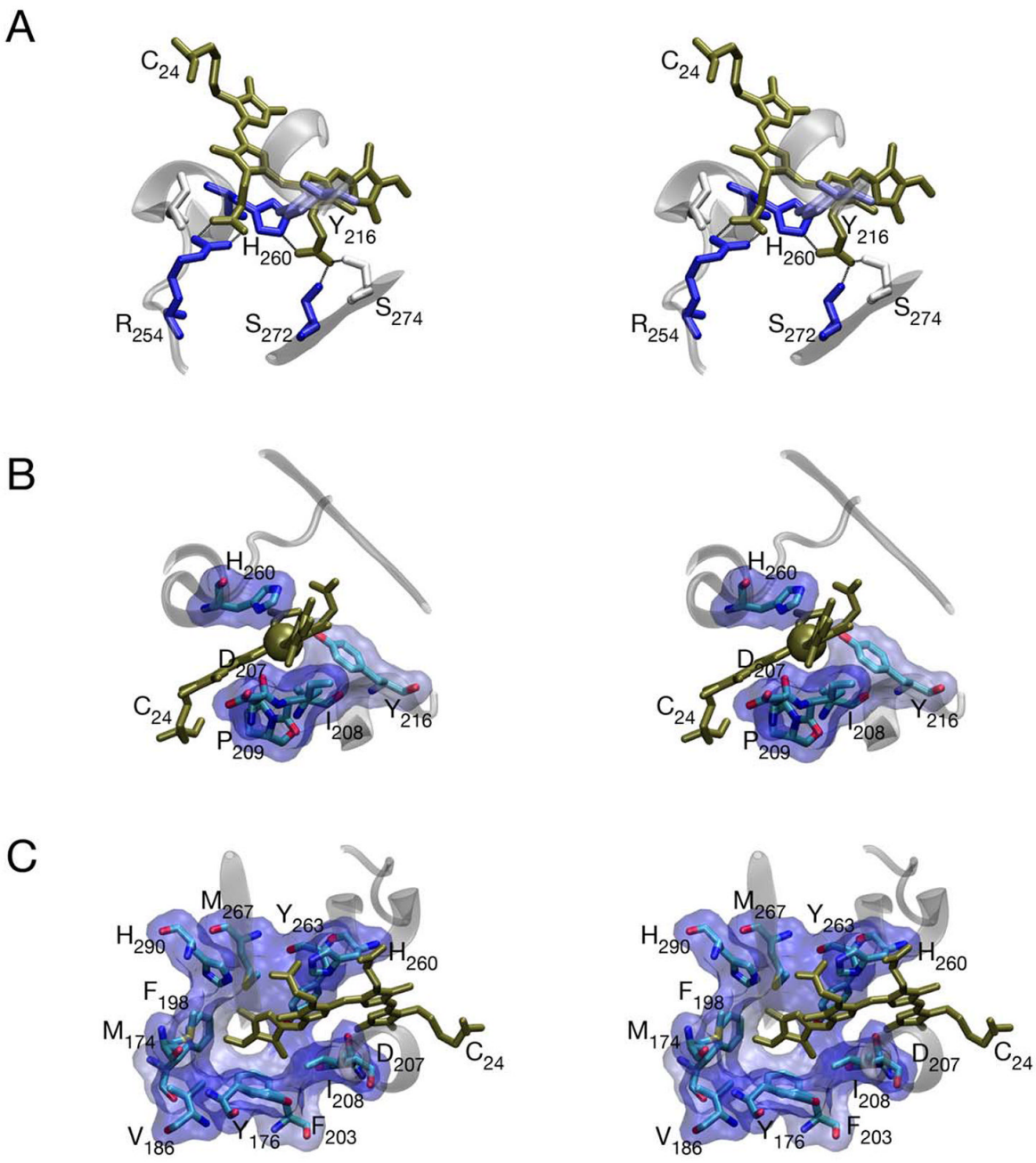

Figure 4. Chromophore-protein interactions in DrBphP are conserved

(a) Interaction of the buried carboxylate side chains of BV (bronze) with DrBphP (103). The B ring carboxylate interacts with the conserved Arg254, which is part of the trefoil knot, while the C ring carboxylate interacts with conserved Ser272 and His260. All protein residues within 3.5 Å of the carboxylate oxygens are shown colored by similarity as in Figure 2. Secondary structure elements are shown in transparent grey for residues 214–218, 254–262, and 271–275 for reference. (b) Environment of the C10 bridging carbon (bronze sphere). This atom is held in place by the B and C rings of biliverdin along with the conserved Asp207, Ile208, Tyr216, His260, and Pro209 (shown as sticks colored by atom type and as solvent-accessible surface, with the surface colored by similarity as in Figure 2). (c) Environment of the D ring. Residues

within 5 Å of the chromophore D ring and/or C15 methine bridge are shown as sticks and surface as in part (b).

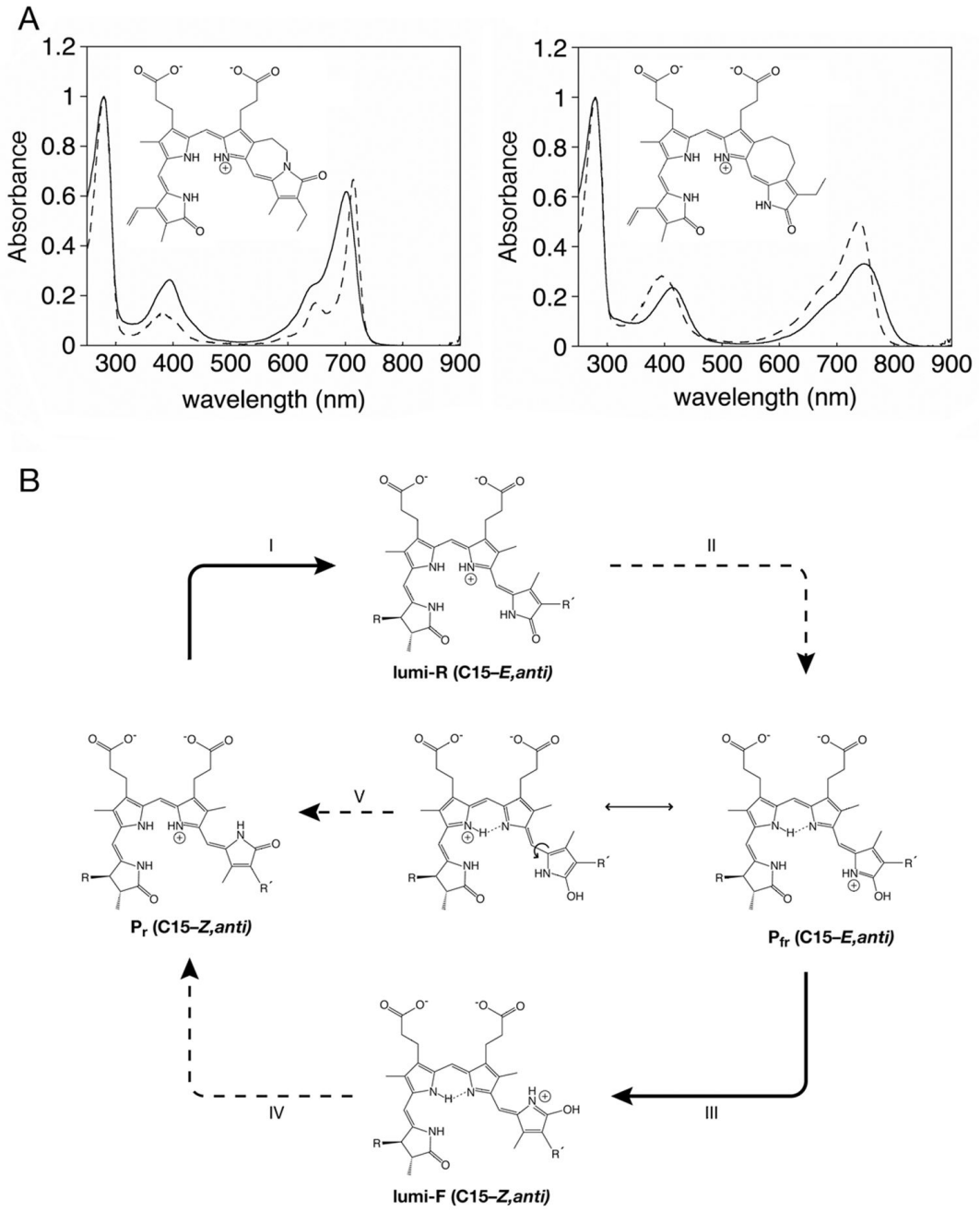

Figure 5. Chemical delineation of the phytochrome photocycle

(a) Structures and spectra for synthetic, sterically locked bilins (44) assembled with the bacteriophytochrome Agp1 from *Agrobacterium tumefaciens*. (left) Spectra for the C15–*Z,anti* locked bilin (dashed) and P_r biliverdin (solid) adducts. (b) Spectra for the C15–*E,anti* locked bilin (dashed) and P_{fr} biliverdin (solid) adducts. Spectra in (a) and (b) are courtesy of Drs. Tilman Lamparter and Katsuhiko Inomata. (c) Proposed photocycle for phytochromes utilizing PCB or PΦB. The P_r conformation is assigned based on the crystal structure of DrBphP, the locked bilin data presented in part (a), and the known stereochemistry of the 3 stereocenters in these molecules. Illumination with red light triggers photoisomerization about the C15–C16 double bond (**I**) to give the C15–*E,anti* primary photoproduct lumi-R, which is

subsequently converted to P_{fr} in several light-independent steps (**II**). As discussed in the text, the proposed P_{fr} is hypothetical but would account for the observed instability of the P_{fr} chromopeptide, the red-shifted P_{fr} absorbance maximum, and the observed P_{fr} dark reversion. Illumination of P_{fr} with far-red light (**III**) triggers the reverse photoisomerization to yield the C15–*Z,anti* lumi-F primary photoproduct, which is subsequently converted to P_r in a series of light-independent steps (**IV**). Dark reversion would proceed through the P_{fr} resonance form with single-bond character about C15–C16, which would readily undergo thermal rotation about this bond and then convert to P_r(**V**).

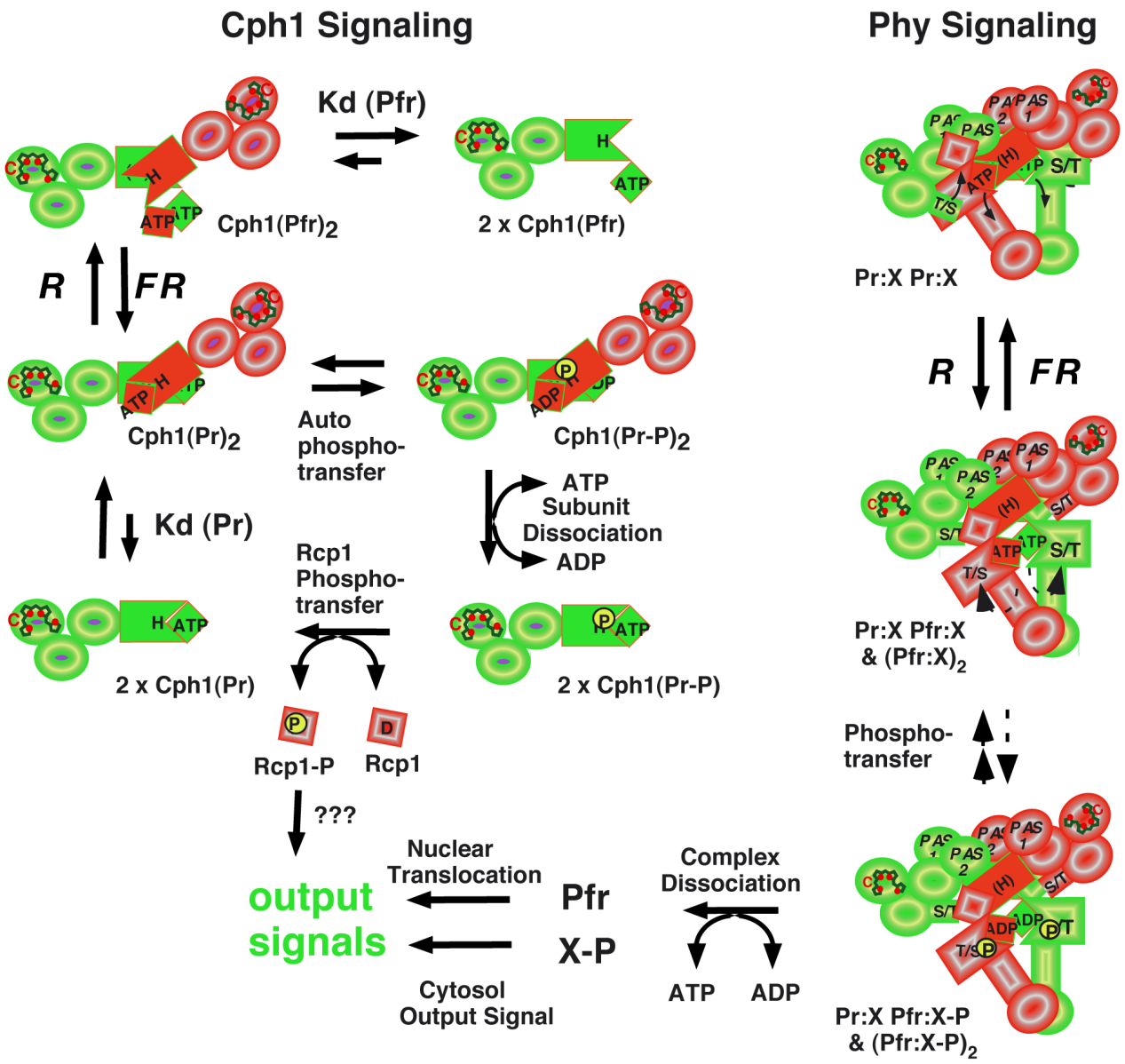

Figure 6. Hypothetical signaling mechanisms for prokaryote and eukaryote phytochromes

Homodimerization of the prokaryote phytochrome (Cph1) is dynamic and light-dependent (upper left) since autophosphorylation is favored by the formation of homodimers in the P_r form ($Cph1(P_r)_2$) and inhibited by conversion to P_{fr} ($Cph1(P_{fr})_2$) which dissociates to an inactive monomer (($Cph1(P_{fr})$). Exchange of bound ADP with ATP, a process that promotes dissociation of the phosphorylated P_r dimer ($Cph1(P_r\text{-}P)_2$) by inhibiting reassociation of the phosphorylated P_r monomer ($Cph1(P_r\text{-}P)$), stimulates histidine to aspartate phosphotransfer to Cph1's substrate Rcp1. The dephosphorylated P_r monomer ($Cph1(P_r)$) reassociates to form the active homodimer ($Cph1(P_r)_2$). Eukaryote phytochromes (Phys) are obligate homodimers that are associated with a cytosolic anchoring protein X in an ATP-dependent protein complex (upper right). Photoconversion yields a $P_r\text{-}P_{fr}$ heterodimer/$P_{fr}\text{-}P_{fr}$ homodimer mixture (P_r:X P_{fr}:X & $(P_{fr}:X)_2$) which results in activation of the Ser/Thr kinase activity and the stimulation

of phosphotransfer to anchoring protein X. The exchange of bound ADP with ATP favors dissociation of the P_{fr}:X complexes, enabling free P_{fr} to move to the nucleus and phosphorylated X to mediate a cytosolic output signal.