On the (un)coupling of the chromophore, tongue interactions and overall conformation in a bacterial phytochrome

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Running title: The (un)coupling of the chromophore and structure in DrBphP

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Keywords: phytochrome, photoreceptor, cell signaling, mutagenesis, photoconversion, protein structure, X-ray crystallography, structural biology, protein conformation, chromophore-binding domain

ABSTRACT

Phytochromes are photoreceptors in plants, fungi, and various microorganisms and cycle between metastable red light-absorbing (Pr) and far-red light-absorbing (Pfr) states. Their light responses are thought to follow a conserved structural mechanism that is triggered by isomerization of the chromophore. Downstream structural changes involve refolding of the so-called tongue extension of the phytochrome-specific GAF-related (PHY) domain of the photoreceptor. The tongue is connected to the chromophore by conserved DIP and PRxSF motifs and a conserved tyrosine, but the role of these residues in signal transduction is not clear. Here, we examine the tongue interactions and their interplay with the chromophore by substituting the conserved tyrosine (Tyr-263) in the phytochrome from the extremophile bacterium Deinococcus radiodurans with phenylalanine. Using optical and FTIR spectroscopy, X-ray solution scattering, and crystallography of chromophore-binding domain (CBD) and CBD–PHY fragments, we show that the absence of the Tyr-263 hydroxyl destabilizes the β-sheet conformation of the tongue. This allowed the phytochrome to adopt an α-helical tongue conformation regardless of the chromophore state, hence distorting the activity state of the protein. Our crystal structures further revealed that water interactions are missing in the Y263F mutant, correlating with a decrease of the photoconversion yield and underpinning the functional role of Tyr-263 in phytochrome conformational changes. We propose a model in which isomerization of the chromophore, refolding of the tongue, and globular conformational changes are represented as weakly coupled equilibria. The results also suggest that the phytochromes have several redundant signaling routes.

Introduction

Phytochromes are a family of red/far-red light-sensing photoreceptors found in plants, fungi, cyanobacteria and eubacteria. They play a role for example in seed germination and shade avoidance in plants and chromatic adaptation in bacteria (1). They bind a linear bilin chromophore that is phytochromobilin (PΦB) in plants, phycocyanobilin (PCB) in cyanobacteria, and biliverdin (BV) in bacteria. In response to red
light, the phytochromes cycle between two metastable states. In prototypical phytochromes, the resting state absorbs red light (Pr) and the activated state absorbs far-red light (Pfr). Some phytochromes however have Pfr as a resting state and are called bathy phytochromes. Phytochromes can be reversibly switched between the states with red light (Pr→Pfr) and far-red light (Pfr→Pr). In dark, they thermally revert to their resting state.

Phytochromes are usually dimeric proteins which consist of two modules (2, 3). The photosensory module (PSM) contains a PAS (Per/Arnt/Sim), a GAF (cGMP phosphodiesterase, adenylyl cyclase, FhlA) and a PHY (phytochrome-specific GAF-related) domain (Fig. 1). In plants, the PΦB chromophore is bound to a conserved cysteine in GAF domain whereas in bacteria this cysteine resides N-terminal to the PAS domain (4). The chromophore itself, consisting of four rings (A–D), is nested in a chromophore-binding pocket that is formed by PAS and GAF domains (5). This PAS-GAF entity is therefore often called a chromophore-binding domain (CBD). The PSM is followed by an output module that is often a histidine kinase (HK) domain in bacteria, making these phytochromes act as sensors in two-component signaling systems (6). The output module is more variable in plants, which includes for example additional PAS domains and a histidine kinase-related domain.

Upon red light activation the bilin chromophore isomerizes, which leads to changes in the rest of the phytochrome (Fig. 1). It has been shown that several residues surrounding the chromophore are important for the initial photoreactions. These include a conserved DIP motif in the GAF domain (3). The initial photoreactions in the immediate chromophore surroundings lead to changes in a so-called tongue extension of the PHY domain. In the Pr state this PHY tongue adopts a β-hairpin conformation, whereas in the Pfr state it adopts an α-helix and a coil (7-10). The tongue contains a conserved PRxSF motif that binds the DIP motif of the GAF domain. In the Pr state, a conserved Asp residue of the DIP motif, D207 in the Deinococcus radiodurans phytochrome (DrBphP), forms a salt bridge with an Arg residue (R466) of the tongue (3, 9, 11). In the Pr→Pfr transition this salt bridge is broken, the entire PHY tongue refolds, and the Asp-interacting arginine is replaced by a serine (S468) of the tongue helix (9, 10, 12). Light-induced refolding of the PHY tongue affects the orientation of the PHY domain, opening the entire PSM dimer (9). In full-length phytochromes, this large-scale change is then relayed by the long connecting helices to the output module, causing changes in its structure and activity (13-15). The output activity is often a kinase activity but may vary depending on the species, and its changes will lead to biological responses.

The important DIP and PRxSF motifs are closely associated to a conserved tyrosine (Y263). The tyrosine resides close to the chromophore. A Y263F mutation obstructs the photocycle in the CBD fragment of DrBphP, leading to relatively high fluorescence quantum yield (16, 17). The same mutation has also been introduced to CBD-PHY fragment of cyanobacterial phytochrome Cph1 and plant PhyB (18, 19). In this study, we show how Y263 in DrBphP plays an important role in the structural and spectroscopic fine-tuning of the phytochrome activity. By a Y263F mutation we pinpoint the multiple roles that this tyrosine has for the photocycle and for stabilizing the tongue interactions. The results indicate that the coupling between the conformational states of the chromophore and protein is redundant and weaker than previously assumed and that the two entities may under certain conditions even be uncoupled.

**Results**

**Absorption and fluorescence spectroscopy indicate a role of Y263 in photocycle**

First, we studied UV-Vis absorption properties in the CBD and CBD-PHY fragments of DrBphP and their Y263F variants. The absorption spectra of these proteins (Fig. 2A) resemble the spectrum of the full-length DrBphP in the Pr form with absorption maxima at 698 nm (CBD), 700 nm (CBD-PHY) and 702 nm (CBD-Y263F and CBD-PHY-Y263F) (16, 18, 20, 21).

Generally, red illumination reversibly bleaches the 700 nm absorbance and the absorption maximum shifts to far-red region (Fig. 2C). In the CBD-PHY fragments the absorbance maxima of the illuminated spectra appear at around 755 nm, but in the CBD fragments this far-red peak is blue-shifted around 10 nm. The shift
due to Y263 was hypsochromic in Pfr, as opposed to Pr. This indicates that the interaction of Y263 with the chromophore is different in Pfr compared to Pr. Five times longer illumination times were needed for CBD-PHY-Y263F to reach photoequilibrium compared similar to the wild-type CBD-PHY (Supplementary Figure S1A). Since, the dark conversion rate from Pfr to Pr was hardly affected by the mutation (Supplementary Figure S1B), we conclude that the Y263F mutation reduces the photoconversion (Pr→Pfr) yield and possibly the absorption properties of the Pfr state.

As demonstrated earlier (16, 17, 22), the Y263F mutation influences to the fluorescence properties of the DrBphP. We recorded fluorescence spectra after excitation at 630 nm (Fig. 2D). The spectra have maxima at 720 nm (CBD), 718 nm (CBD-PHY) and 722 nm (CBD-Y263F and CBD-PHY-Y263F). Again, a small bathochromic shift is seen in both Y263F variants, which is consistent with the steady-state absorption measurements for Pr. The fluorescence quantum yields increase from 2.5% (CBD) to 4.8% (CBD-Y263F) and from 1.7% (CBD-PHY) to 4.4% (CBD-PHY-Y263F) due to the Y263F mutation.

The fluorescence decay properties of the biliverdin molecule in the binding pocket were studied by time-correlated single photon counting with an excitation wavelength of 660 nm and monitoring wavelength of 720 nm (Fig. 2E). The fluorescence decays were fitted with exponential functions (23). For Y263F variants, only a single decay component was needed. For the wild type samples, multi-exponential fitting curves were needed. The fluorescence decay of the CBD sample is described with lifetimes (and amplitudes) of 375 ps (77%) and 615 ps (23%), in line with values of (23). The CBD-PHY sample was best described with lifetimes of 173 ps (67%), 570 ps (31%) and 2 ns (2%). The fluorescence yields correlated with the averaged fluorescence lifetimes, which were 420 ps (CBD), 670 ps (CBD-Y263F), 500 ps (CBD-PHY), and 620 ps (CBD-PHY-Y263F) (Fig. 2F).

An increase in fluorescence yield implies a decrease in yield of forming the first ground state intermediate, Lumi-R. Thus, we conclude that the Y263F mutation hinders the Lumi-R formation. As demonstrated in the Supplementary Figure S1B, the thermal reversion rate from the Pfr state is equally slow or slower in the case of CBD-PHY-Y263F when compared to the wild-type CBD-PHY. This means that the CBD-PHY-Y263F sample can be populated to similar photoequilibrium Pfr state with prolonged illumination of the red light as the wild-type CBD-PHY sample, even though the Lumi-R formation is hampered due to mutation of the Y263.

**FTIR spectroscopy reveals reduced PHY long refoiling in the Y263F mutant**

The Fourier Transform Infrared (FTIR) spectroscopic analysis gives information from the light-induced changes in biliverdin and the protein. Figure 3 shows the FTIR difference spectra between the illuminated and the dark states of wild-type CBD-PHY and CBD fragments and their Y263F mutants. The biliverdin-based transitions are distinguished from those originating from the protein by using uniformly $^{13}$C$^{15}$N-apoprotein labeled CBD-PHY and CBD-PHY-Y263F samples. The transitions from the biliverdin molecule remain unchanged in the $^{13}$C$^{15}$N-labeled samples whereas the protein peaks are shifted. As indicated in the Supplementary Figure S1C, the negative bands at 1734 cm$^{-1}$ and 1712 cm$^{-1}$ originate from the carbonyl vibration of the biliverdin rings A and D, respectively (24). The large positive band at 1688 cm$^{-1}$ in the CBD-PHY is an induced absorption in the Pfr state. Most likely, this positive band originates largely from the carbonyl vibration of the D ring. In addition, changes in the biliverdin vibrations can be observed at 1250 cm$^{-1}$ and 1230 cm$^{-1}$ (Supplementary Figure S1C).

The FTIR difference signal of the CBD-Y263F is small compared to the wild-type CBD (Fig. 3A, top), which is a result of that Pr state dominates the photoequilibrium after red light irradiation, as described in the UV-Vis analysis (Fig. 2B). The three-fold multiplication of the signal however still highlights a difference signal for CBD-Y263F variant. In particular, the negative band at around 1712 cm$^{-1}$ shows that the D ring environment is affected by illumination. On the other hand, the 1734 cm$^{-1}$ band is missing in CBD-Y263F variant thus indicating no changes in the A-ring environment under illumination.
Interestingly, many FTIR features originating from the protein environment of CBD are present and similar in CBD-Y263F, but a few missing bands in the variant can be assigned as potential signals from the Y263 in the CBD sample. Signals that are completely missing in the CBD-Y263F compared to the wild type are found at 1514 cm⁻¹ and 1209 cm⁻¹. Signals with reduced intensity were at 1251 cm⁻¹, 1527 cm⁻¹, and 1598 cm⁻¹. These frequencies refer well to Tyr-OH vibrations (25), except the 1527 cm⁻¹, which may report changes in the Asp (COO⁻) interactions.

In contrast to the CBD fragments, the FTIR difference spectrum of CBD-PHY-Y263F variant has a similar amplitude to the wild-type CBD-PHY (Fig. 3A, middle). This reveals that after prolonged irradiation time these two samples have a similar Pfr/Pr ratios, which is in line with the UV-Vis analysis (Fig. 2B and Supplementary Figure S1A). The similar negative features at 1712 cm⁻¹ in the CBD-PHY and CBD-PHY-Y263F variants indicate that their D ring carbonyls experience similar interactions in the Pr state. On the other hand, the D ring signal in the Pfr state is downshifted to 1685 cm⁻¹ in the CBD-PHY-Y263F variant (Fig. 3A, middle and bottom). This indicates that the D ring carbonyl experiences a different chemical environment in the mutant than in the wild type, for example due to altered H-bonding. The slight upshift of the negative 1734 cm⁻¹ carbonyl vibration of the A-ring, observed both in the non-labeled and ¹³C¹⁵N-labeled samples, indicates that Y263 influences the biliverdinin–protein interaction in the Pr state. Small changes are also seen in the region of 1527 cm⁻¹ (-), 1514 cm⁻¹ (-), and 1506 cm⁻¹ (+), suggesting changes in the Tyr protonation state and interactions. The differences between the wild-type and Y263F samples at 1270–1230 cm⁻¹ reflect to large extend the light-induced changes in the biliverdinin–protein interactions. Naturally, the Y263 H-bonding character between the Pr and Pfr states influence these changes as well (26).

The secondary structural changes in the CBD-PHY domains during the photoconversion are more clear in the ¹³C¹⁵N-isotope labeled samples as they separate better from the overlapping signal of the CBD fragment (Fig. 3A, bottom). In particular, the β-sheet to α-helix transition is now clearly pronounced as a positive-negative feature at 1612 cm⁻¹ (+) / 1600 cm⁻¹ (-). This can be translated to the signal of the transition in the tongue region, similar to Kennis and co-workers with a signal pair at 1655 cm⁻¹ (+) / 1633 cm⁻¹ (-) for the unlabeled samples of bacterial phytochromes from Rhodopseudomonas palustris (10, 12). The difference signal amplitude of CBD-PHY-Y263F is about 60% from that of the wild type. This indicates less secondary structural changes, either due to a larger α-helix population in the Pr state or larger β-sheet population in the Pfr state in the mutant. The smaller amplitudes of CBD-PHY-Y263F in the changes of secondary structural are also observed in the Amide II region as indicated by the broad negative peak in the double difference spectrum at 1560 cm⁻¹ (-) and positive signal at 1506 cm⁻¹ (+) that have shifted due to isotope labeling to 1540 cm⁻¹ (-) and 1483 cm⁻¹ (+), respectively. An additional change at 1580 cm⁻¹ (+) in labeled spectra may be related to the absorption of aspartate side chain, which would be likely given the close proximity of Y263 to D207. In summary, the FTIR difference spectra demonstrate that the yield of the refolding of the tongue is lower in the Y263F mutant.

X-ray solution scattering reveals large-scale changes in all PSM variants
To study the role of Y263F mutation in large-scale structural changes in solution (9), we applied difference X-ray scattering for CBD-PHY and CBD-PHY-Y263F. The results shown in Figure 3B and 3C indicate that both variants give highly similar Pfr–Pr difference signal which has been assigned to the CBD-PHY dimer opening (9, 27). The signals were practically identical in shape (Fig. 3B), indicating that CBD-PHY-Y263F undergoes the large-scale movements which are identical to the wild-type protein. The amplitudes of the differences signals however varied, which indicates a reduced yield of dimer opening when compared to the wild-type CBD-PHY (Fig. 3C). This reduction in yield may be due to the lower yield of forming Lumi-R or the reduced yield of tongue refolding. The effect was even more pronounced in D207H mutant (Supplementary Figure S1D) which has a broken D207–R466 salt bridge and therefore impaired interactions between the PHY tongue and GAF domain.
Importantly, the X-ray scattering results, together with FTIR, indicate that in addition to the conserved Y263 (and D207 of the DIP motif) there should be other signaling routes that relay the structural signal from the chromophore to the PHY tongue. To reveal the structural details underlying the impaired photocycle and the alternative signaling routes to the tongue, we crystallized Y263F mutants of the CBD and CBD-PHY fragments, respectively.

**Crystal structure of CBD-Y263F reveals reduced water interactions**

CBD-Y263F was crystallized in the same conditions as the wild-type CBD (28). Crystal data were processed up to 1.34 Å resolution and the electron density appears completely comparable to the corresponding wild-type data. The overall CBD-Y263F structure was highly similar to the wild-type CBD (Fig. 4A) (28) with overall root-mean-square deviation of 0.102 Å. The most notable difference between the structures is a missing water which resides between the pyrrole water and the Y263 phenyl ring. This “transient water” (W2) is present in some of the CBD structures (17, 22, 28) and interacts with so-called pyrrole water (W1), D207 and biliverdin ring A (Fig. 4B). The phenyl ring of the mutated F263 resides slightly closer to the pyrrole water than in the wild-type Y263, which may exclude the transient water from the binding pocket. The slight repositioning of the F263 side chain may also be a result of reduced steric effects and the lack of hydrogen bonding of the hydroxyl moiety of this residue. The hydroxyl appears to bind to one solvent water molecule in the wild-type (W3). This interaction is lost in the Y263F structure and the water density is reduced (Fig. 4C and 4D). Otherwise no large changes between the CBD and CBD-Y263F structures were observed and the pyrrole ring orientations seemed unchanged at this resolution.

**CBD-PHY-Y263F in Pr state crystallized exclusively in Pfr-like conformation**

Although the wild-type CBD-PHY formed Pr-state crystals like previously reported (14), the Y263F mutant did not (Supplementary Figure S2A). To get Pr structures from the CBD-PHY-Y263F variant, we screened for new crystallization conditions in dark. Although CBD-PHY-Y263F could not be crystallized in Pr-like crystallization condition, it crystallized readily in the previously-reported Pfr-like crystallization condition (9). Pre-illumination with red light greatly facilitated the crystal growth (Supplementary Figure S2A). This conditions yielded “pre-illuminated” crystals that diffracted up to 3.3 Å resolution. Surprisingly, we found out that both wild-type CBD-PHY and the Y263F variant could be crystallized in this Pfr-like crystallization condition even if they were kept in the Pr state. We found an additional condition in which the CBD-PHY-Y263F mutant crystallized especially well in complete darkness and without pre-illumination. The resulting “dark” crystals diffracted to 3.6 Å resolution, and had an open PSM conformation, like in the previously reported Pfr structures (Fig. 5) (9, 29).

The relative tendency of the CBD-PHY variants to form the Pfr-like crystals in dark varied, hinting for different propensities to adopt open conformation in dark (Supplementary Figure S2A). CBD-PHY-Y263F crystallized most readily in the “dark” crystallization condition reported here, but also in a published Pfr crystallization condition (9). This propensity to crystallize implies that the CBD-PHY-Y263F adopts an open conformation more easily, probably due to the weakened interactions between the tongue and GAF domain. The wild-type CBD-PHY, on the other hand, crystallized faintly in Pfr-like crystallization conditions without pre-illumination. This implies that it rarely adopts Pfr-like conformation in darkness due to stronger interactions between the GAF domain and the Pr-state tongue. On some occasions, however, even the wild-type CBD-PHY did crystallize in these conditions. The absorption spectra of the dark crystals (Fig. 5B) verified that the PAS-GAF-PHY variants have indeed remained in the Pr state from the biliverdin point of view.

The pre-illuminated and dark CBD-PHY-Y263F crystals had highly similar packing and dimensions with each other and with previously-reported wild-type pre-illuminated Pfr crystals (9, 29) (see Table 2). Indeed, both crystal structures closely resembled these published structures. The CBD-PHY mutant dimers seemed to adopt an “open” conformation with separated sister PHY
domains and a helical PHY tongue conformation (Fig. 5A and 5C).

Closer inspection of the dark CBD-PHY-Y263F structure revealed that the chromophore-binding pocket resembles that of the Pr-state structure but the rest of the protein adopts a Pfr-state-like conformation. Notably, three residues (Y176, H201 and F203) along with R222 adopted exclusively a conformation that is characteristic of the Pr state (Fig. 5D). Although the resolution was not adequate to unequivocally confirm the details of the biliverdin conformation, the chromophore was modelled in Pr-state (15Z) isomer. The electron density distribution (Supplementary Figure S2C) and the absorption spectrum (Fig. 5B) support this biliverdin state. The overall chromophore surroundings was highly similar to our wild-type Pfr structure, which has mixed Pr/Pfr photochromic state and Pr-like side chain orientations (9).

In the pre-illuminated CBD-PHY-Y263F structure, the orientation of three residues (Y176, H201 and F203) was different from that of the dark CBD-PHY-Y263F structure. Whereas in the dark crystals these residues reside in Pr-like conformation, the Pfr-like conformations predominate in the pre-illuminated structure (Fig. 5D). It appears that these residues can adopt both Pr- and Pfr-orientations in this crystal form and these orientations reflect the prevalent Pr/Pfr ratio within the crystals. The R222 residue close to biliverdin propionate B adopted a Pr-like conformation here, although it has been reported to have a different conformation in a saturated Pfr state (8, 29). The chromophore in the pre-illuminated structure is oriented in the pocket as an intermediate of Pr- and Pfr-state biliverdins, closely resembling the Pfr structure published by us (9). Because this crystal form is a result of pre-illumination with red light, the chromophores were modelled as a Pr/Pfr mixture. In addition, the propionate C of the biliverdin shows some flexibility in pre-illuminated crystals as the electron density is diffuse in this site (see Supplementary Figure S2C), which is in accordance with the results with other Pfr structure of DrBphP PSM (29).

The orientation of the Y176-F203-H201 triplet in Pr state led to a structural clash between H201 and the tongue residue A450 (Fig. 5D). This explains the unstructured region in the tongue near A450 that could not be modelled. The dark crystals therefore have substantially more unstructured PHY tongue region than the pre-illuminated crystals (Fig. 5C).

Discussion

The amino acids surrounding the chromophore are highly conserved in phytochromes, which reflects their importance for the function of the protein. Generally, the residues participate in coupling of the chromophore state to the conformation of the rest of the protein. Specifically, a triad of residues, D203 of the DIP motif, S466/R468 of the PRxSF motif, and Y263, is expected to couple chromophore isomerization with conformational changes in the PHY tongue. In this paper, we confirmed by mutational, spectroscopic and structural analysis that Y263 plays a central role in the structural and spectral responses in *Deinococcus radiodurans* phytochrome. It influences the excited-state reactions of the biliverdin and stabilizes the tongue interactions to the GAF domain, thus affecting the structural equilibrium between Pr- and Pfr-like conformations. However, our data show that this coupling is weaker than commonly believed and that it can be modified. The Y263F mutation leads to reduced Pr→Pfr photoconversion of the biliverdin, but also favors a constitutively active conformation of the photoreceptor.

The role of Y263 in early stages of the photocycle

The UV-Vis absorption and fluorescence data indicate that Y263 plays an important role in phytochrome photocycle. The role of this residue is most striking in the CBD-Y263F sample which has a barely detectable photoconversion and an elevated fluorescence quantum yield (16) (Fig. 2). The increased fluorescence yield hints for a role of Y263 in the excited-state reactions of the biliverdin and therefore on the Lumi-R formation. Somewhat surprising is that the structural differences between the CBD-Y263F and the wild-type CBD are the missing or absent hydrogen bonds involving a few water molecules (Fig. 4). One missing water resides between Y263 and pyrrole water and potentially has an important role in the photocycle. Whether the changes in the
hydrogen bonding pattern and biliverdin electrostatics are the cause of the divergent photocycle of Y263F, is a matter of future research.

The mutated PSM fragment (CBD-PHY-Y263F) also has a higher fluorescence yield compared to the wild-type PSM, suggesting an impaired Lumi-R formation (Fig. 2). This is in accordance with the fluorescence studies with the Y263F mutant of Cph1 (18). Due to the long thermal reversion times, prolonged illumination conditions can be used to populate the sample to a similar Pfr state to the wild-type PSM. This is consistent with a previous study with cyanobacterial Cph1 where it was concluded that the aromatic character, but not the hydroxyl group, of the conserved Y263 is critical for the Pfr formation (18). The hydroxyl of Y263 facilitates the photocycle and Pfr formation, but is not indispensable for these events. The resulting Pfr spectrum of CBD-PHY-Y263F closely resembles that of CBD-PHY, speaking for a similar biliverdin end state to the wild-type protein. This is also apparent in the crystal structures reported here (Fig. 5 and Supplementary Figure S2). The ability of CBD-PHY-Y263F to reach the Pfr state indicates that other residues in the binding pocket and/or in the PHY tongue are likely to be engaged in the photocycle.

The coupling of the PHY tongue to the chromophore

Phytochromes switch between the Pr and Pfr states in response to light, and this change in the photoactive state reflects their output activity state. We propose that the photochromic and conformational states of phytochromes do not necessarily go hand-in-hand. Although the Pr/Pfr ratio of the bilin chromophore can be shifted by light, the phytochrome activity may be uncoupled from this light regulation. By mutating the Y263 residue at the GAFPHY interface, we were able to break this coupling. This resulted in a phytochrome which has its chromophore in Pr state, but its PSM equilibrium is shifted towards a Pfr-like conformation.

This spectral and functional uncoupling has also been shown in other phytochrome species. The mutation of this conserved tyrosine in Arabidopsis thaliana PhyB (Y361F) led to greatly enhanced red light sensitivity (19). There is even evidence that the lack of the entire tongue favors the active state of the phytochrome as an IsPadC tongue deletion mutant has high diguanylyl cyclase activity even in its dark state (30). The evidence speaks for the role of the conserved tyrosine in stabilizing the tongue interactions to the GAF domain. If this interaction is lost or weakened, the phytochrome adopts a Pfr-like conformation more likely that is in many cases physiologically active (1).

The absence of Y263 hydroxyl group in the mutant presumably weakens the tongue interactions that involve a salt bridge between the conserved DIP and PRxSF motifs (D207-R466). This effect favors Pfr-like tongue arrangement over the Pr-like and causes a reduced light-responsiveness of the phytochrome molecule. This may explain smaller signals of tongue refolding (Fig. 3A) and large-scale conformational changes of the Y263F mutant (Fig. 3B and C).

The importance of the tongue interactions in light-responsiveness is further underlined by greatly reduced difference X-ray scattering signal of CBD-PHY-D207H (Supplementary Figure S1D). This indicates that D207 plays an even more prominent role in tongue stabilization than Y263.

CBD-PHY-Y263F seemed to crystallize exclusively in an open Pfr-like conformation. This indicates that the mutation makes the PSM to prefer this conformation over Pr conformation in solution. During the crystal formation, packing effects may further favor a Pfr-like conformation by forcing the remaining Pr-state proteins to adopt this form. We note that even the wild-type PSM molecules in the Pr state can be forced to form Pfr-like crystals by the crystallization conditions (Fig. 5B), even though they would unlikely adopt a Pfr-like conformation spontaneously. In contrast to CBD-PHY-Y263F in D. radiodurans, the Y263F mutant of cyanobacterial Cph1 has been successfully crystallized in the Pr-like conformation (18). This Cph1 mutant however has a slightly different tongue arrangement compared to the wild-type Cph1 and other Pr-state PSM structures (7, 9, 11, 14, 30-32). One explanation to this could be the crystal packing effects: Most phytochrome PSM structures in the Pr state, like in DrBphP CBD-PHY (9, 14), have crystal contacts between the tongues of the neighboring symmetry
mating. If the native tongue conformation is disrupted, this packing interaction is compromised. Even slightest changes in the tongue conformation and stability would therefore make the Pr-like crystal formation unfavorable.

The published Pfr-state crystal structures indicate that the Y263F hydroxyl may in theory interact with the biliverdin D ring (Supplementary Figure S3A). However, we did not detect any increase in the rate of dark reversion in the Y263F mutant (Supplementary Figure S1B), which speaks against the role of Y263 in stabilizing the Pfr state. Residue H201 in DrBphP is shown to stabilize the Pfr state by interacting with the biliverdin D ring (29). This histidine however is not conserved in most prototypical phytochromes (Supplementary Figure S3B). This additional interaction in DrBphP would explain its relatively stable Pfr state and slow dark reversion rate (9, 14, 20).

**Redundant routes for coupling the PHY tongue to the chromophore**

Upon photoconversion, three residues close to the D ring, Y176, F203, and H201, re-orient (Fig. 5D). This change in orientation has been noted before (29) but its exact role in the phytochrome signaling remains unclear. Although the re-orientation of H201 enables an interaction with the ring D and a more stable Pfr state in DrBphP, it may have a different role to leucine in other phytochromes. Residue Y176, and the aromatic character of F203 are conserved among all phytochromes (Supplementary Figure S3B), and their mutation obstructs the photoconversion of DrBphP (21, 29).

Although the residue A450 in the helical tongue may affect the re-orienting of the Y176-F203-H201 triplet through steric packing interactions (arrows in Fig. 5D), this effect is too weak to affect the biliverdin spectrum in dark crystals. The three residues may allow more space for the D ring to rotate, which would enable the switching of these crystals between the Pr- and Pfr-like states without radically losing crystal integrity. Our data therefore indicate that both Pr and Pfr orientations of the triplet can co-exist in Pfr-like crystal form and that they mainly reflect the photochromic state of the biliverdin. This indicates that it is the chromophore, not the tongue conformation, which is the main determinant of the Y176-F203-H201 orientation. It is possible that this triplet plays a role in phytochrome signaling by affecting the tongue stability: In the Pr state, H201 points away from the chromophore-binding pocket and de-stabilizes the Pfr-like tongue conformation around residue A450. This is visible as missing electron density at this region in our dark Pr-state structure (Fig. 5C). In the Pfr state, on the other hand, H201 potentially clashes with the \(^{451}\)WGG\(^{453}\) region of the Pr-like tongue, making this combination unlikely. This way the Y176-F203-H201 route can act as a mediator by favoring the tongue conformation dictated by the chromophore. The residue triplet would facilitate the refolding process during the photoconversion and reduce the prospects of a misfolded PHY tongue. Indeed, mutating the PhyA and PhyB tyrosines that correspond residues Y176 and F203 in DrBphP render them constitutively active (35, 36).

We propose that there is a \(\beta\)-sheet/\(\alpha\)-helix equilibrium for the tongue conformation and that it is affected by several means. The conserved D207–R466 salt bridge and Y263 play an important role in the stabilization of Pr conformation, whereas the Y176-F203-H201 route may facilitate the coupling of the chromophore state to the tongue conformation. In addition, a conserved proline in the PRxSF motif (P465 in DrBphP) has been proposed to play a role in conformational trigger in the photoconversion through interaction with the biliverdin A ring (31). Together, these redundant interaction routes may couple the changes in chromophore to the tongue refolding. This coupling can be considered important for phytochromes, because the two states, dark reversion, and phototransition in between them have to be supported by a single protein structure. Redundancy in the signal pathway is also consistent with that these residues are highly conserved (Supplementary Figure S3B) but not entirely indispensable to the structural photoconversion.

**Phytochromes function at several levels that affect each other**

In the light of the presented result, we propose a model in which light-induced responses in photoreceptors occur at several levels (Fig. 6). Phytochromes can be considered to switch
conformation at the levels of the chromophore, the tongue, the PSM and the entire protein. Each level resides in an equilibrium that is affected by external factors and the other levels. For example, light and tongue interactions affect the Pr/Pfr equilibrium of the biliverdin. On the other hand, the tongue is affected by the state of the chromophore and the quaternary PSM arrangement (37). The PSM arrangement finally affects the structure and hence the activity state of the output module (11, 13, 38).

By introducing the Y263F mutation, we were able to shift the equilibria within each level. This caused deviant spectral and structural properties, but did not totally obstruct the function of the PSM. This speaks for redundant signaling routes within this signaling network, which may be an evolutionary conserved feature of phytochromes and other signaling enzymes. These properties would offer a photoreceptor sophisticated means to fine-tune the light-induced responses without compromising the function of the entire protein. A good example for this is a phytochrome from Stigmatella aurantiaca (SaBphP1), which has apparently defective photocycle (biliverdin level) but undergoes normal light-induced quaternary changes (PSM level) (27). The weak coupling and redundancy between the signaling levels has therefore enabled this phytochrome to adapt spectrally for novel purposes.

Experimental procedures

Molecular cloning and sample preparation
The constructs encoding the CBD and CBD-PHY fragments (amino acids 1–321 and 1–502) of the DrBphP bacteriophytochrome are described elsewhere (5, 21). The Y263F mutation was introduced to the wild-type fragments with QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies) and primer

5’-CCATGCACATGCAGTTCTGCGGAACATG
GCGTC-3’ (nucleotides coding F263 underlined). Protein expression and purification was done like previously (9, 12, 20, 23). The purified protein samples in final buffer (30 mM Tris/HCl, pH 8.0) were concentrated to 30 mg/ml concentration and flash-frozen. Just before measurements, the frozen phytochrome samples were quickly thawed and filtered with a 0.22 µm centrifugal filters (Amicon Ultrafree, Millipore) and diluted to the desired concentration with the final buffer. All measurements were carried out in dark at room temperature.

UV-Vis absorption and fluorescence spectroscopy
The steady-state absorption spectra were recorded as described previously (20). The fluorescence quantum yield was determined from the steady-state absorption and emission spectra, and the fluorescence decay was characterized using a time-correlated single photon counting system (22). The fluorescence data was analyzed using Pygspec (39).

X-ray solution scattering
The X-ray scattering data acquisition was performed at the cSAXS beamline of the Swiss Light Source (SLS) like previously described (13). In brief, the sample (ca. 30 mg/ml) was pumped through a 1.0 mm quartz capillary and switched between the Pr and Pfr states with a red diode-pumped solid state (DPSS) laser (671 nm, 5 mJ/mm², Altechna) and a far-red diode laser (789 nm, 7 mJ/mm², Thorlabs). While switching the sample, an overlapping X-ray beam (11.2 keV) was used to probe the difference scattering signal. More details can be found in Table 1 and in the Supporting information.

Fourier transform infrared (FTIR) spectroscopy
The samples were concentrated to an approximate concentration of 2.5 mM. Sample volume of 2 ul was pipetted on a CaF₂-window and sandwiched without any spacer between two window plates, resulting approximately 10 µm sample thickness. The hydration was assessed with absolute IR absorption spectrum in each sample, which resulted a ratio of 0.9/0.35 between Amide I (at 1645 cm⁻¹) and Amide II region (at 1580 cm⁻¹). The difference spectra were recorded with an FTIR spectrometer (Nicolet) by utilizing red (λ = 655 nm) and far-red (λ = 785 nm) light-emitting diodes in a consecutive manner, similar to (12). The base line was recorded during each switch before changing the illumination conditions. About 20 scans of both light-activated states (Pr and Pfr) were acquired.
Protein crystallography

The CBD-Y263F crystals were grown as in (28). The hanging drop vapor diffusion method was applied, in which 20 mg/ml protein was mixed at 1:1 ratio with reservoir solution (67 mM sodium acetate pH 4.95, 3.3% PEG 400, 30% 2-methyl-2, 4-pentanediol, 1 mM DTT). Large crystals formed after 24–48 h incubation at room temperature. For crystallographic data collection, the crystals were flash-frozen with liquid nitrogen in cryo-loops under green light. Diffraction data were collected at beamline ID23-2 of the European Synchrotron Radiation Facility (ESRF), under a 100K cryostream and an X-ray wavelength of 0.873 Å. The data were processed with the XDS program package (40) and cut at 1.34 Å resolution, which corresponds to a correlation coefficient (CC1/2) value of 0.31 (41). The crystal belonged to C121 space group with highly similar cell parameters as the published wild-type CBD structure (28). The CBD-Y263F crystal structure was solved by molecular replacement with Phaser, and structure 2O9C (42) was used as a search model. The structure was refined using REFMAC5 (43). In the refinement, the geometry was restrained with X-ray matrix-weighting term of 0.5 and anisotropic temperature factors, resulting in \( R_{\text{work}}/R_{\text{free}} \) values of 0.143/0.182. The statistics of data collection, processing, structure determination and refinement of all data sets are summarized in Table 2.

The pre-illuminated CBD-PHY-Y263F crystals were acquired by hanging drop vapor diffusion by mixing (1:1) 20 mg/ml protein with reservoir solution (100 mM Tris/HCl pH 8.5, 200 mM NaCl, 25% PEG3350) in identical conditions to (9). Right after setting up the crystallization, drops were pre-illuminated with red light-emitting diode (655 nm, 5 mW/cm²). The crystals were flash-frozen after a week without cryoprotection. Diffraction data were collected like the CBD-Y263F data at beamline ID23-2 of the European Synchrotron Radiation Facility (ESRF). The data were cut at 3.3 Å which corresponded to CC1/2 value 31.2. The pre-illuminated CBD-PHY-Y263F crystal structure was solved by molecular replacement with Phaser. A modified *Deinococcus radiodurans* CBD-PHY structure 5C5K (29) was used as a search model. The side chains of Y176, H201, F203 and R222, and the biliverdin were removed from the search model, and modelled only at later stages of the refinement. Like the wild-type Pfr crystals (9), the pre-illuminated CBD-PHY-Y263F crystals belonged to \( P2_1212_1 \) space group with two dimers in an asymmetric unit. In the refinement, automatically generated local non-crystallographic symmetry restraints were applied and the geometry was restrained tightly with X-ray matrix-weighting term of 0.005, resulting \( R_{\text{work}}/R_{\text{free}} \) values 0.234/0.267.

The dark CBD-PHY-Y263F crystals were grown by hanging drop vapor diffusion method against reservoir condition (100 mM Tris/HCl pH 8.5, 200 mM sodium acetate trihydrate, 30% PEG4000). The Pr state of the protein was ensured by illuminating the crystallization drops briefly with 785 nm LED (30.7 mW) and keeping the drops in complete darkness at room temperature. Crystals were flash-frozen without additional cryoprotection under green-filtered light. Diffraction data were collected at beamline ID23-1 of the European Synchrotron Radiation Facility (ESRF). The data were cut at 3.6 Å (CC1/2 = 34.3). The cell parameters and packing indicated highly similar crystal form as the pre-illuminated CBD-PHY-Y263F, and the molecular replacement was therefore conducted like above. To minimize the bias caused from the initial search model, the biliverdin and the neighboring residues that differ between Pr and Pfr states (Y176, H201, F203 and R222) were removed and added only at the later stages of the refinement. The final refinement steps were conducted with tight non-crystallographic symmetry restraints between the chains and an X-ray matrix-weighting term of 0.003, which gave final \( R_{\text{work}}/R_{\text{free}} \) values of 0.248/0.287. All electron density maps were prepared from the final structure factor files with FFT of the CCP4 interface, and the crystallography figures created with PyMOL Molecular Graphics System (Version 2.0 Schrödinger, LLC.). The RMSD between wild-type CBD and CBD-Y263F was calculated with PyMOL after outlier rejection (13 out of 315 atoms rejected).

Absorption spectroscopy of crystals

The crystal absorption spectra were measured using a home-built micro-focusing device connected by optical fiber to a standard deuterium
lamp and a diode array spectrometer (Ocean Optics), as in (9). The crystals were mounted in cryo-loops in reservoir solution with 15% glycerol and flash-frozen with liquid nitrogen. The absorption measurements were conducted under 100 K cryo stream, which impairs photoconversion. To test the photoswitching of the crystals, the focused detection light was replaced with red light emitting diode (660 nm, 13 mW, Thorlabs) for 5–15 seconds after the cryostream (and other light sources) was temporarily blocked. Visual inspection showed that the crystals remained intact through the brief illumination treatments.
The (un)coupling of the chromophore and structure in DrBphP

Acknowledgements: The atomic coordinates and structure factors have been deposited in the Protein Data Bank (http://wwpdb.org/) with codes 5NFX, 5NM3, and 5NWN. We acknowledge beamline access and personnel at the cSAXS in the Swiss Light Source (SLS) and at European Synchrotron Radiation Facility (ESRF), as well as A. Liukkonen for assistance in laboratory work.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: H.T. cloned, purified and crystallized the proteins, solved the crystal structures and measured the UV-vis spectra. H.L. measured and analyzed the UV-vis and FTIR spectra. R.N. measured the labeled FTIR spectra. O.B., A.H., S.N., M.P., L.H., A.M. measured and O.B. analyzed the X-ray scattering data. H.T., H.L., S.W. and J.A.I. designed and wrote the paper with input from all other authors.
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FOOTNOTES

The research was supported by the Academy of Finland grants 285461 (H.T.), 277194 (H.L.) and 296135 (J.A.I) grants and Foundation of Strategic Research, Sweden, the Swedish and European Research Councils, agreement number 279944 (S.W.) and Emil Aaltonen Foundation (H.L.).

The abbreviations user are: CBD, chromophore-binding domain; PHY, phytochrome-specific GAF-related domain; PSM, photosensory module; Pr, red light-absorbing state; Pfr, far-red light-absorbing state; PDB, Protein Data Bank; DrBphP, Deinococcus radiodurans bacteriophytochrome.
Table 1. X-ray solution scattering data collection

|                           | CBD-PHY      | CBD-PHY-Y263F | CBD-PHY-D207H |
|---------------------------|--------------|---------------|---------------|
| **Sample Details**        |              |               |               |
| Organism                  | D. radiodurans| D. radiodurans| D. radiodurans|
| Source                    | E. coli      | E. coli       | E. coli       |
| Buffer composition        | 30 mM Tris pH 8.0 | 30 mM Tris pH 8.0 | 30 mM Tris pH 8.0 |
| Protein conc.             | 27 mg/ml     | 32 mg/ml      | 31 mg/ml      |
| **Data collection parameters** |            |               |               |
| Beamline                  | cSAXS, dual flight tube with a Pilatus 2M for small angles and Pilatus 300k-W for wide angles. |
| Wavelength                | 11.2 keV, 1.107 Å |              |               |
| X-ray beam size           | 100 x 200 µm |               |               |
| Camera length             | 2167.4 mm (Pilatus 2M) |          |               |
| Detector readout frequency | 25 Hz (35ms X-ray exposure, 5ms readout) |          |               |
| q-measurement range       | 0.006 – 0.655 Å⁻¹ (Pilatus 2M) | 0.509 – 2.742 Å⁻¹ (Pilatus 300k-W) |               |
| Laser excitation          | Pump laser: 671 nm DPSS laser (Altechna), 10ms pulse, 5 mJ/mm² |
|                          | Recovery laser: 789 nm diode laser (Thorlabs), 50 ms pulse, 7 mJ/mm² |
| Flow cell                 | 1 mm, 0.01 mm wall quartz capillary |               |               |
| Sample temperature        | 20 – 22 °C   |               |               |
| **Data reduction parameters** |            |               |               |
| Radial integration        | Software supplied by the beamline |
| Data analysis             | MATLAB (Mathworks) |
| Normalization             | Scattering was normalized to the absolute scattering at 1.4 < q < 1.6 Å⁻¹ |
| Outlier rejection         | Curves deviating from the median absolute scattering in the region 1.7 < q < 2.2 Å⁻¹ by more than 5% were removed. |
Table 2. X-Ray Diffraction data collection and refinement statistics for the DrBphP Y263F structures.

|                     | CBD-Y263F | CBD-PHY-Y263F pre-illuminated | CBD-PHY-Y263F dark |
|---------------------|-----------|-------------------------------|--------------------|
| **Data Collection**a |           |                               |                    |
| Space group         | C121      | P 2, 2, 2                  | P 2, 2, 2          |
| Cell dimensions     |           |                               |                    |
| a, b, c (Å)         | 94.24, 54.58, 70.56 | 85.7, 198.5, 223.6          | 84.1, 197.1, 214.1 |
| α, β, γ (°)         | 90.00, 92.20, 90.00 | 90.0, 90.0, 90.0            | 90.0, 90.0, 90.0   |
| Resolution (Å)      | 19.93–1.34 (1.38–1.34) | 19.98–3.30 (3.38–3.30)      | 19.94–3.60 (3.69–3.60) |
| Rmerge              | 0.059 (1.333) | 0.157 (1.469)              | 0.072 (1.414)      |
| CC1/2               | 0.998 (0.311) | 0.994 (0.312)              | 0.999 (0.343)      |
| I/σ(I)              | 10.51 (0.89) | 7.68 (0.96)                 | 11.59 (0.90)       |
| Completeness (%)    | 99.8 (99.7) | 99.1 (99.9)                 | 96.6 (98.8)        |
| Redundancy          | 4.13 (3.94) | 4.09 (4.31)                 | 4.39 (4.52)        |
| Wilson B factor     | 18.2      | 99.8                         | 164.2              |
| **Refinement**      |           |                               |                    |
| Resolution (Å)      | 19.93–1.34 (1.38–1.34) | 19.98–3.30 (3.38–3.30)      | 19.94–3.60 (3.69–3.60) |
| No. of reflections  | 76,244 (5,617) | 54,898 (3,899)              | 38,682 (2,783)     |
| Rwork/ Rfree       | 0.143/0.182 (0.528/0.530) | 0.234/0.267 (0.389/0.391) | 0.248/0.287 (0.373/0.359) |
| Overall B factor    | 26.0      | 115.0                        | 197.0              |
| No. of atoms        |           |                               |                    |
| Proteinc            | 2,336     | 14,790                       | 14,443             |
| Ligandd             | 63        | 172                          | 172                |
| Water               | 318       | 5                            | 0                  |
| **Geometry**        |           |                               |                    |
| RMSD                |           |                               |                    |
| Bond lengths (Å)    | 0.014     | 0.007                        | 0.007              |
| Bond angles (°)     | 1.754     | 1.172                        | 1.175              |
| Ramachandran        |           |                               |                    |
| Favored (%)         | 99        | 96                           | 95                 |
| Allowed (%)         | 1         | 4                            | 5                  |
| Outliers (%)        | 0         | 0                            | 0                  |
| **PDB Code**        | 5NFX      | 5NM3                         | 5NWN               |

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a Outer shell values are in parentheses.
b Test set for Rfree calculation constitutes 5% of total reflections that were randomly chosen.
c Number of protein molecules in an asymmetric unit: one (CBD-Y263F) and four (CBD-PHY-Y263F dark and pre-illuminated).
d This includes atoms from a biliverdin, three acetates and a (4S)-2-METHYL-2,4-PENTANEDIOL (CBD-Y263F); or four biliverdins (pre-illuminated and dark CBD-PHY-Y263F).
Figure 1. Overall structure of the bacterial phytochrome from *Deinococcus radiodurans*. (A) Simplified domain composition of the full-length phytochrome. The biliverdin bound to the N-terminal extension of the PAS domain and the PHY tongue are depicted. (B) Crystal structures of the photosensory module in closed (Pr, blue) and open (Pfr, white) conformations (9). Tongue, conserved DIP and PRxSF motifs (green), Y263 (red), and the chromophore (orange) are indicated in the figure. The double-headed arrows represent the changes in the PHY domain separation upon illumination. The CBD part has been shown only for the Pr-state structure for clarity reasons. Abbreviations: CBD (chromophore-binding domain); GAF (cGMP phosphodiesterase, adenylyl cyclase, FhI); HK (histidine kinase); PAS (Per/Arnt/Sim); PHY (phytochrome-specific GAF-related); PHY (phytochrome-specific GAF-related).
Figure 2. Absorption and fluorescence spectra of the DrBphP CBD and CBD-PHY fragments and their Y263F variants. (A) Steady-state absorption spectra of CBD (black), Y263F (yellow), CBD-PHY (blue), and CBD-PHY-Y263F (red) in the dark-adapted state. The spectra were normalized to the absorption maximum. (B) Absorption spectra of the variants after irradiation with the 655 nm light. (C) Absorption difference spectra of the variants (red-irradiated spectra have been subtracted from dark-adapted spectra). (D) Fluorescence emission spectra and (E) emission decay of the same variants. The samples were excited at 660 nm and the fluorescence signal was monitored at 720 nm. The solid lines show the multiexponential fits of the data. (F) The fluorescence quantum yield measurements and fluorescence lifetimes. The average lifetimes are calculated from the amplitude-weighted sum of the lifetimes observed in the single-photon counting experiments.
**Figure 3.** FTIR and X-ray scattering analysis of the studied phytochrome complexes. (A) Comparison of the CBD samples (top panel) indicates a very small signal for CBD-Y263F sample. The 3x scaled difference spectra of CBD-Y263F, shows similar features with those of the CBD sample. The FTIR difference spectra (Pfr–Pr) of CBD-PHY and CBD-PHY-Y263F (middle panel) are similar except for the spectral regions around 1550 cm\(^{-1}\) and in range 1600–1660 cm\(^{-1}\), which show small deviations. The comparison of \(^{13}\)C\(^{15}\)N-labeled CBD-PHY and CBD-PHY-Y263F (bottom panel) indicate the β-sheet → α-helix transition to take place with lower rate in the Y263F mutant. The spectra of each sample are normalized to the UV-Vis absorbance value at 700 nm. The peak position mentioned in the text are indicated as arrows, which are color-coded as changes in biliverdin ring (blue), in secondary structure (green), and other changes (black). (B) Difference X-ray scattering of the two CBD-PHY variants. The Pfr–Pr difference signals of wild-type (blue) and Y263F (red). The signals are scaled to the positive 0.1 Å\(^{-1}\) maximum making the signal shape fully comparable with each other. (C) Relative X-ray scattering signal amplitudes of the CBD-PHY variants. The signals are scaled to the absolute scattering of each sample, making the intensities fully comparable with each other. The signal amplitudes indicate a reduced photoconversion efficiency of the Y263F mutant.
Figure 4. Crystal structure of CBD-Y263F and comparison with CBD. (A) Overall cartoon presentation of superimposed CBD-Y263F (orange) and the wild-type CBD structure (gray, PDB code 5K5B) (28). Biliverdin (BV) is presented as sticks. (B) Structural comparison of the CBD-Y263F and CBD in the biliverdin surroundings. Key residues are indicated and the three waters are named accordingly: pyrrole water (W1), transient water (W2) and solvent water (W3). The hydrogen bonds of the wild-type structure are shown as dashed lines, and the interactions missing in Y263F mutant structure are marked as asterisks (*). (C-D) The electron densities of the chromophore surroundings in CBD (C) and CBD-Y263F (D). The 2Fo-Fc density (blue mesh) is plotted at 1.5 rmsd.
Figure 5. The two CBD-PHY-Y263F crystal forms and the absorption spectra of CBD-PHY variant crystals. (A) Overall dimer structure of pre-illuminated (red) and dark (blue) crystal forms of CBD-PHY-Y263F. Biliverdin (BV) is shown in sticks. (B) Absorption spectra of CBD-PHY wild-type CBD-PHY and Y263F variant crystallized in the dark. The wild-type crystal shown here is crystallized in a published Pfr condition (9), and the Y263F variants was crystallized in the “dark” condition reported in this paper. The crystals had characteristic Pr spectra with a maximum at 700 nm (dark). The CBD-PHY crystals could be partially photo-switched with 660 nm light whereas CBD-PHY-Y263 crystals responded only little to red light (illuminated). (C) Electron densities of the tongue region of both crystal structures. (D) Structure of the D ring surroundings in CBD-PHY variants. Dark (left) and pre-illuminated (right) Y263F structures differ in the side chain orientations of residues Y176, H201 and F203. These orientations are supported by the 2Fo-Fc electron density maps. However, weak positive electron density (*) at the sites where these residues would reside in Pr state imply for traces of Pr-state orientations in the pre-illuminated structure. See Supplementary Figure S2B for comparison with published Pr and Pfr structures. The 2Fo-Fc maps (blue) are plotted at 1.0 rmsd and Fo-Fc maps are plotted at 3.0 rmsd.
Figure 6. Schematic presentation of the three levels in phytochrome signaling and their relations. Each level resides in an equilibrium that is affected by the other levels or external factors (denoted by arrows and k-factors). In the case of CBD-PHY-Y263F, the Pr→Pfr transition is impaired but α-helical tongue is favored over β sheet, which increases the prospects of attaining an open PSM that has its biliverdin in Pr (ZZZ) state. Multiple arrows between the “biliverdin” and “tongue” levels illustrates multiple, partly redundant signaling routes between the levels.
On the (un)coupling of the chromophore, tongue interactions and overall conformation in a bacterial phytochrome

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*J. Biol. Chem.* *published online April 5, 2018*

Access the most updated version of this article at doi: 10.1074/jbc.RA118.001794

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