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ORIGINAL PAPER

Domain interaction in cyanobacterial phytochromes as a prerequisite for spectral integrity

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Abstract Two phytochromes, CphA and CphB, from the cyanobacterium *Calothrix* PCC7601, with similar size (768 and 766 amino acids) and domain structure, were investigated for the essential length of their protein moiety required to maintain the spectral integrity. Both proteins fold into PAS-, GAF-, PHY-, and Histidine-kinase (HK) domains. CphA binds a phycocyanobilin (PCB) chromophore at a "canonical" cysteine within the GAF domain, identically as in plant phytochromes. CphB binds biliverdin IXα at cysteine24, positioned in the N-terminal PAS domain. The C-terminally located HK and PHY domains, present in both proteins, were removed subsequently by introducing stop-codons at the corresponding DNA positions. The spectral properties of the resulting proteins were investigated. The full-length proteins absorb at (CphA) 663 and 707 nm (red-, far red-absorbing P_r and P_{fr} forms of phytochromes) and at (CphB) 704 and 750 nm. Removal of the HK domains had no effect on the absorbance maxima of the resulting PAS–GAF–PHY constructs (CphA: 663/707 nm, CphB: 704/750 nm, P_r/P_{fr}, respectively). Further deletion of the "PHY" domains caused a blue-shift of the P_r and P_{fr} absorption of CphA (λ_{max}: 658/698 nm) and increased the amount of unproperly folded apoprotein, seen by a reduced capability to bind the chromophore in photoconvertible manner. In CphB, however, it practically impaired the formation of P_{fr}, i.e., showing a very low oscillator strength absorption band, whereas the P_r form remains unchanged (702 nm). This finding clearly indicates a different interaction between domains in the "typical", PCB binding and in the biliverdin-binding phytochromes, and demonstrates a loss of oscillator strength for the latter, most probably due to a strong conformational distortion of the chromophore in the CphB P_{fr} form.

Keywords Biliverdin · Photoreceptor · Phycocyanobilin · Phytochrome · Protein-protein interaction

Abbreviations

BV	Biliverdin IXα
GAF	Acronym for: cGMP-specific and–regulated cyclic nucleotide phosphodiesterase, Adenylyl cyclase, and *E. coli* transcription factor FhlA
PAS (protein domain)	PerArntSim (protein domain)
PCB	Phycocyanobilin
P_r, P_{fr}	Red-, far red absorbing forms of phytochrome
PHY	Phytochrome-specific protein domain

Introduction

Phytochromes are red-/far-red light sensitive photoreceptors, ubiquitous in plants and recently identified also in many prokaryotic species (Schäfer and Nagy 2006). They regulate a broad variety of photomorphogenetic responses, based on the photochemical properties of their covalently bound chromophore. Whereas plant phytochromes carry phytochromobilin (PΦB), their bacterial counterparts can be grouped into two classes, one binding phycocyanobilin (PCB) and the other biliverdin IXα (BV, for structural

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formulas see Fig. 1) (Gärtner and Braslavsky 2003; Karniol et al. 2005).

Phytochromes exhibit a typical receptor architecture, being composed of an input (i.e., stimulus-detecting) and an output (i.e., signaling) domain. Information on this arrangement derived originally from plant phytochromes, where proteolytic experiments yielded a protein fragment, spanning the N-terminal half, with spectral properties practically identical to those of the full-length protein (Schmidt et al. 1998), but void of any physiological activity when expressed in transgenic plants (Boylan and Quail 1991). This front half of phytochromes can further be dissected into an N-terminally located PAS domain, followed by a GAF domain and a part of the protein, which is unique for phytochromes, the PHY domain. In phytochromes from plants and in the bacterial ones binding PCB, the chromophore is bound to a fully conserved cysteine in the GAF domain (pos. 321 in oat phyA). In the BV-binding bacterial phytochromes the binding site is located in the PAS domain at the front part of the protein. In all cases, the chromophore is covalently bound via a thioether formed between the SH-group of a cysteine and the 3′-position of PΦB and PCB, and the 3″-position of BV (Fig. 1).

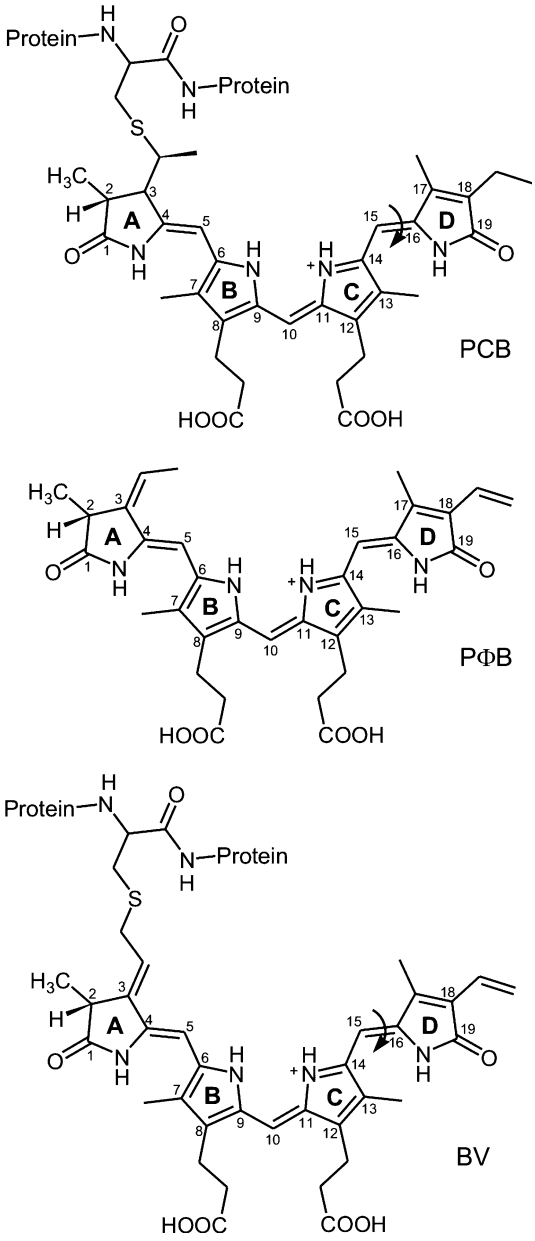

Fig. 1 (*Top*) Structural formula of phycocyanobilin (PCB), covalently bound to a cysteine residue of the protein; (*middle*) structural formula of phytochromobilin (PΦB) in unbound form; the type of covalent binding is identical in both chromophores. PCB and PΦB only differ in the substitution pattern of ring D (ethyl vs. vinyl); (*bottom*) structural formula of biliverdin IXα (BV) in protein bound form. Note that PCB and PΦB are bound at their 3′-position, whereas BV is bound at its 3″-position

The function of the C-terminal part remained uncertain for a long time, since phytochromes are involved in many different photomorphogenic responses. Although, comparison with other sensor domains revealed sequence similarities between the C-terminal part of phytochromes and kinases of bacterial sensors (Schneider-Poetsch 1992). The role of the C-terminal signaling domain became clearer when the bacterial phytochromes were identified. Signatures were found in the initially characterized cyanobacterial phytochromes that are typical for histidine kinases, HKs (Lamparter et al. 1997; Yeh et al. 1997). This enzymatic activity, together with so-called response regulator proteins, constitutes the components of a well-characterized bacterial "two component" signal transduction pathway (Stock and Da Re 2000; Wurgler-Murphy and Saito 1997). In fact, open-reading frames encoding these response regulators are present in the genomes of many phytochrome expressing bacteria, both genes most often being arranged in an operon (Jorissen et al. 2002). On the basis of these findings, similar features were also identified in the signaling domain of plant phytochromes (named "histidine kinase-like domain") (Boylan and Quail 1996; Yeh and Lagarias 1998), although in plants no direct proof for the interaction to response regulator proteins could be presented so far.

Phytochromes adopt two thermally stable, photo-interconvertible states, P_r and P_{fr}, with absorbances at 667 nm (P_r, red-absorbing) and 730 nm (P_{fr}, far red-absorbing; values are given for phyA from oat), of which the P_r state is assumed to be the resting state, whereas P_{fr} induces the photomorphogenic processes. The primary reaction after light absorption is a double bond photoisomerization of the chromophore at the methine bridge between rings C and D of the tetrapyrrole ($C_{15} = C_{16}$, Fig. 1) in the ps time range, which is followed by a series of conformational changes of chromophore and protein, lasting up to several seconds, until the P_{fr} state is completely formed. Under conditions of constant irradiation, a photoequilibrium is established

between both forms. Its ratio only depends on the relative extinction coefficients at the wavelength(s) of irradiation.

The similarity in chromophore structure and in photochemical behavior between plant and bacterial phytochromes, and the more ready preparation of the latter ones has made the prokaryotic phytochromes model compounds for many still open questions concerning chromophore-protein interactions and physiological responses, e.g., the light-induced signal transduction processes (Hübschmann et al. 2001a, b; Remberg et al. 1997; van Thor et al. 2001; Yeh et al. 1997).

However, the finding of two different types of chromophores (PCB and BV) in the bacterial phytochromes, bound to different domains of the protein, raised additional questions about the interactions between chromophores and the binding pockets of the proteins. As yet, no structural information is available for a plant phytochrome, and only a single three dimensional structure has been presented for a truncated bacterial phytochrome (from *Deinococcus radiodurans* (Wagner et al. 2005). This fragment comprised the N-terminal PAS- and the GAF-domain and showed unambiguously the attachment site (cys24) for BV in this protein. The crystal structure also revealed a certain vicinity of the two binding sites (cysteines in the PAS- and in the GAF-domain), which are brought closely together due to the protein fold. It can thus be assumed that both chromophores, PCB and BV, adopt a relatively similar conformation within the protein cavity, although molecular details like single bond conformations or diedric angles can not be decluded from the crystal structure. Interestingly, the environment of the D-ring of the chromophore (the structural element which undergoes the greatest light-induced conformational change) was found relatively unstructured in the crystal structure and part of the D-ring remained exposed to the bulk phase. It was thus not obvious, whether and to what extent the PHY domain, which was deleted for the crystallization experiments, might contribute to a fixation of the D-ring in the P_r and/or P_{fr} form. In order to approach this uncertainty, we performed a comparison of PCB- and BV-binding bacterial phytochromes in full-length or differently truncated form and demonstrate a vital requirement of the PHY domain for the spectral integrity of especially those bacterial phytochromes that bind a biliverdin chromophore.

Experimental

Generation of cDNA encoding the full-length and truncated fragments of CphA and CphB

The generation of the DNA encoding full-length CphA and CphB was described elsewhere (Jorissen et al. 2002). The PAS–GAF and the PAS–GAF–PHY encoding DNAs were obtained by PCR from the full-length DNA using the following primers (sequences for the restriction enzymes used for cloning are underlined and the enzyme names are given in brackets):

CphA: Forward primer (restriction site: *Nco*I) 5′-tccggtc catggtgaatagcttaaaagaagca-3′, reverse primer for PAS–GAF (*Xho*I) 5′-aactcgagagccgcaccttgagcgctagt-3′, reverse primer for PAS–GAF-PHY (*Xho*I) 5′-aaactcgagcgcattcgagcgttcta agtc-3′; CphB: Forward primer (*Nco*I) 5′-tataccatgggcttaag tcctgaaaattctccag-3′, reverse primer for PAS–GAF (*Xho*I) 5′-aaactcgagaacagccgcgcctgtagcatt-3′, reverse primer for PAS–GAF–PHY (*Xho*I) 5′-aaactcgagatcgttgctgcgctgcagttc-3′.

The PCR products were cloned into *pET28a* (Novagen). The such generated plasmids were transformed into *Escherichia coli* strain BL21 DE3 RIPL (Stratagene).

Chromophore preparation, expression of recombinant proteins, assembly and purification of holoproteins

Phycocyanobilin was extracted from freeze-dried *Spirulina platensis* cells, following literature protocols (Kufer and Scheer 1979). Biliverdin IXα was synthesized from bilirubin IXα by DDQ oxidation, followed by HPLC purification. Recombinant full-length CphA and CphB were heterologously expressed in *E. coli* cells (BL21 DE3 RIPL, Stratagene), as recently described (Jorissen et al. 2002; Quest and Gartner 2004). C-terminally truncated CphA and CphB were obtained by heterologous expression in *E. coli* BL21 cells, as described above for the full-length DNAs. In all cases, the encoding DNA was furnished with an octadecanucleotide at the 3′-end, encoding a hexa-histidine tail for affinity chromatography. Since holo-phytochromes generally show a greater stability than the unreconstituted apoproteins, recombinant proteins were in all cases assembled with their chromophores already in the "crude lysate", i.e., immediately after opening the cells and removing the cell debris by ultra-centrifugation. Affinity purification followed published procedures (Mozley et al. 1997). The quality of the eluted proteins was controlled in all cases by gel electrophoresis.

UV-Vis spectroscopy

Prior to the recording of absorbance spectra, the recombinant proteins, eluted from the affinity chromatography resin, were irradiated with a cut-off filter, $\lambda > 715$ nm (PCB-carrying proteins) or with a cut-off filter, $\lambda > 730$ nm for BV-carrying proteins, to ensure a complete conversion into the P_r states. After recording the P_r spectra, the samples were irradiated with light from interference filters (655 ± 7 nm, PCB-containing samples), or with light of 680 ± 7 nm for BV-containing samples in order to obtain a

maximal conversion into the P_{fr} forms. After recording the P_{fr} spectra, difference spectra (P_r–P_{fr}) were generated. Irradiation conditions were as in (Remberg et al. 1999). UV-Vis spectra were recorded with a Shimadzu spectrophotometer (2401 PC), and data treatment was performed by the software from Shimadzu.

Results and discussions

The cyanobacterial phytochromes CphA and CphB were heterologously expressed in full-length and in truncated form. The various constructs had lengths of 768 (full-length), 517 (PAS–GAF–PHY), and 357 amino acids (PAS–GAF) in case of CphA, and 766 (full-length), 536 (PAS–GAF–PHY), and 376 amino acids (PAS–GAF) in case of CphB. Whereas all CphB constructs were obtained in good yields, especially the CphA PAS–GAF construct expressed poorly and the majority of this recombinant protein was found in insoluble form in inclusion bodies. In all cases, however, sufficient amounts of the samples could be accumulated and purified for the spectral analysis. Full-length CphA absorbs at 663 nm and 707 nm in its P_r and P_{fr} forms, respectively, with roughly similar oscillator strength of both forms (Fig. 2 and Table 1). CphB, binding BV, shows bathochromically shifted absorbance maxima with respect to those from CphA at 704 and 750 nm (P_r and P_{fr}), again, both absorbance bands were of similar intensity in the difference spectrum [Fig. 2 and Table 1; for a discussion on the origin of the bathochromic shift in BV-binding bacterial phytochromes see (Quest and Gärtner 2004)]. Removal of the signaling HK domain from CphA had virtually no effect on the absorbance maxima and the relative intensity of the P_r and the P_{fr} forms (λ_{max} = 663 and 707 nm, P_r and P_{fr}, respectively, Fig. 3). Also in CphB, the removal of the corresponding HK domain did not cause any change in the absorbance of P_r or P_{fr} form (λ_{max} = 704 and 750 nm, respectively, Fig. 3), nor did it, in either case, interfere with the photoreversibility of the samples. It can thus be concluded that a construct comprising the PAS-, GAF- and PHY-domains is sufficient to preserve the spectral properties of bacterial phytochromes, quite similar as in the plant phytochromes, and as also has been demonstrated for the cyanobacterial phytochrome Cph1 (Esteban et al. 2005). Taking phyA of oat as an example, the removal of the C-terminal half (amino acids 600–1,125) yields a phytochrome fragment with spectral (and kinetic) properties virtually identical to those of the full-length protein, whereas any removal of amino acids form the N-terminal part of a plant phytochrome (historically named "large" and "small" phytochrome) results in a pronounced hypsochromic of the P_{fr} absorption maximum) (Gärtner et al. 1996; Schmidt et al. 1998).

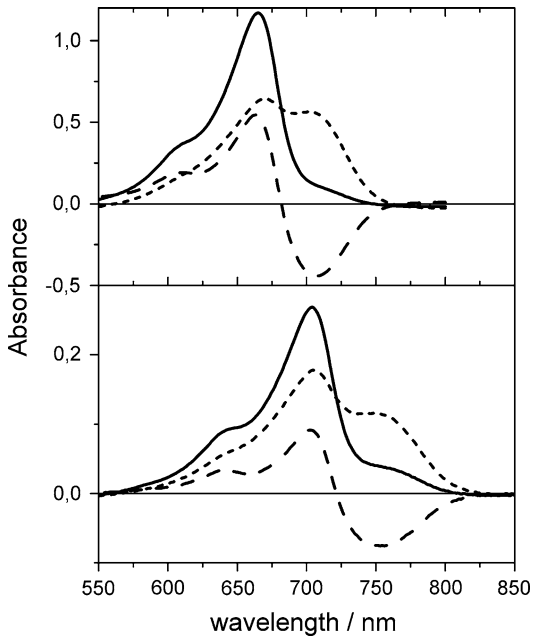

Fig. 2 Absorption and absorption difference spectra (P_r–P_{fr}) for full-length (*top*) CphA and (*bottom*) CphB. For irradiation conditions see Sect. "Experimental"

A further reduction in size, i.e., the removal of the HK- and in addition also of the PHY-domain from either CphA or CphB causes significant changes in the absorption properties of these truncated proteins. The CphA PAS–GAF construct showed a reduced capability to incorporate the chromophore, even though the electrophoretic control indicated a relatively pure protein preparation. This construct also required an extended incubation time with the chromophore (usually over night) for complete formation of the chromoprotein, whereas the longer construct (PAS–GAF–PHY) and the full-length protein assembled within several minutes. Apparently, a large fraction of the apoprotein does not incorporate the chromophore, as can be seen from the relatively small chromophore absorption bands and the high background due to the large excess of free PCB chromophore that had to be used (broad absorption band centering around 585 nm). Still, the resulting P_r- and P_{fr}-absorbances and the generated difference spectrum indicate a relatively "normal" photochemistry with similarly intense absorbances of both P_r and P_{fr} forms, a slight hypsochromic shift of 5–10 nm for the absorbance maxima (λ_{max} = 658 and 698 nm for P_r and P_{fr}, respectively), and full photoreversibility (Fig. 4). This behavior of reduced and slowed-down chromophore binding capacity is probably caused by a misfolding of a relatively large fraction of the apoprotein, which might derive from an exposure of hydrophobic surfaces after the removal of the PHY-domain and a reorientation of the remaining protein. This construct (CphA PAS–GAF) also showed a lower thermal stability of its P_{fr}

Table 1 P_r and P_{fr} absorbance maxima and ratio of the relative oscillator strength of both forms

Protein	Construct								
	Full-length protein			PAS–GAF–PHY			PAS–GAF		
	P_r λ_{max} (nm)	P_{fr} λ_{max} (nm)	Rel. intens. P_r:P_{fr}	P_r λ_{max} (nm)	P_{fr} λ_{max} (nm)	Rel. intens. P_r:P_{fr}	P_r λ_{max} (nm)	P_{fr} λ_{max} (nm)	Rel. intens. P_r:P_{fr}
CphA	663	707	0.8	663	707	0.67	658	698	0.75
CphB	704	750	0.82	704	750	0.67	702	(746)[a]	0.12–0.14

[a] this value shows some variation in repeated preparations due to a very low extinction coefficient

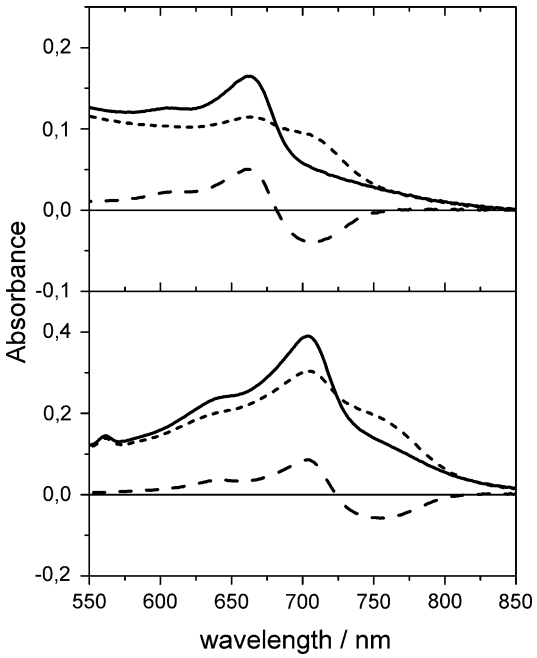

Fig. 3 Absorption and absorption difference spectra (P_r–P_{fr}) for PAS–GAF–PHY fragments of (*top*) CphA and (*bottom*) CphB

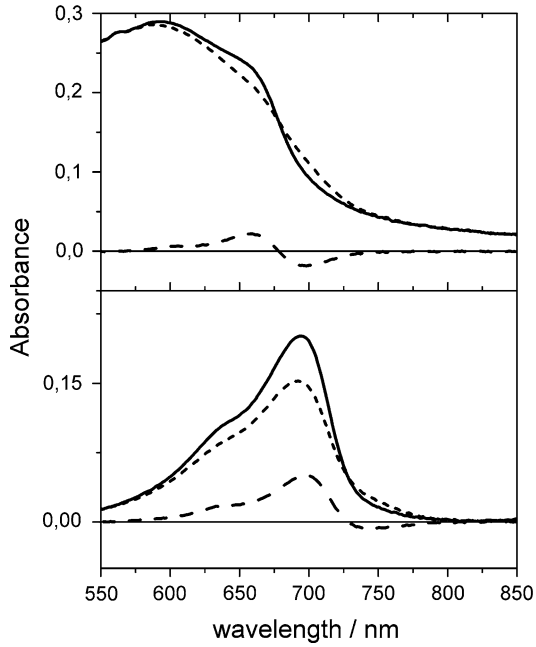

Fig. 4 Absorption and absorption difference spectra (P_r–P_{fr}) for PAS–GAF fragments of (*top*) CphA and (*bottom*) CphB. The absolute spectra still contain a high amount of free chromophore (broad, unstructured absorbance centered around 570 nm)

form, but still was photoreversible. Whereas in all other CphA and CphB constructs the P_{fr} form remained stable in the dark for several days, the P_{fr} form of this fragment (CphA PAS–GAF) reverted back to the P_r form within several hours. Albeit, it showed a bilobal difference spectrum with nearly equal intensity of both bands.

Significantly different spectral properties are found for the CphB counterpart of this construct, i.e., CphB PAS–GAF. Interestingly, the intensity and position of the P_r form of this chromopeptide remained unchanged (λ_{max} = 702 nm), when compared to the extended PAS–PAF–PHY protein. The P_{fr} absorbance was slightly hypsochromic (λ_{max} = 746 nm), but has lost practically all its intensity (Fig. 4). Whereas in all other chromoproteins, irrespective of full-length or C-terminally truncated constructs, the relative intensity of the P_r and the P_{fr} peaks in the difference spectra is roughly similar, the P_{fr} absorbance of the CphB PAS–GAF protein shows only between 10 and 12% of the P_r intensity. This behavior is not due to a reduced photochemical activity of this fragment, as can be deduced from the relatively strong loss in absorbance of the P_r peak upon irradiation with red light, aiming at the conversion into the P_{fr} form (compare the intensity loss around 700 nm upon red light irradiation in the absolute spectrum), but has clearly to be ascribed to a lower extinction coefficient of the resulting P_{fr} form. This construct, as its CphA counterpart, requires an extended (over night) incubation time with the BV chromophore for assembly, and also shows a reduced thermal stability of the low intensity P_{fr} form.

Irrespective of the differences in the shape and position of the absorption bands of the various protein constructs, a fully reproducible forward and backward photoreactivity was found.

It should be noted here that similar difference spectra are obtained in plant phytochromes, if the removal of protein domains from the C-terminal end extends into the chromophore binding domain. The so-called “39-kDa fragment” of

oat phyA, spanning amino acids 66–425, which is obtained by proteolytic treatment [and which has been generated also as a recombinant chromoprotein (Gärtner et al. 1996)] shows an only slightly modified P_r peak, but a very broad, unstructured P_{fr} form with a reduced thermal stability and a rapid thermal reversion into the P_r form. Similar spectral changes had recently been reported for the protease-mediated, C-terminally truncated cyanobacterial phytochrome Cph1 (Esteban et al. 2005).

The known differences of the two proteins CphA and CphB (binding of different chromophores, PCB and BV, at different sites in the protein and the resulting bathochromic shifts of roughly 50 nm for both forms of CphB) raise the question whether it is meaningful at all to compare these two proteins. We are convinced, however, that these differences do not interfere with the interpretation and discussion of our results for several reasons: an inspection of the recently published phytochrome structure (Wagner et al. 2005) reveals that both chromophore-binding amino acids, cys24 in the PAS domain for the BV-binding- and met260 in the GAF domain (being the chromophore-binding cysteine position for the PCB-binding phytochromes) are positioned close to each other in the three-dimensional structure (if a similar folding of the GAF domain is assumed). Also the interactions between chromophore and protein can be assumed to be similar in both types of proteins. This holds true for the fixing of the propionate side chains at rings B and C, as also for the strong interactions between the nitrogen atoms of rings A, B, and C, and the carbonyl group of Asp207, centrally positioned in between the A, B, and C rings, leaving flexibility only for the (photoisomerizing) ring D. One can thus propose a similar conformation for both types of chromophores. The similarity extends also for the protein structure, as far as it can be extracted from presently known data. As well the direct sequence comparison as also the predicted secondary structure for the GAF domains demonstrates the strong similarity of both protein domains, and a similarly strong relation between the GAF domain of CphB and that from *D. radiodurans*.

As outlined in Sect. "Introduction", the inspection of the crystal structure of the PAS–GAF construct of *D. radiodurans* and its similarity to the corresponding fraction of CphB was a rationale for these investigations since the protein region around the D-ring of the chromophore appeared fairly loose in the crystal, leaving this part of the chromophore exposed to the bulk phase. It is, however, generally accepted that the D-ring of the bilin has to be strongly fixed in both its conformations (P_r and P_{fr}) in order to ensure the high thermal stability that both forms of phytochromes exhibit in the dark. Besides the cited reference by Esteban et al. (Esteban et al. 2005) dealing with proteolytic truncation of Cph1, also site directed mutagenesis experiments on that protein have been reported (Hahn et al. 2006). However, a detailed comparison of essential amino acids between all the proteins addressed here, Cph1, CphA, CphB, and that from *D. radiodurans*, would reach too far into speculation.

The here reported data offer an interesting explanation also for the course of the P_r-to-P_{fr} conversion. If the various intermediates in the P_{fr} formation are considered, which are described for plant and PCB-binding phytochromes (Gärtner and Braslavsky 2003; Schmidt et al. 1998; van Thor et al. 2001), the molecule travels, after decaying from the excited state, through a red shifted intermediate ("I_{700}" in oat phyA) into an intermediate with a very low extinction coefficient ("I_{bleach}"). This form is further converted into one or more intermediate states similar to P_{fr} (P_{fr}'), before arriving at this final state. It is thus proposed here that the removal of the PHY domain from the BV-binding phytochromes impairs the complete conversion of the P_r into the P_{fr} state (in contrast to the PCB-binding phytochromes, where a pronounced P_{fr} form can be generated also for the truncated proteins). Moreover, the chromophore remains in an intermediate form on its way into P_{fr}. It can not be discriminated between two possibilities—whether the chromophore in the long-term irradiation, as applied here, accumulates in the P_{fr} form, but falls back into the observed intermediate immediately after the irradiation, or—whether the chromophore, due to missing stabilizing residues from the PHY domain, arrives only at the observed intermediate with the reduced oscillator strength.

Apparently, the situation of domain interaction and chromophore conformational stabilization is different for the two types of bacterial phytochromes, or more generally spoken, for the PΦB- and PCB-binding phytochromes on one side and the BV-binding phytochromes on the other. If the BV-binding phytochromes are taken as the phylogenetically more ancient forms of this photoreceptor, since only the heme oxygenase is required to generate the chromophore in contrast to the PΦB- and PCB-binding ones (where at least one additional enzymatic activity is required to generate this type of chromophores), the here reported results may also be understood as an evolutionary improvement of the phytochromes structure. Yet, only an ongoing structural investigation may shed further light on this interesting observation.

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