Crystal structure of a prokaryotic (6-4) photolyase with an Fe-S cluster and a 6,7-dimethyl-8-ribityllumazine antenna chromophore

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The (6-4) photolyases use blue light to reverse UV-induced (6-4) photoproducts in DNA. This (6-4) photorepair was thought to be restricted to eukaryotes. Here we report a prokaryotic (6-4) photolyase, PhrB from Agrobacterium tumefaciens, and propose that (6-4) photolyases are broadly distributed in prokaryotes. The crystal structure of photolyase related protein B (PhrB) at 1.45 Å resolution suggests a DNA binding mode different from that of the eukaryotic counterparts. A His-His-X-X-Arg motif is located within the proposed DNA lesion contact site of PhrB. This motif is structurally conserved in eukaryotic (6-4) photolyases for which the second His is essential for the (6-4) photolyase function. The PhrB structure contains 6,7-dimethyl-8-ribityllumazine as an antenna chromophore and a [4Fe-4S] cluster bound to the catalytic domain. A significant part of the Fe-S fold strikingly resembles that of the large subunit of eukaryotic and archaeal primases, suggesting that the PhrB-like photolyases branched at the base of the evolution of the cryptochrome/photolyase family. Our study presents a unique prokaryotic (6-4) photolyase and proposes that the prokaryotic (6-4) photolyases are the ancestors of the cryptochrome/photolyase family.

DNA repair | UV light | energy transfer

The cryptochrome/photolyase family (CPF) encompasses structurally highly conserved flavoproteins with divergent functions. Whereas cryptochromes regulate growth and development of plants and circadian rhythm of plants and animals, photolyases repair UV-induced DNA lesions thereby preventing growth delay, mutagenesis, cell death, and cancer (1–3). Based on their substrate specificities, two classes of photolyases can be distinguished, cyclopyrimidine dimer (CPD) photolyases and (6-4) photolyases. CPD photolyases have been identified in all three domains of life and are generally accepted as the common ancestors of the CPF. By contrast, (6-4) photolyases have only been found in eukaryotes and are therefore presumed to have formed later during evolution (1, 4, 5).

Agrobacterium tumefaciens is a soil bacterium famous for its gene transfer mechanism (6). The genome of A. tumefaciens bears two genes encoding the photolyase-related proteins A and B (PhrA and PhrB) (7). PhrA is a prototypical CPD class III photolyase, whereas PhrB belongs to a group of photolyase-like proteins that has recently been denominated as iron-sulfur bacterial cryptochromes and photolyases (FeS-BCP) and is distantly related to other CPFs. Mutant studies have shown that both PhrA and PhrB are required for the full photoreactivation in A. tumefaciens and that the roles of PhrA and PhrB with respect to in vivo DNA repair are not redundant (7). We therefore reasoned that PhrB could function as a (6-4) photolyase.

We describe here a functional study of recombinant PhrB with an HPLC-based in vitro DNA repair assay and confirmed it to be a (6-4) photolyase, which contains a 6,7-dimethyl-8-ribityllumazine (DMRL) chromophore and an iron-sulfur cluster. We also determined the crystal structure of PhrB at 1.45 Å resolution. Significant differences and essential common features were identified in a comparative analysis of the crystal structures of (6-4) photolyases including PhrB and its eukaryotic counterparts. Because PhrB homologs of the FeS-BCP type are widely distributed in prokaryotes, structural considerations lead us to suggest that (6-4) photorepair plays a significant role in bacteria and archaea. The structural homology between PhrB and the large subunit of eukaryotic and archaeal primases (PriL), sheds unique light on the evolution of the CPF.

Results and Discussion

Function of PhrB. Previous DNA repair assays with PhrA and PhrB based on restriction digest and electrophoresis showed that PhrA but not PhrB repairs CPD photoproducts in vitro. A spectral assay in which the specific absorption of the (6-4) photoproduct at 325 nm was followed photometrically gave inconclusive results due to spectral overlap with the PhrB chromophores. In the present work, we used HPLC-purified single stranded (ss) or double stranded (ds) DNA oligonucleotides with a thymine-thymine (TT) (6-4) lesion (8) as substrate and monitored DNA repair by HPLC, combined with continuous recording of UV-visible (UV-vis) spectra. As shown in Fig. 1A and B and Fig. S1, PhrB incubation with ssDNA and dsDNA TT (6-4) photoproducts resulted in a characteristic mobility change on the HPLC column and a loss of the 325-nm absorption peak. The mobility of the product was always identical with that of control DNA without the lesion. This repair of ssDNA and dsDNA (6-4) photoproducts was blue-light and incubation-time dependent, as expected for a typical photolyase (9). We have thus shown that PhrB is a (6-4) photolyase. This finding strongly suggests that prokaryotes are also capable of the photolyase-catalyzed repair of (6-4) lesions.

Considering that PhrB homologs share the same function, the (6-4) photorepair should be broadly distributed among bacteria. In a Uniprot-KB BLAST survey, we found 462 bacterial and archaeal PhrB homologs (FeS-BCPs), which form a group separate from other CPFs (Table S1). In the catalytic region between amino acids 276 and 474 that encompasses the FAD binding and putative DNA binding regions, residues are highly conserved. Therefore, we propose that most if not all FeS-BCP members are (6-4) photorepairers.
photolyases, which is further supported by detailed analysis of sequences and in context with our crystal structure.

Another FeS-BCP member that has been investigated is cryptochrome B (CryB) of Rhodobacter sphaeroides. CryB has been identified as a photoreceptor that regulates photosynthesis genes (10). Tests for in vitro DNA repair activity were negative (11), but this could result from inappropriate in vitro conditions.

Several features are in support of a repair activity of CryB. It is required for the full in vivo photorepair in R. sphaeroides (12), as is PhrB in A. tumefaciens (7). Surface charge and DNA binding properties of CryB (10, 11) are also compatible with DNA repair activity.

Crystal Structure of PhrB. We determined the crystal structure of PhrB at 1.45 Å resolution, the best resolution for a CPF protein structure published so far. The presence of the [4Fe-4S] cluster was confirmed by a strong anomalous scattering signal in the X-ray diffraction data collected at the iron absorption edge (Table S2). The refined model (Fig. 1C) closely matches (rmsd = 1.3 Å for 502 Cα positions) the structure of CryB (11). Superpositions with other members of the CPF yielded rmsds between 2.8 and 3.5 Å (13–22). Remarkably, the two largest rmsds of 3.2 Å and 3.5 Å were obtained with Drosophila melanogaster and Arabidopsis thaliana (6-4) photolyases [Drome (6-4) PL and Arath (6-4) PL, respectively. In the phylogenetic analysis, the prokaryotic and eukaryotic (6-4) photolyases appear as the most distantly related proteins within CPF (Fig. S2A). Besides the FAD chromophore, which assumes a prototypical U-shaped conformation, another organic cofactor was identified. Its electron density does not match with known antenna chromophores of other photolyases, but could be fitted to DMRL (Fig. 2A). We confirmed its identity by HPLC analysis. In the elution profiles, the major peak was found at the same position as that of synthetic DMRL and the UV-Vis spectral properties of both compounds are identical (Fig. S3).
cofactor was also found at a homologous binding site in CryB (11). Another feature in common with CryB is the strict structural conservation of aromatic residues that define the intraprotein electron transfer route for the FAD photoreduction (11).

**His365-His366-X-X-Arg369 Motif.** In a structural alignment (23) of PhrB and two eukaryotic (6-4) photolyases (15, 22), we have identified 34 identical amino acids, 10 of which are conserved to 99–100% in all FeS-BCPs. Three of these amino acids cluster together in the His365-His366-X-X-Arg369 motif. These amino acids are located at the bottom of the DNA lesion’s entrance channel. His365 and Arg369 are conserved in (6-4) photolyases and in many CPD homologs. His366 is conserved in FeS-BCPs, in eukaryotic (6-4) photolyases, and in animal cryptochromes; CPD photolyases have an Asn at the homologous position. The His366 homolog of eukaryotic (6-4) photolyases is essential for DNA repair; it serves as a donor/acceptor in catalytic proton transfer, which accompanies the light-driven electron transfer (22, 24). His366 in PhrB is stabilized by van der Waals contacts with Leu370 and Met410 (Fig. 3A), whereas the homologous His in eukaryotic (6-4) photolyases is stabilized by a hydrogen bonding network (15, 22). The orientation of His366 in PhrB, however, matches with the homologous His residue of Drome (6-4) PL (Fig. 3A), suggesting functional identity despite the different environments of these residues. Gln306, conserved in FeS-BCPs and eukaryotic (6-4) photolyases, is likely to form hydrogen bonds to the DNA lesion (Fig. 3A and Table S3). This residue has a conserved Glu as its counterpart in CPD photolyases. Overall, already the high degree of conservation within FeS-BCPs of amino acid residues that are probably in contact with the DNA lesion in PhrB (Fig. 3A and Table S3) supports the idea that all FeS-BCPs are (6-4) photolyases.

**(6-4) Photoproduct Binding.** According to a DNA–protein interaction model that was obtained using the Hex-Server (http://hexserver.loria.fr) (25), eight amino acids of PhrB that are all conserved within FeS-BCPs interact with the DNA lesion (Table S3). One of these, Arg183, is part of the loop region connecting α7 and α8; this loop is longer in FeS-BCPs than in other CPFs. Three DNA-interacting amino acids of this loop, Ala180 to Asn182, are not visible in the electron density map (Fig. 4A and B). These amino acids might be stabilized by the DNA to acquire a more ordered conformation. Arg183 could change its orientation to interact via its positively charged guanidinium group by forming a salt bridge with the phosphate within the DNA lesion, thereby stabilizing the flip out of the lesion (Fig. 4A and B). In this respect, PhrB differs from prototypical CPFs, in which another Arg residue [Arg421 in Drome (6-4) PL] within the region corresponding to the α17–α18 connecting loop in PhrB stabilizes the flip out of the lesion (Fig. 4C and D) (17, 20, 26). This Arg is missing in FeS-BCPs. We therefore suggest that FeS-BCPs bind UV-damaged DNA in a mode significantly different from prototypical photolyases.

**C-Terminal Extension of PhrB.** Prototypical photolyases have a common set of helices corresponding to α1–α19 in PhrB, and the last common helix α19 forms a part of the DNA binding groove. Arath (6-4) PL has an additional α-helix (15), referred to as C-terminal extension. This helix forms an antiparallel two-helix bundle with α19 and (assuming a prototypical binding mode) faces away from bound DNA. The C terminus of PhrB is extended by two additional helices α20 and α21 (Fig. S4), of which α21 displays sequence similarity with the C-terminal helix α20 of Arath (6-4) PL. Sequence alignments show that α20 and α21 are present in all FeS-BCPs. According to a PhrB–DNA interaction model, which is compatible with a prototypical photolyase–DNA binding mode, helix α20 of PhrB interacts with the DNA, whereas α21 faces away from the DNA. The surface region of α20 displays a positive charge (Fig. S5) that is consistent with its predicted role in DNA binding. Because the C-terminal extension is absent in Drome (6-4) PL and most other photolyases, we suppose that it is dispensable for repair activity and consider a regulatory role for this region.

**Chromophore Binding.** In the neutral radical (FADH*) and the anionic fully reduced (FADH−) states of the FAD cofactor in prototypical photolyases, the N5 atom of the isoalloxazine moiety is protonated and hydrogen bonded to an Asn residue (17, 27). Apparently, this stabilizing function is taken over by a conserved water molecule in PhrB that is held in place by additional hydrogen bonds with the side chain of Arg369 and the backbone carbonyl group of Tyr391 (Fig. 3B). The binding sites for the dicyclic DMRL in PhrB and for the tricyclic antenna chromophores 8-hydroxy-7,8-didemethyl-5-deazariboflavin, FAD, or FMN in other photolyases (14, 21, 28) are in homologous positions (Fig. 2B). His43 of PhrB prevents binding of tricyclic cofactors by steric hindrance, and is in van der Waals contact with the two methyl groups of DMRL (Fig. 2A). This residue is 67% conserved in FeS-BCPs, and is replaced by bulky Tyr and Phe residues in other FeS-BCPs. We therefore predict that none of these proteins binds a tricyclic antenna chromophore.

**[4Fe-4S] Cluster of PhrB.** The [4Fe-4S] cluster of PhrB is coordinated by Cys350, Cys438, Cys441, and Cys453. Structural homology between the carboxyl-terminal domain of PriL (PriL-CTD) of eukaryotic and archaeal primases that also contain [4Fe-4S] clusters and CPF members suggests that both groups of proteins have a common ancestor (29). Structural similarity can be seen between PhrB and PriL for a stretch from amino acids 348–434 of PhrB, which comprises helices α13–α19 (Fig. 5A; the

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Fig. 3. Catalytic center. (A) The active site of PhrB. Sections of Drome (6-4) PL in complex with photodamaged DNA (PDB code 3CVU, DNA in magenta, amino acids in purple, and FAD in blue) and of PhrB (amino acids in green and FAD in yellow) are shown after superposition of the FAD molecules. Amino acid residues in Drome (6-4) PL, which interact with the DNA lesion and the homologous residues in PhrB, are shown. Potential hydrogen bonds are represented by dashed lines. (B) Water W731 of PhrB (green) forms a potential hydrogen bond with the N5 atom of FAD (C atoms in yellow), replacing the role of Asn378 of the class i CPD photolyase from E. coli (PDB code 1DNP, drawn in purple). Its PhrB homolog, Glu403, faces away from FAD and interacts with His388.
rmsd for PhrB and *Saccharomyces cerevisiae* PriL [Protein Data Bank (PDB) code 3LGB] is 3.1 Å. The structurally most conserved core region stretches from amino acids 348 to 398. The iron-sulfur clusters are found at almost the same positions in the superimposed structures, but are tilted against each other (Fig. 5B). This is consistent with the fact that only the first cysteines in the core regions of both structures are strictly conserved. The side chains of the remaining three Cys residues are located only at roughly similar positions, and the sequential order of corresponding cysteines differs (PriL: 336/417/434/474; PhrB: 350/454/438/441). We therefore assume that outside the core region the protein fold diverged and the coordinating Cys residues were exchanged during evolution.

Besides primases and FeS-BCPs, several other DNA-interacting proteins contain Fe-S clusters (30–32). Fe-S clusters are regarded as ancient features. Their typical function is electron transport, and they are also often required for protein folding and stability (30). The Fe-S cluster of human PriL is specifically required for initiation of primer synthesis, but the molecular details of its function are as yet unknown (33). For the base excision repair Mutator Y (MutY, a DNA glycosylase) and Endonuclease III (EndoIII), a charge transfer between DNA and the Fe-S cluster has been proposed to be involved in detecting DNA lesions (34). The present study does
not yet provide insight into the function of the Fe-S cluster of PhrB apart from it being an integral component of the protein fold, but knowledge of the high-resolution structure of PhrB might help to solve this task in the future.

Evolutionary Scenario of CPF. To gain a deeper insight into evolutionary relationships between primases and CPF, we performed phylogenetic studies based on structurally aligned sequences, using parsimony and distance-based algorithms. Irrespective of whether the entire homologous region (348–434) or the more conserved core region (348–398) were used as input, primases and PhrB were placed in one monophyletic group (Fig. S2B). We conclude that the primases, FeS-BCPs, and other CPFs diverged early in the evolution and that the first common ancestor of CPF was a (6-4) photolyase with an Fe-S cluster. The Fe-S cluster was lost in the early evolution of non–Fe-S-BCP CPF members. In several subsequent independent steps, antenna chromophores were replaced, (6-4) repair changed to CPD repair, and DNA repair activity was replaced by the signal transduction that represents a transition from photolyases to cryptochromes (Fig. 6).

Photolyases are regarded as ancient DNA repair enzymes already present before the formation of the oxygen-rich atmosphere (35), and our results imply that the (6-4) photorepair occurred early in the evolution of photolyases. However, CPD repair might have also evolved so early. Most essential features such as DNA binding, the cavity for the DNA lesion, photoreduction, an FAD chromophore close to that cavity, and the overall protein fold are identical in CPD- and (6-4) photolyases. The combination of CPD and (6-4) photorepair provides a major evolutionary advantage. Our survey suggests that a large number of prokaryotes living today still rely on the combined photorepair of CPD and (6-4) photoproducts: 80% of those prokaryotes that contain an FeS-BCP member (Table S1) also contain a CPD photolyase homolog.

Materials and Methods

Protein Purification and Crystallization. Recombinant His-tagged PhrB protein was expressed in Escherichia coli and purified via Ni-affinity chromatography and size-exclusion chromatography as described (7) and used for crystallization at concentrations of 4–6 mg/mL under dim light conditions or in darkness. Crystallization screens were carried out by the sparse matrix method (36) in a sitting-drop vapor diffusion approach testing more than 1,000 crystallization conditions at 277 K and 298 K in 96-well Medical Research Council (MRC) plates. Promising conditions were systematically screened further by changing protein concentration, pH, and the concentration of precipitation agents. After optimization, crystals were grown by hanging drop vapor diffusion at 298 K in 24-well Linbro plates. Each hanging drop was prepared on a siliconized glass plate by mixing 5 μL protein solution with 5 μL reservoir solution. The protein solution contained 12.5 mM Tris (hydroxymethyl) amino methane, 1.25 mM EDTA, 2.5% (vol/vol) glycerol, 75 mM sodium chloride, pH 7.8. The reservoir solution contained 2–6% (wt/vol) polyethylene glycol 400, 100 mM 2-(N-morpholino)ethanesulfonic acid buffer, pH 6.0. Yellow rectangular PhrB crystals appeared within 3 d and grew further for 7 d. PhrB crystals were flash frozen in liquid nitrogen after cryo-soaking with 4 M trimethyamine N-oxide for a few minutes (37). Fully grown crystals had dimensions of ~0.9 × 0.3 × 0.7 mm².

Structure Analysis. Diffraction data collection was performed at 100 K using synchrotron X-ray sources at the European Synchrotron Radiation Facility (Grenoble, France) and the Berlin Radiation Synchrotron Source II (BESSY II) (Berlin, Germany). Best diffraction data for optimized anomalous dispersion and highest resolution were collected at beamline BL 14.2 (38) at BESSY II, Helmholtz Zentrum Berlin für Materialien und Energie with an MAR-225 CCD detector at λ = 1.7409 Å and λ = 0.91841 Å, respectively. All images were indexed, integrated, and scaled using the XDS program package (39) and the CCP4 program SCALA (40, 41). The single-wavelength anomalous dispersion data collection at λ = 1.7409 Å (peak) was collected to 1.9 Å resolution. Initial experimental phases were determined to 1.95 Å resolution by the SAD method based on this dataset by using SHELXD (42) and a beta version of SHELXC-D (43) to solve the iron-atom substructure. Substructure solution with SHELXD was successful for space group P212121, yielding four iron sites (with occupancies greater than 0.62). With this solution a mean figure of merit of 0.640 was obtained after initial phasing, polyalanine chain-tracing, and density modification with SHELXE (43) that increased to 0.770 after density modification with the program DM (44). The electron density map was readily interpretable, and the phases were input for initial automated model building using ARP/WARP 7.0.2 (45). Subsequent steps of crystallographic refinement, consisting of torsion angle molecular dynamics, simulated annealing using a slow-cooling protocol and a maximum likelihood target function, energy minimization, and B-factor refinement by the program CNS (46) were carried out using a high-resolution native dataset in the resolution range of 19.95–1.45 Å. The [4Fe-4S] cluster, the FAD, and 6,7-dimethyl-8-ribityllumazine were clearly visible in the electron density of a σc – weighted 2Fobs − Fcal map already after the first round of refinement, as well as in the σc – weighted simulated annealed omitting density maps that were calculated in the final stage of refinement. Restrained, individual B-factors were refined and the crystal structure was finalized using the CCP4 program REFMAC5, another program of the CCP4 suite (40), and PHENIX (47). The final model has agreement factors Rmerge and Rfree of 18.0% and 13.8%. Table S2 summarizes the statistics for crystallographic data collection and structural refinement. Manual rebuilding of the PhrB model and electron density interpretation was performed after each refinement cycle using the program COOT (48) and WHAT_CHECK (49). Potential hydrogen bonds and van der Waals contacts were analyzed using the programs HBPLUS (50) and LIGPLOT (51). All crystal structure superpositions of backbone α carbon traces were performed using the DALI server (23). The PhrB structure was analyzed for salt bridges using the WHAT IF server (52). Electrostatic potentials were calculated through solution of the Poisson-Boltzmann equation using the program APBS (53). All molecular graphics representations were created using PyMOL (54).

Analytical HPLC. An Agilent 1200 Series HPLC system managed by Chem Station software, equipped with a diode array detector (DAD) (Agilent) and a Nucleosil 100-5 analytical column (250 × 4.6 mm; Macherey and Nagel) was used for analysis of organic cofactors and the single stranded DNA (6-4) photoprotein repair assay, whereas equipped with a Nucleodur 100–3 column (250 × 4.0 mm; Macherey and Nagel) for the double stranded DNA (6-4) photoprotein repair assay.

A protocol established for Rhodobacter C184 (10) was used for analyses of organic cofactors. Purified PhrB was mixed with 7.2% trichloroacetic acid (TCA) followed by 1-h shaking at 100 rpm on ice, and then mixed with 5% (vol/vol) of 0.1% formic acid (HCO₂H) in acetonitrile (ACN) and 0.35 mM NaOH (final concentrations). The mixture was centrifuged at 13,000 × g for 10 min, and the supernatant was analyzed by HPLC. Synthetic DMRL (a generous gift from A. Bacher, Technical University Munich, Germany) and FAD were used as references. For the mobile phase, we used solution A (0.1% HCO₂H in ACN) and solution B (0.1% HCO₂H in H₂O). After injection of 10 μL samples, separations were achieved at 298 K and 0.35 mL/min. A 5-min isocratic run with 95% (vol/vol) of solution B was followed by a gradient from 95% to 25% solution B within 20 min. Elution was monitored at 264 nm and UV-Vis spectra were automatically recorded and stored.
For the DNA (6-4) photoproduct repair assay, HPLC was conducted as described (55). The single stranded or double stranded DNA probe comprising the (AGGT(6-4)TGCC or GCGT(6-4)TGGCG pair with TCGCCAACCGCT, 100 μmol) was dissolved in the repair buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM DTT, 1 mM EDTA, 5% glycerol, pH 7.5) and incubated with PhrB (300 μmol) in the repair buffer. The reaction mixture was irradiated with a 405 nm light-emitting diode (6333UVU1C1L, Farmall Diodes) at an intensity of 40 μmol·m⁻²·s⁻¹. Controls in which the TT (6-4) lesion comprising probes incubated with PhrB in darkness were performed in parallel. The reaction was stopped by heating to 368 K for 10 min and the sample was centrifuged at 13,000 g for 10 min. The supernatant was subjected to HPLC at a temperature of 298 K for single strand or 333 K for double strand DNA. For comparisons, untreated DNA probes with or without TT (6-4) lesion were processed similarly. The mobile phase consisted of solution C [0.1 M triethylamino- methane (TEA) in H₂O] and solution D (0.1 M TEA in H₂O/ACN 20/80). The gradient was 0–17% solution D in 60 min with a flow rate of 0.5 μL/min. Elution was monitored at 260 nm and 325 nm. Based on the retention time and UV-Vis spectral properties, the TT (6-4) lesion-comprising strand was easily distinguished from the repaired and complementary strand as well as from the standard undamaged DNA strand (Fig. 51).

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Supporting Information

Zhang et al. 10.1073/pnas.1302377110

Fig. S1. UV-visible (UV-Vis) spectra of HPLC fractions as recorded by the HPLC-coupled diode array detector (DAD) detector. (A) Spectra from the repair assay with single stranded DNA under blue light for 120 min (Fig. 1A). (B) Spectra from the repair assay with double stranded DNA under blue light for 15 min (Fig. 1B).

Fig. S2. Phylogenetic trees based on structural alignments. Both trees were constructed with the PROTPARS program of the PHYLIP program package (1). Bootstrap values (in percent) are printed along the branches if <95%. (A) Analysis based on a structural alignment of full-length cryptochrome/photolyase families (CPFs). The weak relationship between photolyase related protein B (PhrB) and eukaryotic (6-4) photolyases is also obtained with maximum likelihood and distance-based algorithms. (B) Analysis based on a structural alignment of the protein fold common to primase large subunit (PriL) and CPFs, core region (amino acid residues 348–398 of PhrB). PhrB and PriL are placed in one monophyletic group, a result that is also obtained with distance-based algorithms, and with the same algorithms and a longer homologous stretch (amino acids 333–465). Color code: red, (6-4) photolyases; cyan, CPD class I photolyases; green, CPD class II photolyases; black, plant cryptochromes and Cry-DASH proteins; blue, primase PriL subunits. AgrtuPhrB: (6-4) photolyase of Agrobacterium tumefaciens, Protein Data Bank (PDB) code 4DJA; Anani CPD I: CPD class I photolyase of Anaocystis nidulans, PDB code 1OWL; ArathCry1: cryptochrome 1 of Arabidopsis thaliana, PDB code 1U3C; ArathCry3: cryptochrome 3 (Cry-DASH) of Arabidopsis thaliana, PDB code 2J4D; Arath (6-4) PL: (6-4) photolyase of Arabidopsis thaliana, PDB code 3FY4; Drome (6-4) PL: (6-4) photolyase of Drosophila melanogaster, PDB code 3CVW; Escco CPD I: CPD class I photolyase of Escherichia coli, PDB code 1DNP; HomsaPriL: DNA primase large subunit of Homo sapiens, PDB code 3Q36; Metma CPD II: CPD class II photolyase of Methanosarcina mazei, PDB code 2XRZ; SaccoPriL: DNA primase large subunit of Saccharomyces cerevisiae, PDB code 3LGB; Sulto CPD I: CPD class I photolyase of PDB code Sulfolobus tokodaii, PDB code 2E0I; SynspDASH: Cry-DASH of Synechocystis strain PCC 6083, PDB code 1NP7; Theth CPD I: Thermus thermophilus CPD class I photolyase, PDB code 1IQR.

Fig. S3. The antenna chromophore of PhrB is 6,7-dimethyl-8-ribityllo-lumazine (DMRL). All spectra are normalized to $A_{264\text{nm}}$. (A) HPLC profiles, released PhrB cofactors (black line) and mixture of authentic FAD and DMRL (red line). Peaks 1 and 8 refer to DMRL; peak 9 could relate to a DMRL-like derivative. (B) UV-Vis spectra of DMRL (-like) fractions (HPLC peaks 1, 8, and 9). (C) UV-Vis spectra of FAD (-like) fractions (HPLC peaks 4, 5, 7, 11, 12, and 13). (D) UV-Vis spectra of UV-absorbing compounds of unknown identity (HPLC peaks 2, 3, 6, and 10).

Fig. S4. Comparison of C-terminal helices in superimposed (6-4) photolyases. Drome (6-4) PL and Arath (6-4) PL are illustrated in ribbon representation in blue and orange, respectively; PhrB is drawn in ribbon representation and transparent surface in green. Helix $\alpha_{19}$ of PhrB is common to all three photolyases, Drome (6-4) PL ends with this helix. Arath (6-4) PL has one additional helix and PhrB has two additional helices, $\alpha_{20}$ and $\alpha_{21}$.
**Fig. S5.** Electrostatic surface representation of PhrB. Electrostatic surface potentials were calculated using the program APBS (1) with the nonlinear Poisson–Boltzmann equation and contoured at ±5kT/e. Negatively and positively charged surface areas are colored in red and blue, respectively. The cofactors DMRL, FAD, and Fe-S cluster are illustrated in ball-and-sticks representation. The DNA binding cavity is characterized by its positive charge; the helix α20 of the C-terminal extension with its positive surface charge appears to delimit this cavity.


### Other Supporting Information Files

- Table S1 (DOCX)
- Table S2 (DOCX)
- Table S3 (DOCX)