Structural and Evolutionary Aspects of Antenna Chromophore Usage by Class II Photolyases*

Received for publication, December 19, 2013, and in revised form, May 4, 2014. Published, JBC Papers in Press, May 21, 2014, DOI 10.1074/jbc.M113.542431

Stephen Kiontke, Petra Gnau, Reinhard Haselsberger, Alfred Batschauer,* and Lars-Oliver Essen*

From the Biomedical Research Centre/FB15, Unit for Structural Biochemistry, Philippus-University, Hans-Meerwein-Strasse, D-35032 Marburg, Germany, the School of Physical and Mathematical Sciences, Division of Physics and Applied Physics, Nanyang Technological University, SPMS-PAP-03-11, 21 Nanyang Link, Singapore 637371, and the Faculty of Biology, Department of Plant Physiology and Photobiology, Philippus-University, Karl-von-Frisch-Strasse 8, D-35032 Marburg, Germany

Background: Photolyases are light-driven DNA repair enzymes harboring a catalytic FAD cofactor and usually an antenna chromophore. Results: 8-Hydroxydeazaflavin is the cognate antenna of the Methanosarcina mazei photolyase, an archaeal representative of the clade of otherwise metazoan class II photolyases. Conclusion: Phylogenetically, photolyases lost 8-hydroxydeazaflavin as antenna only in higher plants. Significance: 8-Hydroxydeazaflavin occurs as cofactor within major parts of the metazoan phylome.

Light-harvesting and resonance energy transfer to the catalytic FAD cofactor are key roles for the antenna chromophores of light-driven DNA photolyases, which remove UV-induced DNA lesions. So far, five chemically diverse chromophores have been described for several photolyases and related cryptochromes, but no correlation between phylogeny and used chromophore has been found. Despite a common protein topology, structural analysis of the distantly related class II photolyase from the archaeon Methanosarcina mazei (MmCPDII) as well as plantal orthologues indicated several differences in terms of DNA and FAD binding and electron transfer pathways. For MmCPDII we identify 8-hydroxydeazaflavin (8-HDF) as cognate antenna by in vitro and in vivo reconstitution, whereas the higher plant class II photolyase from Arabidopsis thaliana fails to bind any of the known chromophores. According to the 1.9 Å structure of the MmCPDII-8-HDF complex, its antenna binding site differs from other members of the photolyase-cryptochrome superfamily by an antenna loop that changes its conformation by 12 Å upon 8-HDF binding. Additionally, so-called N- and C-motifs contribute as conserved elements to the binding of deprotonated 8-HDF and allow predicting 8-HDF binding for most of the class II photolyases in the whole phylum. The 8-HDF antenna is used throughout the viridiplantae ranging from green microalgae to bryophyta and pteridophyta, i.e. mosses and ferns, but interestingly not in higher plants. Overall, we suggest that 8-hydroxydeazaflavin is a crucial factor for the survival of most higher eukaryotes which depend on class II photolyases to maintain their genomic integrity. Only a few evolutionary later evolved organisms such as the placental mammals afford the loss of photolyase orthologues by relying on alternative DNA repair pathways for the maintenance of their genomic integrity (3, 4).

The catalytic mechanism of photolyases generally employs the photo-induced injection of an electron from a fully reduced and typically U-shaped flavin adenine dinucleotide cofactor (FADH\(^{-}\)) onto the DNA lesion. This electron transfer triggers cleavage of CPD or (6-4) photoproducts inside duplex DNA (1, 5) within less than a nanosecond and achieves quantum yields close to one for CPD repair (6, 7). Spectroscopic and structural studies (8–10) showed that class I, class II, and (6-4) photolyases catalyze transient electron transfer to these DNA lesions by binding the lesions next to the FADH\(^{-}\) cofactor in the catalytic α-helical C-terminal domain. The N-terminal domain of these photolyases adopts a Rossmann fold, which can accommodate additional prosthetic groups, so called antenna chromophores. Compared with other members of the

* This work was supported by the LOEWE (Landesoffensive zur Entwicklung Wissenschaftlich-Ökonomischer Exzellenz) Center for Synthetic Microbiology (Marburg) and Deutsche Forschungsgemeinschaft Grant BA985/12-1. The atomic coordinates and structure factors (codes 4CDM and 4CDN) have been deposited in the Protein Data Bank (http://wwpdb.org/).

1 Present address: Dept. of Biology/Chemistry, Structural Biology, University of Osnabrück, Barbarastr. 13, D-49076 Osnabrück, Germany.

2 To whom correspondence should be addressed: Biomedical Research Centre/FB15, Philippus-University Marburg, Hans-Meerwein-Str., D-35032 Marburg, Germany. Tel.: 4964212822032; Fax: 4964212822191; E-mail: essen@chemie.uni-marburg.de.

3 The abbreviations used are: (6-4), pyrimidine(6-4)pyrimidone dimer; CPD, cyclobutane pyrimidine dimer; FO synthase, 7,8-didemethyl-8-hydroxy-5-deazariboflavin synthase; 8-HDF, 8-hydroxydeazaflavin; MmCPDII, class II CPD-photolyase from Methanosarcina mazei; MTHF, 5,10-methenyltetrahydrofolate; PDB, Protein Data Bank.
photolyase-cryptochrome family, class II photolyases are evolutionary distant with pairwise sequence identities of <16%. Nevertheless, structures of class II photolyases from a methanogenic archaeon (8) as well as from a higher plant (11) indicate a similar bilobal architecture as class I and (6-4) photolyases and cryptochromes. Although class II photolyases catalyze like class I CPD photolyases and DASH cryptochromes light-driven DNA-repair and photoreduction of the catalytic FAD cofactor, they show marked differences for their active site structures, the mode of DNA binding, the electron transfer pathways required for photoreduction as well as for the discrimination between intact and UV lesion-containing DNA.

A shortcoming of the catalytic FADH$^\bullet$ cofactor is its poor absorption in the blue to near-UV range with extinction coefficients between 2800 M$^{-1}$cm$^{-1}$ (400 nm) and 5600 M$^{-1}$cm$^{-1}$ (360 nm). For efficient light-driven repair in the blue/near-UV region, photolyases hence rely on a second auxiliary chromophore with large absorption cross-sections to broaden up their limited spectral properties. So far, five different classes of antenna chromophores have been identified from members of the photolyase-cryptochrome superfamily (Fig. 1). These antenna chromophores comprise aromatic moieties absorbing in the range between 380 and 420 nm and include (i) 5,10-methenyltetrahydrofolate (MTHF) as found in many microbial class I photolyases (12) and DASH cryptochromes (13); (ii) 8-hydroxydeazaflavin (8-HDF) from (6-4) photolyases (14, 15) and several class I photolyases (16); (iii and iv) flavin mononucleotide (FMN) and FAD in the class I photolyases from Thermus thermophilus (17) and Sulfolobus tokodaii (18), respectively; and finally (v) 6,7-dimethyl-8-ribityl-lumazin in a novel class of proteo-/cyanobacterial cryptochromes (19). For light harvesting the antenna chromophores absorb a photon and transfer its energy via a Förster mechanism to the catalytic active FADH$^\bullet$. All nucleotide-like chromophores (FAD, FMN, and 8-HDF) are bound within the N-terminal α/β domain of photolyases with its Rossmann-like fold in a distance of 17–18 Å to the catalytic FAD cofactor. In contrast to the deeply buried nucleotide-like antenna, the pterin derivative MTHF is bound close to the protein surface along the interface of the N- and C-terminal domains (20).

Like MTHF, the riboflavin derivatives FAD, FMN, as well as the riboflavin biosynthesis intermediate 6,7-dimethyl-8-ribityllumazin are commonly found in archaea, bacteria, and eukaryotes, whereas the occurrence of deazaflavin cofactors such as 8-HDF is more limited. For example, deazaflavins are signature molecules of methanogenic archaea, where 8-HDF in its oligoglutamylated and protonated F420 form plays an important role as a low potential hydride carrier (21). Nevertheless, deazaflavins are also found in several other bacterial clades such as the actinobacteria (22) as well as in unicellular green algae. The biological role of 8-HDF in photolyases was demonstrated in the green algae Chlamydomonas reinhardtii. Here, inactivation of the gene for the deazaflavin synthase PHR1 caused a loss of the photorepair of UV lesions as catalyzed in C. reinhardtii by the class II photolyase PHR2 (23). Interestingly, the (6-4) and class II CPD photolyases from the insect Drosophila melanogaster utilize 8-HDF as antenna chromophore as well (15), although this organism misses like other animals a genomic copy of the deazaflavin synthase gene. Instead, the bacterial symbiont Wolbachia was suggested to supply 8-HDF as a vitamin essential for photolyase function (14).

Here, we investigate the antenna chromophore of the class II CPD photolyase from Methanosarcina mazei (MmCPDII) and the spectroscopic and structural characterization of its complex with the 8-HDF antenna. Together with phylogenetic data the structure of the MmCPDII-8-HDF complex and its differences from higher plant photolyases allow us to predict the occurrence of 8-HDF in the whole metazoan branch of life.

**EXPERIMENTAL PROCEDURES**

Cloning of Streptomyces coelicolor FO Synthase—The gene SCO4429 (foIC) encoding the FO synthase (7,8-didemethyl-8-hydroxy-5-deazariboflavin synthase) of Streptomyces coelicolor (UniProt entry Q9K2Z7) was amplified from genomic DNA using the Phusion® DNA polymerase (Finnzymes) with primers 5’-GCATTGAAATTCCGATGACGACTTCCCGACCTCC-3’ and 5’-GATATTCTCGAGTTAGTCCAGGACCCGGACAGCAG-3’ (Metabion). The PCR product was cloned into pCR®2.1-TOPO® vector (Invitrogen) and subcloned via EcoRI

![FIGURE 1. In vitro reconstitution of apoMmCPDII with synthetic 8-HDF and overview of known antenna chromophores of members of the photolyase-cryptochrome family. Binding of 8-HDF to the M. mazei class II photolyase (red curve) causes a red shift by 15 nm compared with free 8-HDF at pH 8 (gray curve), where free 8-HDF is deprotonated at its 8-OH group (pKₐ 6.3) like MmCPDII-bound deazaflavin.](image-url)
and XhoI sites (underlined) into pCDFDuet-1 (Novagen) thus yielding the cofactor plasmid pCDFDuet-His<sub>6</sub>ScFbiC, which encodes an N-terminally His<sub>6</sub>-tagged ScFbiC fusion.

**Cloning of Photolyases for Co-expression Studies**—The gene *Sympec7942_0112* encoding the class I CPD photolyase (UniProt entry Q31S25) of *Synechococcus elongatus* PCC 7942 (*Anaecistis nidulans* R2) was amplified from isolated genomic DNA with primers 5′-GGTTTTCGATATGGGCTCCGATTCTGTTTGGG-3′ and 5′-CCAAACCAGGCTATGATGCGGTGCCTAGCCTTCC′, thus introducing restriction sites for NdeI and XhoI (underlined). Accordingly, the PCR product was cloned into expression vector pET-28a (Novagen) to give the plasmid pET28a-His<sub>6</sub>ATII. The latter encodes N-terminally His<sub>6</sub>-tagged *An* CPDI. Likewise, the gene fragment (bases 1–1560) coding for the (6-4) photolyase domain from *D. melanogaster* (UniProt entry Q6E8P0) was amplified from the pDEST007 plasmid (9) with primers 5′-GGTTTTCGATATGGGCTCCGATTCTGATTCTC-3′ and subcloned via NdeI and XhoI sites into pET-28a to produce pET-28a-His<sub>6</sub>Dm(6-4). The class I CPD photolyase from *T. thermophilus* HB8 (UniProt entry P61497) was amplified from genomic DNA with primers 5′-GGAAATCCCTCGGGCGAGATCC-3′ and 5′-GCCCGTGTTCCAGTCCAAGGC-3′ and subcloned via NdeI and BamHI into pET-28a to yield pET-28a-His<sub>6</sub>TtCPDI.

**Co-expression and Purification of Photolyases (PHR)—Escherichia coli** BL21 (DE3) Gold cells (Strategene) were transformed with either pET28a-His<sub>6</sub>MmCPDII (8), pET28a-His<sub>6</sub>ATII, pET28a-His<sub>6</sub>Dm(6-4), pET28a-His<sub>6</sub>TtCPDI, or pET28a-His<sub>6</sub>AtCPDI and the cofactor plasmid pCDFDuet-His<sub>6</sub>ScFbiC. Autoinduction-triggered co-expression was performed in terrific broth medium at 25 °C (*Mm*CPDII, *Tt*CPDI, *At*CPDI), 21 °C (*An* CPDI), and 18 °C (*Dm* (6-4)), respectively. Apart from *At*CPDI the putative photolyase-8-HDF complexes were purified according to the protocol of apoMmCPDII apart that cell disruption was performed with a French press. For the purification of *At*CPDI buffer AT<sub>1</sub> (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM NaCl, 20% (v/v) glycerol, pH 7.4) was used for affinity chromatography, and elution was performed by addition of 75 mM imidazole. Size exclusion chromatography was done in buffer AT<sub>2</sub> (20 mM Tris-HCl, 300 mM NaCl, 10% (v/v) glycerol, pH 7.4). The His<sub>6</sub>-tagged FO synthase was efficiently removed by the size exclusion chromatography step.

**Generation and Preparation of MmCPDII Mutants—Photolyase mutants MmCPDII-S26L and MmCPDII-S26F** were obtained from pET28a-MmCPDII by site-directed mutagenesis (QuikChange II; Agilent); resulting plasmids were verified by dyeoxy-sequencing (Qiagen). Co-expression and purification of the Ser<sup>26</sup> mutants were performed as described for the wild type photolyase.

**In Vitro Reconstitution of ApoMmCPDII with Antenna Chromophores**—ApoMmCPDII (53 μM) was mixed with the equimolar amount of either FAD, FMN, MTHF, F420, or chemically synthesized 8-HDF. Afterward, all mixtures as well as negative controls were dialyzed at 4 °C against 1.5 ml of buffer I (10 mM Tris-HCl, 100 mM NaCl, pH 8.0) in a pre-pressured VDX<sup>TM</sup> plate (Hampton Research) using 10-μl microdialysis rods (Hampton Research) and a 3.5-kDa cutoﬀ SnapSkin<sup>®</sup> dialysis membranes (Pierce). After 24 h, reconstitution was analyzed by UV/visible spectroscopy using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Likewise, the 8-HDF reference spectrum was recorded with the NanoDrop 1000 spectrophotometer and normalized to the MmCPDII concentration used before.

**Structure Determination of ApoMmCPDII Crystals Soaked with 8-HDF—MmCPDII crystals grown in 0.5 M lithium sulfate and 7.5% PEG 8000 were soaked for 30 min in crystallization buffer supplemented with 100 μM synthetic 8-HDF. During soaking changes in the integrity of MmCPDII crystals were observed and are most likely reﬂected by the lower resolution limit of 2.7 Å compared with apoMmCPDII crystals (1.5 Å). Before flash-freezing in liquid nitrogen crystals were soaked in the crystallization solution supplemented with 30% glycerol as cryoprotectant. Diffraction data were collected from a single crystal at 100 K at beamline ID23-2, European Synchrotron Radiation Facility (ESRF). Data processing was carried out using XDS and XSCALE (24) and further reﬁnement using COOT (25) and REFMAC5 (26) at 2.7 Å resolution led to refinement statistics are summarized in Table I.

**Crystallographic Statistics of the MmCPDII-8-HDF Complex**—An initial 96-well format crystallographic screening was performed with a Cartesian robot system and commercially available crystalization screens (Qiagen) using the sitting drop vapor diffusion method at 18 °C. First crystals were obtained in a crystallization condition containing 0.1 M lithium sulfate, 0.1 M trisodium citrate, pH 5.6, and 12% PEG 6000 within 48 h. Using the hanging drop vapor diffusion method and this condition, crystals suitable for data collection were grown in a 24-well plate at a protein concentration of 11.8
Antenna Chromophore Usage by Class II Photolyases

mg/ml. For cryoprotection, glycerol was added to a final concentration of 33%, and MmCPDII-8-HDF crystals were flash-frozen in liquid nitrogen. X-ray data were collected from a single crystal at 100 K at beamline ID32-1, ESRF. MmCPDII-8-HDF crystallized in the orthorhombic space group P212121, with unit cell parameters a = 78.97 Å, b = 114.7 Å, c = 141.4 Å. Diffraction data were processed using XDS and XSCALE (24), and initial phases were obtained by molecular replacement using PHASER (27) and the structure of MmCPDII in a tetragonal crystal form (2XRY) as search model. The correct solution with data up to 2.5 Å had two molecules per asymmetric unit with z scores of 25.7 for rotation and 45.9 for translation function for the first molecule and z scores of 26.2 and 79.7 for the second molecule, respectively. After initial automated model building from experimental phases as done by ARP/wARP (28), further refinement using COOT (25) and REFMAC5 (26) at 1.9 Å resolution led to F-values of 26.2 and 79.7 for the first molecule and 14.9% and 17.8% for the second molecule, respectively. After initial automated model building from experimental phases as done by ARP/wARP (28), further refinement using COOT (25) and REFMAC5 (26) at 1.9 Å resolution led to F-values of 26.2 and 79.7 for the first molecule and 14.9% and 17.8% for the second molecule, respectively. After initial automated model building from experimental phases as done by ARP/wARP (28), further refinement using COOT (25) and REFMAC5 (26) at 1.9 Å resolution led to F-values of 26.2 and 79.7 for the first molecule and 14.9% and 17.8% for the second molecule, respectively. After initial automated model building from experimental phases as done by ARP/wARP (28), further refinement using COOT (25) and REFMAC5 (26) at 1.9 Å resolution led to F-values of 26.2 and 79.7 for the first molecule and 14.9% and 17.8% for the second molecule, respectively. After initial automated model building from experimental phases as done by ARP/wARP (28), further refinement using COOT (25) and REFMAC5 (26) at 1.9 Å resolution led to F-values of 26.2 and 79.7 for the first molecule and 14.9% and 17.8% for the second molecule, respectively.

In Vitro Reconstitution of MmCPDII with Synthetic 8-HDF—When purified via the E. coli expression system, recombinant class II photolyase from M. mazei (MmCPDII) possesses the catalytic FAD cofactor but lacks any bound and host-derived antenna chromophore as shown before by UV/visible spectroscopy and structural analysis (8). To identify the cognate light-harvesting prosthetic group of MmCPDII, we performed in vitro reconstitution of this so-called apoMmCPDII with known antenna chromophores such as FAD, FMN, MTHF, 8-HDF, and F420. Only for synthetic 8-HDF, the UV/visible spectroscopic analysis reveals a characteristic modified absorbance spectrum of the M. mazei class II photolyase. The absorbance spectrum of oxidized apoMmCPDII with peaks at 362, 377, 421, 444, and 469 nm is dominated in the reconstituted MmCPDII-8-HDF complex by a significant peak at 435 nm (Fig. 1). Furthermore, incorporation of deprotonated 8-HDF (λ max 420 nm) into MmCPDII causes a 15-nm red shift of the chromophore absorbance. This red shift of bound 8-HDF complies well with data reported for D. melanogaster (6-4) photolyase (14) and A. nidulans class I photolyase (AnCPDII), the latter either purified directly from A. nidulans cells (16) or in vitro reconstituted with 8-HDF using recombinant enzyme (30, 31).

In Vivo Assembly of the MmCPDII-8-HDF Holoenzyme—To confirm these results and to rule out that incorporation of synthetic 8-HDF during in vitro reconstitution and crystal soaking is enforced by the chosen experimental conditions, an in vivo reconstitution system of MmCPDII with endogenously produced 8-HDF was established. For this purpose, a cofactor plasmid encoding the S. coelicolor FO synthase (ScFbiC), a bifunctional, S-adenosylmethionine-dependent enzyme (26), was constructed that enables the biosynthesis of 8-HDF in E. coli from 4-hydroxypyruvate and 5-amino-6-ribityl-amino-2,4(1H,3H)-pyrimidinedione, an intermediate of the riboflavin synthesis pathway (Fig. 2A). After nickel-nitritolriacetic acid affinity chromatography, the MmCPDII-8-HDF complex was separated from ScFbiC by size exclusion chromatography (Fig. 2B). Like in vitro reconstituted MmCPDII, the UV/visible spectrum of the MmCPDII-8-HDF holoenzyme in the oxidized state is hallmarked by the distinct absorption maximum at 435 nm (Fig. 2C, black line) indicating that 8-HDF is bound to the photolyase. Moreover, the fluorescence emission spectrum of the complex exhibits a strong fluorescence peak at λ emo 467 nm (Fig. 2C, thick red line) and differs significantly from apoMmCPDII with the latter exhibiting only broader emission at λ emo of approximately 528 nm for the oxidized FAD cofactor (Fig. 2C, dotted red line). In accordance with data from AnCPDII (16, 31) and Dm(6-4) (14), the fluorescence maximum of MmCPDII-bound 8-hydroxydeazafavin resembles the maximum of deprotonated 8-HDF in solution with its λ emo of 470 nm (Fig. 3A). In contrast, the fully reduced FADH⁻ state exhibits an emission peak for the 8-HDF antenna that is blue-shifted by 12 nm to 455 nm, thus suggesting some interaction between the redox state of the FAD cofactor and the structure of the antenna binding site (Fig. 2D). Furthermore, excitation spectra recorded at λ emo = 533 nm, the emission maximum of the reduced FADH⁻ chromophore, demonstrate efficient Förster energy transfer between the antenna and the flavin cofactor, as the resulting spectra are clearly dominated by the 8-HDF antenna with its λ max of 435 nm (Fig. 2D).

To clear up the question, whether Methanosarcinales are generally capable of synthesizing 8-HDF as cognate antenna of their photolyases, we performed a protein-protein BLAST search with Methanocaldococcus jannaschii CoF and CoH, which have been used before to produce artificial 8-HDF in E. coli (32). Indeed, M. mazei harbors like other methanosarcinal species, e.g., Methanosarcina acetivorans or Methanosarcina Barkeri, one CoF and two CoH orthologues (SwissProt entries COFG, METMA, COFH1_METMA, COFH2_METMA).

Validation of the Heterologous in Vivo 8-HDF Reconstitution System—To rule out that our in vitro reconstitution system generates noncognate antenna-photolyase hybrids, class I and (6-4) photolyases were co-expressed with the ScFbiC FO synthase. AnCPDII was chosen as reliable positive control for successful incorporation of the 8-HDF antenna chromophore, because AnCPDII that is directly purified from A. nidulans cells harbors 8-HDF as cognate antenna (16). This cyanobacterium
Antenna Chromophore Usage by Class II Photolyases

Antenna Chromophore Usage by Class II Photolyases

FIGURE 2. In vivo reconstitution of MmCPDII with an 8-HDF antenna prosthetic group. A, the heterologous synthesis of 8-HDF in E. coli as catalyzed by the S. coelicolor FO synthase (ScFbiC) starting from endogenous metabolites (4-hydroxyphenylpyruvate, compound 6: 5-amino-6-ribityl-amino-2,4(1H,3H)-pyrimidinedione). The reaction mechanism is according to Graham et al. (32). B, SDS-PAGE analysis showing the purification of the MmCPDII-8-HDF complex. C, spectral properties of MmCPDII and its complex with 8-HDF in the oxidized state. The UV/visible spectrum of the MmCPDII-8-HDF complex (black line) features the identical absorbance maximum as the in vitro reconstituted photolyase. Unlike apoMmCPDII (λ<sub>max</sub> 420 nm, dotted red line), the MmCPDII-8-HDF complex features a prominent fluorescence emission maximum at 467 nm (λ<sub>max</sub> 420 nm, solid red line). The λ<sub>max</sub> of 428 nm for the excitation spectrum of the MmCPDII-8-HDF complex (emission measured at 467 nm, thin red line) indicates a bathochromic shift by about 8 nm for the absorption of the antenna chromophore due to its interactions with the photolyase. D, spectral properties of MmCPDII and its complex with 8-HDF in the fully reduced state. The fluorescence excitation spectrum at 533 nm emission corresponds to 90% of the intensity when recorded at the emission maximum of the 8-HDF antenna of 455 nm.

The UV/visible spectrum of the apoMmCPDII complex (λ<sub>max</sub> 420 nm, dotted red line), the MmCPDII-8-HDF complex features a prominent fluorescence emission maximum at 467 nm (λ<sub>max</sub> 420 nm, solid red line). The λ<sub>max</sub> of 428 nm for the excitation spectrum of the MmCPDII-8-HDF complex (emission measured at 467 nm, thin red line) indicates a bathochromic shift by about 8 nm for the absorption of the antenna chromophore due to its interactions with the photolyase. D, spectral properties of MmCPDII and its complex with 8-HDF in the fully reduced state. The fluorescence excitation spectrum at 533 nm emission corresponds to 90% of the intensity when recorded at the emission maximum of the 8-HDF antenna of 455 nm.

Carries genomic copies of the 8-HDF synthase genes cofG and cofH (UniProt entries Q5N4D1, Q5N3T7), on which biosynthesis of the 8-HDF chromophore depends. For comparison, the (6-4) photolyase from D. melanogaster as well as the class I CPD photolyase from T. thermophiles (TtCPDI) were used, as the corresponding organisms lacking CofG/CofH orthologues. Here, 8-HDF has been successfully incorporated by either in vitro reconstitution of Ds(6-4) (14) or crystal soaking of TtCPDI (33).

After purification, all putative recombinant photolyase complexes were analyzed by UV/visible spectroscopy to analyze for in vivo reconstitution with endogenously produced 8-HDF. In case of AnCPDI and Dm(6-4) the UV/visible spectra exhibit prominent absorbance maxima at approximately 440 – 450 nm (Fig. 3B). Whereas the absorbance maximum of the AnCPDI-8-HDF complex at λ<sub>max</sub> 438 nm corresponds closely to spectra already published (16, 30, 31), the maximum at λ<sub>max</sub> 448 nm for the in vivo reconstituted Dm(6-4)-8-HDF complex is red-shifted by 8 nm compared with the in vitro reconstituted photolyase with a λ<sub>max</sub> of 440 nm (14). Interestingly, in vivo reconstitution with 8-HDF failed for TtCPDI, although the FO synthase was solubly expressed (Fig. 3A–C). Accordingly, the co-crystal structure of TtCPDI with synthetic 8-HDF, which was generated by soaking TtCPDI crystals with millimolar concentrations of 8-HDF (33), corresponds to a noncognate complex of this photolyase. However, the lack of endogenous 8-HDF biosynthesis pathways is not per se an indicator for 8-HDF playing no role as a cognate antenna chromophore of the respective photolyase. The fruit fly D. melanogaster lacks the genes required for 8-HDF biosynthesis, but its (6-4) and CPD photolyases are nevertheless capable of accommodating 8-HDF as antenna chromophore (15). This insect species may derive 8-HDF from a bacterial, hitherto unknown commensal as suggested before (14).

Structures of the MmCPDII-8-HDF Holoenzyme—To characterize 8-HDF antenna binding by MmCPDII we derived co-crystal structures either by crystallization of in vivo reconstituted MmCPDII-8-HDF holoenzyme at 1.9 Å resolution or by soaking apoMmCPDII crystals with 8-HDF and subsequent structural analysis at 2.7 Å resolution. Interestingly, the holoenzyme crystallized in a novel, orthorhombic crystal form with 0.20 and 0.28 Å (330, 353 C<sub>α</sub> atoms), respectively, relative to the apoMmCPDII structure. In both reconstitutions, the 8-HDF chromophore is buried deeply...
within the N-terminal domain in a distance of 16.7 Å to the catalytic FAD cofactor, which is well in range for efficient Förster energy transfer (Fig. 4, A and B). According to difference electron density the in vivo reconstituted MmCPDII-8-HDF holoenzyme shows quantitative incorporation of the 8-HDF chromophore, whereas soaking achieved under our conditions only 70% occupancy (Fig. 4, C and D). The inhomogeneity of the latter is reflected by the alternative conformations taken up by parts of the antenna loop (Leu57-Ala64) that links β-strand β2 with helix α2. Here, the short helical turn (Glu60-Glu63) that is found in 8-HDF-free MmCPDII structures (PDB codes 2XRY and 2XRZ (8)) undergoes a conformational change by closing the entrance to the 8-HDF binding site. The largest change within the antenna loop is made by Phe61-Leu62, which swivel their packed side chains by a distance of over 12 Å to form π-π-interaction between Phe61 and the middle ring of the deazaflavin (Fig. 4B).

The in vivo reconstituted MmCPDII-8-HDF holoenzyme shows even larger conformational changes compared with the apo-structures of MmCPDII. The rearrangement of the
antenna loop involves the region between Asp59 and Ala64 and seals the 8-HDF binding site from bulk solvent access. Furthermore, major parts of the linker between the N- and C-terminal domains of this photolyase (Val186-Glu231) either adopt a conformation different from in 8-HDF lacking MmCPDII structures or are disordered as in complex B (Glu189-Leu217). In complex A, helix α7 (Leu200-Glu214), which is structurally also conserved in the class II photolyase from rice (Oryza sativa, PDB code 3UMV, Asp221-Glu232 (11)), breaks down into three shorter helical segments (α7A, Leu200-Glu205; α7B, Val208-Leu211; α7C, Glu214-Lys219). The newly formed α7C segment hereby stabilizes the closed conformation of the antenna loop by several, newly formed van der Waal interactions. As a result Asp222 of the linker that is exposed before as part of a loop becomes buried and forms a salt bridge with Arg67. Overall, these structural adaptations of the second shell around the antenna chromophore binding site most likely impede reopening and hence release of bound 8-HDF.

The Antenna Binding Site of MmCPDII—Although the 8-HDF chromophore locates in the same region of the N-terminal domain of MmCPDII as in the cognate 8-HDF complexes of class I and (6-4) photolyases (Fig. 5), there are several peculiar differences for its binding site. First, the re-side of the aromatic ring system of 8-HDF, i.e. in Fig. 5 the backside, makes extensive van der Waal interactions with the bulky side chains of Phe273 and Phe65 as well as with Met274 via its middle ring. In other photolyases, the latter residue is exchanged by a leucine, whereas the walling by phenylalanines is conserved. More

FIGURE 5. Cognate and noncognate 8-HDF binding sites in the N-terminal antenna domain of DNA photolyases. For clarity, the 8-HDF chromophore is shown as a ball-and-stick model.
Antenna Chromophore Usage by Class II Photolyases

**A**

| Photolyase | N-motif | C-motif | 8-HDF synthesis |
|-----------|---------|---------|-----------------|
| MmCPDII   | NMS     | CRYM   | CofG, CofH      |
| McCPDII   | NQM     | CRYL   | CofG, CofH      |
| CzCPDII   | NMS     | CRYM   | none            |
| DmCPDII   | NMS     | CRYM   | none            |
| OsCPDII   | NML     | CRYL   | none            |
| AtCPDII   | NML     | CRYL   | none            |

**B**

![Structural features of the 8-HDF binding pocket within class II photolyases](image)

**C**

**D**

![UV/visible spectroscopic analysis](image)

**E**

![SDS-PAGE analysis](image)

**FIGURE 6. Structural features of the 8-HDF binding pocket within class II photolyases.**

A, multiple sequence alignment of selected class II photolyases shows characteristic sequence motifs for 8-HDF binding like the N-motif presenting a polar side chain close to the ribityl moiety and the basic C-motif for salt bridge formation with 8-oxy group. B, schematic overview shows structural determinants of the 8-HDF binding motif. C, structural comparison of MmCPDII and OsCPDII (rose) identifies differences crucial for 8-HDF antenna chromophore binding. D, UV/visible spectroscopic analysis shows 8-HDF binding to MmCPDII mutants. Replacement of the polar serine Ser26 within the WMS motif for a nonpolar leucine causes only a slight shift in the absorption maximum (MmCPDII-S26L, dashed line), whereas the bulky, hydrophobic phenylalanine completely impeded 8-HDF incorporation (MmCPDII-S26F, dotted line). E, SDS-PAGE analysis shows MmCPDII mutants/ScFbiC co-expressions. The asterisks mark the bands for the recombinant, co-expressed His6-ScFbiC. M, protein marker; AC, nickel-nitrilotriacetic acid affinity chromatography; SEC, size exclusion chromatography.

**Antenna Chromophore Usage by Class II Photolyases**

important, the si-side of the chromophore aromatic system is covered by Thr58 and Phe61 from the nonregular antenna loop, the indole of Trp126 as well as the side chain of Arg411. In other photolyases, the antenna loop is replaced by a short helical segment, which points only with a leucine toward the middle ring of the 8-HDF chromophore. Second, Trp126 replaces in class II photolyases an otherwise highly conserved arginine (AnCPDI, Arg109; Ds(6-4), Arg118), which forms in class I and (6-4) photolyases an H-bond to the C2-carbonyl of the deazaflavin moiety. In MmCPDII, this role is taken by Ser26, which forms hydrogen-bonds to the C2-carbonyl as well as the 3’-hydroxy group of the ribityl moiety (Fig. 5). Finally, two basic residues surround the phenolic ring of the ribityl moiety (Fig. 5). One of these residues, His272, is similar in class I and 6-4 photolyases (AnCPDI, Lys248; Ds(6-4), Lys266), where it forms a salt bridge to the 8-oxy group of 8-HDF. The conformation of this histidine is stabilized by a salt bridge to Asp512 from the C-terminal domain of MmCPDII (Fig. 5). Interestingly, its preceding residue, Arg411, makes the second salt bridge to the 8-oxy group. This residue has no counterpart in class I and 6-4 photolyases and is replaced by an arginine from the N-terminal domain (AnCPDI, Arg51; Ds(6-4), Arg60).

Given other structural differences, which are crucial for function, e.g., the distinct electron-transfer pathways for photoreduction or the binding sites of the catalytic FAD chromophore (8), these observations corroborate the notion of a large evolutionary gap between class II photolyases and other members of the photolyase-cryptochrome superfamily. For example, class I photolyases that lack 8-HDF as cognate antenna such as TtCPDI, miss all of the ascribed motifs of the 8-HDF binding site (Fig. 5).

Conservation of the 8-HDF Antenna Chromophore within Class II Photolyases—Apart from the structural differences described above, a comparison of the MmCPDII-8-HDF complex and the recently published O. sativa class II CPD photolyase (OsCPDII) shows no other major deviations. Nevertheless, heterologously expressed O. sativa photolyase lacks any kind of an additional antenna such as TtCPDI, miss all of the ascribed motifs of the 8-HDF binding site (Fig. 5).
square deviation 0.60 Å for 338 C\(\text{H9251}\) atoms) shows that although most amino acids lining the antenna binding pocket are either conserved (Os\(\text{CPDII}:\) Trp 43, Leu 76, Ala 85, His 289, Phe 290, Arg 429) or have at least similar biochemical properties (see Fig. 6\(\text{C}\)). However, there are several peculiar differences correlating with the loss of 8-HDF binding. First, the “lower” part of the pocket, where the aromatic ring moiety of 8-HDF is accommodated, lacks in Os\(\text{CPDII}\) the aromatic residues from the antenna loop that are responsible for \(\pi-\pi\) stacking from the \(\text{si}\)-side (Mm\(\text{CPDII},\) Phe61; Os\(\text{CPDII},\) Leu82) as well as the edge-to-face...

**Antenna Chromophore Usage by Class II Photolyases**

**JULY 11, 2014 • VOLUME 289 • NUMBER 28 • JOURNAL OF BIOLOGICAL CHEMISTRY 19667**
interaction with the Cys6 of the deazaflavin (MmCPDII, Tyr69; OsCPDII, Leu90 (35)). Instead, the binding pocket is partly filled in OsCPDII by the bulky side chain of Phe77 (MmCPDII: Thr58) from the antenna loop. Second, in OsCPDII a leucine (Leu49) replaces the polar residue Ser26 of MmCPDII, which is crucial for the recognition of 8-HDF via the H-bonds to the C2-carbonyl and 3′-hydroxy group. In contrast, the basic residues His272 and Arg411 of the C-terminal catalytic domain, which stabilize the deprotonation of the 8-hydroxy group, are preserved in the higher plant photolyases like AtCPDII or OsCPDII and are hence only weak indicators for antenna chromophore binding.

Taken together, we can now predict the signature motifs, by which members of the class II photolyase family are capable of utilizing 8-HDF as a cognate antenna (Fig. 6, A and B). First, the C-motif harboring the basic residues His272 and Arg411 has to be intact for interacting with the deprotonated 8-oxo group of 8-HDF. Second, the N-motif (WMS) has to provide a polar residue for H-bonding interactions with 8-HDF as well as formation of the binding site wall. A replacement of this residue by a bulky aromatic like in the S26F mutant of MmCPDII is crucial for the recognition of 8-HDF via the H-bonds to the C2-carbonyl and 3′-hydroxy group. In contrast, the basic residues His272 and Arg411 of the C-terminal catalytic domain, which stabilize the deprotonation of the 8-hydroxy group, are preserved in the higher plant photolyases like AtCPDII or OsCPDII and are hence only weak indicators for antenna chromophore binding.

CONCLUSION

8-HDF is an apparently widespread antenna chromophore of photolyases not only among microbial organisms, but also in plants and animals. In the latter, it may fulfill a role as a vitamin as suggested before for the (6-4) photolyases from D. melanogaster (14, 15). Likewise, other diphtheria, e.g. the vectors for malaria and West Nile fever, Anopheles gambiae and Aedes aegypti, encode class II photolyases compatible with 8-HDF antenna chromophores (Fig. 7). Interestingly, the earlier notion that endosymbiotic Wolbachia or Spiroplasma species may be the source of 8-HDF for D. melanogaster photolyases (14) is not supported by genomic data, which predict a lack of 8-HDF biosynthesis pathways in these bacteria. Accordingly, class II photolyases compatible with 8-HDF antennas are not a general trait in the insect class, because the class II photolyases from hemiptera and hymenoptera like the honey bee (Apis mellifera) show substitutions similar to those found in the higher plant photolyases. The same diversity also holds for the vertebrates. Here, many amphibia, reptilians, and even non-pterocentric mammals encode 8-HDF utilizing class II photolyases (Fig. 7), whereas others like birds and bony fishes show substitutions in their photolyases genes, which ablate 8-HDF binding.

Acknowledgments—We thank Annegret Wilde (Justus-Liebig University, Giessen) and Verena Helmetag (Philips-University, Marburg) for genomic DNA, Elvira Happel and Emine Kaya (Ludwig-Maximilians University, Munich) for plasmids, Seigo Shima (Max-Planck Institute for Terrestrial Microbiology, Marburg) for a sample of F. g. and Christoph Schwarz, Dennis Walczyk, and Sophie Franz for technical assistance. Synthetic 8-HDF was a gift from Thomas Carell (Ludwig-Maximilians University, Munich). We thank the beamline staff of ID23-1 and ID23-2 at the European Synchrotron Radiation Facility (ESRF Grenoble, France) for excellent support during data collection.

REFERENCES

1. Essen, L. O. (2006) Photolyases and cryptochromes: common mechanisms of DNA repair and light-driven signaling? Curr. Opin. Struct. Biol. 16, 51–59
2. Sancar, A. (2003) Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors. Chem. Rev. 103, 2203–2237
JOURNAL OF BIOLOGICAL CHEMISTRY

Antenna Chromophore Usage by Class II Photolyases

3. Kato, T., Jr., Todo, T., Ayaki, H., Ishizaki, K., Morita, T., Mitra, S., and Ikenaga, M. (1994) Cloning of a marsupial DNA photolyase gene and the lack of related nucleotide sequences in placental mammals. *Nucleic Acids Res.* **22**, 4119–4124

4. Lucas-Lledó, J. I., and Lynch, M. (2009) Evolution of mutation rates: phylogenetic analysis of the photolyase/cryptochrome family. *Mol. Biol. Evol.* **26**, 1143–1153

5. Müller, M., and Carell, T. (2009) Structural biology of DNA photolyases and cryptochromes. *Curr. Opin. Struct. Biol.* **19**, 277–285

6. Liu, Z., Tan, C., Guo, X., Kao, Y. T., Li, J., Wang, L., Sancar, A., and Zhong, D. (2011) Dynamics and mechanism of cyclobutane pyrimidine dimer repair by DNA photolyase. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 14831–14836

7. Thiagarajan, V., Byrdin, M., Eker, A. P., Müller, P., and Brettel, K. (2011) Kinetics of cyclobutane thymine dimer splitting by DNA photolyase directly monitored in the UV. *Proc. Natl. Acad. Sci. U.S.A.* **108**, 9402–9407

8. Kiontke, S., Geisselbrecht, Y., Pokorny, R., Carell, T., Batschauer, A., and Essen, L. O. (2011) Crystal structures of an archaeal class II DNA photolyase and its complex with UV-damaged duplex DNA. *EMBO J.* **30**, 4437–4449

9. Maul, M. J., Barends, T. R., Glas, A. F., Cryle, M. J., Domratcheva, T., Schneider, S., Schlichting, I., and Carell, T. (2008) Crystal structure and mechanism of a DNA (6-4) photolyase. *Angew. Chem. Int. Ed. Engl.* **47**, 10076–10080

10. Mees, A., Klar, T., Gnau, P., Hennecke, U., Eker, A. P., Carell, T., and Essen, L. O. (2004) Crystal structure of a photolyase bound to a CPD-like DNA lesion after *in situ* repair. *Science* **306**, 1789–1793

11. Hitomi, K., Arvai, A. S., Yamamoto, J., Hitomi, C., Teranishi, M., Hirouchi, T., Yamamoto, K., Iwai, S., Tainer, J. A., Hidema, J., and Getzoff, E. D. (2012) Eukaryotic class II cyclobutane pyrimidine dimer photolyase structure reveals basis for improved ultraviolet tolerance in plants. *J. Biol. Chem.* **287**, 12060–12069

12. Johnson, J. L., Hamm-Alvarez, S., Payne, G., Sancar, G. B., Rajagopalan, K. V., and Sancar, A. (1988) Identification of the second chromophore of *Escherichia coli* DNA photolyase and its complex with UV-damaged duplex DNA. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2046–2050

13. Klärt, T., Pokorny, R., Moldt, J., Batschauer, A., and Essen, L. O. (2007) Cryptochrome 3 from *Arabidopsis thaliana*: structural and functional analysis of its complex with a folate light antenna. *J. Mol. Biol.* **366**, 954–964

14. Glas, A. F., Maul, M. J., Cryle, M., Barends, T. R., Schneider, S., Kaya, E., Schlichting, I., and Carell, T. (2009) The archaeal cofactor F0 is a light-harvesting antenna chromophore in eukaryotes. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 11540–11545

15. Selby, C. P., and Sancar, A. (2012) The second chromophore in *Drosophila* photolyase/cryptochrome family photoreceptors. *Biochemistry* **51**, 167–171

16. Eker, A. P., Kooiman, P., Hessels, J. K., and Yasui, A. (1990) DNA photoreactivating enzyme from the cyanobacterium *Anacystis nidulans*. *J. Biol. Chem.* **265**, 8009–8015

17. Ueda, T., Kato, A., Kuramitsu, S., Terasawa, H., and Shimada, I. (2005) Identification and characterization of a second chromophore of DNA photolyase from *Thermus thermophilus* HB27. *J. Biol. Chem.* **280**, 36237–36243

18. Fujihashi, M., Numoto, N., Kobayashi, Y., Mizushima, A., Tsujimura, M., Nakamura, A., Kawarabayashi, Y., and Miki, K. (2007) Crystal structure of archaeal photolyase from *Sulfolobus tokodaii* with two FAD molecules: implication of a novel light-harvesting cofactor. *J. Mol. Biol.* **365**, 903–910

19. Geisselbrecht, Y., Frühwirth, S., Schroeder, C., Pieirc, A. J., Klug, G., and Essen, L. O. (2012) CryB from *Rhodobacter sphaeroides*: a unique class of cryptochromes with new cofactors. *EMBO Rep.* **13**, 223–229

20. Chaves, I., Pokorny, R., Byrdin, M., Hoang, N., Ritz, T., Brettel, K., Essen, L. O., van der Horst, G. T., Batschauer, A., and Ahmad, M. (2011) The cryptochromes: blue light photoreceptors in plants and animals. *Annu. Rev. Plant Biol.* **62**, 335–364

21. Walsh, C. (1986) Naturally occurring 5-deazaflavin coenzymes: biological redox roles. *Acc. Chem. Res.* **19**, 216–221

22. Isabelle, D., Simpson, D. R., and Daniels, L. (2002) Large-scale production of coenzyme F420–5s by using *Mycobacterium smegmatis*. *Appl. Environ. Microbiol.* **68**, 5750–5755

23. Petersen, J. L., and Small, G. D. (2001) A gene required for the novel activation of a class II DNA photolyase in *Chlamydomonas*. *Nucleic Acids Res.* **29**, 4472–4481

24. Kabsch, W. (1993) Automatic processing of rotation diffraction data from crystals of initially unknown symmetry and cell constants. *J. Appl. Crystallogr.* **26**, 795–800

25. Emsley, P., Lohkamp, B., Scott, W. G., and Cowtan, K. (2010) Features and development of COOT. *Acta Crystallogr. D Biol. Crystallogr.* **66**, 486–501

26. Murshudov, G. N., Vagin, A. A., and Dodson, E. J. (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr. D Biol. Crystallogr.* **53**, 240–255

27. McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C., and Read, R. J. (2007) PHASER crystallographic software. *J. Appl. Crystallogr.* **40**, 658–674

28. Langer, G., Cohen, S. X., Lamzin, V. S., and Perrakis, A. (2008) Automated macromolecular model building for x-ray crystallography using ARP/WARP version 7. *Nat. Protoc.* **3**, 1171–1179

29. Delano, W. L. (2002) *The PyMOL Molecular Graphics System*, Delano Scientific, San Carlos, CA

30. Kort, R., Komori, H., Adachi, S., Miki, K., and Eker, A. (2004) DNA apophotolyase from *Anacystis nidulans*: 1.8 Å structure, 8-HDF reconstitution and x-ray-induced FAD reduction. *Acta Crystallogr. D Biol. Crystallogr.* **60**, 1205–1213

31. Malhotra, K., Kim, S. T., Walsh, C., and Sancar, A. (1992) Roles of FAD and 8-hydroxy-5-deazaflavin chromophores in photoreactivation by *Anacystis nidulans* DNA photolyase. *J. Biol. Chem.* **267**, 15406–15411

32. Graham, D. E., Xu, H., and White, R. H. (2003) Identification of the 7,8-dideimethyl-8-hydroxy-5-deazariboflavin synthase required for coenzyme F(420) biosynthesis. *Arch. Microbiol.* **180**, 455–464

33. Klar, T., Kaiser, G., Hennecke, U., Carell, T., Batschauer, A., and Essen, L. O. (2006) Natural and non-antenna chromophores in the DNA photolyase from *Thermus thermophilus*. *ChemBioChem* **7**, 1798–1806

34. Okafuji, A., Biskup, T., Hitomi, K., Getzoff, E. D., Kaiser, G., Batschauer, A., Bacher, A., Hidema, J., Teranishi, M., Yamamoto, K., Schleicher, E., and Weber, S. (2010) Light-induced activation of class II cyclobutane pyrimidine dimer photolyases. *DNA Repair* **9**, 495–505

35. Chakrabarti, P., and Bhattacharyya, R. (2007) Geometry of nonbonded interactions involving planar groups in proteins. *Progr. Biophys. Mol. Biol.* **95**, 83–137

36. Rausher, M. D. (2006) In *The Science of Flavonoids* (Grotewold, E., ed) pp. 175–211, Springer, New York