Cryptochromes and DNA photolyases are related flavoproteins with flavin adenine dinucleotide as the common cofactor. Whereas photolyases repair DNA lesions caused by UV radiation, cryptochromes generally lack repair activity but act as UV-A/blue light photoreceptors. Two distinct electron transfer (ET) pathways have been identified in DNA photolyases. One pathway uses within its catalytic cycle, light-driven electron transfer from FADH\(^{\text{+}}\) to the DNA lesion and electron back-transfer to semireduced FADH\(^{\text{+}}\) after photoproduct cleavage. This cyclic ET pathway seems to be unique for the photolyase subfamily. The second ET pathway mediates photoreduction of semireduced or fully oxidized FAD via a triad of aromatic residues that is conserved in photolyases and cryptochromes. The 5,10-methenyltetrahydrofolate (5,10-methenylTHF) antenna cofactor in members of the photolyase family is bleached upon light excitation. This process has been described as photodecomposition of 5,10-methenylTHF. We show that photobleaching of 5,10-methenylTHF in Arabidopsis cry3, a member of the cryochrome DASH family, with repair activity for cyclobutane pyrimidine dimer lesions in single-stranded DNA and in Escherichia coli photolyase results from reduction of 5,10-methenylTHF to 5,10-methyleneTHF that requires the intact tryptophan triad. Thus, a third ET pathway exists in members of the photolyase family that remained undiscovered so far.

DNA photolyases and cryptochromes (cry)\(^2\) form a large family of related flavoproteins with DNA repair activity and photoreceptor function, respectively. Members of this protein family were identified in all kingdoms of life and can be grouped in at least nine subclades (1). DNA photolyases repair cytotoxic and mutagenic DNA lesions that are formed during exposure of DNA to UV-B. These DNA lesions are cyclobutane pyrimidine dimers (CPDs) or pyrimidine-pyrimidone (6-4) photoproducts. According to their substrate specificity, DNA photolyases are designated as CPD photolyases or (6-4) photolyases (2). The repair of both types of DNA lesions by photolyase requires the catalytic fully reduced and anionic flavin cofactor FADH\(^{\text{+}}\) that, when photoexcited, injects an electron directly into the DNA lesion (1) as shown in Fig. 1A (electron transfer pathway 1). During extraction from the cell and purification under aerobic conditions the flavin cofactor is usually oxidized to the semireduced and eventually to the fully oxidized form. Reduction of these flavin species to FADH\(^{\text{+}}\) in vitro can be achieved by illumination of the enzyme in the presence of reducing agents such as dithiothreitol or \(\beta\)-mercaptoethanol. This process is named photoactivation (1). Photoactivation in vitro requires photoactivation of the flavin and a triad of redox-active residues in the protein moiety that is highly conserved in DNA photolyases (3, 4) as shown in Fig. 1A (electron transfer pathway 2). These residues are generally tryptophans that allow transport of an electron from the protein surface to the U-shaped flavin cofactor, which is buried within the C-terminal \(\alpha\)-helical domain (5–9). Whether the same mechanism is used by photolyase to photoreduce FAD in vivo is a matter of debate (10). Photoreduction of the flavin cofactor was also observed in cryptochrome blue/UV-A photoreceptors. However, instead of fully reduced flavin, semireduced flavin species (either anionic flavin semiquinone radical or neutral semiquinone radical) accumulate. This form of the photoreceptor is considered as the signaling state (11–14).

A recently discovered subclade of the DNA photolyase/cryptochrome family are DASH cryptochromes, which have members in plants, bacteria, and aquatic animals (6, 15–17). Because DASH cryptochromes were found to lack repair activity for CPDs in double-stranded DNA, they were considered as cryptochrome-type photoreceptors (6, 16). However, it was recently shown that DASH cryptochromes repair CPDs in single-stranded DNA (18) and loop structures of double-stranded DNA (19) and, thus, belong to the CPD photolyase group. In contrast to conventional CPD photolyases, DASH cryptochromes are unable to flip the CPD lesion out of the DNA duplex (7).

Besides the flavin cofactor that is essential for enzymatic activity, DNA photolyases and most likely all cryptochromes contain a second chromophore (1). Like the catalytic flavin, the second chromophore is non-covalently attached to the protein moiety. The majority of DNA photolyases and, as far as studied, the cryptochromes including the DASH-type like cry3 from...
Arabidopsis thaliana contain polyglutamated 5,10-methenyltetrahydrofolate (5,10-methenylTHF) as the second chromophore (1, 12, 17, 20, 21) (see Fig. 1B for folate structures). Several organisms like the cyanobacterium Anacystis nidulans (Synechococcus elongatus) produce deazariboflavins (7,8-didemethyl-8-hydroxy-5-deazariboflavin) and utilize them as second cofactors (22). In photolyases of thermophilic bacteria and Archaea of the genus Sulfolobus, FMN and FAD, respectively, were found as second cofactors (23, 24). The sole function of the second cofactors demonstrated at present is transfer of excitation energy to the catalytic flavin cofactor via a Förster-type mechanism. The crystal structures of DNA photolyases and DASH cryptochromes revealed that the second chromophores are located in a cleft between the N-terminal α/β domain and the C-terminal α domain (7–9). The centroid distances between the catalytic FAD and the second chromophore are in the range of 15–18 Å. The close distances and the angles between the transition dipole moments of the two cofactors are favorable for efficient energy transfer. Indeed, energy transfer efficiencies are about 70% for Escherichia coli photolyase (25), close to 100% for A. nidulans photolyase (26), and between 78% (dark-adapted) and 87% (light-adapted) for Arabidopsis cry3 (27). Although the second cofactors are not essential for catalysis (28, 29), they increase the efficiency of repair and possibly of photoinhibition by having higher extinction coefficients than FADH₂ in the near UV and blue region (30). The spectral overlap between 5,10-methenylTHF emission and the absorption of the different flavin redox states is on the order FADH² > FADOx > FADH⁺ (31).

Illumination in vitro of photolyase that contains fully oxidized or semireduced flavin results in light-induced absorbance changes. The decrease in absorption in the 450–470-nm region reflects a decrease in the amount of fully oxidized FAD concomitant with transient increase in absorption above 500 nm, which indicates the formation of a neutral semiquinone radical. Excitation of the 5,10-methenylTHF antenna chromophore at its absorption peak at 380 nm causes a likewise photoreduction of the catalytic FAD (1, 27, 28, 30, 31). However, irreversible bleaching of the 380-nm peak is observed under high irradiance UV-A or camera flash illumination (28, 30). This irreversible bleaching goes along with release of the folate cofactor from the protein moiety (30) and was named photodecomposition of 5,10-methenylTHF (28). However, the identity of the formed folate species remained unknown (30). In our previous spectroscopic characterization of Arabidopsis cry3, a similar bleaching of the 380-nm peak was observed (27).

Here we show that a third electron transfer pathway exists in photolyase and DASH cryptochrome, where the 5,10-methenylTHF cofactor is photoreduced to 5,10-methylene-THF. Thus, bleaching at 380 nm does not simply reflect destruction but is a specific chemical conversion of the second chromophore.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Flavin adenine dinucleotide disodium salt hydrate (FAD) was purchased from Sigma and was used without further purification. Riboflavin (RF) was purchased from Duchefa Biochemie B.V. RF was used as internal standard for HPLC analysis. (6R,S)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid chloride (5,10-methenylTHF), (6R,S)-5,10-methylene-5,6,7,8-tetrahydrofolic acid calcium salt (5,10-methyleneTHF), (6S)-5,6,7,8-tetrahydrofolic acid (THF), and (6R,S)-5-methyl-5,6,7,8-tetrahydrofolic acid calcium salt (5-methylTHF) were purchased from Schircks Laboratories. They were used as delivered. Chemical structures of folates are shown in Fig. 1B. All folate and flavin stock solutions were prepared immediately before usage, and their concentrations were determined by their molar absorption coefficients: FAD, \( \varepsilon_{450\text{ nm}} = 11,300 \text{ M}^{-1}\text{cm}^{-1} \); RF, \( \varepsilon_{445\text{ nm}} = 12,500 \text{ M}^{-1}\text{cm}^{-1} \); 5,10-methenylTHF, \( \varepsilon_{354\text{ nm}} = 24,900 \text{ M}^{-1}\text{cm}^{-1} \); 5,10-methyleneTHF, \( \varepsilon_{294\text{ nm}} = 25,000 \text{ M}^{-1}\text{cm}^{-1} \); THF, \( \varepsilon_{297\text{ nm}} = 29,100 \text{ M}^{-1}\text{cm}^{-1} \); 5-methylTHF, \( \varepsilon_{290\text{ nm}} = 31,700 \text{ M}^{-1}\text{cm}^{-1} \). β-Nicotinamide adenine dinucleotide phosphate sodium salt (NADP⁺) was purchased from AppliChem. NADP⁺ stock solution was prepared immediately before usage, and the concentration was determined by the molar absorption coefficient of NADP⁺, \( \varepsilon_{260\text{ nm}} = 18,000 \text{ M}^{-1}\text{cm}^{-1} \).

Site-directed Mutagenesis of CRY3—To introduce Trp-356 → Phe (W356F) and Trp-432 → Phe (W432F) substitutions into the CRY3 gene, mutagenesis was performed using the QuikChange® II site-directed mutagenesis kit (Stratagene). As the template, the CRY3 gene (amino acids Met-1—Pro-526; GenBank™ protein accession code 568461) in the pQE-60 vector (Qiagen) was used (16). The following primer sets were used with mutated codons shown in small letters: W356F, 5′-GGGGGGAAGtttAGCCAAGATCAG-3′ and 5′-CTGATCTTTGCTaaaCTTCCCTGAC-3′; 5′-CTGATCTTGCCCTaaaCTTCCCTGAC-3′; W432F, 5′-CTAATTATTGGAAACtttACCATGAGCAGGAGA-3′ and 5′-CTCCTGCTCCATAGGaaGTTTCCATAATTAG-3′. The correctness of the final expression constructs was verified by sequencing.

Expression and Purification of cry3—Expression and purification of proteins were done under red light conditions as described (21). However, the size-exclusion chromatography step was omitted, and the final NaCl concentration was adjusted to 200 mM during the concentrating step on Amicon ultra centrifugal filter concentrator with a 30-kDa cutoff (Millipore).

Spectroscopic Studies—UV-visible absorption spectra of purified cry3 wild type and mutant proteins were recorded using a 2-channel UV-2401 PC spectrophotometer (Shimadzu). For direct comparison of cry3 wild type and mutants, absorption cross-section spectra were calculated (8). Photoreduction of fully oxidized FAD by blue light and UV-A illumination and photobleaching of 5,10-methenylTHF by UV-A treatment in the protein samples were followed by recording difference spectra during illumination at 15°C as already described (8). Interference filters (Schott) were used for monochromatic radiation with blue light (450 ± 6 nm; 50 μmol m⁻² s⁻¹) or UV-A (386 ± 5 nm; 112 μmol m⁻² s⁻¹). Kinetics were plotted for the 450-nm absorption peak of fully oxidized FAD and for the 384-nm absorption peak of 5,10-methenylTHF. The data were fitted by mono-exponential decay curves. Excitation and emission spectra of cofactors released from cry3 wild type and mutants after trichloroacetic acid precipitation of the protein (27) and after trichloroacetic acid treatment of cofactor standards, respectively, were recorded in 50 mM sodium phos-
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phosphate buffer, pH 7.0, containing 200 mM NaCl, 10 mM β-mercaptoethanol, and 10% (v/v) glycerol using an RF-5301 PC spectrofluorophotometer (Shimadzu) in a three-window glass fluorescence cuvette (Hellma GmbH & Co. KG).

Cofactor Determination by HPLC and ESI-MS—The cofactor composition and the ratios of cofactor to protein were determined by HPLC reverse phase chromatography analysis as already described (27). Cofactors were separated and eluted from the column using a continuous linear gradient of methanol (60–80%), and separation was monitored by absorption at 360 nm (5,10-methenylTHF and oxidized flavins), at 450 nm (only oxidized flavins), and at 295 nm (oxidized flavins and all folates except 5,10-methenylTHF). For comparison, 5 nmol of each standard were processed analogously. For ESI-MS analysis cofactor standards (5,10-methenylTHF and 5,10-methylene-THF, both monoglutamated) were separated by liquid chromatography, and mass spectra were recorded online by ESI-MS as described below. A 36 μM E. coli photolyase protein solution was treated with sodium borohydride (final concentration 50 mM) to reduce the polyglutamated 5,10-methenylTHF and release the formed 5-methylTHF (29). The enzyme completely depleted of folate by chromatography on a Sephadex PD-10 column was reconstituted with monoglutamated 5,10-methenylTHF by incubating 10 nmol of the enzyme with a 10-fold molar excess of 5,10-methenylTHF at 10 °C for 75 min. The incorporation of 5,10-methenylTHF into E. coli photolyase was monitored spectroscopically and seen by the increase in absorbance at 380 nm. After removal of unbound 5,10-methenylTHF by repeated concentration and dilution of the protein solution in 500 μl of Vivaspin ultrafiltration spin columns (Sartorius Stedim Biotech), the reconstituted protein solution (43 μM) was used for UV-A (386 ± 5 nm, 120 μmol m⁻² s⁻¹) treatment followed by recording the absorption spectra. For liquid chromatography-MS measurements to 100 μl of the E. coli photolyase protein solutions (concentration, 48 μM for reconstituted dark sample and 43 μM for reconstituted UV-A irradiated sample), 1 ml acetonitrile was added. After centrifugation for 15 min (18,000 × g), the supernatants containing the extracted cofactors were dried by lyophilization. The pellet was redissolved in 50 μl of H₂O and subjected to liquid chromatography-MS analysis. For chromatography, a 125/2 Nucleodur C18ec column (Macherey-Nagel) was utilized with an Agilent 1100 chromatography system (Agilent). The following gradient was applied at a flow rate of 0.2 ml/min and a column temperature of 25 °C with buffer A (H₂O, 0.05% formic acid) and buffer B (acetonitrile, 0.045% formic acid), holding B for 5 min at 2% followed by a linear increase of B to 10% within an additional 5 min and to 30% within 15 min. Finally, buffer B was increased to 95% B within 2 min. Mass spectra were recorded online by ESI-MS using a LTQ-FT mass spectrometer (ThermoFinnigan). High resolution mass spectra were obtained using the ICR detection cell, and MSMS data were generated in the linear ion trap.

Enzymatic Characterization of Folate Species in cry3 and E. coli Photolyase—5,10-MethenylTHF dehydrogenase/5,10-methenylTHF cyclohydrase was purified from E. coli cells (BL21 (DE3)) following established procedures (32). In brief, the enzyme was purified from total soluble protein extracts by protamine sulfate (2%) and ammonium sulfate (35, 55, and 70%) precipitation in 50 mM Tris-HCl, pH 7.5, 50 mM β-mercaptoethanol followed by heparin and anion exchange chromatography in the same buffer system with 1 m KCl for elution. The specificity of the purified enzyme for the two substrates, NADP⁺ and 5,10-methyleneTHF, was tested and is shown in supplemental Fig. S1B. The release of the cofactors from cry3 or E. coli photolyase by trichloroacetic acid precipitation, heat treatment, or incubation of the sample in 20% acetonitrile (as used for reverse phase chromatography analysis) inhibited the enzyme assay. Thus, the cofactors were released from the protein moiety by incubation in imidazole (33), resulting in intact cofactor (supplemental Fig. S1A). Thereto the protein sample was incubated at 4 °C for 5 h in the dark in 50 mM sodium phosphate buffer, pH 7.5, 200 mM NaCl, 10 mM β-mercaptoethanol, and 10% (v/v) glycerol containing 500 mM imidazole. After removal of precipitated proteins by centrifugation (20,000 × g, 4 °C, 10 min) the released cofactors were separated from the protein by a concentrating step on an Amicon ultra centrifugal filter concentrator with 30-kDa cut-off (Millipore) and tested in the enzyme assay. Cofactor standard solutions were treated and analyzed the same way, but the concentrating step was omitted (supplemental Fig. S1C). The conversion of 5,10-methyleneTHF to 5,10-methenylTHF by the dehydrogenase activity was traced spectroscopically by the increase in 340-nm absorbance caused by the formation of NADPH (supplemental Fig. S1D) (34). Unless otherwise stated, the standard enzyme assay mixture contained 0.2 mM NADP⁺, 50 mM sodium phosphate, pH 7.5, 200 mM NaCl, 500 mM imidazole, 10 mM β-mercaptoethanol, and 10% (v/v) glycerol in a final volume of 130 μl. The mixture was prepared without NADP⁺; after the addition of the enzyme solution, the mixture was incubated for 5 min at 20 °C. Afterward, NADP⁺ was added to the mixture to start the reaction. For spectroscopic measurements the reference contained the same enzyme assay mixture of released cofactors or cofactor standards like the measured sample except that no NADP⁺ was added.

Structural Analysis of Photobleached cry3-T5 Cocrystals—cry3 was cocryrstallized with a thymine dimer-comprising oligonucleotide (T-T<->T-T-T) as previously described (19). Bleaching of cry3-T5 cocrystals and recording of fluorescence and UV-visible spectra was achieved at the Cryobench, European Synchrotron Radiation Facility. X-ray data were collected for photobleached cry3 cocrystals at beamline ID4-1, European Synchrotron Radiation Facility and processed and scaled by XDS and XSCALE (35, 36). Refinement of the cry3 structure utilized COOT and REFMAC5 (37) (supplemental Table 1).

RESULTS

Irreversible photobleaching of the 5,10-methenylTHF antenna chromophore has been described before for E. coli DNA photolyase and was named photodecomposition (28, 29). However, the identity of the formed product(s) remained elusive (30). In cry3 we have observed the same bleaching of the 5,10-methenylTHF cofactor when the protein was illuminated with UV-A (27). Two tyrosine residues (Tyr-429, Tyr-423) are positioned between the two cofactors in cry3 (8). The distances of the phenyl rings of Tyr-423 and Tyr-429 from the centroid of
 FIGURE 1. Electron transfer pathways in cry3 and structures of folates. A, indicated are the distances of the tryptophans in the tryptophan triad (Trp-356, -409, -432) of Trp-432 to FADH\(^+\) and of FADH\(^+\) to the 5,10-methenylTHF (MTHF) cofactor in cry3. Shown are also the two established routes of electrons from FADH\(^+\) to the DNA lesion (Route 1) and within the tryptophan triad to FAD (Route 2). The third electron transfer pathway from FADH\(^+\) to 5,10-methenylTHF (Route 3) is the subject of this study. B, structural features of folates and their molecular masses. Polyglutamate molecules have a pteridin and a p-aminobenzoate moiety linked with a glutamate chain with a variable number of glutamic acids. The various THF species differ in their oxidation state of the C1 unit that is attached at the N-5 or N-10 position or form a bridge between both.

the FAD isoalloxine ring are 8.45 and 9.80 Å, respectively. Tyr-423 is within H-bonding distance of the N1 and N8 nitrogen atoms of 5,10-methenylTHF. Trp-409, the central tryptophan of the tryptophan triad, is 7.21 Å away from Tyr-429, and the distance from Tyr-429 to Tyr-423 is 6.44 Å (see Fig. 1A for a schematic illustration of steric order of the residues in cry3). We, therefore, reasoned that the light-induced redox changes of FAD could be coupled to changes in side-chain orientation, protonation, and/or redox state of these two aromatic residues as well as of 5,10-methenylTHF itself (8). To test for a role of the tryptophan triad on photobleaching of 5,10-methenylTHF, we performed site-directed mutagenesis of Trp-356 (at the protein surface) to phenylalanine, which is considered to be redox inactive in the process of FAD photoreduction within DNA photolyases (4, 38). Absorption cross-section spectra indicate that the W356F mutant contains both cofactors, 5,10-methenylTHF and FAD, close to stoichiometric amounts (supplemental Fig. S2A). The strong decrease of absorption in the region between 450 and 470 nm (caused by photoreduction of FAD\(_{ox}\)) seen for wild type cry3 during blue light irradiation (Fig. 2A and C) completely lacks the W356F mutant (Fig. 2, B and C), whose spectra after illumination (dashed curve) and after the same period in the dark (dotted curve) overlap well. In general these results demonstrate an essential role of the tryptophan triad for FAD photoreduction in cry3 in vitro as expected. Illumination with UV-A (386 nm) causes a strong and irreversible decrease in the 380-nm peak in wild type cry3 (Fig. 2, D and F) as described before (27). The 380-nm peak originates mostly from 5,10-methenylTHF (27). The bathochromic shift of the absorption maximum of 5,10-methenylTHF in solution (360 nm) and associated to the protein was found before in E. coli photolyase and cry3 (20, 27). In contrast to wild type, the absorption peak of fully oxidized FAD increases in the W356F mutant upon UV-A treatment, and the final decrease in 380-nm absorption is very small (Fig. 2, E and F). This increase in the amount of FAD\(_{ox}\) in the W356F mutant under UV-A irradiation can be explained by electron donation to 5,10-methenylTHF by residual FAD\(^+\) but a lack of photoreduction of the flavin caused by the interrupted tryptophan triad (see below). It should be noted that the increase of the 450-nm absorption peak of FAD\(_{ox}\) is accompanied with an increase in absorption at 380 nm (\(\epsilon_{450} = 11,140 \text{ M}^{-1} \text{cm}^{-1}\); values for FAD\(_{ox}\) in cry3) and shown for cry3 in supplemental Fig. S2B. The extinction coefficients of FAD\(^+\) and FAD\(^-\) at 380 nm are in the range of 4.49 \times 10^3 \text{ M}^{-1} \text{cm}^{-1}. Had no photobleaching of 5,10-methenylTHF occurred in the W356F mutant under UV-A irradiation, one would have expected some increase in the 380-nm absorption (caused by the formation of FAD\(_{ox}\)) instead of the detectable slight decrease. Thus, the minor change seen in 380-nm absorbance (Fig. 2E) between the original spectrum (solid curve) and the spectrum after UV-A irradiation (dashed curve) signifies less change in the 5,10-methenylTHF content than actually occurred. However, the contribution of 5,10-methenylTHF to the 380-nm absorption is at least 2.8 higher that that of FAD. Taking into account the contribution of the various FAD redox states to absorbance at 380 nm, it is evident that photobleaching of 5,10-methenylTHF under UV-A irradiation is strongly reduced in the W356F mutant compared with wild type. The amount of 5,10-methenylTHF after UV-A irradiation is reduced by 64% for wild type and by 20% for the W356F mutant (Fig. 2F).
Thus, an intact tryptophan triad is required for efficient photobleaching of 5,10-methenylTHF in cry3. Photobleaching of 5,10-methenylTHF could result from stepwise reduction to 5-methylTHF via 5,10-methyleneTHF as observed before with chemical reduction using sodium borohydride (30, 39, 40) (see Fig. 1 for folate structures). Because the photodecomposition products of 5,10-methenylTHF have not been identified unambiguously in either *E. coli* photolyase or any other member of the photolyase/cryptochrome family, we further analyzed the folate cofactors of cry3 and of *E. coli* DNA photolyase before and after photobleaching.

We performed reverse phase chromatography (RPC) of the folates released from cry3 under acidic conditions. To assign the cry3 cofactors after RPC, the standards RF, FAD, 5,10-methenylTHF, 5,10-methyleneTHF, and THF were used (Fig. 3C). The retention times of the cofactors released from the cry3 dark sample are very similar to those of standard FAD and 5,10-methenylTHF (Fig. 3A). In contrast, the UV-A-irradiated sample showed two novel peaks with prominent absorption at 295 nm besides the 5,10-methenylTHF peak. One peak eluted faster (peak 1) and the other one slower (peak 2) than 5,10-methenylTHF (Fig. 3B). Peak 1 can be assigned to THF, whereas peak 2 most likely corresponds to 5,10-methyleneTHF. The shift in retention times between the standard folates (THF, 5,10-methenylTHF, 5,10-methyleneTHF) and the folates released from UV-A-irradiated cry3 (peak 1 and peak 2) is presumably caused by the fact that the standard folates were monoglutamated in contrast to the folates of cry3 that are polyglutamated (data not shown).

In general, the analysis of folates by RPC has the problem of pH-dependent non-enzymatic interconversions of some folate species (41, 42). 5-MethylTHF is relatively stable over a broad range of pH values (42). In contrast, 5,10-methenylTHF is mostly stable under acidic conditions (as used in our experiments), whereas it is converted to 5-formylTHF or 10-formylTHF at neutral and alkaline pH (42). 10-FormylTHF as well as 10-formiminoTHF are cyclized back to 5,10-methenylTHF under acidic conditions (41). 5,10-MethyleneTHF is unstable under acidic conditions and converted to THF and formaldehyde (41). However, this conversion of 5,10-methenyl-
THF can be diminished by the addition of β-mercaptoethanol (39). Indeed, the presence of β-mercaptoethanol causes a reduction of the novel folate peak 1 and an increase of peak 2 (Fig. 3 D) similar to stabilization of the 5,10-methyleneTHF standard by β-mercaptoethanol (Fig. 3 E). Thus, RPC analysis suggests, as the fluorescence excitation and emission spectra shown in supplemental Fig. S3, that cry3 converts 5,10-methenylTHF to 5,10-methyleneTHF under UV-A irradiation. The molecular mass of 5,10-methyleneTHF is 1 Da higher than of 5,10-methenylTHF (see Fig. 1 B). Thus, we used ESI-MS analysis to check for the expected mass change of the folate cofactor after UV-A treatment. MALDI-TOF MS analysis has shown that the folate cofactor in cry3 is polyglutamated with two to seven glutamate residues (data not shown). This polyglutamation strongly complicates the mass determination by ESI-MS. Thus, sodium borohydride treatment of the enzyme was performed to reduce and release the folate and reconstitute it with the monoglutamated 5,10-methenylTHF as described before for E. coli photolyase (29, 30). However, sodium borohydride treatment of cry3 led only to partial release of the folate (data not shown), in contrast to E. coli photolyase that was treated exactly the same (Fig. 4 A). Folate-depleted and reconstituted E. coli photolyase showed the 380-nm peak (Fig. 4 A) typical for protein-bound 5,10-methenylTHF. Therefore, we

FIGURE 3. RPC analysis of cofactors released from cry3. A, elution profile of cofactors released from cry3 dark sample recorded with absorbance at 295 nm (solid curve) and 360 nm (dashed curve). Except RF, no other internal standard was included. B, elution profile of cofactors released from UV-A-treated (386 ± 5 nm, 110 μmol m⁻² s⁻¹, 240 min) cry3 sample recorded with absorbance at 295 nm (solid curve) and 360 nm (dashed curve). RF as internal standard was included. Compared with the dark sample, two novel compounds with strong absorption at 295 nm were detected (Peak 1 and Peak 2). C, for comparison, elution profiles of the individually separated standard cofactors RF, THF, 5,10-methenylTHF, and 5,10-methyleneTHF are shown. All standard folates were monoglutamated. Detection was at 295 nm (solid curve for 5,10-methenylTHF and dotted curve for THF) and 360 nm (dashed curve for 5,10-methyleneTHF). D, effect of β-mercaptoethanol on the cofactors of UV-A-treated cry3 (386 ± 5 nm, 116 μmol m⁻² s⁻¹, 210 min). Cofactors were released and afterward separated by RPC in the presence of 200 mM β-mercaptoethanol. The height of peak 1 decreased coincidental with an increase of peak 2. Detection was done at 295 nm (solid curve) and 360 nm (dashed curve). RF was added as internal standard. E, effect of β-mercaptoethanol on standard 5,10-methyleneTHF. 5,10-MethyleneTHF was preincubated with 200 mM β-mercaptoethanol under the same conditions as in D. RF was added as internal standard. Detection was at 295 nm. For convenience, the peaks were marked with the assigned cofactors in all figure parts. mAU, milliabsorbance units.
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A

![Graph showing absorbance vs. wavelength for sodium borohydride reduced Ec PHR and reconstituted Ec PHR.

B

![Graph showing absorbance vs. wavelength for reconstituted Ec PHR before and after 120 min UV-illumination.

C

![Graph showing overlap with 13C isotopes of 5,10-methenylTHF before UV irradiation.

D

![Graph showing overlap with 13C isotopes of 5,10-methenylTHF after UV irradiation, with extract ion chromatogram of 458 m/z.

E

![Mass spectrum showing m/z 456.1626 (calculated: 456.1626) and 457.1659, 458.1678.

F

![Mass spectrum showing m/z 458.1779 (calculated: 458.1783) and 456.1621, 459.1812, 457.1700, 329.3, 301.2.]
analyzed folates released from E. coli and 5,10-methyleneTHF (data not shown). Therefore, the spectra of standard monoglutamated 5,10-methenylTHF and (Fig. 4) residual detection of 5,10-methenylTHF in the same sample compared with 5,10-methenylTHF is seen in ESI mass spectra. The performed in positive ion mode, a mass difference of 2 Da compared with 5,10-methyleneTHF species before and after UV-A treatment (Fig. 4). Prominent peak at 456.1626 Da (Fig. 4, UV-A-treated sample showed a peak at 456.1626 Da (Fig. 4, methenylTHF, whereas the UV-A-treated sample had a prominent peak at 458.1779 Da (Fig. 4, 5,10-methylene-THF beside 5,10-methenylTHF. Because ESI-MS was performed in positive ion mode, the ionization efficiency of 5,10-methenylTHF is higher than that of 5,10-methyleneTHF. ESI mass spectrum of extracted 5,10-methenylTHF before irradiation (upper panel; see C) and the corresponding gas phase fragmentation data of 5,10-methyleneTHF (lower panel); F, ESI mass spectrum of extracted 5,10-methyleneTHF after irradiation (upper panel; see D) and the corresponding gas phase fragmentation data of 5,10-methyleneTHF (lower panel), which is significantly different from 5,10-methenylTHF.

When cocrystals of cry3 and a T5 oligonucleotide containing a synthetic CPD-like lesion (19) were exposed to UV-A at 180 K, a decrease in absorption at 380 nm together with the accumulation of semiquinoid and probably also fully reduced FAD was observed (Fig. 5A). In agreement with FAD photoreduction, the fluorescence emission signal of fully oxidized FAD at 520 nm progressively decreased upon 355-nm laser excitation (Fig. 5B). The initial strong fluorescence emission of 5,10-methenylTHF at 440–460 nm seen for cry3 in solution is decreased in the crystal. This suggests enhancement of energy transfer from 5,10-methenylTHF to FAD in the crystal that could be caused by the crystalline nature of the probe, the bound substrate, or the low temperature during spectroscopy. Together these data indicate that essentially the same photochemistry takes place in solution as in cry3 crystals; however, with better energy transfer from 5,10-methenylTHF to FAD in the crystal. X-ray diffraction analysis of a single UV-A-treated cry3 cocrystal revealed that the methine bond between N5 of the pteridine moiety and N10 of the para-aminobenzoate moiety remained intact (Fig. 5C, supplemental Table 1). This data set precludes the formation of 5-methylTHF (or any other ring opening process) as a product of 5,10-methenylTHF photobleaching in cry3 and supports our above statement that 5,10-methyleneTHF could have been formed.

In a final test to identify unequivocally the folate species formed in cry3 and E. coli photolyase during UV-A irradiation, we used the enzyme 5,10-methyleneTHF dehydrogenase (EC 1.5.1.5) that converts (6R)-5,10-methyleneTHF to (6S)-5,10-methenylTHF+ according to (Equation 1),

\[
(6R)-5,10\text{-methyleneTHF} + \text{NADP}^+ \rightleftharpoons (6S)-5,10\text{-methenylTHF}^+ + \text{NADPH}
\] (Eq. 1)

FIGURE 5. Absorption (A) and fluorescence emission (B) spectra changes in cry3-T5 cocrystal upon UV-A illumination are shown. A single crystal mounted on a goniometer head of the microspectrophotometer was illuminated with 355 nm of laser light at 180 K for the indicated times. During illumination the crystal was rotated at 36 degrees s\(^{-1}\). C, view on the folate-binding site of photobleached cry3 (ribbon: green). The 5,10-methylene-THF molecule (MTHF, stick model, shown in cyan) is shown together with its SIGMAA-weighted 2F\(_{\text{obs}}\)–F\(_{\text{calc}}\) electron density (contouring level, 1 \sigma). Despite the spectral differences, there is no significant structural difference to non-bleached cry3 comprising 5,10-methenylTHF (yellow).

used E. coli photolyase for the ESI-MS analysis of the folate species before and after UV-A treatment (Fig. 4B). Standard monoglutamated 5,10-methenylTHF showed in ESI-MS a peak at 456.1627 Da (calculated 456.1626) and 5,10-methyleneTHF at 458.1785 Da (calculated: 458.1783) (data not shown). Because of protonation of 5,10-methyleneTHF during ESI-MS performed in positive ion mode, a mass difference of 2 Da compared with 5,10-methenylTHF is seen in ESI mass spectra. The folate released from E. coli photolyase that was not exposed to UV-A-treated sample showed a peak at 456.1626 Da (Fig. 4, C and E), matching exactly the mass of monoglutamated 5,10-methenylTHF, whereas the UV-A-treated sample had a prominent peak at 458.1779 Da (Fig. 4, D and F) that corresponds exactly to the mass of the 5,10-methyleneTHF standard residue detection of 5,10-methenylTHF in the same sample (Fig. 4D). Gas phase fragmentation spectra of the ESI-MS analyzed folates released from E. coli photolyase (Fig. 4, E and F, lower panels) correspond to gas phase fragmentation spectra of standard monoglutamated 5,10-methenylTHF and 5,10-methyleneTHF (data not shown). Therefore, the parasitic CPD-like lesion (19) were

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A, sodium borohydride treatment and reconstitution of E. coli (Ec) photolyase (PHR) with 5,10-methenylTHF. Spectra were recorded from a 20 \(\mu\)M protein sample after sodium borohydride treatment (solid line) and after reconstitution of the reduced sample with 5,10-methenylTHF (dashed line). B, UV-A illumination of reconstituted E. coli DNA photolyase. A reconstituted protein solution (43 \(\mu\)M) was irradiated with UV-A (386 ± 5 nm, 120 \(\mu\)mol m\(^{-2}\) s\(^{-1}\), 10°C) for 120 min and used afterward for ESI-MS analysis. C–F, liquid chromatography-MS data of the extracted cofactors from non-irradiated (48 \(\mu\)M) and irradiated samples (43 \(\mu\)M) are presented. C, extract ion chromatograms of the reconstituted non-irradiated sample showing that only 5,10-methenylTHF is present. D, extract ion chromatogram of the reconstituted irradiated sample (see B) showing 5,10-methylene-THF beside 5,10-methenylTHF. Because ESI-MS was performed in positive ion mode, the ionization efficiency of 5,10-methenylTHF is higher than that of 5,10-methyleneTHF.
The *E. coli* 5,10-methyleneTHF dehydrogenase is a bifunctional enzyme (32) with 5,10-methenylTHF cyclohydrolase (EC 3.5.4.9) activity according to (Equation 2),

\[
(6R)-5,10\text{-methenylTHF} + H_2O \rightleftharpoons (6R)-10\text{-formylTHF} + H^+ \tag{Eq. 2}
\]

The dehydrogenase activity has a pH optimum at 8.5 with a 1:0.17 ratio between dehydrogenase and cyclohydrolase activity (32).

Equation 1 shows that NADPH is formed by 5,10-methylene-THF dehydrogenase activity. The formation of NADPH can be followed spectroscopically by an increase in absorbance at 340 nm (34). When this enzyme assay was performed with cofactors released from dark-adapted cry3, no increase in 340-nm absorption was detected upon the addition of NADP⁺. Likewise and as expected, no increase at 340 nm was observed when standard 5,10-methenyl-THF was present in the reaction mixture. In contrast, standard 5,10-methylene-THF as well as cofactor released from UV-A-irradiated cry3 led to a significant increase in 340 nm absorption (Fig. 6A). These data clearly show that 5,10-methylene-THF is present in UV-A-treated cry3 in contrast to the dark sample.

To exclude the possibility that the lack of NADPH formation seen for the dark cry3 sample resulted from the presence of compounds that inhibit the 5,10-methylene-THF dehydrogenase activity, we added 5,10-methylene-THF to both reaction mixtures. A similar rate of NADPH formation was seen for the cry3 samples kept in darkness or irradiated with UV-A (Fig. 6B) when the reaction mixture was supplemented with 5,10-methylene-THF. This result excludes that compounds in the dark sample inhibited the enzymatic reaction. In line with the above-described formation of NADPH seen specifically in the reaction assay with cofactors from UV-A-treated cry3 are the difference spectra shown in Fig. 6C and 6D. Only the assay mixture that contains the cofactors of UV-A-treated cry3 is the difference spectra before (NADP⁺) and after (+ NADP⁺) the addition of NADP⁺. In C spectra are shown for the UV-A-treated sample and in D for the dark control.

FIGURE 6. Enzymatic identification of the folate species formed in cry3 during UV-A irradiation. Cofactors were released from cry3 samples that were kept in darkness or treated with UV-A (386 ± 5 nm, 113 μmol m⁻² s⁻¹) for 240 min. All samples had the same protein concentrations. Release of cofactors was performed by incubation in 400 mM imidazole in darkness at 4 °C for 5 h (supplemental Fig. S1) followed by concentration of the cofactors. A, cofactors released from UV-A-irradiated cry3 (dashed curve) or dark sample (solid curve) were incubated with 5,10-methyleneTHF dehydrogenase. After 5 min of preincubation, NADP⁺ was added to a final concentration of 0.2 mM, and changes in absorbance at 340 nm were monitored continuously to trace the formation of NADPH. For control, the same reaction was performed with standard 0.1 mM 5,10-methenylTHF (short dashed curve) and 0.1 mM 5,10-methyleneTHF (dotted curve). B, control reactions where 5,10-methyleneTHF was added to a final concentration of 0.09 mM to the reaction mixture of cofactors from the dark control (solid curve) and the UV-A-treated cry3 sample (dashed curve). C and D, difference spectra of reaction mixtures taken before (−NADP⁺) and after (+ NADP⁺) the addition of NADP⁺.
The formed 5,10-methenylTHF is rapidly and non-enzymatically converted to 10-formylTHF ($\epsilon_{300\text{ nm}}$ 11,000 M$^{-1}$cm$^{-1}$, $\epsilon_{258\text{ nm}}$ 22,000 M$^{-1}$cm$^{-1}$) (43). Thus, the spectral changes caused by the actual decrease of 5,10-methyleneTHF are partially hidden by the formed 10-formylTHF and NADPH.

The data shown above demonstrate that cry3 photoreduces 5,10-methenylTHF to 5,10-methyleneTHF, whereas 5,10-methyleneTHF dehydrogenase catalyzes the opposite reaction. The equilibrium of the reaction catalyzed by 5,10-methyleneTHF dehydrogenase can be shifted toward 5,10-methenylTHF and NADPH by removing 5,10-methenylTHF from the equilibrium under high NADP$^+$ ratios. We reasoned that cry3 lacking its folate cofactor could deplete the reaction mixture of 5,10-methenylTHF by incorporating it in its folate binding pocket. To test this hypothesis, we performed coupled enzyme tests with UV-A-treated cry3 in the presence of 5,10-methyleneTHF dehydrogenase and NADP$^+$. Indeed, we observed an increase in the 380-nm peak (Fig. 7A) that is completely missing when 5,10-methyleneTHF dehydrogenase is omitted (Fig. 7B). The recovery of the 380-nm peak depends specifically on both the presence of 5,10-methyleneTHF dehydrogenase and/or NADP$^+$, as expected. Because E. coli 5,10-methyleneTHF dehydrogenase is a bifunctional enzyme that has also cyclohydrolase activity (32, 34), some of the formed 5,10-methenylTHF is probably also converted to 10-formylTHF (see Equation 2). However, the cyclohydrolase activity is much lower than the dehydrogenase activity (32), thus removing only a small fraction of 5,10-methenylTHF from the equilibrium between cry3 and 5,10-methyleneTHF dehydrogenase. Furthermore, cry3 is able to bind 10-formylTHF and cyclize it within its binding site to 5,10-methenylTHF (data not shown), as already reported for E. coli DNA photolyase (30).

As outlined above, irreversible photobleaching of the 5,10-methenylTHF cofactor has been described before for E. coli photolyase (28–30). When comparing UV-A-driven photobleaching of 5,10-methenylTHF in cry3 and E. coli photolyase, we observed that this process is even faster in E. coli photolyase (Fig. 8A). Likewise the photoreduction of FAD under blue light irradiation is faster in E. coli photolyase than in cry3 (Fig. 8B). As shown in Fig. 4 by mass spectrometry, E. coli photolyase forms 5,10-methyleneTHF when treated with UV-A. This result is confirmed by the data shown in Fig. 8C. UV-A-pretreated E. coli photolyase builds up the 380-nm peak when incubated with 5,10-methyleneTHF dehydrogenase in the presence of NADP$^+$. In the absence of 5,10-methyleneTHF dehydrogenase and NADP$^+$, this increase in 380-nm absorbance is not detectable (Fig. 8D). When UV-A treatment of
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(A) UV-A illumination
- cry3
- Ec PHR

(B) blue light illumination
- cry3
- Ec PHR

(C) Absorbance
- original
- UV-illumination
- + dehydrogenase + NADP⁺
  - 270 min dark

(D) Absorbance
- original
- UV-illumination
- 270 min dark

(E) Absorbance
- Ec PHR + dehydrogenase + NADP⁺
  - 0 min
  - 90 min UV-illumination

(F) Absorbance
- cry3 + dehydrogenase + NADP⁺
  - 0 min
  - 240 min UV-illumination

(G) Metabolic pathway
- cry3
- Ec PHR
- UV-A
- 5,10-methenylTHF
- 5,10-methyleneTHF
- 10-formylTHF
- pH
- THF
- NADPH
- NADP⁺

[Diagram showing the metabolic pathway involving photoreduction of folate with UV-A and blue light illumination, highlighting the changes in absorbance with and without dehydrogenase and NADP⁺.]
E. coli photolyase or cry3 is done in the presence of 5,10-methyleneTHF dehydrogenase and NADP⁺, the decrease in 380-nm absorbance is strongly reduced (Fig. 8E) or completely lacking (Fig. 8F), respectively. The continuous increase in absorbance at 340 nm reflects the formation of NDPH. The enzymatic coupling of UV-A-driven 5,10-methyleneTHF formation catalyzed by cry3 or E. coli photolyase and the reverse reaction catalyzed by 5,10-methyleneTHF dehydrogenase is schematically shown in Fig. 8G.

**DISCUSSION**

The mechanisms by which DNA photolyases repair CPDs and the (6–4) photoproducet and well established. Biochemical and structural analyses have proven that the only cofactor in photolyase essential for catalysis is FAD (1, 44). For both CD photolyase and (6–4) photolyase, the flavin cofactor is needed in its fully reduced and anionic form (FAD⁻) to allow single electron transfer from its light-activated form (FADH²) to the UV lesion (25, 45, 46). Cocrystal structures of DNA photolyases with bound substrate show distances between FAD and the UV lesion (25, 46). Cocrystal structures of DNA photolyases with bound substrate show distances between FAD and the UV lesion (25, 46). However, photolyases of most organisms contain riboflavin antenna. In cry3 this energy transfer efficiency was close to 100% for the 382-nm absorbing 5,10-methenylTHF as antenna pigment (1, 2, 44). The energy transfer efficiencies between the antenna chromophore and the catalytic flavin are very high and were determined to be in the range between 70% for E. coli (5,10-methenylTHF antenna) and close to 100% for A. nidulans photolyase (8-hydroxy-5-deazariboflavin antenna). In cry3 this energy transfer efficiency was calculated to be between 78 and 87% (27). Thus, the role of 5,10-methenylTHF as antenna chromophore in photolyase is well established. Former studies on E. coli photolyase have shown that its 5,10-methenylTHF cofactor shows bleaching under high light and camera flash irradiation that is irreversible, in contrast to the photoreduction of the catalytic flavin cofactor that is accompanied by a decrease in absorption in the 450 – 470-nm range (28, 29). This bleaching was considered as photodecomposition of 5,10-methenylTHF as a result of ring opening between N5 of the pteridine and N10 of the p-aminobenzoato moiety concomitant with the release of formaldehyde that is eventually oxidized to formic acid (Ref. 30; see Fig. 1B for folate structures). We have observed the same photobleaching of 5,10-methenylTHF in cry3 (Fig. 2) and confirmed that the light conditions used for these studies were such that the 5,10-methenylTHF chromophore in E. coli photolyase was also bleached and at even higher rates than in cry3 (Fig. 8A).

The conserved tryptophan triad in cry3 is not only required for the photoreduction of FAD but also for efficient photobleaching of 5,10-methenylTHF, as shown in Fig. 2. These observations are in line with previous studies on E. coli photolyase showing essentially no photobleaching of the folate cofactor in the W306F mutant and in the absence of reducing agents (38).

Our spectroscopic, biochemical, mass spectroscopic, and structural analyses together show unequivocally that cry3 and E. coli photolyase form 5,10-methyleneTHF during UV-A irradiation. These data are in conflict with the previously published ones for E. coli photolyase that suggested the release of a C1 unit (formaldehyde or formic acid) in the process of photodecomposition (30). A simple explanation for this discrepancy could be the fact that in the previous study the folate was released from the protein under acidic conditions. This might have caused rapid conversion of 5,10-methyleneTHF to THF and formaldehyde as 5,10-methyleneTHF is known to be unstable under acidic conditions (49). Indeed, we have also detected THF after RPC separation of the chromophores released from UV-A-irradiated cry3 (Fig. 3). However, in the presence of β-mercaptoethanol as stabilizer, we identified 5,10-methyleneTHF using the same RPC separation technique (Fig. 3).

The conversion of 5,10-methenylTHF to 5,10-methyleneTHF requires the transfer of two electrons and one proton. As outlined above, we found that the tryptophan triad, which is conserved essentially in all members of the photolyase/cryptochrome family, organizes the flavin chromophore close enough for efficient electron transfer (Fig. 1) (47, 48).

**FIGURE 8.** Photobleaching of E. coli photolyase and cry3 and 5,10-methyleneTHF dehydrogenase-driven reconstitution of the enzymes with 5,10-methyleneTHF. A, photobleaching of 5,10-methyleneTHF by UV-A treatment. Absorbance changes of cry3 (closed squares) and E. coli (Ec) photolyase (PHR) (open squares) at 382 nm under continuous illumination with UV-A (386 ± 5 nm; 113 μmol m⁻² s⁻¹; 15 °C) were recorded over 180 and 60 min, respectively. Half-lives of the 382-nm absorbing 5,10-methyleneTHF were calculated as described before (8). The protein samples did not contain the same ratios of flavin redox states after purification. Therefore, the protein samples were pre-illuminated with blue light (450 ± 6 nm; 95 μmol m⁻² s⁻¹; 15 °C, 60 min) to reach the full content of fully reduced FAD before UV-A treatment. UV-A illumination led to a decrease in 382 nm absorption with a half-life τ₁/₂ of 63 ± 1 min for cry3 and τ₁/₂ of 7 ± 0.2 min for E. coli photolyase. B, photoreduction of oxidized FAD by blue light illumination. The protein samples were stored at 4 °C in darkness to gain oxidized FAD in the samples before blue light illumination. Absorbance changes of cry3 (closed squares) and E. coli photolyase (open squares) at 450 nm under continuous illumination with blue light (450 ± 6 nm; 95 μmol m⁻² s⁻¹; 15 °C) were recorded over 180 and 210 min, respectively. Half-lives of the 450-nm absorbing flavin species were calculated. Blue light illumination led to a decrease in 450-nm absorbance. With a half-life τ₁/₂ of 36 ± 2 min for cry3 and τ₁/₂ of 27 ± 2 min for E. coli photolyase. C, absorbance changes of UV-A-treated (386 ± 5 nm, 103 μmol m⁻² s⁻¹; 60 min, 10 °C) E. coli photolyase (36 μm) during incubation with 0.24 mM NADP⁺ and 5,10-methyleneTHF dehydrogenase. The increase in absorbance at 380 nm reflects the increase in the amount of E. coli photolyase reconstituted with 5,10-methyleneTHF. D, same as C but without 5,10-methyleneTHF dehydrogenase and NADP⁺. E, UV-A treatment (386 ± 5 nm; 113 μmol m⁻² s⁻¹; 15 °C, 90 min) of E. coli photolyase (12 μm) in the presence of 12 μM 5,10-methyleneTHF dehydrogenase and 0.2 mM NADP⁺. F, UV-A treatment (386 ± 5 nm; 113 μmol m⁻² s⁻¹; 10 °C, 240 min) of cry3 (12 μm) in the presence of 12 μM 5,10-methyleneTHF dehydrogenase and 0.2 mM NADP⁺. The rise in absorbance at 340 nm in E and F (upward arrows) indicates the formation of NDPH, and the decrease at 450 nm and 580 nm (downward arrows) indicates the decrease in FAD₆ and FADH, respectively. G, scheme of the light-driven folate cycle and NDPH formation by coupled reactions of cry3 or E. coli photolyase with 5,10-methyleneTHF dehydrogenase.
chromophore, of FADH

methenylTHF is initiated by photoexcitation of the antenna similar to the wild type (data not shown). THF. The reconstituted enzyme showed photobleaching very but the enzyme could be reconstituted with 5,10-methenyl-

Second, mutation of both tyrosines to phenylalanine in cry3 led point redoxpotential of the 5,10-methenylTHF/5,10-methylene-

reductant with a redox potential that is estimated as low as absorption peak of 5,10-methenylTHF in cry3 (and other pho-

fer pathway 1 onto CPD lesions (redox potential about V (52, 53) and sufficient to inject an electron via electron trans-

(52, 54)) (Fig. 1). This again supports the notion that FADH

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electron transfer to 5,10-methenylTHF during photoreduction

vin-free

the obligatory electron donor for photoreduction of 5,10-

could be stripped from the protein matrix. Consistent with the residual photobleaching of 5,10-methenylTHF in the fla-

photolyase indicates an alternative pathway for electron transfer to 5,10-methenylTHF during photoreduction that is independent of FADH−. We exclude, however, that the two tyrosines (Tyr-423, Tyr-429) that are sandwiched in cry3 between the two chromophores could be involved in such an alternative electron pathway for two reasons; first, these tyrosines are not conserved in E. coli photolyase, although photobleaching in E. coli photolyase is faster than in cry3 (Fig. 8A). Second, mutation of both tyrosines to phenylalanine in cry3 led to the loss of 5,10-methenylTHF binding during purification, but the enzyme could be reconstituted with 5,10-methyl-

THF. The reconstituted enzyme showed photobleaching very similar to the wild type (data not shown).

Likewise it is yet not clear whether photoreduction of 5,10-

methenylTHF is initiated by photoexcitation of the antenna chromophore, of FADH−, or of both chromophores as the absorption peak of 5,10-methenylTHF in cry3 (and other photo-
ylases) is at 380 nm, where FADH− also absorbs. The mid-

point redox potential of the 5,10-methenylTHF/5,10-methylene-

THF couple is −300 mV (50). The estimated redox potential for the FAD/FADp pair is −153 mV for Arabidopsis cry1 and −48 mV for the FADH/FADH− pair in E. coli photolyase (51). Upon photoexcitation, FADH−* represents an extremely powerful reductant with a redox potential that is estimated as low as −2.8 V (52, 53) and sufficient to inject an electron via electron transfer pathway 1 onto CPD lesions (redox potential about −2.2 V (52, 54)) (Fig. 1). This again supports the notion that FADH−* is the most likely electron donor for 5,10-methenylTHF reduc-
tion along pathway 3 (Fig. 1). However, the reduction of 5,10-

methenylTHF to 5,10-methyleneTHF requires a second elec-

tron. The origin of this second electron remains elusive and could be stripped from the protein matrix. Consistent with the model that FADH− donates an electron to 5,10-methenylTHF is our observation that the bleaching of 5,10-methenylTHF in cry3 is decelerated in the presence of T < > T containing singlestranded DNA, the substrate of cry3 (data not shown). In this situation a competition for electron transfer exists where FADH− can donate the electron either to the CPD lesion or to 5,10-methenylTHF. Reduced photobleaching of 5,10-methenyl-

THF in the presence of CPD-containing substrate was also found for E. coli photolyase (31). Together our results demonstr-
strate that cry3 and other photolases are able to photoreduce 5,10-methenylTHF using the photoexcited FADH−* cofactor.

Folate (vitamin B), in particular the THF species 10-formylTHF, 5,10-methenylTHF, 5,10-methyleneTHF, and 5-methylTHF, have a crucial role in the C1 metabolism. They are needed for the formation of formylmethionyl-tRNA, purines, thymidylate, and pantothenate, respectively (55, 56). 5-MethylTHF is the direct precursor of methionine, which can be converted to S-adenosyl methionine (57), the methyl group donor for synthesis of polyamines and of the phytohormone ethylene; S-adenosyl methionine is also required for the methylation of proteins and DNA. Furthermore, the biosynthesis of chlorophylls, lignins, and other compounds depends on S-adenosyl methionine (57). It is intriguing to speculate that cry3 could play a role in the folate pathway by catalyzing the forma-
tion of 5,10-methyleneTHF in a light-dependent step. Indeed, it is known that the synthesis of folates is up-regulated in dividing tissues such as meristems (58) and in developing seedlings that synthesize chlorophylls (59).

Light-induced expression of early enzymes in THF synthesis such as dihydropterin pyrophosphokinase-dihydropterate syn-
thase was described before (58). We have detected a significant up-regulation of the cry3 transcript and protein levels in Arabidopsis seedlings that are controlled by phytochrome A (data not shown). The requirement of light for both enhanced cry3 expression and catalytic activity would fit with a model where cry3 plays a role in seedling development in the transition phase between dark and light.

Considering the redundant pathways by which 5,10-

methyleneTHF can be formed in plants, it is likely that the blocking of one pathway (e.g. by mutation) can be compensated at least in part by the others. There are three established path-

ways for the formation of 5,10-methyleneTHF in plants; that is, via the C1-THF synthase pathway, by glycine decarboxylase, or by serine hydroxymethyltransferase (56). In plants, the C1-THF synthase pathway depends on a bifunctional enzyme with 5,10-
methenylTHF cyclohydrolase (EC 3.5.4.9) and 5,10-methylene-

THF dehydrogenase (EC 1.5.1.5) activity (60) and a 10-formyTHF synthetase (EC 6.3.4.3) (60). C1-THF synthase isoenzymes were found in the cytosol, chloroplasts, and mito-

chondria (61). The mitochondrial glycine decarboxylase mul-
tienzyme system (EC 1.4.7.1, 1.8.1.4, 2.1.2.10) catalyzes the irre-

versible conversion of two glycines into one serine, an important step in photorespiration (62). Serine hydroxymethyl-

transferase (EC 2.1.2.1) catalyzes the reversible conversion of glycine into serine (62). Isoenzymes of serine hydroxymethyl-

transferase are also present in the cytosol, chloroplasts, and mitochondria. The data we present here show that cry3 possi-
bly constitutes a fourth pathway in 5,10-methylene-

THF formation. The unique feature of cry3 is, however, its dependence on light energy for the reduction of 5,10-methenyl-

THF. The extent to which cry3 contributes to the formation of 5,10-methyleneTHF in vivo remains unclear. Based on previous in vitro spectroscopic studies, the quantum efficiency for the photobleaching of 5,10-methenylTHF in cry3 was found to be in the range of 3.7 × 10−5 (27) and, thus, is quite low. Therefore, it is possible that cry3 contributes to 5,10-methyleneTHF forma-
tion only under very high irradiance conditions. Further-
more, under standard conditions the midpoint redox potential of the NADP+/NADPH couple is −320 mV and of the 5,10-
methenylTHF/5,10-methyleneTHF couple is −300 mV (50). Thus, there is an equilibrium between NADPH formation and 5,10-methyleneTHF consumption that depends on the NADP+/NADPH ratio.

Another aspect of our studies is the discovery that the light-
driven formation of 5,10-methyleneTHF by cry3 and E. coli

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photolyase can be coupled with the formation of NADPH in the presence of 5,10-methyleneTHF dehydrogenase (Fig. 8G). Because cry3 is located in chloroplasts and mitochondria (16), where 5,10-methyleneTHF dehydrogenase activity is present, these compartments might be able to form NADPH in a lightdependent way but independent of photosynthesis.

Acknowledgments—We thank D. Bourgeois for support at the cryobench laboratory and synchrotron beamline ID14-1, European Synchrotron Radiation Facility, Grenoble, France and V. Koogle for help in editing the manuscript.

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