Purification and Characterization of a Type III Photolyase from *Caulobacter crescentus*[†]

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ABSTRACT: The photolyase/cryptochrome family is a large family of flavoproteins that encompasses DNA repair proteins, photolyases, and cryptochromes that regulate blue-light-dependent growth and development in plants, and light-dependent and light-independent circadian clock setting in animals. Phylogenetic analysis has revealed a new class of the family, named type III photolyase, which cosegregates with plant cryptochromes. Here we describe the isolation and characterization of a type III photolyase from *Caulobacter crescentus*. Spectroscopic analysis shows that the enzyme contains both the methenyl tetrahydrofolate photoantenna and the FAD catalytic cofactor. Biochemical analysis shows that it is a bona fide photolyase that repairs cyclobutane pyrimidine dimers. Mutation of an active site Trp to Arg disrupts FAD binding with no measurable effect on MTHF binding. Using enzyme preparations that contain either both chromophores or only folate, we were able to determine the efficiency and rate of transfer of energy from MTHF to FAD.

Photolyases are DNA repair enzymes that repair UV-induced DNA lesions, cyclobutane pyrimidine dimers (Pyr<>Pyr), or pyrimidine-pyrimidone photoproducts [(6−4) photoproduct] using light energy as a cosubstrate (*1, 2*). As a rule, photolyase contains two chromophores. One is $FADH^-$ which is the catalytic cofactor and is universal to all photolyases identified to date. The other, which is also called "the second chromophore", is methenyl tetrahydrofolate (MTHF)[1] in most photolyases and 8-hyroxy-5-deazaflavin, FAD, or FMN in a few enzymes. The second chromophore, as a rule, has higher absorption than $FADH^-$ in the near UV−visible range and functions as a photoantenna absorbing light and transferring the excitation energy to $FADH^-$ by fluorescence resonance energy transfer to initiate catalysis. Photolyases are closely related to another group of proteins called cryptochromes. Cryptochromes regulate growth and development in plants and the circadian clock in animals (*3, 4*). The sequence homology among some cryptochromes and photolyases is so high that it is not possible to ascertain by sequence inspection alone whether a newly discovered member of the cryptochrome/photolyase family is a cryptochrome or a photolyase. In the absence of genetic data, a newly discovered member of the family is assigned to the photolyase subgroup if it exhibits repair activity and it is called a cryptochrome if it lacks repair function. This assignment by default sometimes can lead to errors. A class of cryptochrome/photolyase enzymes, called Cry-DASH, that are found in bacteria, plants, and animals was originally called the cryptochrome class because no photolyase activity could be detected in these enzymes by conventional assays (*5, 6*). However, later work revealed that Cry-DASH proteins are in fact photolyases with high specificity for Pyr<>Pyr in single-stranded DNA and are now designated ssDNA photolyase (*7*).

The recent advances in genomics and metagenomics have greatly increased the number of photolyase/cryptochrome family members. A phylogenetic analysis of more than 250 member genes by neighbor joining and maximum parsimony methods grouped these genes into eight major classes (*8*). A simplified phylogenetic tree based on this analysis is shown in Figure 1A. Of these eight classes, the one named class III photolyase (*8*) was of special interest to us because members of this class that were identified in more than 20 bacteria constituted a sister class to plant cryptochromes, raising the possibility that these may be the long sought-after bacterial cryptochromes. However, a survey of the existing data for the bacterial species carrying these genes revealed that at least four of them exhibited biological photoreactivation (*9–11*) and possessed only a single member

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[1] Abbreviations: FAD, flavin adenine dinucleotide; MTHF, methenyl tetrahydrofolate; Cry, cryptochrome; Phr, photolyase; T<>T, cyclobutane thymine dimer; T[6-4]T, pyrimidine (6-4) pyrimidone photoproduct; WT, wild type.

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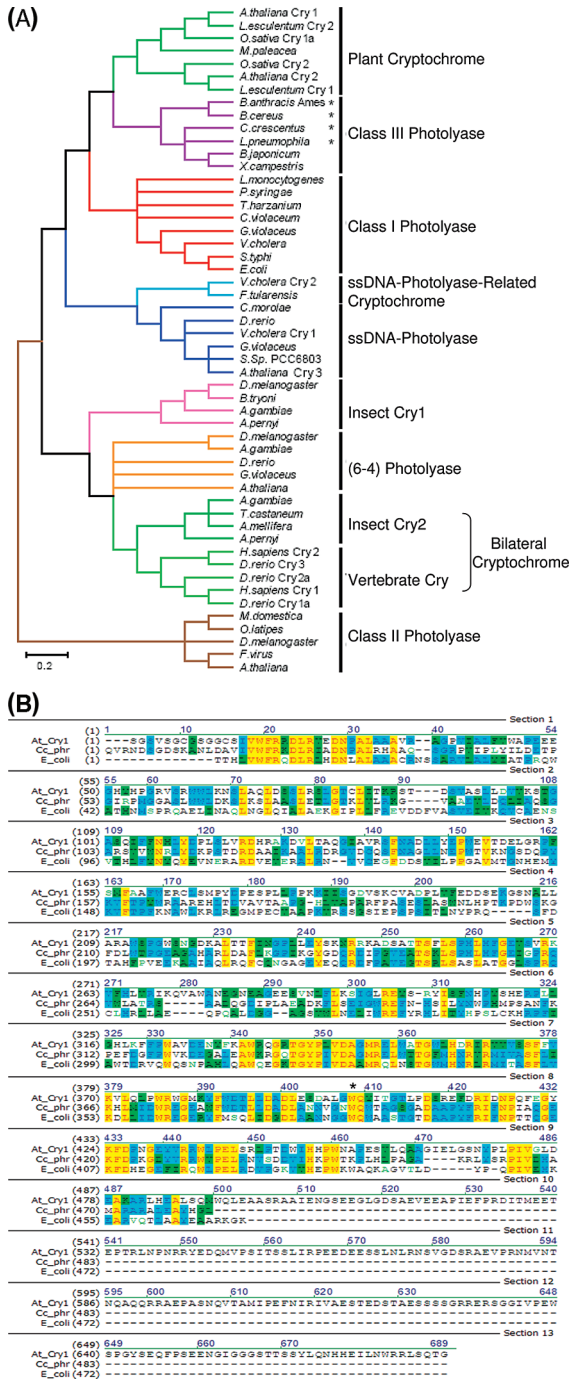

FIGURE 1: (A) Unrooted phylogenetic tree of cryptochrome/photolyase family members generated using neighbor joining methods. Eight major classes are identified, including a novel group, class III (purple), from which plant cryptochromes (green) evolved. Asterisks denote organisms in which biological photoreactivation has been reported. The scale bar represents residue substitution per site. This figure was modified from ref *8*. (B) Sequence alignment of the *C. crescentus* photolyase/cryptochrome member protein (CcPhr) with *E. coli* photolyase (EcPhr) and *Arabidopsis thaliana* Cry1 (AtCry1). The level of sequence identity between CcPhr and EcPhr (38%) is slightly lower than that between CcPhr and AtCry1 (39%). Additionally, the level of sequence identity between EcPhr and AtCry1 is only 30%. Therefore, CcPhr is between EcPhr and AtCry1, but AtCry1 is closer to CcPhr than EcPhr. Sequence alignment was generated using Vector NTI. The color code is as follows: identical residues, red text on yellow background; weakly similar, dark green text on a white background; block of similar, black on a light green background; conservative, dark blue on a light blue background; and nonsimilar, black on a white background.

of the photolyase/cryptochrome family, strongly suggesting that these genes encoded photolyases, though it did not eliminate the possibility that encoded enzymes may have photosensory (cryptochrome) functions as well. For these reasons, we decided to purify and characterize a member of the class III photolyase family. We chose the gene from *Caulobacter crescentus* because this model organism has been extensively studied from genetic and physiological perspectives (*12*) and it has a rather sophisticated sensory system and developmental program. Figure 1B shows that the putative phr protein of *C. crescentus* has a level of sequence identity similar to that of a plant cryptochrome protein and to that of the prototypical prokaryotic photolyase, *Escherichia coli* Phr. Here we describe our purification and characterization of the *C. crescentus* photolyase protein.

MATERIALS AND METHODS

Cloning of Caulobacter Photolyase. Wild-type *C. crescentus* strain CB15 was grown in ATCC Medium 36. The CcPhr gene coding sequence was amplified by PCR from purified genomic DNA. The primers used for amplification were as follows: forward, CAAGGATCCATGCAAGTGCGGAACGACT; and reverse, TTGAAGCTTCTAGAGGCCATGATAGGC. The amplified sequence was cloned into the BamHI and HindIII sites of pMAL-c2 (New England Biolabs). The plasmid construct expresses the photolyase protein fused to the carboxy terminus of the maltose binding protein (MBP). The plasmid isolates were used to sequence the insert in its entirety. The first isolate contained a T → C transition in the first base of codon 395, resulting in the W395R mutation. A second isolate yielded the wild-type gene.

Expression, Purification, and Spectroscopic Analyses of Recombinant Proteins. MBP-tagged wild-type and mutant CcPhr were expressed in *E. coli* UNC523 (phr::kan uvrA::Tn10) and purified by affinity chromatography on amylose resin as described previously (*13*). The presence and stoichiometry of the chromophores were determined by spectroscopic analysis of purified proteins. The concentration of the apoenzyme was determined from absorption at 280 nm using the theoretical extinction coefficient ($\varepsilon_{280} = 1.2 \times 10^5$ M^{-1} cm^{-1}), and the concentration of MTHF was estimated from the absorption of the native enzyme at 387 nm and using an extinction coefficient ($\varepsilon_{387} = 2.5 \times 10^4$ M^{-1} cm^{-1}) (see ref *14*). This calculation gives relatively precise values for the mutant enzyme that lacks FAD; however, it does not take into consideration the contribution of the FAD to absorption at 387 nm, and therefore, the value for the wild-type enzyme can be off by up to 20%. However, because this is the degree of uncertainty in these types of measurements, no correction was made for the contribution of FAD at this wavelength. It should be noted that when the flavin is in the $FADH^-$ form there is no significant contribution to absorption at 387 nm. To determine the FAD concentration, the holoprotein was heated at 95 °C for 5 min in a buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM EDTA, and 1 mM DTT. The denatured protein was removed by centrifugation before the absorption spectrum of released material was recorded. Under these conditions, MTHF is converted to 10-formyl tetrahydrofolate which does not absorb at >300 nm and therefore does not

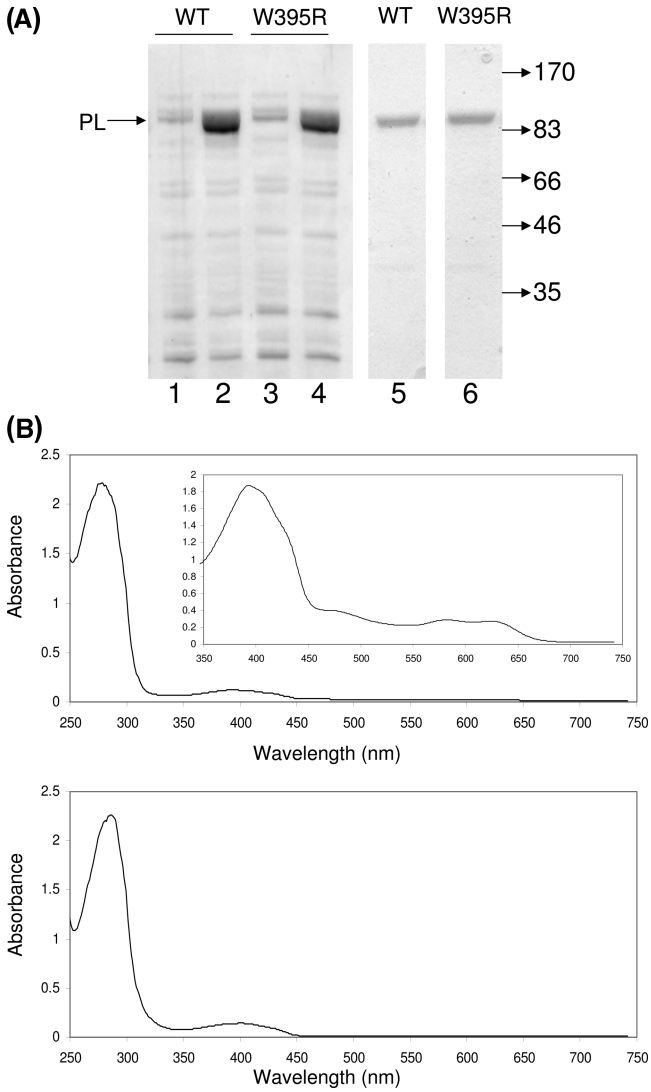

FIGURE 2: Purification and spectroscopic analysis of CcPhr. (A) For purification, CcPhr (WT) and CcPhr (W395R) were expressed in the forms of MBP fusion proteins and purified by affinity chromatography on amylose resin: lane 1, *E. coli* expressing WT CcPhr before induction; lane 2, cells induced by IPTG; lane 3, *E. coli* expressing CcPhr (W395R) before induction; lane 4, cells induced by IPTG (lanes 1−4 contained approximately 200 μg of total protein); lane 5, affinity-purified WT CcPhr; lane 6, affinity-purified CcPhr (W395R) (lanes 5 and 6 contained 5 μg of protein each). The samples were analyzed by SDS−PAGE and Coomassie Blue staining. The mobility of molecular mass size markers, in kilodaltons, is indicated. PL is photolyase. (B) Absorbance spectra of CcPhr (WT) (top panel) and CcPhr (W395R) (bottom panel). The inset in the top panel shows absorption in the 450−650 nm region contributed by FADH° in the enzyme. CcPhr (W395R) shows no detectable absorption in this range (data not shown).

interfere with flavin absorbance in the 400−500 nm range (*14*). The FAD concentration was calculated from the 450 nm absorbance using a molar extinction coefficient (ε_{450}) of 1.13×10^4 M^{-1} cm^{-1}. Absorption spectra were recorded using a Shimadzu UV-1601 spectrophotometer. FAD was also quantitated by fluorescence emission at 520 nm following excitation at 440 nm using a Shimadzu RF5000U spectrofluorimeter. In this case, the concentration of FAD was obtained by interpolation from a standard curve plotting fluorescence intensity versus known amounts of FAD.

Electrophoretic Mobility Shift Assay. Duplexes of 48 bp DNA containing an internal ^{32}P label and either a T<>T or a T[6-4]T in the center were prepared as described previously (*7*). Binding reaction mixtures contained (in 25 μL) 15 mM Tris-HCl (pH 7.5), 20 mM NaCl, 5 mM DTT, 50 μg/mL BSA, 2 nM substrate, and the indicated amounts of enzyme. The reaction mixture was incubated for 30 min on ice, and the protein−DNA complexes were separated on nondenaturing 5% polyacrylamide gels in 0.5× TBE (Tris-Borate-EDTA). The gels were run in the dark at 4 °C for 90 min.

Photorepair Assay. The T<>T and T[6-4]T in the radiolabeled 48 bp DNA substrates are located in an MseI site (TTAA). The photoproducts block digestion by MseI; photorepair regenerates the restriction site. Repair reaction mixtures (20 μL) contained 50 nM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 0.28 nM substrate, and the indicated concentrations of the enzyme. Reaction mixtures were incubated in the dark at 30 °C for 30 min and then exposed to 366 nm light from two black light lamps (F15J8-BLB, General Electric) filtered through a glass plate to cut off light of <300 nm. Irradiation was carried out at a rate of 2 mW/cm^2 for the indicated times. Following photoreactivation, the DNA was extracted with phenol, precipitated with ethanol, and resuspended in 40 μL of buffer containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. To the sample was added 40 units of MseI, and digestion was carried out at 37 °C overnight. Products were analyzed on 8% polyacrylamide sequencing gels.

Image Quantitation. Data from gel shift (binding) and MseI digestion (repair) assays were quantified by densitometry using ImageQuant 5.0 (Molecular Dynamics).

Ultrafast Spectroscopy. Time-resolved fluorescence spectroscopy was carried out using the fluorescence up-conversion method as described previously (*15, 16*).

Phylogenetic Analysis. Protein alignments were performed on the ClustalW server at EBI (www.ebi.ac.uk/clustalw), viewed with ESPript (us.expasy.org), and imported into MEGA 3.0 (www.megasoftware.net) for phylogenetic analysis. Phylogeny was tested by neighbor joining and maximum parsimony methods using 1000 bootstrap replicates to test the reliability of the inferred tree, and nodes with less than 50% bootstrap support were condensed. The sequence alignment was generated by using Vector NTI (Invitrogen).

RESULTS

Purification and Spectroscopic Properties. We amplified the CcPhr gene from wild-type *C. crescentus* strain CB15 grown in ATCC Medium 36 and cloned the gene into the pMal-c2 vector to obtain a construct that expresses CcPhr fused to the C-terminus of maltose binding protein (MBP), which aids in solubilization and purification of the photolyase. During characterization of the cloned Phr, we realized that during amplification and cloning of the CcPhr gene, we had introduced the W395R mutation into the gene. W395 of CcPhr corresponds to W382 of EcPhr which is the proximal Trp residue in the Trp triad for intraprotein electron transfer and is in van der Waals contact with the FAD cofactor in *E. coli* photolyase (*17*). Hence, we decided to purify both the wild-type and mutant proteins to investigate the contribution of this residue in CcPhr structure and function.

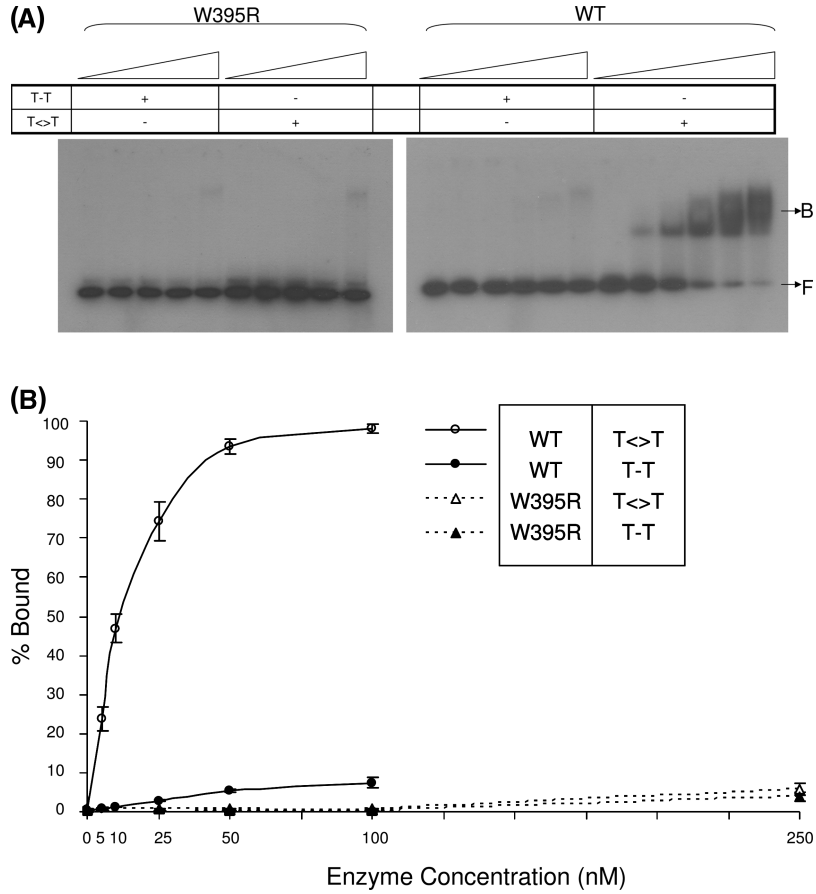

FIGURE 3: Binding of CcPhr (W395R) and CcPhr (WT) to undamaged DNA and a T<>T-containing duplex. The radiolabeled 48 bp duplex (2 nM) was incubated with increasing amounts of enzyme, and products were separated via 5% PAGE: T-T, undamaged duplex; T<>T, duplex with a centrally located cyclobutane thymine dimer. The enzyme concentrations were 0, 25, 50, 100, and 250 nM for the mutant and 0, 5, 10, 25, 50, and 100 nM for the wild-type enzyme. (A) Representative autoradiogram of the gel mobility shift assays. (B) Quantitative analysis of the gel mobility shift assays. Averages of the three experiments are plotted. The error bars are smaller than some of the data point symbols.

The MBP-tagged proteins were expressed in UNC523 (*uvrA*$^-$ *phr*$^-$) and purified to near homogeneity by affinity chromatography on amylose affinity resin (Figure 2A). Because CcPhr represented a novel class of the cryptochrome/photolyase family, we had no a priori assumption about what type of a second chromophore it may possess. To find out, we analyzed the protein by absorption and fluorescence spectroscopy. Figure 2B shows the absorption spectra of both wild-type CcPhr and mutant CcPhr (W395R). Both enzymes exhibit 280 nm peaks of the apoenzyme and 390 nm peaks typical of MTHF. In addition, the wild-type enzyme shows absorption peaks at 580 and 625 nm typical of FADH° flavin neutral radical observed in *E. coli* photolyase and numerous other photolyases (*1*). Thus, we concluded that CcPhr contains MTHF and FADH$^-$ as cofactors and the FADH$^-$ is oxidized to FADH° during purification which has been observed in most photolyases with the rare exception of the *Saccharomyces cerevisiae* enzyme (*1*). Furthermore, from the absorption values at 280, 380, and 580 nm and the known extinction coefficients of the apoenzyme, MTHF, and FADH°, we determined that both chromophores are present in the holoenzyme in essentially stoichiometric amounts. We find this rather interesting because in the majority of MTHF-containing photolyases, this chromophore is bound weakly and a significant fraction of it dissociates from the enzyme during purification. Of more interest was the absorption spectrum of the CcPhr (W395R) mutant (Figure 2B, bottom panel). The mutant contains stoichiometric MTHF but no detectable flavin as evidenced by the absorption spectrum of the native enzyme. We reasoned that the flavin might be in its reduced state in the mutant, in which case its absorption would be obscured by the much higher absorption of MTHF in the 300−400 nm range. Therefore, we heat denatured the enzyme at neutral pH and measured absorption. At neutral pH, the methenyl ring of the released MTHF is hydrolyzed and the product, 10-methyl tetrahydrofolate, no longer absorbs at wavelengths above 300 nm, and thus, any absorption in the 400−700 nm range would be due to oxidized flavin. Even under this condition, we did not detect any flavin (<1% of the stoichiometry relative to that of the apoenzyme). Furthermore, fluorescence was measured to detect any flavin present in the soluble material obtained from the denatured portion of the CcPhr (W395R) protein. The amount of flavin detected was 0.2% of the enzyme. The flavin detected is assumed to be a contaminant, not specifically bound to enzyme, because we found that the same amount of flavin, 0.2%, was obtained from MBP prepared in the same manner. Thus, we conclude that the W395R mutation compromised FAD binding of CcPhr without significantly affecting the overall conformation of photolyase as evidenced by the fact that the mutant enzyme has an MTHF absorption spectrum indistinguishable

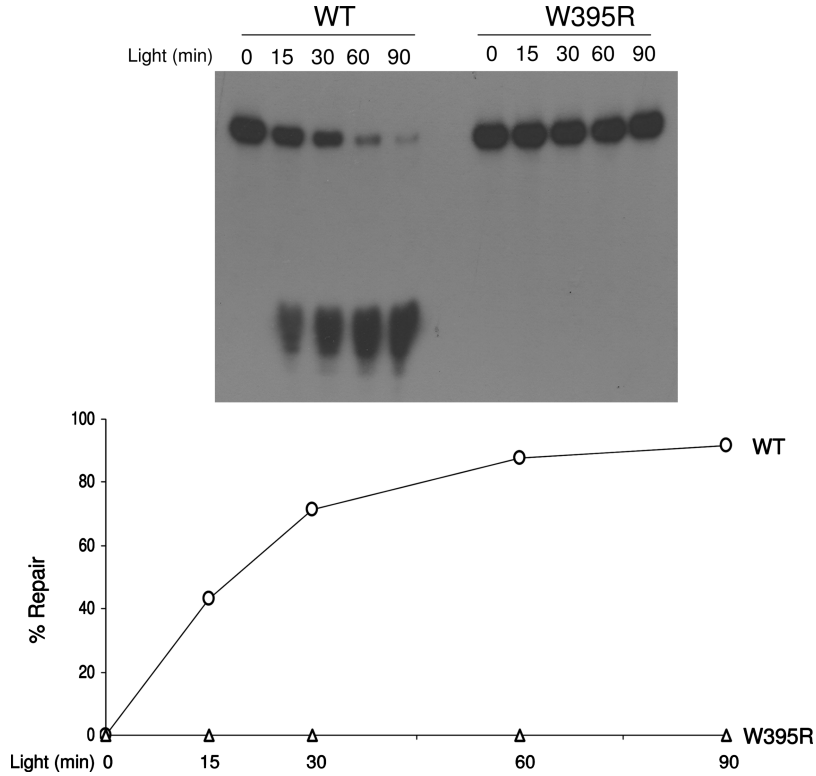

FIGURE 4: Repair of the cyclobutane thymine dimer by CcPhr. A 48 bp duplex (0.28 nM) with a T<>T in the TTAA sequence (MseI recognition site) in the center was mixed with either 50 nM CcPhr (WT) or 2 μM CcPhr (W395R) and exposed to 366 nm at a rate of 2 mW/cm^2 for the indicated times. Then, the DNA was extracted, treated with MseI, and separated on an 8% polyacrylamide gel. The top panel shows an autoradiogram of the gel. The bottom panel shows a quantitative analysis of the data. There was no detectable repair by CcPhr (W395R).

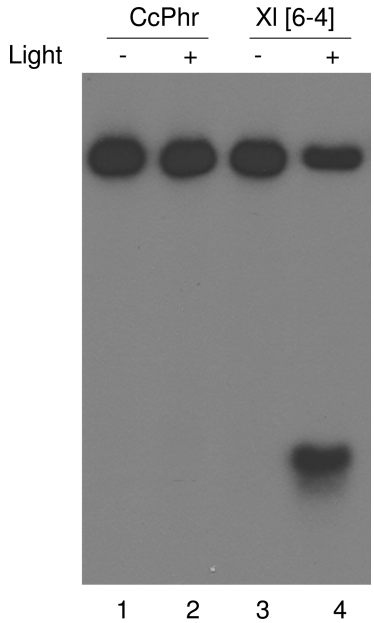

FIGURE 5: Lack of repair of the (6-4) photoproduct by CcPhr. A 48 bp duplex with a T[6-4]T in the TTAA sequence in the middle of the radiolabeled strand was mixed with either 1 μM CcPhr or 50 nM *Xenopus laevis* (6-4) photolyase and irradiated with 366 nm at 2 mW/cm^2 for 90 min. The samples were deproteinized by phenol extraction and digested with MseI and separated on an 8% PAGE gel. An autoradiogram of the gel is shown. Quantitative analysis of the data revealed no repair by CcPhr and 55% repair by *X. laevis* (6-4) photolyase.

from that of the wild type in the 240−420 nm range. As noted above, the W395 equivalent in *E. coli* photolyase makes van der Waals contacts with the flavin and replacement of this residue with another aromatic residue such as F is still compatible with FAD binding, but a charged residue such as R may directly clash with the isoalloxazine ring to interfere with flavin binding or alternatively may change the overall structure of the FAD binding cavity so that the cofactor no longer fits in the active site.

DNA Binding and Repair Properties of Wild-Type and Mutant Enzymes. It has been reported that photolyase that lacks FAD has drastically reduced DNA binding activity and loses its specificity for damaged DNA (*18*). However, these data were obtained with denatured/refolded enzyme; moreover, the refolded enzyme lacked the folate cofactor, and therefore, the previous data had certain limitations. The availability of photolyase in its native conformation and with a full complement of MTHF and no FAD provided an opportunity to test the role of flavin in DNA binding and repair under more appropriate conditions.

The results of binding experiments are shown in Figure 3. The W395R mutant has essentially lost its DNA binding activity, and the residual activity that remains cannot discriminate between damaged and undamaged DNA. In contrast, the wild-type enzyme binds to a 48 bp duplex with a T<>T with high affinity ($K_D \sim 10^{-8}$ M) and specificity as evidenced by the fact that under our experimental conditions there was only marginal binding to undamaged DNA ($K_D > 10^{-4}$ M). To recapitulate, the MTHF cofactor does not affect substrate binding, whereas FAD is essential for binding with high selectivity and specificity. In agreement with the binding data, when the wild-type and mutant enzymes were tested for repair, the wild type repaired nearly

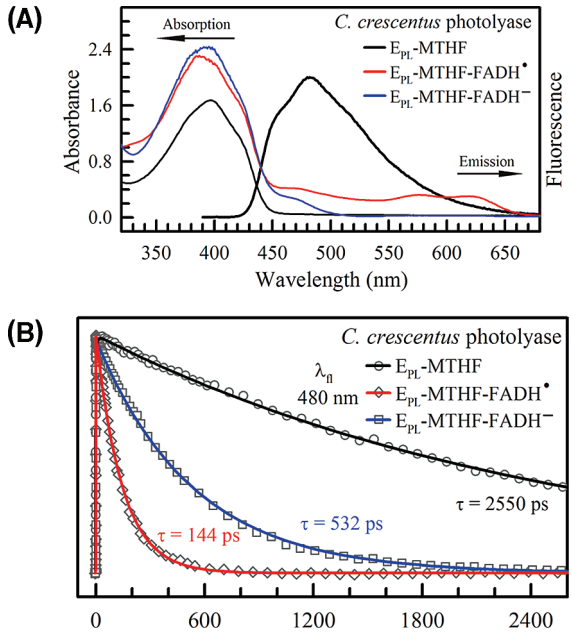

FIGURE 6: Ultrafast dynamics of CcPhr. (A) Absorption and emission spectra of the enzymes used in the ultrafast fluorescence and spectroscopy. The absorption spectra of CcPhr in semiquinone (red) and hydroquinone (blue) forms and the absorption and emission spectra of CcPhr (W395R) (black) are shown. (B) Femtosecond-resolved fluorescence transients of (○) CcPhr (W395R) and of CcPhr containing (◇) FADH° or (□) $FADH^-$ are shown. The transients were gated at 480 nm. The lifetimes of decay dynamics for the various forms are indicated in the figure. The dynamics of both the E_{PL}−MTHF−FADH° and E_{PL}−MTHF−$FADH^-$ forms of the wild-type enzyme contained a small amount (5−25%) of a long component which was ascribed to the enzyme with MTHF and no flavin. This component has been omitted for the sake of clarity.

all of the T<>T whereas the mutant failed to repair the DNA under identical reaction conditions (Figure 4). In fact, even with micromolar concentrations of the mutant enzyme, we were unable to detect any repair. These data confirm the conventional view of the photolyase action mechanism which posits that the sole function of MTHF is to gather light and that FAD is essential both for substrate binding with high specificity and for catalysis.

Phylogenetic analysis of CcPhr places it in a clade closer to plant cryptochromes than any other clade of the phylogenetic tree. As some (6-4) photolyases exhibit a higher degree of sequence homology to cryptochromes than other members of the photolyase/cryptochrome family, we tested CcPhr for (6-4) photolyase activity as well and detected none (Figure 5). Thus, these results identify CcPhr as a folate-type photolyase with specificity for the cyclobutane pyrimidine dimer.

Ultrafast Spectroscopic Analysis of CcPhr. A critical parameter in the efficiency of DNA repair by photolyase is the efficiency with which MTHF transfers energy to flavin. To quantify energy transfer efficiency, it is necessary to know the MTHF lifetime in photolyase, both in the absence and in the presence of flavin. Such experiments were previously conducted with *E. coli* photolyase that was dissociated from its cofactor by mild denaturation and then reconstituted with synthetic MTHF under renaturing conditions (*19*). While the values obtained in that report have been used as reference values in subsequent studies, they have certain limitations arising from the fact that the apoenzyme was unfolded and refolded and that the folate used in reconstitution did not have the oligoglutamate side chain found in the native enzyme (*14, 20*).

The availability of photolyase with a point mutation that causes the release of FAD without causing a major conformational change and without affecting the binding of MTHF enabled us to address the issue under more physiologically relevant conditions.

Figure 6 shows fluorescence transients of CcPhr and CcPhr (W395R) enzymes, representing the fluorescence dynamics of MTHF in the presence and absence of flavin, respectively. In the absence of flavin, MTHF* has a lifetime of 2550 ps. In the presence of FADH°, the folate singlet-state lifetime is 144 ps, and in the presence of $FADH^-$, the MTHF* lifetime is 532 ps. From these values and using the formula

$$\Phi_{ET} = 1 - \tau_2/\tau_1$$

(where Φ_{ET} is the quantum yield of energy transfer, τ_2 the lifetime of MTHF* in the presence of flavin, and τ_1 the lifetime of MTHF* in the absence of flavin), the following quantum yields of energy transfer are calculated: Φ(MTHF*→FADH°) = 0.94 and Φ(MTHF*→$FADH^-$) = 0.79. These values are considerably different from those obtained with the reconstituted EcPhr where τ(MTHF*) = 350 ps and τ(MTHF*) = 140 ps in the absence of flavin and in the presence of $FADH^-$, respectively (*19*). From the latter value, a Φ(MTHF*→$FADH^-$) of 0.62 was calculated. Even though it is possible that these differences may reflect intrinsic differences between the two folate-type photolyases, we believe the value obtained with CcPhr (0.79) is more likely to be applicable to all folate-type photolyases for the following reason. The quantum yield of DNA repair by photolyase is equal to the product of quantum yield of energy transfer from folate to flavin times the quantum yield of electron transfer from flavin to Pyr<>Pyr times the efficiency of splitting of the pyrimidine dimer anion. As a consequence, the efficiencies of the various steps in the pathway cannot be lower than the overall quantum yield of repair. The quantum yield of repair in folate-type photolyases is 0.70−0.75 (*13, 20*). Therefore, we conclude that the MTHF* → $FADH^-$ energy transfer efficiency we obtained in this paper with CcPhr (Φ = 0.79) is more likely to reflect the actual efficiency of energy transfer in this class of enzymes and that the lower value obtained with the reconstituted *E. coli* enzyme reflects the limitations of reconstitution experiments. This statement must be qualified, however, as the crystal structure of photolyase shows FAD bonding to 14 amino acids (*17*) and the absence of these interactions is likely to have some effect on the enzyme structure, at least locally at the flavin binding site. This limitation notwithstanding, we believe the overall enzyme conformation is unperturbed as evidenced by the identical shapes of the MTHF absorption in the wild-type and mutant enzymes, and the fact that the energy transfer efficiency we report in this study is closer to the actual value.

CONCLUSIONS

The data presented in this paper allow us to reach the following conclusions. First, a phylogenetic clade of the photolyase/cryptochrome family that segregates with plant

cryptochromes is in fact a clade of cyclobutane pyrimidine dimer-repairing photolyases. Therefore, this clade has been named "class III photolyase". Second, the fortuitous introduction of a W → R mutation into the FAD binding site of a type III photolyase led to the discovery that this replacement reduces the affinity of the enzyme for FAD so drastically that the purified enzyme contains no FAD within our detection limit (<0.1%) and no photorepair activity. Because this residue is conserved in all photolyase/cryptochrome family members, we predict that the corresponding mutations would result in production of FAD-lacking apoenzymes of the other members of the family as well. Such a mutation would be useful for structural and functional characterizations of the family members. Third, within the limits of our tests, it appears that the lack of FAD in the binding site cavity does not affect the affinity of MTHF for the apoenzyme. Conversely, it has been previously shown that the lack of the second chromophore does not affect the binding and function of flavin (*20, 21*), reinforcing the view that even though two chromophores are functionally coupled they are structurally independent. Fourth, the availability of an otherwise native photolyase lacking FAD but containing MTHF enabled us to demonstrate that FAD is essential for specific binding of the enzyme to DNA with a Pyr<>Pyr and that MTHF does not contribute to the binding specificity. Finally, we were able to obtain photolyase with both chromophores and photolyase with only MTHF [the CcPhr (W395R) mutant]. These forms of the enzyme made it possible to obtain the most reliable values to date for interchromophore energy transfer.

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