# Purification and Characterization of a Type III Photolyase from Caulobacter crescentus †

**ARTICLE** *in* BIOCHEMISTRY · OCTOBER 2008

Impact Factor: 3.02 · DOI: 10.1021/bi801085a · Source: PubMed

Published in final edited form as:
*Biochemistry*. 2008 September 30; 47(39): 10255–10261. doi:10.1021/bi801085a.

# Purification and Characterization of a Type III Photolyase from Caulobacter crescentus

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## Abstract

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 branch of the family which co-segregates with plant cryptochromes. Here we describe the isolation and characterization of a member of this family named 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 or both chromophores we were able to determine the efficiency and rate of energy transfer from MTHF to FAD. 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 branch of the family which co-segregates with plant cryptochromes. Here we describe the isolation and characterization of a member of this family named 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 or both chromophores we were able to determine the efficiency and rate of energy transfer from MTHF to FAD.

## INTRODUCTION

Photolyases are DNA repair enzymes that repair UV-induced DNA lesions, cyclobutane pyrimidine dimers (Pyr<>Pyr) or (6-4) pyrimidine-pyrimidine photoproducts [(6-4) photoproduct] using light energy as a co-substrate (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 methenyltetrahydrofolate (MTHF) 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 and functions as a photoantenna absorbing light and transferring

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the excitation energy to $FADH^-$ by fluorescence resonance energy transfer to initiate catalysis. Photolyases are closely related to another group of proteins called cryptochrome. Cryptochromes regulate growth and development in plants and circadian clock in animals (3, 4). The sequence homology among some cryptochromes and photolyases is so high that, often, by sequence inspection it is not possible to ascertain 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 sub-group 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 were originally called cryptochromes 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 photolyases/cryptochrome family members. A phylogenetic analysis of over 250 member genes by neighbor joining and maximum parsimony methods grouped these genes into 8 major classes (8). A simplified phylogenetic tree based on this analysis is shown in Figure 1A. Of these 8 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 maybe the long sought-after bacterial cryptochromes. However, a survey of the existing data on the bacterial species carrying these genes revealed that at least 4 of them exhibited biological photoreactivation (9-11) and possessed only a single member of the photolyase/cryptochrome family, strongly suggesting that these genes encoded photolyases, though it did not eliminate the possibility that encoded enzymes may be have photosensory (cryptochrome) functions as well. For these reasons we decided to purify and characterize a member of Class III photolyase. We chose the gene from *Caulobacter crescentus* because this model organism has been extensively studied from genetic and physiological perspectives (12). It has a rather sophisticated sensory system and developmental programs. Figure 1B shows that the putative *phr* gene of C. crescentus has higher level of sequence identity to plant cryptochrome gene than to the prototype prokaryotic photolyase, *E. coli phr*. Here we describe our purification and characterization of the *C. crescentus* photolyase gene.

## 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 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→A transversion in the first base of codon 396 resulting in the W396R mutation. A second PCR 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 of $\varepsilon_{280}=1.2\times10^5\ M^{-1}cm^{-1}$ and the concentration of MTHF was estimated from the absorption of the native enzyme at 387 nm and using an extinction coefficient of $\varepsilon_{387}=25{,}000\ M^{-1}cm^{-1}$ (see 14). To determine the FAD concentration the holoprotein was heated at 95°C for 5 min in a buffer containing 50 mM Tris-HCl, ph7.5, 50 mM NaCl, 5 mM EDTA and 1 mM DTT. The denatured protein was removed by centrifugation before recording the absorption spectrum of released material. Under these conditions MTHF is converted to 10-methyltetrahydrofolate that does not absorb at $\lambda>300$ nm and therefore does not interfere with flavin absorbance in the 300-500 nm range (14). The FAD concentration was calculated from 450 absorbance using a molar extinction coefficient of $\varepsilon_{450}=11{,}300\ M^{-1}cm^{-1}$. Absorption spectra were recorded using a Shimadzu UV-1601 spectrophotometer.

### Electrophoretic mobility shift assay

Duplexes of 48-bp containing an internal P-32 label and either a T<>T or a T[6-4] in the center were prepared as described previously (7). Binding reactions contained (in 25 μl) 15 mM Tris-HCl, pH7.5, 20 mM NaCl, 5 mM DTT, 50 μg/ml BSA, and 2 nM substrate, and the indicated amounts of enzyme. The reaction mixture was incubated 30 min on ice and the protein-DNA complexes were separated on non-denaturing 5% polyacrylamide gels in 0.5X TBE (Tris-Borate-EDTA). The gels were run in dark and at 4°C for 90 min.

### Photorepair Assay

The reaction mixture (20 μl) contained 50 nM Tris-HCl, pH7.5, 100 mM NaCl, 1mM EDTA, 10 mM DTT, 0.28 nM substrate, and the indicated concentrations of enzyme. Reaction mixtures were incubated in 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 <300 nm. Irradiation was at a rate of $2\ mW.cm^{-2}$ for the indicated times. Following photoreactivation the DNA was extracted with phenol, precipitated with ethanol, resuspended in 40 μl of buffer containing 10 mM Tris-HCl, pH7.9, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT. To the sample 40 units of MseI were added 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 software (Molecular Dynamics).

### Ultrafast Spectroscopy

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

## 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 W396R mutation in the gene. W396 of Ccphr corresponds to W382 of EcPhr which is the proximal trp residue in the Trp triad for intra-protein electron transfer and is in van der Waals contact with the FAD

cofactor (17). Hence, we decided to purify both the wild-type and mutant proteins in order to investigate the contribution of this residue in CcPhr structure-function.

The MBP-tagged proteins were expressed in UNC523 (*recA*$^-$ *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 opinion as to 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 of CcPhr (W396R). 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 nm and 625 nm peaks 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 as has been observed in most photolyases with the rare exception of the *S. cerevisiae* enzyme (1). Furthermore, from the absorption values at 280 nm, 380 nm, and 580 nm and the known extinction coefficients of the apoenzyme, MTHF, and FADH° we calculate 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 significant fraction of it dissociates from the enzyme during purification. Of more interest was the absorption spectrum of the CcPhr (W396R) 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 reduced 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 denatured the enzyme at neutral pH and measured absorption. At neutral pH the methenyl ring of MTHF is hydrolyzed and the product 5-methyltetrahydrofolate no longer absorbs at $\lambda > 300$ nm and thus any absorption in the 300-700 nm range would be due to oxidized flavin. Even under this condition we did not detect any flavin (<1% of stoichiometry relative to apoenzyme). Thus we conclude that W396R 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 from the wild-type in 240-420 nm range.

## 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 and 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 W396R 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 all of the T<>T whereas the mutant failed to repair the DNA under identical reaction conditions (Figure 4). In fact, even with micromolar concentration of the mutant enzyme we were unable to detect any repair. These data confirm the conventional view of 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 higher 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 cyclobutane pyridine 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 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 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 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 (W396R) enzymes, representing the fluorescence dynamics of MTHF in the presence and absence of flavin, respectively. In the absence of flavin MTHF* has a life time of 2550 ps. In the presence of FADH° the folate singlet-state life time is 144 ps and in the presence of $FADH^-$ the MTHF* life time is 532 ps. From these values and using the formula

$$\Phi_{ET}=1-\tau 2/\tau 1$$

(where $\Phi_{ET}$=quantum yield of energy transfer, τ2=life time of MTHF* in the presence of flavin and τ1=life time of MTHF* in the absence of flavin) quantum yields of energy transfer of Φ(MTHF*→ FADH°)=0.94 and $\Phi(MTHF^*\rightarrow FADH^-)=0.79$ are calculated. These values are considerably different from those obtained with the reconstituted EcPhr in which τ(MTHF*) =350 ps and τ(MTHF*)=140 ps were obtained in the absence of flavin and in the presence of FADH-, respectively (19). From the latter value $\Phi(MTHF^*\rightarrow FADH^-)=0.62$ was calculated. Even though it is possible that these differences may reflect intrinsic differences between 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 to 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 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 $MTHF^*\rightarrow FADH^-$ 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 reconstituted *E. coli* enzyme reflects the limitations of reconstitution experiments.

## CONCLUSIONS

The data presented in this paper allow us to make the following conclusions. First, a phylogenetic clade of the photolyase/cryptochrome family that segregates with plant cryptochromes is in fact a clade of cyclobutane photolyase. Therefore, this clade has been named “Class III photolyase”. Second, the fortuitous introduction of a W→R mutation in the FAD binding site of a Type III photolyase led to the discovery that this replacement reduces the affinity of the enzyme to FAD so drastically such 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 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 to 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, photolyase with only MTHF [(the CcPhrW396R) mutant], and photolyase with only FAD and no MTHF (by selective photodecomposition of folate in the haloenzyme). These forms of the enzyme made it possible to obtain the most reliable values to date for interchromophore energy transfer between the two chromophores in enzyme containing the flavin in various redox states.

## ACKNOWLEDGMENTS

We thank Lucy Shapiro (Stanford University) for advice on *Caulobacter crescentus* biology. This work was supported by NIH grants GM31082 and GM74813.

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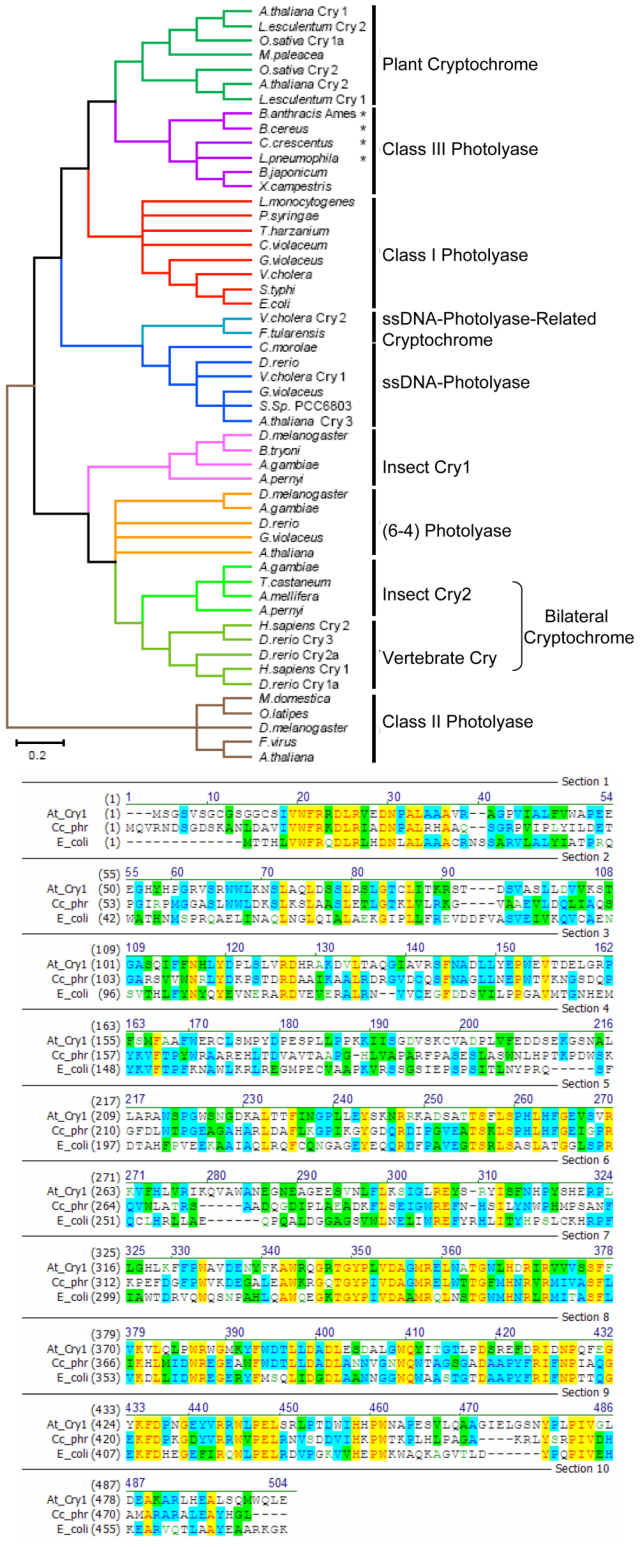
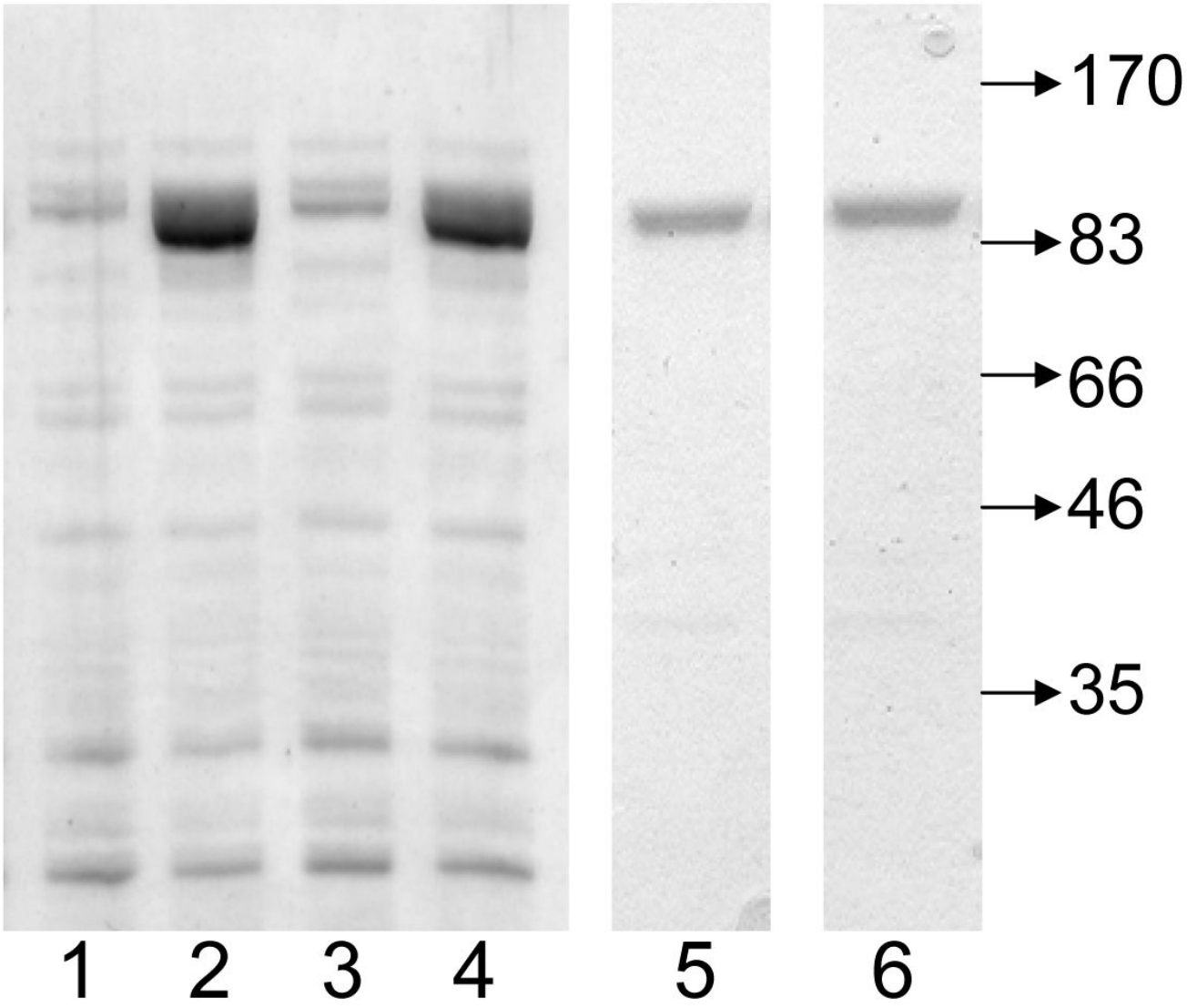



**Figure 1.**

(**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 indicate organisms in which biological photoreactivation has been reported. Scale bar represents residue substitution per site. (**B**) Sequence alignment of the *C. crescentus* photolyase/cryptochrome member protein (CcPhr) with E. coli photolyase (EcPhr) and A. thaliana Cry1 (AtCry1). Note that CcPhr exhibits higher sequence identity to AtCry1 (39%) than to EcPhr (30%). The C-terminal extension of AtCry1 beyond the photolyase homology region (PHR) is not shown. Sequence alignment was generated using the Vector NTI software program.



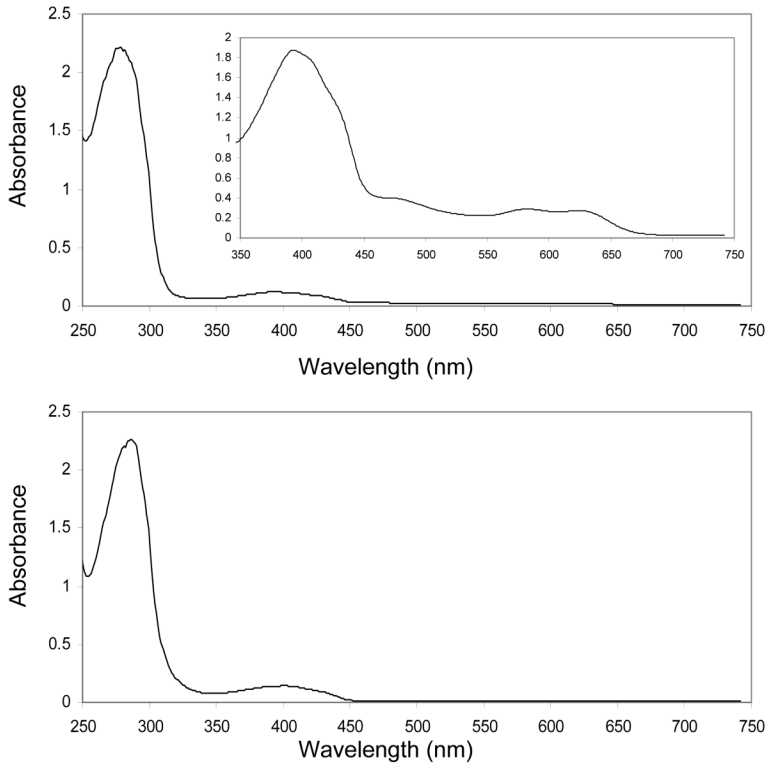



**Figure 2.**

Purification and spectroscopic analysis of CcPhr (**A**) Purification: CcPhr (WT) and CcPhr (W396R) 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 (W396R) 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 (W396R). Lanes 5 and 6 contain 5 μg of protein each. The samples were analyzed by SDS-PAGE and Coomassie Blue Staining. (**B**) Absorbance spectra of CcPhr (WT) (top panel) and CcPhr (W396R) (bottom panel). The inset in the top spectrum shows absorption in the 450-650 nm region contributed by FADH° in the enzyme. CcPhr (W396R) shows no detectable absorption in this range (data not shown).

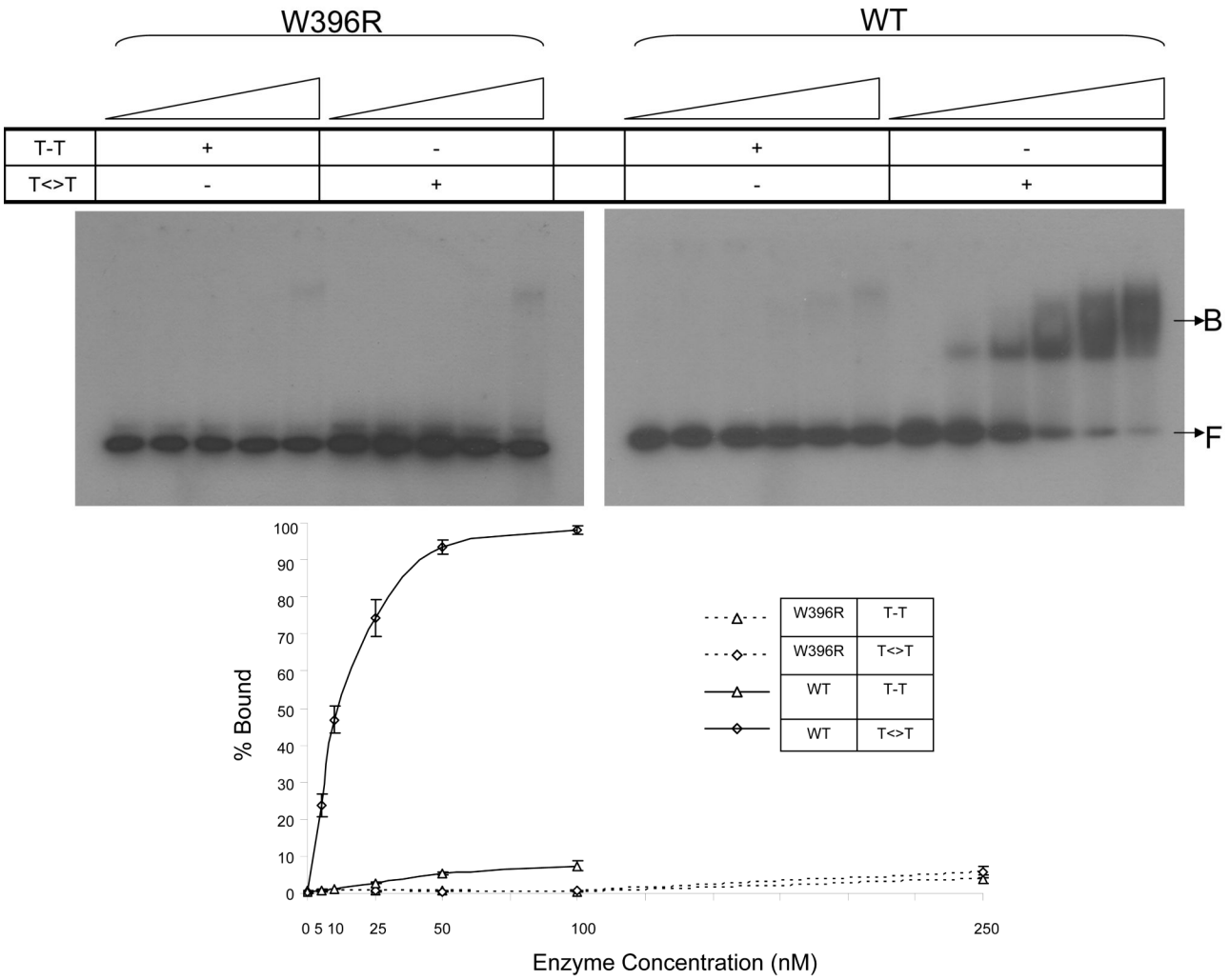



**Figure 3.**

Binding of CcPhr (W396R) and CcPhr (WT) to undamaged DNA and a T<>T-containing duplex. Radiolabeled 48-bp duplex (2 nM) was incubated with increasing were separated on 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 tree experiments are plotted. The error bars are smaller then data point symbols.

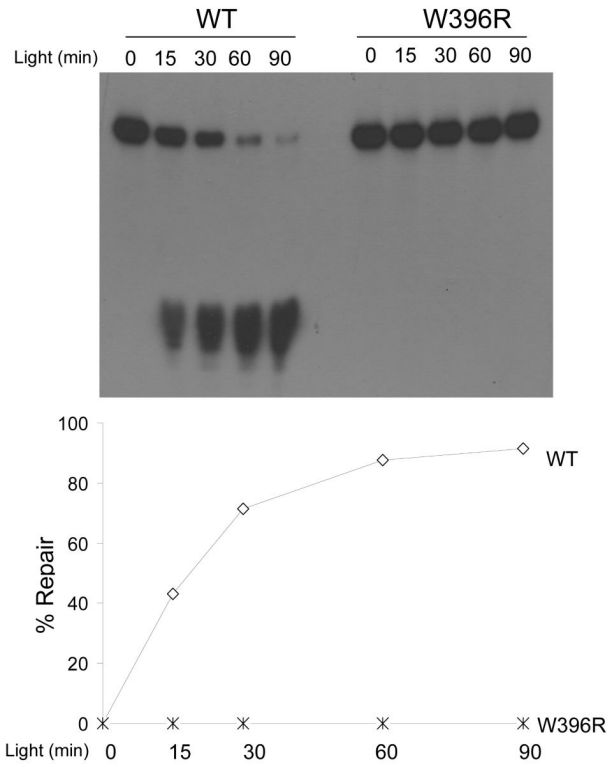



**Figure 4.**

Repair of cyclobutane thymine dimer by CcPhr. A 48-bp duplex (2nM) with a T<>T in the TTAA sequence (MseI recognition site) in the center was mixed with either 50 nM of CcPhr (WT) or 2 μM CcPhr (W396R) 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 8% polyacrylamide gel. Top panel: autoradiogram of the gel. Bottom panel. Quantitative analysis of the data. There was no detectable repair by CcPhr (W396R).

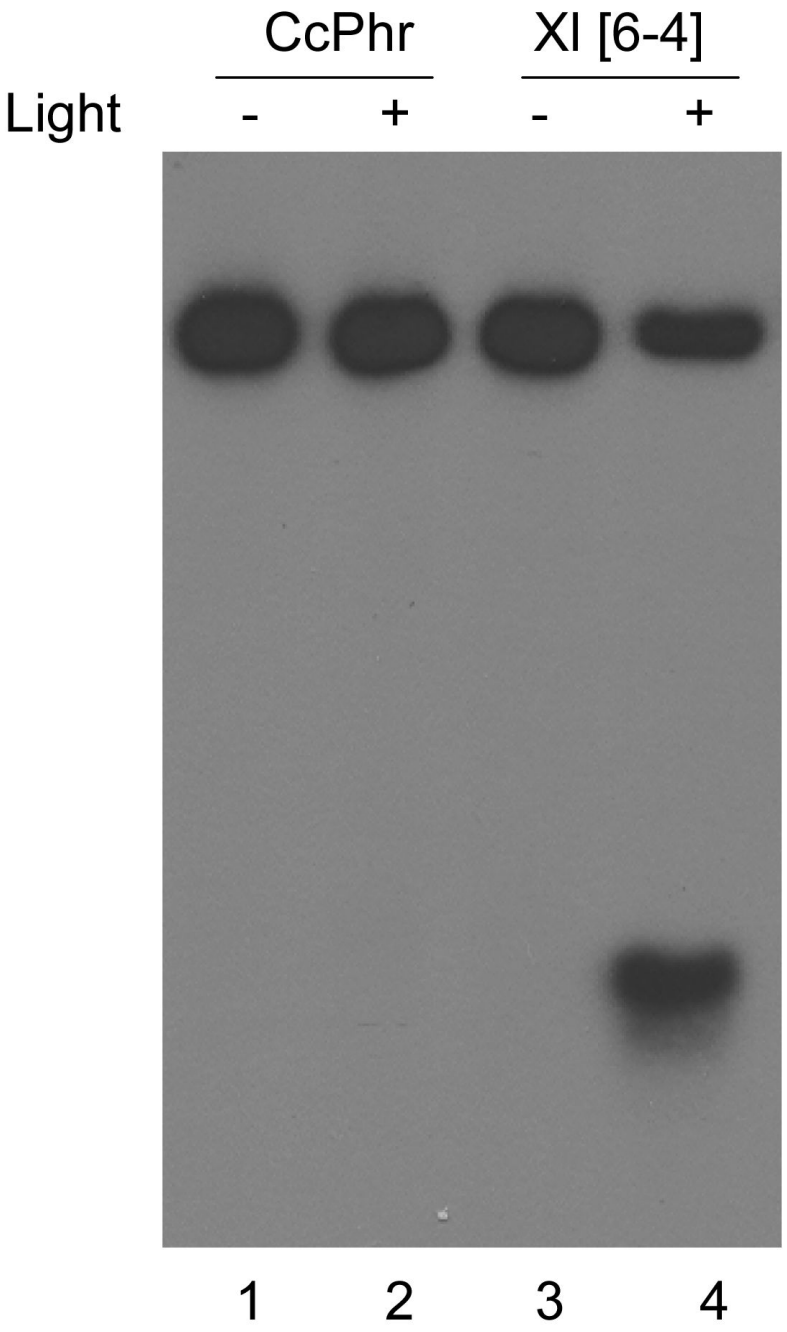



**Figure 5.**
Lack of repair of [6-4] photoproduct by CcPhr. A 48-bp duplex with a T[6-4]T in the TTAA sequence in the middle of the radiolabeled strand was mixed with either of 1 μM of CcPhr or 50 nm X. laevis [6-4] photolyase and irradiated with 366 nm at 2 $mW.cm^{-2}$ for 90 min. The samples were deproteinized by phenol extraction and digested with MseI and separated on 8% PAGE. 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.

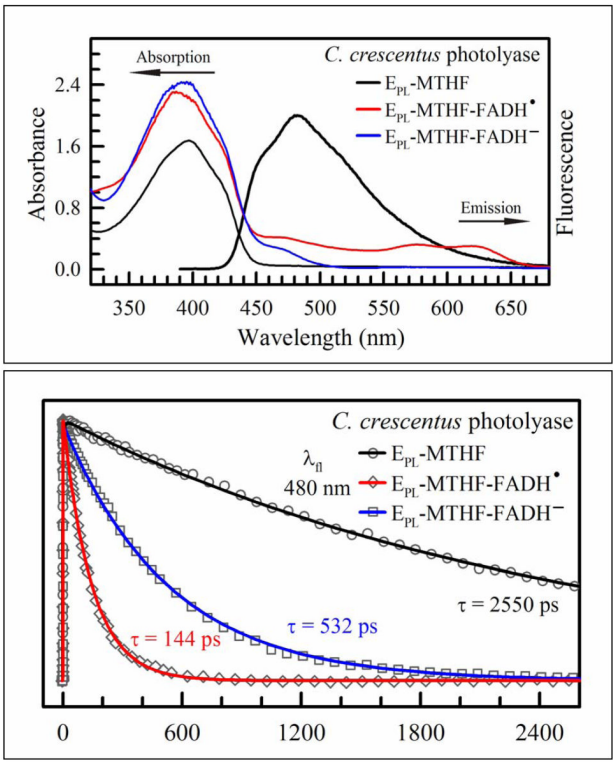



**Figure 6.**

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 (W396R) (black) are shown. (**B**) Femtosecond-resolved fluorescence transients of (o) CcPhr (W396R) 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 $E_{PL}$-MTHFFADH° and the $E_{PL}$-MTHF- $FADH^-$ forms of the wild-type enzyme contained a small (5-25%) of long component which was ascribed to the enzyme with MTHF and no flavin. This component has been removed in this figure for clarity.