Characterization of a Medium Wavelength Type DNA Photolyase: Purification and Properties of Photolyase from *Bacillus firmus*†

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ABSTRACT: The gene for the apoenzyme of *Bacillus firmus* photolyase was cloned and sequenced. The enzyme was overproduced in *Escherichia coli*, purified, and characterized. It has the unique property of having the maximum activity over a wavelength range where all other known photolyases exhibit modest activity. The enzyme contains reduced FAD and methenyltetrahydrofolate and has an absorption and action spectrum peak at 410 nm, and it repairs DNA with a quantum yield of $\phi \sim 0.75$.

Photoreactivation is the prevention of the harmful effects of far-UV light (200–300 nm) by subsequent exposure of cells to near-UV–visible light (300–500 nm). The phenomenon is mediated by DNA photolyase, which splits pyrimidine cyclobutane dimers (Pyr<>Pyr) into monomers. Photoreactivation on whole cells reveals that photolyases fall into three groups depending on the wavelengths of maximum activity (Jagger *et al.*, 1970; Minato & Werbin, 1971): short wavelength (360–390 nm), medium wavelength (390–420 nm), and long wavelength (420–460 nm) type photolyases.

In recent years, several short and long wavelength type photolyases have been purified and characterized [see Sancar (1994)]. It has been found that both types contain $FADH_2$ as the catalytic cofactor and that the short wavelength type contains methenyltetrahydrofolate and the long wavelength type contains 5-deazariboflavin as the major light-gathering chromophore. Accordingly, all known photolyases have been classified as folate or deazaflavin class enzymes. The *Escherichia coli* photolyase (λ_{max} = 384 nm) and the *Saccharomyces cerevisiae* photolyase (λ_{max} = 377 nm) belong to the folate class. Photolyases from *Anacystis nidulans* and *Streptomyces griseus* (λ_{max} = 440 nm) belong to the deazaflavin class. No medium-wavelength photolyase had been characterized previously, and therefore it was conceivable that these enzymes represented a third class of chromophores. In this study we have investigated a medium-wavelength photolyase isolated from *Bacillus firmus* with regard to its chromophore/cofactor composition, structure, and photochemical properties.

Bacillus firmus is a facultative alkaliphile which can grow at pH 10 and above. As a consequence, ATP synthesis by this organism operates against a strong pH gradient, and it is thought that oxidative phosphorylation in *B. firmus* deviates from the chemiosmotic model. For these reasons there has been considerable interest in the structure of F_1F_o-ATPase and cytochrome oxidases from this organism. Recently, Quirk *et al.* (1993) cloned and sequenced the operon that encodes cytochrome caa_3 oxidase and identified an incomplete 339 amino acid open reading frame upstream of the operon which had 42% sequence identity with the C-terminal 335 amino acids of *E. coli* photolyase. In this paper we report the cloning, expression, and characterization of the entire *B. firmus* photolyase and demonstrate that even though it represents a third wavelength type of enzyme, it falls into the folate class on the basis of chromophore composition.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *E. coli* strain UNC523 F′lacI^q (*phr*::*kan uvrA*::*Tn10*) was used as the host strain for the plasmids with *B. firmus phr* gene. Plasmid pTTQ18 (Stark, 1987) was used to construct pUNC1995, which overproduces the *B. firmus* photolyase.

Cloning of B. firmus phr Gene. The gene was cloned by PCR using primers based on the published sequence of the *cta* genes and upstream sequence (Quirk *et al.*, 1993) and a degenerate primer based on the consensus sequence near the amino-terminal region of other photolyases. The chromosomal DNA of *B. firmus* was kindly provided by Dr. Terry A. Krulwich (Mount Sinai School of Medicine, New York). The nucleotide sequences of the primers were as follows: PCR1 (35-mer): GGG GCC ATG GT(A/C/T) (C/T)(G/T)N TT-(T/C) (A/C)(C/G)N (A/C/T)(A/C/G)N GA(T/C) (C/T)TN (A/C)GN (C/T)T. PCR2 (33-mer): CCC GGG CTG CAG TTT ATC TAT TTC ATA CAC CCT. PCR3 (21-mer): TGC ATG ACG GTT TGA AAA AAA. PCR4 (6-mer): NNN NNN. PCR5 (30-mer): GGG GAA TTC GGG GGT GCA TGT TGG GAA TAG.

PCR1 was based on the consensus sequence of a region near the amino terminus of known photolyases (positions 6–14 of *E. coli* photolyase). PCR2 is based on the nucleotide sequence six nucleotides beyond the terminal codon of the *phr*-like open reading frame of *B. firmus* identified by Quirk *et al.* (1993). Using PCR1 and PCR2, a 1464-bp fragment was amplified, cloned into PUC18, and sequenced. This added 132 amino acids to the open reading frame identified by Quirk *et al.* (1993). To obtain the remaining N-terminal sequence, we employed PCR3 (corresponding to the region covering amino acids 64–70 of *B. firmus* photolyase) and the random primer PCR4. A fragment of 300-bp was amplified and sequenced. This sequence revealed that the *phr* gene initiates with a GTG codon 225-bp upstream of the PCR3 primer. PCR5, which corresponds to the region 6-bp upstream of the initiation codon, was then used together with PCR2 to amplify the entire gene on an *Eco*RI–*Pst*I casette, and the resulting

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fragment was inserted into pTTQ18 to obtain the expression plasmid pUNC1995.

Overproduction and Purification of Photolyase. The enzyme was purified from the overproducing strain UNC523/pUNC1995 essentially as described previously for other photolyases (Malhotra *et al.*, 1992a,b). Briefly, the proteins were concentrated from cell-free extract by ammonium sulfate precipitation, and then photolyase was purified by sequential chromatography on Blue Sepharose and DNA–cellulose resins. The binding to DNA–cellulose (Sigma) was especially strong: 0.7 M KCl was required to elute the enzyme. As a result, after this step the enzyme was essentially free of contaminants.

Substrate. Two different substrates were used. One was a synthetic 49-bp duplex with a terminal ^{32}P label and a centrally located T<>T (Smith & Taylor, 1993). The photodimer is at an *Mse*I site (T<>TAA) and renders the duplex resistant to cleavage by the restriction enzyme. Photoreactivation restores susceptibility of the DNA to *Mse*I. The second substrate was prepared by acetone-photosensitized irradiation of oligo(dT)$_{15}$, which converts about 70% of thymines into dimers (Kim & Sancar, 1991) with the corresponding decrease of absorption of the oligonucleotide at 260 nm. Photoreactivation is measured by the increase in absorption at 260 nm.

Spectroscopic Measurements. The absorption and fluorescence measurements were made with a Hewlett-Packard Model 8451A spectrophotometer and a Shimadzu RF5000U spectrofluorometer, respectively. Holoenzyme, or enzyme containing flavin only, was analyzed. Photolyase containing flavin but no folate was prepared by sodium borohydride reduction of the folate followed by removal of the released cofactor by a spin column. In order to remove the second chromophore, 20 μL of 15 mM sodium borohydride in 50 mM sodium borate, pH 9.5, was added to 100 μL of 300 μM photolyase in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 20% (v/v) glycerol. The mixture was incubated on ice until the bubbling stopped. Then, the sample was passed through a Penefsky column equilibrated with the same buffer.

The purified enzyme contains the flavin in the blue neutral radical form (E-FADH°-MTHF).[1] The E-FADH$^-$-MTHF and E-FADH$^-$ forms were prepared by selective photoreduction of FADH° with filtered camera flashes ($\lambda > 580$ nm) in the presence of dithiothreitol, using enzyme with or without the second chromophore, respectively. The extinction coefficients of E-FADH°(ϵ_{580} = 5000) and E-FADH$^-$ (ϵ_{355} = 5800) were determined on the basis of the amounts of FAD_{ox} ($\epsilon_{450} = 1.15 \times 10^4$ M^{-1} cm^{-1}) released upon denaturation of the enzyme with 0.7% sodium dodecyl sulfate. The stoichiometry of FAD to apoenzyme was unity as calculated from the extinction coefficents of the apoenzyme plus FAD at 274 nm (ϵ_{274} = 101 740 + 35 000 = 136 740) and of FAD at 450 nm. The extinction coefficient of E-FADH$^-$-MTHF at 410 nm (ϵ_{410} = 20 000) was calculated relative to the absorption of the E-FADH°-MTHF form at 580 nm prior to photoreduction.

Action Spectrum. First, we determined the absolute values for the photolytic cross sections at 355 and 410 nm for the E-FADH$^-$-MTHF form of the enzyme under enzyme excess conditions and with a 49-bp duplex containing a single T<>T. The photolytic cross section was then determined by the Rupert plot of the repair reaction [see Payne and Sancar (1990)]. The determination of the entire action spectrum by this method is cumbersome, and therefore the action spectrum was determined by conducting repair reactions at other wavelengths with the readily available oligo(dT)$_{15}$–(T<>T) substrate under enzyme limiting conditions. The repair rates at various wavelengths were converted to photolytic cross sections ($\epsilon\phi$) by comparison with repair rates at 355 and 410 nm obtained under identical conditions (Malhotra *et al.*, 1992b).

RESULTS

Sequence of B. firmus phr Gene. Quirk *et al.* (1993) previously reported the sequence of a 339 amino acid open reading frame with 42% sequence identity to the carboxy-terminal 335 amino acids (total size, 472 amino acids) of *E. coli* photolyase. We cloned and sequenced the missing 5′-terminal region of the putative *B. firmus phr* gene. The sequence reveals a ribosomal binding site located 6-bp upstream from an open reading frame of 1455 bp (485 amino acids) and initiating with a GTG codon. Figure 1 shows a comparison of this sequence with those of seven other microbial photolyases. It has the highest homology to the *E. coli* enzyme with an overall amino acid identity of 37% and a similarity of 55%. The sequence identity is most extensive in the carboxy-terminal region, which is known to be the flavin binding domain in other photolyases (Malhotra *et al.*, 1992a). The amino-terminal halves of photolyases are thought to bind the second chromophore, and this has been demonstrated experimentally in the case of *S. cerevisiae* photolyase (Malhotra *et al.*, 1992a).

Along these lines the amino-terminal halves of photolyases of the folate and deazaflavin classes show a higher degree of homology to other photolyases within the same class. The residues Y_{35}, H_{45}, I_{71}, P_{72}, K_{89}, E_{110}, and N_{310}, with the corresponding positions in *E. coli* photolyase in subscript, are uniquely conserved in the folate class. Even though *B. firmus* photolyase has higher overall homology to this class of enzymes, the amino acids at the corresponding positions are F_{48}, A_{55}, G_{81}, D_{82}, L_{99}, G_{118}, and D_{319}, with no sequence identity to the consensus. Similarly, the residues F_{35}, D_{38}, G_{78}, A_{88}, V_{96}, G_{121}, P_{245}, F_{249}, and T_{360}, with the corresponding positions in *A. nidulans* photolyase in subscript, are uniquely conserved in the deazaflavin class enzymes. The amino acids at the corresponding positions of *B. firmus* photolyase are F_{48}, D_{51}, G_{88}, F_{102}, V_{108}, S_{133}, P_{253}, T_{257}, and T_{362}. Thus, six of the nine residues uniquely conserved in the deazaflavin class are identical in *B. firmus* photolyase [see Kobayashi *et al.* (1989), Sancar (1990), and Yajima *et al.* (1991)]. It seemed possible that the *B. firmus* photolyase may be a deazaflavin class enzyme or a representative of yet another class of photolyases as defined by the identity of the second chromophore. We decided to purify the enzyme and characterize it with regard to its chromophore composition and photochemical properties.

Overproduction and Purification of Photolyase. The *B. firmus phr* gene was joined to the *tac* promoter in the *E. coli* expression vector pTTQ18 to obtain pUNC1995 (Figure 2). This plasmid complemented an *E. coli phr*$^-$ mutant (Figure 3), indicating the production of a functional protein. Following induction of cells carrying the plasmid, a protein of M_r = 56 725 was produced to a level of about 7% of total cellular proteins (Figure 4). The protein was purified by a two-column procedure. For unknown reasons the yield was low at both steps; nevertheless, the binding to the DNA–cellulose column was relatively strong, which enabled us to obtain essentially pure protein that can be used for physical and enzymatic characterization (Table 1).

[1] Abbreviations: MTHF, 5,10-methenyltetrahydrofolate; 8-HDF, 7,8-didemethyl-8-hydroxy-5-deazariboflavin; E-FADH°, E-FADH°-MTHF, etc., photolyase apoenzyme bound to the indicated chromophores.

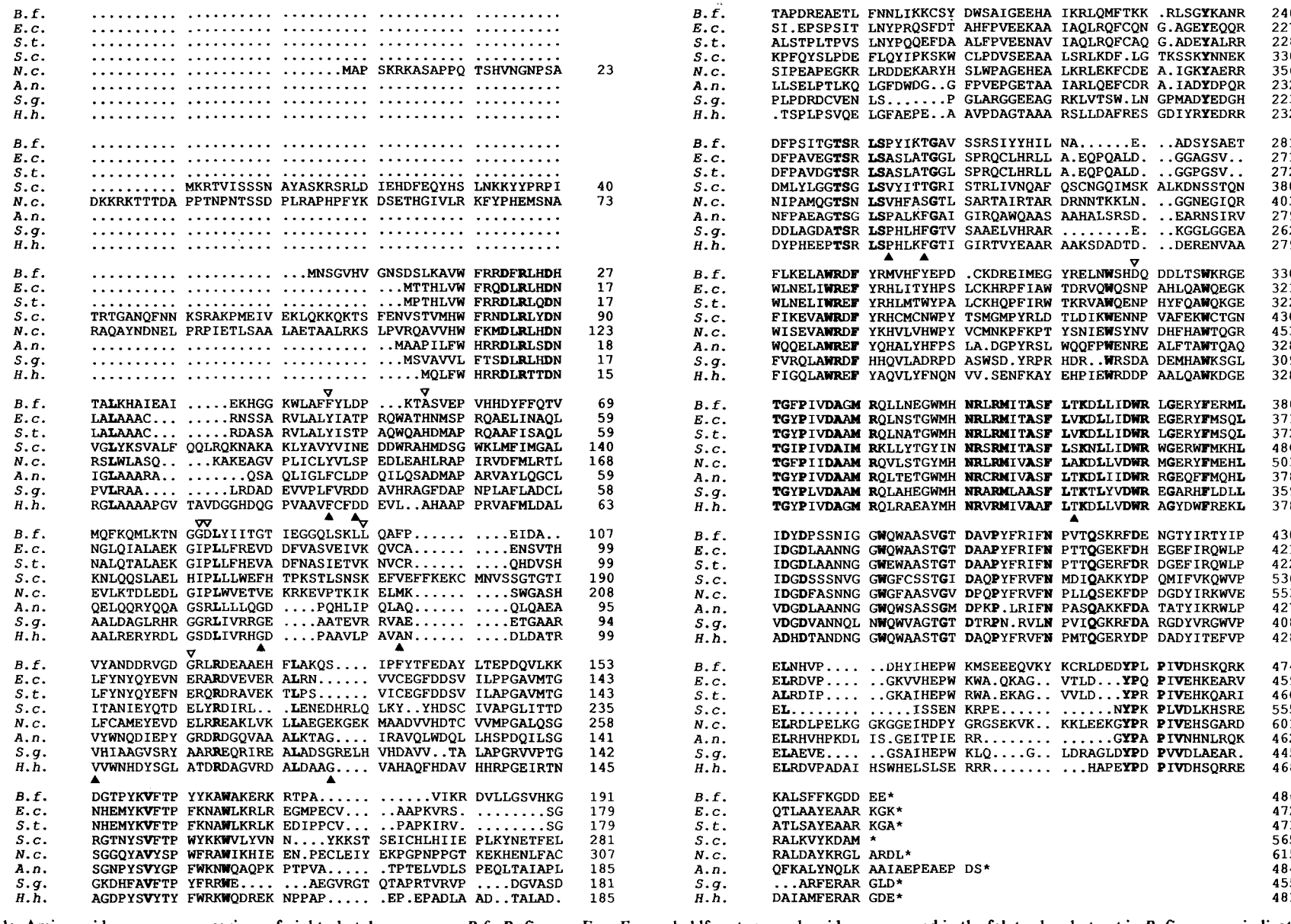

FIGURE 1: Amino acid sequence comparison of eight photolyases genes. *B.f.*, *B. firmus*; *E.c.*, *E. coli*; *S.t.*, *S. typhimurium*; *S.c.*, *S. cervisiae*; *N.c.*, *Neurospora crassa*; *A.n.*, *A. nidulans*; *S.g.*, *S. griseus*; *H.h.*, *Halobacterium halobium*. Amino acids conserved in all photolyases are shown in boldface type, and residues conserved in the folate class but not in *B. firmus* are indicated by open triangles. Amino acids conserved within the deazaflavin class photolyases (the last three) are indicated by solid triangles. The alignment of photolyase genes was done by using the GCG software.

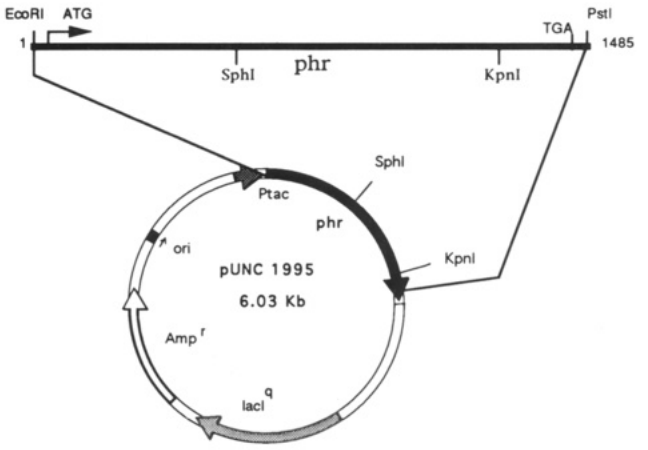

FIGURE 2: Schematic representation of plasmid pUNC1995, which overproduces *B. firmus* photolyase in *E. coli*. Outlined and solid arrows indicate the direction of transcription of the *amp* and *phr* genes, while the *tac* promoter is indicated by a stippled arrowhead.

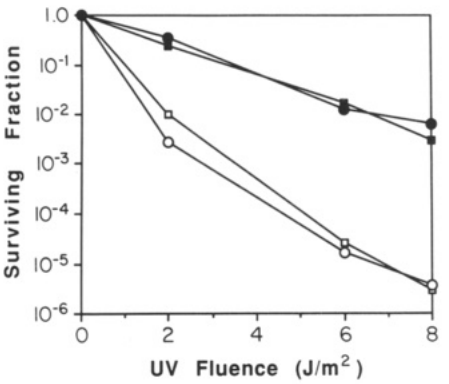

FIGURE 3: Photoreactivation of *E. coli* UNC523(*uvrA*⁻ *phr*⁻) containing *B. firmus* or *E. coli phr* genes. Open and filled circles (*B. firmus*) and open and filled squares (*E. coli*) indicate survival rates after photoreactivation of cells containing the *phr* plasmids or cells containing the vector.

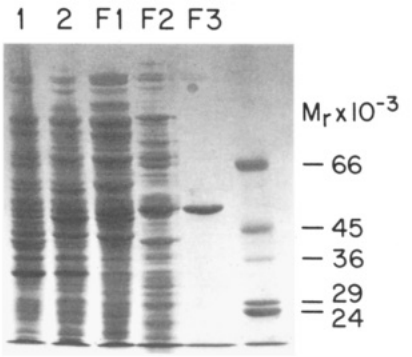

FIGURE 4: Purification of *B. firmus* photolyase. The protein samples were denatured and separated on a 10% SDS–PAGE gel. UNC 523 F′lacI^q/pUNC1995 cultures were induced with IPTG and grown for an additional 10 h. Lane 1, uninduced cells; lane 2, cells induced with IPTG (150 μL); lane F1, crude extract (85 μg); lane F2, peak fraction after Blue Sepharose column (50 μg); lane F3, peak fraction after DNA–cellulose column (7 μg); lane 6 (not labeled), molecular weight standards. The gel was stained with Coomassie Blue.

Spectroscopic Properties of B. firmus Photolyase. The absorption spectrum of *B. firmus* photolyase is shown in Figure 5A. Two features of this spectrum are of interest. First, in the 450–650-nm region the spectrum is the typical flavin neutral radical spectrum as has been seen with all other photolyases which are known to contain FADH⁻ *in vivo* as the catalytic cofactor. The FADH⁻ becomes oxidized to the semiquinone form during purification (Payne *et al.*, 1987; Eker *et al.*, 1990). Thus, we conclude that *B. firmus* photolayse contains FADH⁻ like other photolyases. Second, the absorption maximum of the enzyme in the near-UV–visible is at 410 nm. Since in all photolyases the absorption maximum in the 350–450-nm range is dominated by that of the second chromophore [see Sancar (1994)], we ascribe this absorption band to the second chromophore.

Table 1: Purification of *B. firmus* Photolyase[a]

fraction	total volume, mL	protein, mg	photolyase %	photolyase mg	yield, %
cell-free extract	160	3360	7.6	256	100
Blue Sepharose	35	133	11.0	14.7	5.7
DNA–cellulose	10	3	93.7	2.5	0.9

[a] The starting material was an 8-L culture of UNC523/pUNC1995 induced with 1 mM IPTG for 10 h.

The folate class enzymes show absorption maxima at 375–385 nm, while the deazaflavin class enzymes have a sharp peak at 440 nm. Thus, the presence of a 410-nm absorption peak in the *B. firmus* enzyme raised the possibility of a new class of second chromophores. However, it was also possible that the enzyme belonged in one of the two known classes but that the interaction of the second chromophore was unusually strong to cause a drastic shift in the peak position. Since *E. coli* does not synthesize deazaflavin, even though the apoenzyme has higher sequence homology to the putative second chromophore binding site of the deazaflavin class, we only considered the possibility of a folate chromophore (MTHF) with a 50-nm red shift in the absorption spectrum. Indeed, the fluorescence emission spectrum of the holoenzyme (Figure 5B) is consistent with that of enzyme-bound MTHF (Johnson *et al.*, 1988).

Identification of the Second Chromophore. To ascertain whether or not the second chromophore is MTHF, we applied two tests which are highly specific for this form of folate, namely, the sensitivity of the 5,10-double bond to cleavage by sodium borohydride (Jorns *et al.*, 1990) and by alkali (Hamm-Alvarez *et al.*, 1990). Sodium borohydride reduces 5,10-MTHF to 5- and 10-formyl folate, which do not absorb in the 300–500-nm region. Similarly, at pH > 9 the 5,10-methenyl bond is highly susceptible to hydrolysis: hence the disappearance of the characteristic near-UV absorption band at high pH. Figure 5A, curve 2, shows the absorption spectrum of the enzyme following borohydride treatment. The 410-nm peak has disappeared, and the remaining spectrum is identical to the absorption spectrum of the E-FADH° forms of photolyases. At low pH the enzyme is denatured and the chromophores are released. Figure 6 (spectrum 1) shows that enzyme denatured with SDS in acidic pH retains its high absorption in the 350–500-nm region, although the absorption peaks at longer wavelengths have disappeared because of oxidation of FADH° to FAD_{ox}. In contrast, denaturation by alkali (Figure 6, spectrum 2) results in a drastic reduction in the near-UV absorption, and the resulting spectrum is that of FAD_{ox} with peaks at 370 (partially obscured by the apoenzyme "tail" absorption) and 450 nm.

More direct evidence for the identity of the second chromophore was sought by attempting to reconstitute the second chromophore-depleted enzyme with the monoglutamate form of MTHF. However, several trials were unsuccessful, and the limited amount of the enzyme prevented us from attempting to reconstitute the holoenzyme from the denatured apoenzyme and the two chromophores. Therefore, we decided to isolate the second chromophore and identify its chemical

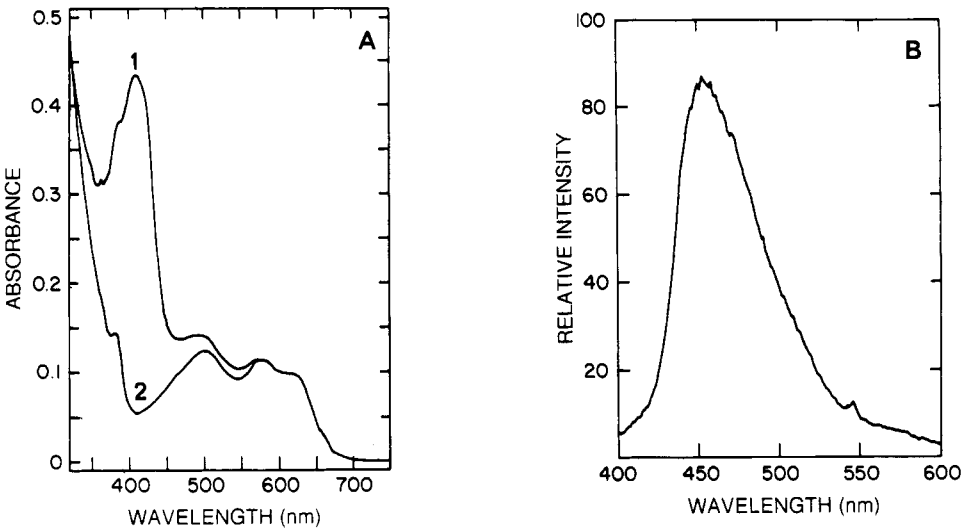

FIGURE 5: (A) Absorption spectra of *B. firmus* photolyase before and after addition of sodium borohydride. Curve 1: Absorption spectrum of *B. firmus* holophotolyase expressed in *E. coli*. Curve 2: Absorption spectrum of photolyase after sodium borohydride reduction of the second chromophore. The latter absorption spectrum is typical of E-FADH° as shown in other photolyases. For both spectra, the photolyase concentration was 24 μM. (B) Emission spectrum of *B. firmus* holophotolyase. The excitation wavelength was 380 nm. The bandwidths for the excitation and emission slits were 5.0 nm. The emission spectrum was not calibrated.

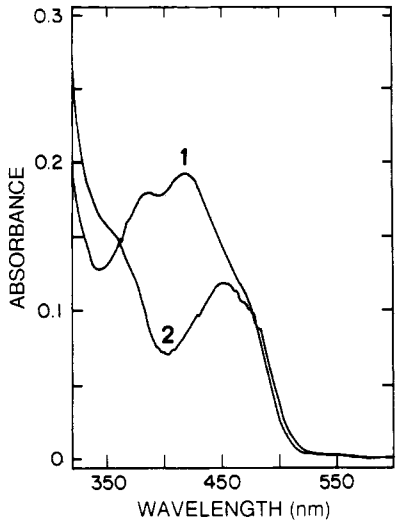

FIGURE 6: Denaturation of *B. firmus* photolyase with SDS (spectrum 1) and NaOH (spectrum 2). Spectrum 1 was obtained 20 min after addition of SDS (0.5%) at 25 °C. Spectrum 2 was obtained 30 min after addition of SDS (0.5%) followed by addition of 5 M NaOH to adjust the pH to 10.0 at 25 °C.

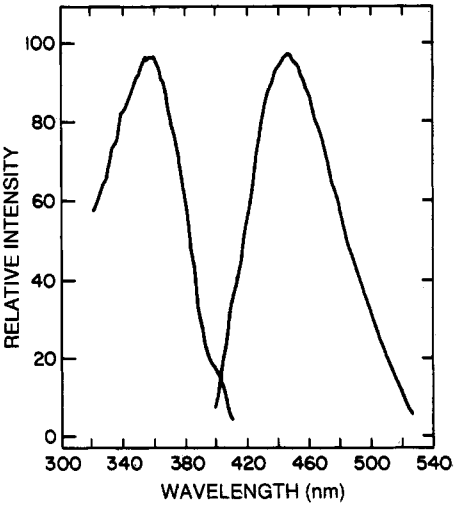

FIGURE 7: Excitation and emission spectra of the second chromophore isolated from *B. firmus* photolyase by silica gel thin-layer chromatography (solvent system: ethyl acetate/methaol/acetic acid, 100:10:1, v/v/v). The excitation spectrum was taken at an emission wavelength of 460 nm; the emission spectrum was taken at an excitation wavelength of 350 nm.

structure by studying its properties in the absence of flavin and the apoenzyme.

The enzyme was denatured in 0.1 M HCl and 0.5% SDS at 45 °C and applied to a Sephadex G-15 column equilibrated with 0.1 M HCl. The fractions containing the chromophores were located by fluorescence with excitation at 350 nm. The fractions were combined and lyophilized to dryness, and the two chromophores were separated by thin-layer chromatography (TLC) in two different solvent systems: solvent A, *n*-butanol/acetic acid/water (5/2/3); solvent B, ethyl acetate/methanol/acetic acid (100/10/1). By using authentic FAD as a reference, the released FAD band and the second chromophore band were easily located, and the second chromophore was scraped off the TLC plates. The excitation and emission spectra of the purified second chromophore are shown in Figure 7 and are identical to those of authentic 5,10-MTHF. Thus we conclude that the second chromophore of *B. firmus* photolyase is 5,10-MTHF.

Substrate Binding and Catalysis. In all photolyases that have been characterized the second chromophore has no effect on substrate binding but drastically affects the efficiency of repair per incident photon. Therefore, we wished to investigate the role of the unusual second chromophore of *B. firmus* photolyase in binding and catalysis. In particular, since the second chromophore appears to interact with the apoenzyme in a unique way (compared to other folate class enzymes), we were interested to find out whether it affected substrate binding. However, we found a binding constant (K_A) of 1.8 × 10^9 M^{-1} for both the E-FADH° and E-FADH°-MTHF forms of the enzyme, suggesting that the folate does not contribute to binding interactions. The absolute action spectra of the E-FADH⁻ and E-FADH⁻-MTHF forms are shown in Figure 8. The repair quantum yield of the E-FADH⁻ form is $\phi = 0.9$ and is identical to the quantum yield of this form of all other photolyases characterized (Figure 8A). The photolytic cross section ($\epsilon\phi$) of the E-FADH⁻-MTHF form is relatively low compared to those of other folate class

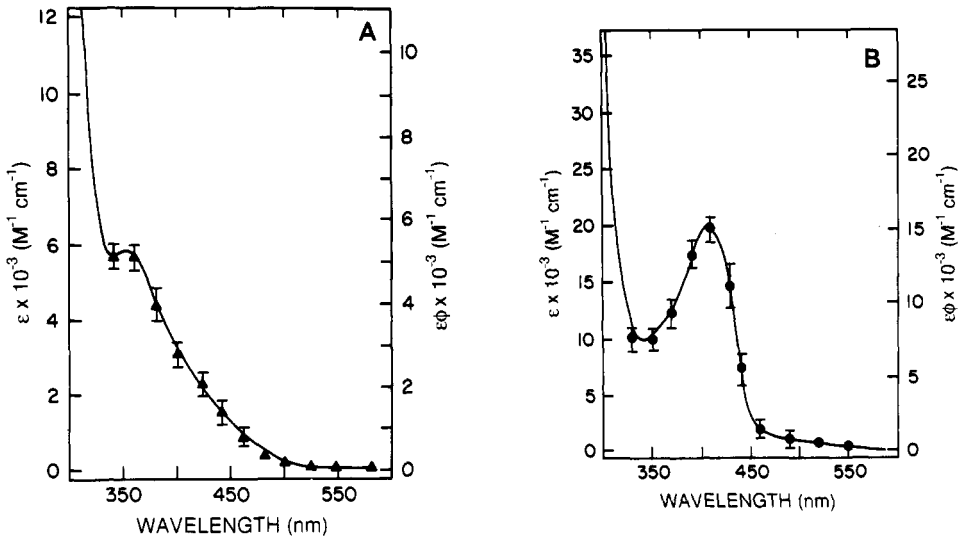

FIGURE 8: Absorption and absolute action spectra of (A) E-FADH⁻ and (B) E-FADH⁻-MTHF forms of *B. firmus* photolyase.

photolyases (Figure 8B). However, this is due to the low extinction coefficient of this form of the enzyme. The extinction coefficient of folate in other folate class enzymes is typically around 25 000 M^{-1} cm^{-1} [see Sancar (1994)], whereas the extinction coefficient of the *B. firmus* holoenzyme at 410 nm is only 20 000 M^{-1} cm^{-1}. Whether this is due to a substoichiometric amount of MTHF in the enzyme we cannot tell at present because the limited amount of enzyme available made it impractical to isolate large quantities of the second chromophore and determine the stoichiometry. Even though there is some uncertainty regarding the extinction coefficient of the holoenzyme at 410 nm, the photolytic cross section could be determined independently, from a Rupert plot of repair *vs* fluence, and the results are shown in Figure 8B. The absolute action spectrum perfectly matches the absorption spectrum in this wavelength range where the contribution of the FADH⁻ is minimal. The quantum yield of the holoenzyme is $\phi = 0.75$, and it does not change with the wavelength. This is the highest quantum yield reported for a folate class enzyme. The other folate class photolyases have overall quantum yields of $\phi = 0.6$ (*E. coli* and *Salmonella typhimurium*) and 0.55 (*S. cerevisiae*).

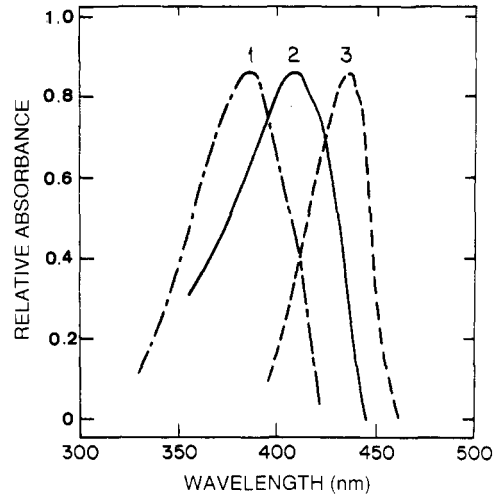

FIGURE 9: Absorption and action spectra of the three types of DNA photolyases belonging to two classes. Spectrum 1: Photolyase from *S. cervisiae* (folate class). Spectrum 2: Photolyase from *B. firmus* (folate class). Spectrum 3: Photolyase from *A. nidulans, S. griseus,* or *H. halobium* (deazaflavin class). The scales are relative to the maximum of each enzyme.

DISCUSSION

Photolyases have been classified as short wavelength, medium-wavelength, and long wavelength types according to the action spectrum maxima (Jagger *et al.*, 1970), and folate class and deazaflavin class, according to the chemical identity of the second chromophore (Sancar & Sancar, 1987). All of the well-characterized long-wavelength types belong in the deazaflavin class, and all of the short-wavelength types belong in the folate class. In this report we show that at least one member of the medium-wavelength type also belongs in the folate class. While it is necessary to characterize other members of the medium-wavelength type before making a general statement regarding the chromophore composition of the medium-wavelength type enzymes, it is possible that these enzymes also fall into the folate class. It is of interest to note that the absorption (and action spectrum) maxima of the folate class range from 377 (yeast) to 410 nm (*B. firmus*), in contrast to those of the deazaflavin class enzymes, all of which have a sharp peak at 440 nm (Figure 9). So it is conceivable that all of the medium-wavelength type enzymes belong in the folate class.

The features of MTHF that make it possible for it to have absorption spectra ranging from 355 (free form) to 410 nm is an interesting question. The methenyl bridge connects the π systems of the aminobenzoyl group and the pterin rings through an immonium group. Thus interaction of the resulting chromophore, which is responsible for the 355-nm peak in the free form, and the apoenzyme determines the wavelength and magnitude of the absorption peak in the enzyme-bound form. It is interesting to note that the three folate class photolyases (yeast, *E. coli, S. typhimurium*) which have their folate absorption maxima within 10 nm of one another share sequence homology unique to the folate class at seven positions, while the amino acids are different at all these positions in *B. firmus* photolyase. We strongly suspect that these six amino acids interact with the folate and that this interaction is much stronger in the *B. firmus* enzyme and results in the reduced absortivity and the shift to longer wavelength of the absorption spectrum of the *B. firmus* enzyme.

How the amino acid substitutions cause this change is not known at present. It is known that the absorption maximum of free MTHF is very dependent on the solvent polarity with a strong bathochromic shift in nonpolar solvents (Kim *et al.*,

1991). Along these lines, in going from the folate class consensus YHIPKEN sequence to the *B. firmus* sequence FAGDLGD, there is a change from a fairly polar set of amino acids to a group of nonpolar residues and a net change in charge of −2 which may favor stronger intractions with the positively charged methenyl group. This may explain the difficulties we encountered in our attempts to remove the folate from the enzyme. It may also explain the lower MTHF extinction coefficient in the *B. firmus* enzyme. Ionic interactions (or hydrogen bonding) can perturb the probability of electronic transitions by affecting the transition dipole moment and thus result in substantial hypochromism.

Finally, it is worth commenting on the presence of photolyase in *B. firmus* and its absence in *Bacillus subtilis* and many other *Bacillus* species. Detecting photolyase activity *in vivo* in wild-type (with regard to excision and recombination repair) cells is difficult in many species, including *E. coli*, because of the efficiency of the dark repair systems. Therefore, a report of the absence of the enzyme in a certain organism based on *in vivo* photoreactivation must be supported by independent tests. In this regard, as pointed out by Quirk *et al.* (1993), we find the organization of the *cta* operon in *B. subtilis* and *B. firmus* rather illuminating. In *B. firmus* the *phr* gene is immediately upstream of the *cta* operon with only a few base pairs separating it from the *ctaA* gene. In contrast, in *B. subtilis* the pyruvate carboxylase gene *pycA* is present at the position equivalent to that of *phr* (Mueller & Tabor, 1989). Thus, it appears that the lack of photoreactivation in *B. subtilis* at least, and possibly in other organisms, is simply due to the deletion of the gene and is not caused by the lack of expression of an existing gene or the lack of an essential chromophore.

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