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Cloning, sequencing, expression and characterization of DNA photolyase from *Salmonella typhimurium*

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ABSTRACT

We have cloned the *phr* gene that encodes DNA photolyase from *Salmonella typhimurium* by *in vivo* complementation of *Escherichia coli phr* gene defect. The *S.typhimurium phr* gene is 1419 base pairs long and the deduced amino acid sequence has 80% identity with that of *E.coli* photolyase. We expressed the *S.typhimurium phr* gene in *E.coli* by ligating the *E.coli* trc promoter 5′ to the gene, and purified the enzyme to near homogeneity. The apparent molecular weight of *S.typhimurium* photolyase is 54,000 dalton as determined by SDS-polyacrylamide gel electrophoresis, which is consistent with the calculated molecular weight of 53,932 dalton from the deduced *phr* gene product. *S.typhimurium* photolyase is purple-blue in color with near UV-visible absorption peaks at 384, 480, 580, and 625 nm and a fluorescence peak at 470 nm. From the characteristic absorption and fluorescence spectra and reconstitution experiments, *S.typhimurium* photolyase appears to contain flavin and methenyltetrahydrofolate as chromophore-cofactors as do the *E.coli* and yeast photolyases. Thus, *S.typhimurium* protein is the third folate class photolyase to be cloned and characterized to date. The binding constant of *S.typhimurium* photolyase to thymine dimer in DNA is $K_D = 1.6 \times 10^{-9}$ M, and the quantum yield of photorepair at 384 nm is 0.5.

INTRODUCTION

DNA photolyase catalyzes the photoreversal of pyrimidine dimers in UV damaged DNA. All photolyases catalyzed to date fall into two classes according to their chromophore compositions (1). Both classes contain flavin adenine dinucleotide in apparently reduced form ($FADH_2$). In addition, the folate class contains methenyltetrahydrofolate (2) and the deazaflavin class contains 8-hydroxy-5-deazaflavin (3) as second chromophores (4). Deazaflavin class photolyases have been sequenced or purified and characterized from several organisms (5,6,7). In contrast only two examples of the folate class are known at present, the *E.coli* and yeast photolyases (8). These two enzymes which are 35% homologous at the amino acid sequence level (9,10,11) interact with their chromophores rather differently. *E.coli* photolyase loses 50–70% of its folate during purification (12), and its flavin cofactor, although remaining stoichiometrically bound, is quantitatively oxidized to flavin neutral radical (13). In contrast, the yeast photolyase retains a stoichiometric amount of folate and of flavin in its native $FADH_2$ form (14).

For better understanding of photolyases in general, and of the folate class in particular, and of the factors affecting the stability of the chromophores, it would be useful if other members of this class were available for study. Towards this end we cloned and expressed the photolyase gene, *phr*, of *Salmonella typhimurium*, a close relative of *E.coli*, which we suspected might have a folate class enzyme. Sequence, biochemical, and photochemical properties of the purified enzyme show that Salmonella has a folate class photolyase which binds to a T<>T in DNA with high affinity ($K_D = 1.6 \times 10^{-9}$ M) and repairs it with a quantum yield of $\phi = 0.5$ at 384 nm.

EXPERIMENTAL PROCEDURES

Materials

Bacterial strains and plasmids. *E.coli* strain CSR06 (*uvrA6 phr-1*) was used as host strain for cloning the *S.typhimurium phr* gene; CSR603 (*uvrA6 phr-1 recA1*) was used to test complemention and overexpression of *phr* gene; DH5αF′ (*recA– hsdR17*) was used for preparing single strand DNA template for sequencing and double strand plasmid; CJ236 (*dut– ung–*) was used for preparing uracil containing single strand DNA template for site-specific mutagenesis (15). Plasmid pKK233-2 (Pharmacia LKB, Inc.) was used to construct the overexpressing plasmid pUNC1992 of *S.typhimurium phr* gene. pIBI24 and 25 (International Biotechnology, Inc.) were used to construct the plasmids for mutagenesis and sequencing.

Salmonella genomic library

A Salmonella genomic library in pUC18 plasmid was kindly provided by Dr. Nicholas M. Kredich (Duke University). The library was constructed by partial Sau3A digestion of *S.typhimurium* LT2 DNA and fragments of average size of 5.4 kbp were ligated into the BamHI site of pUC18 (16).

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Enzymes

All of the restriction enzymes, T4 DNA polymerase and ligase were purchased either from Promega or Boehringer Mannheim companies.

Chromatography

Blue Sepharose and single-stranded DNA cellulose were obtained from Sigma. Bio-gel HT was obtained from Bio-Rad company. The following buffers were used in purification of *S.typhimurium* photolyase. Buffer A (0.1 M KCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 20% glycerol), Buffer B (2 M KCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 20% glycerol), Buffer C (67 mM potassium phosphate, pH 6.8, 1 mM EDTA, 20% glycerol), Buffer D (330 mM potassium phosphate, pH 6.8, 1 mM EDTA, 20% glycerol), Cell lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% sucrose), Storage buffer (50 mM Tris, pH 7.4, 1 mM EDTA, 50 mM NaCl, 50% glycerol).

METHODS

Cloning

DNA from the Salmonella library (0.1 μg) was transformed into *E.coli* strain CSR06 (*phr*− *uvrA*−). The transformants were expressed in Luria broth (LB) for 2 hours, then plated on LB agar plates with 200 μg/ml ampicillin. The transformants were subjected to 100 erg/mm^2 254 nm UV light (Sylvania, GTE incorporated) irradiation, followed by 20,000 erg/mm^2 365 nm black light (Sylvania, GTE incorporated) photoreactivation (at a rate of 6 ergs/mm^2/sec), and incubated at 37°C for 12 hours. Individual colonies were picked and grown overnight in LB/ampicillin medium. DNA from each clone was extracted and retransformed into *E.coli* CSR603 (*phr*− *uvrA*− *recA*−), individually. The transformants were plated on LB/ampicillin plates and irradiated with 5 erg/mm^2 UV light before separating into two sets. One set was kept in dark without further treatment, the other was exposed to 20,000 erg/mm^2 365 nm light photoreactivation. Cells that showed at least 10^2-fold higher surviving fraction upon photoreactivation were presumed to contain the *S.typhimurium phr* gene.

Sequencing

XbaI-KpnI and HincII-EcoRI fragments spanning the entire *phr* gene of *S.typhimurium* were subcloned into the corresponding sites of pIBI24 and pIBI25. Single strand DNA was obtained by superinfection with M13K07 (17) in DH5αF′ and sequenced by the dideoxy method using Sequenase (U.S. Biochemicals).

Overproduction of *S.typhimurium* photolyase

An NcoI site was created at the *S.typhimurium* phr gene starting codon (GTATGC→CCATGG), resulting in changing the second amino acid from proline to alanine, by site-directed mutagenesis (18). The NcoI-PstI fragment carrying the *phr* gene was then ligated with NcoI-PstI digested plasmid pKK233-2 to obtain pUNC1992. Following transformation of pUNC1992 into CSR603F′laci^Q, the ampicillin resistant colonies were isolated and grown in LB/ampicillin medium and tested individually for overproduction of photolyase after induction with IPTG. Cultures that appeared to have photolyase at > 5% of total cellular proteins were used for purification.

Purification of *S.typhimurium* photolyase

S.typhimurium photolyase was purified by a modified form of previously published procedures for *E.coli* photolyase (19). All purification steps were carried out at 4°C and the samples of chromatographic fractions were analyzed both by SDS electrophoresis and absorption spectra. Two liters of induced cell culture were harvested by centrifugation and the pellet was resuspended in 40 mL of cell lysis buffer. The cells were then frozen in dry ice−ethanol bath and stored at −80°C. Cells were thawed at 4°C and sonicated 10×10 sec with a Branson model W185 sonifier set at maximum output for the small tip. The sonicate was centrifuged at 20,000 g for 10 min and then 100,000 g for 1 hour. The proteins in the supernatant (Fraction 1, 55 ml) were precipitated by adding ammonium sulfate to 65% saturation and resuspended in 4 ml of buffer A and dialyzed against buffer A for 4 hours. The dialysate was loaded onto a 40 ml of Blue sepharose column equilibrated with buffer A. The column was washed with 80 ml of same buffer and proteins were eluted with buffer B. Fractions that contained photolyase were combined and dialyzed against buffer C (Fraction 2, 15 ml) overnight. Fraction 2 was loaded onto a 5 ml hydroxylapatite column equilibrated with buffer C. The column was washed with 2 column volumes of the same buffer and the proteins were eluted with a 25 ml gradient of buffer C to buffer D. Photolyase came off the column at around 200 mM potassium phosphate. Fractions containing photolyase were combined and dialyzed against buffer A (Fraction 3, 7 ml). Fraction 3 was loaded onto a 5 ml single strand DNA cellulose column equilibrated with buffer A. The column was washed with 2 column volumes of same buffer and the proteins were eluted by buffer B. Fractions containing photolyase were combined and dialyzed into storage buffer (Fraction 4, 14 ml). Fraction 4 was aliquoted and frozen in a dry ice-ethanol bath, and stored at −80°C.

Spectroscopy

The absorption spectra were measured with a Hewlett-Packard 8451A spectrophotometer. The fluorescence spectra were recorded with Shimadzu RF5000U spectrofluorophotometer. Spectrophotometric measurements of E-FADH$_2$-MTHF and of E-FADH$_2$ forms were conducted under anaerobic condition. To obtain enzyme containing reduced flavin, DTT was added to photolyase holoenzyme (E-FADH°-MTHF) to a final concentration of 70 mM in an anaerobic cuvette. Flash light (Vivitar 2500 flash units covered with 630 nm cutoff filters) was then used to photoreduce the flavin radical as monitored by absorption spectrum. Photolyase in this form (E-FADH$_2$-MTHF) was used to measure the folate fluorescence excitation and emission spectra. To obtain folate-free enzyme, the same enzyme solution was irradiated with black light (Spectroline, model B-100, longwave ultraviolet lamp) for 5 min at 4°C to decompose the folate as determined by the disappearance of the 384 nm peak. Flavin fluorescence spectra were then measured for this form (E-FADH$_2$) of the protein.

ASSAYS

Substrate

The substrate used in gel retardation and quantum yield assays was a ^{32}P-labeled 48 mer duplex DNA with a T<>T in the middle as described before (20,21). Poly (dT)$_{12-18}$ containing T<>T was prepared as described elsewhere (22).

Gel retardation

This assay was used to determine the DNA binding constant as described previously (20). We calculated the ES concentration by quantifying the radioactivity with free DNA (unbound) by an Ambis scanner (Ambis Radioanalytic Imaging System).

Quantum yield of DNA repair

The measurement was the same as described previously (23,24). Holoenzyme (E-FADH$_2$-MTHF) which was reconstituted by supplementing purified enzyme with MTHF (12) was used and the irradiation was conducted at 384 nm. Briefly, 350 μl reaction mixture contained 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 20 mM DTT, 100 μg/ml bovine serum albumin, 0.86 unit/ml Oxyrase (Oxyrase, Inc.), 150 mM lactate, 0.5 nM substrate, 4−5 μM photolyase. The mixture was subjected to 384 nm irradiation anaerobically at fluence rate of 20 erg/mm^2/s by a Quantacount monochromator (Photon Technology International) and samples taken at various fluences were analyzed on a 12% sequencing gel after T4 endonuclease V digestion. The bands were cut out and quantitated by Cerenkov counting and the data were analyzed by the method of Rupert (1962) (25).

Catalytic constant measurement

The substrate used here was polydT$_{(12-18)}$, in which ~ 70% of Ts were converted to T<>T by acetone-sensitized irradiation (22). The reaction mixture included 50 mM Tris, pH 7.4, 1 mM EDTA, 100 mM NaCl, 75 mM DTT, 30 μM T<>T, 10% glycerol and 2−3 μM defolated photolyase (E-FADH$_2$). The mixture was exposed to 3 flashes anaerobicly in order to photoreduce the radical flavin (which also resulted in repair of about 10% of T<>T), then the sample was exposed to 366 nm light irradiation at a fluence rate of 110 ergs/mm^2/sec. Absorption spectra of samples were measured at several fluences and the repair was quantified from the level of increase at 264 nm (26).

RESULTS

Cloning of *S.typhimurium phr* gene

Based on the notion that DNA photolyase is a monomer and functionally independent of other cellular proteins, we used *in vivo* complementation of the *phr* defect in *E.coli* to clone the *S.typhimurium phr* gene. Intially, *E.coli* strain CSR603 (*recA1 uvrA6 phr-1*) was used as the host strain; however, the clones that survived after UV and photoreactiving light irradiation were those predominately containing the Salmonella *recA* gene, apparently because of higher resistance of *recA*$^+$ *phr*$^-$ compared to *recA*$^-$ *phr*$^+$ to these treatments. Therefore, CSR06 (*uvrA6 phr-1*) was used in subsequent experiments. We transformed 0.1 μg DNA of the Salmonella library into CSR06. The expressed transformants were plated on LB/ampicillin plates and irradiated with 100 erg/mm^2 of 254 nm UV light followed by 20,000 ergs/mm^2 of 365 nm photoreactivating light. DNA was isolated from colonies which survived these treatments and transformed into *E.coli* CSR603. The transformants were screened by irradiating with 5 ergs/mm^2 UV light and 20,000 ergs/mm^2 of 365 nm light. Several plasmids complementing the *phr* defect in *E.coli* were identified. One of these was chosen for further study. It contained an insert of about 3 kbp and complemented the *phr* defect of *E.coli* CRS603 (**Table I**).

Table I. Complementation of *E.coli* (E.c.) *phr*$^-$ *by S.typhimurium* (S.t.) *phr* gene.

Strain	UV (5ergs/mm^2)$^+$ No PR	PR (2×10^4 ergs/mm^2)
CSR603	1.1×10^{-6}	2.2×10^{-6}
CSR603/PMS1310 (E.c.)	1.9×10^{-6}	7.9×10^{-3}
CSR603/pUC18-phr (S.t)	4.1×10^{-7}	1×10^{-3}

Surviving fractions after UV and UV plus photoreactivation (PR).

Sequence of S.typhimurium phr gene

To obtain the sequences of both strands, the XbaI-KpnI fragment encompassing the whole Salmonella *phr* gene and the HincII-EcoRI fragment containing about one third of the DNA fragment were used for sequencing by dideoxy-chain termination method using Sequenase. A 1419 base-pair long open reading frame was found in the DNA fragment sequenced. The open reading frame is 73% homologous to the *E. coli phr* gene and the translated gene product yields a protein of amino acid sequence with 80% identity with *E.coli* photolyase (**Figure 1**). There are potential promoter sites within 200 bp 5′ to the coding region and the

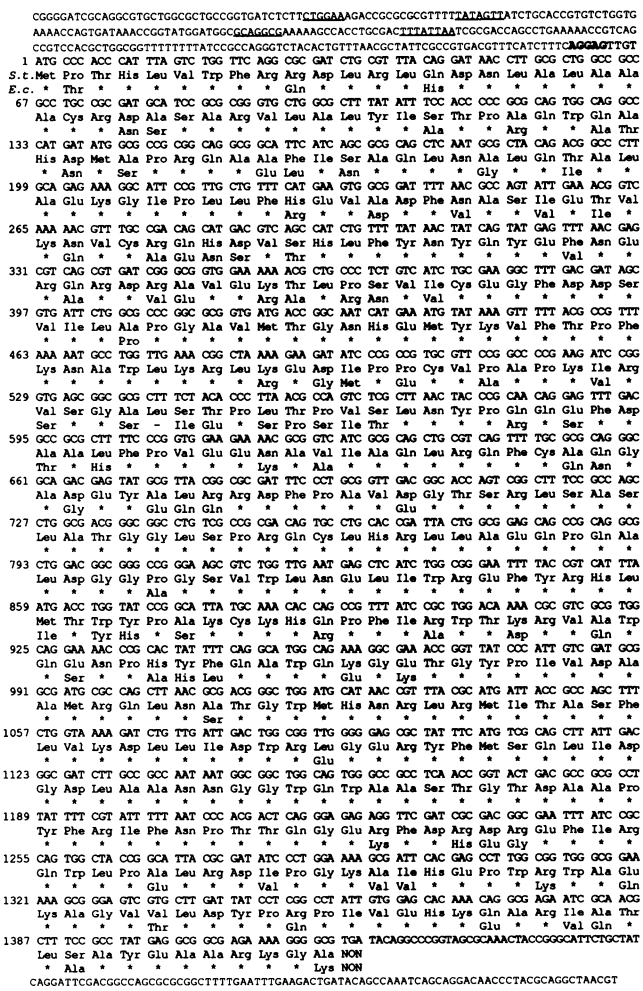

Figure 1. Nucleotide sequence of *S.typhimurium phr* gene and the amino acid sequence of encoded polypeptide. The possible Pribnow box and −35 sequences are underlined and the ribosome binding site is highlighted. Numbers indicate the nucleotides position, starting from the first nucleotide of the *phr* gene. *E.coli* photolyase amino acid sequence is shown under that of *S.typhimurium* protein. The identical residues are represented by an asterisk.

initiation codon is preceded by 4 bp with a good match to the ribosome binding site. These findings combined with the *in vivo* complementation test lead us to conclude that we have cloned the *S.typhimurium* DNA photolyase gene. Interestingly, the DNA sequence homology rapidly falls off 5′ and 3′ to the *phr* gene.

Overproduction of photolyase

To study the *in vitro* characteristics of the Salmonella photolyase, an overproducing plasmid pUNC1992, was constructed. An NcoI site was created by site-specific mutagenesis at the starting codon of the Salmonella *phr* gene, followed by NcoI and PstI digestion, the NcoI-PstI fragment including the whole *phr* gene was ligated to trc promoter containing plasmid pKK233-2 to obtain pUNC1992 (**Figure 2**).. *E.coli* CSR603F′lacI^Q carrying this plasmid overexpressed the *S.typhimurium* photolyase to ~7% of total cellular protein after IPTG induction (**Figure 3**).

Purification of Salmonella photolyase

Salmonella photolyase was purified from 2 liters of IPTG induced overnight culture of CSR603FlaciQ/pUUNC1992. Cell free extract (Fraction 1) was loaded onto a Blue-Sepharose column and the bound photolyase was eluted with 2 M KCl buffer. Following dialysis the sample was loaded onto Bio-Gel hydroxylapatite column which was developed with a gradient of 67 mM−330 mM potassium phosphate buffer. Photolyase was eluted at 150 mM K-phosphate. The photolyase fraction from this column was further purified by chromatography on single-stranded DNA cellulose column. After this step the enzyme was 92% pure and the overall recovery was 15% (**Table II**). *S.typhimurium* photolyase migrated on SDS gel at apparent M.W. of 54 kDa, which is consistent with the calculated M.W. from amino acid content (**Figure 3**). A summary of the purification steps is shown in **Table II**.

Spectroscopy

The purified *S.typhimurium* photolyase is purple-blue in color with an absorption spectrum similar to that of *E.coli* protein. Both proteins absorb between 300−700 nm with a maximum peak at 384 nm and a series of long wavelength (400−600 nm) well-defined bands. The purified *S.typhimurium* photolyase, in addition to the 384 nm peak, has absorption maxima at 480, 580, and 625 nm. As the absorption spectrum at λ >425 nm is typical of flavin neutral radical we conclude that Salmonella photolyase contains FADH° (**Figure 4**). When the flavin radical is reduced chemically with dithionite or photochemically by irradiation with

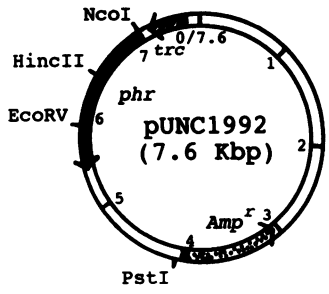

Figure 2. Partial restriction map of the photolyase overproducing plasmid pUNC1992. The positions of the trc promoter and the *phr* and Amp^R genes are indicated.

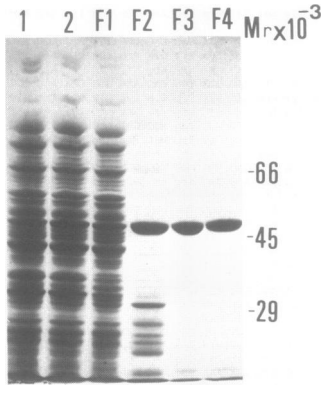

Figure 3. Purification of *S. tyhphimurium* photolyase. Samples from each step of purification were analyzed on a 10% SDS-polyacrylamide gel which was stained with Coomassie blue and photographed. Lanes 1 and 2 contain cells from 200 μl of overnight cultures of pUNC1992/CSR603F′laciQ that were grown without and with 1 mM IPTG induction, respectively. The other lanes contained samples from successive steps of purification as described in the text. The amounts loaded were approximately 50, 10, 13, and 11 μg of protein for F1 through F4, respectively. Molecular weight markers are indicated on the right.

Table II. Purification of *S.typhimurium* photolyase

Fraction	Total Protein (mg)	%	mg	Yield (%)
I. Cell-free extract	677	7	44	100
II. Blue-sepharose	42	35	15	33
III. Hydroxylpatite	9.8	80	7.8	18
IV. DNA cellulose	7.1	92	6.5	15

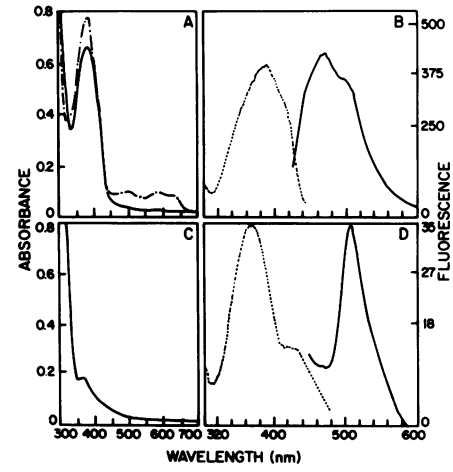

Figure 4. Spectral characteristics of the chromophores in *S.typhimurium* photolyase. The samples were prepared as described in the text. The photolyase concentration was 25 μM. **(A)** Absorption spectrum of folate supplemented holoenzyme, in radical flavin form (E-MTHF-FADH°) (dot-dashed line) and fully reduced flavin form (E-MTHF-FADH$_2$) (solid line). **(B)** Excitation and emission spectra of folate supplemented holoenzyme with fully reduced form of flavin (E-MTHF-FADH$_2$). Excitation spectrum (dotted line) was taken at emission wavelength 470 nm. Emission spectrum (solid line) was taken at excitation wavelength 384 nm. **(C)** Absorption spectrum of fully reduced flavin form protein (E-FADH$_2$), after the folate was photodecomposed by black light. **(D)** Excitation and emission spectra of E-FADH$_2$. Excitation spectrum (dotted line) was taken at emission wavelength 505 nm; emission spectrum (solid line) was taken at excitation wavelength 366 nm.

λ >425 nm in the presence of dithiothreitol, the long wavelength absorption peaks disappear with the 384 nm absorption basicly unchanged. We ascribe the 384 nm absorption to MTHF. At this stage (E-FADH$_2$-MTHF) the enzyme has a fluorescence excitation maximum at 384 nm and emission maximum at 470 nm which are characteristic of the folate cofactor (**Figure 4B**). Irradiation of the enzyme with strong black light results in a drastic decrease in the 384 nm peak, apparently because of photodecomposition of MTHF as was observed with the *E.coli* photolyase (29) and the absorbance peak shifts to 366 nm (**Figure 4C**), which is exclusively due to fully reduced flavin (E-FADH$_2$). The fluorescence excitation and emission spectra of enzyme at this stage yield λ_{ex}=366 nm and λ_{em}=505 nm (27,28), typical of enzyme-bound fully reduced flavin (**Figure 4D**). All these properties are shared by the *E.coli* photolyase. Therefore, we conclude that *S.typhimurium* photolyase like the *E.coli* enzyme has two chromophores, flavin and MTHF. This was also confirmed by the observation that *S.typhimurium* photolyase can be supplemented by methenyltetrahydrofolate (monitored by increase of absorption at 384 nm) and flavin radical was photoreduced by DTT (disappearance of long wavelength absorption after light irradiation). These are photochemical reactions characteristics of folate (MTHF) and flavin (FADH°) which occur with *E.coli* photolyase (29,12).

Catalytic properties

We investigated the substrate binding and catalysis by Salmonella photolyase. The equilibrium binding constant of photolyase was determined by a gel retardation assay with a defined duplex DNA substrate containing a single T<>T. The K_D obtained was 1.6×10^{-9} M. The quantum yield of photorepair of Salmonella photolyase holoenzyme (E-FADH$_2$-MTHF) was determined only at the λmax in near-UV (384 nm) and was found to be 0.5 for the holoenzyme. Both K_D and quantum yield of *S.typhimurium* photolyase are close to those of the E.coli enzyme. We also measured the kcat by a spectrophotometric assay (26) with T<>T containing oligo $(dT)_{12-18}$ as substrate. Under enzyme-limiting conditions and at a fluence rate of 110 ergs/mm^2/sec, a value of 1.8 min^{-1} was obtained, which is similar to the value of *E.coli* protein measured by same method and under the same conditions (S.T. Kim, unpublished observation). The k_{cat} of photolyase is light-intensity-dependent and increased linearly with light intensity up to the maximum fluence rate we could achieve with our light source. It is likely that the actual k_{cat} is close to 50 min^{-1} under near-saturating light as was found with *E.coli* photolyase (unpublished observation). Properties of the *S. typhimarium* photolyase are summarized in **Table III**.

Table III. Properties of *S.typhimurium* photolyase

Gene size	1419 bp
Protein size	473 amino acids
Mr	53,932
Color	Blue-purple
Absorption maxima (nm)	
E-FADH°-MTHF	384,480,580,625
E-FADH$_2$-MTHF	384 (ε=29,500)
E-FADH$_2$	366 (ε=5,700)
Fluorescence excitation (nm)	
E-FADH°-MTHF and E-FADH$_2$-MTHF	384
E-FADH$_2$	366
Fluorescence emission (nm)	
E-FADH°- and E-FADH$_2$-MTHF	470
E-FADH$_2$	505
Specific binding constant (K_D)	1.6×10^{-9} M
Quantum yield of repair (E-FADH$_2$-MTHF)	0.5
k_{cat} (at fluence rate of 110 ergs/mm^2/sec)	1.8 min^{-1}

DISCUSSION

Photolyase from *S.typhimurium* is the third enzyme isolated from eubacteria. The other two are from *E.coli* (30) and *Streptomyces griseus* (31). *S.griseus* photolyase is a deazaflavin class protein (λmax 440 nm), which has 36% amino acid homology with *E.coli* protein. Our results show that photolyase from *S.typhimurium* belongs to the folate class as does the *E.coli* enzyme (λmax 384 nm). *S.typhimurium* and *E.coli* photolyases have 80% identity and 88% homology in amino acid sequence. This is comparable to the homology levels found between other genes of these two organisms (16,32,33). Interestingly, however, the sequences immediately 5′ and 3′ to the *phr* gene of Salmonella have no significant homologies to those in *E.coli*. Especially noteworthy is the lack of the orf169 5′ to the Salmonella *phr* gene. In *E.coli*, an orf169 which encodes a 20 kDa protein is located 5′ to *phr*, the termination codon of orf169 overlaps the initiation codon of *phr* (9) and the two genes are apparently co-transcribed (34) and thus constitute an operon.

Purified *S.typhimurium* protein has neutral radical form flavin and substoichiometric amount of folate as does the *E.coli* protein. Because of the similarities between the two enzymes regarding the behavior of the chromophores, the amino acids which are different between the two proteins cannot be important in maintaining the *in vivo* status (stoichiometric MTHF and fully reduced flavin) of the chromophores. It is especially interesting that of the 6 photolyases belonging to the two classes that have been purified to date (*E.coli*, *S.typhimurium*, *S.cerevisiae*, *A.nidulans*, *S.griseus*, and *M.thermoautotrophium*) only the yeast enzyme retains its flavin cofactor in its catalytically active state (FADH$_2$) suggesting that certain amino acid residues unique to the yeast enzyme might be involved in maintaining the flavin in the fully reduced form.

Most of the nonconserved amino acid residues between *S.typhimurium* and *E.coli* protein are distributed at the N-terminal part of the protein. This is consistent with the observation that all photolyases sequenced to date are highly conserved at the C-terminal half. The structural and functional importance of the C-terminal 'domain' is currently under investigatioin in several laboratories. We have shown that in *E.coli* photolyase, W277 and W384 are involved in substrate binding (Li and Sancar, 1990), and W306 is the intrinsic electron (H atom) donor for flavin photoreduction (Li *et al.*, 1991). Since the chromophores in *E.coli* and *S.typhimurium* photolyases behave the same and since all the tryptophan residues have been conserved between these two proteins, it is conceivable that the corresponding tryptophans in *S.typhimurium* protein might perform the same function in DNA substrate binding and catalysis.

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