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The Folate Cofactor of *Escherichia coli* DNA Photolyase Acts Catalytically*

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Escherichia coli **DNA photolyase catalyzes the light-driven (300–500 nm) repair of pyrimidine dimers formed between adjacent pyrimidine bases in DNA exposed to UV light (200–300 nm). The light-driven repair process is facilitated by two enzyme-bound cofactors, $FADH_2$ and 5,10-methenyltetrahydrofolate. The function of the folate has been characterized in greater detail in this series of experiments. Investigations of the relative binding affinities of photolyase for the monoglutamate and polyglutamate forms of 5,10-methenyltetrahydrofolate show that the enzyme has a greater affinity for the naturally occurring polyglutamate forms of the folate and that the exogenously added monoglutamate derivative is less tightly associated with the protein. Multiple turnover experiments reveal that the folate remains bound to photolyase even after 10 turnovers of the enzyme. Examination of the rates of repair by photolyase containing stoichiometric folate in the presence or absence of free folate under multiple turnover conditions and at micromolar concentrations of enzyme also demonstrates that the folate acts catalytically. The stimulation of turnover by exogenous folate seen at low concentrations of photolyase is shown to be due to the lower affinity of photolyase for the monoglutamate derivative used in reconstitution procedures. These results demonstrate that the folate of *E. coli* DNA photolyase is a bona fide cofactor and does not decompose or dissociate during multiple turnovers of the enzyme.**

Exposure of DNA to UV (200–300 nm) light causes the formation of pyrimidine dimers between adjacent pyrimidines in DNA. One cellular defense against these lesions is the enzyme DNA photolyase (deoxyribodipyrimidine photolyase, EC 4.1.99.3). DNA photolyase binds to *cis-syn* pyrimidine dimers in a light-independent step and then catalyzes the photoreversal of these dimers to monomers in a light-dependent (300–500 nm) step. Purified photolyase from *Escherichia coli* and *Saccharomyces cerevisiae* contains two prosthetic groups, $FADH_2$ and CH^+-H_4 folate[1] (1, 2), which facilitate the repair reaction. We have previously shown that the folate cofactor of the enzyme serves to transfer light energy to the flavin, which then catalyzes the (2 + 2) cycloreversion of the photodimer by electron transfer (3–5). However, it was unclear from these studies whether the folate lost its functionality after a single event or could participate in multiple turnovers. Indeed, we have observed that at low concentrations of enzyme (5–10 nM) and under limiting light and single turnover conditions, photolyase can be stimulated to turn over more rapidly in the presence of excess folate. This observation combined with the fact that the enzyme-bound folate is unstable under prolonged exposure to high intensity light (3, 4) led us to address the question of whether the folate functioned catalytically in the enzyme activity. The results of these studies are presented in this paper.

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[1] The abbreviations used are: CH^+-H_4folate, 5,10-methenyltetrahydrofolate; HPLC, high performance liquid chromatography; DTT, dithiothreitol; 10-CHO-H_4folate, 10-formyltetrahydrofolate; 10-CHO-PteGlu$_n$, 10-formylfolylpolyglutamate with n glutamate residues.

MATERIALS AND METHODS

Chemicals and Reagents—Sodium borohydride, 5-CHO-H_4folate, folic acid, and bovine serum albumin were from Sigma. ^3H-Labeled 3′,5′,7,9-folic acid (43 mCi/μmol) was purchased from Amersham Corp. Oligo(dT)$_{15}$ was from Operon Biotechnology Inc. All reagents for gel electrophoresis were from Bio-Rad. HPLC grade methanol and ammonium acetate were from Fisher.

Enzyme Purification—Purification of DNA photolyase from *E. coli* MS09 (CSR603 F′ *lac*I^Q/pMS969) (6) induced for overproduction of the enzyme was as previously described up to the blue Sepharose step (7). After elution from blue Sepharose, the fractions containing photolyase were pooled, dialyzed overnight against 10 mM potassium phosphate, pH 6.8, containing 1 mM dithiothreitol, 20% glycerol, 1 mM EDTA and mixed with 40–60 ml of a $Ca_3(PO_4)_2$ gel suspension in the same buffer with stirring for 20 min on ice. The mixture was centrifuged in a Sorvall SS34 rotor at 10,000 rpm for 5 min, and the supernatant was discarded. The gel was triturated with 30–40 ml of 10 mM potassium phosphate, 1 mM DTT, 1 mM EDTA and centrifuged, and the supernatant was discarded. Similar treatments with 20 and 50 mM potassium phosphate containing 1 mM DTT and 1 mM EDTA were each carried out in duplicate, and the supernatants were discarded. Finally, the enzyme was eluted from the gel by using 400 mM potassium phosphate, 1 mM DTT, 1 mM EDTA, dialyzed overnight against 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, 50% glycerol (storage buffer), and stored at −20 °C. All treatment of photolyase was at 4 °C unless otherwise indicated in the text.

Synthesis of Folates—Synthesis of ^3H-labeled 3′,5′,7,9-CH^+-H_4folate was performed as described previously (4). Final specific activity was between 15 and 20 mCi/mmol. The labeled CH^+-H_4folate was purified twice by HPLC on a C-18 column (Alltech Associates, Los Altos, CA) using a gradient of 20–40% methanol at pH 2 and subjected to vacuum centrifugation to remove the methanol. The ^3H-labeled 3′,5′,7,9-CH^+-H_4folate was resuspended in 0.01 N HCl and stored at 4 °C in the dark. Unlabeled CH^+-H_4folate and 10-CHO-H_4folate were prepared as previously described (4).

Dissociation Experiments—Borohydride treatment of DNA photolyase was based on the procedure described previously (4, 8). 1 ml of photolyase in storage buffer (~1 mg/ml) was diluted with 1 ml of 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, and 300 μl of 340 mM sodium borohydride in 50 mM ammonium bicarbonate, pH 9.0, was added. The mixture was incubated on ice for 2 h and then

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chromatographed on a Sephadex PD10 column (Pharmacia LKB Biotechnology Inc., 9.1-ml bed volume) to remove unreacted borohydride. The excluded protein fraction (2.5 ml) was examined spectrophotometrically using a Shimadzu 260 spectrophotometer to ensure that all folate was removed, and the folate-free enzyme was reconstituted with ^{3}H-labeled CH$^+$-H$_4$folate as previously described (4), with the inclusion of a potassium ferricyanide treatment (10–30 μl of a 20 mM solution) to convert any FADH$_2$ to the neutral blue flavin radical, FADH· (9). This mixture was chromatographed again on a PD10 column equilibrated in the desired buffer. The reconstituted enzyme containing ^{3}H-labeled CH$^+$-H$_4$folate was treated by dilution into different buffers, incubated at different temperatures, and then centrifuged through spin columns (4, 10, 11) equilibrated in the same buffer. The excluded and included fractions were collected as previously described (4) and counted in a Beckman model LS1801 scintillation counter. The amount of ^{3}H-labeled CH$^+$-H$_4$folate remaining bound to undiluted enzyme (1-5 μM) was taken as 100%, since dialysis experiments at this concentration failed to remove folate (data not shown). Background counts of buffer were subtracted as well.

Gel Retardation Assays—All gel retardation assays and multiple turnover assays were performed under yellow light. Gel retardation assays of photolyase activity were performed as described previously (4). The substrate, a 48-mer duplex containing a single thymine dimer at a specific internal site, was prepared as described previously (12). The concentration of excess free CH$^+$-H$_4$folate ranged from 2 to 5 μM when present. Repair was measured by locating the repaired and unrepaired DNA by autoradiography followed by excision of the band corresponding to repaired DNA and quantitation by Cerenkov counting.

T4 Endonuclease V Assays—T4 endonuclease V is an endonuclease specific for pyrimidine dimers and cleaves the glycosidic bond of the 5′ thymine of thymine dimer and the intradimer phosphodiester bond. When assayed with ^{32}P-labeled 48-mer dimer-containing DNA as substrate, cleavage of unrepaired DNA generates two radiolabeled 24-mers which can be seen on a 12% sequencing gel. T4 endonuclease V can therefore be utilized with photolyase in a coupled assay to measure DNA repair (13).

Reaction mixtures containing 1 μM photolyase in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 20 mM DTT, 16% glycerol, 100 μg/ml bovine serum albumin (photolyase reaction buffer) were incubated with 20 μM dimer-containing oligo(dT)$_{15}$ and trace amounts of double-stranded 48-mer substrate such that oligo(dT)$_{15}$ was in 2×10^6-fold excess over the 48-mer. Mixtures were incubated for 10 min at room temperature to allow binding to dimers, after which the repair of the 48-mer by photolyase was quantitated by the T4 endonuclease V assay as follows. Aliquots of the reaction mixture were withdrawn after exposure to photoreactivating light from a black lamp (General Electric), and the DNA was isolated by phenol extraction, ether extraction, and ethanol precipitation. The photoreactivated DNA for each sample was resuspended in 12 μl of H$_2$O, 2 μl of 10 mM dithiothreitol, 2 μl of 1 mg/ml bovine serum albumin, and 2 μl of 10-fold concentrated T4 endonuclease V reaction buffer salts (0.5 M Tris-HCl, pH 8.0, 1 M NaCl, 10 mM EDTA). After addition of 2 μl of T4 endonuclease V (~20 units), the samples were incubated at 37 °C for 1 h. The samples were then lyophilized and resuspended in 10 μl of formamide containing 0.05% bromphenol blue and 0.05% xylene cyanol, heated at 95 °C for 3–4 min, cooled on ice, and loaded onto a 12% sequencing gel. After electrophoresis, the gel was autoradiographed and the bands corresponding to full-length (repaired by photolyase) DNA and incised (unrepaired) DNA were quantitated by gel scanning. Such assays were used to define the appropriate irradiation conditions for all of the multiple turnover experiments for the determination of potential folate release and also for the folate exchange experiments. Alternatively, repair of the oligo(dT)$_{15}$ in the reaction mixtures was measured by the spectrophotometric assay (14), which confirmed the results obtained with the coupled assay.

Exchange Experiments—Photolyase that had been borohydride-treated and reconstituted with ^{3}H-labeled CH$^+$-H$_4$folate as described in the previous section was incubated in reaction buffer with and without dimer-containing oligo(dT)$_{15}$ such that dimers were in 20-fold molar excess (20 *versus* 1 μM photolyase). The mixture was incubated with and without irradiation and with and without 25-fold excess unlabeled CH$^+$-H$_4$folate. The duration of irradiation was determined by the T4 endonuclease V assay described above. After the reaction, samples were loaded on Centricon 30 microconcentrators (Amicon, Millipore Co.) and centrifuged at 6000 rpm for 15 min at 4 °C in an SS34 rotor. Samples were rinsed with 500 μl of reaction buffer, and the centrifugation was repeated. The filtrate (small molecule) and retained (enzyme-bound) fractions were counted. The ^{3}H-labeled CH$^+$-H$_4$folate remaining bound to 1 μM enzyme incubated without DNA, irradiation, or excess unlabeled CH$^+$-H$_4$folate was taken as 100% retention, and other numbers were normalized to this value.

Catalytic Folate—Photolyase (1 μM) that had been borohydride-treated and reconstituted with ^{3}H-labeled CH$^+$-H$_4$folate as described in the previous section was incubated in reaction buffer with and without excess dimers (20 μM in oligo(dT)$_{15}$). Mixtures were exposed to photoreactivating light from a black lamp such that 10 enzyme turnovers would occur as determined by the T4 endonuclease V assay. Samples were then applied to Centricon 30 microconcentrators and treated as described in the previous section. The enzyme-bound radioactivity was determined by counting the filtrate (small molecule) and retained (enzyme-bound) fractions. The 100% retention value was set as the percentage of counts retained by ^{3}H-labeled CH$^+$-H$_4$folate-containing photolyase in the absence of DNA and irradiation, and the data were normalized to this value.

The T4 endonuclease V assay was also performed as described above using borohydride-treated photolyase, supplemented photolyase, and supplemented photolyase plus excess folate. These assays were designed so that the rate of repair under limiting light conditions could be assessed after 10 enzyme turnovers. Repair was quantitated by gel scan of the autoradiograms of each assay.

Analysis of Folylpolyglutamates—Analysis of the distribution of folylpolyglutamates bound to photolyase was based on a procedure described previously (2). Briefly, photolyase containing a mixture of bound CH$^+$-H$_4$folylpolyglutamates and ^{3}H-CH$^+$-H$_4$folate was incubated without added CH$^+$-H$_4$folate or with a 5- or 25-fold excess of CH$^+$-H$_4$folate for 10 min at room temperature and then passed over Sephadex G-25 columns (PD10) in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA (PD10 buffer) to remove unbound folate. A solution of KI/I$_2$ (1 g of I$_2$ and 2 g of KI in 100 ml of water) was then added to the protein, and the mixture was incubated for 3 h or more to oxidize CH$^+$-H$_4$folate and CH$^+$-H$_4$folylpolyglutamates to 10-CHO-folate and 10-CHO-folylpolyglutamates. After this time, mixtures were acidified to pH 1, centrifuged in an Eppendorf microcentrifuge, centrifuged again through Spin-X columns (Costar), and applied to a C-18 HPLC column (Alltech Associates, Los Altos, CA) equilibrated in 50 mM ammonium acetate at pH 6.8. The HPLC solvent delivery system and Fluoromonitor III were from LDC.

Reconstitution of Photolyase with a Heterogeneous Mixture of CH$^+$-H$_4$folylpolyglutamates—A mixture of CH$^+$-H$_4$folylpolyglutamates was obtained by releasing the cofactor from unsupplemented photolyase. A solution containing several milligrams of photolyase in storage buffer was acidified to pH 1 with 4 N HCl, centrifuged for 5 min in an Eppendorf microcentrifuge, neutralized immediately with 4 N NaOH, and centrifuged again for 5 min. The supernatant (1 ml) was added to 1 ml of photolyase in storage buffer (0.5 mg, containing 0.3 mol of folate/mol of enzyme) and incubated for 30–60 min on ice in the dark. The mixture was then chromatographed on a PD10 column equilibrated in photolyase reaction buffer to remove all unbound material.

RESULTS

Folate Effect—When *E. coli* DNA photolyase fully supplemented with CH$^+$-H$_4$folate was assayed by the gel retardation technique under enzyme excess conditions but at low (5–10 nM) concentrations, the repair kinetics shown in the *upper gel* in Fig. 1 were observed. However, if excess CH$^+$-H$_4$folate was added to the reaction mixture, the rate of repair was stimulated as shown in the gel in the *lower panel* of Fig. 1. This reproducible phenomenon was designated the "folate effect." Controls showed that DNA incubated with excess of CH$^+$-H$_4$folate and exposed to photoreactivating light in the absence of enzyme did not produce such an effect (data not shown). Preirradiation of the DNA in the presence of excess CH$^+$-H$_4$folate followed by addition of photolyase and examination by the gel shift assay also failed to produce any visible change in the ability of the DNA to be bound by photolyase (Fig. 1, *bottom*, *lanes 9* and *10*).

The observed folate effect might be attributed to one of two possibilities. It was possible that at low (nM) concentrations

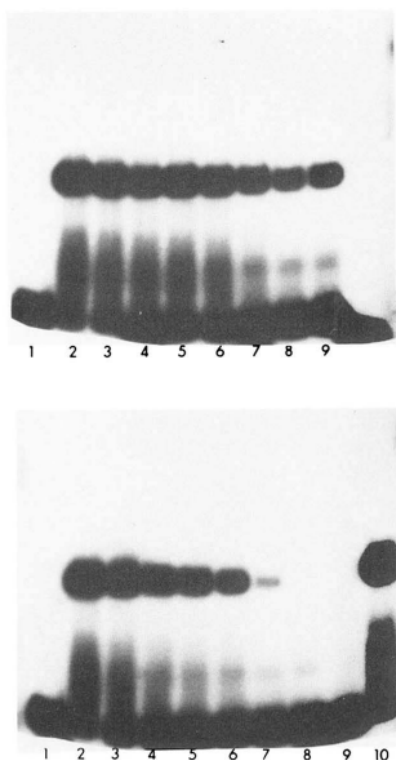


FIG. 1. **Gel retardation assays (single turnover) for photolyase supplemented with stoichiometric folate in the absence (*top*) or presence (*bottom*) of excess CH+-H4folate.** *Top, lane 1* contained DNA only. *Lanes 2–9* contained DNA and a 10-fold excess of photolyase. The sample in *lane 2* was not photoreactivated. The photoreactivating fluences for *lanes 3–9* were 750, 1500, 2250, 3000, 6000, 9000, and 9000 erg/mm^2, respectively. *Bottom, lanes 1–8* received identical fluences to the comparable lanes in the *top gel. Lane 9* contained DNA incubated with excess CH^+-H_4folate and was given a fluence of 9000 erg/mm^2. *Lane 10* contained an aliquot of the DNA from *lane 9* that was then incubated with 10-fold excess photolyase for 20 min. The *upper bands* on the gel represent the enzyme-DNA complexes (unrepaired), while the *lower bands* represent free DNA (repaired). Photoreactivations were performed with a black lamp with maximum output at 365 nm.

of photolyase the folate dissociated from the enzyme and that the presence of excess folate shifted the binding equilibrium in the direction of enzyme-bound folate. It also seemed possible that absorption of light by the folate could degrade the folate and cause its release from the enzyme. In this case, the excess folate in the reaction mixture would replace the modified folate and lead to a higher rate of repair. The observation that the folate cofactor is photodecomposed by prolonged exposure to high intensity light (3, 4) might also be attributed to the lability of a high energy folate intermediate. This folate effect therefore raised the question of whether the folate of photolyase was noncatalytic or catalytic. This issue is of particular importance in folate biochemistry, as there are no known examples of reactions which use folate catalytically.

Binding Curves—In order to simplify the analysis of the dissociation of folate from photolyase, the binding of the monoglutamate derivative of the folate cofactor, CH^+-H_4folate, to photolyase was examined. Traditional techniques like equilibrium dialysis could not be used to determine the binding constant of this derivative because of the instability of free CH^+-H_4folate in the pH range within which the enzyme is stable. Instead, photolyase was borohydride-treated to remove endogenous folylpolyglutamates (4) and reconstituted with ^{3}H-labeled 3′,5′,7,9-CH^+-H_4folate. Dilutions of the fully reconstituted enzyme were made into appropriate buffers, incubated at various temperatures, and the excluded (protein-bound) and included (small molecule) fractions were separated by centrifugation on spin columns and analyzed for the distribution of radioactivity. Results from several experiments are shown in Fig. 2. These results indicate that dissociation is dependent on the buffer composition and the temperature. The *upper curve* represents the dissociation of the folate from enzyme incubated on ice in the photolyase reaction buffer. The *lower curve* represents two sets of points obtained either at room temperature in photolyase reaction buffer or after incubation on ice in PD10 buffer. The conditions of the gel retardation assay when the folate effect is observed are similar to those of the room temperature incubations and indicate that under these conditions at 100 nM photolyase at least 50% of the CH^+-H_4folate originally bound by the enzyme has been released. The fact that the buffer appears to affect the dissociation indicates that perhaps the equilibrium between enzyme-bound and free CH^+-H_4folate is affected by the conversion of free CH^+-H_4folate to 10-CHO-H_4folate as well as the resulting oxidation of the labile 10-CHO-H_4folate. Oxidation of the 10-CHO-H_4folate species is prevented by DTT, and the presence of DTT would be expected to maintain a higher concentration of CH^+-H_4folate and thus cause a lesser extent of dissociation.

Exchange of Enzyme-bound CH^+-H_4folate—In view of the observed concentration-dependent dissociation of CH^+-H_4folate under the assay conditions used to obtain the folate effect, the possible exchange between the enzyme-bound CH^+-H_4folate and free folate in solution was examined using ^{3}H-labeled CH^+-H_4folate-containing enzyme and excess unlabeled CH^+-H_4folate. These experiments showed that in the presence or absence of dimers and the presence or absence of light and after 10 and 20 min of photoreactivation sufficient to induce multiple turnovers, only 1–8% of the ^{3}H-labeled CH^+-H_4folate remained bound to the enzyme.

Polyglutamate Exchange Experiments—Previous results had demonstrated that the folate cofactor released from purified photolyase is a heterogeneous mixture of polyglutamate derivatives of CH^+-H_4folate (2) containing the novel $(\gamma_3)(\alpha_n)$ linkage identified in folylpolyglutamates of *E. coli* (15, 16). It was not possible to directly address the issue of dissociation of the CH^+-H_4folylpolyglutamates from the enzyme by using binding curves with ^{14}C- or ^{3}H-labeled CH^+-H_4folylpolyglutamates since the *E. coli* folylpolyglutamate derivatives were not available. However, an alternative procedure was developed to examine the exchange of the polyglutamate derivatives of CH^+-H_4folate. Enzyme containing a heterogeneous mixture of folylpolyglutamates (approximately 0.3 mol of folate/mol of enzyme) was incubated under conditions similar to those used for the monoglutamate exchange experiments. Excess monoglutamate was separated from the enzyme by chromatography on PD10 columns, and the enzyme was treated with iodine to oxidize enzyme-bound CH^+-H_4folates to 10-CHO-folate derivatives. By using HPLC to resolve the polyglutamate derivatives of 10-CHO-folate, it was possible to see if the polyglutamates were replaced by monoglutamate.

The results of such an experiment are shown in Fig. 3. In this experiment, photolyase containing 30% of the folate as heterogeneous CH^+-H_4folylpolyglutamate was reconstituted in the remaining apofolate sites with ^{3}H-labeled CH^+-H_4folate and then incubated with different amounts of excess unlabeled CH^+-H_4folate. Under conditions in which 65–70% of the ^{3}H-labeled monoglutamate was exchanged in the presence of 5- and 25-fold excess CH^+-H_4folate (data not shown), at least 50–60% of the triglutamate and tetraglutamate folate derivatives are exchanged. However, neither the pentagluta-

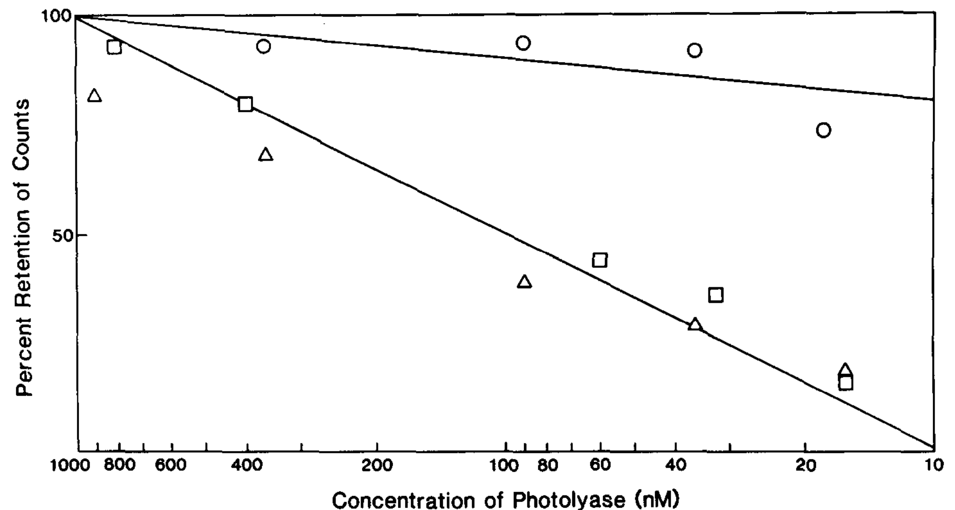


FIG. 2. Photolyase containing ^{3}H-labeled CH^+-H_4folate was diluted into 50 mM Tris-HCl, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol for 60 min on ice (□, *lower curve*) or into photolyase reaction buffer for 30 min at room temperature (Δ, *lower curve*) or on ice (O, *upper curve*). After the incubations, samples were centrifuged through spin columns and the excluded (enzyme-bound) and included (small molecule) fractions were collected and counted.

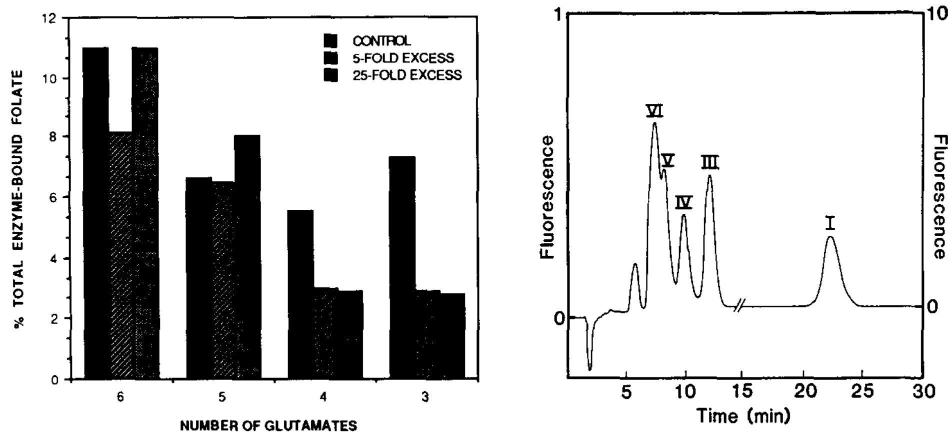


FIG. 3. Photolyase (7 μM) containing approximately 0.3 mol of CH^+-H_4folylpolyglutamate and 0.7 mol of ^{3}H-labeled CH^+-H_4folate/mol of enzyme was incubated with 5- or 25-fold excess unlabeled CH^+-H_4folate in 50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA for 10 min at room temperature. The mixtures were chromatographed over PD10 columns in the same buffer to remove unbound folate. The excluded (enzyme) fractions (2.0 ml) were incubated with 300 μl of stock I_2/KI for 2 h, acidified to pH 1 with 4 N HCl, centrifuged in an Eppendorf microcentrifuge, and centrifuged again through Spin-X columns. One-ml samples were injected onto an HPLC C-18 column equilibrated in 50 mM ammonium acetate at pH 6.8 at a flow rate of 2 ml/min. *Left*, distribution of the folylpolyglutamates remaining bound to photolyase after incubations with excess CH^+-H_4folate. Analysis of the composition of enzyme-bound folate was by comparison of the HPLC chromatograms for each sample. *Right*, representative HPLC chromatogram. The *labeled peaks* are as follows: *VI*, 10-CHO-PteGlu$_6$; *V*, 10-CHO-PteGlu$_5$; *IV*, 10-CHO-PteGlu$_4$; *III*, 10-CHO-PteGlu$_3$; and *I*, 10-CHO-PteGlu.

mate nor the hexaglutamate derivatives are exchanged to any significant extent. The exchange of these folylpolyglutamates is not stimulated by the addition of photodimers and photoreactivation of these enzyme-substrate mixtures (data not shown). We interpret this as evidence that these polyglutamate derivatives of the folate cofactor are bound more tightly to the enzyme than is the monoglutamate derivative and that the polyglutamate moiety contributes substantially to the binding energy.

Catalytic Folate—Though the folate effect seemed likely to be attributable to reconstitution with the nonphysiological monoglutamate derivative and dissociation of this derivative at low concentrations, it was necessary to address the issue of whether the folate was catalytic. All of the assays so far had been performed under conditions involving a single turnover of the enzyme. In fact, quantum yield measurements of photolyase are typically conducted under single turnover conditions (13). It remained to be established whether the photoexcited folate might be released when the enzyme is allowed to undergo several cycles of reaction.

In order to address this question, photoreactivation experiments were conducted under multiple turnover conditions and at micromolar concentrations of photolyase so that the amount of CH^+-H_4folate that would freely dissociate from the enzyme would be negligible. Photolyase that had been treated with sodium borohydride and reconstituted with ^{3}H-labeled 3′,5′,7,9-CH^+-H_4folate was incubated in the presence and absence of single-stranded oligo(dT)$_{15}$-containing thymine dimers such that dimers were in 20-fold excess over photolyase. Mixtures were exposed to photoreactivating light sufficient to allow 10 enzyme turnovers and then concentrated on Centricon 30 microconcentrators and analyzed for enzyme-bound ^{3}H-labeled 3′,5′,7,9-CH^+-H_4folate. The data clearly showed that even under conditions of multiple turnover, the folate remains bound to enzyme. That the double-stranded 48-mer and single-stranded oligo(dT)$_{15}$ were repaired at approximately equal rates was verified by a spectrophotometric photolyase repair assay (14). Comparison of the repair of oligo(dT)$_{15}$ by the spectrophotometric assay and repair of the 48-mer by T4 endonuclease V assay revealed that when all 48-mer had been repaired in the multiple turnover experiments, 10 enzyme turnovers had occurred (data not shown).

Previous studies have demonstrated that the *E. coli* DNA photolyase has equal affinity for pyrimidine dimers in single-stranded DNA and double-stranded DNA (17) and that the dimer in the 48-mer double-stranded DNA substrate is repaired with the same quantum yield as dimers in oligo(dT) (18).

T4 Endonuclease V Assays—Further verification of the catalytic nature of the folate cofactor of *E. coli* DNA photolyase was provided by T4 endonuclease V assays. It has previously been shown that folate-free photolyase exhibits a decreased photolytic cross-section relative to photolyase-containing stoichiometric folate (4), indicating that the light absorbed by the folate is used for dimer repair. By utilizing the T4 endonuclease V assay under the multiple turnover conditions with reaction mixtures containing oligo$(dT)_{15}$ and trace 48-mer with dimer, the rates of repair by borohydride-treated photolyase, fully supplemented photolyase, and fully supplemented photolyase with additional excess folate were measured. It is evident from Figs. 4 and 5 that the repair by the borohydride-treated enzyme is significantly lower than that by the fully supplemented enzyme or the fully supplemented enzyme with excess folate. These data further confirm the catalytic nature of the folate, since under the conditions of the assay (10 turnovers), if the folate were lost after a single turnover, the repair curve for fully supplemented photolyase in the absence of added folate should have resembled that of the borohydride-treated enzyme. The fact that there is a slight stimulation of turnover in the presence of excess folate is again attributable to a slight folate effect (dissociation) of CH^+-H_4folate even at 1 μM enzyme. These assays also validate under multiple turnover conditions the conclusions made earlier, based on observations of single turnover experiments performed with limiting light (4), that the presence of the folate increases the photolytic cross-section of the enzyme.

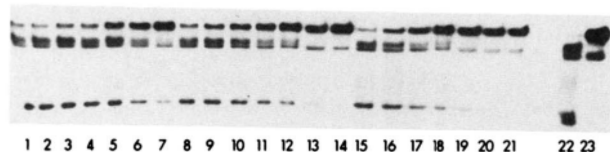


FIG. 4. **T4 endonuclease V assays of folate-free photolyase (*lanes 1–7*), photolyase containing stoichiometric CH^+-H_4folate (*lanes 8–14*), and photolyase containing stoichiometric CH^+-H_4folate plus excess free CH^+-H_4folate (*lanes 15–21*).** Photoreactivation times: *lanes 1, 8,* and *15,* not photoreactivated; *lanes 2, 9,* and *16,* 30 s; *lanes 3, 10,* and *17,* 1 min; *lanes 4, 11,* and *18,* 2 min; *lanes 5, 12,* and *19,* 3 min; *lanes 6, 13,* and *20,* 5 min; and *lanes 7, 14,* and *21,* 10 min. *Lanes 22* and *23* contained ^{32}P-labeled dimer treated with T4 endonuclease V and not exposed to the endonuclease, respectively.

Assays of Photolyase Supplemented with CH^+-H_4folate and CH^+-H_4folylpolyglutamate—To test the hypothesis that the folate effect is in fact primarily a dissociation effect, samples of photolyase that had been reconstituted with either CH^+-H_4folate or heterogeneous CH^+-H_4folylpolyglutamate were assayed by gel retardation assay under single turnover conditions and under limiting light for repair of the 48-mer containing a thymine dimer. The results of a representative experiment are shown in Fig. 6. As predicted, the folate effect was evident at low concentrations (6 nM), but the magnitude of the stimulation by excess folate was much greater for the photolyase samples that had been reconstituted with CH^+-H_4folate. This finding was consistent with our observation that dissociation of the monoglutamate form of the folate cofactor occurs in this range and that the presence of excess folate permits saturation of the folate site on the enzyme. The observation of a lesser magnitude of the folate effect for the folylpolyglutamate-reconstituted enzyme corroborates the results of binding studies showing tighter binding of the longer chain folylpolyglutamates to photolyase. The stimulation observed with the folylpolyglutamate-reconstituted enzyme is probably due to the dissociation of the triglutamate and tetraglutamate derivatives of CH^+-H_4folate. Of interest also was the HPLC profile of 10-CHO-folylpolyglutamates isolated from the CH^+-H_4folylpolyglutamate-reconstituted photolyase. Reconstitution of photolyase with the heterogeneous mixture of CH^+-H_4folylpolyglutamates yielded an enzyme preparation containing a higher proportion of the hexa- and pentaglutamate derivatives than were present in the reconstitution mixture (data not shown). This again demonstrates the higher affinity for the longer chain CH^+-H_4folylpolyglutamates inferred from the exchange experiments.

DISCUSSION

The goal of these studies was to determine whether the CH^+-H_4folate cofactor of *E. coli* DNA photolyase behaves catalytically or noncatalytically during the reaction catalyzed by the enzyme. Earlier observations that the enzyme-bound folate, but not the free species, was photodecomposed by prolonged exposure to high intensity light (3, 4) suggested the possibility that during the light-harvesting reaction, photoexcited folate might be chemically altered and released from the enzyme. The observation that the catalytic efficiency of photolyase supplemented with stoichiometric folate and assayed at low enzyme concentrations under single turnover conditions was stimulated by excess folate was in accord with this

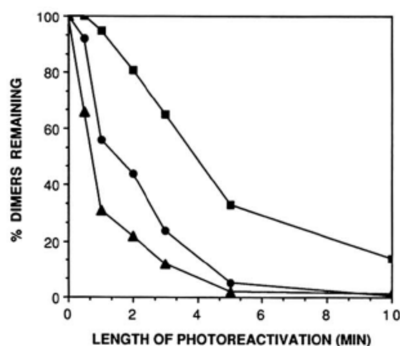


FIG. 5. **Repair rate of photolyase with or without excess folate.** The data points were obtained from the autoradiogram shown in Fig 4. The rates of repair for folate-free photolyase (■), photolyase containing stoichiometric CH^+-H_4folate (●), and photolyase containing stoichiometric CH^+-H_4folate plus excess CH^+-H_4folate (▲) were obtained by gel scan of the autoradiogram in Fig. 4.

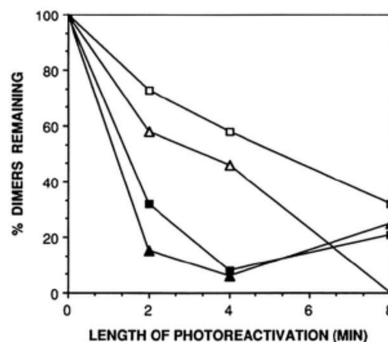


FIG. 6. **Repair rates (single turnover) of photolyase (6 nM) containing mono- or polyglutamyl forms of folate.** Assays were performed with photolyase reconstituted with stoichiometric CH^+-H_4folate in the presence (■) or absence (□) of excess CH^+-H_4folate or with photolyase reconstituted with CH^+-H_4folylpolyglutamate in the presence (▲) or absence (△) of excess CH^+-H_4folate.

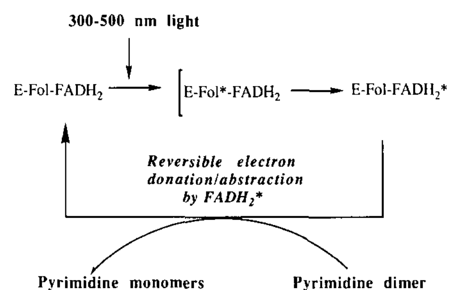
possibility. The failure to observe this folate effect with limiting substrate but at higher concentrations of enzyme was explainable as being due to replacement of released folate (that must be turned over with a quantum yield of less than 1) with folate cofactor from the excess enzyme in solution. However, it also seemed feasible that this folate effect was not due to decomposition of the folate after each catalytic event but rather to its dissociation from the enzyme.

Purified *E. coli* DNA photolyase contains a heterogenous mixture of CH^+-H_4folylpolyglutamates (2) but exhibits tighter binding to CH^+-H_4folate derivatives containing 5 or 6 glutamate residues. This was evident from the exchange experiments which demonstrate dissociation of mono-, tri-, and tetraglutamate derivatives but not of the penta- and hexaglutamate derivatives. The greater affinity of photolyase for the longer side chain derivatives of CH^+-H_4folate is not unexpected, since these folylpolyglutamate derivatives are more prevalent in *E. coli* (15). The presence of subsaturating amounts of folate in purified photolyase has been attributed partly to an insufficiency of intracellular folate to meet the demand of the overproduced photolyase and partly to dissociation of this chromophore from the apoenzyme during purification (4). The heterogeneity of the folylpolyglutamates bound to the purified photolyase can also be attributed to the cellular overproduction of photolyase. It would be interesting to see whether CH^+-H_4folylpolyglutamates containing five or six glutamates in γ-linkage rather than the $(\gamma_3)(\alpha_n)$ linkage peculiar to *E. coli* also have high affinity to the *E. coli* DNA photolyase.

The loss of the more loosely associated monoglutamate derivative, CH^+-H_4folate, upon dilution of photolyase under the conditions which lead to the folate effect explains our observation that the catalytic efficiency of the enzyme is stimulated by the addition of excess folate. That this effect is based on the different affinities of CH^+-H_4folate and CH^+-H_4folylpolyglutamates for the folate-binding site of photolyase is also evidenced by the fact that less of a folate effect is observed for enzyme reconstituted with folylpolyglutamates than with the monoglutamate as determined by gel retardation assay.

The issue of folate turnover was not resolved by these binding studies, though they did explain the folate effect satisfactorily. To truly address the issue of folate turnover, it was necessary to use multiple turnover experiments. By using photolyase reconstituted with ^{3}H-labeled CH^+-H_4folate at micromolar concentrations to minimize dissociation of this monoglutamate derivative of the folate, it was determined after multiple turnovers that the ^{3}H label remained bound to the enzyme. In addition, the use of coupled T4 endonuclease V assays revealed that photolyase at micromolar concentrations and containing stoichiometric folate exhibited the same rate of repair in the presence or absence of excess folate. This rate was greater than that exhibited by folate-free photolyase assayed under identical conditions. This was further verification that enzyme-bound CH^+-H_4folate behaves catalytically in the *E. coli* DNA photolyase.

The presence of a catalytic folate in photolyase is consistent with other observations. Payne (18) found that photolyase assayed under multiple turnover conditions did not exhibit any changes in absorbance at 384 nm. Other studies have revealed that the CH^+-H_4folylpolyglutamates of the yeast DNA photolyase are not photodecomposed by excess light (19).[2] Though we have demonstrated that the folate is a catalytic cofactor, it is still possible that the structure of the folate is reversibly altered during the light harvesting process; for instance, the energy transfer to the flavin could be driven by cleavage of the methenyl bridge of the CH^+-H_4folate to yield 10-CHO-H_4folate. The results obtained by folylpolyglutamate exchange assay with photoreactivation in the presence of excess dimers strongly suggest that there is no transient release of a 10-CHO-H_4folate derivative, as CH^+-H_4folylpolyglutamate exchange with free monoglutamate is not stimulated during enzyme turnover. Therefore, if cleavage to the 10-CHO-H_4folate derivative occurs, the latter must be rapidly converted to CH^+-H_4folate.



Scheme 1. **Model for catalysis by photolyase.**

Scheme 1 illustrates our model for catalysis by the *E. coli* DNA photolyase. The enzyme is shown to contain $FADH_2$ and folate, since the $FADH^{\cdot}$ seen in the purified enzyme is apparently photoreduced before the catalytic cycle is initiated (7, 20, 21). Most of the light absorption is by the folate cofactor since the extinction of enzyme containing folate is 6-fold higher at 384 nm than that of folate-free enzyme (4). It has been postulated that the photoexcited folate can transfer energy to the $FADH_2$ (3–5). In the absence of the folate, apparently the $FADH_2$ itself can absorb sufficient light to become photoexcited, as enzyme containing only the flavin cofactor has been demonstrated to show activity, though with a reduced photolytic cross-section (3, 4). The photoexcited flavin is proposed to directly catalyze the dimer photoreversal by a mechanism involving electron donation/abstraction (5, 22). The reaction catalyzed by the *E. coli* DNA photolyase is the only known reaction which utilizes a tightly bound folate cofactor to harvest light. It is also the only known reaction which makes catalytic use of the folate rather than utilizing the carbon or hydrogen donor capabilities of this cofactor.

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