DNA photolyase catalyzes the photoreversal of pyrimidine dimers. The enzymes from *Escherichia coli* and yeast contain a flavin chromophore and a folate cofactor, 5,10-methylenetetrahydropteroylpolyglutamate. *E. coli* DNA photolyase contains about 0.3 mol of folate/mol flavin, whereas the yeast photolyase contains the full complement of folate. *E. coli* DNA photolyase is reconstituted to a full complement of the folate by addition of 5,10-methylenetetrahydrofolate to cell lysates or purified enzyme samples. The reconstituted enzyme displays a higher photolytic cross section under limiting light. Treatment of photolyase with sodium borohydride or repeated camera flashing results in the disappearance of the absorption band at 384 nm and is correlated with the formation of modified products from the enzyme-bound 5,10-methenyltetrahydrofolate. Photolyase modified in this manner has a decreased photolytic cross section under limiting light. Borohydride reduction results in the formation of 5,10-methylenetetrahydrofolate and 5-methyltetrahydrofolate, both of which are released from the enzyme. Repeated camera flashing results in photodecomposition of the enzyme-bound 5,10-methenyltetrahydrofolate and release of the decomposition products. Finally, it is observed that photolyase binds 10-formyltetrahydrofolate and appears to cyclize it to form the 5,10-methenyltetrahydrofolate chromophore.

Irradiation of DNA with UV light causes the formation of dimers between adjacent pyrimidines. One cellular defense against these mutagenic lesions is the enzyme DNA photolyase (deoxyribopyrimidine photolyase, EC 4.1.99.3). DNA photolyases bind to cis-syn pyrimidine dimers in a light-independent step and then catalyze the removal of these dimers to pyrimidine monomers in a light-dependent step driven by near-UV or visible light. DNA photolyases have been purified from many different organisms and shown to contain intrinsic chromophores. The action spectrum of each enzyme varies according to the nature of the chromophores, thus implicating these chromophores in catalysis.

*Escherichia coli* DNA photolyase is a monomeric protein of molecular mass 54,000 daltons (1). The purified protein contains FAD as a neutral blue flavin radical (2) as well as the recently identified 5,10-methylenetetrahydropteroylpolyglutamate, CH⁻H,PteGlu⁴⁺ (3), which was previously referred to as the "second chromophore." The flavin radical displays a series of absorption bands above 400 nm and contributes about one-third of the absorbance at 384 nm in purified enzyme. Free CH⁻H,PteGlu has an absorption maximum at 355 nm at pH 2 with ε = 25,000 M⁻¹ cm⁻¹ (4). The protein-bound chromophore has an absorption maximum at 384 nm at neutral pH. The folate is the major absorbing component at 384 nm, accounting for at least two-thirds of the extinction (5). The semiquinone nature of the flavin appears to be a purification artifact, because it is not detected by EPR in vivo and photochemical or dithionite reduction of the radical increases the photolytic cross-section and the quantum yield to levels comparable to the in vivo value (6).

The action spectrum of the purified blue *E. coli* enzyme corresponds to the absorption spectrum of the enzyme with a maximum at 384 nm (7), indicating that the bound CH⁻H,PteGlu participate in the repair reaction. One possible mechanism has been proposed in which light is absorbed by this chromophore and transferred as energy to the reduced flavin (9). Reversible electron transfer could then occur directly from the flavin to the dimer, producing a dimer radical anion which could spontaneously monomerize and transfer its extra electron back to the flavin. The role of the flavin as the electron donor is supported by photochemical studies of free flavins and dimers (9, 10).

Experimental procedures for selective modification of the second chromophore have been described. Exposure of the *E. coli* photolyase to repeated camera flashing (11) or excess sodium borohydride (12) results in elimination of the folate absorption band at 384 nm. While no quantitative data are available on the borohydride-reduced enzyme, analysis of camera-flashed enzyme indicates that although φ, the photolytic cross-section (ε = molar extinction coefficient and φ = quantum yield), decreases, φ remains constant (11). Because the flavin appears to remain intact after each treatment, it seems to be capable of sustaining activity in the absence of the folate absorption band.

In this study of the role of the folate cofactor of *E. coli* photolyase, the structural changes in the folate cofactor that are brought about by camera flashing and sodium borohydride are characterized. The amount and chemical state of the

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1 The abbreviations used are: CH⁻H,PteGlu, 5,10-methylenetetrahydropteroylpolyglutamate with n glutamyl residues; pABA,Glu, p-aminobenzoic acid with n glutamyl residues; CH⁻H,folate, 5,10-methylenetetrahydrofolate; CH⁻H,PteGlu, 5,10-methylenetetrahydrofolate; CH⁻H,folate, 5-methyltetrahydrofolate; H₂,folate, tetrahydrofolate; 10-CHO-H,folate, 10-formyltetrahydrofolate; 5-CHO-H,folate, 5-formyltetrahydrofolate; H₂,folate, dihydrofolate; HPLC, high-performance liquid chromatography; DT, diithothreitol.
enorme-bound folate were varied by using these techniques. It is shown that the catalytic efficiency and photochemical cross-section of the enzyme can be manipulated by altering its folate content by supplementation or chemical and photochemical modification. These results clearly implicate the folate chromophore in the enzyme activity.

MATERIALS AND METHODS

Enzyme Preparation—Purification of E. coli DNA photolyase from the overproducing strain MS09 (C89003/F' lac/pvPS8969) (1) was based upon the procedure described previously (6). Photolyase was greater than 95% pure as measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis. When free CH2-Hfolate was added to the crude cell lysate, the procedure was modified by performing the lysis in 0.0 m citrate buffer, pH 6.0, 50 mM NaCl, 10% sucrose, 1 mM EDTA, 10 mM 2-mercaptoethanol with 0.05 ml of CH2-Hfolate (0.7 mM in 0.01 N HCl) added per ml of crude extract. The mixture was incubated on ice for 30 min before the purification was initiated. Purified enzyme was stored in stock buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM DTT, 1 mM EDTA, 50% glycerol) at −70 or −20 °C. Protein concentration was determined by the absorbance at 580 nm using the extinction of 5000 M−1 cm−1 reported previously (6). Yeast photolyase was the generous gift of Dr. Gwendolyn Sancar, University of North Carolina, Chapel Hill.

Chemicals and Reagents— Sodium cyanoborohydride was purchased from Aldrich. [32P]ATP was from ICN Radiochemicals, and T4 polynucleotide kinase was obtained from Bethesda Research Laboratories. HPLC grade methanol was from Fisher. [14C]Formic acid was obtained from Du Pont-New England Nuclear. 9, 9', 7, 9'-[8H] Optic acid (sodium salt) was from Amersham Corp. Formic acid was from Nutritional Biochemicals. Sodium borohydride, tetrabutylammonium hydroxide, bovine liver diphosphodiestrase, Hfolate, 5-CHO-Hfolate, and CH2-Hfolate were obtained from Sigma.

Synthesis of Folate—CH2-Hfolate was synthesized from 5-CHO- Hfolate by the method of Rabinowitz (13) and stored at −20°C in the dark at 0.1% HCl. It was stable for several weeks. CH2-Hfolate was synthesized by a modification of the method of Rowe (14). Briefly, 20 mg of Hfolate was added to 1.6 ml of glacial acetic acid containing 2% 2-mercaptoethanol, 0.01 ml of 88% formic acid, and 0.59 ml of [14C]formic acid (42 mM, 1.8 mCi/ml) in a closed vial under N2 and heated at 70 °C for 16 h in a water bath. The product was precipitated with diethyl ether and washed successively with diethyl ether and tetrahydrofuran. It was stored as a crystalline solid at −20°C. Spectral and HPLC analyses confirmed the identity of the product as CH2-Hfolate. CH2-Hfolate was synthesized by the method of Jungeldahl et al. (15) and stored frozen in liquid suspension under N2.

Quantitation of CH2-HPteGlu, Bound to Photolyase—Photolyase was incubated in 0.1 N HCl for 1 h at 65°C and then centrifuged in an Eppendorf model 5415 microcentrifuge for 5 min at 14,000 rpm. The supernatant was injected directly onto an HPLC reverse-phase column equilibrated with 20% methanol at pH 2. Chromatography was performed at a gradient of 20-50% methanol at pH 2 for 20 min at 1 ml/min. All photolyase derivatives of CH2-Hfolate elute together under these gradient conditions. HPLC was performed on a 10-μm reverse-phase C18 column from Alltech Associates (Los Altos, CA). The solvent delivery system was a Hewlett-Packard 1090 liquid chromatograph. Spectra of the HPLC eluate were monitored continuously using a Hewlett-Packard 1040A diode array detector. The area under the CH2-HPteGlu peak was measured by integration and compared to a synthetic standard prepared under similar conditions. The identity of the isolated species as CH2-HPteGlu, was confirmed by spectrophotometry. Quantitation of total folate was performed according to Schan (18) by preparation of PABA-Glu, derivatives from all samples, and used standards, and was determined by fluorescence measurements of oxidized PAB in protein-free supernatant obtained from enzyme samples treated with 50% trichloroacetic acid.

Supplementation of E. coli DNA Photolyase with CH2-Hfolate—Photolyase in storage buffer was transferred to 50 mM citrate buffer, pH 6.0, containing 50 mM NaCl, 10 mM DTT, 1 mM EDTA either by chromatography on a Sephadex G-25 column (PD-10, Pharmacia) or by passage over a Penefsky column (19, 20). Penefsky columns containing Sephadex G-50 Fine (Pharmacia LKB Biotechnology Inc.) equilibrated in the appropriate buffer were prepared in small (0.6 × 6.8 cm) dialysis tubing, and tuberculin syringes fitted with porous supports to retain the gel. The syringes were packed with the gel until the level of the gel had reached the top of the syringe, and then the gravity packed columns were placed in receiving tubes and prepared for sample application by spinning in a Sorvall GSA rotor equipped with PN00939 adaptors (21). A 0.2-ml sample was applied to the column and the centrifugation was repeated, effecting transfer of the gel-excluded fraction to the receiving tube. When specified, the included column fraction was isolated after removal of the excluded fraction by washing the gel with 0.1 ml of buffer followed by application of 1.0 ml of buffer and centrifugation to collect the included fraction. For the binding reaction, 0.05 ml of CH2-Hfolate stock solution (0.5–1 mM in 0.01 N HCl) was added per ml of enzyme solution (5–15 μM). The mixture was incubated for 30 min on ice and then passed through Penefsky columns in 0.2-ml aliquots to remove unbound CH2-Hfolate. All absorption spectra were obtained on a Shimadzu UV-240 spectrophotometer.

Analysis of DC1-Folate-supplemented Photolyase after Borohydride Treatment or Camera Flashing—Enzyme was supplemented with a single DC1-Hfolate using the procedure described above. Supplemented enzyme samples were either treated with borohydride or exposed to repeated camera flashes. The samples were passed through Penefsky columns, and the included and excluded fractions from each Penefsky column were isolated and analyzed for radioactivity. Sodium borohydride treatment of photolyase was by the method of Jones et al. (12). Flashing experiments were adapted from the method of Heelis et al. (11) and were performed with a Hanimex integrated flash system. Supplemented photolyase was placed in a quartz cuvette and flushed with a nitrogen atmosphere. The cuvette was immersed in ice water every 10 flashes. The absorption spectrum of the sample was monitored, and flashing was considered complete when there was no further decrease in the absorbance at 384 nm. Free CH2-Hfolate was flashed in 50 mM citrate, pH 6.0, 50 mM NaCl, 10 mM DTT, 1 mM EDTA.

Analysis of DC1-Labeled Folate from Supplemented/Borohydride-treated and Supplemented/Camera-flashed Enzyme—Borohydride-treated and camera-flashed photolyase samples were acidified to pH 1, centrifuged for 5 min at 14,000 rpm in an Eppendorf centrifuge, and the supernatants were analyzed immediately by HPLC. To allow measurement of the possible identity of the total folate bound to enzyme was equal to CH2-HPteGlu, bound to enzyme. The flavin contents of the yeast and E. coli DNA photolyases were determined by fluorescence measurements of oxidized PAB in protein-free supernatant obtained from enzyme samples treated with 50% trichloroacetic acid.

Folate in DNA Photolyase
gradient of 10–20% methanol from 10 to 30 min, and isocratic elution in 20% methanol from 30 to 40 min. The flow rate was 1 ml/min.

The second set of conditions was modified from the method of McMartin et al. (21) and employed isocratic elution in 5 mM tetrahydroammonium phosphate, pH 7.4–7.8, containing 20–35% methanol. Flow rates of 1.0–1.8 ml/min were used, depending upon the desired resolution. Under both sets of chromatography conditions, the peaks due to CH$_2$H$_4$folate, CH$_3$H$_4$folate, CH$_3^+$H$_4$folate, and H$_4$folate were resolved.

Assay of Photolyase—E. coli DNA photolyase was assayed by a gel retardation assay. A 48-base pair duplex containing a single dimer in a specified site synthesized by the method of Husain et al. (22) was kindly provided by Dr. Intisar Husain. The 48-mer was end-labeled with $^{32}$P using T4 polynucleotide kinase. Excess photolyase was mixed with substrate so that at equilibrium all dimers were bound by the enzyme, and the mixture was irradiated with light of limiting intensity. Under these conditions, repair can be quantitated as a function of fluence. The photoreactivation cross-section, $\phi$, is obtained using the following equation (23):

$$\phi (M^{-1} \cdot cm^{-1}) = 5.2 \times 10^9 k_p (mm^2 \cdot erg^{-1})/\lambda (nm)$$

where $k_p$ is the photolytic rate constant and is obtained from the plot of fraction of dimers remaining versus fluence (Rupert plot), as described by Sancar et al. (7). The $k_p$ for the photolyase reaction is defined in the following equation:

$$E + S \underset{k_3}{\overset{k_2}{\rightleftharpoons}} ES \underset{1}{\rightarrow} E + P$$

where I = intensity of illumination.

All manipulations were performed under illumination from General Electric gold fluorescent lamps. No photoreactivation occurs under these conditions. Reaction mixtures containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 20 mM DTT, 100 $\mu$g/ml bovine serum albumin in addition to $^{32}$P-labeled substrate (1 nM) and excess (10–40 times) enzyme were incubated at room temperature in the dark for 30–60 min. After the incubation, the sample was photoreactivated at 384 nm with a Quantacount monochromator–actinometer from Photon Technology International (Princeton, NJ) calibrated by potassium ferrocyanate actinometry (24). The Quantacount was equipped with a Fisher circulating water bath which maintained a constant sample temperature of 23°C. All 384 nm irradiations were performed through a Balzers 10.2% filter and a BP570 filter from Schott Glass Technologies Incorporated.

Aliquots of 50 $\mu$l were removed at various fluences during the photoreactivation and run on a 5% acrylamide gel as described previously (25). Under these assay conditions all dimers are complexed by photolyase unless repaired and released during photoreactivation (26). The free (repaired) DNA and enzyme-DNA complexes migrate differently and are located by autoradiography. The bands due to repaired DNA are more compact and are typically used for quantitation instead of the more diffuse enzyme-DNA bands. By excision and quantitation by Cerenkov counting of the bands due to repaired DNA, both the extent of repair and the fraction of dimers remaining at any given fluence can be determined. Because some of the enzyme-DNA complexes dissociate during electrophoresis, some “free” DNA is present even when the photolyase is in excess and all substrate is enzyme bound (as determined by flash photolysis) prior to electrophoresis. Therefore a correction is made by assuming 100% retardation for nonphotoreactivated samples. When it was necessary, individual assays were corrected for nonspecific binding by determining the amount of protein in the same excess that was bound to unmodified 48-mer.

RESULTS

Folate Content of the E. coli DNA Photolyase—We previously reported that E. coli DNA photolyase contains tightly bound CH$_3^+$H$_4$PteGlu, but did not determine its stoichiometry relative to the flavin cofactor. Quantitative analysis of CH$_3^+$H$_4$PteGlu, bound to photolyase revealed that the amount of the folate cofactor varied from one preparation to another, supporting an earlier observation that the ratio of the absorbance at 384 nm (due primarily to the folate) to the absorbance at 580 nm (due entirely to radical flavin) is not constant (6). Photolyase purified by the standard procedure contained on average 0.3 mol of CH$_3^+$H$_4$PteGlu/mol of flavin (values ranged from 0.19 to 0.35 mol of folate).

The finding that purified enzyme does not have a stoichiometric complement of folate is not surprising. CH$_3^+$H$_4$folate has an extinction of 25,000 M$^{-1}$·cm$^{-1}$ at 355 nm at pH 2. In contrast, the extinction of a typical photolyase preparation at 384 nm is only 18,100 M$^{-1}$·cm$^{-1}$ and this extinction is due to the absorption of both the folate and the radical flavin (5). This discovery also explains the variability in the absorbance ratios at 384 and 580 nm that was previously reported (6). In contrast, yeast DNA photolyase purified from an overexpressing E. coli strain has a single peak which is considerably larger than the corresponding 384 nm peak for the E. coli enzyme even after subtraction of the contributions of the respective flavin chromophores (5, 27). This suggested that the yeast enzyme might have a higher folate content. HPLC analysis of the CH$_3^+$H$_4$PteGlu, from yeast photolyase indicated that the CH$_3^+$H$_4$PteGlu, was present in a 1:1 stoichiometry with flavin. Quantitation of total folate verified that all bound folate was accounted for by CH$_3^+$H$_4$PteGlu,.

Supplementation of E. coli DNA Photolyase with CH$_3^+$H$_4$folate—Reconstitution of photolyase to its full complement of folate required the use of conditions under which both CH$_3^+$H$_4$folate and enzyme were stable. It was found that the use of 50 mM citrate buffer, pH 6.0, with 50 mM NaCl, 1 mM EDTA, 10 mM DTT met these requirements. The results of a reconstitution experiment using CH$_3^+$H$_4$folate are presented in Fig. 1. The 384 nm peak of the supplemented enzyme increased dramatically, indicating that the added CH$_3^+$H$_4$folate was bound by the enzyme and exhibited the same bathochromic shift from 355 to 384 nm as the native chromophore. The bound CH$_3^+$H$_4$folate was not removed from the enzyme by chromatography on Sephadex G-50 as evidenced by the retention of the strong 384 nm absorption band on the enzyme after chromatography. In addition, when CH$_3^+$H$_4$folate was used to supplement the enzyme, the enzyme retained the radioactivity after chromatography on Sephadex G-50 (data not shown). Analysis of supplemented enzyme indicated that it contained a full complement of the CH$_3^+$H$_4$folate derivative. From these binding experiments, it is also apparent that the polyglutamate side chain is not critical for
the stable binding of CH*-H,folate to photolyase.

In order to understand the basis for the folate-deficient state of purified photolyase, the purification procedure was modified slightly by lysing the *E. coli* cells in a citrate buffer at pH 6.0 which contained additional CH*-H,folate. The absorption band at 384 nm of photolyase purified using this procedure clearly showed that the CH*-H,folate added to the crude extract had supplemented the enzyme in that extract (data not shown). HPLC analysis of this supplemented photolyase indicated that the enzyme contained a stoichiometric complement of CH*-H,PteGlu. It seems, then, that the primary cause of the lack of stoichiometric folate in standard purified enzyme is an insufficiency of the appropriate folate species in *vivo*, caused by the gross overproduction (15% of the total cellular protein) of the apoenzyme.

**Borohydride Treatment of Free and Enzyme-bound CH*-H,folate**—Jorns et al. (12) have shown that the addition of sodium borohydride to *E. coli* photolyase results in a dramatic decrease in the 384 nm absorption, suggesting that the second chromophore (folate) was in fact eliminated by this treatment. The borohydride reacted selectively, as evidenced by the lack of change in the spectrum of the enzyme-bound radical flavin.

We have examined the effect of sodium borohydride on the enzyme-bound CH*-H,PteGlu, in greater detail by analyzing the chemical nature of the products of the reaction and establishing whether these products were still enzyme-bound. Reaction of free CH*-H,folate with sodium borohydride has been demonstrated to result in conversion to CH3-H,folate or CH3-H,folate, depending upon the amount of borohydride used in the reaction (4). The observed effect of borohydride on the enzyme, i.e., elimination of the folate absorption band at 384 nm, was consistent with reduction of the enzyme-bound CH*-H,folate to one of these reduced derivatives with absorption maxima below 300 nm.

To facilitate analysis of the products of borohydride reduction of photolyase, the enzyme was reconstituted with 4CH*-H,folate such that it contained approximately 70% of the bound folate as the 14C-labeled monoglutamate. Fig. 2 shows the absorption spectrum of photolyase supplemented with 4CH*-H,folate before and after treatment with sodium borohydride. The elimination of the 384 nm absorption band of the bound CH*-H,PteGlu, is apparent. The results from HPLC analysis of the acid-released products of the borohydride reaction in an acid/methanol gradient are shown in Fig. 3. The folate from supplemented photolyase showed a single peak of CH*-H,folate as judged spectrally and by recovery of radioactivity. After 2 h of borohydride treatment, the CH*-H,folate peak had almost completely disappeared and two new peaks were observed. These peaks were identified as CH3-H,folate and CH3-H,folate by their spectra and by HPLC coelution with the appropriate standards. The yields of CH3-H,folate and CH3-H,folate were about 60 and 30%, respectively. The remaining counts appeared in the excluded volume of the column and were probably due to free 14C-formaldehyde formed from the acid-catalyzed degradation of the CH3-H,folate to H,folate and formaldehyde (28). All of the initial counts from the enzyme-bound CH*-H,folate were recovered in these three products. These conclusions were confirmed by HPLC analysis of the products in tetrabutylammonium phosphate/methanol. Chromatography in the second solvent revealed the same products in the same distribution.

To determine whether CH3-H,folate and CH3-H,folate remained enzyme-bound, photolyase that had been supplemented with 4CH*-H,PteGlu and treated with borohydride was centrifuged through Penefsky columns containing Sephadex G-50. The included and excluded column volumes were recovered and analyzed for radioactivity. All of the counts in the untreated sample were recovered in the excluded volume, whereas 85% of the counts for the borohydride-reduced sample were present in the included volume. These findings indicate that the affinity of photolyase for the CH3-H,folate and CH3-H,folate derivatives produced by the borohydride reaction is much less than for the CH*-H,folate derivative.

**Modification of Free and Enzyme-bound CH*-H,folate by**
Camera Flashing—Prolonged flashing of *E. coli* photolyase causes a reversible reduction of the enzyme-bound flavin radical (29) but an irreversible decline in absorption at 384 nm attributable to the "selective decomposition" of the second chromophore (11). To examine the nature of the chemical modification of the folate chromophore caused by flashing, photolyase supplemented with "CH"\(^\text{+}\)-H\(_\text{folate}\) was flashed repeatedly until no further decrease in the absorbance at 384 nm was observed. The spectra in Fig. 4 demonstrate the decrease in the 384 nm band that occurred with exposure to camera flashes. The photoreduction of the flavin radical with the concomitant appearance of a peak at 360 nm due to FADH\(_2\) was also evident. Supplemented/flashed photolyase was examined for its ability to be supplemented again with CH\(^\text{+}\)-H\(_\text{folate}\) and was found to be reconstituted to a full complement of the CH\(^\text{+}\)-H\(_\text{folate}\) under standard conditions. This indicates that the folate-binding site on the enzyme remains intact during this treatment.

HPLC analysis in an acid/methanol gradient of the acid-released products from unflashed enzyme demonstrated that all of the "\(^\text{14}\)C" counts were associated with the CH\(^\text{+}\)-H\(_\text{folate}\). Analysis of the flashed sample by HPLC in an acid/methanol gradient indicated that no CH\(_2\)-H\(_\text{folate}\) or CH\(_3\)-H\(_\text{folate}\) were present. Chromatography in tetrabutylammonium phosphate/methanol at pH 7.4 (Fig. 5) revealed that the majority of the counts eluted in the excluded volume of the column, and not with CH\(_2\)-H\(_\text{folate}\) or CH\(_3\)-H\(_\text{folate}\) standards. The appearance of all of the counts in the excluded volume of the column is consistent with photodecomposition of the CH\(^\text{+}\)-H\(_\text{folate}\) and release of free "\(^\text{14}\)C"formic acid. Similar treatment of free CH\(^\text{+}\)-H\(_\text{folate}\) in 50 mM citrate, pH 6.9, 50 mM NaCl, 10 mM DTT, 1 mM EDTA revealed that no decomposition of the CH\(^\text{+}\)-H\(_\text{folate}\) had occurred (data not shown).

Quantitative analysis of "\(^\text{14}\)C" in the camera-flashed enzyme that had been supplemented with "CH"\(^\text{+}\)-H\(_\text{PteGlu}\) was performed with Penefsky columns and revealed that 92% of the "\(^\text{14}\)C" was recovered in the included fraction. Because HPLC analysis had shown that the counts were not recovered in CH\(_2\)-H\(_\text{folate}\) and CH\(_3\)-H\(_\text{folate}\), it was important to determine whether the pABAGlu and pterin moieties were still protein-bound. For this purpose, photolyase was reconstituted with 3',5',7,9,'"\(^\text{14}\)H"CH\(^\text{+}\)-H\(_\text{folate}\). When this reconstituted sample was flashed and analyzed on Penefsky columns it was found that 83% of the initial counts were lost from the excluded fraction. Because the "H label was present in both the pterin and p-aminobenzoic acid moieties, it is evident that both of these components of the folate, in addition to the formate, were released by flashing. Although the exact nature of the folate products of flashed enzyme remains to be established, it is clear that camera-flashed enzyme has lost most of its folate.

Activity of Supplemented, Supplemented/Borohydride-treated, and Supplemented/Flashed Enzyme—With the availability of fully supplemented enzyme it was possible to examine further the role of the folate in the repair reaction. Supplemented photolyase was assayed by a gel retardation assay. The autoradiogram of such a gel is shown in Fig. 6, and a Rupert plot of data from several experiments is presented in Fig. 7. The photolytic cross-section for supplemented enzyme was calculated by extrapolating the initial phase of the

**Fig. 4.** Spectra of photolyase supplemented with CH\(^\text{+}\)-H\(_\text{folate}\) and camera-flashed continuously. Photolyase (2 \(\mu\)M) was flashed in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 20% glycerol. Spectra were taken after 6, 100, 200, 300, and 400 flashes. The arrow indicates the direction of change in the spectra during the flashing reaction.

**Fig. 5.** HPLC elution profile of radioactive species released from photolyase after camera flashing. Photolyase (0.1 mg in 1.0 ml) was supplemented with "CH"\(^\text{+}\)-H\(_\text{folate}\), flashed 400 times, acidified to pH 1, and centrifuged in an Eppendorf model 5415 microcentrifuge for 5 min at 14,000 rpm. The supernatant was neutralized with NaOH to pH 7.4 and chromatographed in 5 mM tetrabutylammonium phosphate, pH 7.4, containing 30% methanol at 1.8 ml/min.

**Fig. 6.** Gel retardation assay for supplemented (lanes 1-5), supplemented/borohydride-treated (lanes 6-10), and supplemented/camera-flashed (lanes 11-16) photolyase. Lanes 1, 6, and 11 contain DNA only. The other lanes contain DNA and a 10-fold excess of photolyase. The samples in lanes 2, 7, and 12 were not photoreactivated. The other samples were exposed to 384 nm monochromatic light of the following fluences: lane 13, 1500; lanes 3, 8, and 14, 3000; lanes 4, 9, and 15, 6000; and lanes 5, 10, and 16, 9000 erg/mm\(^2\).
Repair curve is determined by the biophysical activity of the reaction with supplemented enzyme but its cause is unknown. The photolytic cross-section for unsupplemented photolyase was reported previously (7) and is shown here for comparison by the dashed and dotted line in Fig. 7. It is evident that supplemented photolyase exhibits a higher photolytic cross-section under limiting light than does unsupplemented photolyase. This increase is not due to an increase in the affinity of supplemented enzyme for the substrate, because the same amount of DNA is bound by supplemented and unsupplemented enzyme (data not shown). The increased catalytic efficiency must therefore be due to an increase in the overall efficiency of the photochemical reaction per incident quantum, leading to the conclusion that the externally added CH'-H,folate enhances the photochemical reaction of the enzyme.

The decrease in absorbance of the enzyme at 384 nm due to formation and release of the CH, H,PteGlu, and CH', H,PteGlu, upon treatment with borohydride should then be reflected in a lower photolytic cross-section at 384 nm than that of supplemented or unsupplemented photolyase under these conditions. To test this proposal, supplemented/borohydride-treated photolyase was also assayed by the gel retardation technique under enzyme excess conditions. Fig. 6 shows the autoradiogram of a typical gel assay, and the results of several assays are shown in the Rupert plot in Fig. 8. It is apparent from these results that the removal of CH', H,PteGlu, by reduction with borohydride does dramatically decrease the photolytic cross-section of the enzyme.

Supplemented/flashed enzyme showed an attenuated efficiency under limiting light (Fig. 6, gel, and Fig. 8, plot). Also of interest was the finding that enzyme that had been supplemented, flashed, and resupplemented with folate had a catalytic efficiency comparable to that of supplemented enzyme, indicating that the decrease observed for the flashed sample was not due to damage to the enzyme from the flashing procedure. Table I summarizes the relative photolytic cross-sections of the various forms of blue photolyase that have been examined.

Reconstitution of Photolyase with 10-CHO-H,folate—In E. coli, CH'-H,PteGlu, is formed during the conversion of CH', H,PteGlu, to 10-CHO-H,PteGlu,. This reaction is catalyzed by the bifunctional enzyme complex CH', H,PteGlu dehydrogenase-CH', H,PteGlu cyclohydrolase (30). The probable existence of this folate as an enzyme-bound intermediate and the instability of free CH'-H,folate in solution at neutral pH make it unlikely that free CH'-H,PteGlu, accumulates to any extent in the cell. This raised the question of how photolyase is able to sequester large quantities of the CH', H,folate, especially under the conditions of photolyase overproduction. One possibility is that photolyase binds 10-CHO-H,PteGlu, in vivo and induces its cyclization to CH', H,PteGlu,. To test this possibility, photolyase was incubated with an excess of 10-CHO-H,folate. Since the εₘₐₓ of 10-CHO-H,folate is below 300 nm, formation of the 384 nm absorption band in the presence of photolyase at neutral pH would indicate binding.

**Fig. 7.** Plot of dimers remaining versus fluence at 384 nm for supplemented photolyase. Unsupplemented photolyase is represented by the dashed and dotted line (7).

**Fig. 8.** *Top panel,* plot of dimers remaining versus fluence at 384 nm for supplemented/borohydride-treated photolyase (■) and supplemented photolyase (▲). *Bottom panel,* plot of dimers remaining versus fluence at 384 nm for supplemented/flashed photolyase (■) and supplemented photolyase (▲).

**Table I**

| εₘₐₓ (10⁻⁴ M cm⁻¹) | εₙₐₜ | εₘₚ | εₘₙ |
|-------------------|------|-----|-----|
| Supplemented      | 2.4  | 5.4 | 6.2 |
| Supplemented/borohydride | 5.4×10⁻⁵ | 1.2 | 1.0 |
| Supplemented/flashed | 4.4×10⁻⁴ | 1.0 | 1.8 |
| Supplemented/flashed/supplemented | 4.4×10⁻⁴ | 9.9 | 6.2 |
| Unsupplemented* | 8.3×10⁻⁴ | 1.9 | 3.0 |

*See Ref. 7.
and cyclization. This phenomenon was in fact observed immediately after mixing photolyase and 10-CHO-H₄folate; not only did the 384 nm peak appear but its magnitude indicated that there was a stoichiometric conversion to CH⁺⁻H₄folate by the enzyme. This new absorption band was present on the enzyme after separation on Penefsky columns, verifying that the chromophore was protein-bound (data not shown). The instantaneous generation of the 384 nm band upon mixing photolyase with 10-CHO-H₄PteGlu at pH 7.4 suggests that the folate-binding site of the E. coli DNA photolyase has a 10-CHO-H₄PteGlu cyclodihydrolase activity. Addition of the other potential precursor of CH⁺⁻H₄folate, 5-CHO-H₄folate, to photolyase did not result in the formation of the 384 nm peak.

**DISCUSSION**

Binding of exogenous CH⁺⁻H₄folate to folate-deficient E. coli photolyase preparations occurs specifically, as evidenced by the long term stability of the supplemented complex at neutral pH where the free compound is unstable. The bathochromic shift of the absorption maximum of the folate from 355 to 384 nm is further evidence for specific binding. The dramatically higher photolytic cross-section of supplemented enzyme under conditions of limiting light at 384 nm attests to the productive nature of the binding of the added chromophore.

As summarized in Fig. 9, reduction of supplemented photolyase with excess sodium borohydride for 2 h results in the formation of CH₂⁻H₂folate and CH₃⁻H₂folate, both of which are released from the enzyme. The distribution of these two products is probably a function of the incubation time with borohydride, with longer incubations of photolyase with borohydride producing a greater percentage of CH₂⁻H₂folate. The photolytic cross-section for borohydride-treated photolyase under limiting light at 384 nm is considerably lower than that of supplemented and unsupplemented photolyase. Jorns et al. (12) have reported that borohydride-treated enzyme shows virtually no change in catalytic activity, in contrast to the results presented here. It is to be noted that under high intensity illumination where the light-independent step, ES complex formation, becomes rate limiting, photolyases with different amounts of folate will have the same activities. The assay conditions utilized here, however, are designed specifically to assess differences in light-harvesting capability.

![Diagram of the products of borohydride-treated and camera-flashed enzyme-bound CH⁺⁻H₄PteGlu](image)

**FIG. 9.** Schematic depiction of the products of borohydride-treated and camera-flashed, enzyme-bound CH⁺⁻H₄PteGlu.

Camera flashing of supplemented photolyase also results in release of the counts associated with the enzyme-bound CH⁺⁻H₄folate, indicating that virtually no bound folate remains. Camera-flashed enzyme is also active, but its photolytic cross-section is lower than that of supplemented or unsupplemented photolyase. This finding is consistent with the data reported by Heelis et al. (11) that the photolytic cross-section of flashed enzyme decreases. As the data summarized in Table I indicate, it is evident that in the absence of folate the enzyme is able to catalyze dimer repair. However, it is obvious that both the photolytic cross-sections and catalytic efficiencies increase with increasing contents of folate.

The presence of less than stoichiometric amounts of folate in the purified enzyme is surprising, considering the ease and rapidity of supplementation with CH⁺⁻H₄folate and 10-CHO-H₄folate. The finding that addition of CH⁺⁻H₄folate to crude cell lysates as a prelude to the purification procedure yields a fully supplemented preparation indicates that overproduction of photolyase in the genetically engineered strain is the primary cause of the insufficiency of folate in the enzyme. The broad distribution of polydopaglutamites bound to photolyase (3) might also be attributed to the overproduction of photolyase.

It has been proposed that the role of the second chromophore is that of a light harvester which could garner more light energy than reduced flavin because of its high extinction (8). The folate does have a light harvesting function because the increase in the extinction at 384 nm results in a corresponding increase in the photolytic cross-section of the enzyme. It is conceivable that the excited CH⁺⁻H₄PteGlu transfers energy to reduced flavin and that excited reduced flavin reversibly transfers an electron to the dimer to complete the repair reaction. Energy transfer from the CH⁺⁻H₄PteGlu might occur through orbital overlap. Nonradiative energy transfer, or Förster energy transfer, is known to occur in biological systems. This type of energy transfer requires overlap by the fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor (31). Because the fluorescence emission of the donor (folate) is approximately at 470 nm (2) and the lowest energy absorption band of the acceptor (FADH₂) is at 360 nm, this type of mechanism does not seem to be feasible.

Assays of borohydride-treated and camera-flashed photolyase demonstrate that enzyme containing only flavin can catalyze the repair reaction. The CH⁺⁻H₄PteGlu, might also be able to catalyze repair independently of the flavin either by electron donation or abstraction to form a stable folate radical and an unstable dimer radical that would collapse to monomers. This might occur by excitation of the enzyme-bound CH⁺⁻H₄PteGlu, with cleavage at the bridging carbon and production of 10-CHO-H₄PteGlu, with the bond cleavage providing energy to drive radical formation. The enzyme could then spontaneously recyclize the 10-CHO-H₄PteGlu, that would eventually be regenerated to CH⁺⁻H₄PteGlu.

All of the possibilities mentioned so far invoke a catalytic function for the folate. However, folates typically function noncatalytically. The E. coli and yeast DNA photolyases belong to a small subset of enzymes that not only use folate but are actually purified with tightly bound folate. Two other enzymes, the rat liver sarcosine dehydrogenase and dimethylglycine dehydrogenase (32, 33) are purified with tightly bound H₄PteGlu. A reaction mechanism for these two dehydrogenases has been proposed in which the bound H₄PteGlu serves to trap the carbon unit produced during the oxidative demethylation of dimethylglycine and sarcosine (33, 34, 35). The CH₂⁻H₂PteGlu, that is formed from this reaction is
released from the enzyme and more H,PteGlu, then binds. It is conceivable that the biphasicity of the rate that is observed with the supplemented preparations is due to formation of a folate species on the enzyme that requires replacement by free folate. However, there is no evidence for folate release from photolyase during catalysis.

Until now, CH+-H,PteGlu, has not been identified as a participant in a folate-requiring reaction. Our results demonstrate definitively that light energy absorbed by the enzyme-bound CH+-H,PteGlu, is used to drive dimer repair. The mechanism of the reaction involving this folate cofactor is still unclear but further investigations along the lines described should enable us to elucidate the mechanism of this intriguing reaction.

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