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Identification of the second chromophore of *Escherichia coli* and yeast DNA photolyases as 5,10-methenyltetrahydrofolate

(10-formylfolic acid/α- and γ-glutamate/flavin/DNA repair)

JEAN L. JOHNSON*†, SARAH HAMM-ALVAREZ*, GILLIAN PAYNE‡, GWENDOLYN B. SANCAR‡, K. V. RAJAGOPALAN*, AND AZIZ SANCAR‡

*Department of Biochemistry, Duke University Medical Center, Durham, NC 27710; and ‡Department of Biochemistry, University of North Carolina School of Medicine, Chapel Hill, NC 27599

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ABSTRACT **Denaturation of DNA photolyase (deoxyribodipyrimidine photolyase, EC 4.1.99.3) from *Escherichia coli* with guanidine hydrochloride or acidification to pH 2 released, in addition to FAD, a chromophore with the spectral and chromatographic properties of a reduced pterin. Treatment of the enzyme with iodine prior to acidification converted the chromophore to a stable, oxidized derivative, which was resolved by HPLC into four species with identical spectral properties. The same species, in the same distribution, were obtained from the yeast enzyme. The material isolated from the iodine-oxidized enzyme was shown to be a pterin by conversion to pterin-6-carboxylic acid with alkaline permanganate and was found to release glutamate upon acid hydrolysis. The presence of 10-formylfolate in the isolated, oxidized chromophore was demonstrated by absorption and fluorescence spectroscopy and by deformylation and conversion to folic acid. Analysis of the distribution of polyglutamates revealed that the four species identified by HPLC corresponded to the tri-, tetra-, penta-, and hexaglutamate derivatives of 10-formylfolate. The results were consistent with γ linkages in the triglutamate derivative with additional glutamates linked via the α-carboxyl group of the preceding residue. Treatment with rat plasma hydrolase produced the monoglutamate derivative of 10-formylfolate. The native, enzyme-bound form of the folate cofactor was identified as 5,10-methenyltetrahydrofolylpolyglutamate by effecting release and isolation at low pH to protect the 5,10-methenyl bridge and preserve the reduced pyrazine ring structure.**

DNA photolyase (deoxyribodipyrimidine photolyase, EC 4.1.99.3) catalyzes the repair of pyrimidine dimers that are formed between adjacent pyrimidine bases in the DNA as a result of damage by UV light. The repair reaction is driven by visible or long wavelength UV light and appears to depend on electron transfer from a photoexcited reduced flavin in the photolyase to the dimer (1). DNA photolyase has been purified from a number of different organisms. The action spectrum of the enzyme varies somewhat with the species as does the state of the flavin in the isolated enzyme. The enzyme purified from *Escherichia coli* contains a blue neutral flavin semiquinone radical, but the flavin can be reduced with a concomitant increase in activity (1, 2). The flavin in the isolated yeast enzyme is in the fully reduced state (3). The mechanism by which visible or long wavelength UV light energy is gathered, transferred to the flavin, and subsequently utilized in the DNA repair reaction is not fully understood. It is becoming clear, however, that a second enzyme-bound cofactor participates in catalysis. In the experiments described below, the second chromophore of *E. coli* and yeast photolyase is characterized as a conjugated pterin. A stable, oxidized species was isolated and identified as 10-formylfolic acid; however, evidence is presented that indicates that in the enzyme the folate cofactor exists in a reduced, fluorescent state with spectral properties consistent with those of 5,10-methenyltetrahydrofolate.

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MATERIALS AND METHODS

DNA photolyase was purified from a genetically engineered strain of *E. coli* in which the enzyme can be induced to constitute 15% of the total cellular protein (4). The enzyme was purified and was shown to be homogeneous by several criteria as described earlier (4). Yeast DNA photolyase was purified from *E. coli* cells containing the cloned *PHR1* gene of *Saccharomyces cerevisiae* (3). For isolation of the second chromophore, enzyme in storage buffer (50 mM Tris·HCl, pH 7.4/50 mM NaCl/10 mM dithiothreitol/1 mM EDTA/50% glycerol) was transferred to 50 mM Tris·HCl, pH 7.4/100 mM NaCl by chromatography on a column of Sephadex G-25 (PD-10, Pharmacia) at 4°C.

Ultrapure guanidine hydrochloride was obtained from Schwarz/Mann, HPLC grade ammonium acetate was from Fisher, and methanesulfonic acid with 0.2% 3-(2-aminoethyl)-indole was from Pierce. The *o*-phthaldialdehyde reagent solution (complete), standard amino acids, pterin, pterin-6-carboxylic acid, folinic acid, and carboxypeptidase Y were obtained from Sigma. Folic acid was from Nutritional Biochemicals.

Fluorescence spectra were recorded on an Aminco–Bowman SPF spectrofluorometer. Absorbance spectra were obtained on a Shimadzu UV-240 spectrophotometer. HPLC was carried out using a 10 μm C-18 column obtained from Alltech Associates (Los Altos, CA). The solvent delivery system was an SP8700 system from Spectra Physics (Santa Clara, CA). Absorbance was monitored with a Kratos Spectroflow 773 variable wavelength detector, and fluorescence was monitored with a Laboratory Data Control Fluoromonitor III. The release of 5,10-methenyltetrahydrofolate from the enzyme was monitored directly in the HPLC effluent using a Hewlett–Packard 1040A diode array detector.

Analysis for organic phosphate was carried out as described by Ames (5). Hydrolysis in methanesulfonic acid, derivatization with *o*-phthalaldehyde, and HPLC chromatography of the derivatized amino acids were as described by Jones *et al.* (6). Standard 10-formylfolate was synthesized by heating folate with formic acid and acetic anhydride (7). The product was >95% pure as judged by HPLC chromatography in several solvents. Standard 5,10-methenyltetrahydrofolate was prepared from folinic acid as described by Rabinowitz (8). Pteroylpolyglutamate standards were prepared by C. M. Baugh and were kindly provided by Robert Ferone

†To whom reprint requests should be addressed.

of the Wellcome Research Laboratories. Rat plasma hydrolase was prepared as described by Wilson and Horne (9).

RESULTS

Release of the Chromophore. Earlier work has established that DNA photolyases from *E. coli* and yeast contain $FADH_2$ and a second chromophore, which is responsible for the intrinsic blue fluorescence of the enzyme and the absorption band at 385 nm (1, 10). In an attempt to separate this chromophore from the protein and begin its characterization, a sample of the *E. coli* enzyme was denatured in 5 M guanidine hydrochloride at room temperature for 5 min and applied to a column of Sephadex G-15 equilibrated with a solution of 6 M guanidine in H_2O. As can be seen in Fig. 1*A*, a peak of fluorescent material with excitation at 365 nm and emission at 455 nm (at pH 10) was detected eluting slightly ahead of the FAD released from the enzyme. The fluorescence was weak when fractions were monitored immediately after elution from the column but increased in intensity after overnight incubation at room temperature. The fluorescence properties of the material released (Fig. 2) were similar to those expected for the second chromophore, but were unlike those of FAD, and were suggestive of a pterin species. Increasing fluorescence upon aerobic oxidation is characteristic of a reduced pterin being converted to a more fluorescent oxidized species. Release of the cofactor by guanidine denaturation of the protein indicates a noncovalent attachment of the chromophore; its elution position on Sephadex G-15 indicates that the species released is larger than FAD

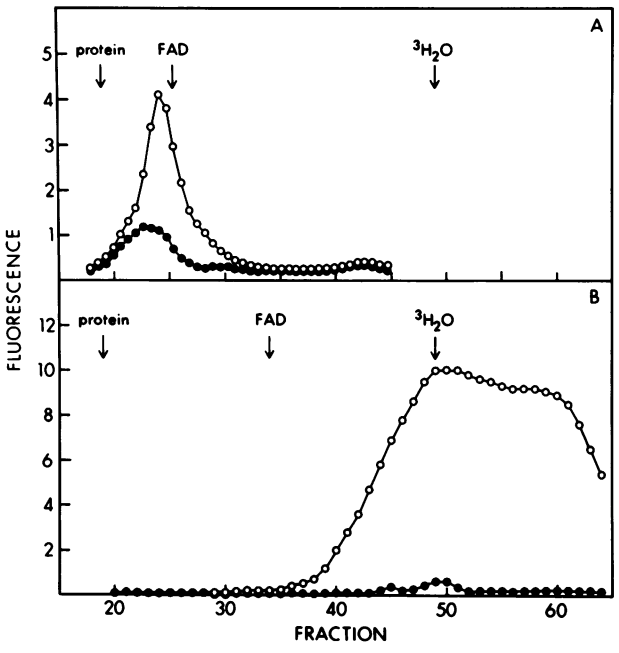

FIG. 1. Sephadex G-15 chromatography of the second chromophore released from DNA photolyase. Samples were denatured and chromatographed in 6 M guanidine hydrochloride (*A*) or in 0.01 M HCl (*B*). Fractions eluting from the column were adjusted to pH 10 with NaOH and monitored for fluorescence (excitation wavelength = 365 nm and emission wavelength = 455 nm) immediately (●) and after overnight oxidation in air (○). Protein was monitored by tryptophan fluorescence (excitation wavelength = 290 nm and emission wavelength = 340 nm), and FAD was monitored by fluorescence using excitation and emission wavelengths of 450 and 520 nm, respectively. ^3H_2O was used as a column-included-volume marker and was quantitated by liquid scintillation counting of fraction aliquots in Safety-Solve (Research Products International, Mount Prospect, IL).

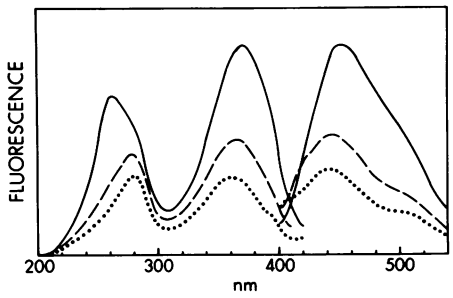

FIG. 2. Fluorescence excitation and emission spectra of fluorescent material released from DNA photolyase by guanidine denaturation. Spectra were recorded of samples at pH 11 (—), 6.8 (– –), and 3 (···). Excitation spectra (scans from 200–420 nm) were recorded with the emission wavelength adjusted to yield maximal fluorescence (440–450 nm). Emission spectra (scans from 400–540 nm) were recorded with the excitation wavelength at 360–370 nm.

(786 daltons) but smaller than the exclusion limit of the column (1500 daltons).

Fluorescent material with identical spectral properties was obtained when the enzyme was denatured by acidification to pH 2.0 with HCl and chromatographed on G-15 in 0.01 M HCl. In this case, however, the fluorescence of the second chromophore, which was low in fractions analyzed immediately after elution (monitored at pH 10) and increased upon aerobic incubation, was eluted much later, in fractions near and after the column-included volume (Fig. 1*B*). This behavior is also characteristic of pterins, particularly reduced pterins, which exhibit a strong interaction with the Sephadex resin in solutions of low ionic strength. The flavin also binds to the resin in 0.01 M HCl and is eluted a few fractions later than with guanidine as the eluant.

Multiple Forms of the Chromophore. Because air oxidation of reduced pterins can sometimes generate side products, a solution of I_2 (1%) and KI (2%) was added to the enzyme solution prior to denaturation to direct the course of oxidation. The following protocol was adopted to convert the chromophore to a stable, oxidized derivative. Enzyme in 50 mM Tris·HCl, pH 7.4/100 mM NaCl was incubated at room temperature with a solution of I_2 and KI (100 μl/mg of protein). After 3 hr, the mixture was acidified to pH 2.0 with HCl and centrifuged to remove precipitated protein, and the supernatant was injected onto a C-18 reverse-phase HPLC column in 50 mM ammonium acetate (pH 6.8). A typical elution profile is shown in Fig. 3. Four major peaks of fluorescent material, each with a corresponding 280 nm absorption, were obtained. Material from each peak was collected and analyzed. The fluorescence spectra of peaks I–IV were identical to one another and indistinguishable from the spectra shown in Fig. 2. None of the peaks eluting from the C-18 column with ammonium acetate contained FAD.

A sample of yeast photolyase purified from *E. coli* containing the yeast *PHR1* gene was analyzed for the presence of the second chromophore. The same four fluorescent peaks were obtained in the same ratio as seen for the *E. coli* enzyme, and each exhibited a fluorescence spectrum identical to that of the *E. coli* species (data not shown).

Demonstration That the Chromophore Is a Pterin. Material from each of the four peaks obtained from the *E. coli* enzyme was treated with potassium permanganate in 0.1 M NaOH at 100°C for 15 min. Each of the four species was converted to an identical, new fluorescent molecule that coeluted with authentic pterin-6-carboxylic acid. The purified permanganate product exhibited absorption and fluorescence spectra that were identical to those of the standard pterin-6-carboxylic acid (data not shown). The fluorescent species

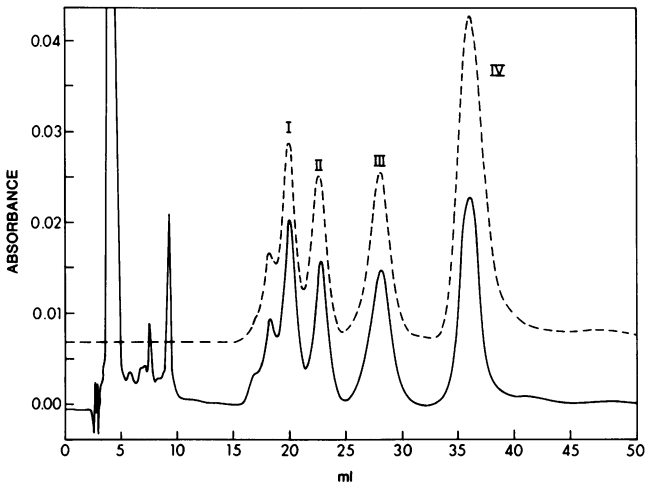

FIG. 3. HPLC elution profile of fluorescent species released from DNA photolyase. The enzyme was treated with iodine at neutral pH for 3 hr and then denatured by acidification to pH 2. Chromatography was on a C-18 reverse-phase column with 50 mM ammonium acetate as the mobile phase. Absorbance at 280 nm (—) and fluorescence (– –) were monitored. The flow rate was 1 ml/min.

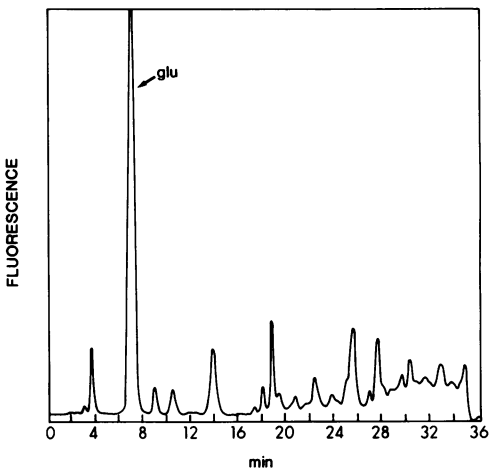

FIG. 4. HPLC elution of orthophthalaldehyde derivatives of amino acids released from the oxidized photolyase pterin by hydrolysis with methanesulfonic acid. The large peak at 7.5 min elutes at the same position as the *o*-phthalaldehyde derivative of glutamic acid (glu).

released from photolyase were not altered by alkaline permanganate at room temperature.

Presence of Glutamic Acid in the Chromophore. Each of the four species released from photolyase and resolved in ammonium acetate (Fig. 3) was anionic; that is, each was eluted from a C-18 column in the breakthrough fractions with 20% methanol as the mobile phase and was retained when the methanol/water was acidified to pH 2. Similarly, elution from QAE-Sephadex was effected when the pH of the eluent was lowered sufficiently to protonate a phosphate ester or carboxylic acid. To test for the presence of phosphate, material in peaks I–IV was pooled and was subjected to chemical analysis for phosphate. The sample was found to contain no organic phosphate.

The anionic character and the relatively high apparent molecular weight of the pterin released from photolyase suggested the possible presence of amino acids as components of the 6-alkyl substituent. For amino acid analysis, a sample containing a mixture of peak I and peak II material was hydrolyzed for 22 hr with methanesulfonic acid, neutralized, and reacted with orthophthalaldehyde. When the mixture was chromatographed on a C-18 HPLC column, as shown in Fig. 4, a single large fluorescent peak was seen at the elution position of the phthalaldehyde derivative of glutamic acid. This immediately suggested that the photolyase pterin might be, in fact, a folate derivative and that the heterogeneity could be explained by various numbers of glutamate residues.

Identification of the Stable, Oxidized Form of the Chromophore as 10-Formylfolate. A number of properties of the pterin released from iodine-oxidized photolyase, which have already been described, are not typical of oxidized, unsubstituted folic acid. Folic acid is readily cleaved by permanganate in alkaline solution at room temperature (11), whereas the photolyase pterin is not. Moreover, folic acid exhibits little fluorescence relative to the unconjugated 6-alkyl pterins, and the pH dependence of the fluorescence is quite distinct, with maximal fluorescence at acidic and basic pH values and no fluorescence at pH 6 (12). As already presented (Fig. 2), the fluorescence of the oxidized photolyase pterin is typical of the unconjugated pterins, with maximal fluorescence at pH 10–11, very little fluorescence in acid, and characteristic shifts in spectral peaks with changes in pH. The one known folic acid derivative with properties similar to those established for the oxidized photolyase pterin is the 10-formyl species. The formyl group decreases the lability of the C-9–N-10 bond and abolishes the effects of conjugation on the fluorescence of the pterin component (11, 12). To examine further similarities to 10-formylfolate, photolyase pterin was prepared from a large sample of enzyme (16 mg), and material in peaks I–IV was pooled and chromatographed on QAE-Sephadex. The absorption spectra of the sample at various pH values were obtained and compared to those of authentic 10-formylfolate. As shown in Fig. 5, the similarities were quite striking.

The formyl substituent of 10-formylfolate is labile to base. Incubation in 0.1 M NaOH overnight or in more concentrated NaOH for shorter times leads to quantitative conversion to folic acid (13). The time course of the deformylation reaction of standard 10-formylfolate in 0.5 M NaOH was followed spectrally as shown in Fig. 6. Also shown is the effect of 0.5 M NaOH on the oxidized photolyase pterin. In both cases there is a dramatic shift in the UV portion of the spectrum. The absorbance of the single peak at 255 nm decreases with time in NaOH, while a new peak at 280 nm, characteristic of the *p*-aminobenzoic acid component of folic acid, appears. These results leave little doubt that the material isolated from photolyase is indeed a conjugated pterin with a formyl or chemically similar substituent at the N-10 position.

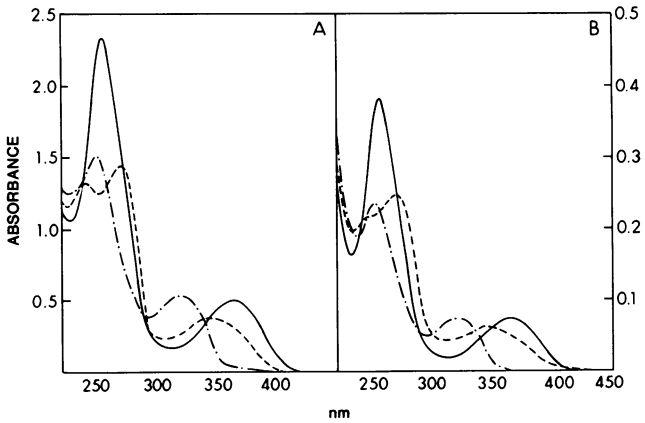

FIG. 5. Absorption spectra of 10-formylfolate (*A*) and oxidized chromophore (*B*). Samples were in 0.1 M NaOH (—), 0.1 M HCl (–·–), or 10 mM ammonium acetate, pH 6.8 (- - -).

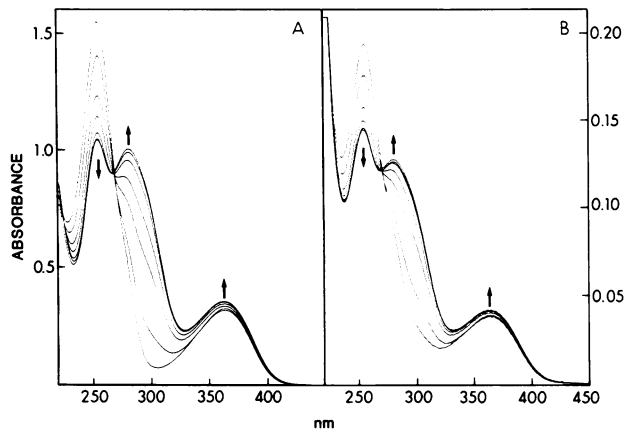

FIG. 6. Effect of 0.5 M NaOH on 10-formylfolate (*A*) and oxidized chromophore (*B*). Samples were prepared in 0.01 M NaOH (initial spectra). Spectra were recorded immediately after the addition of NaOH to a final concentration of 0.5 M and after incubations of 5, 10, 20, 40, and 140 min at room temperature. The directions of change in the spectra at various wavelengths during the course of deformylation are indicated by the arrows.

Characterization of Polyglutamates Bound to the Chromophore. Material from each of peaks I–IV was deformylated by incubation for several hours in 0.5 M NaOH. Samples and pteroylpoly-γ-glutamate standards were adjusted to pH 0.5 with HCl and reductively cleaved to the corresponding *p*-aminobenzoylpolyglutamate derivatives using zinc dust (14). The elution profiles of these derivatives chromatographed on a reverse-phase column in acetonitrile/trifluoroacetic acid are shown in Fig. 7. As can be seen, material derived from photolyase pterin peak IV coeluted with that obtained from the pteroyltriglutamate standard. The elution position of material derived from peak III eluted close to, but slightly slower than, the *p*-aminobenzoyltetraglutamate standard. Greatly enhanced retention of the peak II product relative to the *p*-aminobenzoylpentaglutamate standard and an even greater retention of the peak I product relative to *p*-aminobenzoylhexaglutamate are also apparent. These results suggest that, in fact, peak IV material contains three

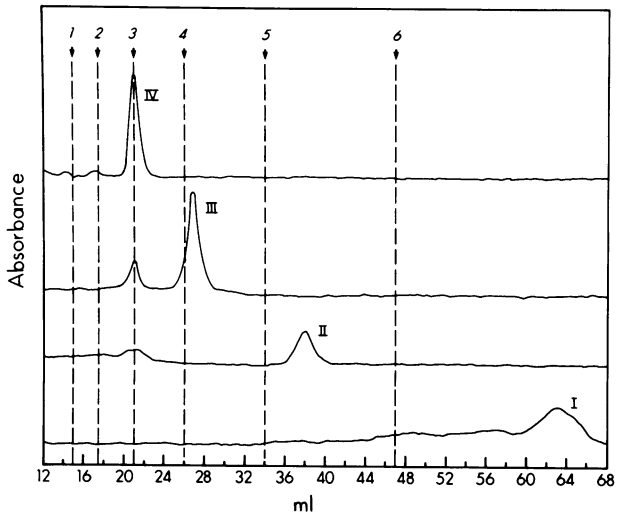

FIG. 7. HPLC elution profiles of *p*-aminobenzoylpolyglutamate derivatives obtained by reductive cleavage of deformylated, oxidized photolyase pterin peaks I–IV. The elution positions of the derivatives obtained by reductive cleavage of folylpolyglutamate standards are indicated by dashed vertical lines. The mobile phase was 4% acetonitrile containing 0.1% trifluoroacetic acid. Absorbance was monitored at 305 nm.

glutamate residues in a γ-carboxyl linkage to formylated pteroic acid. The observation that those species from the photolyase with more than three glutamates show progressively longer retention times than the γ-carboxyl standards is consistent with a report from Ferone *et al.* (15), which demonstrates that glutamate residues 4–8 in the folylpolyglutamates of *E. coli* are linked to the polyglutamate chain at the α-carboxyl group of the preceding glutamate. Additionally, it was found (data not shown) that material from photolyase peak I was cleaved by carboxypeptidase Y but only as far as the triglutamate species (peak IV). From these data it can be concluded that photolyase peaks I, II, III, and IV correspond to the hexa-, penta-, tetra-, and triglutamate species, respectively. Trace amounts of the hepta- and octaglutamate species may be identified in the original HPLC profile (Fig. 3), but no species with fewer than three glutamates were detected.

If the pterin species isolated from photolyase are indeed polyglutamate derivatives of 10-formylfolate, they should yield the monoglutamate derivative when cleaved with the appropriate hydrolase. A pteroylpolyglutamate hydrolase that cleaves pteroylglutamates to the monoglutamate derivative was prepared from rat plasma and incubated at 37°C with photolyase pterin peak IV material. The course of the reaction was followed by monitoring aliquots of the incubation mixture on HPLC in 50 mM ammonium acetate. With time, in the presence of hydrolase, the peak IV material eluting at 36 ml was converted to a new species eluting at 80 ml. As expected, the product of the hydrolase reaction coeluted with synthetic 10-formylfolate.

Thus far we have shown that the oxidized pterin product isolated from photolyase has the characteristics of 10-formylfolylpolyglutamate. Its absorption and fluorescence properties, its decay in base to folic acid, and coelution of the monoglutamate hydrolase product with authentic 10-formylfolate substantiate the identification.

Evidence That the Chromophore in the Enzyme Is 5,10-Methenyltetrahydrofolate. In view of the known interconversions of the various substituted folic acid derivatives, further studies were carried out to discern whether the pterin in native photolyase exists as oxidized 10-formylfolate or as some other species that yields 10-formylfolate when subjected to our isolation procedure. The fluorescent properties of the pterin in the native enzyme are not inconsistent with those of oxidized 10-formylfolate. However, a potential precursor of 10-formylfolate, 5,10-methenyltetrahydrofolate, also exhibits blue fluorescence (12). The 5,10-methenyl bridge is stabilized by acidic conditions but is readily cleaved to yield 10-formyltetrahydrofolate at neutral or basic pH (13). To test for the possible presence of 5,10-methenyltetrahydrofolate, a sample of *E. coli* photolyase was acidified to pH 2 (omitting the usual step of iodine oxidation prior to acidification). The acidified mixture was chromatographed on a C-18 reverse-phase column at pH 2 using a gradient of 20% to 100% methanol. A peak of 360-nm absorbing material was eluted at 10 min (approximately 60% methanol). Its absorption spectrum was scanned on-line by diode array detection and is shown in Fig. 8. A preparation of standard 5,10-methenyltetrahydrofolate chromatographed under identical conditions displayed an absorption spectrum identical to that of the material released from the photolyase (see Fig. 8). Chromatography of the material released from the enzyme using an isocratic mobile phase of 20% methanol at pH 2 resolved the single peak of 360-nm absorbing material into multiple peaks, which is consistent with the distribution of polyglutamate residues established for the 10-formylfolate derivative.

The possibility that the enzyme-bound form of the chromophore might be 10-formyltetrahydrofolate, which would cyclize to 5,10-methenyltetrahydrofolate upon acidification,

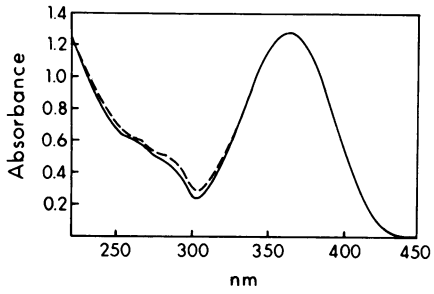

FIG. 8. Absorption spectra of 5,10-methenyltetrahydrofolate released from *E. coli* photolyase (—) and the standard compound (– –) prepared from 5-formyltetrahydrofolate. Spectra were obtained by diode-array detection of the HPLC effluent during the course of gradient elution from 20% to 100% methanol at pH 2.

is considered highly unlikely since the spectral properties of the enzyme-bound chromophore (absorbance at 385 nm and blue fluorescence) are not characteristic of 10-formyltetrahydrofolate. Thus, we conclude that the second chromophore in DNA photolyase is 5,10-methenyltetrahydrofolylpolyglutamate.

DISCUSSION

Spectral studies on DNA photolyase from *E. coli* carried out by Jorns *et al.* (1, 10) indicated the presence of a second chromophore in the enzyme in addition to the flavin cofactor. This second chromophore appeared to be responsible for the intrinsic blue fluorescence of the enzyme and to contribute to a strong absorption band at 385 nm. These properties were suggestive of the presence of an oxidized pterin species; thus, our initial characterization was undertaken with this possibility in mind. The results, as documented above, have revealed that the chromophore is indeed a pterin but not a simple oxidized 6-alkyl species. Rather the isolation and characterization of 10-formylfolic acid indicate that the chromophore is a conjugated pterin with appended polyglutamate residues. Further studies, demonstrating the release of 5,10-methenyltetrahydrofolate, suggest that the enzyme-bound form of the chromophore is in fact this reduced, fluorescent folate derivative.

The demonstration that the enzyme-bound chromophore is a reduced fluorescent pterin species explains certain ambiguities found in the early guanidine denaturation experiments. The simple interpretation that an oxidized pterin on the enzyme was becoming reduced upon enzyme denaturation and then reoxidized with prolonged exposure to air was not consistent with the known chemistry of simple pterins since no source of adequate reducing power was evident in the system. A much better interpretation of the results is now possible, which is in accord with established folate chemistry. We can conclude that the 5,10-methenyltetrahydrofolate released from the enzyme was rapidly converted at pH 10 to the nonfluorescent 10-formyltetrahydrofolate. This in turn was gradually air oxidized to 10-formylfolate. In the protocol adopted to release and purify the photolyase pterin for structural characterization, iodine was added to the native enzyme to maintain the pterin in the oxidized, fluorescent state. It now appears that addition of the oxidizing agent to the chromophore *in situ* forces pyrazine ring oxidation concomitant with methenyl bridge cleavage with the resultant conversion of one blue fluorescent species (5,10-methenyltetrahydrofolate) to another (10-formylfolate).

The results presented are consistent as well with earlier observations (10) on the photolyase second chromophore. It was shown, for instance, that denaturation of the enzyme with sodium dodecyl sulfate at pH 3 led to a shift in the absorption band of the second chromophore from 385 to 360 nm, which is indicative of a transition from the bound to the free form, and that the 360 nm absorbing species was stable at pH 3 for at least 7 hr. In contrast, when the denaturation was carried out at pH 7.4, a slow decomposition of the chromophore was observed. This decomposition, which was seen to be greatly accelerated at pH 10, is consistent with base-catalyzed methenyl bridge hydrolysis. The product, 10-formyltetrahydrofolate, absorbs in the 250–260 nm region, where its presence would be obscured by protein.

The identification of 5,10-methenyltetrahydrofolate in DNA photolyase from yeast as well as in the enzyme from *E. coli* is important in that it demonstrates the presence of this cofactor in a eukaryotic enzyme. In addition, it supports fully the conclusion reached earlier (3) that the chromophore composition of the yeast enzyme is identical to that of the fully reduced *E. coli* enzyme. The reaction catalyzed by the enzyme from both sources is complex and intriguing especially in terms of light-driven electron transfer mechanisms. The structural characterization of the second chromophore will contribute significantly to attempts aimed at understanding the mechanism of catalysis of this unusual reaction. Whether the folate cofactor serves mainly as a light-harvesting chromophore, transferring the absorbed energy to the flavin, or whether it serves a more direct role in catalysis as an electron or carbon transfer agent can now be addressed in detail. The observation that absorption of light by the reduced folate at 385 nm corresponds very closely with the action spectrum determined for the enzyme (16) strongly suggests that the second chromophore participates in the catalytic reaction; however, the possibility that the folate serves a regulatory or structural function cannot be ruled out.

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