Reconstitution of *Escherichia coli* DNA Photolyase with Various Folate Derivatives†

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ABSTRACT: DNA photolyase from *Escherichia coli* contains both flavin and pterin. However, the isolated enzyme is depleted with respect to the pterin chromophore (0.5 mol of pterin/mol of flavin). The extinction coefficient of the pterin chromophore at 360 nm is underestimated by a method used in earlier studies which assumes stoichiometric amounts of pterin and flavin. The extinction coefficient of the pterin chromophore, determined on the basis of its (*p*-aminobenzoyl)polyglutamate content ($\epsilon_{360} = 25.7 \times 10^3$ M^{-1} cm^{-1}), is in good agreement with that expected for a 5,10-methenyltetrahydrofolate derivative. Also consistent with this structure, the pterin chromophore could be reversibly hydrolyzed to yield a 10-formyltetrahydrofolate derivative or reduced to yield a 5-methyltetrahydrofolate derivative. The isolated enzyme could be reconstituted with various folate derivatives to yield enzyme that contained equimolar amounts of pterin and flavin. Similar results were obtained in reconstitution studies with the natural pterin chromophore, with 5,10-methenyltetrahydrofolate, and with 10-formyltetrahydrofolate. The results show that the polyglutamate moiety, previously identified in the natural chromophore, is not critical for binding. Reconstitution with the natural pterin chromophore did not affect catalytic activity. The latter is consistent with our previous studies which show that, although the pterin chromophore acts as a sensitizer in native enzyme, it is not essential for dimer repair which can occur at the same rate under saturating light with flavin (1,5-dihydro-FAD) as the only chromophore.

Exposure of DNA to ultraviolet light results in the formation of cyclobutane dimers between adjacent pyrimidine residues. This DNA damage can be repaired by an enzyme, DNA photolyase, in a rather unusual reaction which requires visible light. Catalysis proceeds via a photosensitized process involving enzyme-bound chromophores (Jorns, 1987). DNA photolyase from *Escherichia coli* contains two chromophores. In vivo, the flavin chromophore is present as 1,5-dihydro-FAD,[1] but oxidation to a stable, neutral FAD radical occurs during enzyme purification (Sancar et al., 1987a). The second chromophore has recently been identified as a pterin derivative since oxidation with permanganate yields 6-carboxypterin (Wang et al., 1988; Johnson et al., 1988). Treatment of the enzyme with I_2 yields another oxidized derivative which has been identified as 10-formylpteroylpolyglutamate [10-HCO-Pte$(Glu)_n$] (Johnson et al., 1988). Johnson et al. (1988) have proposed that the natural chromophore is 5,10-methenyltetrahydropteroylpolyglutamate [5,10-CH^+-H_4Pte$(Glu)_n$]. The magnitude of the extinction coefficient of 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4folate) at its absorption maximum (λ_{max} = 360 nm) is a very characteristic feature since the observed value [$\epsilon_{360} = 25.1 \times 10^3$ M^{-1} cm^{-1} (Rabinowitz, 1963)] is at least 2-fold larger as compared with other pterin derivatives that exhibit absorption maxima in this region (Pfleiderer, 1985). Although the pterin chromophore from *E. coli* photolyase exhibits an absorption maximum at 360 nm, the extinction coefficient previously reported for the chromophore (Jorns et al., 1984) is less than half the value expected for a 5,10-CH^+-H_4folate derivative.

In this paper, we show that photolyase isolated from *E. coli* is depleted with respect to the pterin chromophore, an observation which can explain why the extinction coefficient of the chromophore was underestimated in earlier studies. Photolyase preparations that contain equimolar amounts of pterin and flavin have been prepared by reconstituting the isolated enzyme with the natural pterin chromophore, with 5,10-CH^+-H_4folate, or with 10-formyltetrahydrofolate (10-HCO-H_4folate).

Experimental Procedures

Materials. 5-Formyltetrahydrofolate (5-HCO-H_4folate) was purchased from Sigma. Sodium cyanoborohydride was from Aldrich.

Methods. The blue form of *E. coli* photolyase was purified as previously described (Jorns et al., 1987a). The enzyme was assayed by the procedure described by Jorns et al. (1985). The flavin content of the enzyme was estimated on the basis of the absorbance of the flavin radical at 580 nm. The extinction coefficient of the enzyme-bound radical ($\epsilon_{580} = 4.8 \times 10^3$ M^{-1} cm^{-1}) was determined on the basis of the amount of oxidized FAD released upon aerobic denaturation of the enzyme with sodium dodecyl sulfate (SDS), following a procedure identical with that described in earlier studies (Jorns et al., 1984). (A somewhat lower value for ϵ_{580} was obtained previously, possibly because some of the flavin in the earlier preparations was present as oxidized FAD rather than as flavin radical.) Protein concentration was determined on the basis of the absorbance of the enzyme at 280 nm after denaturation with SDS and complete loss of the pterin absorption band at 360 nm. The absorbance at 280 nm was corrected for the contribution expected from free FAD (ϵ_{280} = 21 200 M^{-1} cm^{-1}) and the free pterin chromophore (ϵ_{280} = 16 000 M^{-1} cm^{-1}) under the experimental conditions (aerobic, pH 7.4). An extinction

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[1] Abbreviations: FAD, flavin adenine dinucleotide; 10-HCO-Pte$(Glu)_n$, 10-formylpteroylpolyglutamate; 10-HCO-H_4folate, 10-formyltetrahydrofolate; 5,10-CH^+-H_4Pte$(Glu)_n$, 5,10-methenyltetrahydropteroylpolyglutamate; 5,10-CH^+-H_4folate, 5,10-methenyltetrahydrofolate; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; 5-HCO-H_4folate, 5-formyltetrahydrofolate; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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coefficient for the protein moiety (ϵ_{280} = 100 100 M^{-1} cm^{-1}) was calculated by using the number of tryptophans (15) and tyrosines (14) determined from the DNA sequence of the photolyase gene (Sancar et al., 1984) and the extinction coefficients for tryptophan and tyrosine (Fasman, 1976). The isolated enzyme was found to contain 0.85 mol of flavin/mol of protein. Unless otherwise noted, enzyme concentration is expressed on the basis of flavin concentration.

The pterin chromophore was isolated from *E. coli* photolyase and purified as described by Wang et al. (1988) except that the starting enzyme was required to be homogeneous rather than about 80% pure. [The latter enzyme preparations do not contain chromophores other than those bound to photolyase but do contain two polypeptides (M_r 5200 and 40 000) that are readily soluble in methanol and not completely removed by the pterin purification procedure despite a trichloroacetic acid protein precipitation step (Wang and Jorns, unpublished results).] To determine the extinction coefficient of the purified chromophore, the concentration of the pterin was estimated on the basis of its (*p*-aminobenzoyl)polyglutamate content. In this procedure, the absorbance of the purified chromophore at 360 nm was first measured in 1.0 × 10^{-2} M HCl. The sample was then lyophilized and degraded as described by Foo et al. (1980) to release (*p*-aminobenzoyl)-polyglutamate. The latter was converted to a pink azo dye and quantified as described by Blakley (1957). The extinction coefficient determined by this procedure (ϵ_{360} = 25.7 × 10^3 M^{-1} cm^{-1}) was used in subsequent experiments to determine the concentration of the chromophore in aqueous solutions in the pH range from pH 2 to neutral pH. [This range of pH does not affect the absorption spectrum of the chromophore (Wang et al., 1988).] The extinction coefficient of the pterin chromophore was also determined after its release from the enzyme with SDS at pH 7.4, as previously described (Jorns et al., 1984).

5,10-CH^+-H_4folate was prepared as described by Rabinowitz (1963). 10-Formyltetrahydrofolate (10-HCO-H_4folate) was prepared by incubating 5,10-CH^+-H_4folate under anaerobic conditions in 6.0 mM sodium borate, pH 9.0, for 150 min at room temperature. For reconstitution experiments with 5,10-CH^+-H_4folate, photolyase was incubated with excess coenzyme as described in the legend to Figure 2, and the reaction mixture (520 μL) was then applied to a Sephadex G-25 column (1.2 × 23 cm), previously equilibrated with 50 mM sodium phosphate, pH 7.4, containing 50 mM sodium chloride, 10 mM β-mercaptoethanol, and 20% glycerol. In a separate reconstitution experiment, the enzyme (57.5 μM) was incubated with the purified pterin chromophore (259 μM) in 50 mM sodium phosphate buffer, pH 7.4, containing 50 mM sodium chloride, 10 mM dithiothreitol (DTT), and 50% glycerol. After 6 h at 4 °C, the sample (500 μL) was applied to a Sephadex G-25 column as described above. In an attempted reconstitution experiment with 5-HCO-H_4folate, photolyase (11.8 μM) was incubated for 4 h at 4 °C with 5-HCO-H_4folate (30 μM) in 50 mM sodium phosphate buffer, pH 7.4, containing 50 mM sodium chloride, 10 mM DTT, and 50% glycerol.

Absorption spectra were recorded by using a Perkin-Elmer Lambda 3 spectrophotometer. Anaerobic spectral experiments were conducted as previously described (Jorns & Hersh, 1975).

Results

Spectral Properties of the Pterin Chromophore and Its Derivatives. Two methods were used to determine the extinction coefficient of the pterin chromophore at 360 nm. Following a procedure described by Jorns et al. (1984), both chromophores were released from the enzyme by denaturation with SDS under aerobic conditions at neutral pH. Immediate oxidation of the released flavin radical was followed by a slower reaction which resulted in the complete loss of the absorption of the free pterin chromophore at 360 nm. The extinction coefficient of the pterin chromophore (ϵ_{360} = 13.0 × 10^3 M^{-1} cm^{-1}) was calculated on the basis of its absorption immediately after SDS addition and the assumption that the isolated enzyme contained equimolar amounts of flavin and pterin. In the second approach, measurements were made with the purified pterin chromophore. Since the chromophore can be oxidized to yield 10-HCO-H_4Pte(Glu)$_n$ (Johnson et al., 1988), the intact chromophore should contain a stoichiometric amount of (*p*-aminobenzoyl)polyglutamate. In this approach, the isolated chromophore was degraded by a procedure (Foo et al., 1980) which yields quantitative release of (*p*-aminobenzoyl)polyglutamate from all known naturally occurring folate derivatives. The amount of (*p*-aminobenzoyl)polyglutamate released was then determined colorimetrically after conversion to a pink-colored azo dye. Values for the extinction coefficient obtained by this procedure with the isolated pterin chromophore (ϵ_{360} = 25.7 × 10^3 M^{-1} cm^{-1}) and with authentic 5,10-CH^+-H_4folate (ϵ_{360} = 25.0 × 10^3 M^{-1} cm^{-1}) are in good agreement with the literature value reported for 5,10-CH^+-H_4folate (ϵ_{360} = 25.1 × 10^3 M^{-1} cm^{-1}) (Rabinowitz, 1963). The value obtained for the extinction coefficient of the pterin chromophore by the (*p*-aminobenzoyl)polyglutamate method is nearly twice the value estimated by the SDS procedure. The results suggest that the isolated *E. coli* enzyme contains only 0.5 mol of pterin/mol of flavin, rather than the equimolar ratio assumed by the SDS method.

Further evidence to support the proposal that the pterin chromophore is 5,10-CH^+-H_4Pte(Glu)$_n$ was sought by preparing derivatives. The 360-nm absorption band of 5,10-CH^+-H_4folate is lost upon exposure of the compound to weakly alkaline conditions (e.g., pH 9.0), owing to a reversible ring opening reaction to form 10-HCO-H_4folate. Ring opening is pH dependent and can be quantitatively reversed upon acidification but only when reactions are conducted under anaerobic conditions since 10-HCO-H_4folate is air-sensitive (Kay et al., 1960; May et al., 1951). Exposure of the pterin chromophore to pH 9.0 under anaerobic conditions converts the compound to an air-sensitive species with spectral properties similar to those reported for 10-HCO-H_4folate (Table I). The original chromophore can be regenerated upon acidification, as shown by Wang et al. (1988). Reaction of the pterin chromophore with sodium cyanoborohydride eliminates the chromophore's absorption band at 360 nm (Jorns et al., 1987b; Wang et al., 1988). The spectral properties observed for the pterin chromophore or 5,10-CH^+-H_4folate after reaction with cyanoborohydride are similar to those reported for 5-methyltetrahydrofolate (5-CH_3-H_4folate) (Table I). Formation of 5-CH_3-H_4folate upon reaction of 5,10-CH^+-H_4folate with excess cyanoborohydride is expected since borohydride will reduce 5,10-CH^+-H_4folate to 5,10-methylenetetrahydrofolate and the latter is further reduced to yield 5-CH_3-H_4folate (Osborn et al., 1960; Blair & Saunders, 1971).

Interaction of Photolyase with Various Pterin Derivatives. That *E. coli* photolyase was found to contain only 0.5 mol of 5,10-CH^+-H_4Pte(Glu)$_n$/mol of flavin suggested that the isolated enzyme might be able to bind additional pterin. In an initial experiment, the enzyme was mixed with an amount of 5,10-CH^+-H_4folate approximately equal to the estimated amount of unoccupied pterin binding sites in the enzyme. The absorption spectrum recorded immediately after the addition

Table I: Spectral Properties of the Pterin Chromophore from *E. coli* Photolyase and Its Derivatives

compound	pH	λ_{max} (nm)	$\epsilon \times 10^{-3}$ (M^{-1} cm^{-1})
pterin chromophore			
unmodified[a]	0	346	25.7
	2	360	25.7
alkali-treated, anaerobic[b]	9	260	24.6
$CNBH_4$-reduced[c]	2	268, 290	25.8, 24.4
	7	289	30.6
5,10-CH^+-H_4folate			
unmodified[d]	0	345	26.0
	3	360	25.1
alkali-treated, anaerobic[b]	9	260	22.3
$CNBH_4$-reduced[c]	2	268, 290	26.2, 24.4
	7	289	33.8
10-HCO-H_4folate[e]	9	260	18.8
5-CH_3-H_4folate[f]	1	270, 294	24.0, 23.2
	7	290	32.0

[a] Wang et al. (1988) reported that ϵ_{360}(pH 2) = ϵ_{346}(pH 0) = 7.8 × 10^3 M^{-1} cm^{-1}. The value for ϵ_{346}(pH 0) shown in the table has been corrected on the basis of the value determined for ϵ_{360}(pH 2) in this paper. [b] Product formation was complete after 140 min in 5.0 mM borate buffer, pH 9.0, at room temperature. [c] The compounds (7.4 μM) were reduced at room temperature with excess sodium cyanoborohydride (2.8 mM) in 1.0 × 10^{-2} M HCl. The pH 7.0 data were obtained in 8 mM potassium phosphate. Absorption maxima at 264 nm (pH 2) and 284 nm (pH 8) were previously reported for $CNBH_4$-reduced pterin chromophore (Wang et al., 1988). However, these results were obtained for a pterin chromophore preparation that contained two UV-absorbing polypeptide contaminants (Wang and Jorns, unpublished results). [d] Rabinowitz (1963). [e] A value of 18.1 × 10^3 M^{-1} cm^{-1} was observed by Kay et al. (1960) at pH 7.0 where 10-HCO-H_4folate exists in equilibrium with a significant amount (8.3%) of 5,10-CH^+-H_4folate (ϵ_{260} = 10.2 × 10^3 M^{-1} cm^{-1}) whereas at pH 9 the contribution from 5,10-CH^+-H_4folate is negligible. The value reported by Kay et al. (1960) at pH 7 has been corrected for the contribution from 5,10-CH^+-H_4folate. [f] Blair & Saunders (1971).

(Figure 1, curve 2) showed that the intensity of the absorption band observed for the isolated enzyme at 384 nm was enhanced but that the position of the absorption maximum was unaffected. In contrast, a hypsochromic shift of the absorption maximum from 384 to 370 nm was predicted (Figure 1, curve 4) on the basis of the assumption that 5,10-CH^+-H_4folate would not interact with the enzyme. Our previous studies showed that the natural pterin coenzyme absorbs at longer wavelengths when enzyme-bound (λ_{max} = 390 nm) as compared with the free chromophore (λ_{max} = 360 nm) (Jorns et al., 1984, 1987b). This suggested that the absence of the predicted hypsochromic shift might be due to a rapid binding of at least a portion of the added 5,10-CH^+-H_4folate. Further evidence was provided by the fact that the reaction mixture showed only a small decrease (5%) in the intensity of the absorption band at 384 nm after a 5-h incubation (Figure 1, curve 3). In the absence of binding, a much larger decrease would be expected (30%) owing to the instability of free 5,10-CH^+-H_4folate under the reaction conditions (Figure 1, inset). In a separate experiment, the isolated enzyme was incubated for 6 h with excess 5,10-CH^+-H_4folate, and the reaction mixture was then chromatographed on a Sephadex G-25 column. As judged by the elution profile (Figure 2A), the chromatography procedure yielded a base-line separation of the reconstituted enzyme from unbound small molecules. As compared with the starting enzyme (Figure 2B, curve 2), the reconstituted enzyme (Figure 2B, curve 1) showed enhanced absorbance at 380 nm relative to its absorbance at 580 nm. The increase in the value observed for the ratio, A_{380}/A_{580}, after reconstitution (A_{380}/A_{580} = 6.6) as compared with the starting enzyme (A_{380}/A_{580} = 4.2) is attributed to the binding

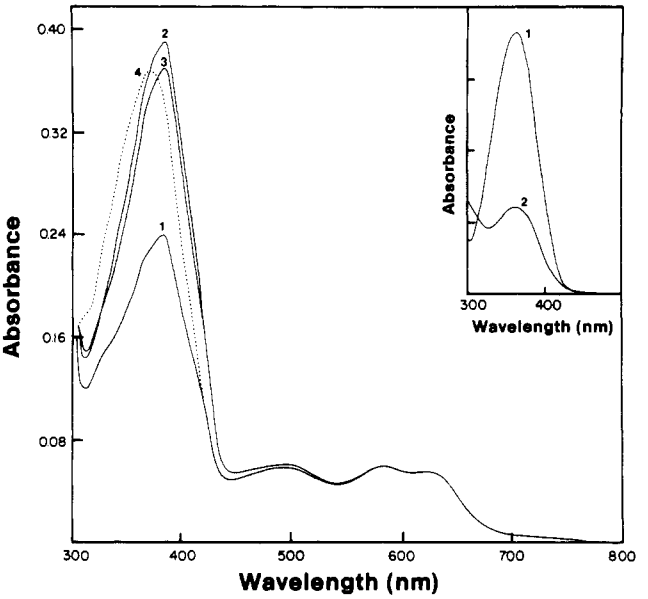

FIGURE 1: Binding of 5,10-CH^+-H_4folate to *E. coli* photolyase. Curve 1 is the absorption spectrum recorded for the isolated enzyme (12.5 μM) at 4 °C in 50 mM sodium phosphate buffer, pH 7.4, containing 50 mM sodium chloride, 10 mM DTT, and 50% glycerol. Curve 2 was recorded immediately after adding 5.8 μM 5,10-CH^+-H_4folate. Curve 3 was recorded after a 5-h incubation. The calculated spectrum shown in curve 4 is the sum of the absorption spectra observed for the starting enzyme plus free 5,10-CH^+-H_4folate (5.8 μM). Inset: Curve 1 is the initial spectrum observed for 5,10-CH^+-H_4folate (5.8 μM) in the same buffer as described above. Curve 2 was recorded after a 5-h incubation at 4 °C.

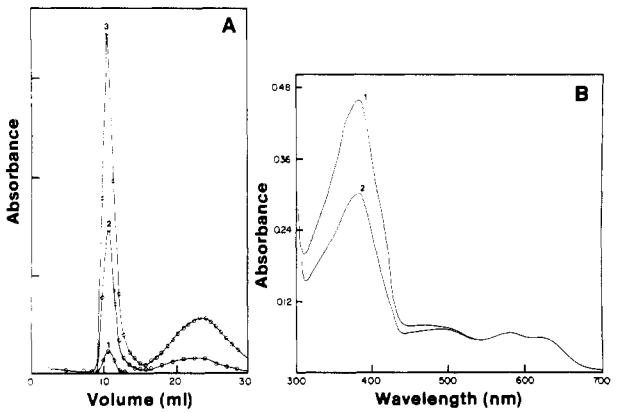

FIGURE 2: Reconstitution of *E. coli* photolyase with 5,10-CH^+-H_4folate. The enzyme (55.3 μM) was incubated with 490 μM 5,10-CH^+-H_4folate in 63 mM sodium phosphate, pH 7.4, containing 63 mM sodium chloride, 4.8 mM DTT, and 24% glycerol. After 6 h at 4 °C, the sample was applied to a Sephadex G-25 column. (Panel A) Curves 1–3 show elution profiles obtained when the fractions were monitored at 580, 380, and 280 nm, respectively. Readings at 580 and 380 nm are shown expanded 2-fold relative to readings at 280 nm. The peak protein fraction eluted at 10.5 mL. (Panel B) Curve 1 is the spectrum of the reconstituted enzyme and was recorded by using the peak protein fraction in the Sephadex G-25 column eluate. Curve 2 is the spectrum of the enzyme before reconstitution, normalized to the same absorbance at 580 nm.

of additional 5,10-CH^+-H_4folate. The increased value for the ratio cannot be due to a selective loss of flavin radical since quantitative recovery of the initial absorption of the enzyme at 580 nm was observed after reconstitution. Taking into account the known contribution of the enzyme-bound flavin radical at 380 nm (Jorns et al., 1987b), it was calculated that the observed increase in the value for the ratio, A_{380}/A_{580}, corresponded to a 1.9-fold increase in the pterin content of the enzyme. Since the starting enzyme contained 0.5 mol of pterin/mol of flavin, the results show that the reconstituted

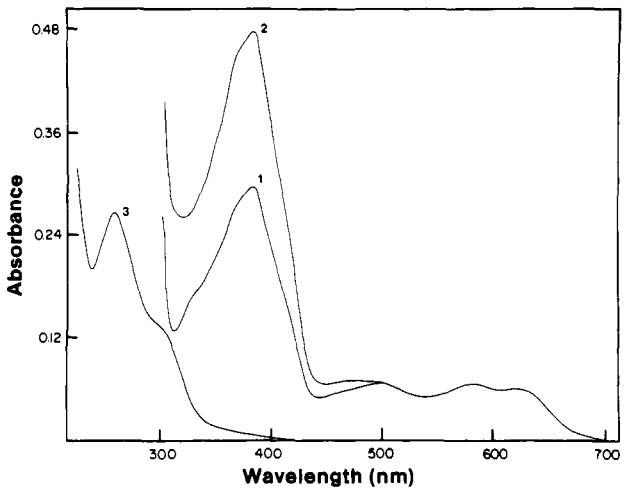

FIGURE 3: Interaction of *E. coli* photolyase with 10-HCO-H_4folate. Curves 1 and 2 show absorption spectra recorded for enzyme (12.3 μM) before and after, respectively, an overnight incubation with 10-HCO-H_4folate (14.1 μM) under anaerobic conditions at 4 °C in 27 mM sodium phosphate buffer, pH 7.4, containing 27 mM sodium chloride, 5.5 mM DTT, and 27% glycerol. (The reaction was 50% complete within 125 min, as judged by the increase in absorbance at 380 nm.) For comparison, curve 3 shows the spectrum recorded for a solution containing only 10-HCO-H_4folate (14.1 μM).

enzyme must contain stoichiometric amounts of pterin and flavin.

Since the foregoing results were obtained with a pteroylmonoglutamate derivative, additional evidence was sought to determine whether pterin binding might be affected by the polyglutamate moiety [$(Glu)_n$, n = 3, 4, 5, and 6] (Johnson et al., 1988) in the natural cofactor. A similar reconstitution experiment was therefore conducted using the pterin chromophore isolated from *E. coli* photolyase in place of 5,10-CH^+-H_4folate. The spectral properties of the reconstituted enzyme (A_{380}/A_{580} = 6.4) suggested that the binding of the natural chromophore was similar to that observed with 5,10-CH^+-H_4folate. The activity of the enzyme reconstituted with the natural pterin chromophore was measured and found to be identical with that observed for the starting enzyme.

At neutral pH, solutions of 10-HCO-H_4folate contain only a small amount of 5,10-CH^+-H_4folate at equilibrium [3.7% at pH 7.4, Kay et al. (1960)]. Nevertheless, the ability of photolyase to rapidly bind 5,10-CH^+-H_4folate (e.g., see Figure 1) suggested that a comparable reconstitution reaction might be achieved by incubating the enzyme with 10-HCO-H_4folate in place of 5,10-CH^+-H_4folate. Anaerobic solutions of 10-HCO-H_4folate are stable at neutral pH and exhibit negligible absorbance at 380 nm. Therefore, in reconstitution experiments with 10-HCO-H_4folate, it should be possible to accurately measure binding of 5,10-CH^+-H_4folate, without gel filtration, by directly monitoring the increase in absorbance of the enzyme at 380 nm. As shown in Figure 3, incubation of photolyase with 10-HCO-H_4folate did not affect the absorbance of the enzyme at 580 nm but did cause a pronounced increase in the absorbance at 380 nm. The ratio, A_{380}/A_{580}, increased from a value of 4.2 to 7.0, a change which corresponds to a 2.0-fold increase in the pterin content of the enzyme. When an analogous reconstitution experiment was attempted, using 5-HCO-H_4folate in place of 10-HCO-H_4folate, no incorporation of 5,10-CH^+-H_4folate into the enzyme was detected.

Discussion

In this paper, we show that DNA photolyase, isolated from *E. coli*, does not contain stoichiometric amounts of pterin and flavin. The extinction coefficient of the pterin chromophore at 360 nm is underestimated when measured by using an earlier procedure which assumes equimolar quantities of pterin and flavin. The extinction coefficient of the pterin chromophore, determined on the basis of its (*p*-aminobenzoyl)polyglutamate content (ϵ_{360} = 25.7 × 10^3 M^{-1} cm^{-1}), is in good agreement with that expected for a 5,10-CH^+-H_4folate derivative. We also show that the pterin chromophore can be reversibly hydrolyzed to yield a 10-HCO-H_4folate derivative or reduced with sodium cyanoborohydride to yield a 5-CH_3-H_4folate derivative. The downfield region of the ^1H NMR spectrum of the pterin chromophore was found to exhibit a pair of doublets [δ 7.99 (d, 2 H, J = 8.7 Hz), 7.58 (d, 2 H, J = 8.9 Hz)] that arise from the *p*-aminobenzoyl moiety. A signal attributable to the 5,10-methenyl carbon [δ 9.58 (s, 1 H)] was also detected (Wang and Jorns, unpublished observations). These results are consistent with the observed oxidation of the pterin chromophore to yield 10-HCO-Pte-$(Glu)_n$ (Johnson et al., 1988) and with the proposal that the natural chromophore is 5,10-CH^+-H_4Pte$(Glu)_n$.

Most of the studies described in this paper were conducted with an enzyme preparation which contained 0.5 mol of pterin/mol of flavin and exhibited a value of 4.2 for a spectral ratio (A_{380}/A_{580}) which reflects the relative amounts of pterin and flavin chromophores in the preparation. A survey of the spectral properties observed for nine other enzyme preparations (A_{380}/A_{580} = 4.3 ± 0.2) indicates that the ratio of pterin to flavin reported in this paper is likely to be fairly representative of the enzyme as it is currently isolated in our laboratory. Our results (repeated with two different enzyme preparations) show that the isolated enzyme is able to bind additional pterin to yield enzyme that contains equimolar quantities of pterin and flavin chromophores. This suggests that the low pterin content observed for the isolated *E. coli* enzyme may not be an inherent property but rather that the isolated enzyme is depleted with respect to the pterin chromophore. Since the enzyme is isolated from an overproducing *E. coli* strain, where photolyase constitutes 15% of total protein, it is conceivable that protein synthesis might outstrip the capacity of the organism to supply the pterin chromophore. Alternatively, a portion of the pterin chromophore might be lost during enzyme purification. That the latter is at least partly responsible is suggested by inspection of the spectral properties observed for the enzyme prior to the last chromatography step. At this stage, the enzyme is about 80% pure but does not contain any contaminants that absorb in the visible region. On the basis of results obtained with nine preparations (A_{380}/A_{580} = 5.1 ± 0.2), it is estimated that the partially purified enzyme contains somewhat more pterin (0.65 mol of pterin/mol of flavin) as compared with the final enzyme preparation.

Yeast photolyase exhibits many similarities with the *E. coli* enzyme. The yeast enzyme contains 1,5-dihydro-FAD (Jorns et al., 1987b; Sancar et al., 1987b) and a pterin chromophore (Wang et al., 1988; Johnson et al., 1988) that is probably also 5,10-CH^+-H_4Pte$(Glu)_n$. We estimated the extinction coefficient of the pterin chromophore in yeast photolyase, using the earlier SDS procedure. The observed value [ϵ_{360} = 18.9 × 10^3 M^{-1} cm^{-1} (Chanderkar and Jorns, unpublished results)] and a similar value reported by Sancar et al. (1987b) (ϵ_{360} = 19.7 × 10^3 M^{-1} cm^{-1}) are low as compared with the value expected for a 5,10-CH^+-H_4folate derivative. This suggests that the isolated yeast enzyme is also depleted with respect to the pterin chromophore, containing about 0.75 mol of pterin/mol of flavin. Although the estimated fraction of unoccupied pterin sites is smaller as compared with the isolated

E. coli enzyme, yeast photolyase did not retain the ability to bind additional pterin, as judged by an attempted reconstitution experiment with 5,10-CH^+-H_4folate (Chanderkar and Jorns, unpublished observations).

Although the natural pterin chromophore in yeast and *E. coli* photolyase contains a polyglutamate moiety, the latter does not appear to be critical for binding as judged by the similar results obtained in reconstitution experiments with *E. coli* photolyase and 5,10-CH^+-H_4folate or the natural pterin chromophore. A similar increase in the ratio, A_{380}/A_{580}, was observed in reconstitution experiments with the *E. coli* enzyme when 5,10-CH^+-H_4folate was replaced by 10-HCO-H_4folate. It is assumed that the results obtained with 10-HCO-H_4folate reflect a binding of the small amount of 5,10-CH^+-H_4folate which exists in equilibrium with 10-HCO-H_4folate at neutral pH. However, binding of 10-HCO-H_4folate, followed by conversion of the bound pterin to 5,10-CH^+-H_4folate, cannot be excluded as a possibility. No incorporation of 5,10-CH^+-H_4folate was detected in a reconstitution experiment with 5-HCO-H_4folate. This result would appear to reflect certain critical differences between the two isomers. Thus, although 5-HCO-H_4folate also exists in equilibrium with 5,10-CH^+-H_4folate, unlike 10-HCO-H_4folate, interconversions with 5-HCO-H_4folate are very slow except at elevated temperatures. Also, the amount of 5,10-CH^+-H_4folate, present at equilibrium with 5-HCO-H_4folate at pH 7.4, is more than 3 orders of magnitude smaller as compared with that observed with 10-HCO-H_4folate (Kay et al., 1960).

Reconstitution of *E. coli* photolyase with the natural pterin chromophore nearly doubled the pterin content of the enzyme but did not affect catalytic activity. This result is consistent with our previous studies. We have shown that the pterin chromophore in native enzyme can act as a sensitizer in catalysis via a pathway that requires 1,5-dihydro-FAD (Jorns, 1987). However, dimer repair in native enzyme can also occur via a pterin chromophore-independent pathway where 1,5-dihydro-FAD acts as the sensitizer. It should be noted that catalytic activity is measured under saturating light where the same rate of dimer repair is observed with 1,5-dihydro-FAD or with the pterin chromophore as sensitizer. As a consequence, the pterin chromophore can be completely eliminated without affecting catalytic activity (Jorns et al., 1987b). This means that a loss of the pterin chromophore during purification would not be detectable on the basis of the recovery of enzyme activity. The pterin chromophore does act as a sensitizer in vivo, as evidenced by the action spectrum determined for dimer repair with *E. coli* cells (Harm, 1970; Jagger et al., 1969).

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Registry No. 5,10-CH^+-H_4folate, 10360-12-0; 10-HCO-H_4folate, 2800-34-2; DNA photolyase, 37290-70-3.

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