Identification of a Pterin Derivative in *Escherichia coli* DNA Photolyase†

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ABSTRACT: DNA photolyase from *Escherichia coli* contains reduced flavin adenine dinucleotide plus a second chromophore, partially characterized in previous studies. Both chromophores function as sensitizers in catalysis. The second chromophore has been identified as a 6-substituted pterin derivative. The compound is oxidized with permanganate to yield 6-carboxypterin or reduced with sodium cyanoborohydride to yield a 5,6,7,8-tetrahydropterin derivative. The second chromophore exhibits spectral properties (λ_{max} = 360, 255 nm, pH 2) similar to that observed for 7,8-dihydropterin cations. The compound does not exhibit a spectrally detectable pK_a around 4 but is converted to a dication (λ_{max} = 346, 255 nm) in strong acid (pK_a ~ 1). Similar ionization behavior is observed with 7,8-dihydropterin derivatives that are alkylated at N(5). The instability of the second chromophore in weakly alkaline solution is due to a fully reversible conversion to a labile bleached form. As compared with other pterin derivatives, the hydrolytic instability is unusual but is very similar to that observed for 5,6-dialkyl-7,8-dihydropterinium salts. It is proposed that the second chromophore is a 7,8-dihydropterin with substituents at positions 5 and 6. The discovery that a pterin derivative functions as a photosensitizer in DNA repair is apparently the first example of a photobiological function for pterins.

Photoreactivating enzymes (DNA photolyases) monomerize dimers that are formed between adjacent pyrimidine residues when DNA is exposed to ultraviolet light. The enzymatic reaction is rather unusual since catalysis requires visible light. The catalytically and physiologically significant form of the enyzme in *Escherichia coli* contains 1,5-dihydro-FAD[1] plus a partially characterized second chromophore (Jorns et al., 1987a,b; Sancar et al., 1987a). A similar pair of chromophores is present in yeast photolyase (Jorns et al., 1987a; Sancar et al., 1987b). Enzymic dimer repair with *E. coli* photolyase exhibits a quantum yield of 1.0 (Sancar et al., 1987a). This means that every absorbed quantum of light is used to split dimers and that either of the two chromophores can act as a sensitizer in catalysis. We have proposed that catalysis with the reduced flavin as sensitizer involves transfer of an electron from the excited flavin to form an unstable pyrimidine dimer radical anion that spontaneously monomerizes (Jorns et al., 1987b; Jorns, 1987). We initiated studies to determine the structure of the second chromophore in *E. coli* photolyase in an effort to elucidate its role in catalysis. In this paper we show that the second chromophore contains a 6-substituted pterin ring and can be oxidized by permanganate to yield 6-carboxypterin or reduced with cyanoborohydride to yield a 5,6,7,8-tetrahydropterin derivative. As a pterin derivative, the hydrolytic instability of the second chromophore is rather unusual but remarkably parallels reactions observed with 5,6-dialkyl-7,8-dihydropterinium salts (Pfleiderer & Mengel, 1971; Jordan and Jorns, unpublished experiments). On the basis of these and other observations, we propose that the second chromophore is a 7,8-dihydropterin with substituents at positions 5 and 6. The results appear to provide the first example of the involvement of pterin in a photobiological process.

Experimental Procedures

Materials. 6-Carboxypterin, 7-carboxypterin, and 5,6,7,8-tetramethyl-7,8-dihydropterinium-*p*-toluenesulfonate were gifts from Dr. Wolfgang Pfleiderer. Biopterin, 7,8-dihydrobiopterin, 6,7-dimethyl-5,6,7,8-tetrahydropterin, and Sephadex G-25 were purchased from Sigma. Potassium permanganate was from Fisher. Sodium cyanoborohydride was obtained from Aldrich. Dowex AG 1-X8 and Dowex AG 50W-4 were from Bio-Rad Laboratories. Silica gel 60 F254 TLC plates were from Merck. Cellulose (Cel 300-10 UV 254) TLC plates were purchased from Brinkmann.

Methods. The blue form of *E. coli* photolyase was purified by a procedure similar to that previously described (Jorns et al., 1987a). All steps of the isolation of the second chromophore were conducted at 0–4 °C. Purified enzyme was desalted by chromatography on Sephadex G-25. The protein was precipitated with 5% trichloroacetic acid, and the precipitate was removed by centrifugation. The supernatant was applied to a Dowex AG 1-X8 column equilibrated with 1.0×10^{-2} M HCl. FAD and trichloroacetic acid stick to the column and are separated from the purified second chromophore which passes through the column. The eluate containing the purified second chromophore was lyophilized and stored at −20 °C. The overall yield varied between 60 and 70% as judged by the recovery of the absorbance of the second chromophore at 360 nm. An extinction coefficient previously reported by Jorns et al. (1984) ($\epsilon_{360} = 7.8 \times 10^3$ M^{-1} cm^{-1}) was used to calculate the second chromophore concentration. Oxidation of the second chromophore with potassium permanganate was performed by heating a sample (80 μL) for 15 min at 100 °C in 0.1 N NaOH containing 0.055 M potassium permanganate. Excess reagent was destroyed with ethanol (10 μL), and MnO_2 was removed by centrifugation. 6,7-Dicarboxypterin, used as a standard in TLC studies, was prepared from 6,7-dimethyl-5,6,7,8-tetrahydropterin as described by Keltjens et al. (1983). A Perkin-Elmer Lambda 5 spectrophotofluorometer and a Perkin-Elmer Lambda 3 spectrophotometer were used for fluorescence and absorption studies, respectively. Anaerobic spectral experiments were conducted as previously described (Jorns & Hersh, 1975). Attempted reduction of the second chromophore with sodium dithionite was conducted under anaerobic conditions with

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[1] Abbreviations: FAD, flavin adenine dinucleotide; TLC, thin-layer chromatography.

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Table I: Identification of the Pterin Formed by Permanganate Oxidation of the Second Chromophore[a]

compound	R_f in solvent system[b]				
	A	B	C	D	E
6-carboxypterin	0.58	0.05	0.80	0.18	0.31
oxidized second chromophore	0.58	0.05	0.80	0.18	0.31
7-carboxypterin	0.46	0.37	0.75	0.21	0.26
6,7-dicarboxypterin	0.39	0.76	0.80	0.27	0.31

[a] Permanganate oxidation was performed as described under Methods. [b] The following solvent systems were used: A, 3% NH_4Cl in water; B, ethanol–water (80:20); C, pyridine–ethyl acetate–water (4:3:3); D, 1-butanol–acetic acid–water (12:3:5); E, 2-butanol–formic acid–water (6:1:2). Silica thin-layer plates were used for all solvents except for solvent E where cellulose plates were used.

methanol containing 5% water as solvent. (Reaction of dithionite with biopterin under the same conditions yielded 7,8-dihydrobiopterin.) A pH titration with the cyanoborohydride-reduced second chromophore was conducted under aerobic conditions in 18 mM sodium pyrophosphate buffer containing 10 mM NaCl at 6 °C.

Results

Oxidation of the Second Chromophore with Permanganate. As compared with other compounds known to function as prosthetic groups in enzymes, the spectral properties of the second chromophore most closely resemble those observed for pterin derivatives. Oxidation with alkaline permanganate converts even large, complex substituents on the pterin ring to a simple carboxylic acid group (Keltjens et al., 1983). Accordingly, the second chromophore was treated with permanganate in an attempt to convert the compound to a known, carboxy-substituted pterin derivative. When the second chromophore was added to 0.1 N NaOH, the absorption band of the compound at 360 nm immediately disappeared owing to a bleaching reaction (vide infra). The alkaline sample was mixed with permanganate and heated for 15 min at 100 °C. A single, blue fluorescent product was formed that comigrated with 6-carboxypterin in TLC studies using five different solvent systems (Table I). The absorption spectrum observed after permanganate oxidation of the second chromophore exhibited peaks at 363 and 260 nm, similar to that reported for 6-carboxypterin under alkaline conditions [λ_{max} = 363, 263 nm, pH 13 (Pfleiderer et al., 1971)]. The yield of 6-carboxypterin, calculated on the basis of the absorption of the product at 363 nm, was 34%. That the blue fluorescent product detected in TLC studies was responsible for the absorption spectrum observed for permanganate-treated second chromophore was evidenced by the fact that the fluorescence excitation spectrum of the treated sample (λ_{max} = 365, 261 nm, pH 13; Figure 1) nearly superimposed with its absorption spectrum. 6-Carboxypterin exhibits characteristic spectral changes as a function of pH owing to ionizations in the pterin ring [pK_a = 7.78, 2.88 (Pfeiderer et al., 1971)]. Fluorescence excitation spectra recorded for the permanganate-treated second chromophore at pH 13.0, pH 6.5, and pH 1.0 show that lowering the pH results in a pronounced hypsochromic shift of the 365-nm band while the 261-nm peak shifts to longer wavelengths, similar to that observed for 6-carboxypterin (Figure 1). Changes in excitation spectra were mirrored by corresponding changes in absorption spectra (data not shown). Both the permanganate-treated second chromophore and 6-carboxypterin exhibit a fluorescence emission maximum at 450 nm and maximal fluorescence intensity at neutral pH. That 6-carboxypterin is formed upon permanganate oxidation indicates the second chromophore is a 6-substituted pterin derivative.

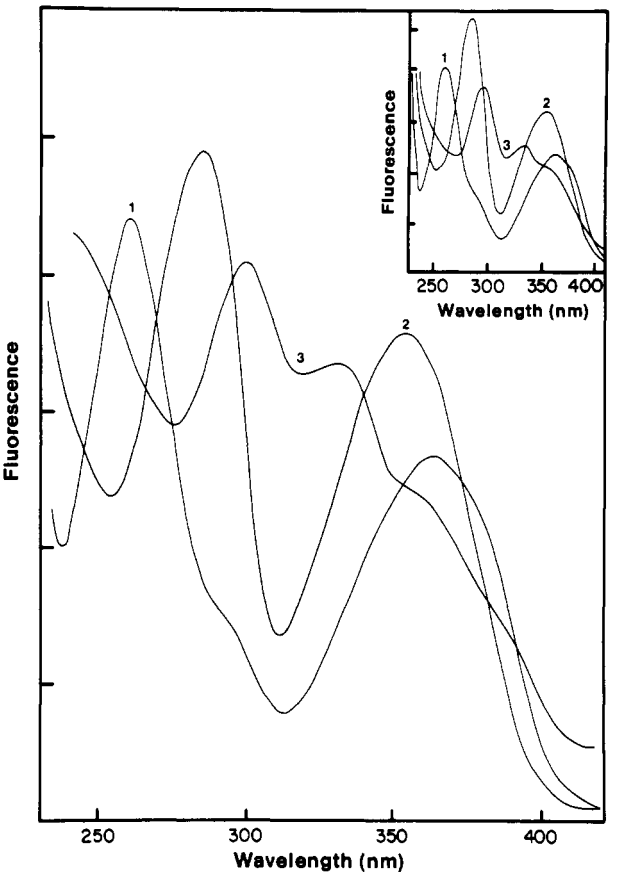


FIGURE 1: Comparison of the spectral properties of the permanganate-oxidized second chromophore with 6-carboxypterin. Curves 1–3 are fluorescence excitation spectra (emission at 450 nm) recorded for the permanganate-oxidized second chromophore at pH 13, pH 6.5, and pH 1.0, respectively. Curves 1 and 2 are plotted with the same arbitrary units for fluorescence intensity whereas curve 3 is shown expanded 10-fold to facilitate comparison of excitation maxima. The inset shows the corresponding data obtained with 6-carboxypterin.

Effect of pH on the Absorption Spectrum of the Second Chromophore. At pH 2.0 the purified second chromophore exhibits absorption maxima at 360 [$\epsilon_{360} = 7.8 \times 10^3$ M^{-1} cm^{-1} (Jorns et al., 1984)] and 255 nm ($\epsilon_{255} = 10.1 \times 10^3$ M^{-1} cm^{-1}) (Figure 2). That the second chromophore is unlikely to be a 5,6,7,8-tetrahydropterin derivative is evidenced by the presence of an absorption band at 360 nm since tetrahydropterin derivatives typically do not exhibit absorption maxima at wavelengths greater than 305 nm (Pfleiderer, 1985). 7,8-Dihydropterin derivatives exist as monocations at pH 2.0 [pK_a ~ 4 (Pfleiderer, 1985)] and exhibit spectral properties similar to those observed for the second chromophore. For example, the monocation formed with 6-methyl-7,8-dihydropterin exhibits maxima at 361 and 252 nm (Pfleiderer & Zondler, 1966). That the second chromophore is positively charged at pH 2.0 is evidenced by the fact that the compound sticks to a cation-exchange column (Dowex AG 50W-4) at this pH but passes through an anion-exchange column (Dowex AG 1-X8). (The results obtained with the cation-exchange resin cannot be attributed to hydrophobic interaction with the matrix since the same styrene–divinylbenzene matrix was present in the anion-exchange resin.) Oxidized pterin derivatives are generally partially protonated at pH 2.0 (pK_a ~ 2) and absorb at shorter wavelengths (λ_{max} ~ 330 nm (Pfleiderer, 1985)]. 7,8-Dihydropterin derivatives typically exhibit a hypsochromic shift of the near-UV peak when the pH is raised from acidic to neutral owing to proton loss from N(5), whereas a bathochromic shift is expected with oxidized pterin derivatives due

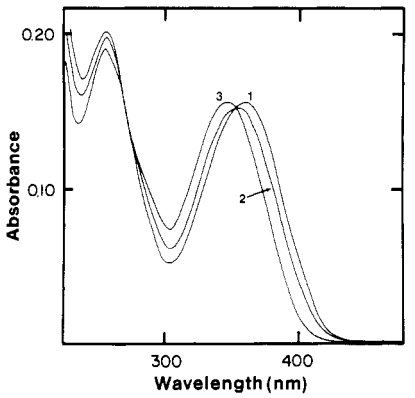


FIGURE 2: Effect of pH on the absorption spectrum of the second chromophore. Curves 1–3 were recorded in 1.0×10^{-2}, 0.10, and 1.0 M HCl, respectively. The spectrum shown in curve 3 was unaffected when the HCl concentration was raised from 1.0 to 6.0 M. Spectra identical with that shown in curve 1 were obtained at pH 3.0 and pH 7.0. The latter spectra were recorded immediately after pH adjustment at 4 °C, as described in the text.

to loss of ionization at N(1) (Pfleiderer, 1985). This difference normally provides a useful criterion for distinguishing between oxidized versus 7,8-dihydropterin derivatives but is not applicable in the case of 7,8-dihydropterins that are alkylated at N(5) since the resulting 5-alkyl-7,8-dihydropterinium salts cannot ionize and exist as monocations under both neutral and weakly acidic conditions (Pfleiderer & Mengel, 1971). Although the second chromophore is labile at pH values greater than 2 (vide infra), spectra recorded immediately after the pH of an acidic solution at 4 °C was raised revealed no significant spectral changes in the range pH 2–7. On the other hand, titration of the second chromophore from pH 2 to pH 0 (Figure 2) resulted in a reversible hypsochromic shift of the 360-nm peak to 346 nm ($pK_a \sim 1$). A similar hypsochromic shift (e.g., from 361 to 350 nm) is observed when 5-alkyl-7,8-dihydropterinium monocations are converted to the corresponding dications in strong acid (Pfleiderer & Mengel, 1971). The spectral properties of the second chromophore at various pH values are similar to those expected for a 5-alkyl-7,8-dihydropterinium salt.

Reduction of the Second Chromophore. The purified second chromophore is readily reduced by sodium cyanoborohydride at pH 2.0. The conversion does not involve any spectrally detectable intermediates, as evidenced by the isosbestic course of the reaction (Figure 3). The reaction results in a loss of the absorption band at 360 nm accompanied by a shift of the band at 255 nm to 264 nm ($\epsilon_{264} = 11.6 \times 10^3$ M^{-1} cm^{-1}). When the reduced second chromophore was titrated in the range pH 2–9, an isosbestic shift of the 264-nm band to 284 nm ($\epsilon_{284} = 10.9 \times 10^3$ M^{-1} cm^{-1}) was observed. The shift was fully reversible and complete by pH 8 (pK_a = 6.0; data not shown). 5,6,7,8-Tetrahydropterin derivatives also exhibit a pK_a near 6 due to ionization at N(5), and the monocation and neutral forms are expected to be the predominant species in solution at pH 2 and pH 8, respectively (Pfleiderer, 1985). The absorption maxima and extinction coefficients observed for the reduced second chromophore at pH 2 and pH 8 are similar to those observed for the monocation and neutral forms of tetrahydropterin derivatives, respectively. For example, the monocation formed with 5,6-dimethyl-5,6,7,8-tetrahydropterin exhibits a peak at 265 nm ($\epsilon_{265} = 12.6 \times 10^3$ M^{-1} cm^{-1}) while the neutral form shows a band at 286 nm ($\epsilon_{286} = 11.0 \times 10^3$ M^{-1} cm^{-1}) (Armarego & Shou, 1978). The results indicate that the reaction with sodium cyanoborohydride converts the second chromophore

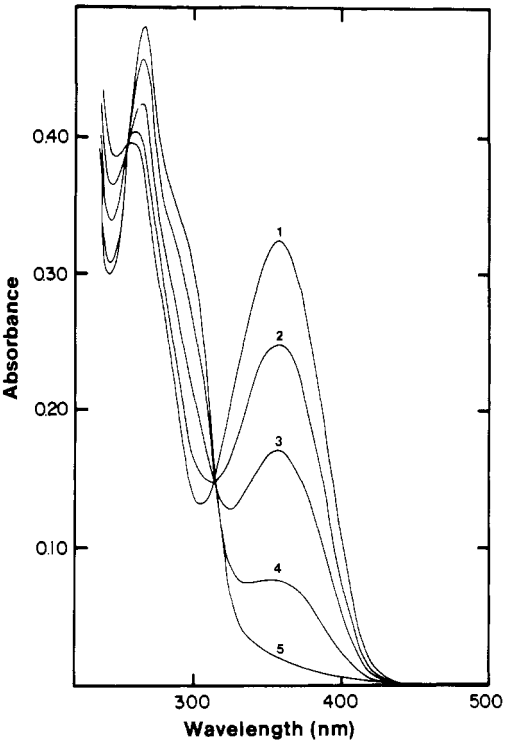


FIGURE 3: Reaction of the second chromophore with sodium cyanoborohydride. Curve 1 shows the absorption spectrum of the second chromophore in 1.0×10^{-2} M HCl at 6 °C. Curves 2–5 were recorded 10, 25, 60, and 180 min, respectively, after adding 1.59 mM sodium cyanoborohydride.

to a 5,6,7,8-tetrahydropterin derivative. The reaction observed with the purified second chromophore is similar to that observed in earlier studies with crude extracts (Jorns et al., 1987b) where, due to UV-absorbing contaminants, the spectral course of the reaction could only be monitored at wavelengths greater than 300 nm. In contrast to the facile reduction observed with cyanoborohydride, the second chromophore was resistant toward reduction by dithionite. This observation provides further evidence to support the proposal that the second chromophore is a dihydropterin derivative since oxidized pterins react readily with dithionite to yield the corresponding dihydro derivative, which is resistant toward further reduction by dithionite (Fukushima & Akino, 1968; Futterman, 1957). On the other hand, both oxidized and 7,8-dihydropterin derivatives are converted to the corresponding 5,6,7,8-tetrahydropterin via reaction with borohydride (Albert & Matsuura, 1962; Viscontini & Möhlmann, 1959; Kaufman, 1964). That a similar reaction occurs with cyanoborohydride is evidenced by the isosbestic formation of 5,6,7,8-tetrahydrobiopterin, observed when either biopterin or 7,8-dihydrobiopterin was reacted with cyanoborohydride under conditions similar to those described for the reaction with the second chromophore (data not shown). That 7,8-dihydrobiopterin was not detectable as an intermediate during reduction of biopterin is due to its relatively slow formation as compared with a much faster further reduction to the tetrahydro level. Therefore, an isosbestic reaction with cyanoborohydride may not distinguish between oxidized versus 7,8-dihydropterin derivatives.

Bleaching of the Second Chromophore. Except at acid pH, aqueous solutions of the second chromophore appear to be unstable, as evidenced by the loss of the absorption band at 360 nm. Figure 4 shows the isosbestic spectral course of the reaction at pH 9.1. The rate of the reaction, as measured at 360 nm, is oxygen-independent (Jorns et al., 1984) but is both

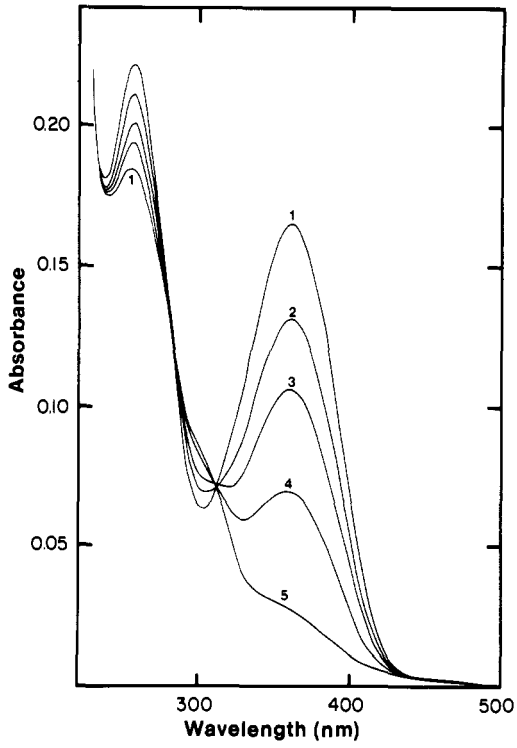


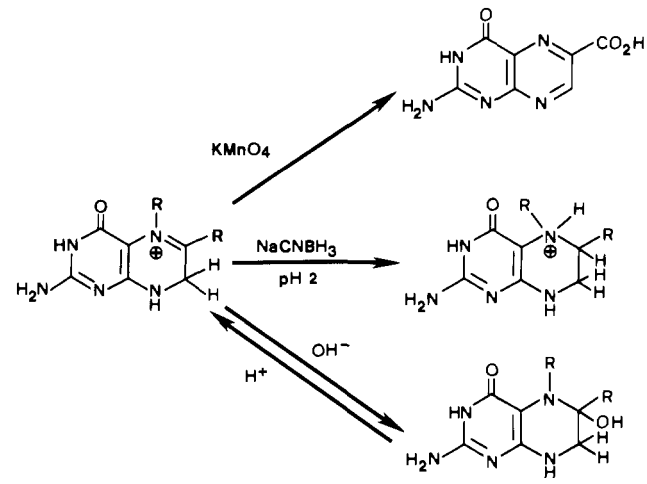
FIGURE 4: Bleaching of the visible absorption of the second chromophore. A small aliquot of a concentrated stock solution of the second chromophore in 1.0×10^{-2} M HCl was diluted into 50 mM sodium borate buffer, pH 9.1, at 5.8 °C. (The pH of the buffer was unaffected by the addition.) Curves 1–5 were recorded 0.5, 5, 10, 20, and 50 min, respectively, after mixing.

pH- and temperature-dependent, being faster at higher temperatures and pH values. 7,8-Dihydropterin derivatives are known to add various nucleophiles (such as sulfite and, in certain cases, even OH^-) across the 5,6 double bond to yield 6-substituted tetrahydropterin derivatives (Stuart et al., 1966; Pfleiderer & Mengel, 1971; Vonderschmitt et al., 1967). The reactions are reversible and result in the loss of the near-ultraviolet absorption band of the 7,8-dihydropterin. In the latter regard, the nucleophilic addition reactions resemble the spectral changes observed for the bleaching of the second chromophore. This prompted us to determine whether the bleaching of the second chromophore might also be a reversible reaction. In one experiment the second chromophore was allowed to bleach at 5 °C under anaerobic conditions at pH 9.0 (50 mM sodium borate), and then the solution was acidified to pH 0 with HCl. Acidification initiated a time-dependent increase in absorption at 360 nm in an isosbestic reaction that was complete in 45 min at 5 °C (data not shown). The recovery of the starting compound was quantitative, as evidenced by the return to an absorption spectrum identical with that observed for the untreated second chromophore and by the fact that the recovered material would undergo a normal reaction with sodium cyanoborohydride. The recovery of the second chromophore was less if bleaching was allowed to proceed under aerobic conditions. Also, pH 9 appears to be an optimal pH since lower recoveries were obtained if bleaching was conducted at either higher or lower pH values. The results indicate that the bleaching of the second chromophore at 360 nm in weakly alkaline solution is a fully reversible process but that the bleached form of the second chromophore is labile and can undergo secondary, irreversible changes.

Discussion

The second chromophore isolated from *E. coli* DNA photolyase is a 6-substituted pterin derivative, as evidenced by the

Scheme I



fact that the compound can be oxidized with permanganate to yield 6-carboxypterin. That the second chromophore is a 7,8-dihydropterin derivative alkylated at position N(5) (Scheme I) is consistent with the following observations. The spectral properties of the second chromophore in the range pH 2–7 are similar to those observed for the cationic form of 7,8-dihydropterin derivatives. Neither the second chromophore nor 5-alkyl-7,8-dihydropterinium salts exhibit a pK_a near 4 which is characteristically observed with 7,8-dihydropterins that are not alkylated at position 5. 5-Alkyl-7,8-dihydropterinium salts are converted to a dication in strong acid. A spectrally similar ionization is observed with the second chromophore. The second chromophore is reduced with sodium cyanoborohydride to yield a 5,6,7,8-tetrahydropterin derivative. Unlike oxidized pterin derivatives, the second chromophore cannot be reduced with dithionite, either when free in solution or, as previously shown, when bound to DNA photolyase (Jorns et al., 1987a). The bleaching of the second chromophore is a reversible reaction that exhibits a spectral course similar to that observed for the reversible addition of nucleophiles across the 5,6 double bond in 7,8-dihydropterin derivatives. Since the bleaching of the second chromophore occurs in the absence of added nucleophiles, it would seem reasonable to propose that the reaction might involve addition of OH^- (Scheme I). Alternatively, bleaching might involve intramolecular addition of a nucleophilic residue in the side chain at position 6 of the pterin ring. 6-Substituted-7,8-dihydropterin derivatives exhibit restricted reactivity toward nucleophiles as compared with analogues lacking a substituent at position 6 and generally do not react with OH^- unless the N(5) position is alkylated. Alkylation at position N(5) renders even 6-substituted-7,8-dihydropterin derivatives particularly susceptible toward nucleophilic addition (Pfleiderer & Mengel, 1971). For example, at alkaline pH the 5,6,7,8-tetramethyl-7,8-dihydropterinium cation does not undergo the normally expected loss of a proton at N(3) but instead adds OH^- at position 6 to form a pseudobase (Pfleiderer & Mengel, 1971), analogous to the reaction proposed for the second chromophore in Scheme I. Similar to the bleached form of the second chromophore, the pseudobase formed with the 5,6,7,8-tetramethyl-7,8-dihydropterinium cation is not stable in alkaline solution. The latter is evidenced by the fact that complete reversibility of pseudobase formation was observed only when the sample was acidified after a brief exposure to alkali (Jordan and Jorns, unpublished experiments). The bleaching and reversal reactions observed with the second chromophore are relatively slow as compared with the im-

mediate formation and dissociation of the pseudobase observed with the model compound (Jordan and Jorns, unpublished experiments). The basis for this difference is not known but might be related to steric factors.

Further studies on the structure of the second chromophore are in progress. The identification of the precise nature of the substituents on the pterin ring may provide insight regarding the biosynthesis of a prosthetic group that is stable when enzyme bound but not when free in solution under physiological conditions. Yeast photolyase contains reduced FAD plus a second chromophore that exhibits spectral properties similar to those of the second chromophore from *E. coli* photolyase (Jorns et al., 1987a; Sancar et al., 1987b). The yeast chromophore is reducible by cyanoborohydride and undergoes a bleaching reaction similar to that of the second chromophore from the *E. coli* enzyme (Chanderkar and Jorns, unpublished experiments). The results suggest that the yeast enzyme may also contain a pterin derivative. That pterin derivatives are found concentrated in areas of organisms that are exposed to light, such as the skin and eyes, has led to the suggestion that these compounds might be involved in photobiological processes (Chahidi et al., 1981). This hypothesis is supported by our discovery that a pterin derivative functions as a photosensitizer during enzymic repair of UV-damaged DNA.

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