Heyn, M. P., Cherry, R. J., & Dencher, N. A. (1981) *Biochemistry 20*, 840–849.
Himmelblau, D. M. (1972) *Applied Nonlinear Programming*, p 341, McGraw-Hill, New York.
Jap, B. K., Maestre, M. F., Hayward, S. B., & Glaeser, R. M. (1983) *Biophys. J. 43*, 81–89.
Kendrew, J. G. (1962) *Brookhaven Symp. Biol. No. 15*, 215–226.
Kendrew, J. G., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Philips, D. C., & Shore, V. C. (1960) *Nature (London) 185*, 422–427.
Leifer, D., & Henderson, R. (1983) *J. Mol. Biol. 163*, 451–466.
Lewis, B. A., & Engelman, D. M. (1983) *J. Mol. Biol. 166*, 203–210.
Long, M. M., Urry, D. W., & Stoeckenius, W. (1977) *Biochem. Biophys. Res. Commun. 75*, 725–731.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Magar, M. E. (1968) *Biochemistry 7*, 617–620.
Mao, D. (1984) Ph.D. Thesis, Columbia University.
Mao, D., Wachter, E., & Wallace, B. A. (1982) *Biochemistry 21*, 4960–4968.
Oesterhelt, D., & Stoeckenius, W. (1971) *Nature (London) 233*, 149–152.
Provencher, S. W., & Glöckner, J. (1981) *Biochemistry 20*, 33–37.
Racker, E. (1973) *Biochem. Biophys. Res. Commun. 55*, 224–230.
Rehorek, M., & Heyn, M. P. (1979) *Biochemistry 18*, 4977–4983.
Reich, C., Maestre, M. F., Edmondson, S., & Gray, D. M. (1980) *Biochemistry 19*, 5208–5213.
Reynolds, J. A., & Stoeckenius, W. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2803–2804.
Schneider, A. S., & Harmatz, D. (1976) *Biochemistry 15*, 4158–4162.
Urry, D. W. (1972) *Biochim. Biophys. Acta 265*, 115–168.
Wallace, B. A., Kohl, N., & Teeter, M. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1406–1410.

Identification of a Neutral Flavin Radical and Characterization of a Second Chromophore in *Escherichia coli* DNA Photolyase†

Marilyn Schuman Jorns,* Gwendolyn B. Sancar, and Aziz Sancar

ABSTRACT: DNA photolyase from *Escherichia coli* is a blue protein exhibiting absorption maxima at 580, 475, and 384 nm. One of the two chromophores present in this enzyme has been identified as the blue neutral flavin adenine dinucleotide (FAD) radical on the basis, in part, of visible absorption and electron spin resonance (ESR) data. The enzyme-bound radical ($\epsilon_{580} = 3.6 \times 10^3$ M^{-1} cm^{-1}) is stable toward O_2 or $K_3Fe(CN)_6$, is reversibly reduced by dithionite, and is converted to oxidized FAD upon aerobic denaturation. Disproportionation of the radical is observed upon anaerobic denaturation, consistent with an N-5 unsubstituted radical. The absorbance of the enzyme at $\lambda > 500$ nm is due only to the FAD radical whereas the band at 384 nm reflects contributions from both the radical and a second chromophore. The latter is labile when protein free at neutral pH ($\lambda_{max} = 360$ nm, $k = 5.5 \times 10^{-2}$ $min^{-1} \pm O_2$), a reaction that is readily monitored by the loss of an intense absorption band at 360 nm following enzyme denaturation under conditions where radical oxidation is immediate. This decomposition is pH dependent and the chromophore is stable at acid pH. Native photolyase is fluorescent (emission $\lambda_{max} = 470$ nm, excitation $\lambda_{max} = 398$ nm). An unlikely fluorescent flavin radical can be excluded by the position of the emission maximum. The enzyme fluorescence is attributed to the second chromophore.

Ultraviolet light causes formation of cyclobutane dimers between adjacent pyrimidine bases in DNA. The reaction, which can cause mutation, cancer, and death (Harm, 1981), is photochemically reversible. The dimer absorbs at shorter wavelengths that favor monomer formation while dimer formation is favored at longer wavelengths (Rupert, 1962) (eq 1). Action spectra observed for DNA photolyase vary

$$\text{Py} + \text{Py} \underset{240\text{ nm}}{\overset{280\text{ nm}}{\rightleftharpoons}} \text{Py–Py} \qquad (1)$$

somewhat depending on the source, but all exhibit maxima in the near-UV or visible region [418 nm, *Anacystis nidulans* (Saito & Werbin, 1970); 366 nm, yeast (Rupert & To, 1976); 360–380 nm, *Escherichia coli* (Jagger & Latarjet, 1956; Setlow, 1966); 400 nm, human (Sutherland et al., 1974); 435 nm, *Streptomyces griseus* (Jagger et al., 1969)]. Since direct excitation of pyrimidine dimers does not occur with near-UV or visible light, it has been proposed that the reaction might be a photosensitized process involving an enzyme-bound chromophore that absorbs in the near-UV or visible region. This hypothesis has not been easy to evaluate since DNA photolyase is usually present in very low levels in cells and isolation of even very modest amounts of enzyme has required heroic efforts. Nevertheless, Eker and his co-workers (Eker et al., 1981) recently showed that the enzyme from *S. griseus* does contain a chromophore that was identified as an 8-hydroxy-5-deazaflavin derivative. Studies with yeast DNA photolyase I (one of two photorepair enzymes present in this organism) show that FAD[1] is released when the enzyme is

† From the Department of Biological Chemistry, Hahnemann University School of Medicine, Philadelphia, Pennsylvania 19102 (M.S.J.), and the Department of Biochemistry, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27514 (G.B.S. and A.S.). *Received January 26, 1984.* This work was supported in part by Grants GM 31704 (M.S.J.) and GM 31082 (A.S.) from the National Institutes of Health.

0006-2960/84/0423-2673$01.50/0

heated (Iwatsuki et al., 1980; Madden & Werbin, 1984). The structure of the chromophore in the intact enzyme is not known. It cannot be FAD itself since the absorption spectrum of the enzyme (λ_{max} = 380 nm) is very different from that expected for oxidized FAD (λ_{max} = 450, 375 nm). The yeast chromophore is probably a labile FAD derivative that decomposes upon denaturation.

Sancar and his co-workers recently cloned the *E. coli* photolyase gene and joined it to a strong inducible promoter. In contrast to the parent strain, which contains only 10–20 copies of enzyme per cell, DNA photolyase constitutes 15% of total protein in induced cells containing the restructured gene (Sancar & Rupert, 1978; Sancar et al., 1983; Sancar et al., 1984). Preliminary studies on the cloned enzyme showed that it exhibited an absorption spectrum similar to that of the yeast enzyme and that FAD was also released upon heating (Sancar & Sancar, 1984).

In this paper, we report studies which show that the blue neutral FAD radical is the flavin derivative present in the *E. coli* enzyme. This enzyme also contains a second chromophore that contributes to the absorption of the enzyme at 380 nm and that is probably responsible for the visible fluorescence of the enzyme.

Experimental Procedures

Materials. Horseradish peroxidase, catalase, and glucose oxidase were purchased from Sigma. Glucose oxidase was purified before use as previously described (Jorns et al., 1983). $NaDodSO_4$ was obtained 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. Cloned *E. coli* DNA photolyase was purified to homogeneity, as judged by polyacrylamide gel electrophoresis, by a procedure that will be described elsewhere (Sancar et al., 1984). The specific activity of the enzyme (4.0×10^6 pmol h^{-1} (mg of protein)$^{-1}$, as determined by the method described by Sancar et al., 1984) was measured before and after recording absorption spectra and was found to be unchanged. Similar results were obtained for two separate preparations of the enzyme that were used in these studies. Concentrated stock solutions of enzyme were stored at −80 or −20 °C in 50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 50% glycerol. All handling of the enzyme was done under yellow light. Except where noted, reactions were conducted in 50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 1.0 mM DTT, 5% glycerol, and 10% sucrose. This solution will be referred to as standard buffer. Protein concentration was calculated by using the absorbance of the enzyme at 280 nm (corrected for the contribution of the flavin chromophore) and an extinction coefficient for the protein moiety (ϵ_{280} = 82 500 M^{-1} cm^{-1}) calculated by using the number of tryptophans (15) determined from the DNA sequence of the gene for photolyase apoenzyme (A. Sancar et al., unpublished experiments) and the extinction coefficient for tryptophan (Fasman, 1976). The flavin contribution was estimated by using an extinction coefficient determined for free FAD (ϵ_{280} = 21 200 M^{-1} cm^{-1}).

For ESR experiments samples were placed in 100-μL capillary tubes, frozen in liquid nitrogen, and then inserted into a standard 4 mM quartz ESR tube, which was located in the Varian E-109B cavity Dewar liner. For each spectrum eight scans were made. Spectra were computer averaged with an SD systems S-100 microcomputer, which was interfaced to the ESR spectrometer. Spectra were recorded at −90 °C at the following instrument settings: microwave power, 0.3 mW; modulation amplitude, 2.5 G; microwave frequency, 9.07 GHz.

Fluorescence measurements were made on an Aminco-Bowman spectrophotofluorometer. Absorption spectra were recorded on a Beckman 25 spectrophotometer.

For anaerobic experiments, a specially constructed cuvette was made anaerobic as previously described (Jorns & Hersh, 1975). In control experiments to check for residual oxygen, the main compartment of the cuvette contained 1.0 mL of standard buffer plus BSA (2 mg/mL), *o*-dianisidine (0.01%), horseradish peroxidase (5 μg/mL), and 5 μL of a saturated glucose solution. The cell was made anaerobic and then mixed with glucose oxidase (0.15 nmol) from a side arm. Oxidation of *o*-dianisidine was monitored at 440 nm [$\epsilon \sim 1.2 \times 10^4$ M^{-1} cm^{-1} (Duley & Holmes, 1975)] but was not detectable, indicating residual oxygen at less than 1 μM. As an extra precaution in experiments with photolyase, glucose and glucose oxidase were added to scavenge oxygen.

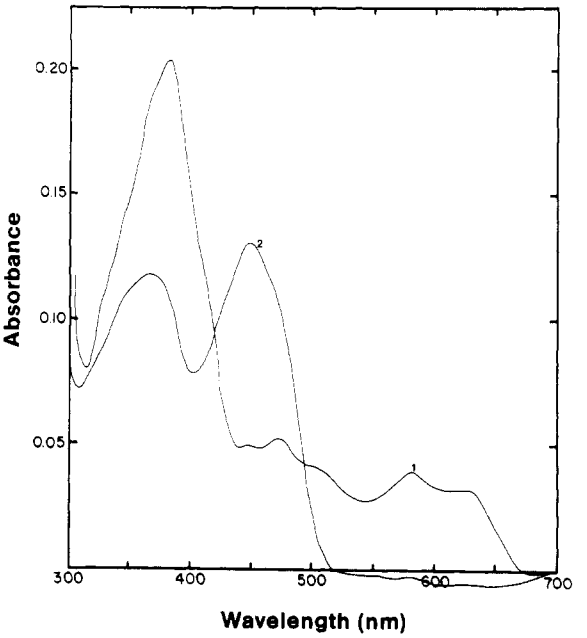

FIGURE 1: Heat denaturation of photolyase. Curve 1 shows the spectrum of native enzyme at 5.5 °C in 50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 1 mM DTT, 5% glycerol, and 10% sucrose. This buffer solution will be referred to as standard buffer. Curve 2 was obtained after heating for 5 min in a boiling water bath. Spectra are not corrected for a small negative deviation in the instrument base line at >515 nm.

Results

Concentrated (8 mg/mL) solutions of *E. coli* DNA photolyase are blue. The absorption spectrum of native enzyme (Figure 1) exhibits maxima at 580, 475, and 384 nm and prominent shoulders at 625, 505, and 450 nm. The spectrum of the enzyme, particularly in the 500–700-nm range [a region overlooked in preliminary studies (Sancar & Sancar, 1984) when the spectrum of the enzyme was zeroed at 500 nm], is similar to that observed for the blue neutral radicals bound to other flavoproteins (Müller et al., 1972). However, relative to absorbance at 580 nm, the absorbance of the photolyase at 384 nm is about twice the intensity expected for a neutral flavoprotein radical. Heat denaturation of the enzyme (Figure 1) results in the disappearance of the long-wavelength ab-

[1] Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TLC, thin-layer chromatography; ESR, electron spin resonance; DTT, dithiothreitol; Fl, flavin.

Table I: Identification of the Flavin Component Liberated by Heat Denaturation of DNA Photolyase[a]

solvent[b]	matrix	R_f FAD	R_f heat extract	R_f FMN
A	silica	0.52	0.52	0.35
B	silica	0.046	0.046	0.10
C	silica	0.079	0.079	0.15
B	cellulose	0.12	0.12	0.21
D	cellulose	0.55	0.54	0.50

[a] DNA photolyase was heated for 5 min at 100 °C and denatured protein was removed by centrifugation. The heat extract was desalted by phenol extraction (Jorns & Hersh, 1975) before TLC. [b] The following solvent systems were used: A, butanol–ethanol–0.01 M sodium acetate, pH 5.5 (1:1:1); B, butanol–acetic acid–water (4:1:5), upper layer; C, butanol–acetic acid–water (12:3:5); D, 5% NaH_2PO_4 in water.

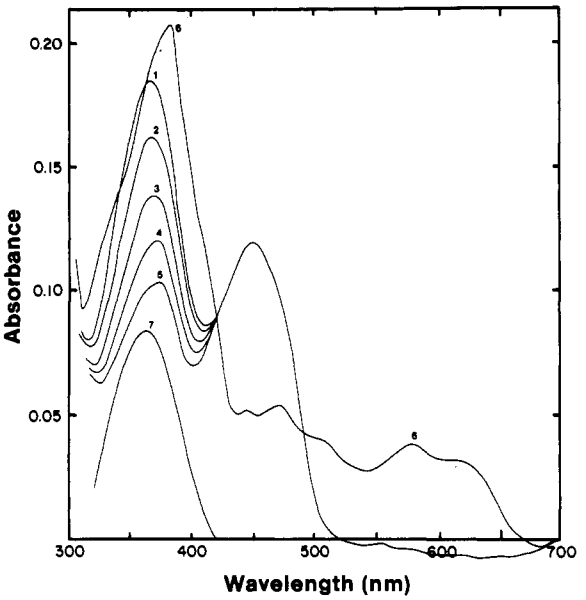

FIGURE 2: Denaturation of photolyase with $NaDodSO_4$. A stock solution of photolyase was diluted into standard buffer containing 0.77% $NaDodSO_4$ at 25 °C. Curves 1–5 were recorded after 50 s and 7, 16, 30, and 101 min, respectively. For comparison, curve 6 shows the spectrum obtained for a comparable dilution of native enzyme in standard buffer at 5.5 °C. Curve 7 is the difference spectrum obtained when curve 5 is subtracted from curve 1.

sorption band and the appearance of a typical oxidized flavin spectrum without any indication of a second chromophore in the 380-nm region. TLC studies show that the heat extract contains a single fluorophore that comigrates with FAD (Table I).

Evidence for a second chromophore was obtained by studying the denaturation of the enzyme with $NaDodSO_4$. Addition of 0.77% $NaDodSO_4$ at 25 °C results in the immediate disappearance of the absorption band around 580 nm and the appearance of oxidized flavin, as judged by the increase in absorption at 450 nm (Figure 2). The initial spectrum after $NaDodSO_4$ addition also shows an intense band at 360 nm, which decays in a relatively slow first-order reaction ($k = 5.59 \times 10^{-2}$ min^{-1}) to yield a typical flavin spectrum ($A_{450}/A_{375} = 1.17$ vs. $A_{450}/A_{375} = 1.22$ with FAD), similar to that observed after heat denaturation. The results indicate that the photolyase contains a second chromophore that decomposes upon denaturation at neutral pH to product(s) that do not absorb significantly in the visible region. The spectrum of the protein-free second chromophore can be calculated by correcting the spectrum observed immediately after $NaDodSO_4$ addition for the contribution due to oxidized flavin. The calculated

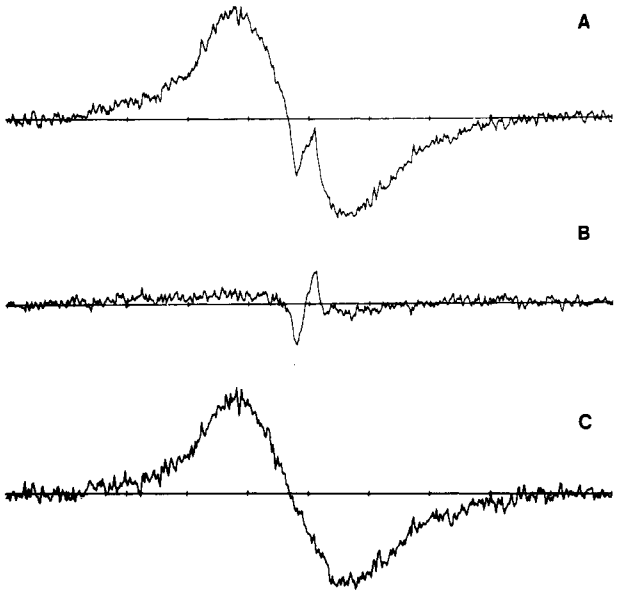

FIGURE 3: ESR spectrum of photolyase. Panel A shows the spectrum observed with photolyase in 50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM DTT, and 50% glycerol. The signal observed for buffer alone is shown in panel B. Panel C shows the difference spectrum of enzyme minus buffer. Each division on the X axis represents 10 G.

spectrum (Figure 2, curve 7) exhibits a peak at 360 nm and an intensity, relative to the initial native enzyme, which suggests that about 50% of the absorbance of the enzyme in this region is attributable to the second chromophore.

Rapid denaturation of the enzyme also occurs at 5.5 °C with 0.77% $NaDodSO_4$ as evidenced by the immediate appearance of oxidized flavin. (The slow decay of the 360-nm chromophore cannot be readily followed to completion under these conditions owing to a delayed precipitation of $NaDodSO_4$.) A slow unfolding of the enzyme appears to occur at 5.5 °C upon addition of $NaDodSO_4$ at a 10-fold lower concentration (0.077%) as evidenced by the slow first-order disappearance of absorption at 580 nm ($k = 1.33 \times 10^{-2}$ min^{-1}) accompanied by a similar rate of appearance of oxidized flavin as monitored at 450 nm ($k = 1.35 \times 10^{-2}$ min^{-1}). Under these conditions flavin oxidation occurs at a rate similar to that observed for the decay of the second chromophore at 360 nm ($k = 1.17 \times 10^{-2}$ min^{-1}). This indicates that enzyme denaturation is rate limiting for both reactions and is consistent with a model where both chromophores are attached to the same enzyme molecule.

The amount of oxidized FAD released in denaturation experiments corresponds to 0.70 mol of flavin/mol of enzyme and can be used to calculate an extinction coefficient for the enzyme at 580 nm ($\epsilon = 3.6 \times 10^3$ M^{-1} cm^{-1}), which falls within the range [(3.4–5.4) $\times 10^3$ M^{-1} cm^{-1}] (Müller et al., 1972) observed with other blue flavoprotein radicals. Assuming a 1:1 stoichiometry of flavin and the second chromophore, the data can also be used to estimate an extinction coefficient for the protein-free second chromophore ($\epsilon_{360} = 7.8 \times 10^3$ M^{-1} cm^{-1}).

The ESR spectrum observed for DNA photolyase (Figure 3) exhibits a prominent signal with a line width of 19 G, similar to that observed for neutral flavoprotein radicals, whereas red anionic flavin radicals show a bandwidth of 14–15 G (Palmer et al., 1971). The spectrum of the enzyme also contains an unexpected narrow signal superimposed on the broader flavin spectrum. This narrow signal is also observed with buffer alone (possibly due to sulfur radical from DTT) and is completely eliminated in the difference spectrum of enzyme minus buffer.

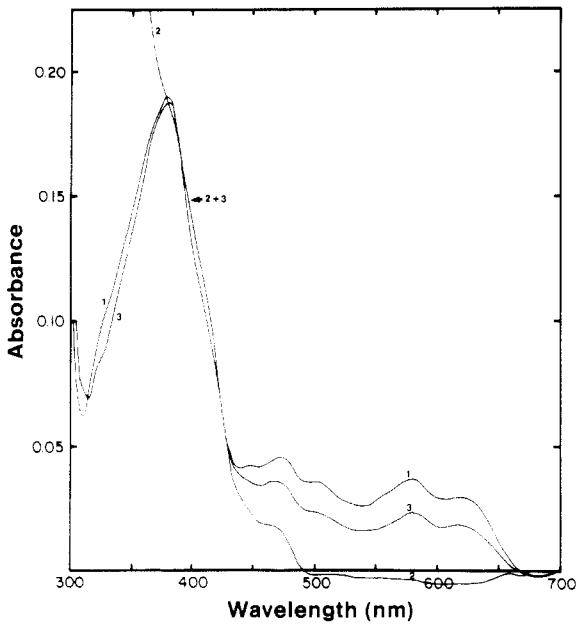

FIGURE 4: Effect of dithionite on photolyase. Curve 1 is the spectrum of the starting enzyme at 5.5 °C in standard buffer. Curve 2 was recorded immediately after the addition of a few crystals of sodium dithionite under aerobic conditions. Curve 3 was recorded 35 min later. The spectrum recorded after 15.5 h was identical with the starting spectrum.

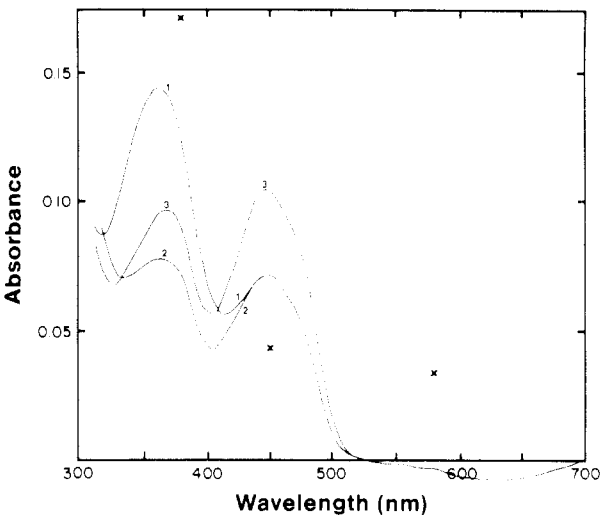

FIGURE 5: Anaerobic denaturation of photolyase. After degassing, the main compartment of an anaerobic cuvette, containing 0.9 mL of buffer plus 10 μL of a saturated glucose solution, was mixed with the contents of a side arm, which contained a mixture of catalase (1480 Sigma units), glucose oxidase (0.3 nmol), and photolyase in 120 μL. Except for enzymes and glucose, the final composition of the buffer corresponded to standard buffer. After a 15-min incubation at 0 °C, the absorbance of photolyase was quickly checked at several wavelengths (shown by ×) to verify that no denaturation had occurred during the degassing procedure. The sample was warmed to 25 °C and $NaDodSO_4$ (0.77%) was added from a second side arm. Curves 1 and 2 were recorded 2 and 60 min, respectively, after mixing. The sample was made aerobic after 64 min and curve 3 was recorded.

The radical concentration in the sample used for EPR studies was estimated at 1.09×10^{-4} M with potassium nitrosodisulfonate (Fremy's salt) as standard. This is in excellent agreement with that predicted from visible absorption studies (1.08×10^{-4} M) where experiments were typically conducted with 10-fold lower enzyme concentrations.

A stable neutral radical is not formed with free FAD where disproportionation of the radical to a mixture of oxidized and fully reduced flavin is favored (eq 2). Neutral radicals can

$$2\mathrm{FlH}\cdot \rightleftarrows \mathrm{FlH_2} + \mathrm{Fl} \qquad (2)$$

be stabilized against disproportionation when bound to certain flavoproteins, but it is rather unusual to isolate an enzyme such as DNA photolyase where the radical must also be extremely stable toward air oxidation. In the case of NADPH cytochrome P-450 reductase, the FMN component forms a radical that is stable toward air oxidation but is easily oxidized with stoichiometric amounts of $K_3Fe(CN)_6$ (Iyanagi et al., 1974; Vermilion & Coon, 1978). In tests with DNA photolyase, no evidence for radical oxidation was obtained with a 10-fold excess of $K_3Fe(CN)_6$ or upon mixing the enzyme (10^{-5} M) with phenazine methosulfate (10^{-6} M) as a potential mediator to oxygen. On the other hand, the flavin radical in DNA photolyase is readily reduced by dithionite (Figure 4). The fully reduced enzyme is not stable toward air oxidation, which results in the return of the original radical spectrum.

In addition to enzyme-bound flavin, radicals stable toward disproportionation can be formed with free flavin derivatives containing an alkyl substituent at position N-5. Since photolyase contains a second chromophore along with the flavin radical, it is conceivable that the second chromophore might be attached to the 5-position of the radical and that the protein-free radical might be stable in the absence of oxygen. (In this case, the substituent must be released upon radical oxidation since the oxidized flavin recovered from denatured DNA photolyase comigrates with authentic FAD in TLC studies.) To test this hypothesis, the enzyme was denatured in an anaerobic experiment under conditions (0.77% $NaDodSO_4$, 25 °C) where the radical is immediately oxidized when oxygen is present. As shown in Figure 5, anaerobic denaturation results in the immediate decay of the radical, as judged by the disappearance of the long-wavelength absorption band. An immediate increase in absorbance at 450 nm is also observed, but the magnitude of this change is less than that observed in aerobic experiments. No further changes are observed at 450 nm upon incubation under anaerobic conditions. Slow decomposition of the second chromophore (monitored by decreases in absorption at 360 nm) does occur under anaerobic conditions at a rate ($k = 5.54 \times 10^{-2}$ min^{-1}) similar to that observed in aerobic experiments ($k = 5.59 \times 10^{-2}$ min^{-1}). After complete decomposition of the second chromophore, the sample was made aerobic, which caused a 32% increase in absorbance at 450 nm. The results show that the protein-free radical is not stable under anaerobic conditions but rather disproportionates to a mixture of oxidized and reduced flavin as would be expected for an unsubstituted FAD radical. The percent of oxidized flavin recovered upon air oxidation is 18% less than expected on the basis of the stoichiometry of the disproportionation reaction. This might reflect a limited oxidation of the reduced flavin by small amounts of disulfide or sulfur radical associated with the DTT (1 mM) component of the buffer. No oxygen ($<10^{-6}$ M) was detected in control tests of the anaerobic system (see Methods).

Yeast photolyase I is fluorescent, exhibiting an emission maximum at 475–480 nm and an excitation maximum at 375–380 nm (Iwatsuki et al., 1980; Madden & Werbin, 1984). As shown in Figure 6, similar fluorescence properties are observed for the *E. coli* enzyme. Excitation at 380 nm yields an emission spectrum with a peak at 470 nm and shoulders at 445 and 505 nm. The excitation spectrum obtained by monitoring emission at 470 nm exhibits a maximum at 398 nm and a shoulder at 380 nm. A similar peak is seen in the excitation spectrum when emission is monitored at 520 nm, a wavelength where the enzyme fluorescence is considerably

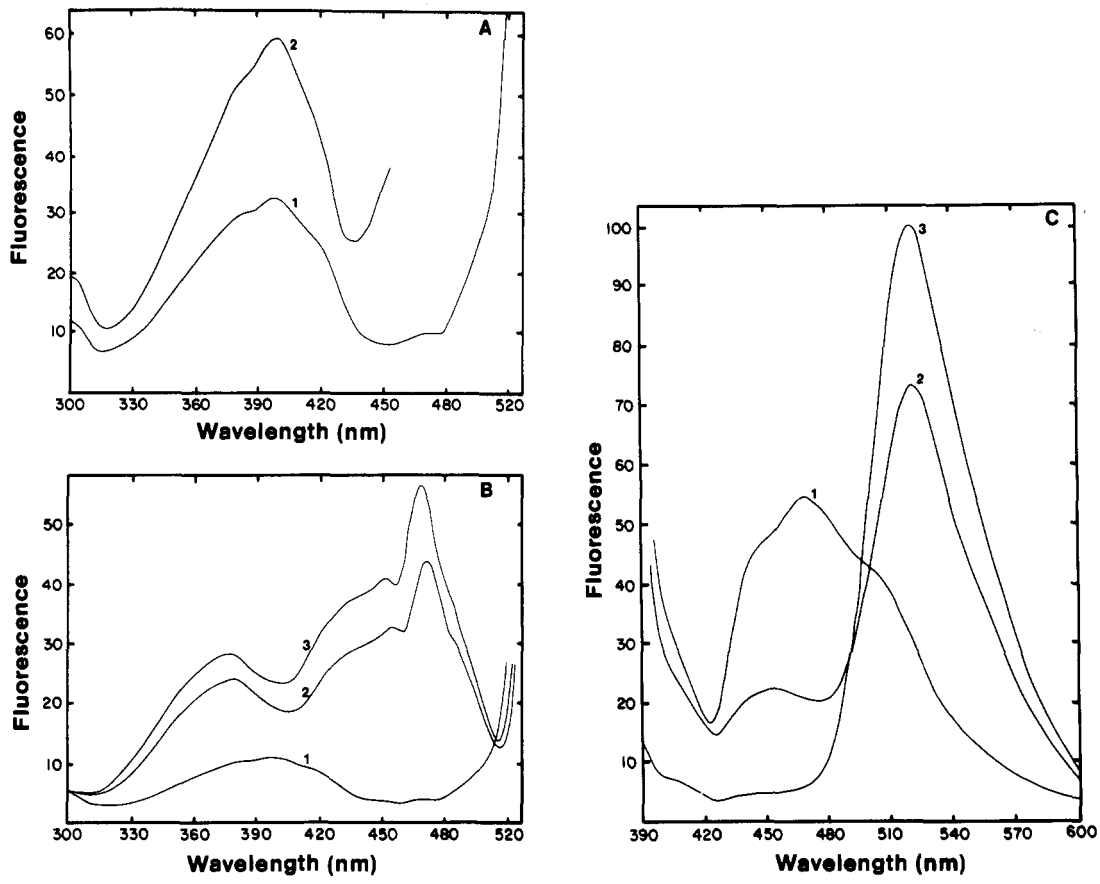

FIGURE 6: Fluorescence properties of photolyase. Except where noted, all enzyme data were collected at 5.5 °C in standard buffer. The same buffer, minus glycerol and sucrose and with 10 mM instead of 1 mM DTT, was used with FAD. Panel A: Excitation spectra of photolyase, recorded by monitoring emisssion at 520 and 470 nm, are shown in curves 1 and 2, respectively. Panel B: Excitation spectra of the enzyme (emission at 520 nm) were recorded before (curve 1) and after (curve 2) heating for 5 min at 100 °C. Curve 3 is the excitation spectrum of authentic FAD with emission monitored at 520 nm. Panel C: Emission spectra of photolyase (excitation at 380 nm) were recorded before (curve 1) and after (curve 2) heat denaturation. Curve 3 is the emission spectrum (excitation at 380 nm) recorded at 25 °C after denaturation with 0.77% $NaDodSO_4$ in standard buffer containing 0.1 M sodium citrate, pH 3.0, in place of 50 mM Tris, pH 7.4.

diminished but where oxidized FAD emits maximally. This spectrum does show a weak band near 470 nm, which probably reflects a trace amount of oxidized flavin. Aside from the fact that fluorescence spectra are uncorrected, the observation that the excitation maximum (398 nm) does not precisely match the absorption maximum (384 nm) is not unexpected since it is known that the absorption of the enzyme in this region reflects contributions from two chromophores. While there are no known examples of fluorescent flavin radicals, it is unlikely that the fluorescence of the enzyme is due to the radical simply because the emission band occurs at higher energy than the lowest energy band in the radical absorption spectrum. The results indicate that the fluorescence of the enzyme is most probably due to the second chromophore, which exhibits an absorption maximum at 360 nm when free in solution.

Heat denaturation of the enzyme results in the disappearance of the 470-nm emission band and the appearance of a band at 519 nm (Figure 6), as expected for free FAD. The heat extract also shows a weak band at 455 nm [similar to that observed in preliminary studies (Sancar & Sancar, 1984)] that is not seen with authentic FAD. This component is not detectable in the excitation spectrum of the heat extract, which is similar to that observed with FAD.

Unlike the slow decomposition of the second chromophore observed upon denaturing the enzyme with $NaDodSO_4$ at pH 7.4 ($t_{1/2}$ = 12.4 min, 25 °C), the same reaction is complete immediately after mixing the enzyme with $NaDodSO_4$ at pH 10.1 whereas at pH 3.0 the free chromophore is stable for at least 7 h (Figure 7). The results indicate that the decomposition reaction is pH dependent. Companion fluorescence experiments show that denaturation at pH 3.0 results in the immediate disappearance of the emission band of native enzyme at 470 nm accompanied by the appearance of a band at 519 nm due to oxidized FAD (Figure 6). The spectrum is stable and does not show a peak at 455 nm. A slow appearance of an emission peak at 455 nm, similar to that observed for the heat extract, is seen with enzyme samples denatured with $NaDodSO_4$ at pH 7.4 where the chromophore is labile. The results suggest that the minor band at 455 nm is associated with the decomposition of the second chromophore. The loss of the emission band at 470 nm when the sample is denatured at pH 3.0 suggests that the fluorescence of the chromophore is quenched in acidic aqueous solution, a point that will be pursued in further studies.

Discussion

That one of the two chromophores in *E. coli* DNA photolyase is the neutral FAD radical is supported by visible absorption and ESR data. The enzyme-bound radical is reducible with dithionite and stable against oxidation by O_2 [or $K_3Fe(CN)_6$], which does occur upon aerobic denaturation, yielding oxidized FAD. Disproportionation of the radical, observed upon anaerobic denaturation, is consistent with an N-5 unsubstituted semiquinone although a hydrolytically labile substituent cannot be ruled out. The second chromophore slowly decays when free in solution at pH 7.4 (λ_{max} = 360 nm) but is stable at acidic pH. Along with the radical, it con-

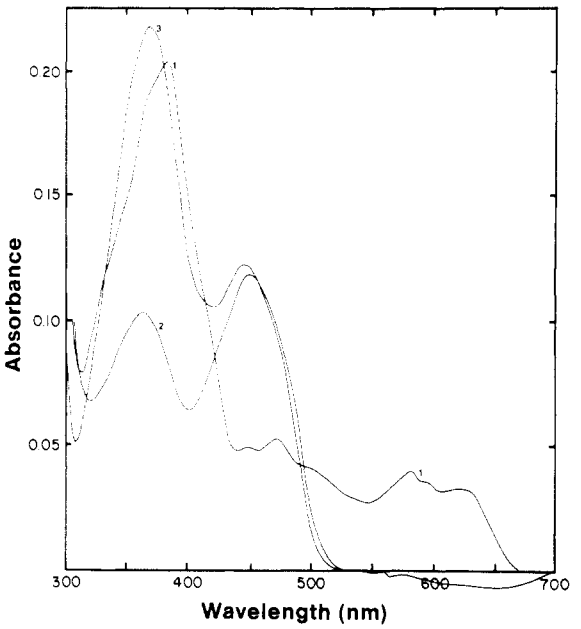

FIGURE 7: Effect of pH on the stability of the second chromophore from photolyase. Identical dilutions of a stock solution of the enzyme were made into various buffers. Curve 1 is the spectrum of native enzyme at 5.5 °C in standard buffer. Curve 2 was obtained immediately after diluting the enzyme into standard buffer at 25 °C containing 0.77% $NaDodSO_4$ and 100 mM sodium carbonate, pH 10.1, in place of 50 mM Tris, pH 7.4. Curve 3 was obtained when the denaturation was conducted at 25 °C in standard buffer containing 0.77% $NaDodSO_4$ and 100 mM sodium citrate, pH 3.0, in place of Tris.

tributes significantly to the intense absorption band of the enzyme at 384 nm and is probably responsible for all of the fluorescence of the enzyme at 470 nm.

Assuming the enzyme repairs DNA via a photosensitized reaction, it is reasonable to suppose that the light required for catalysis is absorbed by one of two chromophores. Action spectra could be used to discriminate between the two chromophores, but available data, obtained with crude enzyme or in in vivo studies (Jagger & Latarget, 1956; Setlow, 1966), are not useful since they show a maximum in the region (360–380 nm) where both chromophores absorb and were not extended into the 500–700-nm region where only the flavin radical absorbs. Evidence in favor of the second chromophore is provided by the fact that no dimer repair is observed for "dark" controls when assay mixtures are prepared under yellow light, which would be absorbed only by the flavin radical. While speculations regarding the mechanism of the *E. coli* enzyme are clearly premature, the presence of a flavin radical as a potential electron donor or acceptor is particularly tantalizing since it is known (Helene & Charlier, 1971, 1977; Roth & Lamola, 1972) from model reactions that one-electron reduction or one-electron oxidation results in monomerization of thymine dimers.

Aside from the fact that the spectrum of yeast DNA photolyase I in the 500–700-nm region is not known, the enzyme exhibits an absorption spectrum similar to that of the *E. coli* enzyme. Both enzymes exhibit similar fluorescence properties and yield oxidized FAD upon denaturation. On the basis primarily of the absorption band at 380 nm it was suggested that the yeast enzyme might contain a 4a,5-reduced FAD derivative (Iwatsuki et al., 1980). A similar proposal was made for the *E. coli* enzyme in preliminary studies (Sancar & Sancar, 1984) when the absorbance of the enzyme in the 500–700-nm region was not known. While further work is needed to determine whether the yeast enzyme contains a pair of chromophores similar to those of the *E. coli* enzyme, the results with these enzymes, along with the data on the *S. griseus* enzyme, point to an important role for flavin derivatives in enzymic photorepair of DNA and suggest that flavin-containing photolyase enzymes may be isolated from other organisms. The photolyase from *E. coli* described in this paper (M_r = 53994) has been referred to as photolyase F (Sancar et al., 1984) in order to distinguish it from an entirely different RNA-containing protein (photolyase R, M_r = 36800) also isolated from *E. coli* (Sutherland et al., 1973; Snapka & Sutherland, 1980; Cimino & Sutherland, 1982). Photolyase F appears to be the major enzyme in *E. coli* since it is 1000-fold more active than photolyase R and cells mutated at the gene, which codes for the apoenzyme of photolyase F, have very little photoreactivating activity (Sancar & Rupert, 1978).

Flavin-containing photolyase enzymes may be grouped with several other flavoenzymes that also catalyze reactions that do not involve a net oxidation–reduction. In oxynitrilase, flavin functions as a structural component at the active site (Jorns, 1979). Flavin is not bound at the active site in glyoxylate carboligase where it apparently functions to maintain the gross conformation of the enzyme (Chromartie & Walsh, 1976). Although the oxidation–reduction properties of flavin are not directly involved in catalysis with either enzyme, these properties may be important in regulation since the enzymes are inhibited when the flavin is reduced. *N*-Methylglutamate synthetase may represent the closest model for the photolyase reaction in the sense that catalysis with the synthetase involves a cyclic oxidation–reduction process where flavin functions as a typical redox catalyst (Jorns & Hersh, 1975).

Acknowledgments

We thank Dr. Theodore Taraschi for assistance in ESR experiments.

Registry No. DNA photolyase, 37290-70-3.

References

Cimino, G. D., & Sutherland, J. C. (1982) *Biochemistry 21*, 3914–3921.

Cromartie, T. H., & Walsh, C. T. (1976) *J. Biol. Chem. 251*, 329–333.

Duley, J., & Holmes, R. S. (1975) *Anal. Biochem. 69*, 164–169.

Eker, A. B. M., Dekker, R. H., & Berends, W. (1981) *Photochem. Photobiol. 33*, 65–72.

Fasman, G. D., Ed. (1976) *Handb. Biochem. Mol. Biol., 3rd ed., 1975–1977 1* (Proteins), 188.

Harm, W. (1980) *Biological Effects of Ultraviolet Radiation*, Cambridge Unniversity Press, London.

Helene, C., & Charlier, M. (1971) *Biochem. Biophys. Res. Commun. 43*, 252–256.

Helene, C., & Charlier, M. (1977) *Photochem. Photobiol. 25*, 429–434.

Iwatsuki, N., Joe, C. O., & Werbin, H. (1980) *Biochemistry 19*, 1172–1176.

Iyanagi, T., Makino, N., & Mason, H. S. (1974) *Biochemistry 13*, 1701–1710.

Jagger, J., & Latarjet, R. (1956) *Ann. Inst. Pasteur 91*, 858–873.

Jagger, J., Stafford, R. S., & Snow, J. M. (1969) *Photochem. Photobiol. 10*, 383–395.

Jorns, M. S. (1979) *J. Biol. Chem. 254*, 12145–12152.

Jorns, M. S., & Hersh, L. B. (1975) *J. Biol. Chem. 250*, 3620–3628.

Jorns, M. S., Ballenger, C., Kinney, G., Pokora, A., & Vargo, D. (1983) *J. Biol. Chem. 258*, 8561–8567.
Madden, J. J., & Werbin, H. (1984) *Photochem. Photobiol.* (in press).
Müller, F., Brüstlein, M., Hemmerich, P., Massey, V., & Walker, W. H. (1972) *Eur. J. Biochem. 25*, 573–580.
Palmer, G., Müller, F., & Massey, V. (1971) in *Flavins and Flavoproteins* (Kamin, H., Ed.) pp 123–137, University Park Press, Baltimore, MD.
Roth, H. D., & Lamola, A. A. (1972) *J. Am. Chem. Soc. 94*, 1013–1014.
Rupert, C. S. (1962) *J. Gen. Physiol. 45*, 703–724.
Rupert, C. S., & To, K. (1976) *Photochem. Photobiol. 24*, 229–235.
Saito, W., & Werbin, H. (1970) *Biochemistry 9*, 2610–2620.
Sancar, A., & Rupert, C. S. (1978) *Gene 4*, 294–308.
Sancar, A., & Sancar, G. B. (1984) *J. Mol. Biol. 172*, 223–227.
Sancar, A., Smith, F. W., & Sancar, G. B. (1984) *J. Biol. Chem.* (in press).
Sancar, G. B., Smith, F. W., & Sancar, A. (1983) *Nucleic Acids Res. 11*, 6667–6678.
Setlow, J. K. (1966) *Curr. Top. Radiat. Res. 2*, 195–248.
Snapka, R. M., & Sutherland, B. M. (1980) *Biochemistry 19*, 4201–4208.
Sutherland, B. M., Chamberlin, M. J., & Sutherland, J. C. (1973) *J. Biol. Chem. 248*, 4200–4205.
Sutherland, B. M., Runge, P., & Sutherland, J. C. (1974) *Biochemistry 13*, 4710–4715.
Vermilion, J. L., & Coon, M. J. (1978) *J. Biol. Chem. 253*, 8812–8819.

Preferential Stimulation of the in Vivo Synthesis of a Protein by Polyamines in *Escherichia coli*: Purification and Properties of the Specific Protein†

Kazuhiro Mitsui, Kazuei Igarashi,* Tomohito Kakegawa, and Seiyu Hirose

ABSTRACT: The possibility that polyamines can stimulate the synthesis of special kinds of proteins has been examined by using a polyamine-requiring mutant of *Escherichia coli*. It was found that the synthesis of some proteins, particularly one with a molecular weight (M_r) of 62K, was significantly stimulated following polyamine supplementation of polyamine-starved cells. The preferential stimulation of the synthesis of this polyamine-induced protein of M_r 62K (PI protein) was followed by the stimulation of overall protein synthesis by polyamines. PI protein was purified to homogeneity and some of its properties were examined. From studies on the effect of PI protein on MS2 RNA directed protein synthesis, it was shown that this protein stimulated the synthesis of RNA replicase by 2.2-fold in the presence of 1 mM spermidine.

Polyamines have been implicated in numerous growth processes (Cohen, 1971; Tabor & Tabor, 1976). Since polyamines are polycationic, they probably participate in many cellular processes through their binding to DNA, RNA, ribosomes, and phospholipid (Igarashi et al., 1982). It is well-known that polyamines exhibit not only a sparing effect on the Mg^{2+} requirement for polypeptide synthesis but also a stimulating effect, which cannot be equalled by any amount of Mg^{2+} (Igarashi et al., 1974; Atkins et al., 1975; Hunter et al., 1977). The stimulation of polypeptide synthesis by polyamines occurs mainly due to a stimulation of the initiation complex formation of polypeptide synthesis (Konecki et al., 1975; Salden & Bloemendal, 1976; Igarashi et al., 1980a). In addition, we have recently shown that, in QB RNA and MS2 RNA directed protein synthetic systems, spermidine caused a marked stimulation in the synthesis of RNA replicase without a significant effect on the coat protein (Watanabe et al., 1981). Therefore, we have examined the possibility of polyamines enhancing the synthesis of special kinds of proteins in vivo using a polyamine-requiring mutant of *Escherichia coli*.

In this paper, we present evidence for the preferential synthesis of a M_r 62K protein under the influence of polyamines, along with its purificatiion and properties.

Materials and Methods

Bacterial Strain and Culture Conditions. A polyamine-requiring mutant of *E. coli* used in this experiment was MA261 (*thr*, *leu*, *ser*, *thi*, *speB*, *speC*), which was kindly supplied by Dr. W. K. Maas. The cells were cultured in the presence or absence of 100 μg/mL putrescine (media A and B, respectively) by the method of Algranati et al. (1975). When growth was sufficient to give an absorbance of 0.45 at 540 nm, the cells were harvested by centrifugation at 15000*g* for 15 min. About 7 and 15 h of incubation were required when media A and B were used, respectively. The cells were washed once with a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 60 mM NH_4Cl, and 6 mM 2-mercaptoethanol, centrifuged as above, and stored at −80 °C until used. The cells cultivated in media A and B were named, tentatively, unstarved and starved bacteria, respectively.

Materials. The fractions containing elongation factors and aminoacyl-tRNA synthetases (S-100 protein) and 0.3 M NH_4Cl washed ribosomes were prepared from *E. coli* Q13 as described previously (Watanabe et al., 1981). Initiation factor 2 was purified according to the method of Hershey et al. (1977). Bacteriophage MS2 was grown and purified according to the method described by Loeb & Zinder (1961). MS2 RNA was prepared from the phage by phenol extraction and alcohol precipitation according to the procedure of Gierer & Schramm (1956). The ribosomal S1 protein was purified as described previously (Igarashi et al., 1981) according to the

† From the Faculty of Pharmaceutical Sciences, Chiba University, Yayoi-cho, Chiba, Chiba 260, Japan. *Received August 4, 1983.* This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and by a grant from Takeda Shorei Zaidan, Japan.

* Address correspondence to this author at the Department of Enzymology, Research Institute for Chemobiodynamics, Chiba University, 1-8-1, Inohana Chiba 280, Japan.

0006-2960/84/0423-2679$01.50/0