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DNA Repair Catalyzed by *Escherichia coli* DNA Photolyase Containing Only Reduced Flavin: Elimination of the Enzyme's Second Chromophore by Reduction with Sodium Borohydride†

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ABSTRACT: DNA photolyase from *Escherichia coli* contains FAD plus a partially characterized, second chromophore. In vivo, the flavin is fully reduced ($FADH_2$), but oxidation to a stable, blue radical ($FADH^{\bullet}$) occurs during enzyme isolation. The second chromophore is irreversibly reduced by reaction of the enzyme with sodium borohydride or by photoreduction in the presence of dithiothreitol. A similar reaction occurs with the protein-free chromophore and sodium cyanoborohydride. Reduction of the second chromophore is accompanied by a complete loss of the chromophore's visible absorption and fluorescence but does not significantly affect catalytic activity. The results show that the enzyme can repair dimers by a pathway involving only $FADH_2$. Enzyme-bound $FADH_2$ is fluorescent and exhibits emission (505 nm) and absorption (360 nm) maxima similar to that expected for a 1,5-dihydroflavin derivative. It is proposed that dimer cleavage via the second chromophore independent pathway involves electron donation from excited $FADH_2$ to pyrimidine dimer. Pyrimidine dimer radicals are unstable and spontaneously monomerize. Unmodified second chromophore can also act as a sensitizer in a pathway that requires $FADH_2$. This pathway may be similar to that proposed for the second chromophore independent reaction except that excited $FADH_2$ would be produced via energy transfer from the excited second chromophore. The fluorescence observed for enzyme-bound, unmodified second chromophore is quenched by $FADH^{\bullet}$ and increases 6-fold when the latter is reduced, but the absorption spectrum (λ_{max} = 390 nm, $\epsilon_{390} = 12.7 \times 10^3$ M^{-1} cm^{-1}) is independent of the redox state of the flavin. The latter observation indicates that, if the enzyme-bound chromophores constitute a single molecule, they must be separated by an "insulating" link.

The principal damage resulting from exposure of DNA to ultraviolet light is the formation of cyclobutane dimers between adjacent pyrimidine residues. Photoreactivating enzymes (DNA photolyases) repair UV-damaged DNA by splitting dimers in a rather unusual reaction that requires visible light. DNA photolyase from *Escherichia coli* contains FAD[1] plus a partially characterized fluorescent, second chromophore. In

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; $FADH^{\bullet}$, flavin adenine dinucleotide neutral radical; $FADH_2$, fully (two electron) reduced flavin adenine dinucleotide; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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vivo, the flavin is present as fully reduced FAD ($FADH_2$), but oxidation to a stable blue neutral flavin radical ($FADH^{\bullet}$) occurs during enzyme isolation (Jorns et al., 1984, 1987; Sancar et al., 1987). Yeast photolyase also contains $FADH_2$ plus a second chromophore similar to the *E. coli* enzyme (Jorns et al., 1987; Iwatsuki et al., 1980).

Recent studies indicate that $FADH^{\bullet}$ cannot act as a sensitizer in the *E. coli* enzyme and that the dimer repair observed with radical-containing enzyme is actually due to a rapid photoreduction under assay conditions which generates the active $FADH_2$ enzyme (Jorns et al., 1987; Sancar et al., 1987; Heelis & Sancar, 1986). A quantum yield of 1.0 is observed when the flavin is present as $FADH_2$. A quantum yield of 1.0 means that every absorbed quantum of light is used to split dimers and that either the reduced flavin or the second chromophore can act as a sensitizer in catalysis. It has been proposed that the enzyme can split dimers either via a mechanism involving both $FADH_2$ and the second chromophore or via a mechanism that requires only $FADH_2$ (Jorns et al., 1987; Sancar et al., 1987). Indirect evidence in support of the latter pathway is provided by recent model studies that show that reduced flavin alone can act as a photosensitizer in the cleavage of thymine dimers (Jorns, 1987).

In this paper we show that sodium borohydride selectively and irreversibly reduces the second chromophore in *E. coli* photolyase. The reaction is accompanied by a loss of the chromophore's visible absorption and fluorescence but does not significantly affect catalytic activity. The results show that the enzyme can repair dimers by a pathway involving only $FADH_2$. The borohydride reaction has also provided an opportunity to characterize the absorption and fluorescence properties of each of the chromophores in the enzyme as isolated and in its physiologically significant form.

Experimental Procedures

Materials. Sodium borohydride was purchased from Fisher. Sodium cyanoborohydride was obtained from Aldrich. SDS was bought from Bio-Rad Laboratories.

Enzyme Purification and Assay. Purification of *E. coli* photolyase was performed as previously described (Jorns et al., 1987). Enzyme activity was measured with UV- (254 nm) irradiated oligo$(dT)_{18}$ as substrate, according to the method of Jorns et al. (1985).

Reaction of Photolyase with Sodium Borohydride. Except where noted, reactions were conducted in 50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 1.0 mM dithiothreitol, and 5% glycerol. This solution will be referred to as standard buffer. Sodium borohydride stock solutions were prepared in cold 0.05 M sodium borate, pH 9.5, and used immediately. To correlate the extent of the borohydride reaction with the second chromophore content of the enzyme, separate samples of enzyme were reacted with 4.2×10^{-4}, 2.5×10^{-3}, or 1.6×10^{-2} M sodium borohydride in standard buffer at 5.5 °C. The extent of the borohydride reaction, $[(A_i - A)(A_i - A_f)^{-1}(100)]$, was calculated on the basis of the absorbance of the sample at 380 nm after a given extent of reaction (A), the initial absorbance at 380 nm (A_i), and the final absorbance at 380 nm (A_f). The second chromophore content was determined by reaction with SDS, as previously described (Jorns et al., 1987). To determine whether the borohydride reaction might be photochemically reversible, borohydride-treated enzyme was irradiated under standard assay conditions (Jorns et al., 1985), and the second chromophore content was measured by the SDS method.

Reaction of Protein-Free Second Chromophore with Sodium Cyanoborohydride. For preparation of a protein-free chromophore extract, a concentrated stock solution of photolyase in storage buffer (50 mM Tris, pH 7.4, containing 50 mM NaCl, 1.0 mM EDTA, 10 mM dithiothreitol, and 50% glycerol) was diluted 10-fold into cold water and then desalted by chromatography on a Sephadex G-25 column equilibrated with water. The enzyme was denatured with 1.0 N HCl, and the protein precipitate was removed by centrifugation. The supernatant was evaporated. The residue was washed with acetonitrile. The chromophores were dissolved in 100 mM sodium citrate buffer at pH 2.1 or pH 3.0. Stock solutions of sodium cyanoborohydride were prepared in cold methanol immediately before use.

Photoreduction of Photolyase. The sample was irradiated at a distance of 5 cm from two germicidal lights (Sylvania G15T8, 15 W) in a specially constructed cuvette under conditions as further described in the legend to Figure 7.

Spectroscopy. Fluorescence measurements were made with a Perkin-Elmer Lambda 5 spectrophotofluorometer. All spectra are reported with fluorescence intensity expressed in the same arbitrary units. For fluorescence studies, enzyme-bound $FADH_2$ was generated by adding 3.0 mM sodium dithionite to an aerobic solution of the enzyme (dithionite solutions are prepared under anaerobic conditions). Previous studies have shown that the flavin in photolyase remains fully reduced under these conditions for long periods unless the solution is vigorously aerated (Jorns et al., 1987). Similar fluorescence spectra are obtained when enzyme-bound $FADH^{\bullet}$ is reduced with a stoichiometric amount of dithionite under anaerobic conditions (Jorns, unpublished experiments). Absorption spectra were recorded with a Perkin-Elmer Lambda 3 spectrophotometer. For absorption studies, enzyme-bound $FADH_2$ was generated by anaerobic titration with dithionite as described by Jorns (1985). Extinction coefficients for enzyme-bound $FADH^{\bullet}$ and $FADH_2$ were calculated on the basis of flavin content. The latter was determined as previously described (Jorns et al., 1987). In calculating extinction coefficients for enzyme-bound second chromophore, it is assumed that the latter is stoichiometric with enzyme flavin.

Results

Effect of Borohydride on the Absorption Spectrum of Photolyase. Reaction of sodium borohydride with the enzyme results in an isosbestic reaction characterized by a large decrease in absorbance in the 384-nm region. The absorption of the enzyme in the 500–700-nm region is unaffected (Figure 1). Both $FADH^{\bullet}$ and the second chromophore contribute to the absorption band at 384 nm whereas absorption in the long-wavelength region is solely attributable to $FADH^{\bullet}$. These results suggested that borohydride caused a selective loss of the absorption due to the second chromophore. Consistent with this hypothesis, when the spectrum at the end of the borohydride reaction is subtracted from the initial spectrum of the enzyme, the resulting difference spectrum (λ_{max} = 390 nm, $\epsilon_{390} = 12.7 \times 10^3$ M^{-1} cm^{-1}) (Figure 1) is similar to that previously reported for the protein-bound second chromophore (λ_{max} = 390 nm, $\epsilon_{390} = 12.0 \times 10^3$ M^{-1} cm^{-1}) (Jorns et al., 1987).

Further evidence to show that the absorbance due to the second chromophore is eliminated by treatment with borohydride was sought by denaturing the enzyme with sodium dodecyl sulfate (SDS) after varying extents of reaction with borohydride. We have shown that the aerobic denaturation of photolyase with SDS results in the immediate oxidation of the flavin radical followed by a slower decomposition of the second chromophore, which can be monitored by following the decrease in absorbance in the 360 nm region. The magnitude

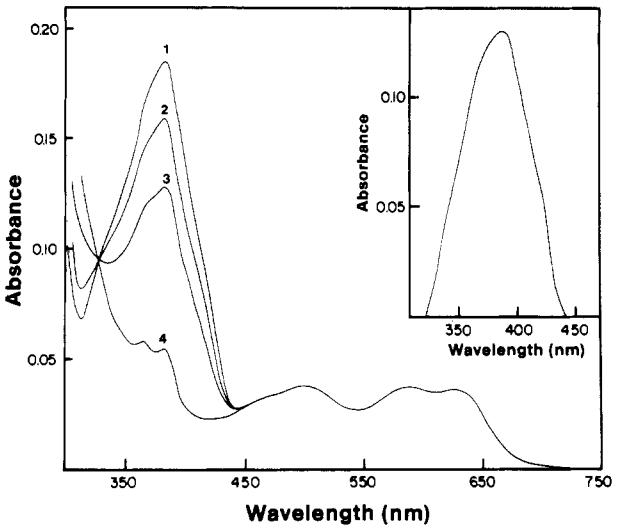

FIGURE 1: Reaction of photolyase with sodium borohydride. Curve 1 is the absorption spectrum of the enzyme in standard buffer at 5.5 °C. Curves 2–4 were recorded after adding borohydride to a concentration of 4.2×10^{-4}, 2.5×10^{-3}, and 1.6×10^{-2} M, respectively. Spectra were recorded 30 min after each addition when the bubbling due to borohydride had stopped. The inset shows the difference spectrum calculated by subtracting curve 4 from curve 1.

of this slow absorbance decrease has been used to estimate the second chromophore content of various enzyme preparations (Jorns et al., 1987). The second chromophore content, as estimated by the SDS method, is found to be inversely proportional to the extent of the borohydride reaction, and no second chromophore is detectable at the end of the reaction.

The absorption spectrum of FADH• bound to photolyase has previously been difficult to evaluate at $\lambda < 450$ nm owing to interference from the strong absorption of the second chromophore in this region. Since the latter is eliminated by reaction of the enzyme with borohydride, the resulting spectrum should correspond to the absorption spectrum of FADH•. In addition to absorption bands in the long-wavelength region, the enzyme-bound radical exhibits peaks at 364 nm ($\epsilon = 5.6 \times 10^3$ M^{-1} cm^{-1}) and 382 nm ($\epsilon = 5.3 \times 10^3$ M^{-1} cm^{-1}) and a minimum centered around 420 nm. Neutral radicals bound to other flavoproteins (Müller et al., 1972) exhibit an absorption maximum around 380 nm [$\epsilon = (4.6–8.2) \times 10^3$ M^{-1} cm^{-1}] similar to photolyase and a second peak around 350 nm [$\epsilon = (7.7–10.2) \times 10^3$ M^{-1} cm^{-1}], which is shifted somewhat as compared to the peak at 364 nm observed for the photolyase radical.

Effect of Borohydride on Enzyme Fluorescence. We have shown that the fluorescence [$\lambda_{max} = 470$ nm (emission), 390 nm (excitation)] observed with photolyase preparations containing FADH• plus the second chromophore is due entirely to the second chromophore (Jorns et al., 1984, 1987). Since borohydride eliminates the visible absorption of the second chromophore, it is expected that the reaction would also result in a loss of fluorescence. As shown in Figure 2, reaction with borohydride does result in a progressive loss of the emission band at 470 nm. The emission spectrum recorded at the end of the reaction is characterless but shifted upward as compared with a spectrum recorded for a buffer control. The reason for this difference is unclear but may possibly reflect the tail end of the emission from aromatic amino acid residues or a small amount of light scattering in the protein sample. The activation spectrum observed for borohydride-treated enzyme shows a small residual peak at 390 nm, which is largely attributable to a low level of fluorescence observed with the buffer alone (see Figure 4).

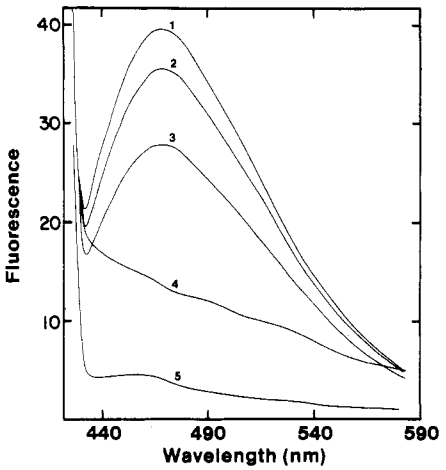

FIGURE 2: Effect of borohydride on the fluorescence of photolyase. Curve 1 shows the emission spectrum (excitation at 398 nm) of the enzyme in standard buffer at 5.5 °C. Curves 2–4 were recorded 30 min after adding borohydride to a concentration of 4.2×10^{-4}, 2.5×10^{-3}, and 1.6×10^{-2} M, respectively. Curve 5 is the spectrum recorded for the buffer alone.

Effect of Borohydride on Catalytic Activity. After reaction with borohydride the enzyme exhibited 80% of the activity observed for an untreated control. Photoreversal of the borohydride reaction under assay conditions is not detectable. The results show that photolyase can repair dimers via a pathway that is independent of the second chromophore. The extensive frothing of the enzyme solution during the borohydride reaction may account for the less than quantitative recovery of activity.

Nature of the Borohydride Reaction. Given the known chemical reactivity of borohydride, it would seem reasonable to conclude that the spectral changes observed in the photolyase reaction are due to reduction of the protein-bound second chromophore. However, it is known that the second chromophore is not stable when released into solution at neutral pH where it decomposes in a reaction accompanied by a complete loss of absorption in the visible region (Jorns et al., 1984). It is therefore conceivable that, instead of acting as a reductant, borohydride causes a selective denaturation of the protein domain associated with the second chromophore, releasing the compound into solution where it decomposes. We have previously shown that the stability of the protein-free second chromophore in aqueous solution increases with decreasing pH and that the compound is stable at pH ≤ 3 (Jorns et al., 1984). In an effort to resolve the ambiguity regarding the nature of the borohydride reaction with photolyase, protein-free extracts of the enzyme containing intact second chromophore plus oxidized FAD were prepared and reacted with sodium cyanoborohydride at pH 2.1 (Figure 3). The initial spectrum observed for the extract shows a peak at 450 nm due to FAD, plus a more intense band at 366 nm that reflects contributions from FAD ($\lambda_{max} = 375$ nm) and the protein-free second chromophore ($\lambda_{max} = 360$ nm) (Jorns et al., 1984). Addition of cyanoborohydride initiates an isosbestic reaction involving a decrease in the intensity of the 366-nm peak, accompanied by a shift in the absorption maximum from 366 to 375 nm. The absorbance of the extract at 450 nm is unaffected by cyanoborohydride. At the end of the reaction, the ratio A_{450}/A_{375} equals 1.22 and is identical with that observed with authentic FAD. The results indicate that cyanoborohydride causes a complete loss of the visible absorption due to the second chromophore but does not affect the absorption spectrum of FAD. In the presence of excess cyanoborohydride (3.2×10^{-4} M), the reaction exhibits apparent

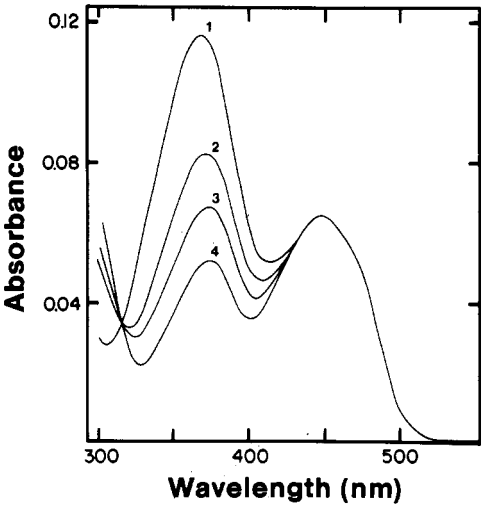

FIGURE 3: Reaction of the protein-free second chromophore with cyanoborohydride. A photolyase extract, containing the second chromophore plus FAD, was prepared as described under Experimental Procedures. Curve 1 shows the absorption spectrum of the extract at 25 °C in 100 mM sodium citrate, pH 2.1. Curves 2–4 were recorded 5, 11, and 60 min, respectively, after adding 3.2×10^{-4} M sodium cyanoborohydride.

first-order kinetics ($k_{obsd} = 9.2 \times 10^{-2}$ min^{-1}). When the reaction was repeated at pH 3.0, similar spectral changes were observed, but the rate was slower ($k_{obsd} = 1.1 \times 10^{-2}$ min^{-1}). Since decomposition of the second chromophore does not occur at these pH values, it is concluded that the observed spectral changes are due to reduction of the protein-free second chromophore and that a similar reaction occurs when the intact enzyme is treated with borohydride at pH 7.4. In the former reaction, an increase in pH from 2.1 to 3.0, corresponding to a 7.9-fold decrease in proton concentration, is accompanied by a comparable decrease (8.4-fold) in reaction rate. That the observed rate appears to be directly proportional to the proton concentration is similar to that reported for the reduction of aldehydes, ketones, and oximes with cyanoborohydride in this pH range (Borch et al., 1971).

Spectral Properties of Enzyme-Bound Fully Reduced Flavin. Since borohydride eliminates the visible absorption and fluorescence due to the second chromophore, experiments were initiated to determine whether the reaction might provide an opportunity to characterize the spectral properties of fully reduced FAD bound to photolyase. It is found that reduction of the radical in borohydride-treated enzyme with dithionite results in the appearance of an emission band with a peak at 505 nm and a shoulder at 475 nm. The shoulder at 475 nm is diminished when the spectrum is corrected for the residual fluorescence observed before dithionite addition (Figure 4). The excitation spectrum observed after reduction exhibits a peak at 390 nm (Figure 4, curve 2) and, except for a decrease in intensity, is curiously similar to that observed for enzyme containing intact second chromophore plus FADH• (Figure 4, curve 3). The results show that $FADH_2$ bound to photolyase is fluorescent, similar to that observed with a number of other flavoproteins (emission λ_{max} = 500–530 nm) (Ghisla et al., 1974). The intensity of the emission observed for $FADH_2$ bound to photolyase is 16% of that observed for protein-free oxidized FAD, or 11% if corrected for the residual fluorescence of the preparation observed before reduction of FADH• (Table I).

The absorption spectrum obtained upon reduction of borohydride-treated photolyase with dithionite shows a peak at 360 nm ($\epsilon = 5.5 \times 10^3$ M^{-1} cm^{-1}) (Figure 5). The observed spectrum is similar to that reported for 1,5-dihydroflavin

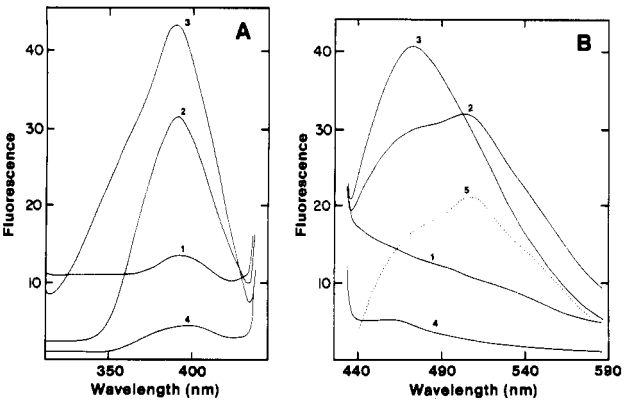

FIGURE 4: Effect of reduction of the flavin radical on the fluorescence properties of borohydride-treated enzyme. Excitation (emission at 470 nm) and emission (excitation at 398 nm) spectra are shown in panels A and B, respectively. In each panel, curve 1 shows the spectrum of the enzyme recorded after reaction with 1.6×10^{-2} M sodium borohydride in standard buffer at 5.5 °C; curve 2 shows the spectrum after reaction of borohydride-treated enzyme with excess dithionite (3.0 mM); curves 3 and 4 show spectra recorded for untreated enzyme and with buffer alone, respectively. Curve 5 in panel B is the difference spectrum obtained by subtracting curve 1 from curve 2.

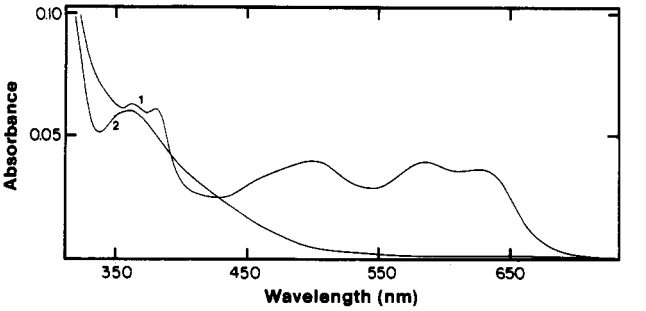

FIGURE 5: Absorption spectrum of $FADH_2$ bound to photolyase. Curve 1 is the absorption spectrum of borohydride-treated (1.6×10^{-2} M borohydride) enzyme in standard buffer under anaerobic conditions at 5.5 °C. Curve 2 was recorded after reduction with a stoichiometric amount of dithionite.

Table I: Fluorescence of Various Photolyase Preparations

chromophore composition		fluorescence intensity (% of FAD)[a]	
flavin	second chromophore	470 nm	505 nm
FADH•	intact	20	
$FADH_2$	intact	119	
FADH•	reduced	6.0	5.0
$FADH_2$	reduced	14	16

[a] Fluorescence emission with various photolyase preparations was measured at the indicated wavelength with excitation at 390 nm. FAD fluorescence at 510 nm was measured with excitation at 450 nm. All measurements were conducted in standard buffer at 5.5 °C.

derivatives bound to other flavoproteins, which typically exhibit maxima in the 350–370-nm region with extinction coefficients in the range $(3.5–6.2) \times 10^3$ M^{-1} cm^{-1} (Ghisla et al., 1974). The absorption maximum observed for $FADH_2$ bound to photolyase does not coincide with the fluorescence excitation maximum at 390 nm observed upon reduction of borohydride-treated enzyme. The reason for this difference is not known.

Effect of the Flavin Redox State on the Fluorescence of the Second Chromophore. The 505-nm fluorescence emission band due to $FADH_2$ in borohydride-treated enzyme is similar (80%) in intensity as compared with the 470-nm emission band observed for the second chromophore in untreated enzyme containing FADH•. This suggested that reduction of the

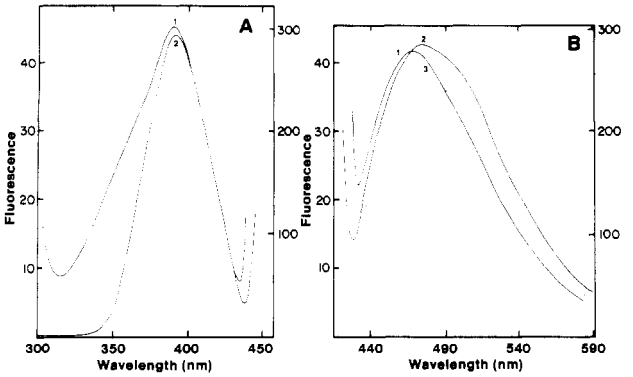

FIGURE 6: Effect of reduction of the flavin radical on the fluorescence properties of native photolyase. Excitation (emission at 470 nm) and emission (excitation at 398 nm) spectra are shown in panels A and B, respectively. In each panel, curve 1 shows the initial spectrum of the enzyme in standard buffer at 5.5 °C and curve 2 shows the spectrum after addition of excess dithionite (3.0 mM). Spectra shown in curves 1 and 2 are plotted according to the scales shown on the left- and right-hand axes, respectively. Curve 3 in panel B is the difference spectrum obtained after subtracting from curve 2 the contribution expected for enzyme-bound $FADH_2$ (curve 2 in panel B of Figure 4).

radical in enzyme containing intact second chromophore might result in an emission spectrum with two peaks, approximately equal in intensity, at 470 and 505 nm. This result is not observed. The major effect observed upon reduction of FADH• is a 6-fold increase in fluorescence intensity. To compensate for the large change in fluorescence intensity, spectra recorded before and after reduction are drawn according to different scales in order to facilitate comparison of band shape and position (Figure 6). Reduction of the radical causes a small bathochromic shift of the emission maximum of the second chromophore from 470 to 475 nm, accompanied by the development of a shoulder at 505 nm. The latter probably reflects the contribution from reduced flavin. The absence of a distinct peak at 505 nm is understandable since the fluorescence intensity expected for enzyme-bound $FADH_2$ at 505 nm is small (13%) as compared with the enhanced fluorescence of the second chromophore at 475 nm. Reduction of the radical does not shift the maximum observed at 390 nm in the excitation spectrum. The results indicate that the flavin radical causes a pronounced quenching of the fluorescence of the second chromophore. The basis for this phenomenon will be considered in a later section.

Photoreduction of the Second Chromophore. Previous studies have shown that the radical in photolyase can be reduced with dithionite (Jorns et al., 1984) or by irradiation with light at $\lambda > 520$ nm in the presence of an electron donor such as dithiothreitol (Heelis & Sancar, 1986). These reactions result in the complete loss of the radical absorption in the 500–700-nm region accompanied by a modest decrease in extinction coefficient at 384 nm from 18.1×10^3 M^{-1} cm^{-1} to 16.4×10^3 M^{-1} cm^{-1} (Jorns et al., 1987). The spectral changes are fully reversible upon admission of oxygen. A significant change in the nature of the photoreaction is observed when the enzyme is irradiated in the presence of dithiothreitol with light in the 300–400-nm region that can excite the second chromophore as well as the flavin radical. Rapid reduction of the radical occurs under these conditions, as evidenced by the loss of absorption in the 500–700-nm region (Figure 7). However, the reduction of the radical is followed by a much slower reaction, which causes a large decrease in the extinction coefficient at 384 nm from 18.1×10^3 M^{-1} cm^{-1} to 4.6×10^3 M^{-1} cm^{-1}. The spectrum observed at the end of the photoreaction exhibits a peak at 360 nm ($\epsilon = 5.6 \times 10^3$ M^{-1} cm^{-1}) and is similar to the spectrum observed after dithionite reduction of FADH• in borohydride-treated enzyme. Quantitative recovery of the radical absorption in the 500–700-nm region is observed when the photoreduced sample is made aerobic. This is accompanied by a small increase in absorption in the 360-nm region and the appearance of peaks at 363 and 381 nm. The spectrum of the reoxidized enzyme is very similar to that observed for borohydride-treated enzyme, except that the former shows a small wedge of increased absorption in the 450–500-nm region. That the photoreduction eliminates the visible absorption due to the second chromophore is confirmed by the fact that no second chromophore is detectable when the reoxidized enzyme is reacted with SDS. The results indicate that photoreduction under these conditions yields a rapid, reversible reduction of FADH•, followed by a slower, irreversible reduction of the second chromophore, similar to that observed with borohydride.

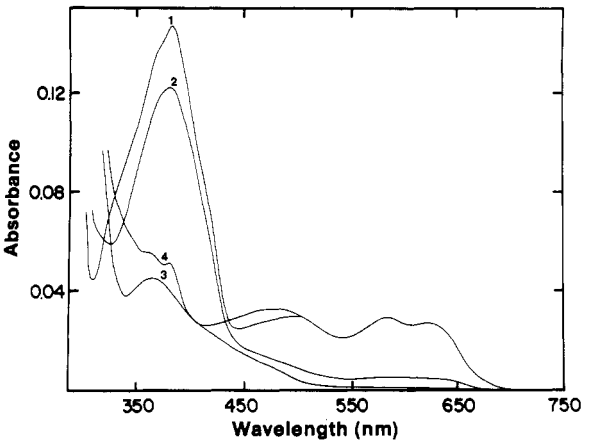

FIGURE 7: Photoreduction of photolyase. Curve 1 shows the absorption spectrum of the enzyme in standard buffer containing 2.7 mM dithiothreitol under anaerobic conditions at 5.5 °C. The sample was irradiated with light in the range from 300 to 400 nm. Curves 2 and 3 were recorded after 150 s and 20 min of irradiation, respectively. Curve 4 shows the spectrum after reoxidation.

Discussion

In this paper we show that sodium borohydride causes an irreversible reduction of the second chromophore in *E. coli* photolyase, accompanied by a complete loss of the chromophore's absorption and fluorescence in the visible region. A similar reaction is observed with sodium cyanoborohydride and the protein-free second chromophore and also upon photoreduction of the enzyme in the presence of dithiothreitol.

The borohydride reaction has provided an opportunity to characterize the spectral properties of each of the chromophores present in the enzyme as it is normally isolated (FADH•, second chromophore) and in its physiologically significant form ($FADH_2$, second chromophore). Whether a covalent link exists between the enzyme-bound chromophores is unknown, although existing evidence indicates that such a linkage must be labile since the protein-free chromophores appear as separate molecules (Jorns et al., 1984; Jorns, unpublished observations). In this regard it is relevant that the absorption spectrum determined in this study for the second chromophore bound to enzyme containing FADH• is virtually identical with a spectrum previously determined for the second chromophore bound to enzyme containing $FADH_2$ (Jorns et al., 1987). The results indicate that the absorption spectrum of the second chromophore is independent of the redox state of the flavin. It is therefore reasonable to conclude that the second chromophore must be electronically isolated from the flavin and, if the enzyme-bound chromophores constitute a

single molecule, they must be separated by an "insulating" link.

Elimination of the absorption due to the second chromophore revealed underlying bands due to the flavin radical at 364 and 382 nm. A similar pair of absorption maxima are observed in this region for neutral flavin radicals bound to other flavoproteins (Müller et al., 1972). Reduction of the radical in borohydride-treated enzyme showed that $FADH_2$ bound to photolyase is fluorescent with an emission maximum at 505 nm and an absorption maximum at 360 nm. The observed spectral properties are similar to those reported for 1,5-dihydroflavin derivatives bound to other flavoproteins (Ghisla et al., 1974). The results suggest that the fully reduced FAD in *E. coli* photolyase is also a 1,5-dihydroflavin derivative.

In recent studies a yellow form of *E. coli* photolyase was isolated that contained a normal amount of FAD but very little second chromophore. The atypical yellow color was due to the fact that most of the flavin was present as yellow oxidized FAD instead of the blue neutral FAD radical. When yellow enzyme was reduced with dithionite, the spectrum of the fully reduced enzyme exhibited a shoulder in the 350–400-nm region instead of the well-defined peak at 360 nm observed for $FADH_2$ in borohydride-treated enzyme. It should be noted that borohydride treatment does not affect the flavin site in photolyase as evidenced by the retention of the unusual stability of the enzyme-bound radical against air oxidation. In contrast, this property was retained by only half of the flavin sites in yellow enzyme. The results suggest that the absorption spectrum of $FADH_2$ in borohydride-treated enzyme provides a better estimate of the spectrum of the chromophore in the physiologically significant form of the enzyme. As a further check, the data in Figures 1 and 5 were used to calculate extinction coefficients for the second chromophore (12.6×10^3 M^{-1} cm^{-1}) and for $FADH_2$ (4.2×10^3 M^{-1} cm^{-1}) at 384 nm. The data for the individual chromophores was then used to estimate the extinction coefficient of the physiologically significant form of the enzyme at its absorption maximum. The calculated value ($\epsilon_{384} = 16.8 \times 10^3$ M^{-1} cm^{-1}) is in very good agreement with the observed value ($\epsilon_{384} = 16.4 \times 10^3$ M^{-1} cm^{-1}) (Jorns et al., 1987).

When the radical in borohydride-untreated enzyme is reduced to $FADH_2$, a 6-fold increase in fluorescence intensity is observed accompanied by a shift in the emission maximum from 470 to 475 and the appearance of a shoulder at 505 nm. The emission spectrum observed for enzyme containing $FADH_2$ plus the second chromophore appears to be dominated by the enhanced fluorescence of the second chromophore while the contribution from $FADH_2$ would appear to be responsible for the shoulder at 505 nm. The latter is diminished upon subtracting the fluorescence expected for enzyme-bound $FADH_2$ (Figure 6), as estimated by the fluorescence observed for the chromophore in borohydride-treated enzyme. The increased fluorescence observed upon reduction of FADH• indicates that the radical acts as a potent quencher of the fluorescence of the second chromophore. In this regard, it should be noted that the absorption spectrum of the radical overlaps with the fluorescence emission spectrum of the second chromophore. This suggests that the observed fluorescence quenching could be due to a transfer of energy from the second chromophore singlet to the nonfluorescent flavin radical. Alternatively, the paramagnetism of the flavin radical might enhance spin–orbit coupling in the nearby second chromophore, similar to the effect observed with paramagnetic species in other systems (Turro, 1967). Enhanced spin–orbit coupling results in an increased rate of intersystem crossing from the singlet to the triplet state, accompanied by a decrease in fluorescence yield. Studies are planned to distinguish between these possibilities.

That photolyase can repair dimers by a pathway that does not involve the second chromophore is evidenced by the fact that the visible absorption and fluorescence of the second chromophore can be eliminated by reaction with borohydride without causing a significant change in catalytic activity. It is known that enzyme-bound FADH• is rapidly converted to $FADH_2$ under assay conditions and that $FADH_2$ (but not FADH•) can act as a photosensitizer in borohydride-untreated enzyme (Jorns et al., 1987; Sancar et al., 1987; Heelis & Sancar, 1986). Model studies with thymine dimers and a variety of sensitizers suggests two principal modes for photosensitized cleavage: (1) electron donation from the excited sensitizer (e.g., indole derivatives) to generate thymine dimer radical anion (Hélène & Charlier, 1971; Charlier & Hélène (1975); (2) electron abstraction by the excited sensitizer (e.g., oxidized flavin, various quinones) to generate thymine dimer radical cation (Ben-Hur & Rosenthal, 1970; Lamola, 1972; Roth & Lamola, 1972; Rokita & Walsh, 1984). Thymine dimer radicals are unstable and spontaneously monomerize. Since $FADH_2$ is unlikely to act as an electron acceptor, we propose that dimer ($\overline{TT}$) cleavage by photolyase in the second chromophore independent pathway proceeds via a mechanism involving electron donation from excited $FADH_2$ (eq 1–4).

$$EFADH_2\cdots\overline{TT} \xrightarrow{h\nu} EFADH_2{}^*\cdots\overline{TT} \quad (1)$$

$$EFADH_2{}^*\cdots\overline{TT} \rightarrow EFADH^\bullet\cdots\overline{TT}^{\bullet-} \quad (2)$$

$$EFADH^\bullet\cdots\overline{TT}^{\bullet-} \rightarrow EFADH^\bullet\cdots T^{\bullet-} + T \quad (3)$$

$$EFADH^\bullet\cdots T^{\bullet-} \rightarrow EFADH_2 + T \quad (4)$$

Evidence for the feasibility of the proposed mechanism is also provided by recent model studies, which show that certain reduced flavin derivatives can act as sensitizers in the splitting of thymine dimers (Jorns, 1987).

A quantum yield of 1.0 is observed with photolyase preparations containing intact second chromophore plus $FADH_2$ (Sancar et al., 1987). This means that every quantum of light that is absorbed by the enzyme is used to split dimers. Therefore, there must exist a second pathway for dimer repair involving the second chromophore. The relative importance of the two pathways will clearly be wavelength dependent since, for example, the second chromophore is the major chromophore at 384 nm whereas only $FADH_2$ absorbs at 450 nm. Since dimer repair is not observed with enzyme containing intact second chromophore plus oxidized FAD (Jorns et al., 1987), it is likely that the second pathway requires both the second chromophore and $FADH_2$. It has been proposed (Jorns et al., 1987; Sancar et al., 1987) that the excited second chromophore (SC*) donates an electron to the pyrimidine dimer. This generates a second chromophore radical cation that is subsequently reduced by $FADH_2$ (eq 5–7) and a pyrimidine dimer radical anion that monomerizes as shown in eq 3 and 4, regenerating $FADH_2$. In this mechanism $FADH_2$ acts only as a redox catalyst, in contrast to the proposed second

$$E^{SC}_{FADH_2} \xrightarrow{h\nu} E^{SC^*}_{FADH_2} \quad (5)$$

$$E^{SC^*}_{FADH_2} \xrightarrow{\overline{TT}\ \ \overline{TT}^{\bullet-}} E^{SC^{\bullet+}}_{FADH_2} \quad (6)$$

$$E^{SC^{\bullet+}}_{FADH_2} \longrightarrow E^{SC}_{FADH^\bullet} \quad (7)$$

chromophore independent pathway where the photochemical reactivity of $FADH_2$ is also important.

As an alternative second pathway, it is relevant to consider the possibility of energy transfer from the excited second chromophore to $FADH_2$ (eq 8). Dimer cleavage would then

$$E^{SC^*}_{FADH_2} \longrightarrow E^{SC}_{FADH_2{}^*} \quad (8)$$

proceed via the same mechanism (eq 2–4) as proposed for the second chromophore independent pathway except that $FADH_2{}^*$ is not generated by direct absorption of a photon. Triplet–triplet energy transfer can occur with 100% efficiency provided (1) the lowest triplet state of the donor lies above that of the acceptor and (2) the donor and acceptor molecules are in close proximity of one another (Turro, 1967). The latter criterion is likely to be satisfied in the case of photolyase since $FADH_2$ and the second chromophore are both bound at the active site of the enzyme. Expected rates for triplet–triplet energy transfer ($100\ s^{-1}$) (Turro, 1967) are 3 orders of magnitude faster than the rate observed for photolyase catalysis under conditions of saturating light and substrate ($k_{cat} = 0.1\ s^{-1}$) (Sancar et al., 1987). Experiments to determine whether triplet–triplet energy transfer occurs during photolyase catalysis are in progress. On the other hand, the following observations suggest that efficient energy transfer from the second chromophore singlet to generate $FADH_2$ singlet is not likely: (1) the absorption spectrum of $FADH_2$ partially overlaps with the emission spectrum of the second chromophore, but the overlap occurs in a region ($\lambda > 450$ nm) where absorption by $FADH_2$ is relatively weak; (2) the emission spectrum observed for enzyme containing intact second chromophore plus $FADH_2$ is dominated by the fluorescence of the second chromophore whereas a prominent band due to $FADH_2$ would be expected in the case of efficient singlet–singlet energy transfer. It is important to emphasize that triplet–triplet energy transfer is not ruled out by the foregoing considerations since this process can occur with high efficiency between molecules where singlet–singlet energy transfer is unlikely (Turro, 1967).

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