linking or following amine incorporation into protein components.

The βBp subunit is a principal component of β-crystallins in the bovine and perhaps in other species too. McFall-Ngai et al. (1986) found that an antiserum to βBp recognized a band with a molecular weight of about 50K on transblots from human lens SDS-PAGE profiles and that the amount of this band increased with the age of the propositus. Though there was no suggestion that this 50K band might have been a product of transglutaminase catalysis and that it might in this sense be similar to the ~55K cross-linked β-crystallin species described earlier (Lorand et al., 1985), we thought it was important to ask the question whether βBp participated in the transglutaminase-mediated generation of such β-crystallin dimers. The experimental results presented in Figure 6 (panel C, lane 2) are entirely clear in this regard. Not only did the antiserum to the bovine βBp react with the corresponding rabbit ~23K subunit but also it recognized the ~55K cross-linked dimeric product essentially with the same sensitivity. This important finding lends added support to the physiological significance of transglutaminase in the aging process in lens. The enzyme, present in a latent form, might become activated through redistribution of Ca^{2+} in the tissue, proteolytic cleavage, release from inhibition, or combination with positive effectors. Though the experiments presented in this paper focused on crystallin subunits as substrates, cross-linking in the aging lens might resemble the situation in Ca^{2+}-enriched human red cells (Lorand et al., 1976, 1978; Bjerrum et al., 1981) and could involve membrane constituents as well. Future efforts will be directed to examining these questions and to further delineate the role of transglutaminase in the irreversible processes associated with cataract formation.

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Photochemical Properties of *Escherichia coli* DNA Photolyase: Selective Photodecomposition of the Second Chromophore†

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ABSTRACT: *Escherichia coli* DNA photolyase contains a stable flavin radical and a second chromophore (SC) of unknown structure. The effects of flash (both conventional and laser) excitation of either the radical alone or both the radical and the second chromophore have been investigated by variation of the excitation wavelengths. Radical excitation leads to an electron abstraction by the lowest excited doublet state of the radical from an amino acid residue, probably a cysteine or tyrosine. On a longer time scale, a back-reaction occurs that can be prevented by the presence of certain electron donors, e.g., thiols, NADH, or tyrosine, but not pyrimidine dimers. Excitation of the second chromophore leads to electronic energy transfer from second chromophore excited states to the ground-state flavin radical doublet state, thus increasing the population of the lowest excited doublet state. Repetitive excitation of the enzyme with white light leads to photodecomposition of the second chromophore but not of the flavin adenine dinucleotide cofactor. Enzyme with photodecomposed SC retains full activity.

DNA photolyases (EC 4.1.99.3) catalyze the photochemical conversion of pyrimidine dimers in DNA into pyrimidines, thus reversing the effect of far-UV (200-300-nm) radiation. The enzyme from *Escherichia coli* contains the FAD[1] blue neutral

Table I: Activities of Various Forms of Photolyase

form of enzyme	rel $\epsilon_{366}\phi$
E-FADH^0	1
E-FADH$_2$	8
SC†-E-FADH$_2$	5

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radical and a second chromophore (SC) of unknown structure (Sancar & Sancar, 1984; Jorns et al., 1984). The second chromophore has an absorption maximum at 360 nm when free in aqueous solution (Jorns et al., 1984) and at 390 nm when enzyme bound (Jorns et al., 1987), as determined by difference spectroscopy. The second chromophore is fluorescent, having an excitation maximum λ = 398 nm and an emission maximum λ = 470–480 nm (Jorns et al., 1984, 1987). Both steady-state rate measurements (Jorns et al., 1987) and absolute action spectrum of the enzyme (Sancar et al., 1987b) indicate that FAD plays an essential role in catalysis while the photolytic cross section at 384 nm for repairing pyrimidine dimers suggests that both the flavin and the second chromophore act as photosensitizers.

We reported previously (Heelis & Sancar, 1986) that excitation of the flavin radical of the enzyme results in a one-electron reduction of the flavin to the fully reduced flavoenzyme. Such a photoreduction may well be an essential first step in the action of photolyase in vitro as the reduced enzyme is markedly more active than the radical form (Jorns et al., 1987; Sancar et al., 1987b). This study represents an extension of our previous work to include excitation of the SC in the near-UV region. In addition, studies of the photoreduction of the radical have been extended to include the effect of a number of biomolecules.

Experimental Procedures

DNA photolyase was prepared as described previously (Sancar et al., 1984; Sancar et al., 1987a). The enzyme was in the "blue form", free of oxidized FAD by visual inspection as well as absorbance and fluorescence measurements. The stock solutions of the enzyme were stored at −20 °C in storage buffer containing 50 mM Tris·HCl, pH 7.5, 50 mM NaCl, 10 mM DTT, 1 mM EDTA, and 50% glycerol. Experiments were carried out by diluting the stock solution 60-fold into a buffer containing 50 mM Tris·HCl, pH 7.4, 100 mM NaCl, and other components as indicated. Enzyme concentrations were in the range (5–10) × 10^{-6} M with respect to the flavin radical as determined from the absorbance of 580 nm with ϵ = 3600 M^{-1} cm^{-1} (Jorns et al., 1984). All experiments were carried out at 4 °C. Chemicals were purchased from either Sigma or British Drug House and were of the purest grade available.

The laser flash photolysis system was based on a JK Lasers System 2000 neodymium:YAG laser emitting pulses at 532 and 353 nm with energies in the range 10–200 mJ and a pulse duration of 20 ns. A pulsed Xe lamp, which produced light of constant intensity for 400 μs after the laser pulse was used as the analyzing light source for measurements in the microsecond time range. For measurements on a millisecond time scale, a quartz–halogen lamp of constant intensity was used as the analyzing source. Measurements of transient changes in absorbance at wavelengths in the range 300–700 nm were recorded on a Phillips PM3311 digital oscilloscope and then transferred to an LSI/2 computer and stored on disk for further analysis. Pulse-to-pulse variations in the laser intensity were corrected for by monitoring the integrated laser intensity on each pulse. Samples were contained in quartz cells of 1-cm optical path length. Appropriate filters were placed in the analyzing light path to reduce photolysis. Transient quantum yields were determined by the comparative technique (Bensasson et al., 1978). For 353-nm excitation, anthracene in oxygen-free cyclohexane was employed as standard, with values of 0.75 for the quantum yield of intersystem crossing (Medinger & Wilkinson, 1965) and 64 700 M^{-1} cm^{-1} for the triplet-state extinction coefficient at 422.5 nm (Bensasson & Land, 1971).

Conventional photolysis experiments were carried out with a Starblitz 2400 T5 photographic flash unit on manual mode and will be termed camera flash experiments in order to distinguish them from the laser flash photolysis experiments. In some cases, the light output was passed through an Oriel OG515 cutoff filter to exclude wavelengths shorter than 520 nm to ensure absorption by the flavosemiquinone only. Otherwise the unfiltered output (termed white light) was employed. These experiments were carried out under aerobic conditions, as we have found that oxygen has no effect on radical photoreduction (Heelis & Sancar, 1986).

The relative activities of the various forms of the enzyme were measured as described elsewhere (Sancar et al., 1987b). The light source in these experiments was a Sylvania F15T8BLB (black light), and the samples were illuminated under argon atmosphere to minimize reoxidation of the photoreduced enzyme during enzymatic photoreactivation.

Results

Camera Flash Photolysis

Photoreduction of $FADH^0$ and Photodecomposition of SC. It was previously shown that flash photolysis of the blue enzyme in the presence of DTT with long wavelengths (λ > 520 nm) leads to photoreduction of the flavin radical (Heelis & Sancar, 1986). Similarly, by use of the full "white output" of the flash lamp, a loss of radical absorption at λ > 450 nm is observed in the presence of DTT. However, in contrast to the results with long-wavelength irradiation, a pronounced decrease in the 380-nm absorption maximum is observed with white light (Figure 1). This is more clearly shown in Figure 2 in which the absorbances at 380 (due to the SC and $FADH^0$) and 580 nm ($FADH^0$ only) are shown during photolysis with filtered and white light. As can be seen for a given degree of photoreduction of the radical (580 nm), a far more pronounced absorbance decrease at 380 nm is observed for irradiation with white light compared to filtered light. We interpret these results to mean that white-light illumination of the blue enzyme leads to photodecomposition of the second chromophore but not the flavin radical (see below).

Activity of Photolyzed Enzyme (SC†–Enzyme). The photolyzed enzyme (Figure 1, curve 3) is fully active as is apparent from the data presented in Table I. The reduced form of the enzyme, with or without the SC, has a higher photoreactivation cross section at 366 nm compared to the blue radical form. This is in qualitative agreement with our earlier report that the $FADH_2$ form of the enzyme (generated by dithionite reduction) has about 15-fold higher quantum yield than the radical form (Sancar et al., 1987b). The photoreactivation cross section ($\epsilon\phi$) at 366 nm of the SC†–E–$FADH_2$ is about half that of the photoreduced enzyme, consistent with the decrease in absorption at this wavelength. However, the quantum yields for both enzymes at this wavelength are comparable, indicating that $FADH_2$ is sufficient for catalysis and that the main function of the SC is to act as a photoreceptor.

[1] Abbreviations: SC, second chromophore; DTT, dithiothreitol; FAD, flavin adenine dinucleotide; E–$FADH^0$, enzyme-bound FAD neutral radical; E–$FADH_2$, enzyme-bound fully reduced FAD; E–†FADH, the lowest excited doublet state of the FAD neutral radical; SC†–enzyme, enzyme with photodecomposed second chromophore; SC†–E–$FADH_2$, photoreduced enzyme with photodecomposed second chromophore; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

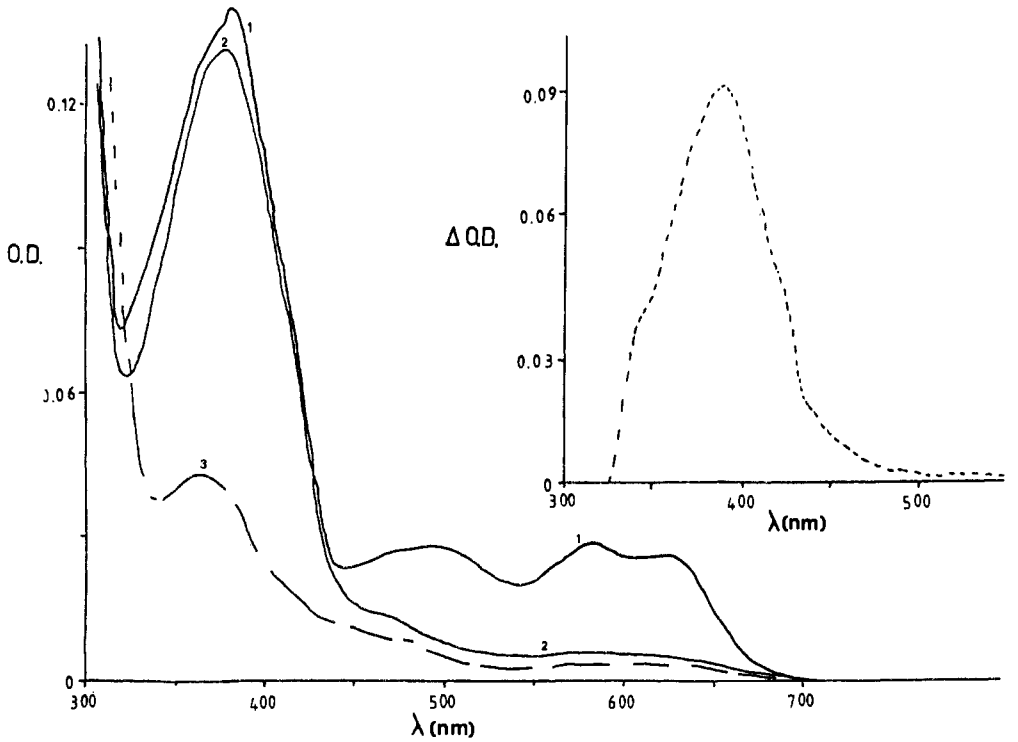

FIGURE 1: Absorption spectrum of the blue enzyme before (curve 1) and after camera flash photolysis with filtered light (curve 2, 40 flashes) or white light (curve 3, 100 flashes). (Inset) Difference spectrum for SC destruction (curve 2 − curve 3).

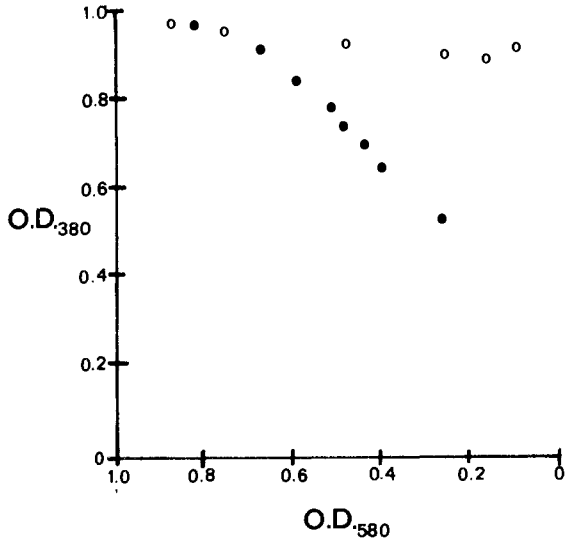

FIGURE 2: Absorbance of the blue enzyme at 380 nm vs. 580 nm during photolysis using filtered (○) or white light (●). Absorbances are expressed as fractions of the original absorbance before irradiation.

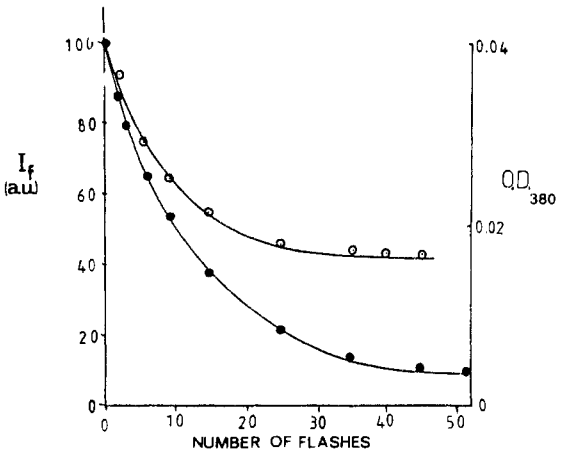

FIGURE 3: Effect of camera flash photolysis using white light on the SC fluorescence emission intensity in arbitrary units [(●); excitation λ = 390 nm, emission λ = 460 nm], left-hand ordinate, and absorbance at 380 nm (○), right-hand ordinate. Enzyme had been previously photoreduced by 40 filtered (λ > 520 nm) flashes.

Effect of Reduction of FADH⁰ on Photodecomposition of SC. Prior photoreduction of the radical with filtered light followed by irradiaiton with white light again resulted in a loss of the SC absorption at 380 nm at a rate comparable to that observed for the blue enzyme. In this case the absorption spectral changes due to SC destruction can be more easily determined. The difference spectrum obtained for white-light irradiation of the photoreduced enzyme is shown in Figure 1 (inset). The maximum at 390 nm agrees well with the suggested absorption characteristic of the SC (Jorns et al., 1987). Of interest is that throughout the white-light photolysis of the reduced enzyme, an isosbestic point is maintained at 330 nm, suggesting that only one photoproduct is formed.

The fluorescence characteristics of the enzyme-bound SC have been previously described (Sancar & Sancar, 1984; Jorns et al., 1984). White-light photolysis of enzyme prereduced with filtered light leads eventually to an almost complete loss of the SC emission (Figure 3) although substantial absorbance at 380 nm remains. This residual absorbance is due to the contribution of the fully reduced flavin chromophore (E–$FADH_2$), and therefore, we conclude that curve 3 in Figure 1 is the absorption spectrum of photolyase with fully reduced flavin but no second chromophore. This spectrum is remarkably similar to the absorption spectrum of L-lactate-reduced lactate oxidase (Ghisla et al., 1974), which has a flavin mononucleotide (FMN) cofactor.

Effect of Reducing Agents on Photolysis of SC. In the absence of DTT, irradiation of the blue enzyme with white light resulted in the loss of SC absorption at an rate essentially identical with that observed in the presence of DTT. As reported earlier (Heelis & Sancar, 1986) minimal photoreduction of the radical occurs in the absence of DTT. In fact,

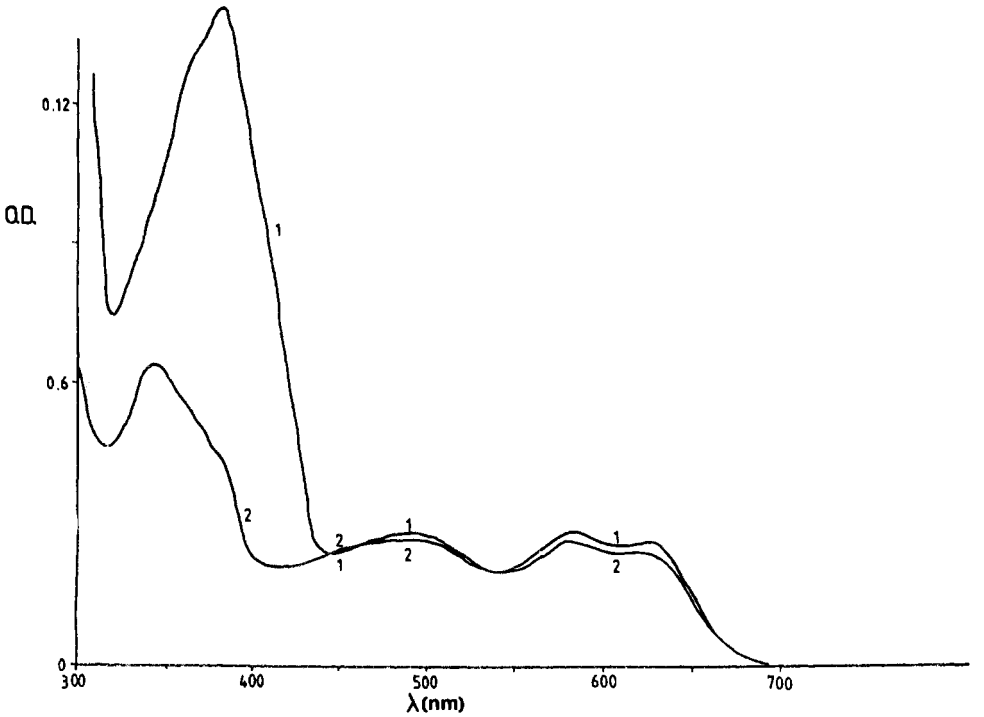

FIGURE 4: Absorption spectrum of the blue enzyme before (curve 1) and after 100 white flashes followed by a 24-h storage at 5 °C in 50% glycerol (curve 2).

with the large number of flashes used to degrade SC, extensive oxidation of the radical to the yellow oxidized form occurred. These results suggest that the SC can undergo photochemical changes quite independently from the flavin chromophore. Storage of white light irradiated enzyme at 5 °C for 10 h resulted in substantial oxidation of the enzyme to the yellow (FAD_{ox}) form as shown by the pronounced increase in absorbance at 450 nm. Hence SC photodamage would appear to increase the susceptibility of the flavin chromophore to oxidation.

Recovery of Flavin Radical in the Presence or Absence of SC. Irradiation of the blue enzyme with white light in 50% glycerol produces spectral changes essentially identical with those observed in the absence of glycerol. However, the enzyme appears to be more stable to denaturation in this medium, as 24 h storage at 5 °C leads to over 95% recovery of the blue radical (Figure 4). No recovery of the SC absorption was, however, detected in either the presence or absence of glycerol. Of interest is that the spectrum of the reoxidized "SC-damaged" enzyme now resembles more closely the typical spectrum of other flavoenzyme semiquinones (Massey & Palmer, 1966). Hence the effect of light on the SC would appear to differ from the effect on the radical in that it is neither reversible nor dependent upon the presence of an electron donor such as DTT. To assess the possible role of the SC in the photoreduction of the radical, the blue enzyme was photoreduced with filtered light in 50% glycerol. The reduced enzyme so formed was then extensively irradiated (100 flashes) with white light until no further changes in SC absorbance were observed. After reoxidation of the reduced flavin component to the radical form (>90% radical recovery), the enzyme was again irradiated with filtered light. The rate of loss of radical absorption and the spectral changes observed were essentially identical with those observed upon irradiation of fresh blue enzyme, allowing the conclusion that the SC is not the electron donor.

Effect of Amino Acids and Thymine Dimer on Photoreduction of $FADH^0$. Having determined that the SC is not an electron donor for photoreduction of the $FADH^0$, we tested some other compounds that may act as natural electron donors. Flash photolysis with filtered light in the presence of a number of amino acids, thiols, and NADH was carried out. An efficient and oxygen-reversible photoreduction of the radical was observed in the presence of all thiols studied, NADH, or glycyltyrosine. Thymine dimers were also tested for their ability to photoreduce the flavin radical. An association constant of 6×10^7 M^{-1} has been determined for the binding of photolyase to dimer containing DNA (Sancar et al., 1987a). Upon the assumption that this association constant applies to oligo(deoxythymidine) (dT_{10}) dimers (Jorns et al., 1985), sufficient dimers (1.5×10^{-5} M) were added to complex over 95% of the enzyme present (5×10^{-6} M). However, no photoreduction of the flavin radical was observed in the presence of dimers (Table II). The photoreduction reaction in the presence of glycyltyrosine and DTT was further investigated. The dependence of the efficiency of photoreduction on the concentration of glycyltyrosine and DTT is shown in Figure 5. As can be seen, glycyltyrosine is clearly saturating at concentrations above 10^{-2} M. It is not clear whether saturation with DTT can be achieved as the enzyme becomes unstable above 3×10^{-2} M dithiothreitol. However, it is clear that the reactivity of DTT is markedly lower than that of

Table II: Relative Rates of Radical Loss upon Irradiation with Filtered (>520-nm) Light in the Presence of Added Compounds

compd added	concn (M)	rel rate
none[a]		0.12
dithiothreitol	10^{-2}	1.0
cysteine	10^{-2}	2.0
2-mercaptoethanol	10^{-2}	0.58
L-tryptophan	10^{-2}	0.11
L-histidine	10^{-2}	0.04
L-glycine	10^{-2}	0.12
NADH	10^{-2}	0.84
L-methionine	10^{-2}	0.11
glycerol	40% v/v	0.10
glycyl-L-tyrosine	10^{-2}	2.0
oligo(deoxythymidine) dimers	1.5×10^{-5}	0.06

[a] The reaction was conducted in 50 mM Tris·HCl, pH 7.4, and 100 mM NaCl.

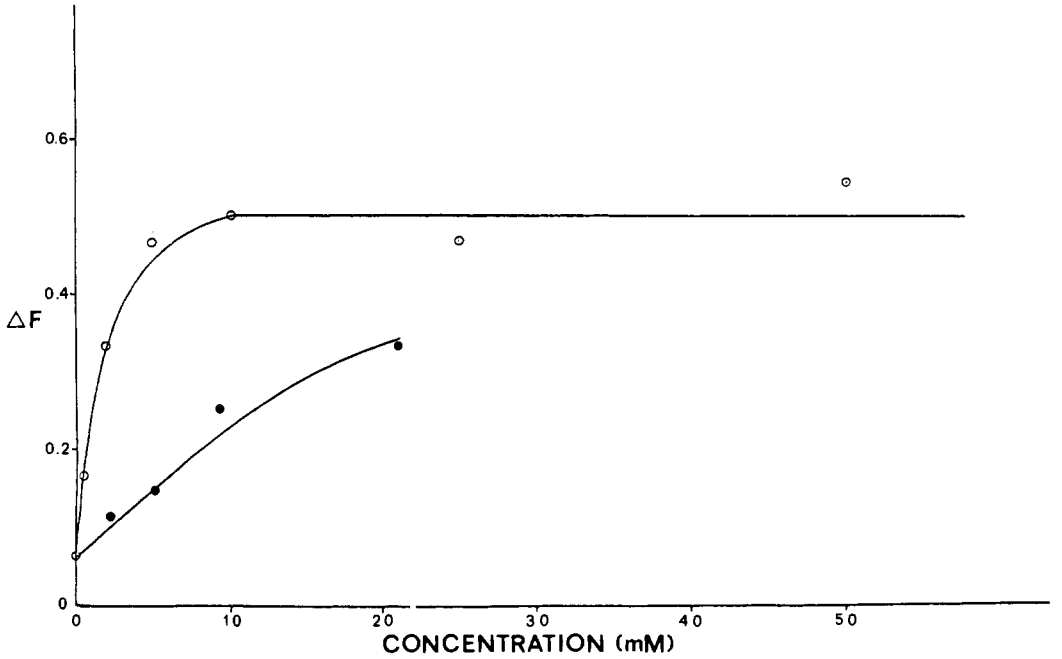

FIGURE 5: Fraction (ΔF) of the flavin radical photoreduced by a single filtered flash as a function of concentration of DTT (●) or glycyltyrosine (○).

glycyltyrosine. We also determined the pH dependence of the photoreduction by DTT or Tyr in the pH range 6–9. We first measured the absorption spectrum of the blue enzyme in the pH range 5–10. We found that identical spectra were obtained throughout the pH range, although denaturation quickly occurred at pH values >9 and <6. A marked pH dependence of photoreduction by DTT was observed in contrast to that by glycyltyrosine (Figure 6). This suggests that the thiolate anion (RS^-) is more reactive than the thiol group (pK_a = 9.4).

Laser Flash Photolysis

Effect of 353- and 532-nm Laser Excitations. Laser excitation of the blue enzyme at 532 nm has previously been described (Heelis & Sancar, 1986). When laser excitation was carried out at 532 nm in the presence of the compounds listed in Table I, the yield of transient bleaching at 580 nm observed 4 μs after the pulse was identical with that observed in the absence of added compounds. Laser excitation at 353 nm produced an initial transient absorption (λ_{max} = 420 nm) essentially identical with that reported previously for 532-nm excitation. This transient species was earlier assigned to the first excited doublet state ($E-{}^2_1FADH^0$). This species decays via first-order kinetics ($t_{1/2}$ = 1 μs) to yield a species with λ_{max} = 420 nm and a more pronounced depletion from 440 to 700 nm, again essentially identical with previous observations with 532-nm excitation. This latter transient species was assigned to the absorption of the reduced enzyme ($E-FADH_2$).

Quantum Yields for Photoreduction by 353 and 532 nm. The quantum yield of formation of the reduced enzyme was determined from the magnitude of the depletion at 580 nm, 4 μs after the pulse (with ϵ = 3600 M^{-1} cm^{-1}). A value of 0.1 ± 0.015 was obtained on the basis of the total absorbance of the enzyme at 353 nm. This value is essentially identical with that previously determined for 532-nm excitation. However, at 353 nm, only approximately 50% of the absorbance is due to the flavin radical. Hence the quantum yield of photoreduction based upon the flavin radical absorption alone would be 0.2 ± 0.03. To determine whether this apparently higher quantum yield at 353 nm was due to energy transfer from the second chromophore to the radical, we carried out 353- and 532-nm laser flash photolyses on blue enzyme possessing

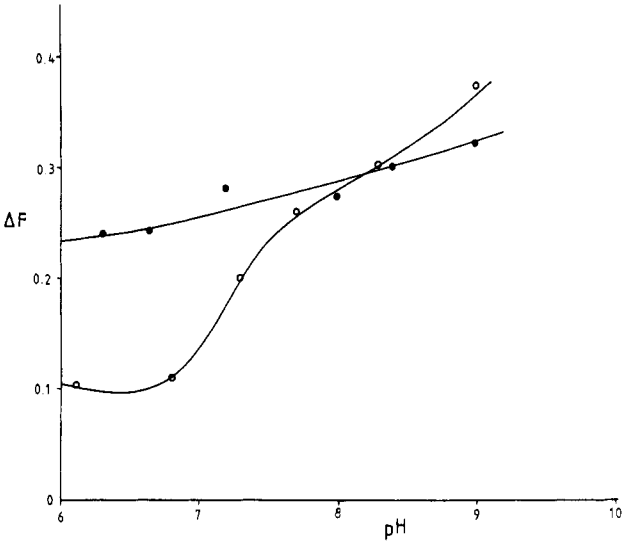

FIGURE 6: Fraction (ΔF) of the flavin radical photoreduced by a single filtered flash as a function of pH in the presence of 10^{-2} M DTT (○) or 1.5×10^{-3} M glycyltyrosine (●).

previously photolyzed SC (termed SC†–enzyme). Such samples had been extensively photolyzed with white light in the presence of DTT and then stored for 12 h at 5 °C until the reduced flavin component had completely (>95%) reoxidized to the radical form. Laser flash photolysis of SC†–enzyme using 532-nm excitation produced quantitatively the same transient spectra as fresh blue enzyme having the same radical concentration. Excitation of SC†–blue enzyme at 353 nm produced qualitatively the same transient spectra as fresh blue enzyme. However, the yield of transient reduction at 580 nm for SC†–enzyme (4 μs after the pulse) was 54% of the yield observed for fresh enzyme. It should be noted that the absorbance of SC†–enzyme at the excitation wavelength (353 nm) was 60% that of the fresh enzyme. Hence it would appear that the yield of transient photoreduction is reduced in proportion to the total absorbance to the enzyme at 353 nm.

Rate of Recovery of $FADH^0$. Laser flash photolysis using 532 nm excitation was also extended into the millisecond time

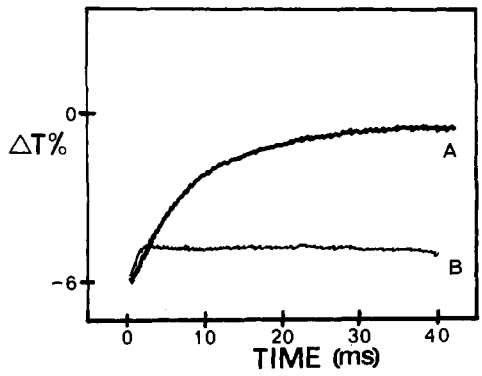

FIGURE 7: Typical oscilloscope traces observed at 580 nm during laser excitation of the blue enzyme at 532 nm: (A) no added compounds; (B) plus 2.5×10^{-2} M glycyltyrosine.

scale in order to measure the rate of semioxidation of photoreduced flavin. The recovery of the radical absorption at 580 nm after photoreduction was approximately 92% after 12 ms (Figure 7A). In contrast, in the presence of glycyltyrosine the recovery was limited in a concentration-dependent manner: to the 55% of the original value (data not shown) at 2.5×10^{-3} M and only 14% (Figure 7B) at 2.5×10^{-2} M glycyltyrosine concentration. These observations support our earlier conclusion (Heelis & Sancar, 1986) that the electron donor in photoreduction is intrinsic to the enzyme and that the apparent lack of photoreduction with camera flash in the absence of extrinsic electron donors (e.g., DTT) in the solution is due to a very fast back-reaction.

Discussion

Earlier studies on the photoreduction of the flavin radical of DNA photolyase suggested that the primary transient species observed 0.1 μs after laser excitation can be identified as the first excited doublet state of E–$FADH^0$, i.e., $^2_1FADH^0$ (Heelis & Sancar, 1986). This excited state completely reacts within 4 μs to yield the reduced flavin (E–$FADH_2$) irrespective of the presence of DTT (eq 1). Hence it was proposed that

$$\begin{matrix} E\text{-}{}^2_1FADH^0 \\ | \\ DH \end{matrix} \rightarrow \begin{matrix} E\text{-}FADH_2 \\ | \\ D^0 \end{matrix} \qquad (1)$$

the electron donor in eq 1 was a constituent of the enzyme itself, either an amino acid residue or the second chromophore. The observations that SC damage does not affect the yield of transient photoreduction (4 μs after a 532-nm laser pulse) or the rate of permanent photoreduction (camera flash experiments in the presence of DTT) strongly suggest that the SC is not the internal donor in eq 1.

Of some interest is that the quantum yields of transient photoreduction based upon the total enzyme absorbance are essentially identical for 353- and 532-nm excitations even though in the former case only 50% of the light is absorbed by the flavin chromophore. This suggests that light absorption by either the flavin radical or the SC is equally effective in producing radical reduction. It is therefore suggested that the most likely mechanism is that absorption of light by the SC is followed by rapid intra-enzyme electronic energy transfer from SC excited states to the flavin radical (eq 2). Such a

$$SC \xrightarrow{h\nu} SC^* \xrightarrow{E\text{-}FADH^0} SC + E\text{-}{}^2_1FADH^0 \qquad (2)$$

process would be energetically feasible as the SC absorbs at much shorter wavelengths than the longest band of the radical. Support for the proposed role of the SC in promoting radical reduction is that SC-damaged enzyme shows a lower yield of transient radical reduction for 353-nm excitation. In contrast, the yield of radical reduction for 532-nm excitation is not impaired by SC damage, thus confirming that the intrinsic ability of the radical to be photoreduced by direct excitation is not impaired.

An alternative explanation for the above observations could be that SC excited states donate a hydrogen atom to the flavin radical (eq 3). However, it would be expected that the kinetics

$$SC^* + E\text{-}FADH^0 \rightarrow SC(-H^0) + E\text{-}FADH_2 \qquad (3)$$

of formation of E–$FADH_2$ via eq 3 would be different for 353- and 532-nm excitations, which is not observed. In addition, the ratio of the initial yield of E–$^2_1FADH^0$ ($t = 0.1$ μs) to the final ($t = 4$ μs) yield of E–$FADH_2$ is constant, irrespective of the wavelength of excitation or whether or not SC damage has occurred. This is consistent only with the proposed scheme involving energy transfer (eq 2).

In our earlier study (Heelis & Sancar, 1986) it was proposed that the initial photoreduction reaction (eq 1) was followed by a back-reaction (eq 4); i.e., long-term photoreduction was not observed in the absence of DTT.

$$\begin{matrix} E\text{-}FADH_2 \\ | \\ D^0 \end{matrix} \longrightarrow \begin{matrix} E\text{-}FADH^0 \\ | \\ DH \end{matrix} \qquad (4)$$

This reaction has now been directly confirmed by the laser flash photolysis experiments on the millisecond time scale. In the absence of added compounds, a near complete recovery of the radical absorption at 580 nm occurs over 12 ms (Figure 7A). This is consistent with the minimal photoreduction observed in the camera flash experiments (Table II). In contrast, increasing concentrations of glycyltyrosine result in an increasing retention of the radical bleaching after 12 ms (Figure 7B). Again this is consistent with the efficient photoreduction observed in the presence of glycyltyrosine in the camera flash experiments (Figure 5 and Table II). It was earlier suggested (Heelis & Sancar, 1986) that the ability of various compounds, e.g., DTT, to promote the long-term photoreduction of the flavin radical was due to the "repair" of the D^0 radical species by (for example) DTT (eq 5), thus preventing the back-re-

$$\begin{matrix} E\text{-}FADH_2 \\ | \\ D^0 \end{matrix} + RSH \longrightarrow \begin{matrix} E\text{-}FADH_2 \\ | \\ DH \end{matrix} + RS^0 \qquad (5)$$

action. The effect of glycyltyrosine on the recovery of the radical bleaching on the millisecond time scale provides a good evidence for the proposed mechanism. The results given in Table I demonstrate that a number of compounds, particularly thiols, NADH, or tyrosine, are also capable of promoting permanent photoreduction and hence the repair of the D^0 radical (eq 5). Hence several cellular components would be capable of performing this function "in vivo". From a thermodynamic viewpoint any compound capable of reducing the D^0 species should also be capable of reducing the excited radical (E–$^2_1FADH^0$). Although the latter process does not occur in reality in the case of free compounds in solution, these results nonetheless make it attractive to speculate that an amino acid residue adjacent to the flavin, possibly a tyrosyl or cysteinyl residue, is the primary electron donor (DH) in eq 1.

Finally, in this paper we have described a well-defined method for obtaining SC-free photolyase: illumination of the enzyme with 100 flashes from a Starblitz flash unit causes the disappearance of the 390-nm absorption and 470-nm fluorescence emission peaks of the second chromophore without significant damage to the flavin radical or the apoenzyme. This is in contrast to the other method that yielded SC†–

enzyme: "lengthy and/or harsh" purification procedures result in the purification of green or yellow enzymes that are partially or completely devoid of the second chromophore but also have a substantial fraction of the flavin in fully oxidized form (Jorns et al., 1987). The photochemical method for obtaining SC†-enzyme is fully reproducible and yields an enzyme with essentially intact flavin chromophore. The availability of such an enzyme should help us discern in more detail the individual roles of the flavin and the second chromophores in the photosensitization reaction mediated by photolyase.

Registry No. FADH, 1910-41-4; DTT, 3483-12-3; NADH, 58-68-4; DNA photolyase, 37290-70-3; thymine dimer, 28806-14-6; cysteine, 52-90-4; 2-mercaptoethanol, 60-24-2; L-tryptophan, 73-22-3; L-histidine, 71-00-1; glycine, 56-40-6; L-methionine, 63-68-3; glycerol, 56-81-5; glycyl-L-tyrosine, 658-79-7.

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Cleavage of Fibrin-Derived D-Dimer into Monomers by Endopeptidase from Puff Adder Venom (*Bitis arietans*) Acting at Cross-Linked Sites of the γ-Chain. Sequence of Carboxy-Terminal Cyanogen Bromide γ-Chain Fragments†

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ABSTRACT: Puff adder venom contains a protease capable of cleaving the γ-chain of cross-linked D-dimer, derived from the plasmin digestion of fibrin, into apparently symmetrical monomers. The cross-linked γ-chains are separated in the process without apparent loss of mass and without loss of the substituent at the glutamine cross-link site, if fluorescent D-dimer (the lysine analogue dansylcadaverine used as substituent) is used as substrate [Purves, L. R., Purves, M., Lindsey, G. G., & Linton, N. J. (1986) *S. Afr. J. Sci. 82*, 30]. The γ-chain from puff adder venom digested D-monomer was isolated and cleaved by cyanogen bromide, and the carboxy-terminal peptide was isolated and sequenced. The carboxy-terminal peptide composition indicated a lower content of histidine, leucine, and glycine than expected. Manual microsequencing by gas-phase Edman degradation demonstrated that two amino-terminal ends were present. By use of the known sequence of the human fibrinogen γ-chain, the sequencing data could be resolved into a dipeptide cross-linked between lysine-406 and either glutamine-398 or -399 (residues 6 and 13 or 14 from the carboxy-terminal end of the γ-chain) with the loss of residues 401–404 that occur between the cross-link sites of both antiparallel cross-linked γ-chains. D-dimer is therefore separated into monomers by cleavage of the γ-chain between the cross-link sites. Two symmetrical fragments are produced consisting of a cross-linked dipeptide with the loss of four amino acids. The sequence is therefore equivalent to the original fibrinogen sequence

$$\begin{matrix} {}_{385}\text{KIIPFNRLTIGEG}(\text{Q}^{*}\text{Q}^{*})\text{H}_{400} \\ \text{X} \\ {}_{405}\text{A}(\text{K}^{*})\text{QAGDV}_{411} \end{matrix}$$

where *X* indicates a cross-link site. There is heterogeneous loss of ^{401}HLGG^{406} and possibly also ^{399}Q and ^{400}H in subfractions of the peptide. The predominant cross-linked glutamine appears to be residue 399, 13 residues from the carboxy-terminal end.

Covalent stabilization of the fibrin clot is brought about by the formation of γ-(ε-glutamyllysine) cross-links between antiparallel γ-chains of fibrin (Lorand, 1983) by the enzyme "transglutaminase"—factor XIIIa of the coagulation cascade (EC 2.3.2.13, glutaminyl-peptide glutamyltransferase). The amino acid sequence of the γ-chain is highly conserved, permitting hybrid cross-links of the γ-chains of fibrin from different species, e.g., bovine and human (Chen & Doolittle, 1971). The cross-link has been assigned to sites lysine-406 and glutamine-398 (6 and 14 residues from the carboxy-ter-

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