Photoreactivating Enzyme from *Escherichia coli*: Appearance of New Absorption on Binding to Ultraviolet Irradiated DNA†

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ABSTRACT: The photoreactivating enzyme, PRE, monomerizes pyrimidine dimers in DNA in a light requiring reaction ($\lambda > 300$ nm). However, the purified PRE from *E. coli* has no well-defined absorption band for $\lambda > 300$ nm. Using absorption difference spectroscopy, we show that when PRE is mixed with ultraviolet-irradiated DNA, new absorption appears in the spectral region required for catalysis. There is a concomitant decrease in the absorption of the mixture for wavelengths less than 300 nm. The hyperchromicity for $\lambda > 300$ nm is true absorption, not an artifact due to light scattering. Both the hyperchromicity ($\lambda > 300$ nm) and hypochromicity ($\lambda < 300$ nm) can be reversed by irradiation at 365 nm with identical first-order kinetics. We estimate the molar extinction coefficient of the new absorption to be 6900 ± 1400 at 350 nm. We conclude that the PRE from *E. coli* does not possess a distinct "chromophore" which by itself is entirely responsible for the absorption of photoreactivating light. Instead, new absorption results when PRE binds its substrate, dimer-containing DNA.

The photoreactivating enzyme monomerizes cyclobutylpyrimidine dimers [also called cyclobutadipyrimidines (Madden et al., 1973; Cohn et al., 1974)] in an enzymatic reaction which requires near-ultraviolet and visible light in the range from 300 to 600 nm (the upper wavelength depends on the species of origin) for catalysis. The PRE thus partially reverses the lethal and mutagenic and presumably the carcinogenic effects of far-ultraviolet ($\lambda < 300$ nm) radiation. (See, e.g., Setlow, 1966; Cook, 1970.)

It has generally been supposed that the purified enzyme would be found to have a cofactor or "chromophore" which absorbs the radiation required for catalysis (see, e.g., Lamola 1966, 1972). Purified PRE from *E. coli*, however, has little absorption above 300 nm compared with its intense absorption below 300 nm (Sutherland and Sutherland, 1972). Either (a) any "chromophore" absorption at $\lambda > 300$ nm must be much less than the 257-nm peak and not easily detected or (b) the purified PRE from *E. coli* is not solely responsible for the absorption of photoreactivating light. We report experiments which support the latter hypothesis.

Experimental Procedure

Photoreactivating enzyme from a strain of *E. coli* W3350 lysogenic for the inducible phage (λ C1857S7 dg D → J) (Sutherland et al., 1972) was purified by a procedure which will be described in detail elsewhere (Snapka et al., in preparation). Briefly, this involved disruption by grinding with glass beads, streptomycin sulfate precipitation, ammonium sulfate fractionation, isoelectric focusing, and dialysis to remove ampholytes. The purified enzyme gave a single band on sodium dodecyl sulfate–polyacrylamide gels.

Calf thymus DNA[1] (type I, D1501, Sigma Chemical Co., St. Louis, Mo.) was purified by phenol extraction (Felsenfeld and Hirschman, 1965) until the ratio of absorbance at 260 nm to that at 280 nm was 1.9. Dimers were introduced into samples of the calf thymus DNA by irradiating at 289 nm to a total exposure of 3×10^4 J/m^2 in a Johns monochromator (Johns and Rauth, 1965a,b). Samples were stirred during irradiation and corrected for internal absorption (Johns et al., 1964). The intensity of irradiation was monitored with a vacuum photodiode connected to a vacuum tube photometer. The monitoring system was calibrated with a radiant flux meter [Hewlett-Packard, Palo Alto, Calif., Model 8330A/8334A(014)] and potassium ferrioxalate actinometry (Hatchard and Parker, 1956). The percent of thymine converted to dimers by this irradiation was determined independently in a parallel experiment using [^{3}H]thymine-labeled bacteriophage T7 DNA. After 289-nm irradiation, the T7 DNA was hydrolyzed and thin-layer chromatographed and counted (Sutherland and Sutherland, 1969). The percentage of radioactivity associated with dimers was 15%.

Absorption and absorption difference spectra were recorded with a Cary 118c spectrophotometer (Varian Associates, Palo Alto, Calif.), equipped with temperature-regulated sample and reference cuvette holders (Models 1844100 and 1844200, respectively, Varian Associates) and a scattered transmission accessory (Model 1862000, Varian Associates).

Difference spectra were recorded using the configurations shown in Figure 1. A cuvette containing only PRE in buffer was placed in position R1, a cuvette containing only DNA in R2, a cuvette containing only buffer in position S1, and a cuvette containing both PRE and DNA in S2. The total concentrations of PRE and DNA in the reference beam were identical with those in the sample beam in all cases. The possible influence of light scattering on difference spectra was determined by shifting the cuvettes at positions S2 and R2 to the "high scatter" positions S2′ and R2′, respectively. Operating the tungsten–halogen lamp at high intensity permitted continuous scans from 800 to 240 nm without changing light

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[1] Abbreviations used: ADS, absorption difference spectroscopy; DNA, deoxyribonucleic acid; PRE, photoreactivating enzyme; $NaDodSO_4$, sodium dodecyl sulfate; UV-DNA, ultraviolet-irradiated DNA; λ, wavelength; OD, optical density.

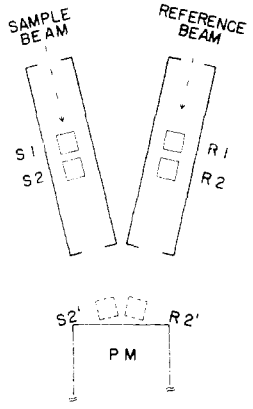

FIGURE 1: Arrangement of cuvettes for difference spectroscopy. Cuvette positions S1, S2, R1, and R2 are located inside temperature regulated holders. Alternate positions S2′ and R2′ are used to evaluate possible effects due to light scattering. The photomultiplier, PM, detects scattered light with a much greater efficiency when the sample is located at position S2′ compared with position S2 and thus any apparent absorption caused by scattering will be reduced.

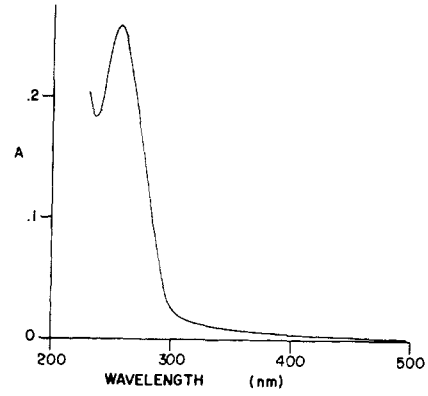

FIGURE 2: Absorption spectrum of PRE from *E. coli*.

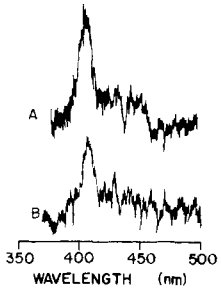

FIGURE 3: Fluorescence emission spectrum of PRE from *E. coli* (top). Excitation was at 360 nm. The lower spectrum is that of buffer E (10 mM Tris (pH 7)–0.1 mM EDTA–0.1 mM dithiothreitol). The peak at 408 nm in both spectra is Raman scattering of water. Using the Raman peak as an internal standard and comparing the observed emission with that of quinine sulfate, we estimate that the product of the molar extinction of the enzyme at 360 nm and the quantum yield for fluorescence is less than 1.0.

sources. Positions S1, S2, R1, and R2 are located inside the thermostated sample and reference chambers and were maintained at approximately 4 °C. Positions S2′ and R2′ were at ambient temperature. Condensation was prevented by purging the sample and detector compartments with dry nitrogen gas.

Fluorescence spectra were determined using an emission and polarization spectrometer described elsewhere (Sutherland et al., 1976). The sample was also maintained at 4 °C while in this instrument and the sample chamber purged with dry nitrogen.

Results and Discussion

Absorption and Fluorescence Spectra. The absorption spectrum of the purified enzyme is shown in Figure 2. There is a well-defined absorption maximum near 257 nm. However, no well-resolved absorption band is detected at wavelengths greater than 300 nm. *E. coli* PRE purified by a different method (Sutherland and Sutherland, 1972) as well as PRE from other organisms such as orchid seedlings (*Cattleya aurantiaca*) (C. W. Painter and B. M. Sutherland, in preparation) and silverfish (*Thermobia domestica*) (B. M. Sutherland et al., in preparation) exhibit absorption spectra similar to that shown in Figure 2.

The lack of well-defined absorption bands at wavelengths greater than 300 nm is surprising since these wavelengths are required for catalysis. It has frequently been assumed that all PRE's contained a "chromophore" responsible for the absorption of the photoreactivating wavelengths (see reviews by Lamola, 1972; Setlow, 1966, 1967). Lack of a well-resolved absorption band at wavelengths greater than 300 nm as exemplified by Figure 2 indicates either that the absorbance of the "chromophore" is much less than the absorbance of the 257-nm band and not detected under these conditions, or that part or all of the absorption responsible for dimer monomerization does not exist unless the enzyme is bound to dimer-containing DNA, a possibility previously suggested by Setlow (1966). The absorption difference spectroscopy (ADS) experiments described below support the second hypothesis.

Other laboratories have reported absorption bands at wavelengths greater than 300 nm associated with purified PRE. These include PRE from bakers yeast (Minato and Werbin, 1971), the blue-green alga [*Anacystis nidulans*] (Minato and Werbin, 1972; Saito and Werbin, 1970) and the bacterium *Streptomyces nidulans* (Eker and Fichtinger-Schepman, 1975). Very recent work on the yeast PRE indicates, however, that the near-UV absorption can be separated from enzymatic activity. The resulting fraction which is enzymatically active exhibits an absorption spectrum which is qualitatively similar to the *E. coli* PRE absorption spectrum shown in Figure 2 (Werbin and Madden, 1976; H. Werbin, personal communication).

However, this more highly purified yeast PRE exhibits a fluorescence excitation spectrum with a maximum between 300 and 400 nm (H. Werbin, personal communication) similar to that previously reported (Minato and Werbin, 1971). Our attempts to detect fluorescence with excitation between 300 and 400 nm which we can unambiguously attribute to the enzyme have been unsuccessful (see Figure 3).

Since the optical properties reported for the algal and *S. griseus* enzymes are considerably different from those we find for the *E. coli*, orchid, and silverfish enzymes, there may be two or more classes of PRE with distinct optical properties and mechanisms of action.

Absorption Difference Spectra. Since the isolated enzyme does not exhibit an apparent absorption maximum between 300 and 400 nm, it seems possible that the absorption responsible for photoreactivation might arise or be enhanced when the enzyme interacts with its substrate, i.e., DNA containing cyclobutylpyrimidine dimers. We tested this hypothesis by measuring the difference between the absorption of PRE mixed with UV-irradiated DNA and the sum of the absorptions of equal concentrations of unmixed enzyme and irradi-

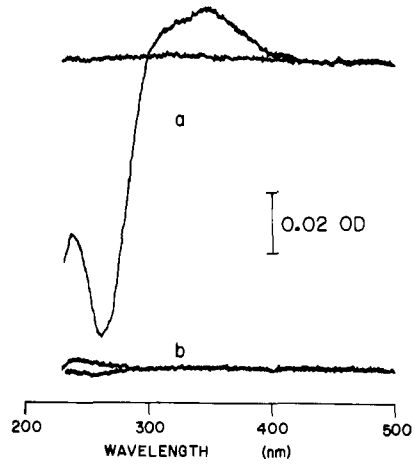

FIGURE 4: (a) A four-cuvette difference spectrum of PRE and UV-DNA. The absorbance of the PRE at 257 nm, when measured separately, was 0.24 and that of the UV-DNA was 1.26 at 257 nm. The baseline was recorded with equal amounts of PRE + UV-DNA mixtures in cuvettes S2 and R2 and cuvettes S1 and R1 filled with buffer. (b) The difference spectrum of PRE and unirradiated DNA. The concentrations of PRE and DNA used were the same as in a.

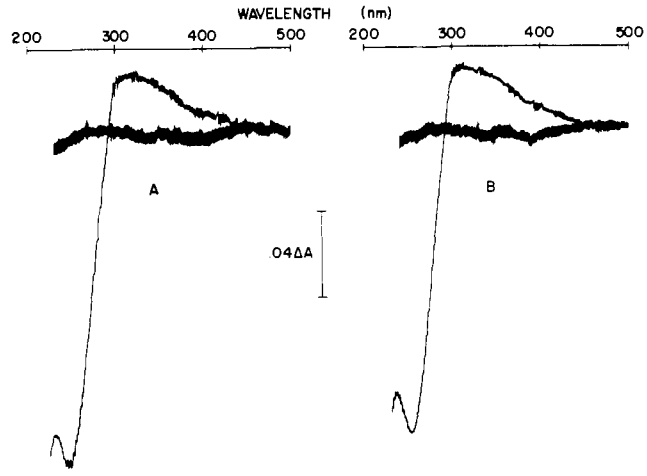

FIGURE 5: (A) The difference spectrum of PRE and UV-DNA with cuvettes containing UV-DNA and (PRE + UV-DNA) in positions R2′ and S2′, respectively (see Figure 1). (B) Difference spectrum of PRE and UV-DNA with cuvettes containing UV-DNA and (PRE + UV-DNA) in positions R2 and S2, respectively (see Figure 1).

ated DNA. The arrangement of cuvettes is shown in Figure 1 and described in Experimental Procedure.

Typical results are shown in Figure 4. The positive absorbance recorded for wavelengths greater than 300 nm reflects an increase in the absorption of the PRE–UV-DNA complex in this region compared with the unmixed components. Similarly the negative values recorded at shorter wavelengths indicate hypochromicity. The baselines in Figure 4 (and Figure 5) were measured by placing identical samples of PRE plus UV-DNA in both the sample and reference beams.

If unirradiated DNA is used instead of irradiated DNA, there is no detectable increase in absorption at wavelengths greater than 300 nm as shown in Figure 4b. A slight hypochromicity is observed at shorter wavelengths. Difference spectra similar to the one shown in Figure 4 have been recorded using several independently purified samples of PRE.

It is important to show that the hyperchromicity observed for $\lambda > 300$ nm is due to true absorption and not to an increase in light scattering resulting from formation of a PRE–UV-DNA complex. Two experiments show that this is not the case. First, irradiation of the complex with 365-nm radiation causes a concomitant decrease in the magnitude of both the positive ($\lambda > 300$) and negative ($\lambda < 300$) portions of the difference spectra (vida infra). The ability of the 365-nm radiation to reverse the difference spectrum implies that these wavelengths must be absorbed rather than scattered. Second, we measured the difference spectrum with the PRE–UV-DNA sample at both positions S2 and S2′, while the cuvette containing UV-DNA was at R2 and R2′, respectively. If the apparent absorption at wavelengths greater than 300 nm was due to scattering rather than absorption, the observed optical density should have decreased when the cuvettes were moved to positions S2′ and R2′. In fact, the difference spectra were essentially the same as shown in Figure 5. There are small changes in ΔA_{257} and ΔA_{350} at the "high scatter" position (see Figure 5A), but the data show that light scattering could have accounted for no more than about 10% of the absorbance changes we have observed in the difference spectrum. Thus we conclude that the positive portion of the difference spectrum is due to absorption and not to scattering.

Extinction Coefficient of the Complex. The molar extinction coefficient of the PRE is not yet known. We can, however, estimate the molar extinction coefficient associated with complex formation. The molar extinction coefficient of calf thymus DNA is 6600 $(M1)^{-1}$ (Mahler et al., 1964); thus the concentration of the DNA, measured in mol of phosphate/L, used in the experiment shown in Figure 4 is 1.9×10^{-4} M. Thymine constitutes 28% of the bases in calf thymus DNA (Chargaff, 1955) and we determined that about 15% of the thymine was in dimers. Thus, since each dimer contains two thymines, the concentration of dimers is approximately 4.0×10^{-6} M. The enzyme concentration was adjusted to saturate the available dimers so the number of PRE–dimer complexes (assuming 1:1 stoichiometry) is equal to the number of dimers. Since the absorbance at 350 nm is 0.020, the molar extinction at the wavelength is approximately 5000. We have repeated the experiment four times with freshly purified enzyme and observed similar results, giving an average apparent molar extinction coefficient of 6900 ± 1400. This value is in reasonable agreement with the value of 7000 to 11 000 estimated by Rupert (1962) from kinetic data.

Photoreversal of the Difference Spectrum. If the new absorption band observed for wavelengths greater than 300 nm is responsible for the absorption of photoreactivating light, then irradiation in this wavelength region should remove dimers from the DNA. Removal of dimers should also reduce the magnitude of the difference spectrum if enzyme is present in excess. Irradiation of the PRE–UV-DNA mixture at 4 °C in the Johns monochromator with 365-nm radiation reduces the magnitude of both the positive and negative portions of the difference spectrum. In Figure 6 we plot $\Delta A(E)/\Delta A(0)$, the ratio of the absorbance difference remaining after exposure (E) to the initial absorbance difference. Absorbance differences were monitored at both 350 and 257 nm. Figure 6 shows that 365-nm radiation reverses the difference absorption at both wavelengths with essentially identical first-order kinetics. Irradiations of isolated pyrimidine dimers under similar conditions did not result in the production of monomeric thymine. Thus, the effect we observe in the PRE–UV-DNA mixture is not due to direct (nonenzymatic) monomerization of dimers.

From the slopes of the lines in Figure 6, we can calculate the product of the molar extinction coefficient for the absorption responsible for the disappearance of the difference spectrum

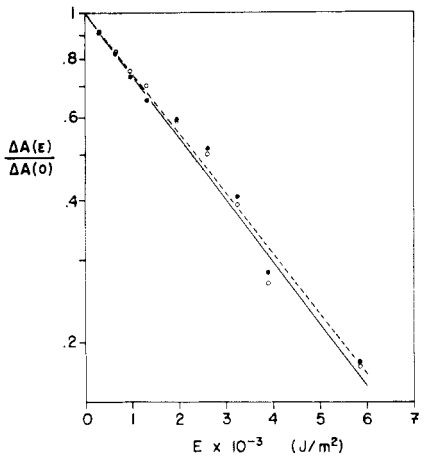

FIGURE 6: Semilogarithmic plots of the fractional changes in difference absorptions as a function of exposures to 365-nm light. The cuvette containing the (PRE + UV-DNA) mixture was irradiated at 365 nm with a constant fluence of 65 J m^{-2} s^{-1} in the Johns monochromator. After irradiating for fixed time intervals, the difference absorbance of the complex, $\Delta A(E)$, was then monitored at 257 nm (●—) and at 350 nm (O---O) by using the same four-cuvette arrangement as described earlier. $\Delta A(0)$ is the initial difference absorbance before any exposure to photoreactivating light. The lines were determined by the method of least-squares.

and the quantum yield for that process (see, e.g., Setlow, 1957). We obtain values of 520 and 640 for the 350 and 257 nm data, respectively. Taking the molar extinction coefficient in this product as 6900 ± 1400 gives a quantum yield of about 0.1, a value we find surprisingly low. Experiments to determine the dependence of $\phi\epsilon$ on experimental parameters such as temperature and ionic strength are in progress.

Our results indicate that there is an increase in absorption in the wavelength region between 295 and 450 nm when PRE is mixed with UV-DNA. While we have no direct experimental evidence on the nature of this increased absorption, it is interesting to speculate how our results can be rationalized with the nonenzymatic monomerization of (isolated) pyrimidine dimers by visible and near-UV light ($\lambda > 300$ nm) in the presence of sensitizers such as anthroquinone (Lamola, 1972, and references cited therein). Anthroquinone absorbs radiation ($\lambda > 300$ nm) and the excited molecule is thought to form a charge-transfer complex with a dimer. Removing an electron renders the dimer unstable and causes it to revert to two monomers. Roth and Lamola (1972) found direct experimental evidence for the existance of a dimer-anthroquinone complex.

The anthroquinone molecule interacts with a dimer only after it has absorbed a photon and been promoted to an excited state. Thus, the absorption spectrum of the anthroquinone-dimer mixture is just the sum of its separate components; no new absorption bands are observed. We can invoke the same charge-transfer mechanism of dimer monomerization and simultaneously explain the appearance of a new absorption band by postulating that a complex is formed between a dimer (in a strand of DNA) and some portion of the enzyme when both components are in their ground electronic states. The lowest excited state of the complex would correspond to (partial) transfer of an electron from one component to the other. This process (1) results in the appearance of a new "charge-transfer" absorption band and (2) renders the dimer partially ionized and thus unstable. The partially ionized dimer would, as in the anthroquinone complex, revert to monomers. This mechanism will be discussed in greater detail elsewhere (Sutherland, 1977).

Acknowledgments

We thank Dr. B. M. Sutherland, Cathy Painter, and Robert Snapka of the Department of Molecular Biology for unpublished results. We also thank Dr. Ronald Rahn, Biology Department, Oak Ridge National Laboratory, for helpful discussions.

References

Chargaff, E. (1955), The Nucleic Acids, Vol. I, Chargaff, E., and Davidson, J. N., Ed., New York, N.Y., Academic Press, pp 307-407.

Cohn, W. E., Leonard, N. J., and Wang, S. Y. (1974), *Photochem. Photobiol. 19,* 89-94.

Cook, J. S. (1970), *Photophysiology 5,* 191-233.

Eker, A. P. M., and Fichtinger-Schepman, A. M. J. (1975), *Biochim. Biophys. Acta 378,* 54-63.

Felsenfeld, G., and Hirschman, S. Z. (1965), *J. Mol. Biol. 13,* 407-427.

Hatchard, C. G., and Parker, C. A. (1956), *Proc. R. Soc. London, Ser. A, 235,* 518-536.

Johns, H. E., Pearson, M. L., LeBlanc, J. C., and Helleiner, C. W. (1964), *J. Mol. Biol. 9,* 503-524.

Johns, H. E., and Rauth, M. (1965a), *Photochem. Photobiol. 4,* 673-692.

Johns, H. E., and Rauth, M. (1965b), *Photochem. Photobiol. 4,* 692-707.

Lamola, A. A. (1966), *J. Am. Chem. Soc. 88,* 813-819.

Lamola, A. A. (1972), *Mol. Photochem. 4 (1),* 107-133.

Madden, J. J., Werbin, H., and Denson, J. (1973), *Photochem. Photobiol. 18,* 441-446.

Mahler, H. R., Kline, B. E., and Mehrotra, B. D. (1964), *J. Mol. Biol. 9,* 801-811.

Minato, S., and Werbin, H. (1971), *Biochemistry 10,* 4503-4508.

Minato, S., and Werbin, H. (1972), *Photochem. Photobiol. 15,* 97-100.

Roth, H. D., and Lamola, A. A. (1972), *J. Am. Chem. Soc. 94,* 1013-1014.

Rupert, C. S. (1962), *J. Gen Physiol. 45,* 725-741.

Saito, N., and Werbin, H. (1970), *Biochemistry 9,* 2610-2620.

Setlow, J. K. (1966), *Curr. Top. Radiat. Res. 2,* 195-248.

Setlow, J. K. (1967), *Compr. Biochem. 27,* 157-209.

Setlow, R. B. (1957), *Adv. Biol. Med. Phys. 5,* 37-74.

Sutherland, B. M., Court, D., and Chamberlin, M. J. (1972), *Virology 48,* 87-93.

Sutherland, B. M., and Sutherland, J. C. (1969), *Biophys. J. 9,* 292-302.

Sutherland, J, C. (1977), *Photochem. Photobiol.* (in press).

Sutherland, J. C., Cimino, G. D., and Lowe, J. T. (1976), *Rev. Sci. Instrum. 47,* 358-360.

Sutherland, J. C., and Sutherland, B. M. (1972), *Prog. Photobiol, Proc. Int. Congr., 6th,* 115.

Werbin, H., and Madden, J. (1976), Abstract of the 4th Annual Meeting of American Society for Photobiology, Denver, Colo.