from phosphocellulose columns as described by Eley (1969). By this technique we have obtained milligram amounts of essentially pure enzyme and there appear no technical limitations to scaling up the process for the production of larger amounts. The uses and future of phosphocellulose columns in the purification of enzymes which act on nucleic acids have been discussed by Eley; however one difficulty arises in the phosphocellulose chromatography of the Lactobacillus enzyme.

After elution of the enzyme with RNA from the first phosphocellulose column and throughout the second chromatography the presence of contaminating RNA interferes dramatically with the enzyme assay. In contrast to Eley's studies with chicken pancreas nuclease the RNA is not removed completely on the second (salt elution) column. We have resorted to extended incubation periods allowing degradation of the accompanying RNA followed by dialysis to eliminate the RNA. Unfortunately contaminating RNA (<2% by weight) is often still retained. However, when fraction IV or V enzymes are subjected to electrophoresis on acrylamide gels the enzyme which can be recovered from the gels is now free of detectable RNA. We anticipate that the use of preparative disc gel electrophoresis will provide us with completely pure protein for our future studies.

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Spectral Properties of the Chromophoric Material Associated with the Deoxyribonucleic Acid Photoreactivating Enzyme Isolated from Baker's Yeast*

Sadamasa Minato and Harold Werbin

ABSTRACT: A DNA photolyase, heretofore referred to as a DNA-photoreactivating enzyme, was purified 70,000-fold from Baker's yeast. The last step in the procedure involved chromatography on far-uv-irradiated DNA noncovalently bound to cellulose. The final enzyme preparation absorbed near-uv light between 350 and 420 nm and exhibited maximum absorbance at about 380 nm. Maxima at 385 and 485–490 nm appeared in its excitation and fluorescence spectra, respectively. The molecular weight of the enzyme determined by gel filtration was 53,000. The photolyase was reversibly inactivated and reactivated by treatment first with ferricyanide and then with 2-mercaptoethanol. The fluorescent material was not removed from the enzyme by either chromatography on hydroxylapatite or Sephadex G-100, provided during the latter procedure the salt concentration in the buffer was maintained at 0.4 M. Partial dissociation of the fluorescent material occurred when salt-free buffer was used to elute the enzyme from the Sephadex column and loss of activity ensued. The spectral properties of the fluorescent materials associated with the DNA photolyase are those expected for the chromophore involved in DNA photoreactivation.

Photoreactivation may be defined as restoration of activity to biological material by illumination with longer wavelengths of radiation than that which engendered the inactivation. Significant contributions to our understanding of this phenomenon at the molecular level have been made during the past decade. Inactivation is caused principally by far-uv damage to deoxyribonucleic acid (DNA) or to ribonucleic acid (RNA) in those organisms such as most plant viruses and RNA phages where the genome is composed only of RNA. This communication is concerned only with DNA photoreactivation. In this case a substantial fraction of the inactivation by far-uv radiations is attributed to the formation of cyclobutane dipyrimidines[1] in DNA (Setlow, 1968) and the re-activation, to re-

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[1] The reasons for preferring the term cyclobutane dipyrimidines instead of pyrimidine dimers are discussed in another publication (Minato and Werbin, 1972). These compounds are engendered in deoxyribonucleotides (Cook, 1967), synthetic deoxypolynucleotides (Setlow *et al.*, 1965), and deoxyoligonucleotides (Setlow and Bollum, 1968) by far-uv-induced cycloaddition of the 5,6 double bond of a pyrimidine residue to the 5,6 double bond of an adjacent pyrimidine and are isolated by acid hydrolysis at 165° (Varghese and Wang, 1967). Because of their isolation by hot acid, it is conceivable that the cyclobutane dipyrimidines are derived rather than primary photoproducts.

formation of native DNA by light-mediated cleavage of these lesions (Setlow *et al.*, 1965; Setlow and Bollum, 1968; Williams *et al.*, 1971) by the DNA-photoreactivating enzyme. This enzyme will be referred to as a DNA photolyase.[2,3] The enzyme, first encountered by Rupert *et al.* (1958), was purified about 3600-fold from both Baker's yeast (Muhammed, 1966) and the blue-green alga *Anacystis nidulans* (Saito and Werbin, 1970). Purification to an apparently homogeneous state of a similar enzyme from *Escherichia coli* was reported recently by Sutherland and Chamberlin (1971).

All action spectra of DNA photoreactivation exhibit maxima in the near-uv or visible region of the spectrum (Jagger *et al.*, 1970). Since the DNA photolyase is known to form a stable complex with its substrate, far-uv-irradiated DNA (Rupert, 1962), the observed maxima can be ascribed to absorption by this complex, or alternatively, to a chromophoric substance either covalently or noncovalently bound to the enzyme. Until recently, because the enzyme had not been extensively purified, a choice between these alternative explanations was not possible. Thus, Muhammed's enzyme preparation exhibited weak absorption bands at 415 nm which he attributed to an impurity and the band at 418 nm in the spectrum of the algal enzyme was explained similarly (Saito and Werbin, 1970). Sutherland and Chamberlin (1971) did not describe the spectral properties of their purified DNA photolyase.

In this communication, we report on the absorption, excitation, and fluorescence spectra of the DNA photolyase isolated from Baker's yeast after 70,000-fold purification. The observed spectra are attributed to a near-uv-absorbing substance associated with the yeast photolyase in DNA photoreactivation.

Methods and Materials

Enzyme Bioassay. The procedure has been described by Saito and Werbin (1970) except that illumination was carried out for 5 min with five 15-W General Electric black lights (F15T8-BLB) and 0.05 M phosphate buffer (pH 7.5) containing 1 mM EDTA and 0.1% bovine serum albumin (hereafter simply referred to as albumin) was substituted for Tris-borate buffer. This assay measures the enhanced capacity of far-uv-irradiated *Haemophilus influenzae* transforming DNA carrying a streptomycin marker to transform competent *H. influenzae* cells after illumination of the mixture of enzyme and DNA.

Protein Determination. Protein was measured by the method of Lowry *et al.* (1951) or by determining absorbance at 280 nm with a Zeiss spectrophotometer (M IV Q III, PM Q II).

Chromatographic Adsorbants. Phosphocellulose (P-1) and cellulose powder (CF-11) were Whatman Chromedia products. Sephadex G-100 was purchased from Pharmacia Fine Chemicals, Inc., and hydroxylapatite (Bio-Gel HTP) from Bio-Rad Laboratories, Richmond, Calif.

Irradiated-DNA Cellulose. A solution of calf thymus DNA (type V from Sigma Chemical Co.), 2 mg/ml, was prepared with 0.01 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and irradiated with a dose of 3.2×10^5 ergs/mm^2 of 254-nm light from a germicidal lamp. The irradiated DNA was noncovalently linked to cellulose powder by the procedures described by Alberts *et al.* (1968). The buffer used to equilibrate and elute the DNA cellulose column is referred to as AC buffer and consisted of 0.05 M Tris-HCl containing 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.02 M K_2HPO_4, 0.1 M KCl, and 5% glycerol (pH 7.5).

Disc Electrophoresis. Electrophoresis on polyacrylamide gel was performed according to Davis (1964) using the Canalco Model 12 apparatus. To assay for photolyase activity after a run, the unstained gel was cut into 5-mm sections and each portion was homogenized at 5° in 0.05 M phosphate buffer (pH 7.5) containing 0.1% albumin. The homogenized gels were centrifuged and their supernatants assayed.

Spectroscopic Procedures. The ultraviolet and fluorescence spectra of the purified enzyme preparation were determined on a Cary 14 spectrophotometer and a Perkin-Elmer Hitachi MPF-2A spectrofluorometer, respectively. Excitation and fluorescence spectra were measured in 5-mm cylindrical cells and emission intensities were corrected for the variation of the light intensities from the Xenon lamp with the wavelengths (Argauer and White, 1964; Melhuish, 1962). Only the data for the spectra in Figure 7 were corrected in this way.

Experimental Details and Results

Enzyme Purification. PREPARATION OF CELL-FREE EXTRACT. Fresh Baker's yeast (50 lb) was air-dried and the dry material (4.5 kg) was suspended in 18 l. of 0.066 M K_2HPO_4 to which 900 ml of toluene had been added. The suspension was stirred for 4 hr at 37° and centrifuged at 10,000 rpm for 20 min. About 12 l. of supernatant was obtained. All steps beyond this stage were carried out in a cold room at 5°.

AMMONIUM SULFATE FRACTIONATION. The supernatant was adjusted to pH 7.0 with 2 N NaOH, 2.4 kg of ammonium sulfate was added to 33% saturation, and stirring was continued for 30 min more. The resulting precipitate was removed by centrifugation at 10,000 rpm for 20 min and discarded. Another 1.48 kg of ammonium sulfate was added to bring the supernatant to 50% saturation and the suspension was left in the cold room overnight. After centrifugation, the precipitate obtained was dissolved in 0.05 M phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. After this solution was dialyzed against the same buffer for 2 days and centrifuged, there was obtained about 2700 ml of supernatant.

COLUMN CHROMATOGRAPHY ON PHOSPHOCELLULOSE. The supernatant was divided in half and each portion was treated similarly. Each was passed through a phosphocellulose column (5.5 × 50 cm) equilibrated with 0.05 M phosphate buffer (pH 7.5) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The column was washed with 2 l. of the same buffer and eluted with a linear gradient of 2 l. each of 0 and 0.6 M KCl in the same buffer. The enzyme was eluted when the salt concentration in the buffer was between 0.25 and 0.3 M. Active fractions were pooled and the enzyme was precipitated by addition of ammonium sulfate to 60% saturation. The precipitate was dissolved in a small amount of AC buffer,

[2] Illumination by near-uv or visible radiations of the mixture of DNA-photoreactivating enzyme and substrate results in cleavage of cyclobutane dipyrimidines and restoration of the adjacent bases to their original configurations (Setlow *et al.*, 1965; Cook, 1967; Williams *et al.*, 1971). In the systematic nomenclature of enzymes (Florkin and Stotz, 1964) those in class 4, referred to as lyases, remove groups from substrates by cleaving C–C, C–O, C–N, C–S, or C–Cl bonds. Although the enzyme does not remove groups from its substrate, it does cleave C–C bonds and hence can be classified as a photolyase. We discuss elsewhere (Minato and Werbin, 1971) reasons for designating the enzyme as a deoxyribonucleate cyclobutane dipyrimidine photolyase.

[3] Gordon *et al.* (1971) recently observed RNA photolyase activity in cell-free extracts of tobacco plant leaves.

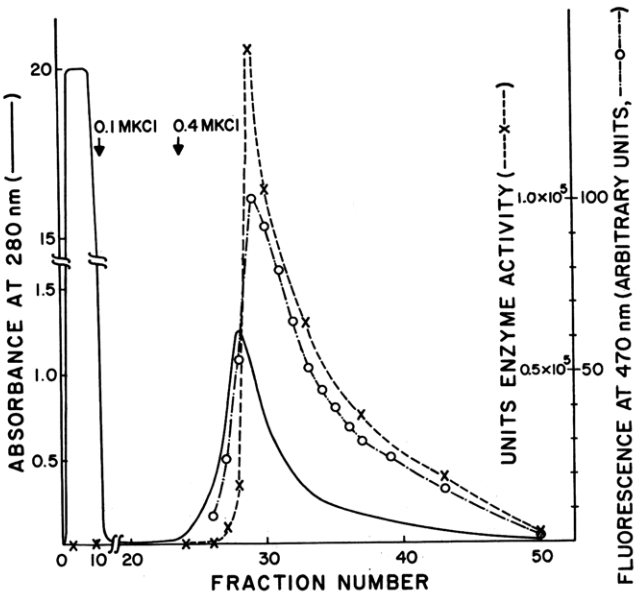

FIGURE 1: Affinity chromatography of DNA photolyase on uv-irradiated DNA noncovalently linked to cellulose. The pooled active fractions from the phosphocellulose column after concentration by ammonium sulfate and dialysis were applied to a column (1.8 × 17 cm) that had been equilibrated with AC buffer. After washing the column with 50 ml of the same buffer, the enzyme was eluted by increasing the KCl concentration in the buffer to 0.4 M. Fluorescence was excited by 380-nm light.

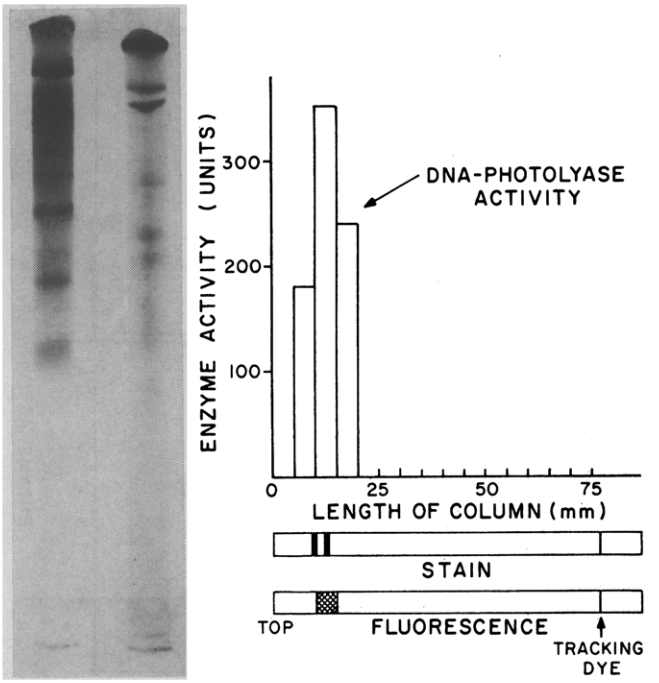

FIGURE 2: (A, left) Protein distribution following electrophoresis on polyacrylamide gels of pooled eluates containing DNA photolyase after chromatography on phosphocellulose (left) and after affinity (right) chromatography. (B, right) Examination of unstained gels for photolyase activity and fluorescence (observation made on the gel) after an electrophoretic run of pooled active fractions from affinity chromatography.

mixed with its counterpart, and dialyzed overnight against AC buffer.

CHROMATOGRAPHY ON IRRADIATED DNA-CELLULOSE. This step was carried out under yellow light to avoid dissociation of the enzyme–substrate complex. The dialyzed enzyme solution from the previous step was passed through an irradiated-DNA-cellulose column (1.8 × 17 cm) that had been equilibrated with AC buffer. The column was washed with 50 ml of the same buffer and then eluted with AC buffer containing 0.4 M KCl. The eluates were monitored for enzyme activity and for protein concentration, the latter by measurement of absorbance at 280 nm (Figure 1). Active fractions when excited by 380-nm light fluoresced at 470 nm and therefore all the eluates were also monitored for this emission (Figure 1). The active fractions 29–42 when pooled and concentrated (collodion bag) yielded about 2.8 ml of clear solution with a specific activity of 647,000. The purification procedures summarized in Table I show that the overall yield of activity was 8.3% and the enzyme was about 70,000-fold purified over that in the cell-free extract.

Some Properties of Purified Enzyme. ELECTROPHORESIS ON POLYACRYLAMIDE GEL. Figure 2A shows the electrophoretic pattern of protein distribution on polyacrylamide gel of pooled active fractions that were run after enzyme purification by phosphocellulose chromatography and affinity chromatography. In active fractions from the latter step, there were two major and several minor protein bands. Figure 2B shows that photolyase activity and fluorescence overlapped the two major protein bands.[4]

[4] In one run, after affinity chromatography there was sufficient protein remaining to permit an additional purification by chromatography on unirradiated DNA bound to cellulose. The material obtained yielded a single protein band in the same region where the two major bands usually appeared.

MOLECULAR WEIGHT BY GEL FILTRATION. When the purified enzyme was chromatographed on Sephadex G-100 with buffer containing 0.4 M KCl, the elution pattern (Figure 3A) showed coincidence of photolyase activity, absorbance at 280 nm, and fluorescence at 470 nm. However, when the chromatography was performed with KCl-free buffer (Figure 3B), while there was still overlapping of eluted activity, fluorescence, and protein at the same position, the recovery of each was only 19, 17, and 11%, respectively. By continuing the elution of the column with buffer containing 0.4 M KCl, more protein and fluorescence could be accounted for, but the elution patterns did not coincide and none of these fractions had photolyase activity (Figure 3B). From these observations it seemed that the molecular weight of the enzyme could be determined by gel filtration using the method of Andrews (1967) provided the standard proteins and the

TABLE I: Summary of Purification Steps.

Purifcn Step	Total Protein (mg)	Total Act.[a]	Sp Act.[b]	Yield (%)
Cell-free crude extract	760,500	6900	9.2	100.0
Ammonium sulfate 33–50%	97,800	5200	53.2	76
Phosphocellulose	2,208	3490	1,580	51
Uv-irradiated DNA cellulose	0.80	574	647,000	8.3

[a] Units × 10^{-3}. [b] Units/mg of protein.

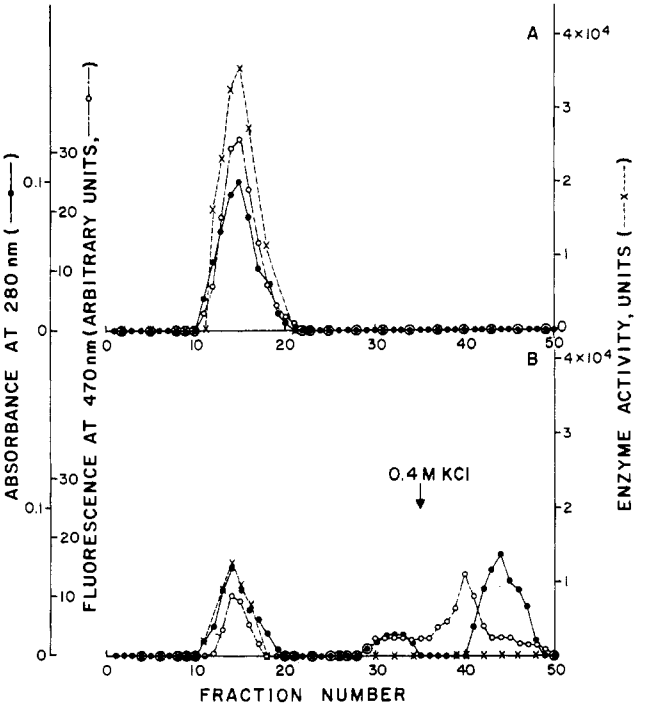

FIGURE 3: Gel filtration of DNA photolyase (purified by affinity chromatography) on a Sephadex G-100 column (1.2 × 30 cm). (A) The column was equilibrated with 0.02 M phosphate buffer (pH 7.5) containing 0.4 mM EDTA, 10 mM 2-mercaptoethanol, and 0.4 M KCl. The elution was performed with the same buffer and 1.5-ml fractions were collected. (B) The column was equilibrated with 0.02 M phosphate buffer (pH 7.5) containing 0.4 mM EDTA and 0.4 mM 2-mercaptoethanol. The elution was performed first with KCl-free buffer, then with 0.4 M KCl in the buffer, and 1.5-ml fractions were collected. (●) Absorbance at 280 nm; (×) DNA photolyase activity; (○) fluorescence at 470 nm.

photolyase were eluted from the Sephadex column with buffer containing 0.4 M salt. This expectation was realized and a molecular weight of 53,000 was calculated from the curve shown in Figure 4. Sutherland and Chamberlin (1971),

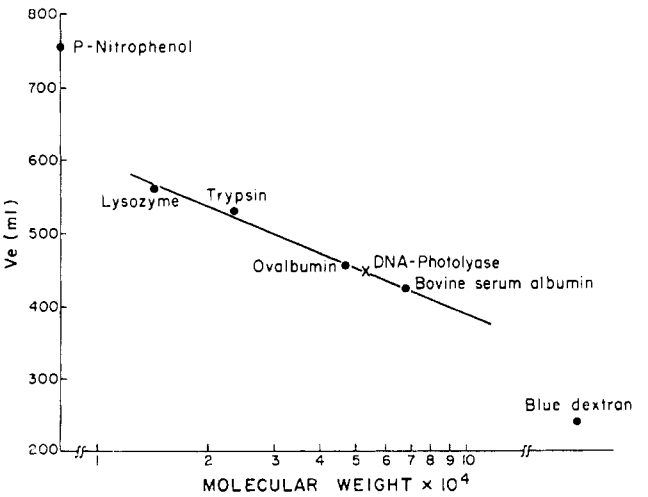

FIGURE 4: Logarithm of protein molecular weights as a function of elution volume (V_e). Chromatography was performed on a Sephadex G-200 column (2.5 × 128 cm) with 0.05 M phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.4 M KCl. Pooled active fractions from affinity chromatography were used for the experiment.

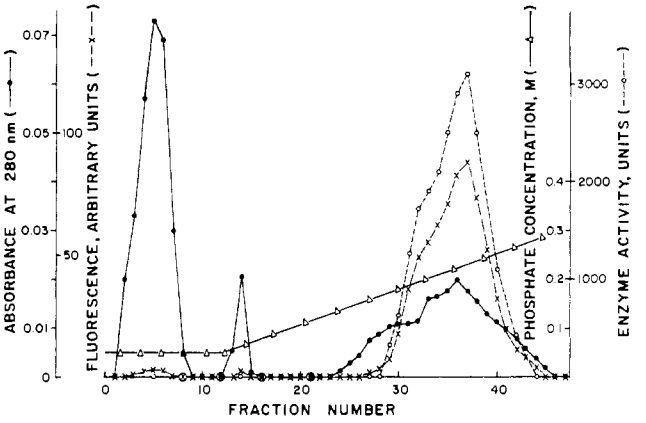

FIGURE 5: Chromatography on hydroxylapatite. The photolyase fraction from affinity chromatography was applied to an hydroxylapatite column (0.8 × 5 cm) and the column was run with a linear phosphate gradient established between 50 ml each of 0.05 and 0.3 M phosphate buffer (pH 7.5). (○) Enzyme activity; (●) absorbance at 280 nm; (×) fluorescence at 470 nm (excitation 380 nm).

making use of sodium dodecyl sulfate–polyacrylamide electrophoresis to determine molecular weights, obtained a value of 45,000 for the *E. coli* enzyme.

ASSOCIATION OF FLUORESCENT CHROMOPHORE WITH DNA PHOTOLYASE ACTIVITY. The overlapping of fluorescence and photolyase activity observed when the purified enzyme was examined by electrophoresis on polyacrylamide gel (Figure 2B) or was chromatographed on Sephadex G-100 (Figure 3A) suggested the association with the active enzyme of a fluorescent substance. This idea received support from the results of the experiment shown in Figure 5. After purification by affinity chromatography the photolyase was chromatographed on an hydroxylapatite column and only eluates with photolyase activity fluoresced.

SPECTRAL PROPERTIES OF ENZYME PREPARATION AFTER AFFINITY CHROMATOGRAPHY. The absorption spectrum of the enzyme shown in Figure 6 reveals protein absorption at 273 nm and a weak broad band between 350 and 420 nm peaking at about 380 nm. The excitation and fluorescence spectra are depicted in Figure 7; sharp maxima appear at 385 nm in the former and at 485–490 nm in the latter.

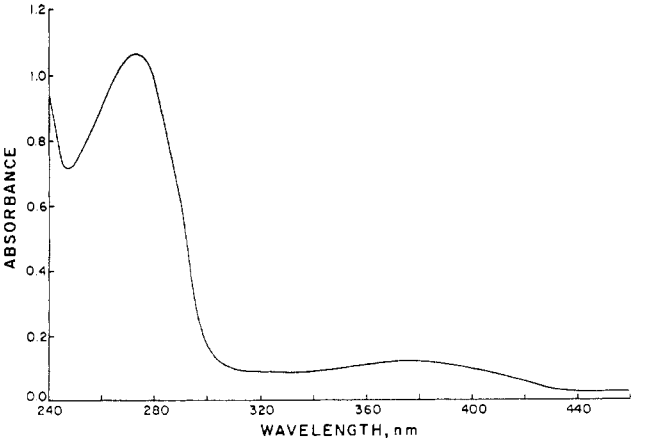

FIGURE 6: Absorption spectrum of DNA photolyase after affinity chromatography. Fractions 29–42 from affinity chromatography were pooled and concentrated to obtain the sample.

TABLE II: Reversible Inactivation of DNA Photolyase by Ferricyanide.[a]

Inactivation (Ferricyanide)	Reactivation (2-Mercaptoethanol)	Act.
−	−	100
+	−	8.5
−	+	234
+	+	220

[a] Inactivation: to 0.2 ml of enzyme solution in 0.05 M phosphate buffer (pH 7.5) containing 0.1% albumin was added 0.2 ml of 1 mM ferricyanide, and the mixture was kept at 0° for 60 min before assay. Reactivation: the inactivated enzyme solution was diluted ten times with 0.05 M phosphate buffer containing 0.1% albumin. To 1.0 ml there was added 1.0 ml of 6 mM 2-mercaptoethanol and the mixture was held at 0° for 60 min before assay.

REVERSIBLE INACTIVATION OF THE ENZYME WITH FERRICYANIDE. The data in Table II reveal that treatment of the enzyme with ferricyanide resulted in almost complete loss of activity, but the latter was completely restored by subsequent incubation with 2-mercaptoethanol.

Discussion

DNA photoreactivation *in vitro* is assayed by the increased transformation capacity imparted to far-uv-irradiated *H. influenzae* transforming DNA when it is illuminated in the presence of DNA photolyase. Action spectra of this process for the yeast enzyme exhibit either two maxima at 355 and 385 nm (Setlow and Boling, 1963) or a single broad maximum in the 355- to 385-nm region peaking at about 366 nm (Harm and Rupert, 1970). Since action spectra generally mimic the absorption spectra of those substances responsible for the actions being measured (McLaren and Shugar, 1964; Jagger, 1967), it is reasonable to expect that there is associated with the yeast photolyase a prosthetic group or cofactor absorbing between 355 and 385 nm. The yeast enzyme after purification by affinity chromatography, although not homogeneous, does exhibit such spectral properties. It absorbs weakly between 350 and 420 nm with a peak at 380 nm. This absorption is intensified in the excitation spectrum of the enzyme which shows a maximum at 385 nm.[5] When the purified photolyase is chromatographed on either Sephadex G-100 or hydroxylapatite, only active fractions fluoresce. If the chromatography on Sephadex G-100 is carried out with salt-free buffer, there is a partial dissociation of fluorescent material and protein, and the latter is devoid of photolyase activity.

[5] The difference in the maxima at 385 and 366 nm of the excitation spectrum of the chromophore and the photoreactivation action spectrum (Harm and Rupert, 1970) may result from either one or a combination of several factors. Thus, both measurements were made with buffers differing in their composition and, perhaps, pH. The action spectrum is drawn through points, whereas the excitation spectrum is continuously recorded. The specific activity of the enzyme preparation used in the action spectrum measurement was about 1×10^4 times less than that used to measure the excitation spectrum. McLaren and Shugar (1964) point out other reasons why action spectra and absorption spectra frequently do not coincide.

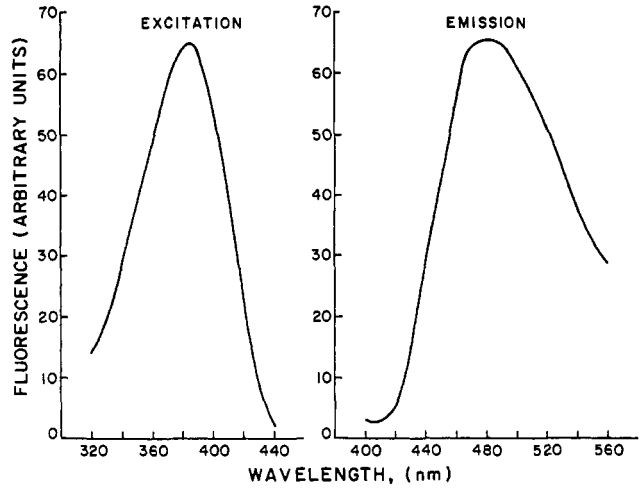

FIGURE 7: Excitation and fluorescence emission spectra (both corrected) of DNA photolyase in AC buffer containing 0.4 M KCl. These spectra were measured on the same enzyme preparation as that used to obtain Figure 6. For the excitation spectrum emission at 470 nm was recorded. The emission spectrum was excited with 380-nm radiation.

Altogether these findings favor the view that the fluorescent material associated with the yeast enzyme after its purification by affinity chromatography is involved in DNA photoreactivation; but they do not prove it. Additional evidence for this view stems from the observations that partially purified photolyase from the blue-green alga *A. nidulans* exhibits an absorption maximum at 418 nm and one at 420 nm in its excitation spectrum (Minato and Werbin, 1971). Both maxima are close to the one at 436 nm in the photoreactivation action spectrum of the algal enzyme (Saito and Werbin, 1970). The fluorescence spectrum of the algal enzyme peaks at 470 nm. Hence, there is also associated with the algal enzyme, purified by affinity chromatography, fluorescent material with spectral properties expected for the chromophore involved in DNA photoreactivation.

Since the DNA photolyase is known to form a stable complex with its substrate (Rupert, 1962), the possibility exists that the maxima in the photoreactivation action spectra at 355 and 385 nm (Setlow and Boling, 1963) or at 366 nm (Harm and Rupert, 1970) can be attributed to absorption by this complex rather than to absorption by the fluorescent substance found with the partly purified yeast enzyme. However, none of the amino acids commonly found in proteins absorb above 310 nm and cyclobutane dipyrimidines in DNA, the substrate, absorb wavelengths considerably below 300 nm. Hence, there would have to be a rearrangement of bonds in the complex or the formation of a charge-transfer complex (Kosower, 1962; Cilento and Tedeschi, 1961) between enzyme and substrate were the formation of the enzyme–substrate complex to account for the maximum(a) in the action spectrum.

Maxima in *in vitro* DNA photoreactivation spectra generally fall in three regions (Jagger *et al.*, 1970): 355–385 nm, *E. coli* (Setlow, 1966), and yeast (Setlow and Boling, 1963; Harm and Rupert, 1970); 400 nm, *Neurospora crassa* (Terry and Setlow, 1967) and pinto bean sprouts (Saito and Werbin, 1969); 435 nm, *A. nidulans* (Saito and Werbin, 1970). If these maxima reflect the formation of enzyme–substrate complexes, then these complexes either have different structures, or if their structures are the same, they must be present in very

different protein environments—the enzyme binding sites must vary markedly.

While these considerations do not rule out the possibility that the maxima in DNA photoreactivation action spectra arise from absorption of an enyzme–substrate complex or charge transfer complex, they do make such explanations unlikely. The data in this and another communication (Minato and Werbin, 1971) support the alternative explanation that the various action spectra can be accounted for by the association with the several photolyases of different fluorescent substances that photosensitize the cleavage of cyclobutane dipyrimidines. If these substances have a common ring structure, then different substituents on the ring could alter their absorption spectra, and thereby, the action spectra. While this idea is still not proven, the finding that the DNA photolyases from yeast and an alga are associated with fluorescent material having different excitation and absorption spectra lends credence to it. The reversible inactivation by ferricyanide of both the yeast and algal DNA photolyases suggests the liability of the same bonds in both enzymes, but we have not determined whether the protein or the chromophore is the target.

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