Purification of a Blue-Green Algal Deoxyribonucleic Acid Photoreactivating Enzyme. An Enzyme Requiring Light As a Physical Cofactor to Perform Its Catalytic Function*

Narimasa Saito and Harold Werbin

ABSTRACT: A DNA-photoreactivating enzyme that uses light as a cofactor to perform its catalytic activity, cleavage of the two cyclobutane carbon–carbon bonds of pyrimidine dimers in far-ultraviolet-treated DNA, has been purified 3760-fold from cell-free sonicates of the blue-green alga *Anacystis nidulans*. A minute band at 418 nm was observed in the absorption spectrum of the enzyme. Using gel filtration, a molecular weight of 93,000 was estimated for the enzyme, and during the early stages of the purification active enzyme aggregates were found. The photoreactivation action spectrum of the enzyme exhibited a sharp peak around 4358 Å.

Oxidizing agents that generally convert protein thiol groups into disulfide linkages inactivated the enzyme but several organic thiols and inorganic reducing agents restored the activity completely. Photoreactivation in the presence of these reducing agents alone was markedly enhanced. Substrate did not protect the enzyme from inactivation by ferricyanide indicating that the groups oxidized were not part of the active center.

Photoreactivation may be broadly defined as the restoration of activity to biological material by wavelengths of light longer than those that caused the inactivation. Generally, far-ultraviolet (germicidal) irradiation is used to inactivate and near-ultraviolet or visible illumination to photoreactivate. Rupert *et al.* (1958) and Rupert (1960) discovered a DNA-photoreactivating enzyme in cell-free extracts of *Escherichia coli* and yeast which they noted could account for many *in vivo* photoreactivating phenomena. The enzyme is unique in being the only one presently known having an absolute requirement for near-ultraviolet or visible light to perform its catalytic function–monomerization of cyclobutane pyrimidine dimers in far-ultraviolet-irradiated DNA. The only other enzyme in which light is known to participate in the catalytic step is ribulose diphosphate carboxylase (Wildner and Criddle, 1969).

We found the enzyme in extracts of the photoautotrophic organism *Plectonema boryanum* and, several varieties of bean sprouts (Werbin and Rupert, 1968; Saito and Werbin, 1969a). These observations and the finding that near-ultraviolet light reduces the number of far-ultraviolet-induced thymine dimers in plant DNA (Trosko and Mansour, 1969) buttress the notion that some *in vivo* photoreactivating phenomena described for plants, such as the prevention of leaf bronzing, restoration of leaf capacity to support viral infection (Chessin, 1958; Cline and Salisbury, 1966), and reduction of potential mutations induced in maize pollen by far-ultraviolet light (Ikenaga and Mabuchi, 1966; Fujii, 1969), may be attributable to this enzyme.

While the enzyme was not particularly stable in cell-free extracts of *P. boryanum* and bean sprouts, we could prepare a very stable preparation from extracts of the unicellular blue-green alga *Anacystis nidulans*. The procedures used to enhance enzyme activity 3760-fold and some of the properties of the purified preparation are described in this report.

Methods and Materials

For Enzyme Purification. ENZYME BIOASSAY. The method is based on the increased capacity of ultraviolet-irradiated *Hemophilus influenzae* transforming DNA, carrying a streptomycin marker, to transform competent cells after its incubation with active enzyme extract in visible light (Rupert *et al.*, 1958; Rupert, 1960). Transforming DNA was prepared by the method of Berns and Thomas (1965) and 2.85 μg/ml in 0.15 M sodium chloride was irradiated with about 3360 ergs/mm^2 of 254-nm light from a germicidal lamp until 1% of its activity remained. Photoreactivation was carried out in 1-ml depressions of a plastic container that floated in a 37° water bath. In the standard assay 0.15 ml of irradiated DNA (0.1 μg/ml) was added to the same volume of enzyme extract in 0.05 M Tris-borate buffer containing 0.1 mM EDTA (pH 7.2). This buffer, which was routinely used in these studies, will be referred to henceforth as TB buffer. The mixture was held in darkness for 5 min at 37° before it was illuminated for 20 min at the same temperature with white fluorescent light. Then 0.2 ml of this solution was used to transform competent cells. The proper controls for the assay have been described (Saito and Werbin, 1969a).

Units of enzyme activity are expressed as the ratio obtained by dividing the number of light transformants less the dark transformants by the latter (Muhammed, 1966; Saito and Werbin, 1969a). Specific activity was calculated by dividing units of enzyme activity by mg of enzyme protein per ml of photoreactivating mixture. Figure 1 shows the activity as a function of the concentration of purified enzyme. In determining activity only the linear portion of the curve is useful,

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hence all assays are carried out with several protein dilutions in order to ensure that the observed activity is linearly related to the enzyme concentration. After the calcium phosphate gel purification step, all dilutions were made with TB buffer containing 0.1% bovine serum albumin.

PROTEIN AND DNA DETERMINATIONS. Protein and DNA were determined by the methods of Lowry *et al.* (1951) and Burton (1956), respectively.

CONCENTRATION OF ENZYME SOLUTIONS. Large volumes were concentrated in a Microconcentrator purchased from Biomed Instruments, Chicago, Ill., while for smaller volumes, a Carl Schleicher & Schuell glass suction apparatus with a collodion bag was employed.

DISC ELECTROPHORESIS. Electrophoresis on polyacrylamide gel was performed according to Davis (1964) using the Canalco Model 12 apparatus. The acrylamide concentration was 7%, and the pH of the Tris-glycine buffer was 8.3. The time required for a run at 300 V and at a current of 4 mA/column (after the tracking dye reached the stacking column) was 6.5 hr.

CULTIVATION OF ALGAL CELLS. The blue-green alga *A. nidulans*, strain No. 625 from the Indiana Culture Collection, was used routinely. The enzyme content was lower in two other strains that we obtained from Dr. Chase Van Baalen and Dr. Mary M. Allen. Algae were cultivated in 20-l. bottles in Kratz and Myers (1954) medium C at room temperature while 1% carbon dioxide in air was bubbled through the culture medium. Illumination was provided externally by four 15-W cool white fluorescent lamps. The cell volume was approximately 1 mm^3/ml when the cells were harvested.

CHROMATOGRAPHIC ADSORBENTS. Calcium phosphate gel was prepared according to the method of Keilin and Hartree (1938). Hydroxylapatite (Bio-Gel HTP powder) was purchased from Bio-Rad Laboratories, Richmond, Calif. Sephadex G-100 and Sephadex G-200 were products of Pharmacia Fine Chemicals, Inc. Other ion-exchange celluloses that were used were all Whatman Chromedia products.

REAGENTS REACTING WITH SH GROUPS. Sodium tetrathionate was purchased from K & K Laboratories, Plainview, N. Y. Iodoacetic acid, iodoacetamide, *o*-iodosobenzoic acid, phenylmercuric acetate, and porphyrindin were from Sigma Chemical Co., St. Louis, Mo.

ENZYMES AND PROTEINS. Lysozyme, deoxyribonuclease, and yeast alcohol dehydrogenase were purchased from Worthington Biochemical Corp., N. J. Horse apoferritin, bovine hemoglobin, and human γ-globulin were obtained from Mann Research Laboratories, Inc., New York.

For Determining Whether the Algal Enzyme Catalyzes the Light-Triggered Monomerization of Pyrimidine Dimers in Ultraviolet-Irradiated DNA (Dr. M. H. Patrick). IRRADIATED DNA. Purified *Escherichia coli* DNA 15T$^-$ ([^{3}H]thymine labeled) with a specific activity of 2×10^5 dpm/μg was dissolved in TB buffer at a concentration of 20 μg/ml. Three milliliters were irradiated (253.7 nm) in a Petri dish while stirring until the DNA received a dose of 15,000 ergs/mm^2.

PHOTOREACTIVATION. Two 1-ml samples of irradiated DNA were each mixed with 0.1 ml of algal enzyme, 2.14 mg of protein/ml, that had been purified through the first hydroxylapatite chromatography. After preincubation for 5 min at 37°, one sample was held in darkness while the other was illuminated for 1 hr at 37°.

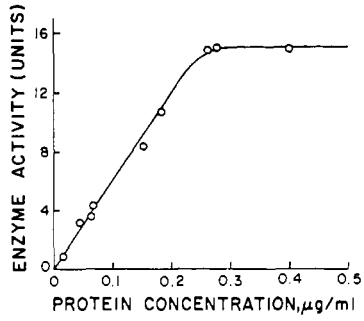

FIGURE 1: Enzyme activity as a function of enzyme protein concentration in the photoreactivation mixture. The enzyme was from the third hydroxylapatite chromatography and was diluted with 0.05 M TB buffer (pH 7.2) containing 0.1% bovine serum albumin and 0.1 mM EDTA.

PYRIMIDINE DIMER ANALYSIS. The samples exposed to light and darkness were dialyzed overnight against distilled water and were then evaporated to dryness. After addition of 0.5 ml of trifluoroacetic acid to each sample, they were hydrolyzed 90 min at 165° in sealed tubes. When cool, the samples were spotted on 3-cm strips of Whatman No. 1 chromatography paper. Chromatography was carried out for 20 hr in 1-butanol–acetic acid–water (80:12:30). The dried strips were cut into 1-cm segments and were left 1 hr at room temperature in a scintillation vial containing 0.5 ml of water. Then 5 ml of scintillation solution (8 g of 2-(4-*t*-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole in 150 ml of Bio-Solv diluted to 1 l. with toluene) was added and the radioactivity was measured on a Nuclear-Chicago Mark II scintillation spectrometer with an ^{3}H counting efficiency of about 40%.

EXTENT OF ACID SOLUBILIZATION OF TRITIUM IN IRRADIATED DNA AFTER PHOTOREACTIVATION WITH ALGAL ENZYME. The irradiation and photoreactivation conditions were the same as those described above. Then to each sample, calf thymus DNA and cold trichloroacetic acid were added to yield respective concentrations of 0.5 mg/ml and 5%. The samples were iced for 5 min and filtered through Millipore filters, 0.45 μ. The supernatants were saved and the filters were washed with cold trichloroacetic acid. The filters were dried and counted in 10 ml of Bray's solution with an efficiency of 23% for ^{3}H while 1-ml amounts of the supernatant were counted in the butylphenylbiphenyloxadiazole–toluene mixture. The quenching of the ^{3}H counting by trichloroacetic acid brought the efficiency down to about the same level as that observed in the counting of the Millipore filters.

Experimental Details and Results

Enzyme Purification. PREPARATION OF CRUDE EXTRACT. Five-day-old cells were harvested by centrifugation in a refrigerated Sharples steam-driven continuous centrifuge and washed by centrifugation twice with TB buffer. Washed cells (950 g) were sonicated below 5° in 3 l. of TB buffer, and the mixture was centrifuged at 6000 rpm for 8 min. The supernatant was saved and, after addition of buffer, the residue was again sonicated and the mixture was centrifuged at 6000 rpm. The sonication and centrifugation were repeated several times. The pooled supernatants were centrifuged at 30,000 rpm yielding about 7.5 l. of clear blue-green solution

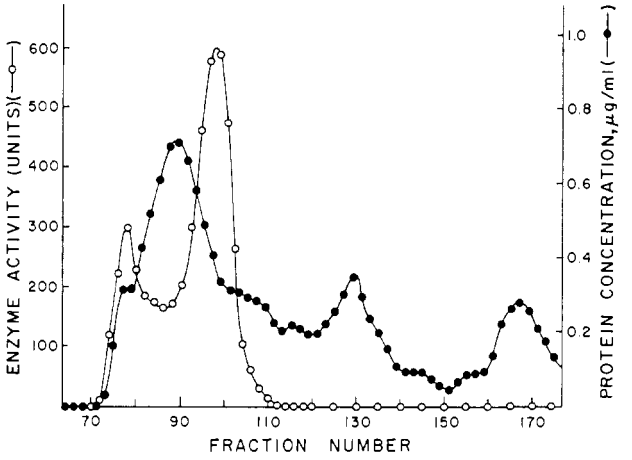

FIGURE 2: Chromatography of ammonium sulfate fraction on a 7.6 × 145 cm Sephadex G-100 column. The column was equilibrated with 0.05 M TB buffer (pH 7.2) containing 0.1 mM EDTA. The elution, at a rate of 80 ml/hr, was performed with the same buffer, and 25-ml fractions were collected. (○) Enzyme activity; (●) protein concentration.

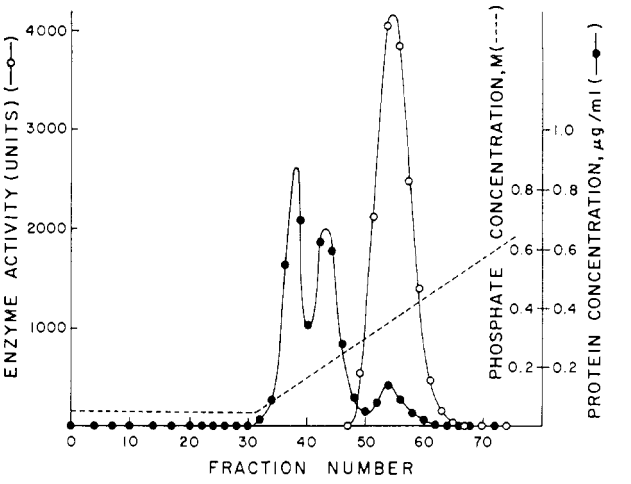

FIGURE 4: Chromatography of active fractions 46–70 from the ECTEOLA-cellulose column on an hydroxylapatite column (2.5 × 12 cm) after equilibration with 0.05 M phosphate buffer (pH 7.0). After washing the column with 100 ml of this buffer, a linear gradient was established between 140 ml each of 0.05 and 0.8 M buffer. (○) Enzyme activity; (●) protein concentration.

with a protein concentration of 8.8 mg/ml and a specific activity of 15.3.

AMMONIUM SULFATE FRACTIONATION. To the clarified supernatant, 1.86 kg of powdered ammonium sulfate was added slowly with stirring to bring the saturation to 43% and the stirring was continued for 20 min. During this operation which was carried out in the laboratory, the mixture was chilled in a large ice bucket. The precipitate was removed by centrifugation at 9000 rpm for 20 min and discarded. Then the supernatant fraction was brought to 85% saturation by adding to it 2.13 kg of powdered ammonium sulfate. The protein precipitating after 20 min, which contained most of the photoreactivating activity, was collected by centrifugation and dissolved in about 100 ml of TB buffer. The solution was dialyzed overnight against the same buffer yielding a dialysate with a protein concentration of 28 mg/ml, which though highly pigmented, had a specific activity of 186. The purification was 12.2-fold over the crude extract and the yield of activity was 76.1%. In its precipitation with ammonium sulfate between 43 and 85% saturation, the *A. nidulans* enzyme behaved like the one in extracts of bean sprouts.

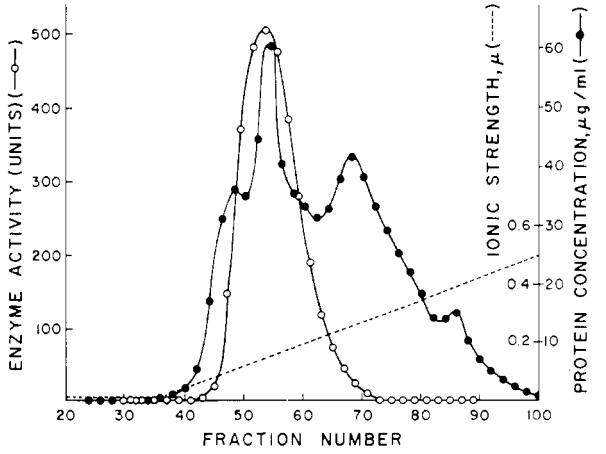

FIGURE 3: Chromatography of active fractions from Sephadex G-100 column on a 2.5 × 35 cm ECTEOLA-cellulose column. The flow rate was 60 ml/hr and for elution a linear gradient of 350 ml each of 0 and 0.6 M KCl in 0.01 M TB buffer (pH 7.2) was used. (○) Enzyme activity; (●) protein concentration.

SEPHADEX G-100 COLUMN CHROMATOGRAPHY. About 1.3 g of the protein in 47 ml from the previous step was fractionated on a large column (7.6 × 145 cm) of Sephadex G-100 after the column had been equilibrated by passing several liters of TB buffer through it. The eluates that were collected in 25-ml volumes contained many of the algal pigments that had separated during the run. Figure 2 shows that enzyme activity appeared in two peaks, of which the first one was excluded from the column. Green active fractions (74–103) from three runs were concentrated to 150 ml by ultrafiltration in a collodion bag or with a Microconcentrator, and were then dialyzed for 24 hr against 0.01 M phosphate buffer (pH 7.0). This fraction had 65% of the original activity and a specific activity of 483.

CALCIUM PHOSPHATE GEL TREATMENT. The 150 ml of enzyme solution with a protein concentration of 8.5 mg/ml was stirred with 15 g (dry weight) of calcium phosphate gel. After centrifugation, the gel was washed by centrifugation with 150 ml each of 0.05 and 0.15 M phosphate buffer (pH 7.0). The enzyme was then extracted twice with 150 ml of 0.4 M phosphate buffer (pH 7.0). The pooled active extracts had a specific activity of 3720 after dialysis against 0.01 M TB buffer.

COLUMN CHROMATOGRAPHY ON ECTEOLA-CELLULOSE. The dialyzed enzyme solution (60 ml) was charged on an ECTEOLA-cellulose column (2.5 × 35 cm). The column was washed with 200 ml of 0.01 M TB buffer and a linear gradient was established in the same buffer between 0 and 0.6 M KCl. Fractions of 10 ml were collected and the enzyme was eluted between 0.1 and 0.3 M KCl (Figure 3). Active

TABLE I: Summary of Purification Steps.

Purification Step	Total Protein (mg)	Total Act. (units × 10^{-4})	Sp Act. (units/mg of protein)	Yield (%)	Purifcn (X)
1. Crude sonicate	62,100	95.0	15.3	100.0	1.0
2. Ammonium sulfate, 43–85%	3,890	72.4	186	76.1	12.2
3. Sephadex G-100	1,280	61.8	483	65.3	31.6
4. Calcium phosphate gel	124	46.1	3,720	48.6	243
5. ECTEOLA-cellulose	56	29.2	5,210	30.7	341
6. Hydroxylapatite I	5	14.4	29,120	15.2	1905
7. Hydroxylapatite II	2.7	13.0	48,300	13.7	3160
8. Hydroxylapatite III	1.6	9.1	57,500	9.5	3760

fractions 46–70 from five runs were concentrated to about 40 ml and this solution after dialysis against 0.01 M phosphate buffer (pH 7.0) had a specific activity of 5210.

HYDROXYLAPATITE COLUMN CHROMATOGRAPHY. The enzyme was now chromatographed repeatedly on hydroxylapatite columns by using a linear phosphate gradient for elution. The concentrated and dialyzed enzyme solution from the previous step was placed on a 2.5 × 12 cm column that had been equilibrated with 0.05 M phosphate buffer (pH 7.0). After washing the column with 100 ml of 0.05 M phosphate buffer (pH 7.0) a linear gradient was established between 140 ml each of 0.05 and 0.8 M phosphate buffer (pH 7.0). Five milliliter fractions were collected. The enzyme was eluted at a phosphate concentration between 0.28 and 0.45 M which is shown in Figure 4. The pooled active fractions 47–65 were concentrated to 10 ml by dialysis against a saturated ammonium sulfate solution and the resulting solution was dialyzed against 0.05 M phosphate buffer. It was applied to a small hydroxylapatite column (0.8 × 10 cm) previously equilibrated with 0.05 M phosphate buffer (pH 7.0). The column was washed with 20 ml of buffer and a linear gradient was established between 30 ml each of 0.05 and 0.8 M phosphate buffer. The enzyme activity was eluted at the same phosphate concentration as it had been in the first run. Active fractions were pooled and dialyzed overnight against 0.05 M phosphate buffer with several changes of buffer.

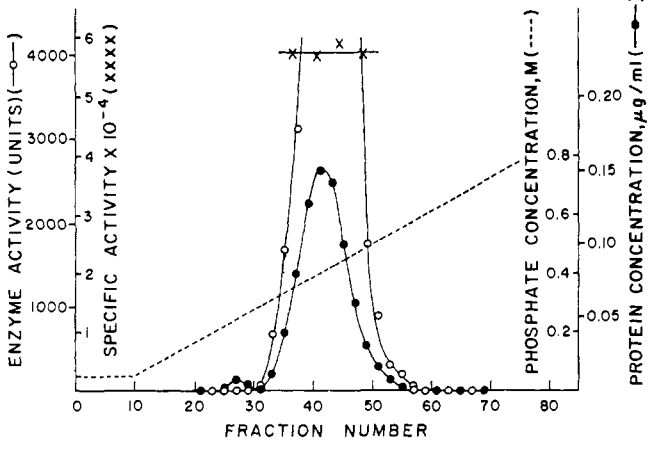

FIGURE 5: The enzyme solution from the second hydroxylapatite chromatography was applied to a hydroxylapatite column (0.8 × 10 cm) and a linear phosphate gradient was established between 45 ml each of 0.05 and 1 M phosphate buffer (pH 7.0). (○) Enzyme activity; (●) protein concentration.

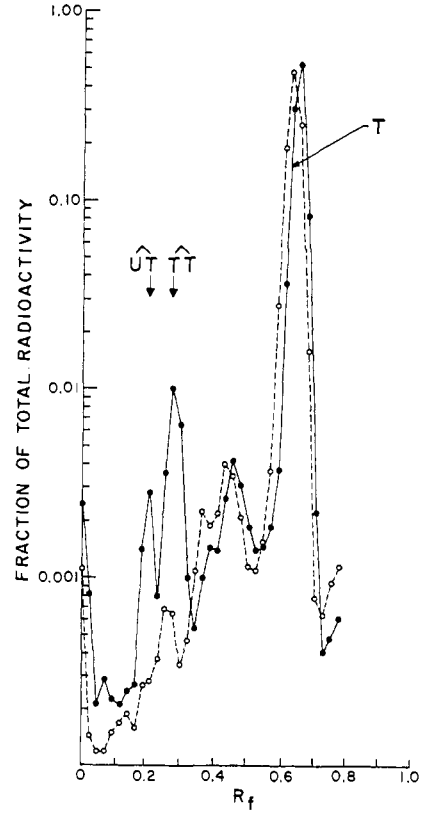

FIGURE 6: Fraction of total ^3H in thymine-derived photoproducts from ultraviolet-irradiated (254 nm) DNA held in darkness or illuminated with white light in the presence of algal DNA-photoreactivating enzyme (●–●) without photoreactivation for 1 hr at 37°, (○–○) with photoreactivation for 1 hr at 37°. ÛT and T̂T are cyclobutane-type dimers. U = uracil, T = thymine. U originates from cytosine which is deaminated during DNA hydrolysis.

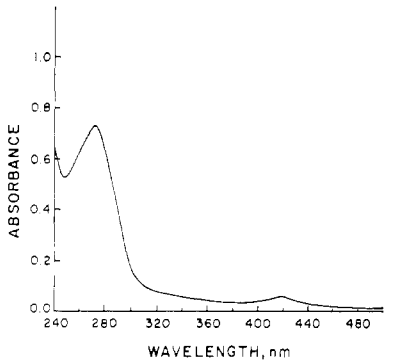

FIGURE 7: Ultraviolet absorption spectrum of the purified enzyme. Active fractions from the third hydroxylapatite chromatography were concentrated to 2 ml by dialysis first in saturated ammonium sulfate solution and then in 0.05 M TB buffer (pH 7.2.) The enzyme solution was centrifuged at 30,000*g* before analysis.

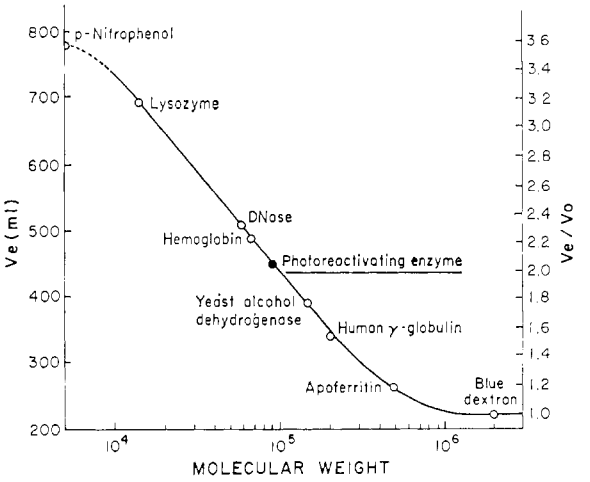

FIGURE 8: Logarithm of protein molecular weights as a function of the elution volume (V_e) and the ratios of the elution volume to the void volume (V_e/V_0). Chromatography was performed on a Sephadex G-200 column (2.5 × 126.5 cm) with 0.05 M TB buffer (pH 7.2) after the column had been equilibrated with the same buffer.

The enzyme was charged on a third hydroxylapatite column (0.8 × 10 cm) and a phosphate gradient between 45 ml each of 0.05 and 1 M phosphate buffer established. Figure 5 shows that in the peak fractions of eluted enzyme there was a coincidence of protein concentration and photoreactivating activity, and a constancy of specific activity, 57,500.

The purification steps, summarized in Table I, disclose a 3760-fold purification over the original extract with a 10% recovery of activity. The amount of protein available for further manipulation amounted to only 1.5 mg. Electrophoresis on acrylamide gel revealed the presence of several protein bands in this material. An attempt to elute active enzyme from the acrylamide gel after electrophoresis failed. However, when we used a modified procedure (Brown, 1969) in which the buffer pH was 7.5, active enzyme that had separated from three or four protein bands could be eluted from the gel after a 17-hr run.

Some Properties of the Purified Enzyme. CATALYTIC FUNCTION. If the algal enzyme acts catalytically in the same way as the yeast DNA-photoreactivating enzyme, it should be possible to observe a reduction in the dimer content of irradiated DNA after photoreactivation. Figure 6 shows the fraction of total DNA radioactivity attributable to uracil–thymine ($\widehat{UT}$) and thymine–thymine ($\widehat{TT}$) dimers before and after photoreactivation. The two dimer peaks are practically eliminated (97–98%) after photoreactivation. Similar results have been obtained by Dr. M. H. Patrick with the yeast enzyme and "black light" illumination.

The data in Table II show there was no difference in the extent of acid solubilization of [^{3}H]DNA when comparison

TABLE II: Extent of [^{3}H]DNA Acid Solubilization after Photoreactivation by Algal Enzyme.

Experimental Conditions	Filter (cpm)	Supernatant (cpm)	^{3}H in Supernatant (%)
Unirradiated DNA + enzyme, darkness	156,000	2200	1.4
Unirradiated DNA + enzyme, light	127,000	2200	1.7
Irradiated DNA + enzyme, darkness	128,000	3000	2.3
Irradiated DNA + enzyme, light	164,000	2000	1.2

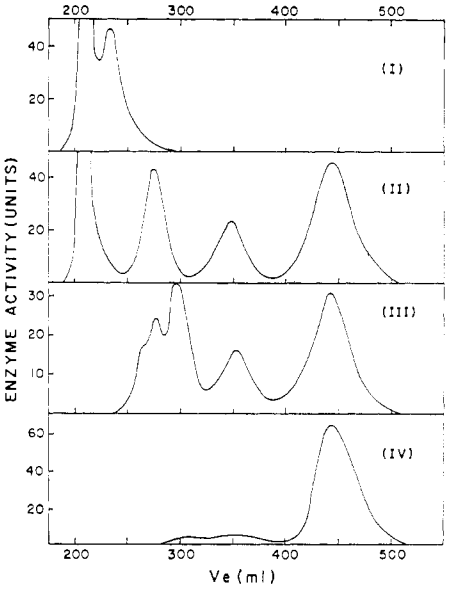

FIGURE 9: Enzyme activity as a function of the elution volumes of enzyme fractions at various stages of purification. The fractions were run on the same Sephadex G-200 column that was used to obtain the data in Figure 8. Purification step: (I) ammonium sulfate fraction (II) calcium phosphate fraction (III) ECTEOLA-cellulose fraction (IV) third hydroxylapatite fraction. The peak at the elution volume of 443 ml represents unaggregated enzyme having a molecular weight of 93,000.

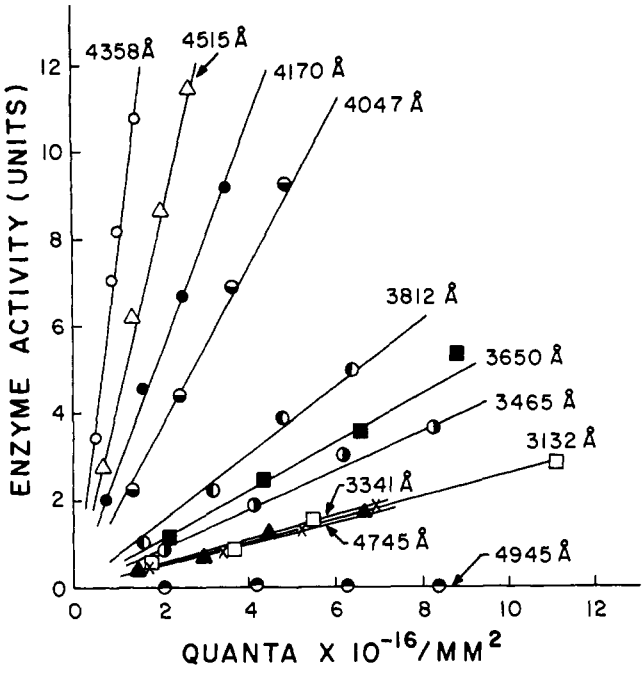

FIGURE 10: DNA-photoreactivating activity as a function of the incident light quanta at various wavelengths. Enough enzyme solution from the first hydroxylapatite chromatography diluted in 0.05 M TB buffer containing 0.1% bovine serum albumin and irradiated substrate were mixed to provide one solution for all the assays and it was held at 0° until used. After 5-min preincubation at 25°, aliquots containing 2 μg of enzyme, 50 μg of bovine serum albumin, and 0.05 μg of substrate per ml were gently stirred magnetically at 25° in cuvets having a 1-cm light path and illuminated with monochromatic light from a Bausch & Lomb grating monochromator (Model 33-86-45-49). A mylar filter was used to ensure exclusion of far-ultraviolet light. The light intensity of each wavelength was adjusted to 100 ergs/mm^2 per sec, an intensity at which the reciprocity relation between time and dose is obeyed (Table I in Saito and Werbin, 1969b). In calculating the number of incident quanta the correction of Morowitz (1950) was used. The transmission of the samples varied between 100 and 97.8%.

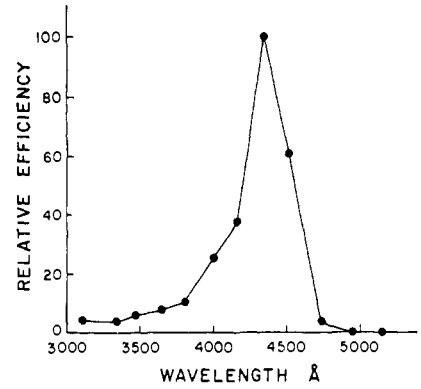

FIGURE 11: Action spectrum for photoreactivation of irradiated *H. influenzae* transforming DNA by *A. nidulans* enzyme. Initial reaction rates relative to the one at 4358 Å arbitrarily assigned a value of 100, were calculated from the slopes of the linear part of the curves in Figure 10, and are plotted as a function of wavelength of illuminating light.

was made among unirradiated and irradiated DNAs and light- and dark-treated samples. These data provide further evidence that the algal enzyme monomerizes rather than excises dimers.

ABSORPTION SPECTRUM. The ultraviolet absorption spectrum of the purified enzyme measured with a Cary 14 spectrophotometer is shown in Figure 7. A small peak was observed at 418 nm as well as a protein peak at 275 nm.

ENZYME STABILITY. The purified enzyme was reasonably stable at 5° provided it was stored at high protein concentrations or as a suspension in saturated ammonium sulfate solution. Heating the enzyme solution at 50° for 5 min or at 40° for 30 min destroyed almost all activity, which the prior addition of 0.1% bovine serum albumin did not prevent. But the presence of substrate afforded considerable protection, an observation made earlier with the yeast and bean sprout enzymes (Rupert, 1962b; Saito and Werbin, 1969a).

MOLECULAR WEIGHT BY GEL FILTRATION. Andrews (1967) has shown that a plot of logarithms of molecular weights for globular proteins against the ratios obtained by dividing their respective elution volumes by the void volume of the column (V_e/V_0) is generally a linear function. We could confirm this relationship when typical globular proteins such as lysozyme, deoxyribonuclease, hemoglobin, yeast alcohol dehydrogenase, human, γ-globulin, and apoferritin were chromatographed on Sephadex G-200. The data shown in Figure 8 were obtained with one column, and when the purified enzyme was run on this column it was eluted as a single band at a V_e/V_0 of 2.05, equivalent to a molecular weight of 93,000. When gel filtration was used to determine the molecular weight of the photoreactivating enzyme during the early stages of its purification, multiple peaks of enzyme activity were eluted indicating that the enzyme was present in several aggregates. The molecular weights of these aggregates appeared to be multiples of the lowest molecular weight, 93,000. Figure 9 shows the gel filtration patterns of the enzyme at several stages of its purification and that after hydroxylapatite chromatography the aggregation was minimal.

PHOTOREACTIVATING ACTION SPECTRUM. In Figure 10 there is plotted units of enzyme activity as a function of the quanta of incident light at various wavelengths and from these data the action spectrum shown in Figure 11 was plotted. There is one sharp peak at 4358 Å in contrast to the one found at 4047 Å with bean sprout photoreactivating enzyme (Saito and Werbin, 1969b). *In vivo* photoreactivation action spectra of *Streptomyces griseus* and of *Agmenellum quadruplicatum* show peaks at 4360 and 4300 Å, respectively (Kelner, 1951; Van Baalen, 1968).

Changes in Activity after Treating Enzyme with Reagents Modifying SH Groups. Table III shows the effects on photoreactivating activity after the enzyme was treated with various reagents that generally modify SH groups in four different ways (Webb, 1966). The per cent inhibition of transformation by these reagents is also shown in the table. This could result from an interaction of the reagent with DNA (Sutherland and Sutherland, 1970; Rahn *et al.*, 1970) preventing either the latter's uptake by the cells or its integration into the cellular genome. Interference by the reagents with cellular metabolism might also inhibit transformation. The data in Tables III and IV on the modification of photoreactivation are probably more reliable in those cases where the transformation was effected minimally.

TABLE III: Effects on Enzymatic Activity Following Treatment of DNA-Photoreactivating Enzyme with Reagents Modifying SH Groups.[a]

	Concn of Reagent during Enzyme Treatment (M)	Time of Action on Enzyme at 0° (min)	Inhibn or (Activation) of Enzyme Act. (%)	Inhibn of Transformation (%)
	Oxidation of SH Groups			
Tetrathionate	2×10^{-4}	30	0	0
	1×10^{-3}	30	20	0
	4×10^{-3}	30	70	19
o-Iodosobenzoate	2×10^{-4}	30	65	0
	6.6×10^{-4}	30	80	0
	2×10^{-3}	30	100	0
Porphyrindin	2×10^{-5}	30	75	0
	2×10^{-4}	30	98	34
Potassium ferricyanide	2×10^{-4}	30	98	0
	8×10^{-4}	30	98	0
	1.6×10^{-3}	30	100	0
	2×10^{-3}	10	100	0
Oxidized glutathione	4×10^{-3}	720	23	0
	2×10^{-2}	720	74	0
Copper sulfate	4×10^{-4}	10	87	0
	1.6×10^{-3}	10	95	17
	2×10^{-3}	10	96	33
Potassium persulfate	6.6×10^{-4}	10	47	0
	2×10^{-3}	10	77	0
Ferric citrate	4×10^{-4}	30	85	0
	1.6×10^{-3}	30	90	0
	Alkylation of SH Groups			
Iodoacetate	1×10^{-5}	10	(73)	5
	2×10^{-5}	10	(23)	25
	2×10^{-4}	10	(3)	33
	2×10^{-3}	10		100
Iodoacetamide	6×10^{-6}	10	(24)	2
	6×10^{-5}	10	7	55
	6×10^{-4}	10	25	96
	Addition of SH Groups to Double Bonds			
Maleate	2×10^{-4}	10	34	0
	2×10^{-3}	10	22	4
	2×10^{-2}	10		17
	Conversion of SH Groups into Mercaptide			
Mercuric chloride	2×10^{-6}	10	0	55
	2×10^{-5}	10	0	67
	2×10^{-4}	10		100
Silver nitrate	2×10^{-6}	10	46	34
	2×10^{-5}	10	61	42
	2×10^{-4}	10	100	49
Sodium arsenate	2×10^{-4}	10	0	11
	1×10^{-3}	10	0	13
	2×10^{-3}	10	0	23
Phenylmercuric acetate	2×10^{-6}	10	(40)	88
	2×10^{-5}	10		100

FOOTNOTES TO TABLE III

[a] To 0.3 ml of enzyme solution obtained after the first hydroxylapatite chromatography was added 0.2 ml of reagent and the mixture was held at 0° for the times designated in the table. Then 0.5 ml of *H. influenzae* transforming DNA (0.1 μg/ml) was added and two 0.3-ml aliquots were held in the dark for 5 min at 37°. Then one was illuminated with white light for 10 min while the other was held in darkness another 10 min. From both mixtures, 0.2 ml was pipetted into two 3-ml quantities of competent cells and transformation was allowed to proceed for 2 hr. Aliquots were plated on streptomycin-containing agar and the number of light and dark transformants was counted the next morning. Details of these assay procedures have been reported. The percentage change in enzyme activity was calculated as follows: units of enzyme activity after reaction with SH reagent/units of enzyme activity of unmodified enzyme × 100. The percentage inhibition of transformation was calculated by dividing the number of dark transformants from the photoreactivating enzyme treated with SH reagent by the number of dark transformants from the untreated enzyme and multiplying by 100. The concentrations of the reagent in the photoreactivating mixture and in the transformation mixture were $^1/_2$ and $^1/_{32}$, respectively, of that in the enzyme-treated solutions.

TABLE IV: Enhancement of Activity by the Action of Reductants on the DNA-Photoreactivating Enzyme.[a]

	Concn of Reagent during Enzyme Treatment (M)	Time of Action on Enzyme at 0°		Enhancement of Enzyme Act. (%)	Inhibn of Transformation (%)
		min	hr		
2-Mercaptoethanol	6×10^{-3}		24	153	0
	1.8×10^{-2}		24	125	0
	6×10^{-3}	10		77	0
Glutathione	6×10^{-3}		25	84	0
	1.8×10^{-2}		24	136	28
	6×10^{-3}	10		35	0
Cysteine	6×10^{-3}		24	93	15
	6×10^{-3}	10		26	13
Hydroxylamine	2×10^{-4}	30		30	0
	1×10^{-3}	30		44	0
	5×10^{-3}	30		43	23
	1.8×10^{-2}	10		50	59
Sodium hydrosulfite	1×10^{-3}	30		76	0
	5×10^{-3}	30		76	0
	2×10^{-2}	30		134	36
Sodium bisulfite	1×10^{-3}	30		85	0
	5×10^{-3}	30		87	0
	2×10^{-2}	30		133	0
Sodium sulfite·$7H_2O$	1×10^{-3}	30		68	0
	5×10^{-3}	30		68	0
	2×10^{-2}	30		63	0

[a] Details of the assay procedures and the methods of calculation are given in the lower legend of Table III.

Those reagents which oxidize thiol groups in proteins to disulfide linkages produced a marked inhibition of activity while barely effecting the transformation. In contrast, two reagents which generally carboxymethylate SH groups showed a tendency to enhance photoreactivation. No distinct pattern of enzyme modification emerged from the action of reagents which convert SH groups into mercaptides, other than their marked inhibitory effect on transformation. While mercuric chloride did not inhibit the enzyme, silver nitrate did so markedly but phenylmercuric acetate actually activated the enzyme. Maleate produced a slight inhibitory effect.

Inhibition of the yeast DNA-photoreactivating enzyme by low concentrations of silver, copper, and mercury ions and by *p*-hydroxymercuric benzoate has been reported previously (Rupert, 1962a; Muhammed, 1966). Rupert (1962a) showed that silver ions and the organic mercurial inhibit noncompetitively and that the presence of substrate

TABLE V: Recovery of Enzymatic Activity by Reduction of Oxidized Enzyme.[a]

Oxidizing Agent	Reducing Agent (6×10^{-3} M)	Enzyme Act. (%)
None	None	100
	2-Mercaptoethanol	216
	Glutathione (reduced form)	221
	Sodium hydrosulfite	250
	Sodium bisulfite	255
o-Iodosobenzoate (6.6×10^{-4} M)	None	8.4
	2-Mercaptoethanol	235
	Glutathione (reduced form)	211
	Sodium hydrosulfite	240
	Sodium bisulfite	176
Ferricyanide (1×10^{-3} M)	None	0.7
	2-Mercaptoethanol	255
	Glutathione (reduced form)	230
	Sodium hydrosulfite	221
	Sodium bisulfite	201

[a] Oxidation: to 0.3 ml of enzyme solution in 0.05 M TB buffer (pH 7.2) containing 0.1% bovine serum albumin, was added 0.2 ml of oxidant at the concentration shown in the table, and the mixtures were kept at 0° for 60 min. Reduction: the oxidized enzyme solution was diluted 10× with 0.05 M TB buffer containing 0.1% bovine serum albumin. To 0.3 ml there was added 0.2 ml of reducing agent and the mixtures were held at 0° for 60 min. After addition of substrate, photoreactivation was carried out for 10 min.

TABLE VI: Failure of Substrate (Ultraviolet-Irradiated DNA) to Prevent Enzyme Oxidation by Potassium Ferricyanide.

Enzyme Treatment		
First Addn (30 min at room temperature)	Second Addn (30 min at 0°)[b]	Act. (%)
0.2 ml of enzyme[a] + 0.2 ml of TB buffer, pH 7.2	0.5 ml of substrate	100
0.2 ml of enzyme + 0.2 ml of 5 mM ferricyanide	0.5 ml of substrate	3
0.2 ml of enzyme + 0.5 ml of substrate	0.2 ml of 5 mM ferricyanide	2

[a] The enzyme was obtained from active fractions of the first hydroxylapatite chromatography. [b] After the second incubation at 0°, the mixtures were held 5 min at 37° in the dark and were then assayed for photoreactivating activity by incubating separately 10 min in the light and dark.

protects the enzyme from inactivation by the organic mercurial.

Many reducing agents had a marked stimulatory effect on enzyme activity (Table IV). This effect was not so noticeable in crude extracts of the photoreactivating enzyme although its occasional observation in previous studies prompted our use of glutathione in the buffer used to prepare cell-free extracts of various bean sprouts (Saito and Werbin, 1969a).

Following oxidation of the enzyme with either *o*-iodosobenzoate or ferricyanide, which resulted in almost complete loss of activity, the latter could be completely restored by reduction with either 2-mercaptoethanol, glutathione, sodium hydrosulfite, or sodium bisulfite (Table V).

The inhibition of enzyme activity by oxidation with ferricyanide was not precluded by first adding substrate (Table VI).

Discussion

Far-ultraviolet irradiation of biological material (Wacker *et al.*, 1962), or of DNA extracted from it (Wacker *et al.*, 1960) induces intrastrand cyclobutane pyrimidine dimers between adjacent pyrimidine bases. Setlow (1968) has adduced convincing evidence that the formation of the dimers leads to biological inactivation of the DNA. The DNA-photoreactivating enzyme complexes with the damaged DNA (Rupert, 1962a,b) and uniquely, using light as a cofactor, monomerizes the dimers thereby restoring the repaired section of the DNA to its native functional state. Proof of dimer monomerization was established by Cook (1967) when he illuminated a mixture of ultraviolet-irradiated *E. coli* DNA ([^{3}H]thymine labeled) and unirradiated *E. coli* DNA ([^{14}C]thymine labeled) in the presence of yeast photoreactivating enzyme and found that the tritium counts that disappeared from the thymine dimers was equivalent to that which appeared in thymine of the DNA. Since one of the substrates, ultraviolet-irradiated *H. influenzae* transforming DNA, for the yeast enzyme, is the same as that used in the algal studies, it is reasonable to assume that the algal enzyme functions catalytically by also monomerizing pyrimidine dimers. The data of Figure 6 and Table II support this contention.

DNA-photoreactivating enzyme(s) from different sources vary in the wavelengths of light they require for most effective action. Thus the photoreactivating action spectra of the bean sprout and *Neurospora crassa* enzymes (Terry and Setlow, 1967) both peak at 4047 Å, while the ones from *A. nidulans* and yeast peak at 4358 Å, and 3550 and 3850 Å (Setlow and Boling, 1963), respectively. Since the substrate used in all of these *in vivo* studies was basically the same,[1] ultraviolet-irradiated *H. influenzae* transforming DNA, the differences observed are most likely attributable to the presence of different chromophoric groups within the enzymes or to structurally different enzymes forming enzyme–substrate complexes with diverse absorption spectra. These alternate explanations also account for the very similar *in vivo* and *in vitro* action spectra of the *N. crassa* enzyme and the small difference of the most efficient wavelengths

[1] It should be noted, however, that different procedures were used in various laboratories to isolate the DNA from the bacterial cells.

in the action spectra obtained with the blue-green algal enzymes from *A. nidulans* (4358 Å, *in vitro*) and *Agm. quadruplicatum* (4300 Å, *in vivo*).

The question of whether the DNA-photoreactivating enzyme possess a chromophore absorbing near-ultraviolet or visible wavelengths remains unresolved at present. Muhammed found that the purified yeast enzyme absorbed minimally at 415, 520, and 550 nm which he attributed to cytochrome impurities not completely removed from the enzyme. Our purified enzyme preparation absorbed weakly at 418 nm in contrast to the sharp peak at 436 nm in the action spectrum. This lack of coincidence favors the view that the 418-nm band can be attributed to enzyme impurities rather than an enzyme chromophore, particularly since our purified preparation showed several protein bands when it was analyzed by acrylamide gel electrophoresis.

The data presented leave no doubt that the DNA-photoreactivating enzyme from the blue-green alga *A. nidulans* has been purified extensively and still retains considerable activity. This was achieved by concentrating the enzyme immediately whenever it was eluted from a chromatographic column. Dilution of highly purified and concentrated enzyme to measure activity generally led to inactivation, but this was circumvented by adding 0.1% bovine serum albumin to the buffer used for dilution.

Metal ions did not appear to be required for enzyme catalysis because activity remained undiminished in the presence of several kinds of metal chelators such as EDTA, α,α'-dipyridyl, *o*-phenanthroline, and 8-hydroxyquinoline.

The molecular weight of 93,000 found by gel filtration for the *A. nidulans* enzyme contrasts with the value of 30,000 obtained by ultracentrifugal methods for the yeast enzyme by Muhammed (1966). The latter value must be taken with reservations since calculations by Harm and Rupert (1968) indicate that the enzyme preparation used by Muhammed for molecular weight determination contained only about 1% photoreactivating enzyme.

Our work has uncovered two interesting differences between the algal and yeast enzymes. The former appears to be negatively charged at pH 7.2 in 0.01 M TB buffer, or even in phosphate buffer, because it is not retained by cation exchange columns prepared from CM- and phosphocelluloses but is on anion exchangers such as TEAE-, DEAE-, and ECTEOLA-celluloses. The retention of the yeast enzyme on phosphocellulose equilibrated with 0.1 M phosphate buffer (pH 7.0) suggests that it is positively charged (Muhammed, 1966). The other difference is in the ammonium sulfate saturation required to precipitate most of the yeast and algal enzymes: the former is precipitated between 30 and 50% saturation, the latter between 43 and 85% saturation.

The easily reversible oxidation and reduction of the photoreactivating enzyme by reagents that usually oxidize SH groups to disulfide linkages suggest that two juxtaposed cysteinyl residues are being acted upon inter- or intramolecularly. Although these residues do not appear to be part of the active center because prior addition of substrate does not afford protection from oxidation (Table VI), nevertheless, their coupling possibly transforms the tertiary structure of the enzyme to a form that either prevents substrate binding or enzyme catalysis.

It is difficult to assess whether *p*-hydroxymercuric benzoate, which inhibits the yeast enzyme noncompetitively, acts on the same cysteinyl residues. The noncompetitive inhibition suggests that these residues are not at the enzyme's active center. In the case of the yeast enzyme the substrate reduces inhibition by the organic mercurial but this may be accounted for by a change in enzyme conformation consequent to the formation of the enzyme–substrate complex.

The marked enhancement of enzyme activity by reducing agents (Table IV) indicates that labile cysteinyl groups may be oxidized during the enzyme purification and that their function is restored by reduction. These observations recall those made by Rupert (1964) that reduced NAD and reduced glutathione restored the activity of a crude photoreactivating enzyme preparation from *E. coli*. Rupert recognized that these reductants were not stoichiometric reactants in the photoreactivating process and considered that they might overcome some kind of inhibition in the crude extract. It is more likely that during the preparation of the enzyme it was oxidized and inactivated and that these reductants restored activity. These data and our own suggest that future work on the purification of the enzyme be carried out with buffers to which an organic thiol has been added.

While it is difficult to assess quantitatively the survival value to algae of the DNA-photoreactivating enzyme, the presence of the enzyme at least does ensure recovery from any pyrimidine dimer damage to DNA that may be induced by sunlight (Harm, 1969) and thus, the enzyme plays some role in maintaining the ecological balance in nature between heterotrophic and autotrophic organisms.

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Subunit Structure of L-Aspartate β-Decarboxylase from *Alcaligenes faecalis**

William F. Bowers,† Valentine B. Czubaroff, and Rudy H. Haschemeyer

ABSTRACT: Sedimentation equilibrium studies of highly purified L-aspartate β-decarboxylase from *Alcaligenes faecalis* show the molecular weight of the 19S holoenzyme to be 675,000. The 19S apoenzyme obtained by resolution at pH 5–6 undergoes dissociation at pH 8.0 to a 6S component which is one-sixth as large as the holoenzyme and is itself composed of two polypeptide chains. The morphological appearance of the holoenzyme revealed by electron microscopy of negatively stained preparations is presented and compared with that expected for possible dodecameric structures.

For this purpose a versatile computer program has been developed to simulate negative staining of macromolecules under a variety of conditions.

L-Aspartate β-decarboxylase from *Alcaligenes faecalis* has been intensively investigated by Meister and coworkers (Novogrodsky *et al.*, 1963; Soda *et al.*, 1964; Novogrodsky and Meister, 1964a,b; Wilson and Meister, 1966; Tate and Meister, 1968, 1969a,b; Tate *et al.*, 1969) with respect to various catalytic and chemical properties of the enzyme. Tate and Meister (1968) demonstrated that the highly purified 19S holoenzyme migrates as a single band on acrylamide gel electrophoresis, and that the resolved apoenzyme dissociates at pH 8 into a 6S component of increased electrophoretic mobility.

Spectrophotometric and optical rotatory dispersion titration studies (Wilson and Meister, 1966) indicated that approximately 1 mole of pyridoxal-5′-P is bound per 52,000 g of enzyme, consistent with earlier minimal pyridoxal-5′-P unit values of 53,000 and 57,000 (Novogrodsky and Meister, 1964a) from microbiological and phenylhydrazone procedures, respectively. Tate *et al.* (1969) found that about 1 mole of β-chloro-L-alanine could be bound per 60,000 g of enzyme in their studies on active-site labeling. This level of binding was also required to totally inactivate the enzyme. The enzyme contains 11 methionine, and 49 lysine and arginine residues per 50,000 g (Tate and Meister, 1968), and cyanogen bromide cleavage yields 12 peptides, while trypsin digestion results in about 43 fragments (M. Jensen, unpublished data). These results are in accord with the view that the enzyme contains a unique amino acid sequence with a total molecular weight of about 50,000–60,000, identical with that required per coenzyme binding site.

We report here the results of sedimentation equilibrium molecular weight studies on the 19S, 6S, and totally dissociated forms of L-aspartate β-decarboxylase from *A. faecalis*. Electron microscopic studies on the enzyme are presented

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