Yeast DNA Photolyase: Molecular Weight, Subunit Structure, and Reconstitution of Active Enzyme from Its Subunits†

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ABSTRACT: Yeast DNA photolyase, purified by affinity chromatography, ran as a single component when analyzed by either electrophoresis on polyacrylamide gradient gels or by sedimentation velocity through 5–20% sucrose gradients containing 0.4 *M* KCl, and, therefore, was considered homogeneous. The molecular weights of photolyase, determined by these methods, were 130000 and 136000, respectively. When the enzyme was examined by electrophoresis on sodium dodecyl sulfate polyacrylamide gradient gels, it dissociated into two bands whose molecular weights were 60000 and 85000. After the enzyme was sedimented through sucrose gradients in the presence of 1.0 *M* KCl, two absorbance maxima, which corresponded to polypeptides of 54000 and 82500, were found in the fractions collected. Thus, the enzyme consists of two dissimilar subunits. When the two fractions that exhibited maximal absorbance were mixed together, a time-dependent increase in activity occurred, demonstrating that active enzyme could be reconstituted from these subunits. Analysis of sucrose gradients containing 1.0 *M* salt for photolyase activity showed that it was present exclusively in the region of the gradient corresponding to 68200 in agreement with a previous report (J. Cook and T. Worthy (1972), *Biochemistry 11*, 388). These active fractions were found in the overlap region between the two subunits, and their activity was attributed to reconstitution of the enzyme during the assay.

DNA photolyase (EC 4.1.99.3) repairs cyclobutadipyrimidine damage introduced into DNA by far-uv irradiation. The formation of these lesions by sunlight and the wide distribution of the enzyme suggest that photolyase may have significant ecological impact in maintaining cellular DNA integrity.

The general enzyme reaction mechanism, first analyzed by Rupert (1962), involves the formation of a stable complex in the dark between the enzyme and the dipyrimidine region of DNA, and the subsequent light-catalyzed splitting of the cyclobutane ring. The photolysis step is most efficient for light in the near-uv and visible regions with the wavelength of maximal efficiency dependent on the source of the enzyme, for example, 360–385 nm for the yeast (Madden and Werbin, 1974) and *Escherichia coli* enzymes (Setlow, 1966) and 435 nm for the *Streptomyces griseus* enzyme (Jagger et al., 1970). It has been postulated (Madden and Werbin, 1975; Eker and Fichtinger-Schepman, 1975) that the enzyme utilizes light at these wavelengths through the mediation of a chromophore or cofactor, varying structurally with each enzyme depending upon its source and giving each enzyme its characteristic action spectrum.

While DNA photolyase from *E. coli* (Sutherland et al., 1973) and *S. griseus* (Eker and Fichtinger-Schepman, 1975) has been purified to a state of homogeneity, the yeast enzyme purified by affinity chromatography on uv-irradiated DNA yielded multiple bands after electrophoresis on polyacrylamide gels. These bands were attributed to enzyme aggregates because each one was active after elution from the gel (Madden and Werbin, 1974).

In this communication we report that a second affinity chromatography provided a photolyase preparation that ran as a single band on polyacrylamide gradient gels. The enzyme's molecular weight of 130000 was determined by comparing its mobility on gradient slab gels with those of a series of proteins of known molecular weight run simultaneously. Electrophoresis of the purified enzyme on gradient gels under dissociating conditions yielded two protein bands that appeared to be subunits and that had molecular weights of 60000 and 85000. Sucrose gradient sedimentation of the enzyme in 1 *M* salt solutions partially separated the subunits which could then be reconstituted to active enzyme.

Materials and Methods

Materials. The following proteins were used as molecular weight standards: *E. coli* β-Gal,[1,2] 520000 (Zipser, 1963), and yeast ADH, 150000 (Harris, 1964), from Sigma Chemical Co.; bovine liver CAT, 232000 (Schroeder et al., 1969), and *E. coli* AP, 90000 (Garen and Leventhal, 1960), from Worthington Biochemical Corp. and bovine HB, 64500 (Braunitzer et al., 1964), from Mann Research Laboratories, Inc. Additional standards were obtained by dissociation of β-Gal (130000), CAT (57500), and AP (45000) into their subunits. Riboflavine, *N,N,N′,N′*-tetramethylmethanediamine, acrylamide, *N,N′*-methylenebisacrylamide, glycine, and HSEtOH were purchased from Eastman Kodak Organic Corp. The acrylamide was recrystallized from warm chloroform (150 g/l.), filtered after standing overnight at 2–4°C, and dried over silica gel.

Photolyase Assay. Enzyme activity was measured by the transformation assay of Saito and Werbin (1970) with the

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[1] Abbreviations used are: β-Gal, β-galactosidase; ADH, alcohol dehydrogenase; CAT, catalase; AP, alkaline phosphatase; HB, hemoglobin; HSEtOH, 2-mercaptoethanol; PL, DNA photolyase; TB, transformation assay buffer.

[2] This enzyme was further purified on Sephadex G-100 by Dr. Horst Brunschede and was a gift from him.

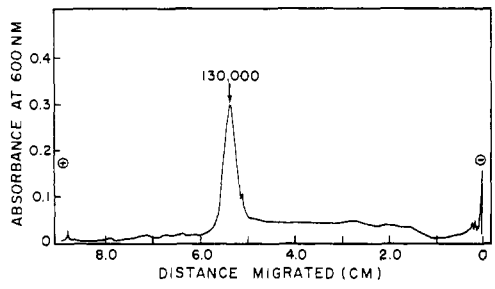

FIGURE 1: Scan of absorbance at 600 nm vs. distance migrated by DNA photolyase (2 × AC) on a 7.5–30% polyacrylamide gradient slab gel. The gel was loaded with 10–20 μl of photolyase (0.5 mg/ml) and electrophoresed at pH 8.3 for 72 hr at 10 mA at 2–4°. After staining the protein with Coomassie Blue, the gel was scanned on a Gilford double beam spectrophotometer with gel adapter, Model 2400.

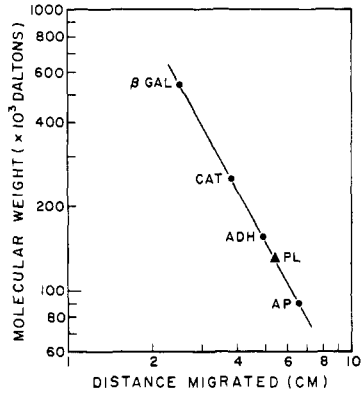

FIGURE 2: Log–log plot of the molecular weight of several protein standards vs. their migration distance on a 7.5–30% polyacrylamide slab gel. Running conditions are described in the legend to Figure 1. The standards and photolyase (2 × AC) were run simultaneously on the same gel.

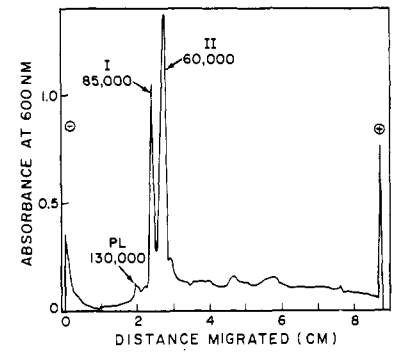

FIGURE 3: Absorbance scan at 600 nm of DNA photolyase (2 × AC) vs. migration distance on a 7.5–30% polyacrylamide gradient slab gel. Photolyase was heated in a boiling water bath for 5 min in 1% dodecyl sulfate and electrophoresed for 48 hr at 10 mA at 22° in gels containing 1% dodecyl sulfate. The gel was stained with Coomassie Blue and scanned as described in Figure 1. The numbers in the figure refer to molecular weights.

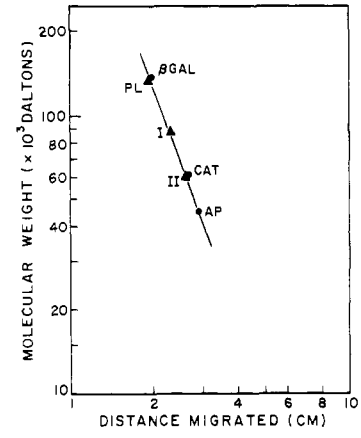

FIGURE 4: Log–log plot of molecular weight of protein standards vs. their migration distance on dodecyl sulfate polyacrylamide gradient gels. The standards and photolyase were prepared and run simultaneously as described in the legend to Figure 3. I and II refer to the 85000 and 60000 photolyase subunits, respectively, and PL to the 130000 undissociated enzyme.

following modifications. The assay buffer (TB) was composed of 0.05 *M* Tris-HCl, 1 m*M* EDTA, and 1 m*M* HS-EtOH (pH 7.5). The incubation in the dark under General Electric gold fluorescent lights was performed for 5 min at 37°C, and the photolysis with five black lights (General Electric, F15T8-BLB) for 5 min at 37°C.

Gradient Gel Electrophoresis. Slab gels, composed of linear 7.5–30% polyacrylamide gradients, were prepared and run by the procedure outlined by Studier (1971). Details of these procedures are described by Boatwright (1975).

Sedimentation Velocity Studies. Sucrose gradients, 5–20% in 0.05 *M* sodium phosphate and 0.01 *M* EDTA (pH 7.5) containing either 0.4 or 1.0 *M* KCl, were centrifuged in a Spinco SW-50L rotor at 45000 rpm at 2–4°. The molecular weights of the proteins in the isolated fractions were determined by the method of Martin and Ames (1961).

Purification of DNA Photolyase. Partial purification of DNA photolyase by affinity chromatography on far-uv irradiated DNA noncovalently bound to cellulose has been described (Madden and Werbin, 1974). This enzyme preparation, designated 1 × AC, was mixed in a column (1 × 5 cm) with irradiated DNA-cellulose in TB containing 0.05 *M* NaCl and the mixture incubated for 30 min at 2–4° in the dark. When the resin had settled, it was washed with 50 ml of 0.05 *M* NaCl, 200 ml of a NaCl gradient between 0.05 and 1.0 *M*, and 50 ml of 2 *M* NaCl each in TB. A yield of 80–90% of the activity was recovered in the fractions eluted in the 0.3–0.6 *M* region of the gradient. Active fractions were concentrated in an Amicon Ultrafiltration concentrator using a PM-10 membrane and stored at −80°C. This 2 × AC preparation lost considerable activity upon storage.

Results

Homogeneity of Photolyase (2 × AC) Determined by Gradient Gel Electrophoresis. When photolyase was electrophoresed on a 7.5–30% polyacrylamide slab gel, a single band was found after Coomassie Blue staining (Figure 1). Several protein standards were run simultaneously and from a log–log plot of their molecular weights vs. their migration distances (Figure 2) a molecular weight of 130000 was determined for photolyase by interpolation.

Dissociation of Photolyase by Dodecyl Sulfate Gel Electrophoresis. To determine whether the molecular weight of 130000 represented a monomeric species or an aggregate of photolyase, possibly a dimer of the 62000–73000 component reported by Cook and Worthy (1972), it was analyzed on a dodecyl sulfate polyacrylamide gradient slab gel (Figure 3). The enzyme dissociated into two sharp bands, I and II, with molecular weights of 85000 and 60000, respectively, values determined by interpolation from a standard curve (Figure 4). Occasionally, a third faint band was

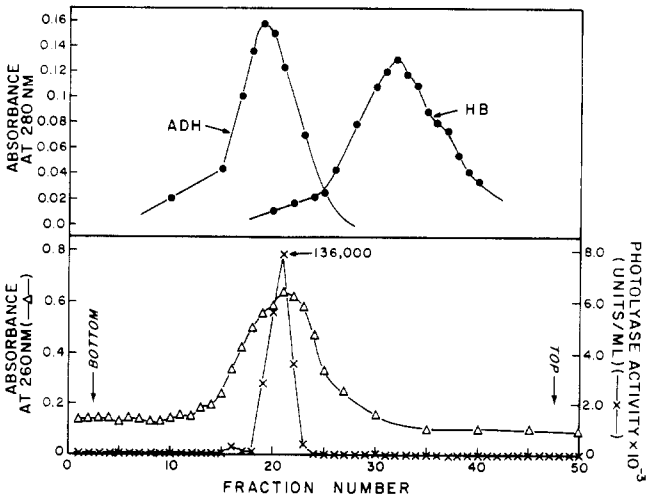

FIGURE 5: Sedimentation velocity on low salt, sucrose gradients. Sucrose gradients (5 ml, 5–20%) which were 0.4 *M* in KCl were overlayered with 0.1 ml of photolyase. The run with 1 × AC enzyme was used to determine activity while that with 2 × AC enzyme was used for 260-nm absorbance. The gradients were centrifuged for 10 hr at 45000 rpm in a Spinco SW-50L rotor at 2–4°C, and samples were collected from the bottom. Photolyase activity (x); photolyase absorbance at 260 nm (Δ); ADH and HB absorbance at 280 nm (●).

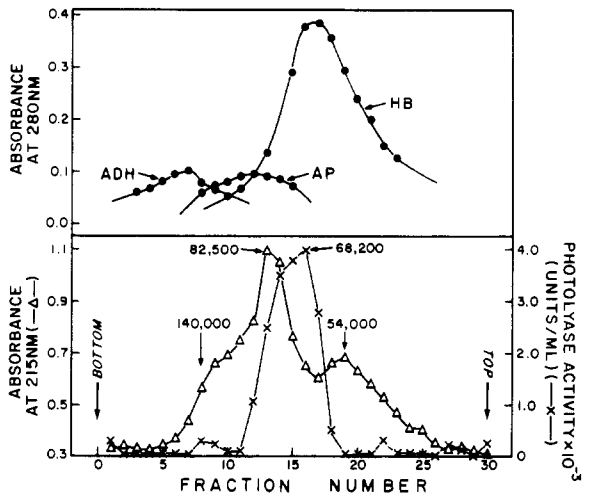

FIGURE 6: Sucrose gradients, 1.0 *M* in KCl, were overlayed with photolyase as described in Figure 5. The gradients were centrifuged for 19 hr at 45000 rpm in a Spinco SW-50L rotor at 2–4°C and samples collected from the bottom. Photolyase activity (x), photolyase absorbance at 215 nm (Δ); HB, ADH, and AP absorbance at 280 nm (●). The numbers in the figures refer to molecular weights.

found at a position in the gel corresponding to that of the undissociated enzyme.

Sedimentation Velocity Studies. Photolyase was sedimented through 5–20% sucrose gradients containing 0.4 *M* KCl. Such gradients are referred to as low salt gradients in contrast to those containing 1.0 *M* salt which are denoted as high salt ones. Photolyase activity and absorbance at 260 nm were measured on the fractions collected. The coincidence of the maxima for both measurements indicated the presence of a single component with a relative mobility (distance migrated/length of gradient) of 0.58 (Figure 5). All the fractions under the absorbance peak were active when assayed at higher relative concentrations than those used in obtaining the data in Figure 5. Several factors, such as the low protein concentration and the presence of interfering substances like Tris and sucrose, precluded the accurate

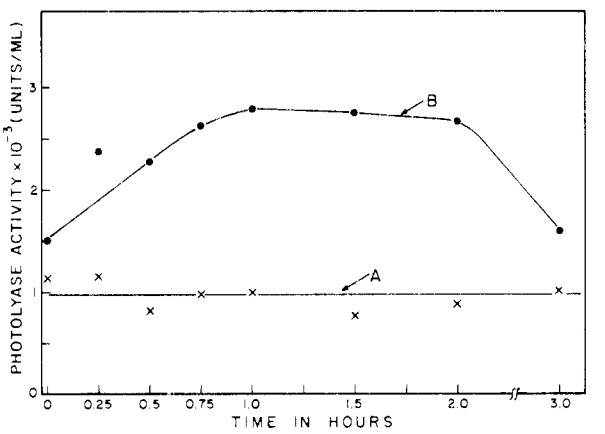

FIGURE 7: Kinetics of reconstitution of active photolyase from its partially separated subunits. Fractions 13 and 19, from the 1 *M* salt, sucrose gradient described in Figure 6, were diluted with TB and assayed both separately and after mixing. In the latter case, 75 μl each of diluted 13 and 19 were mixed with 150 μl of irradiated transforming DNA and incubated for varying periods of time in the dark at 22°C prior to photolysis. When assayed separately, 75 μl of TB was substituted for the second fraction. (A) The sum of the activities of 13 and 19 assayed individually (x); (B) the activity of the mixture of the two (●).

measurement of the protein concentration and specific activity. ADH run simultaneously had a relative mobility of 0.62. From these data and the Martin and Ames (1961) relationship, a molecular weight of 136000 was calculated for photolyase, a value in reasonable agreement with that obtained from the polyacrylamide gradients.

When photolyase was centrifuged on a high-salt, sucrose gradient, the peak of activity corresponded to a protein with a molecular weight of 68200 in excellent agreement with the value measured by Cook and Worthy (1972) who used similar experimental conditions (Figure 6). This peak of activity was bracketed by two absorbance maxima with relative mobilities of 0.37 and 0.57. We assumed that they were the 60000 and 85000 subunits observed on dodecyl sulfate electrophoresis, and, indeed, their calculated molecular weights of 54000 and 82500 compared favorably with those values. From these data we inferred that the observed activity, which was found in the overlap region between the partially separated subunits, might have arisen by the reconstitution of active enzyme from these subunits during the assay.

Reconstitution of Active Photolyase from Its Subunits. Fractions 13 (82500 subunit) and 19 (54000 subunit) from a high salt, sucrose gradient (Figure 6) were assayed for photolyase activity both separately and in combination. The sum of the activities (units/ml) of these fractions assayed separately remained constant for up to 3 hr when substrate was present (Figure 7). When these two fractions were mixed together and incubated with substrate prior to illumination, the activity of the mixture increased almost threefold, reaching a maximum after 1 hr.

Discussion

Molecular weights between 30000 and 73000 have been reported for yeast DNA photolyase. The 30000 value can be disregarded because calculations by Harm and Rupert (1968) indicated that this preparation was highly impure. Minato and Werbin (1971) obtained a molecular weight of 53000 by gel filtration. They observed an affinity of the enzyme for Sephadex which could be overcome by eluting the protein with 0.4 *M* KCl. Using a similar procedure with a

buffer containing 1 *M* salt, Cook and Worthy (1972) obtained values of 71000 and 73000. Their sedimentation velocity studies with sucrose gradients containing 1 *M* KCl yielded 69500, a value agreeing with those obtained by gel filtration.

In this study we found molecular weights for yeast photolyase of 130000 by electrophoresis on gradient slab gels and 136000 by sedimentation velocity on sucrose gradients in 0.4 *M* KCl. The finding of a single protein species by both these methods was a strong indication that the 2 × AC photolyase preparation was homogeneous. Nevertheless, we found it advantageous to follow activity on the sucrose gradients with the 1 × AC preparation rather than the more purified 2 × AC enzyme because of the instability of the latter.

Dodecyl sulfate electrophoresis caused dissociation of photolyase into two polypeptides with molecular weights of 60000 and 85000, a finding confirmed by sedimentation velocity of photolyase in sucrose gradients containing 1 *M* salt where two subunits with estimated molecular weights of 54000 and 82500 were detected by absorbance at 215 nm. The agreement between the molecular weights measured by gel electrophoresis and those from sedimentation velocity was good with the exception of the values calculated for the smaller subunit (60000 and 54000). This discrepancy of approximately 10% is not unreasonable considering that the molecular weights obtained by the Martin and Ames (1961) treatment are only crude estimations.

The closeness of the subunit molecular weights prevented their total separation on sucrose gradients, and several fractions that were collected contained both subunits. It was in the overlap region containing the two subunits that photolyase activity was found, suggesting that the activity arose by reassociation of the subunits into active enzyme. To test this idea, two fractions from the high salt, sucrose gradient, one enriched in the lower molecular weight subunit, and the other in the higher one, were mixed and assayed at varying time intervals. A significant, time-dependent increase in photolyase activity occurred confirming the concept of reconstitution.

This finding and the appearance of two bands when the homogeneous enzyme is electrophoresed on dodecyl sulfate polyacrylamide gels leave little doubt that the yeast enzyme is composed of two dissimilar subunits. Whether either of these subunits alone is fully active or can bind to the substrate, and which one contains the fluorescent chromophore, are questions that await their complete purification.

The present study also points to the versatility of polyacrylamide gradient gels in studying proteins that are poorly resolved on single concentration gels because they aggregate.

Acknowledgments

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