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Purification and Properties of Superoxide Dismutase from *Drosophila melanogaster**

(Received for publication, March 26, 1981, and in revised form, May 4, 1981)

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The major superoxide dismutase ("slow" electromorph) of the fruit fly, *Drosophila melanogaster*, has been purified to homogeneity. This enzyme contains 2 Cu^{2+} and 2 Zn^{2+}/molecule. The ultraviolet absorption spectrum indicates a lack of tryptophan. This enzyme has a molecular weight of 32,000 and is composed of two subunits of equal size, which are joined by noncovalent interactions. Cyanide at 1 and 3 mM inhibits the activity of superoxide dismutase 92 and 100%, but 5 and 10 mM azide caused 15 and 30% inhibition. The isoelectric point, assessed by isoelectric focusing, is 5.3. Amino acid analyses, as well as the spectral and catalytic properties, are reported. The *D. melanogaster* superoxide dismutase does not cross-react with antibodies to bovine erythrocyte Cu-Zn-containing superoxide dismutase nor to *Escherichia coli* manganese- and iron-containing superoxide dismutases.

Superoxide dismutases, which catalytically scavenge O_2^-, appear to be essential components of the biological defense against oxygen toxicity (1-3). These enzymes are neither a part of structural proteins nor involved in intermediate metabolism, providing a unique situation to be studied by population geneticists. Genetic polymorphism of superoxide dismutase has already been discovered in diverse living organisms including humans and the fruit fly, *Drosophila* (4-10). Little is known, however, about the structural basis of such polymorphisms.

Superoxide dismutases have been isolated from several organisms; thus far, only three grossly dissimilar kinds have been found. The structural and functional relationships of these three classes of superoxide dismutases have raised interesting and unresolved questions about their evolution. Copper- and zinc-containing superoxide dismutases have been isolated from various species (11-19) and considered to be characteristic of the cytosol of eukaryotic cells (20), but a similar enzyme has been found in a prokaryote, *Photobacterium leioghathi* (20). Manganese-containing superoxide dismutases have been isolated from several prokaryotes (21-23) and from the mitochondria of chicken liver (14) and of yeast (24). Structural analyses have demonstrated a close relationship between the bacterial and the mitochondrial enzymes (25, 26), supporting of the hypothesis of a symbiotic origin of mitochondria (25-27).

It was believed that the cytosol superoxide dismutases in eukaryotes would contain copper-zinc, while the mitochondria would contain manganese. However, the luminous fungus, *Pleurotus olearius*, has been shown to contain two superoxide dismutases, both of which contain manganese (28). Furthermore, substantial quantities of manganese enzyme have been found in the cytosol of chicken liver and of baboon liver (29). Superoxide dismutase isolated from the cytosol of unicellular red alga, *Porphyridium cruentum*, which is considered to be perhaps the most primitive eukaryote, contain manganese (30). However, blue-green algae, which are considered to be the most advanced prokaryotes, have an iron-containing superoxide dismutase (31, 32). Iron-containing enzymes have also been found in several bacteria (33-35). A survey of progressively more advanced plants has failed to find copper-zinc superoxide dismutase in marine plants, but has found it in land plants such as mosses and ferns (36). Thus, the facts are not easily arranged into a coherent theory of descent.

There have been several reports indicating that superoxide dismutase protects against ionizing radiation damage to DNA, viruses, bacteria, mammalian cells in culture, and even whole animals (37-43). Since insects have been shown to be more resistant to ionizing radiation than mammals, *Drosophila* are reported to survive radiation exposure of 64,000 rads (44), and because a superoxide dismutase has not been isolated from an insect, it seemed important to purify and characterize this enzyme from *Drosophila melanogaster*. We here report the thorough purification and characterization of one of two electrophoretically detectable allozymes of superoxide dismutase from *D. melanogaster* with the expectation that this will relate to the radioresistance of the organism and will also bring us a step closer to disentangling the complex evolutionary history of these enzymes.

MATERIALS AND METHODS AND RESULTS[1]

DISCUSSION

Cell-free extracts of the fruit fly, *D. melanogaster*, contain two superoxide dismutases which are separable on polyacrylamide gel electrophoresis. The major superoxide dismutase activity was inhibited by cyanide. This enzyme, like the bovine erythrocyte superoxide dismutase, survived an unusual purification step which included use of chloroform-ethanol to denature extraneous proteins. However, unlike other Cu-Zn

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[1] Portions of this paper (including "Materials and Methods," "Results," Figs. 1-4, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-717, cite authors, and include a check or money order for $4.00 per set of photocopies. Full size photo copies are also included in the microfilm edition of the Journal that is available from Waverly Press.

superoxide dismutases, the *Drosophila* enzyme ("slow" electromorph) quickly lost its activity when salted out of an ethanol-rich phase with K_2HPO_4.

Isolation of the major superoxide dismutase of *D. melanogaster* revealed that the molecular properties of this enzyme appear to have been rigidly preserved during the evolution of eukaryotes. Thus, the enzyme is similar to the cytoplasmic enzymes of other eukaryotes (11-19) with respect to molecular weight, quaternary structure, metal prosthetic groups, and ultraviolet spectrum, but it does not cross-react with a rabbit antibody to the bovine erythrocyte enzyme. The *D. melanogaster* enzyme was stable to freezing and thawing and was homogeneous by the criteria of polyacrylamide gel electrophoresis and sodium dodecyl sulfate gel electrophoresis.

Since a 245-fold purification from the cell-free extract was homogeneous, and since the net recovery was 8%, we can estimate that this superoxide dismutase constituted 0.4% of the protein of the crude soluble extract. *Drosophila* superoxide dismutase is at least 1.5 times more active than the enzymes of all other species reported in the literature. Because superoxide dismutase has been implicated in protecting against ionizing radiation (37-43) and insects have been shown to be more radio-resistant than most animals (44), it seems reasonable to believe that the high concentrations of highly active superoxide dismutase detected in *Drosophila* could be contributing to the higher resistance of these flies to ionizing radiation. The evolutionary relationships among superoxide dismutases are obviously of great interest. The structural basis of genetic polymorphisms of this enzyme noticed in *Drosophila* (9) needs to be explored.

Acknowledgments—We would like to thank L. Barr for providing the isogenic line of fruit flies, and David Silberman for atomic absorption analysis of the metals.

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Supplementary Material

PURIFICATION AND PROPERTIES OF SUPEROXIDE DISMUTASE FROM DROSOPHILA MELANOGASTER

Young Moo Lee, Francisco J. Ayala, and Hara P. Misra

Materials and Methods

D. melanogaster flies were made isogenic for the whole third chromosome by crossing flies collected in Tunisia, Africa, with the balancer stock TM3 as described (45). One of the isogenic lines was multiplied and collected at 2-4 days of age. These flies were frozen and stored at -70°C until needed. Cytochrome c (type III), xanthine and xanthine oxidase were products of Sigma. Microgranular diethylaminoethyl cellulose and superfine sephadex G-75 were obtained from Whatman and Pharmacia, respectively. Ampholytes were purchased from LKB.

Superoxide dismutase was assayed and units were defined as previously described (11). Electrophoresis on polyacrylamide gels was performed according to Davis (46). Zones of protein were localized by staining with amidoblack (46), while zones of superoxide dismutase were negatively stained by the photochemical procedure previously described (47). Spectrophotometric assays were performed at 25° in a Gilford model 260 spectrophotometer and optical spectra were recorded with a Cary model 219 spectrophotometer. An LKB 2117 multiphor system was used for preparative electrofocusing. Protein was measured by the spectrophotometric procedure of Lowry (48) with recrystalized bovine serum albumin as the standard.

Amino acid analysis was performed on a Durrum model D500 amino acid analyzer using a single column, three buffer elution program. Protein samples were hydrolyzed for 24, 48 and 72 hr at 110° in vacuo in the presence of a small crystal of phenol (49). Half cystine was determined as cysteic acid after oxidation of the protein samples with performic acid (50). For the determination of tryptophan residues, samples were hydrolyzed in 5.7 N HCl -5% β-mercaptoacetic acid (51). Molecular weight was determined by gel filtration on a Sephadex G-75 superfine column (1.5 x 98 cm). Subunit weight was estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (52). Metal analyses were performed by atomic absorption spectrophotometry with a Perkin-Elmer Model 306.

Antisera were prepared by injecting rabbits with 3 booster doses of purified superoxide dismutases, purified from bovine erythrocytes and Escherichia coli, in Freund's adjuvant (53). The rabbit antiserum was fractionated with ammonium sulfate and the precipitate, collected at 40% saturation, was dissolved in water, dialyzed in 20 mM Tris, pH 7.5, passed through a column of DE 52 (2.6 x 15 cm), concentrated on a PM 10 Amicon membrane and then stored at -20°C.

Results

Purification of the Enzyme. Frozen flies were homogenized in a Waring blender with four volumes of 5 mM potassium phosphate, 0.1 mM EDTA, pH 7.8. The homogenate was clarified by centrifugation at 20,000 x g for one hour at 4°. After passing the supernatant through glass wool, the solution was brought to 35% with solid ammonium sulfate. The precipitate which formed after one hour stirring at 4°C was removed by centrifugation and the supernatant was brought to 80% saturation with $(NH_4)_2SO_4$. The precipitate, collected by centrifugation, was suspended in a minimal volume of chilled 50 mM potassium phosphate, 0.1 mM EDTA, pH 7.8, and was dialyzed against several changes of the same buffer. The dialyzed solution was then treated with 0.25 volume of ethanol and 0.05 volume of chloroform. Although, the superoxide dismutase withstood chloroform-ethanol treatment, it quickly lost its activity when salted out by K_2HPO_4, a procedure previously described for Cu-Zn superoxide dismutases (11-18). Therefore, the following modification was adopted to purify the superoxide dismutase from Drosophila: the precipitate, which formed after stirring with chloroform-ethanol mixture for 1/2 hr at 4°C, was removed by centrifugation and the supernatant (∼200 ml) was concentrated by lyophilization. The concentrated sample (∼25 ml) was dialyzed exhaustively against several changes of 2.5 mM potassium phosphate, pH 8.4, in the cold and was then adsorbed onto a column (5 x 20 cm) of DE-52 which had been previously equilibrated with this buffer. The column was washed with 400 ml of the buffer and a linear gradient of potassium phosphate (2.5 mM ⟶ 200 mM at pH 8.4, in a total volume of 2 liter) was then applied and 20 ml fractions were collected. The results of this chromatographic procedure are shown in Figure 1. Fractions having a specific activity in excess of 100 units of superoxide dismutase per mg of protein were pooled (∼200 ml) and concentrated to about 15 ml by ultrafiltration over a Diaflow PM-10 membrane. The enzyme was then applied to a column of Sephadex superfine G-75 (5 x 58 cm) which had been equilibrated with 50 mM potassium phosphate, 0.1 mM EDTA, pH 7.8. The active fractions were eluted as a small peak followed by a symmetrical large peak which was congruent with a peak of 280 nm absorbance (Figure 2). The fractions (20 ml) having a total of 5,000 units or more of superoxide dismutase activity were pooled. SDS acrylamide gel electrophoresis revealed that at this stage of purification, some proteins, noticeably of approximately 27,000 molecular weight, were still not completely removed from the superoxide dismutase

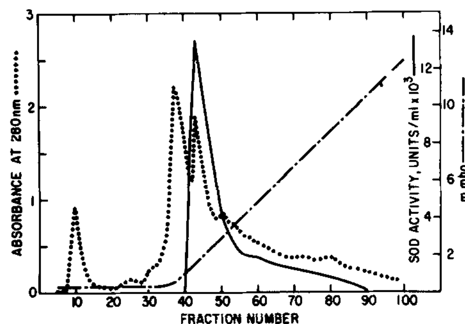

Figure 1. Chromatography of SOD on DE-52 column (5 x 20 cm) as described in the text. Protein (dotted line) and superoxide dismutase (solid line) activity were monitored. The conductivity of the effluent is indicated by the dot-and-dash line.

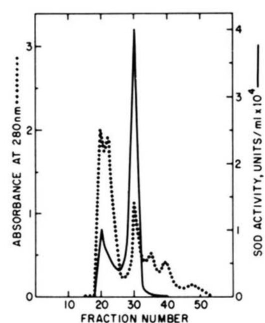

Figure 2. Chromatography of SOD on Sephadex G-75 superfine column, as described in the text. The flow rate of about 40 ml per hour was obtained by using a peristaltic pump. Dotted and solid lines show protein elution profile and superoxide dismutase activity respectively.

preparation. Final purification of superoxide dismutase was accomplished by preparative electrofocusing on Sephadex G-75 superfine gel using a pH range of 3.5 —>5.5 ampholine. The enzyme was freed from ampholyte by gel exclusion chromatography using a Sephadex G-75 column (2 x 55 cm). The results of this purification procedure are summarized in Table I.

Molecular weight and quaternary structure - Molecular weight was determined by gel filtration on a Sephadex G-75 superfine column (1.5 x 98 cm). The column was equilibrated with 5 mM potassium phosphate, 0.2 M KCl, pH 7.8, and calibrated with the following molecular weight standards: bovine serum albumin, 67,000; ovalbumin, 45,000; chymotrypsin A, 25,000; and ribonuclease, 13,700. The molecular weight of the purified superoxide dismutase was found to be 32,000 by this method. Subunit molecular weight was determined by sodium dodecylsulfate gel electrophoresis as previously described (52). The standards used to make a plot of log molecular weight versus mobility of protein band were: phosphorylase b (100,000), bovine serum albumin (67,000), ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (21,000) and lysozyme (14,300). The superoxide dismutase exhibited mobility consistent with a molecular weight of 16,000 in the absence of β-mercaptoethanol and in its presence 16,500. These results imply that the superoxide dismutase of D. melanogaster is composed of two subunits of equal size held together by noncovalent interaction. No other bands were visible on these gels (Figure 3).

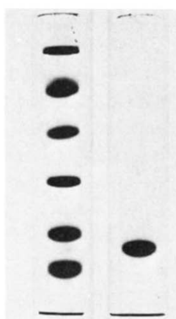

Figure 3. SDS gel electrophoresis. The gel in left has standard proteins as described in the text; the gel in right has the purified Drosophila superoxide dismutase.

Table I. Purification of Superoxide Dismutase from D. melanogaster

Fraction	Volume (ml)	Total Protein (mg)	Total Units	Specific Activity (units/mg)	Yield (%)	Purification
Soluble extract*	1,020	6,120	250,000	40.8	100	1
35% $(NH_4)_2SO_4$* supernatant	1,100	4,400	250,000	56.8	100	1.4
80% $(NH_4)_2SO_4$* precipitation	130	2,500	230,000	92	92	2
Ethanol-chloroform* treatment	36	1,100	230,000	209	92	5
DEAE column	15	367	150,000	408	60	10
Sephadex G-75 column	120	68	80,000	1,170	32	29
Isoelectrofocusing	1	2	20,000	10,000**	8	245

*One milliliter of each fraction was dialyzed against 50 mM potassium phosphate - 0.1 mM EDTA, pH 7.8, in an exhaustive way. The samples were then centrifuged to remove precipitates. After normalizing the sample volume, the assay for SOD was undertaken.

**When based upon absorbance at 215-225 nm, this specific activity was 4,800. In parallel studies when protein and activity of both Drosophila enzyme and bovine erythrocyte enzyme were measured, the specific activity of Drosophila superoxide dismutase was 1.6 times more than the erythrocyte superoxide dismutase.

Amino acid composition and serological cross reactivity - Table II presents the amino acid composition of D. melanogaster superoxide dismutase. In comparison with the amino acid compositions reported for all other superoxide dismutases, D. melanogaster superoxide dismutase exhibits notable differences which suggest differences in structure which might be reflected in immunologic specificity. Therefore the cross reactivity of D. melanogaster superoxide dismutase was tested by a microcapillary precipitin method (53) with rabbit antibodies to bovine erythrocyte Cu-Zn-, E. coli Mn- and E. coli Fe-superoxide dismutases. All of these antisera were reactive with their specific antigens, yet none of them cross-reacted with the D. melanogaster enzyme.

Metals and inhibitors - Atomic absorption spectroscopy indicated 2.1 moles of Cu^{++} and 2.2 moles of Zn^{++} per 32,000 g of this superoxide dismutase. As described previously, the Cu-Zn superoxide dismutases in general are more sensitive to cyanide (54) and are less sensitive to azide (52) than the corresponding iron or Mn superoxide dismutases. The D. melanogaster enzyme behaved like other Cu-Zn superoxide dismutases in this respect. Thus, 1 mM cyanide inhibited 92% of this superoxide dismutase activity when measured by cytochrome c assay (11), and 3 mM cyanide virtually eliminated its activity when analyzed by gel electrophoresis (47). Azide at 5 and 10 mM inhibited the D. melanogaster enzyme by 15 and 30%, respectively.

Isoelectric point - D. melanogaster superoxide dismutase was purified to homogeneity by preparative electrofocusing on Sephadex G-75 superfine gel in a pH 3.5 - 5.5 ampholine buffer. The applied voltage was increased gradually (100 V/4 hrs) from 450 V to a maximum of 1.05 kV. After 12 hr at 1.05 kV the fluid was collected in 1 ml fractions, which were assayed for pH, absorbance at 230 nm and enzymic activity. The zone of protein containing superoxide dismutase activity had an apparent isoelectric point of 5.3.

Ultraviolet spectrum - The purified superoxide dismutase exhibited an absorption spectrum which lacked the 280 nm maximum usually associated with proteins, indicating that the D. melanogaster superoxide dismutase, like the corresponding Cu-Zn enzymes (11-19), is devoid of tryptophan. The spectrum of the enzyme in the ultraviolet region was similar to the absorption spectrum of phenylalanine and is shown in Figure 4. The molar extinction coefficient at 258 nm was 13,200 $M^{-1}cm^{-1}$.

Table II. Amino Acid Analyses[a]

Amino Acid	Residue/16,000 A[b]	Residue/16,000 B[c]
Asx	15.19	-
Thr	7.86	8.25
Ser	7.54	8.10
Glx	9.26	-
Pro	5.29	-
Gly[d]	22	-
Ala	10.21	-
Val	13.13	14.02
1/2 Cys	4.22	-
Met	0.80	-
ILe	7.37	7.98
Leu	6.36	6.69
Tyr	0.88	-
Phe	5.16	-
His	7.28	-
Lys	9.45	-
Arg	3.05	-

a. Values shown are average of two independent analyses.

b. Values obtained from 24 hydrolyzate.

c. Values obtained from 24, 48 and 72 hr hydrolyzate and corrected for time-dependent losses by extrapolating to zero time.

d. The relative number of residues for each amino acid per subunit molecule was calculated by assigning 22 residues to glycine arbitrarily to give the best fit for an enzyme of subunit 16,000.

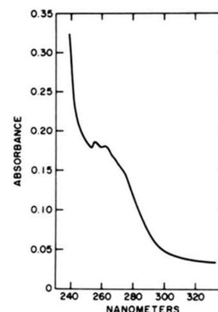

Figure 4. Absorption spectrum of superoxide dismutase in the ultraviolet. The enzyme was at 450 μg per ml in 0.05 M potassium phosphate at pH 7.8.

Polyacrylamide gel electrophoresis - The crude soluble extract of D. melanogaster was analyzed by gel electrophoresis (40), as was the purified superoxide dismutase. Protein was visualized by staining with amidoblack, whereas superoxide dismutase activity was negatively stained by a photochemical procedure as described (47). The crude extract of D. melanogaster exhibited several bands of color during electrophoresis but only one band of cyanide-sensitive superoxide dismutase activity was immediately evident. However, a cyanide-resistant second band (Rf. 1.6) of enzyme activity did appear in the gels by using the crude extract incubated for one week at 4°C. This incubation also precipitated some of the brown colored proteins which were overlapping on the second, minor (<5%) band of superoxide dismutase in the gels. The purified enzyme gave only one discernible band of protein, which coincided with the zone of enzymatic activity.