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Permalink
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Journal
The Journal of biological chemistry, 256(16)

ISSN
0021-9258

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Publication Date
1981-08-01

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Purification and Properties of Superoxide Dismutase from Drosophila melanogaster

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

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

The major superoxide dismutase ("slow" electrophoromorph) of the fruit fly, Drosophila melanogaster, has been purified to homogeneity. This enzyme contains 2 Cu²⁺ and 2 Zn²⁺/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 O₂⁻, 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 leiognathi (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 the hypothesis of a sybetic 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 algae, 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 radioreistance 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

Cell-free extracts of the fruit fly, D. melanogaster, contain two superoxide dismutases which are separable on polycrylamide 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 superoxide dismutases, this enzyme could be purified using the reverse technique.

DISCUSSION

Portions of this paper (including "Materials and Methods," "Results," Figs. 1-4, and Tables 1 and 2) 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 photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Superoxide Dismutase from Drosophila

Superoxide dismutases, the Drosophila enzyme ("slow" electromorph) quickly lost its activity when salted out of an ethanol-rich phase with K₂HPO₄. 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 enzyme from 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 Silverman for atomic absorption analysis of the metals.

REFERENCES

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Supplementary Material

PURIFICATION AND PROPERTIES OF SUPEROXIDE DISMUTASE FROM DROSOPHILA MELANOGASTER

Young-Moo Lee, Francisco J. Ayala, and Hara P. Mitera

Materials and Methods

In-millimeter files were made isogonic for the whole third chromosome by crossing flies collected in Tuxteco, Mexico, with the balancer stock 106R as described (45). One of the isogonic lines was maintained and collected at 24°C days of age. These flies were frozen and stored at -20°C until used. Cytoplasmic (class III), cuticular and sandwich salivary glands were products of Stock. Micrococcus luteus (1) cell culture and superoxide dismutase were obtained from Worthington Biochemicals, respectively. Reagents were purchased from RBI.

Superoxide dismutase was assayed and units were defined as previously described (45). Electrophoresis on polyacrylamide gels was performed according to Davis (46). Zones of pellets were eluted with buffers containing sodium dodecyl sulfate (45), while zones of superoxide dismutase were negatively stained as the photometric procedure previously described (47). Electrophoretic migration was performed at 10°C in a sliding model 285 spectrophotometer and optical spectra were recorded with a Cary model 179 spectrophotometer. An O.D. 210 mullerator system was used for preparative electrophoresis. Protein was measured by the spectrophotometric method of Lowry (48) with recrystallized bovine serum albumin as the standard.

Amino acid analysis was performed on a Durrum model 5200 amino acid analyzer using a single column, three buffer elution program. Protein samples were hydrolyzed for 24, 48 and 72 hr at 110°C in vacuo in the presence of a small crystal of phenol (49). Half system was determined as cysteine and after oxidation of the protein sample with performic acid (50). For the determination of tryptophan residues, samples were hydrolyzed in 6 N HCl at 110°C in vacuo. Tryptophan was determined by gel filtration on a Sephadex G-75 superfine column (1.6 x 90 cm), followed by elution with the presence of water-dodecyl sulfate-negative staining of the protein samples was performed by atomic absorption spectrophotometry with a Perkin-Elmer model 303.

Results

Purification of the enzyme. Frozen flies were homogenized in a Waring blender with four volumes of 0.1 M potassium phosphate, pH 7.0, 0.1 M dithioerythritol, and 0.1 M ethanol. The homogenate was clarified by centrifugation at 25°C, 10,000 g for one hour. 4℃ After passing the sample through a 300-μm diameter syringe needle, the supernatant was poured into a 300-μm diameter syringe needle. The precipitate, collected by centrifugation, was suspended in a minimal volume of chilled 0.1 M potassium phosphate, 0.01 M EDTA, pH 7.0, and was dialyzed against several changes of the same buffer. The dialyzed solution was treated with 0.25% volume of ethanol and 0.05% volume of chloroform. Although the superoxide dismutase was treated with chloroform-ethanol treatment, it quickly lost its activity when stored out by light. The activity was obtained by Sephadex G-75 gel filtration. The following procedure was repeated to purify the enzyme. Two or three enzyme solutions were prepared from Sephadex G-75 column at 25°C, 10,000 g for 45 minutes. The enzyme, after centrifugation, was shown to be 100% active when assayed by spectrophotometrically monitored changes in absorbance. The enzyme was then purified by electrophoresis on a Sephadex G-75 column (1.6 x 90 cm) which was equilibrated with 50 μM potassium phosphate, 0.1 M Tris, pH 7.0. The active fractions were dialyzed a small scale followed by a semi-preparative large scale which was collected with a peak of 280-μm absorbance (Figure 1). The fractions (20 μl) having a total of 3,000 units or more of superoxide dismutase activity were pooled. SDS-acrylamide gel electrophoresis showed that the enzyme is not contaminated with other proteins. The enzyme (ammoniacal phenol) molecular weight, still not completely removed from the superoxide dismutase.

Figure 1. Chromatography of SDS on DE-52 column (5 x 30 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 thick dashed line.
Preparation. Final purification of superoxide dismutase was accomplished by preparative electrophoresis on aephape G-75 superfine gel using a pH range of 3.5–10.8 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.

Table I. Purification of Superoxide Dismutase from D. melanogaster

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Soluble extract*</th>
<th>DEAE column</th>
<th>Sephadex G-75 column</th>
<th>Isoelectric focusing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>1,020</td>
<td>36</td>
<td>120</td>
<td>1</td>
</tr>
<tr>
<td>Total Protein (mg)</td>
<td>6,120</td>
<td>2,560</td>
<td>80</td>
<td>2</td>
</tr>
<tr>
<td>Total Units</td>
<td>250,000</td>
<td>230,000</td>
<td>80,000</td>
<td>20,000</td>
</tr>
<tr>
<td>Specific Activity (units/mg)</td>
<td>40.8</td>
<td>92</td>
<td>1,170</td>
<td>20,000</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>100</td>
<td>92</td>
<td>92</td>
<td>2</td>
</tr>
</tbody>
</table>

Note: *The high viscosity of each fraction was adjusted against 50 mM potassium phosphate - 0.5 mM EDTA, pH 7.0, in an exhaustively lyophilized. The samples were then centrifuged to remove precipitates. After normalizing the sample volume, the assay for SOD was undertaken.

**When based on absorbance at 275–295 nm, this specific activity was 4.000. 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 2.0 times higher than the erythrocyte superoxide dismutase.

Table II. Amino Acid Analysis

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residue/10,000</th>
<th>µC</th>
<th>µC</th>
<th>µC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>15.10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thr</td>
<td>7.94</td>
<td>6.25</td>
<td>6.70</td>
<td>6.70</td>
</tr>
<tr>
<td>Gly</td>
<td>9.26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pro</td>
<td>5.67</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ala</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Val</td>
<td>10.71</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ile</td>
<td>13.13</td>
<td>14.02</td>
<td>14.02</td>
<td>14.02</td>
</tr>
<tr>
<td>Leu</td>
<td>4.22</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Met</td>
<td>7.37</td>
<td>2.93</td>
<td>3.04</td>
<td>3.04</td>
</tr>
<tr>
<td>Phe</td>
<td>6.94</td>
<td>6.69</td>
<td>7.92</td>
<td>7.92</td>
</tr>
<tr>
<td>Trp</td>
<td>5.68</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>His</td>
<td>5.18</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tyr</td>
<td>7.26</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lys</td>
<td>9.45</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Arg</td>
<td>3.05</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

a. Values shown are average of two independent analyses.
b. Values obtained from 24 hours absence.
c. Values obtained from 24, 48, and 72 hours absence.
d. The relative number of residues for each amino acid per subunit mole was calculated by assigning 100 to the one that gives the best fit for an enzyme of 16,000.

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.

Figure 4. Absorption spectrum of superoxide dismutase in the ultraviolet. The enzyme was examined at 450 µm per ml of 0.05 M potassium phosphate at pH 7.0.

Polyacrylamide gel electrophoresis: The crude soluble extract of D. melanogaster was analyzed by gel electrophoresis (40), and was purified as described in the text. Protein was visualized by staining with Coomassie blue, whereas superoxide dismutase activity was negatively stained by a photosensitive procedure as described (47). The crude extract of D. melanogaster cell fractioned at several bands, and only one band of quinone-sensitive superoxide dismutase activity was immediately evident. However, a second enzyme band was also evident (fig. 4). This second band of enzyme activity did appear in the gel by using the semi-dry method to separate the gels, and D. melanogaster band of superoxide dismutase in the gel...