Drosophila Alcohol Dehydrogenase

PURIFICATION AND PARTIAL CHARACTERIZATION*

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SUMMARY

An alcohol dehydrogenase has been purified from two strains of Drosophila melanogaster. This enzyme has a molecular weight of $4.4 \times 10^4$ and shows a broad specificity for secondary alcohols. Crude extracts of flies contain multiple forms of the enzyme. One of these forms has been isolated by ion exchange chromatography.

Drosophila alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) has attracted attention in several laboratories (1-3) recently. Genetic variants of this enzyme were found, and consequently it was possible to determine its structural locus on the genetic map. The specific activity of alcohol dehydrogenase varies greatly along the time axis of development of this organism, and it occurs tissue specifically. Thus alcohol dehydrogenase is of developmental interest. A study of Drosophila alcohol dehydrogenase is also interesting from a comparative viewpoint, since so much is known about mammalian and yeast alcohol dehydrogenase.

The enzyme was first detected through gel electrophoresis of crude fly extracts, and it became immediately apparent that it exists in multiple forms (1-3). When partially purified alcohol dehydrogenase preparations were treated with NAD$^+$, their isozyme patterns could be shifted, indicating that the charge differences of multiple forms of this enzyme might be due to differences in molar ratios of bound coenzyme. In order to test this hypothesis, it was necessary to purify the enzyme.

This report describes the purification and partial characterization of Drosophila alcohol dehydrogenase.

MATERIALS AND METHODS

Organisms—The Oregon-RCII and Dizylic strains of Drosophila melanogaster were used as sources of enzyme in these experiments. The flies were grown in mass culture according to the method of Mitchell and Mitchell (4). They were collected with a vacuum cleaner into a cheesecloth net and immediately etherized and frozen. All experiments reported here were done with flies which had been stored up to 1 month at $-20^\circ$.

Assays—Drosophila alcohol dehydrogenase activity was assayed by a modification of the method of Jacobson and Murphy. The reaction mixture contained NAD$^+$ (1.9 mM), 2-butanol (0.13 M), carbonate buffer, pH 9.5 (0.05 M), and enzyme, in 3 ml. Assays were performed at 340 m$\mu$ in a Zeiss M4 QIII spectrophotometer equipped with a Guilford model 2000 sample absorbance recorder. The cuvette chamber and assay solutions were maintained at 30$^\circ$.

The linearity of the assay with enzyme concentration is shown in Fig. 1. One unit of activity is defined as a change in absorbance at 340 m$\mu$ of 0.001 (1-cm light path) per min. Protein concentration of crude preparations was determined with the biuret procedure of Gornall, Bardawill, and David (5) with bovine serum albumin as a standard. In purified preparations, protein was determined by measuring the adsorption at 280 m$\mu$, assuming an absorbance of 9 for a 1% solution (1-cm light path).

Yeast alcohol dehydrogenase was assayed by measuring the increase in absorbance at 340 m$\mu$ (1-cm light path) for 30 sec of a 3-ml reaction mixture containing pyrophosphate buffer, pH 8.8 (0.01 M), NAD$^+$ (0.0005 M), ethanol (1.1 M), and enzyme. Catalase was assayed by the method of Beers and Sizer (6). One enzyme unit is defined as a change in absorbance at 340 m$\mu$ of 0.001 (1-cm light path) per min in the yeast alcohol dehydrogenase assay and as a change in absorbance at 240 m$\mu$ of 0.001 (1-cm light path) per min in the catalase assay.

Gel Filtration—The Stokes radius of alcohol dehydrogenase was determined according to the method of Ackers (7). Dry Sephadex G-200 was suspended in 0.05 M phosphate buffer, pH 7.5, and allowed to swell for 5 days at 4$^\circ$ during which time fine particles were removed. A 2.5-cm diameter column was filled to a height of 35.5 cm with gel and allowed to run for 24 hours before use.

The sample was applied onto the gel under the buffer with a

1 K. R. Jacobson and J. B. Murphy, in preparation.

3 K. B. Jacobson and J. B. Murphy, in preparation.
Pasture pipette. The final concentration of sucrose in all samples was 2.5%. That of blue dextran 2000 0.1%, in 2 ml of 0.05 M phosphate buffer, pH 7.5. Fractions were collected into preweighed tubes, and the volumes were estimated by weight. Flow rates varied from 25 to 15 ml per hour.

Elution of blue dextran 2000 was used to determine the void volume of the column. The elution positions of yeast alcohol dehydrogenase and catalase were used to calculate the effective gel pore radius of the Sephadex (7). With this parameter, it was possible to calculate the Stokes radius of Drosophila alcohol dehydrogenase.

**Density Gradient Centrifugation**—The procedures of Martin and Ames (8) were followed. Linear 4.6-ml sucrose gradients were generated with 5% and 20% sucrose solutions in 0.05 M phosphate buffer at pH 7.5. The linearity of the gradients was ascertained by addition of 2,6-dichlorindophenol to the 5% sucrose and subsequent measurement of the absorbance of collected fractions at 600 μ. One hundred microliters of sample were applied to each gradient. Centrifugation was performed in the SW-39 rotor of a Spinco model L-2 ultracentrifuge for 12 hours at 4°C. After centrifugation the tubes were pierced with a needle and 10-drop fractions were collected and analyzed.

Additional sedimentation analyses were carried out in the Spinco model E ultracentrifuge at 52,640 rpm at 3.6°C.

**Electrophoresis**—Agar gel electrophoresis was carried out as described previously (3). The procedure for polyacrylamide electrophoresis was essentially that of Davis (9), except that the spacer gel was omitted. Instead we overlaid the sample in 5% sucrose-0.05 M Tris-HCl-0.001 M EDTA, pH 8.3, on the 7.5% acrylamide gel. Electrophoresis was carried out in glass tubes, 0.6 x 9 cm, at 290 volts, 3 ma per tube, for 45 min at room temperature. Gels were removed from the tubes, stained for protein with Amido black (9), and destained by diffusion in 7.5% acetic acid. Alcohol dehydrogenase activity was visualized on the gels with the staining mixture described by Ursprung and Leone (3), except that 2-butanol was substituted for ethanol.

**Chemicals** NAD+ and NADH were purchased from Sigma. Solutions of these coenzymes were prepared just before use. Catalase was purchased from Worthington and Sigma. Yeast alcohol dehydrogenase and protamine sulfate were Sigma products. DEAE-cellulose (1 meq per g) was obtained from Bio-Rad and the Reeve Angel Company. 2-Butanol, cyclohexanol, 2-propanol, and isobutyl alcohol were obtained from Matheson, Coleman, and Rell. These alcohols had a minimal purity of 99% and were used without further purification. Sephadex and blue dextran 2000 were purchased from Pharmacia.

**RESULTS**

**Purification**—All operations were carried out at 0–4°C. Centrifugations were done at 27,000 × g for 30 min. Flies were homogenized in a Servall Omnimixer for 30 to 60 see at approximately 110 volts in 2.5 times their weight of cold, 0.05 M phosphate buffer, pH 7.5. The crude homogenate was centrifuged, the supernatant fluid was removed, and 14 ml of a freshly prepared 2% protamine sulfate solution were added to each 100 ml of the supernatant fluid. After 15 min this solution was centrifuged, the supernatant fluid was removed, and solid ammonium sulfate was slowly added to 40% saturation. After 15 min or more, this solution was recentrifuged, and this time the supernatant fluid was brought to 60% saturation with ammonium sulfate and left overnight in the cold. The next day the protein which had precipitated was centrifuged, the supernatant fraction was discarded, and the yellow-brown precipitate was dissolved in a small volume of 0.05 M phosphate buffer, pH 7.5. This material was then applied to a column of Sephadex G-150 (4.5 × 45 cm) previously equilibrated against 0.05 M Tris-HCl-0.001 M EDTA, pH 8.3 (determined at room temperature). Fractions, 7 ml, were collected at a flow rate of about 50 ml per hour. The fractions containing the bulk of the alcohol dehydrogenase activity were pooled and precipitated by the addition of solid ammonium sulfate to 60% saturation. The precipitated protein was dissolved in a small volume of 0.05 M Tris-HCl-0.001 M EDTA and dialyzed against this buffer overnight. The dialyzed solution was then applied to a DEAE-cellulose column, 2.5 × 30 cm, prepared according to the method of Peterson and Sober (10) and equilibrated with 0.05 M Tris-HCl-0.001 M EDTA, pH 8.3. Protein was eluted with a convex gradient generated from a mixing vessel that contained 250 ml of 0.05 M Tris-HCl-0.001 M EDTA, pH 8.3, and a reservoir containing 250 ml of 0.3 M NaCl in 0.05 M Tris-HCl-0.001 M EDTA, pH 8.3. Fractions of 5 ml were collected at a flow rate of 100 ml per hour.

The summary of the purification procedure is shown in Table I. This represents our best purification to date.

In other experiments the final DEAE-cellulose eluates had specific activities ranging from 30,000 to 87,000 units per mg

**Fig. 1.** Change in absorbance (340 μm, 1-cm light path) per min × 1000 with increasing amounts of enzyme. This enzyme preparation derived from Oregon-RCH flies was purified as described in the text up to and including passage through Sephadex G-150.

**Table I**

<table>
<thead>
<tr>
<th>Purification of Drosophila alcohol dehydrogenase</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg unit/mg</td>
<td>mg unit/mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude supernatant</td>
<td>600</td>
<td>4.1</td>
<td>650</td>
</tr>
<tr>
<td>Protamine sulfate supernatant</td>
<td>1400</td>
<td>4.3</td>
<td>2950</td>
</tr>
<tr>
<td>40 to 60% ammonium sulfate</td>
<td>511</td>
<td>4.4</td>
<td>8630</td>
</tr>
<tr>
<td>Sephadex G-150 eluate</td>
<td>182</td>
<td>3.3</td>
<td>18,000</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>23.4</td>
<td>2.2</td>
<td>94,000</td>
</tr>
</tbody>
</table>
FIG. 2. Staining patterns of alcohol dehydrogenase (Oregon-RCH) after agar gel electrophoresis of aliquots of successive 4-ml fractions eluted from DEAE-cellulose. Pattern to the very right: crude extract of Oregon-RCH.

of protein. This variation is in part due to the different sources of enzyme. The Bethylie strain of Drosophila yields only about half as much alcohol dehydrogenase activity as Oregon-RCH. Also, the specific activity of alcohol dehydrogenase purified from Bethylie is probably lower than that obtained from Oregon-RCH flies.

We have already reported (3) that Bethylie and Oregon-RCH

FIG. 3. Polyacrylamide gel electrophoresis of a purified preparation of alcohol dehydrogenase (Oregon-RCH). The direction of migration was toward the anode. Tube a was stained for protein and tube b for alcohol dehydrogenase activity. This enzyme preparation had a specific activity of 94,000 units per mg; 170 µg were applied to each column in 0.2 ml.

FIG. 4. Absorption spectrum of purified alcohol dehydrogenase at 0.7 mg per ml. This preparation was the same as that in Fig. 3. The buffer was 0.05 M Tris-Cl-0.001 M EDTA, pH 8.3.
SUCROSE DENSITY GRADIENT CENTRIFUGATION
OF DROSOPHILA ADH

**Enzyme Units**

**Fraction Number**

**Sucrose density gradient centrifugation of alcohol dehydrogenase (Bethylie) centrifuged relative to catalase**: 60 µg of alcohol dehydrogenase and 37.5 µg of catalase (Worthington) were applied to the gradient. A 10-µl aliquot was taken from each collected fraction in order to measure alcohol dehydrogenase activity (ADH), and 100 µl in order to measure catalase activity.

**Analytical ultracentrifugation of alcohol dehydrogenase**: The enzyme, at a concentration of 4 mg per ml (specific activity of 46,000 units per mg) in 0.1 M NaCl-0.05 M Tris-HCl-0.001 M EDTA, pH 8.5, was centrifuged at 52,640 rpm. The direction of sedimentation is from left to right. a, b, c, and d represent the sedimentation position at 21, 53, 151, and 183 min after the rotor had attained full speed. The bar angle was 60°.

**Species Elution volume Stokes radius**

<table>
<thead>
<tr>
<th>Species</th>
<th>Elution volume</th>
<th>Stokes radius</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue dextran 2000</td>
<td>58.7 (4)</td>
<td>47.6</td>
</tr>
<tr>
<td>Catalase</td>
<td>84.0 (2)</td>
<td>52.2</td>
</tr>
<tr>
<td>Yeast alcohol dehydrogenase</td>
<td>90.0 (1)</td>
<td>46.2</td>
</tr>
<tr>
<td>Drosophila alcohol dehydrogenase</td>
<td>110.3 (3)</td>
<td>27.2</td>
</tr>
</tbody>
</table>

**SEPHADEX GEL FILTRATION OF DROSOPHILA ADH**

**Gel filtration of alcohol dehydrogenase**: 750 µg of fly alcohol dehydrogenase, 58 µg of catalase, and 5 mg of yeast alcohol dehydrogenase were applied to the column. Fractions, 5 ml, were collected at a flow rate of 25 ml per hour. Samples, 50, 2.5, and 50 µl, were taken from collected fractions in order to measure Drosophila alcohol dehydrogenase, yeast alcohol dehydrogenase, and catalase activity respectively.

**Strains contain multiple forms of alcohol dehydrogenase resolvable by electrophoresis**: Agar gel electrophoresis of crude supernatants or Sephadex G-150 eluates derived from Oregon-RCH flies reveal three bands of enzyme activity (3), designated 1, 3, and 5. When Sephadex G-150 eluates were chromatographed on DEAE-cellulose we were able to separate the fastest cathodally migrating alcohol dehydrogenase band (No. 5) from the remaining bands (Fig. 2). The homogeneity of such fractions was also tested by electrophoresis on polyacrylamide gels. As shown in Fig. 3, both alcohol dehydrogenase and protein stains revealed the presence of one band in the gels.

**Absorption spectrum of purified alcohol dehydrogenase**: The absorption spectrum of purified alcohol dehydrogenase containing only Band 5 is shown in Fig. 4. A 0.1% solution of this alcohol dehydrogenase preparation, prepared by weighing.
FIG. 8. Lineweaver-Burk plot of initial enzyme activity with increasing concentrations of various alcohols. This enzyme preparation was the same as in Fig. 3. Five micrograms of enzyme were added to initiate the reaction. a, cyclohexanol, 2-butanol, and 2-propanol; b, ethanol and isobutyl alcohol.

Determination of Molecular Weight—Early in our study we noticed that the alcohol dehydrogenase isolated from Balthylie and Oregon-RCH strains of D. melanogaster were equivalent in respect to their behavior upon sucrose density centrifugation and gel filtration. With the technique of Martin and Ames (8) we have established the sedimentation coefficient ($s_{20,w}$) of alcohol dehydrogenase to be 3.9 S. This value is an average of four experiments in which Drosophila (Balthylie) alcohol dehydrogenase was centrifuged relative to catalase ($s_{20,w} = 11.3$ (8)) (Fig. 5). Sedimentation velocity studies, performed on the analytical ultracentrifuge, of purified alcohol dehydrogenase obtained from Balthylie flies gave the same values (Fig. 6).

We then estimated the Stokes radius of alcohol dehydrogenase by the method of Ackers (7). Yeast alcohol dehydrogenase and bovine catalase were used to calibrate the columns. With the correlations reported (7) we estimate a Stokes radius of 27.2 Å and assuming a partial specific volume of 0.725 cm$^3$ per g (8), we calculate the molecular weight of Drosophila alcohol dehydrogenase to be 4.4 x 10$^4$.

Substrate Specificity—We have measured the enzymatic reduction of a fixed concentration of NAD$^+$ in the presence of a number of alcohols. Fig. 8 shows a Lineweaver-Burk plot of the data obtained. Notice that 2-butanol, 2-propanol, and cyclohexanol show a marked increase in activity at high substrate concentrations, while the primary alcohols, ethanol and isobutyl alcohol, show little or no increase.

DISCUSSION

Yeast alcohol dehydrogenase and mammalian liver alcohol dehydrogenase have been comprehensively studied and compared with respect to a number of characteristics including substrate specificity and molecular weight (11). We have examined these properties in purified preparations of the alcohol dehydrogenase isolated from D. melanogaster. Drosophila alcohol dehydrogenase shows a marked substrate preference for secondary alcohols. The rate of oxidation is higher for secondary alcohols than for primary alcohols. This is in marked contrast to both the mammalian liver and yeast enzymes, both of which oxidize primary alcohols at a faster rate than secondary alcohols (with the exception that the liver enzyme oxidizes cyclohexanol at about the same rate as ethanol).

With increasing concentrations of some alcohols (cyclohexanol, 2-propanol, and 2-butanol) substrate activation is observed in alcohol dehydrogenase from Drosophila. This phenomenon has also been observed with horse liver alcohol dehydrogenase (12). In this case Dalziel and Dickinson (12) have postulated the formation of an abortive complex of enzyme, NADH, and secondary alcohol. This ternary complex dissociates more rapidly than the normal NADH-enzyme complex. It would be of considerable interest to determine whether this mechanism could account for the substrate activation observed in the present instance.

The molecular weight of Drosophila alcohol dehydrogenase has been found to be about 44,000 as compared to 85,000 reported for liver ADH and 150,000 for the yeast enzyme (11). We cannot draw any conclusions from these values on the subunit composition of the Drosophila enzyme, however, since both liver and yeast alcohol dehydrogenases are tetramers despite vast differences in molecular weight (12).

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REFERENCES
Drosophila Alcohol Dehydrogenase: PURIFICATION AND PARTIAL CHARACTERIZATION
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