Some Physical Properties of Three Sugar Dehydrogenases from a Pseudomonad*

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Recent studies of the evolution of protein molecules have been principally confined to studies of a single protein, such as hemoglobin or cytochrome c, in numerous representatives of the phylogenetic scale (1, 2). Such studies trace the changes in a protein molecule as a function of alterations of its host. A second approach would be to study the evolution of functionally related proteins by comparing their structures. Indeed, investigations of the catalytic sites of enzymes which exhibit esteratic activity have yielded interesting results (3). These latter investigations, however, suffer from interpretive limitations inherent in comparative studies of proteins from various sources; differences may be ascribed to either phylogenetic variation or to alterations in function. Such limitations in interpretation would be minimized if the proteins compared were obtained from a single species.

A number of inducible sugar dehydrogenases are produced by a pseudomonad, and the regulation of these enzymes has been found to be loosely linked (4). Three of these dehydrogenases, aldose dehydrogenase, galactose dehydrogenase, and D-arabinose dehydrogenase, have been highly purified (5), and their catalytic properties have been examined (6). Some of the physical properties of the enzymes are reported here. Because of the marked catalytic similarities of the aldose and galactose dehydrogenases (6), their primary structures have been compared by means of peptide maps, and these comparisons are also reported here.

EXPERIMENTAL PROCEDURE

The enzymes used in these studies were purified and assayed as described previously (5). Protein was determined spectrophotometrically by the method of Warburg and Christian (7).

Measurements of molecular weights, sedimentation coefficients, and diffusion coefficients were obtained by means of a Spinco model E ultracentrifuge. The molecular weights and diffusion coefficients were determined by the method of Ehrenberg (8). For calculations of molecular weights, a partial specific volume of 0.74 was assumed for all the enzymes. Guanidine hydrochloride (purified grade) was recrystallized from water before use. The density of guanidinium chloride solutions was determined pycnometrically.

The estimations of molecular weight by filtration analysis were obtained according to the procedure of Whitaker (9). The cross-linked dextrans employed were Sephadex G-75 and Sephadex G-100 (Pharmacia). The columns (3 x 100 cm) were packed within 6 cm of the top. The packed dextran was first equilibrated with 0.05 M phosphate buffer, pH 6.8, containing 0.001 M EDTA, after which 5 ml of enzyme solution (10 to 50 μg of enzyme per ml) were introduced. The enzyme solution was eluted with the same buffer, and the eluant was collected in 5-ml fractions. The solutions were then assayed for enzyme activity, and the volume required for the elution of the enzyme was determined.

Heat inactivation studies were performed as follows. Two water baths were employed; one was set at the proper temperature; the second was set at a temperature about 10° above the temperature of the experiment. The enzyme solutions (in 0.05 M phosphate, pH 6.8, and 0.001 M EDTA) were heated in the high temperature bath until solution was within 1° of the desired temperature, after which the solution was immediately transferred to the water bath set at the desired temperature. A timer was activated as soon as the transfer was made. The time required to heat 5 ml of enzyme solution from 25° to within 1° of the desired temperature was approximately 30 sec. Aliquots were removed and chilled at given time intervals up to 12 min. The samples were then centrifuged at 10,000 × g for 15 min at 2° and assayed.

Crystalline trypsin (Worthington) was freed of chymotryptic activity by means of the specific inhibitor of chymotrypsin, N-tosylphenylalanylchloromethyl ketone (10). Trypsin (500 mg) was dissolved in 10 ml of Tris-citrate, pH 6.1, with 0.005 M CaCl₂. A 0.1-ml solution of N-tosylphenylalanylchloromethyl ketone (16 mg per ml of absolute ethanol) was then added to the enzyme solution, and the reaction mixture was incubated at 24° for 2½ hours. After incubation, the solution was dialyzed against several changes of distilled water (2 liters each), then lyophilized. This preparation was used for hydrolyzing the enzymes.

Tryptic hydrolysis of enzymes was preceded by the denaturation of the enzymes in 4.2 M guanidinium chloride (containing 0.005 M mercaptoethanol and adjusted to pH 6.8 with NaOH). The guanidinium chloride solution was usually introduced by dialysis of native enzyme solutions against several changes of the denaturant solution. The denaturant was then removed by extensive dialysis against distilled water. The precipitated protein was centrifuged and resuspended in 0.2 M NH₄HCO₃, pH 8.0 (1 ml for each 20 mg of protein). A 0.1-ml solution of saturated phenol red (in 0.2 M NH₄HCO₃, pH 8.0) and a 0.04-ml

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solution of trypsin (27 mg per ml of water) were added to each 0.9 ml of protein suspension. The resulting reaction mixture was then incubated at 37° for 4 hours with occasional shaking. The resulting clear solution was stored at −10° until used.

Peptide map studies were conducted according to the procedures described by Katz, Dreyer, and Anfinsen (11). Descending chromatography in butanol-acetic acid-water (4:1:5, upper phase) was conducted for 38 hours. Electrophoresis in acetic acid-pyridine-water (20:1:288) was carried out for 90 min at 2600 volts. After drying, the chromatograms were stained with ninhydrin (12). In our hands, the absolute reproducibility of the maps was only fair. Peptides were considered to be identical only when cochromatography of hydrolysates of each enzyme gave rise to marked reinforcement of ninhydrin color at the expected positions.

### Table I

<table>
<thead>
<tr>
<th>Dehydrogenases</th>
<th>Property</th>
<th>Mol. wt. in phosphate buffer (ultracentrifuge)</th>
<th>Mol. wt. estimated from Sephadex columns</th>
<th>Mol. wt. in 4.2 M guanidinium chloride (ultracentrifuge)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldose dehydrogenase</td>
<td></td>
<td>6.93</td>
<td>160</td>
<td>40</td>
</tr>
<tr>
<td>Galactose dehydrogenase</td>
<td></td>
<td>7.03</td>
<td>7.0</td>
<td>64</td>
</tr>
<tr>
<td>D-Arabinose dehydrogenase</td>
<td></td>
<td>4.78</td>
<td>4.7</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.60</td>
<td>5.6</td>
<td>50</td>
</tr>
</tbody>
</table>

**Fig. 1.** Molecular weight values as a function of centrifugation time. The molecular weight values were determined according to the method of Ehrenberg (8). Aldose dehydrogenase in phosphate-EDTA, ○; D-arabinose dehydrogenase in phosphate-EDTA, ●; aldose dehydrogenase in 4.2 M guanidinium chloride, (▲); galactose dehydrogenase in 4.2 M guanidinium chloride, □;

**Fig. 2.** Heat inactivation of aldose dehydrogenase. See "Experimental Procedure" for details. Heated in the absence of DPN and assayed with galactose, ●; heated in the absence of DPN and assayed with glucose, ▲; heated in the presence of DPN and assayed with galactose, ○.

**Fig. 3.** Heat inactivation of D-arabinose dehydrogenase. See "Experimental Procedure" for details. Heated in the absence of TPN and assayed with TPN, ●; heated in the absence of TPN and assayed with DPN, ▲; heated in the presence of TPN and assayed with TPN, ○.

**RESULTS AND DISCUSSION**

**Molecular Weights**—There are striking discrepancies between the molecular weight values obtained by sedimentation analysis and those obtained by filtration analysis on columns of cross-linked dextran (Table I). The filtration values for the galactose dehydrogenase and D-arabinose dehydrogenase, 30,000 and 50,000, respectively, are very nearly one-half of the values obtained by sedimentation analysis, 64,000 and 104,000, respectively. For the aldose dehydrogenase the filtration value of
40,000 is one-fourth to one-third of the value of 140,000 obtained by sedimentation analysis. It is probable that these differences reflect a reversible dissociation of enzyme subunits which is concentration-dependent. The initial concentration of enzyme used for filtration analyses varied between 10 and 50 μg per ml, while the concentration used in the sedimentation analyses was about 5 mg per ml. Indeed, Winzor and Scheraga have demonstrated that the observed molecular weights of certain proteins are markedly concentration-dependent when estimated by gel filtration (13, 14). These authors have utilized gel filtration to measure the monomer molecular weights of rapidly associating systems (14). We feel that the molecular weight values obtained by filtration analysis in Table I are those of enzyme subunits. In certain cases, observed molecular weight values measured by the method of Ehrenberg have been observed to decrease with sedimentation time. This decrease has been

![Image](http://www.jbc.org/)
The presence of 0.001 M DPN decreased both the initial aldose dehydrogenase (Fig. 2). The Michaelis constant of DPN molecules do, in fact, exist as such a complex even in highly purified preparations of the enzymes. Such a possibility was tested by subjecting the enzymes to heat inactivation in the presence of DPN. These results are consistent with the view that a single enzyme is involved in each case.

Heat Inactivation—Aldose dehydrogenase has been found to act on both galactose and glucose, and the D-arabinose dehydrogenase has been found to utilize either DPN or TPN (6). Heat inactivation studies were conducted to check whether these multiple activities would decrease to the same relative extent during heating. As shown in Fig. 2, the loss of glucose and galactose activities did occur in a parallel relationship during the heating of aldose dehydrogenase. Similarity, shown in Fig. 3, the DPN and TPN activities of the D-arabinose dehydrogenase decreased in a parallel relationship during heating. These results are consistent with the view that a single enzyme is involved in each case.

The rates of inactivation of aldose and L-arabinose dehydrogenase were biphasic and were each characteristic of two separate first order reactions. These results might be interpreted to mean either that there are two distinct molecular species of each enzyme or that each enzyme exists in two or more configurations at least one of which is significantly less stable than the others. Perhaps the simplest explanation for the biphasic curves is that the enzyme-coenzyme complex is more stable than the free molecules of enzyme, and that a certain fraction of the enzyme molecules do, in fact, exist as such a complex even in highly purified preparations of the enzymes. Such a possibility was tested by subjecting the enzymes to heat inactivation in the presence of 0.001 M coenzyme. As shown by the upper curves in Figs. 2 and 3, the presence of coenzyme did reduce the rates of inactivation, but it did not eliminate the biphasic nature of the curves. The presence of 0.001 M DPN decreased both the initial rapid rate and the secondary slower rate of inactivation of the aldose dehydrogenase (Fig. 2). The Michaelis constant of DPN for aldose dehydrogenase is 0.00025 M (6), and about 80% of the enzyme molecules might be expected to exist as the enzyme-DPN complex in the presence of 0.001 M coenzyme. Extrapolation of the slower rate of inactivation in the presence of coenzyme to zero time yields a value of about 45% of the initial activity. These values, along with the alterations in rates in the presence of DPN, are not consistent with a simple model of a more stable enzyme-DPN complex.

The heat inactivation curves of the D-arabinose dehydrogenase differ from those of the aldose dehydrogenase in one important respect. The slower rate of inactivation in the presence of TPN is very nearly identical with that in the absence of the coenzyme (Fig. 3). Thus, evaluated in terms of stability, the more stable form in the presence of TPN is very similar (if not identical) to that in the absence of the coenzyme. The Michaelis constant of TPN is 0.0022 M (6), and at a concentration of 0.001 M coenzyme, then, approximately 30% of the enzyme molecules would exist as the enzyme-TPN complex. Extrapolation of the upper curve in Fig. 3 to zero time yields a value of approximately 50%.

Peptide Maps—Because of the similarities in their substrate specificities, pH optima, and reaction pathways (6), the peptide maps of galactose dehydrogenase and aldose dehydrogenase have been compared (Figs. 4 and 5). When the tryptic hydrolysates of the enzymes were cochromatographed, a marked increase of intensity was observed for certain spots (Fig. 5). We have tentatively concluded that the two enzymes share 8 to 11 common peptide conformations which are intrinsically more stable.
mon peptide fragments (Fig. 6). It is possible that other common peptides have been masked by the random formation of disulfide bonds since care was not taken to avoid this possibility. The common peptides may reflect the presence of polypeptide subunits common to both enzymes, as has been suggested for the isozymes of lactic dehydrogenase (22), or may indicate common sequences along portions of otherwise distinct polypeptides.

**SUMMARY**

The molecular weights of galactose dehydrogenase, aldose dehydrogenase, and D-arabinose dehydrogenase were found to be 64,000, 140,000, and 104,000, respectively, by ultracentrifugal analysis. The values estimated from gel filtration measurements were considerably smaller, 30,000, 40,000, and 50,000, respectively. A reversible association of subunits was proposed to explain the discrepancy. This proposal was consistent with the low molecular weight values obtained by ultracentrifugal analysis in 4.2 M guanidinium chloride.

The aldose dehydrogenase and D-arabinose dehydrogenase each exhibited biphasic heat inactivation curves which were altered by the presence of coenzyme.

Peptide maps of galactose dehydrogenase and aldose dehydrogenase revealed about eight common peptide fragments, a possible indication of close structural relationships between those two enzymes.

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**REFERENCES**


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