Amino Acid Composition and Properties of Crystalline Lactate Dehydrogenase X from Mouse Testes*

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SUMMARY
Lactate dehydrogenase (LDH) X has been isolated in crystalline form from mouse testes. Homogeneity of the protein preparation was established by disc gel electrophoresis, analytical ultracentrifugation, and immunochemical analyses. LDH-X is clearly distinct in amino acid composition from LDH-1 and LDH-5 with respect to numbers of residues of leucine, glycine, threonine, and methionine. When 6 to 8 moles of p-hydroxymercuribenzoate are bound per mole of LDH-X substantial enzymic activity remains. A molecular weight of 140,000, comparable to that of beef heart LDH-1, was determined for LDH-X by zone velocity sedimentation in an isokinetic sucrose gradient.

When LDH-X is incubated at 65° for 20 min it retains 65% of its original activity as compared to the virtually complete inactivation of LDH-1 and LDH-5.

Substantial evidence is now available that the lactate dehydrogenase (l-lactate:NAD reductase, EC 1.1.1.27) isozyme (LDH-X) of the testis is kinetically, and immunochemically distinct from LDH-1 and LDH-5. These latter two isozymes are homotetrameric products of gene loci which encode polypeptide subunits designated A and B by Markert (1) (M and H by Cahn et al. (2)). Thus, LDH-1 may be represented as BBBA (or HHHH); LDH-5 as AAAA (or MMMM); and LDH-2, 3, and 4 as BBBA, BBAA, BAAA, respectively. A third polypeptide type (C) forms LDH-X, also a tetramer (CCCC), and is the product of a separate gene (3-6). Remarkably, this locus is active only during the primary spermatocyte stage of the spermatogenic cycle and is inactive in all other cells of the organism (7, 8).

Partially purified preparations of LDH-X from the mouse (9, 10), rat (11), rabbit (12, 13), bull (14), and man (15) have been characterized particularly with respect to kinetic properties. While these experiments clearly show that LDH-X is correctly designated as a lactate dehydrogenase, they additionally reveal that this isozyme has distinctive kinetic features, especially with respect to low substrate specificity and capacity to react with coenzyme analogues. The present report presents the crystallization of LDH-X from mouse testes, and the amino acid composition of the protein as well as further observations on the kinetics, substrate specificity, and thermal stability of this isozyme.

EXPERIMENTAL PROCEDURE
Determination of Lactate Dehydrogenase Activity—For routine assay of enzymic activity, the reaction mixture contained (final concentrations): 50 mM sodium phosphate buffer, pH 7.0, 0.27 mM sodium pyruvate, 0.15 mM NADH, and enough lactate dehydrogenase (approximately 0.01 to 0.02 i.u.) to give a decrease in optical density at 25° of between 0.03 and 0.06 per min at a wave length of 340 nm. Catalytic studies were carried out as described previously (9).

LDH-X Purification and Crystallization—Approximately 100 g of testes from Swiss-Webster (CFW) mice were homogenized in 1 liter of 0.02 M Tris-Cl buffer, pH 7.4, in a Waring Blender and allowed to stir at 4° for 1 hr. The extract was poured through a double layer of cheesecloth and centrifuged at 13,000 x g for 45 min. The supernatant was heated in a water bath to 55° and maintained at that temperature for 5 min, after which it was cooled in ice and then centrifuged for 40 min at 13,000 x g. Solid ammonium sulfate was added to 40% saturation and the preparation was allowed to stand overnight at 4°. The precipitate was discarded, ammonium sulfate was added to 70% saturation and the preparation was allowed to stand at 4° for 2 hours before centrifugation at 13,000 x g for 45 min. The precipitate was discarded, ammonium sulfate was added to 70% saturation, and the preparation was allowed to stand overnight at 4°. The precipitate was collected by centrifugation at 27,000 x g for 30 min. The dialysate was clarified by centrifugation at 27,000 x g for 40 min and then layered on a DEAE-Sephadex (Pharmacia, Uppsala) column (3.8 X 20 cm) which had been equilibrated with 0.02 M Tris-Cl buffer, pH 7.4, at 4° and dialyzed against several 20-volume portions of the same buffer for 48 hours. The dialysate was clarified by centrifugation at 27,000 x g for 40 min and then layered on a DEAE-Cellulose column (Pharmacia, Uppsala) column (3.8 X 20 cm) which had been equilibrated with 0.02 M Tris-Cl, pH 7.4, also used as the elution buffer. These fractions containing the highest specific activity were combined and concentrated with an Amicon Diaflo apparatus so that the protein concentration at this stage was approximately 10 mg per ml. Ammonium sulfate was added slowly to 40% saturation and the preparation kept at 4° for about a week until crystals appeared. Subsequently, it was possible to achieve crystallization of LDH-X within 24 hours by seeding with these first crystals.

Analytical Methods—Protein was determined spectrophoto-
metrically according to the method of Warburg and Christian (16). A molar extinction coefficient of 1.88 × 10^4 for rat lactate dehydrogenase (17) was used for crystalline LDH-X. Gel electrophoresis was carried out as described previously (18). The amino acid composition of LDH-X was obtained according to standard procedures in a Beckman-Spinco amino acid analyzer (model 120C), following hydrolysis under reduced pressure in 6 N glass triple distilled HCl for 12, 24, and 72 hours.

Centrifugation of crystalline LDH-X was carried out in a Spinco model E ultracentrifuge at a rotor speed of 60,000 rpm and a temperature of 20°. Zone velocity sedimentation in a sucrose gradient and molecular weight calculation was performed according to the method of Noll (19). The LDH-X crystals were harvested, dissolved in 0.1 M sodium phosphate buffer, pH 7.0, to a concentration of approximately 10 mg per ml, and dialyzed for 24 hours against two changes of this same buffer prior to centrifugation.

Antiserum to LDH-X was produced by immunizing albino female rabbits with injections of crystalline enzyme at a concentration of 2 mg per ml suspended in Freund's adjuvant. Each rabbit received a 0.5-ml injection twice weekly, intramuscularly, for 4 weeks. The first bleeding, by cardiac puncture was done in the 5th week. Subsequent bleedings were at weekly intervals. Antibody specificity was established by the Ouchterlony diffusion method applied as recommended by Stollar and Levine (20).

The reaction of LDH-X sulfhydryl groups with HMB was measured according to the method of Beyer (21). The crystalline enzyme was dissolved in and dialyzed for 24 hours against 0.1 M phosphate buffer at pH 7.0 before use. To assess the effect of sulfhydryl group binding on enzymic activity aliquots of the LDH-X-HMB incubation mixture were removed, quickly diluted in 0.1 M phosphate buffer, pH 7.0, at 4°, and assayed as described above. The HMB solution was standardized against reduced glutathione.

RESULTS

LDH-X accounts for approximately 40% of the total lactate dehydrogenase activity in extracts of mature mouse testes (8) and seems to be the sole catalyst for pyruvate reduction in spermatocytes (18, 22). While mouse LDH-X and LDH-5 have coincident electrophoretic mobilities on polyacrylamide gels (18) there is virtually no detectable LDH-5 in mature testes (23). Thus, LDH-1, -2, and -3 are responsible for the remainder of the lactate dehydrogenase activity in this tissue. During the purification procedure, heating the extract is made possible by the thermal stability of LDH-X (9) while the other isozymes are denatured. The results of a typical purification are presented in Table I.

A single protein band which was coincident in electrophoretic mobility with the zone of LDH-X activity was observed by disc electrophoresis in polyacrylamide gels. Deliberate overloading of gels failed to reveal the presence of contaminating protein. Double diffusion analysis of antisera produced by rabbits injected with crystalline LDH-X indicated immunological homogeneity, since a single precipitation line of identity was obtained from LDH-X and from crude extracts of testis (Fig. 2). In no case was a reaction observed with homogeneous preparations of mouse LDH-1 and LDH-5 at several concentrations of antiserum and of enzyme. Further evidence of the immunological specificity of LDH-X has been presented previously (24).

The amino acid composition of mouse LDH-X is given in Table II. These data for LDH-X are compared with literature
**TABLE II**

Amino acid composition of LDH isozymes*

<table>
<thead>
<tr>
<th>Residue</th>
<th>LDH-X</th>
<th>LDH-1</th>
<th>LDH-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>104±7.4</td>
<td>96</td>
<td>111</td>
</tr>
<tr>
<td>Histidine</td>
<td>32±1.2</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>Arginine</td>
<td>41±2.9</td>
<td>33</td>
<td>41</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>155±2.4</td>
<td>149</td>
<td>127</td>
</tr>
<tr>
<td>Threonine</td>
<td>80±6</td>
<td>57</td>
<td>45</td>
</tr>
<tr>
<td>Serine</td>
<td>108±3</td>
<td>102</td>
<td>95</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>108±3.9</td>
<td>111</td>
<td>114</td>
</tr>
<tr>
<td>Proline</td>
<td>48±1.9</td>
<td>45</td>
<td>47</td>
</tr>
<tr>
<td>Glycine</td>
<td>144±3.7</td>
<td>92</td>
<td>97</td>
</tr>
<tr>
<td>Alanine</td>
<td>83±2.4</td>
<td>81</td>
<td>78</td>
</tr>
<tr>
<td>Valine</td>
<td>130±4.3</td>
<td>144</td>
<td>141</td>
</tr>
<tr>
<td>Methionine</td>
<td>13±6.1</td>
<td>34</td>
<td>27</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>74±2.5</td>
<td>87</td>
<td>90</td>
</tr>
<tr>
<td>Leucine</td>
<td>179±7.0</td>
<td>138</td>
<td>140</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>25±3.1</td>
<td>26</td>
<td>28</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>23±0.0</td>
<td>22</td>
<td>28</td>
</tr>
</tbody>
</table>

*Values in residue per molecule, assuming a molecular weight of 140,000.

Standard deviation of three analyses.

Data of Wieland and Pfleiderer (25) recalculated for a molecular weight of 140,000 for lactate dehydrogenase from rat tissues.

Analysis of data of Pesce et al. (26).

Values were made by comparing values obtained from companion 24- and 72-hour hydrolysates and applying the calculation described by Moore and Stein (27). Thus, S.D. values were not calculated.

The most distinctive comparative difference in amino acid composition of these isozymes is the markedly greater number of residues of leucine, glycine, and threonine, and fewer methionyl residues in LDH-X. Otherwise the values obtained closely approximate those reported for either LDH-1 or LDH-5, with perhaps more similarities seen between the C and B than the C and A polypeptides.
Inhibition decreases from 26% at 32° to 11% at 37°.

The response of LDH-X to this substrate concentration, however, markedly altered at these temperatures with inhibition decreasing from 26% at 32° to 11% at 37°.

Of particular interest is the change in apparent $K_m$ values for LDH-X, -5, and -X in response to temperature (Table III). These values increase virtually linearly for LDH-1 and -5 between 25 and 37°; for LDH-X there is essentially no change in substrate affinity between 25 and 32°, but about a 40% increase in $K_m$ occurs between 32 and 37°.

### Table III

<table>
<thead>
<tr>
<th>Isoenzyme</th>
<th>$K_m$ ( \mu M \times 10^3 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH-X</td>
<td>2.6  6.6</td>
</tr>
<tr>
<td>LDH-1</td>
<td>4.2  6.8</td>
</tr>
<tr>
<td>LDH-5</td>
<td>12.0 22.0</td>
</tr>
</tbody>
</table>

$K_m$ values as well as a decrease in the extent of substrate inhibition. Excess pyruvate has virtually no effect on the degree of inhibition, approximately 15% by 1.0 mM substrate, of LDH-1 at 25, 32, and 37°. The response of LDH-X to this substrate concentration is, however, markedly altered at these temperatures with inhibition decreasing from 26% at 32° to 11% at 37°.

The values in the table were calculated from Lineweaver-Burk plots of substrate saturation data obtained as described previously (9).

#### Discussion

That LDH-X must be related to the other lactate dehydrogenases is suggested by the heterotetramer formation in vitro between C and B as well as C and A subunits (18, 31). The data reported here on the amino acid composition of LDH-X clearly supports this conclusion. The amino acids, histidine, glutamic acid, proline, alanine, valine, isoleucine, leucine, and tyrosine vary only slightly or not at all between LDH-1 and LDH-5 (25, 26). In LDH-X other than for isoleucine which is low and leucine which is exceptionally high, the amino acid composition is very similar to that of the other lactate dehydrogenases. As noted above, the glycine and threonine content of LDH-X is also much higher than in the other isozymes. The differences in primary structure of LDH-X, with the previously reported immunological (24) and genetic (5) data, establish unequivocally that this isozyme is indeed a distinct gene product rather than an epigenetic modification of A or B subunits, or both.

In their study of the function of sulfhydryl groups in 19 species of lactate dehydrogenases Fondy et al. (29) found that generally four thiol groups per molecule were essential for the catalytic operation of the various tetramers. On the basis of the present data the only clear conclusion permissible is that LDH-X differs from other lactate dehydrogenases in reactivity of its thiol groups with HMB. This implies that differences in the active sites of the isozymes exist.

Recently Zinkham et al. (32) demonstrated that the molecular size of mouse LDH-X was identical with LDH-1 and LDH-5 in a Sephadex gel filtration system. This finding is confirmed here by constant velocity sedimentation of crystalline LDH-X in an isokinetic sucrose gradient (10) from which a molecular weight of 140,000 is obtained. In a brief report Wong et al. (33) state that the "crystalline enzyme from mouse testes has a weight average molecular weight of 139,465 ± 5941 and a subunit weight average molecular weight of 37,684 ± 842," as determined by ultracentrifugation. Schatz and Segal (11) report a molecular weight of 125,000 for LDH-X from rat testes.

In general, the kinetic properties obtained for crystalline LDH-X agree with data from previous work (9, 10). When each substrate is assayed at the concentration which gives maximal initial reaction velocities, pyruvate, representing 100% activity, is about 30% more active than a-ketoglutarate and 40% more active than a-ketovalerate. The metabolic significance of this broad substrate specificity is difficult to equate with what is presently known about testes and sperm metabolism (34, 35) and may in fact be of no biological consequence. Schatz and Segal (11) have suggested that a-ketoglutarate reduction might serve a hormonal-like role in the process of fertilization itself. In any case, the primary function of LDH-X most certainly involves the pyruvate-lactate interconversion.

The low turnover number for LDH-X may be of considerable physiological consequence. Production of large quantities of lactate by spermatozoa would inhibit the motility of the cells and effectively impair their functional capacity. In contrast, the lactate dehydrogenases of muscle (LDH-5) have high turnover numbers (26); however lactate is rapidly removed from its site of production via the circulatory system to be metabolized in the liver, under normal circumstances. Obviously, such an arrangement is not available to the spermatozoa enroute to its ultimate destination, the ovum. Thus far, LDH-X has been detected only in animals in which internal fertilization occurs (7, 18, 22, 24).

The metabolic regulation of the pyruvate level by LDH-X could conceivably be related to the adenyl cyclase system of sperm. Garbers et al. (36) found that incubation of bovine epididymal sperm with pyruvate and phosphodiesterase inhibitor resulted in an increased level of cyclic AMP, as well as increased respiration and enhanced motility. The kinetic characteristics of LDH-X indicate that its high affinity for pyruvate could keep the level of this substrate reduced at 32° (scrotal temperature) in epididymal sperm which are nonmotile and have a relatively low respiratory rate. In contrast, ejaculated sperm are subjected to the higher temperature of the female reproductive tract at which time the spermatozoa become vigorously motile and show an increased respiratory rate. At the higher temperature, 37°, LDH-X shows a markedly decreased affinity for substrate.

Thus, LDH-X could serve to regulate pyruvate levels differentially, according to its environment, and this in turn could contribute to the control of cyclic nucleotide levels, sperm respiration, and motility. While it is certainly true that the kinetic properties of an enzyme in vitro cannot directly be extrapolated to explain its biological properties in vivo, nevertheless such suggestions should be made as a basis for study in vivo. Whether its role is strictly metabolic (i.e., in glycolysis) or not, LDH-X undoubtedly plays a significant part in sperm physiology and this must be reflected in its unique properties.

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