Crystallization and Properties of Aldolase from a Transplantable Rat Sarcoma

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SUMMARY

Aldolase was isolated in a crystalline form in a 6% yield from rat Rhodamine sarcomas. The purification involved extraction with Tris-buffer, batchwise treatment with diethylaminoethyl-cellulose, ammonium sulfate fractionation, column chromatography on cellulose phosphate, and crystallization from ammonium sulfate solution (0.35 saturation). The crystalline aldolase preparation behaved as a homogeneous protein during electrophoresis and ultracentrifugal sedimentation. Aldolase of Rhodamine sarcoma was identical to aldolase A of normal rat muscle according to the following criteria: the crystalline aldolase had a specific activity of 12.5 units/mg protein; \( K_m \) values were \( 4 \times 10^{-5} \) M for fructose 1,6-diphosphate and \( 1 \times 10^{-2} \) M for fructose 1-phosphate; the activity for fructose 1,6-diphosphate was depressed to 6% of the original by carboxypeptidase A and inhibited completely by anti-aldolase A antibody but not at all by anti-aldolase B antibody; the fructose 1,6-diphosphate activity was inhibited by adenine nucleotides, most efficiently by adenosine triphosphate and less by adenosine diphosphate and adenosine monophosphate.

INTRODUCTION

In the highly malignant and fast-growing hepatomas of the rat, such as Yoshida ascites hepatomas, we observed that aldolase B (fructose 1,6-diphosphate D-glyceraldehyde-3-phosphate-lyase, EC.4.1.2.7), found in the normal liver, disappeared and aldolase A (EC.4.1.2.13) found in the muscle appeared instead (8, 15). In the less malignant and slowly growing hepatomas of the rat, Morris hepatomas 7793 and 7316A, and both aldolase A and B and their hybrid molecules, were observed (8). These situations were interpreted as a "switch-off" of gene for aldolase B and a "switch-on" of gene for aldolase A during the carcinogenic processes (8, 15). These findings were in agreement with the results reported for human hepatomas by Schapira et al. (13) and for rat experimental hepatomas by Rutter et al. (10), Adelman et al. (1), and Farina et al. (5). Whether or not aldolase found in the malignant tumor is exactly the same as the aldolase A found in the muscle remained to be established.

In this paper, we describe a method of preparation of crystalline aldolase from the highly malignant rat Rhodamine sarcoma (17), which is known to produce aldolase with a fructose 1,6-diphosphate/fructose 1-phosphate ratio of 50. The enzymatic and immunologic properties of crystalline aldolase from this tumor were investigated in comparison with crystalline aldolase A from rat muscle.

MATERIALS AND METHODS

Substrates and Cofactors. Fructose 1,6-diphosphate, crystalline tetracyclohexylammonium salt, and fructose 1-phosphate, crystalline dicyclohexylammonium salt, were obtained from the Boehringer Mannhein Corp., Mannheim, Germany. NADH \(^2\), ATP, ADP, and AMP were products of the Sigma Chemical Co., St. Louis, Mo.

Enzymes. Glyceraldehyde 3-phosphate dehydrogenase and the mixture of glycerol 1-phosphate dehydrogenase and triosephosphate isomerase were obtained from the Boehringer Mannhein Corp. Carboxypeptidase A was from the Worthington Biochemical Corp., Freehold, N. J. Crystalline aldolase A was prepared from rat muscle by the procedure of Taylor et al. (16) and aldolase B from rat liver by the method of Matsushima et al. (7). Protein was determined by the method of Lowry et al. (6) or by measurement of absorbancy at 280 nm.

Tumors. Rhodamine sarcoma (17) was transplanted subcutaneously into the back of 9-10-week-old Wistar strain rats maintained on diet CE-2 (CLEA, obtained from the Central Laboratory of Experimental Animals, Japan). Three weeks later, the rats were killed by decapitation, and their tumors were removed. Necrotic and hemorrhagic materials were carefully trimmed.

Enzyme Assay. Aldolase activity was assayed by the modified method of Blostein and Rutter (2). The reaction mixture (1.0 ml) contained 2 \( \mu \)moles of FDP or 10 \( \mu \)moles of F1P, 0.3 \( \mu \)mole of NADH, 25 \( \mu \)g of the mixture of glycerol 1-phosphate dehydrogenase and triosephosphate isomerase, 100 \( \mu \)moles of Tris-HCl buffer (pH 7.4), and an

\(^{2}\) The following abbreviations are used: NADH, reduced nicotinamide adenine dinucleotide; ATP, adenosine triphosphate, ADP, adenosine diphosphate; AMP, adenosine monophosphate; FDP, fructose 1,6-diphosphate; F1P, fructose 1-phosphate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetate.

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appropriate amount of enzyme solution. The absorbancy change at 340 mÅ was followed at 30°C by a Gilford Multiple Sample Absorbance Recorder Model 2000. One unit of aldolase activity was defined as the cleavage of 1 µmole substrate per minute.

**Limited Hydrolysis with Carboxypeptidase A.** Hydrolysis was carried out according to the method of Blostein and Rutter (2). An ammonium sulfate suspension of carboxypeptidase A was dissolved in 10% LiCl before use. The molar concentration was calculated from its absorbancy at 280 mÅ. One milligram of carboxypeptidase A per ml gave an O.D.280 of 2.3 and a molecular weight of 34,000. The digestion by carboxypeptidase A was carried out in 0.1 M Tris-HCl buffer (pH 8.0) at 30°C for 1 hr with an enzyme:aldolase molar ratio of 1:6.

**Immunologic Assay.** Anti-aldolase was produced in a hen by intramuscular injection of crystalline rat liver or muscle aldolase with Freund’s complete adjuvant and by intravenous booster injection of crystalline enzyme. The anti-aldolase fraction was obtained from serum by ammonium sulfate fractionation and DEAE-cellulose chromatography (3) and stored at -80°C until used. Inhibition of enzyme activity by anti-aldolase was determined as follows. Enzyme and anti-aldolase were mixed at a slight excess of the latter over the equivalent ratio in 0.7 ml of 0.02 M borate buffer (pH 8.4) and 0.4 M NaCl. Incubation was continued at 30°C for 2 hr. The mixture was then kept at 4°C overnight. The supernatant after centrifugation was analyzed for the remaining enzymatic activity.

**Electrophoresis.** Electrophoresis was performed with Veronal buffer (pH 8.6, ionic strength = 0.05) and 10 mM 2-mercaptoethanol on cellulose acetate membranes (Gelman, 1 X 6 inch) at 250 v for 3 hr. Enzyme solution was applied on the center of the strip. The staining of the strip for aldolase activity was carried out according to the method of Penhoet et al. (9) with a slight modification (8). Protein was stained on the strip by a solution of Ponceau 3 R.

**Sedimentation.** The sedimentation velocity experiment was performed at 15°C with a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm at 59,780 rpm by using the An-D rotor.

## RESULTS AND DISCUSSION

### Purification of Aldolase

**Crude Extract.** Rhodamine sarcoma tissues were minced twice through a meat grinder. The tissue mince weighing about 1.5 kg was homogenized in the cold with 2 volumes of 0.02 M Tris-HCl buffer (pH 7.6) by means of Virtis 45 homogenizer for 3 minutes at the top speed. The homogenate was centrifuged for 15 minutes at 18,000 X g, and 3,240 ml of supernatant were obtained as crude extract.

**DEAE-cellulose Treatment.** DEAE-cellulose (100 gm) buffered with 0.02 M Tris-HCl buffer (pH 7.6) was added to the crude enzyme extract, and the mixture was stirred for 30 minutes at 4°C, filtered, and the DEAE-cellulose was washed once with 100 ml of 0.02 M Tris-HCl buffer (pH 7.6).

**Ammonium Sulfate Fractionation.** To 3,250 ml of the filtrate, 813 gm of solid ammonium sulfate and 1.2 gm of solid EDTA (disodium salt) were added to obtain a 0.35 saturation. The pH of the mixture was about 7. After standing 30 minutes at 4°C, centrifugation for 15 minutes at 18,000 X g yielded 3,170 ml of supernatant. To this, 567 gm of solid ammonium sulfate were added to obtain a 0.60 saturation. The precipitate obtained after centrifugation was dissolved in 0.02 M Tris-HCl buffer (pH 7.6)–1 mM EDTA and dialyzed against the same buffer solution.

**First Column Chromatography on Cellulose Phosphate.** The dialyzed enzyme solution was centrifuged at 18,000 X g for 15 minutes, and the supernatant was applied to a cellulose phosphate column (6 x 15 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 7.6)–1 mM EDTA. The column was washed with about 4 liters of the same buffer. The aldolase was then eluted with a linear gradient consisting of 1 liter of 0.02 M Tris-HCl buffer (pH 7.6)–1 mM EDTA and 1 liter of 0.1 M Tris-HCl buffer (pH 7.6)–1 mM EDTA–0.1 M NaCl.

**Second Column Chromatography on Cellulose Phosphate.** The fractions with aldolase activity from the first cellulose phosphate column chromatography were diluted to four volumes with distilled water and rechromatographed on a cellulose phosphate column (6 x 15 cm). The column was washed with 4 liters of 0.02 M Tris-HCl buffer (pH 7.6)–1 mM EDTA–0.05 M NaCl, and eluted with a linear gradient consisting of 1 liter of 0.02 M Tris-HCl buffer (pH 7.6)–1 mM EDTA–0.05 M NaCl, and 1 liter of 0.1 M Tris-HCl buffer (pH 7.6)–1 mM EDTA–0.1 M NaCl. The fractions with specific activity higher than 5 units per E280 were pooled, dialyzed against water, and concentrated to about 10 ml by lyophilization.

**Crystallization.** Saturated ammonium sulfate (pH 7.0) was added to the concentrated enzyme solution to give a 35% saturation at 0°C. The solution was centrifuged, and a small residue was discarded. The supernatant solution was allowed to stand at room temperature. Crystals were produced as hexagonal plates after 12 hr (Fig. 1). Aldolase from Rhodamine sarcoma was purified to about 100-fold (Table 1).

### Properties of Purified Rhodamine Sarcoma Aldolase

**Michaelis-Menten Constant (Km).** The activity of the cleavage for FDP is higher than for F1P, and the activity ratio of FDP to F1P was 50.8. Km values were 4 x 10^-5 M for FDP and 1 x 10^-2 M for F1P; these are very close to the values obtained with crystalline aldolase A from muscle (12).

**Carboxypeptidase A Treatment.** After digestion with carboxypeptidase A, the FDP activity of aldolase of Rhodamine sarcoma was depressed to about 6% of its original value, while the F1P activity remained unchanged (Table 2). This agrees with the effect of carboxypeptidase A digestion on aldolase A from rat muscle, as shown in Table 2, and also with that from rabbit muscle (4, 11).

**Immunologic Properties.** Anti-aldolase A completely inhibited crystalline aldolase of Rhodamine sarcoma as well as crystalline aldolase A from rat muscle but did not inhibit crystalline aldolase B of rat liver at all. Crystalline Rhodamine aldolase and crystalline aldolase A were not inhibited by anti-aldolase B, which completely inhibited crystalline aldolase B.
Aldolase from Rat Sarcoma

Table 1

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (unit)</th>
<th>Recovery (%)</th>
<th>Protein (mg)</th>
<th>Recovery (%)</th>
<th>Specific activity (unit/mg)</th>
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<tbody>
<tr>
<td>Crude extract</td>
<td>14,100</td>
<td>100</td>
<td>103,000</td>
<td>100</td>
<td>0.137</td>
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<tr>
<td>DEAE-cellulose treatment (NH₄)₂SO₄ 0.35–0.60 saturation</td>
<td>14,100</td>
<td>100</td>
<td>95,600</td>
<td>92.8</td>
<td>0.147</td>
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<td>Cellulose phosphate column (I)</td>
<td>8,590</td>
<td>60.9</td>
<td>34,200</td>
<td>33.2</td>
<td>0.251</td>
</tr>
<tr>
<td>Cellulose phosphate column (II)</td>
<td>2,930</td>
<td>20.8</td>
<td>590</td>
<td>0.57</td>
<td>4.97</td>
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<tr>
<td>Crystallization (I)</td>
<td>1,920</td>
<td>13.6</td>
<td>325</td>
<td>0.32</td>
<td>5.91</td>
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<tr>
<td>Crystallization (II)</td>
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<td>8.7</td>
<td>102</td>
<td>0.10</td>
<td>11.8</td>
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<tr>
<td>Crystallization (II)</td>
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<td>6.0</td>
<td>67.1</td>
<td>0.07</td>
<td>12.5</td>
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</tbody>
</table>

Purification of Rhodamine sarcoma aldolase. DEAE, diethylaminoethyl.

Table 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>FDP (%)</th>
</tr>
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<tbody>
<tr>
<td>Crystalline aldolase A</td>
<td>8</td>
</tr>
<tr>
<td>Crystalline Rhodamine sarcoma aldolase</td>
<td>6</td>
</tr>
<tr>
<td>Crystalline aldolase B</td>
<td>53</td>
</tr>
<tr>
<td>Crystalline aldolase A</td>
<td>100</td>
</tr>
<tr>
<td>Crystalline Rhodamine sarcoma aldolase</td>
<td>100</td>
</tr>
<tr>
<td>Crystalline aldolase B</td>
<td>42</td>
</tr>
</tbody>
</table>

Effect of carboxypeptidase A. FDP, fructose 1,6-diphosphate; F1P, fructose 1-phosphate.

Effects of Adenine Nucleotides. Crystalline aldolase of Rhodamine sarcoma was inhibited by adenine nucleotides. The addition of ATP, ADP, and AMP at 15 mM to the assay system inhibited FDP activity 66, 52, and 44% respectively (Table 3). ATP was most effective. This was also found with aldolase A from rat muscle (Table 3) and reported on rabbit muscle (14). Although the concentration of adenine nucleotide needed to produce roughly 50% inhibition was apparently several times higher on Rhodamine sarcoma aldolase or rat muscle aldolase than on rabbit muscle aldolase.

Electrophoretic Properties. The activity and protein of crystalline aldolase of Rhodamine sarcoma migrated on cellulose acetate membranes as a single band with the same mobility as aldolase A of rat muscle.

Table 3

<table>
<thead>
<tr>
<th>Added nucleotide</th>
<th>AMP (%)</th>
<th>ADP (%)</th>
<th>ATP (%)</th>
</tr>
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<tbody>
<tr>
<td>Crystalline aldolase A</td>
<td>43</td>
<td>53</td>
<td>67</td>
</tr>
<tr>
<td>Crystalline Rhodamine sarcoma aldolase</td>
<td>45</td>
<td>52</td>
<td>66</td>
</tr>
<tr>
<td>Crystalline aldolase B</td>
<td>17</td>
<td>3</td>
<td>0</td>
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</tbody>
</table>

Effects of adenine nucleotides. AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

Sedimentation Velocity. In the ultracentrifuge pattern of the crystalline aldolase of Rhodamine sarcoma (0.6% protein solution), only a single homogeneous boundary with an S₂₀, w value of about 7.4 was observed (Fig. 2). This is again very close to the value reported for rabbit muscle aldolase A (12).

The many properties of crystalline aldolase of Rhodamine sarcoma, including Kₘ values for FDP and F1P, loss of FDP aldolase activity by limited hydrolysis with carboxypeptidase A, inhibition by adenine nucleotides, inhibition by anti-aldolase A, and the electrophoretic features, were indistinguishable from those of crystalline aldolase A from rat muscle. At the present time it can be stated that Rhodamine sarcoma is producing aldolase A characteristic of muscle, although a definitive conclusion has to await the complete structural elucidation of both aldolases of the rat. Rhodamine sarcoma is histologically defined as a fibrosarcoma, and its homologous cells are fibroblasts. Normal fibroblast can possibly produce aldolase A (10).

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Fig. 1. Crystalline Rhodamine sarcoma aldolase. X 200.

Fig. 2. Ultracentrifuge pattern of crystalline Rhodamine sarcoma aldolase. Aldolase was dissolved at 0.6% in 0.2 M NaCl and 20 mM Tris-HCl buffer (pH 7.4). Ultracentrifuge was carried out for 40 minutes at 59,780 rpm.
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