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PURIFICATION AND PROPERTIES OF RABBIT HEART MUSCLE ALDOLASE

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Summary

Fructose diphosphate aldolase (D-fructose-1,6-biphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13) from rabbit heart has been purified and obtained in crystalline form. The preparations are homogeneous on the basis of disc gel electrophoresis and ultracentrifugation. The catalytic and the molecular properties indicate that this is aldolase A. A comparison was made between rabbit heart aldolase and the rabbit muscle enzyme. The sedimentation coefficient, energy of activation and Michaelis constant for Fru-1,6-P_2 were found to be identical with the values obtained for the muscle enzyme. As in case of the muscle enzyme, heart aldolase was found to have a broad pH optimum, remarkable stability over a wide pH range, and the ability to form a Schiff base intermediate with dihydroxyacetone phosphate upon reduction with borohydride. Cleavage of the methionyl bonds with CNBr yields the same pattern as obtained with the muscle enzyme.

Introduction

Two types of fructose diphosphate aldolases (D-fructose-1,6-biphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13) have been detected in biological systems, and termed Class I and Class II aldolases, both presenting distinct catalytic and molecular characteristics. Class I aldolases are present in animals, plants, protozoa and green algae. Class II aldolases are present in bacteria, yeast, fungi and blue-green algae, except the aldolase from *Micrococcus aerogenes* which was found to be Class I aldolase [1,2]. Both classes have been found in *Euglena* [3] and *Chlamydomonas* [4].

Rutter and co-workers [5—8] have established that within Class I there are three distinct variants of aldolases in mammalian tissues. The classical muscle aldolase has been termed aldolase A. Aldolase found in kidney and liver has been termed aldolase B, and aldolase C is found in the brain.

Although considerable research has been carried out on the skeletal muscle aldolase [9—23] liver aldolase [10,12,21,24—28], and brain aldolase [21,29, 30] of various species a rather limited amount of information is available on the properties of this enzyme from heart muscle [31].

The present paper describes some catalytical and molecular studies on the purified and homogeneous rabbit heart aldolase.

Materials and Methods

Rabbit hearts were obtained from animals supplied by Granja Campo Novo, Bragança Paulista, Estado de São Paulo. Whatman cellulose phosphate powder P-11 (7.4 mequiv./g) was obtained from Reeve Angel, U.S.A. and was prepared according to Peterson and Sober [32] and equilibrated in 0.05 M Tris · HCl buffer, pH 7.5, containing 0.01 M EDTA and 0.01% β-mercaptoethanol. Sephadex G-75 (particle size 40—120 μm), glycerol-3-phosphate dehydrogenase, triosephosphate isomerase, Fru-1,6-P_2 (tetrasodium salt), NADH, dihydroxyacetone phosphate, $NaBH_4$, CNBr, were obtained from Sigma Chemical Co.; reagent grade $(NH_4)_2SO_4$ and EDTA, from J.T. Baker Co.; iodoacetic acid, from Eastman Organic Chemical Co.

Dihydroxy[^{14}C]acetone phosphate was prepared from uniformly labeled [^{14}C]fructose (Nuclear Research Chemicals, Inc.) [33]. Aldolase assay was performed at 25°C in a Gilford recording spectrophotometer as described by Racker [34] in 1 ml of 40 mM triethanolamine buffer, pH 7.5, containing 10 mM EDTA, 0.1 mM NADH, 1.0 mM fructose diphosphate, and 10 μg of triosephosphate isomerase and of glycerol-3-phosphate dehydrogenase. The protein in crude homogenates was determined by the turbidimetric procedure of Bücher [35]. Protein concentration of purified rabbit heart aldolase solution was determined by the absorbance at 280 nm of samples in 0.1 M NaOH, using the absorbance value of 0.895 for a solution containing 1 mg/ml in a cell with a light path of 1.0 cm ($E^{0.1\%}_{1\,cm}$ = 0.895). This conversion was standardized with a sample of crystallized rabbit heart aldolase, whose dry weight was determined after dialysis against distilled water and drying at 105°C. A unit of enzyme activity is the amount catalyzing the cleavage of 1 μmol of Fru-1,6-P_2 per min in the above assay.

Disc gel electrophoresis was performed according to Davis [36]. Gels were prepared with a 7.5% monomer concentration in Tris/glycine buffer, pH 8.6, and stained with 0.5% analine blue black in 7% acetic acid.

Sedimentation studies were carried out with a Spinco Model E ultracentrifuge. The centrifugations were performed at 42 040 rev./min at 20°C. Pictures were taken with Schlieren optics, 75° bar angle, on Kodak sensitive green films. The enzyme volume was 0.7 ml and the partial specific volume in cm^3 per g assumed was 0.74 [37].

Reduction and *S*-carboxymethylations of cystein residues were performed under the conditions described by Crestfield et al. [38].

Amino acid composition was determined on duplicate samples hydrolyzed in 5.7 M HCl at 110°C for 22, 48 and 72 h. The hydrolysates were analyzed according to Moore and Stein [39] in a Spinco Beckman Model 120C amino acid analyzer.

Cleavage of *S*-carboxymethylated aldolase with CNBr and separation of the resulting peptides by gel filtration on Sephadex G-75 was carried out by the procedure of Gross and Witkop [40] as described by Lai [41].

S-Carboxymethylated rabbit heart aldolase (80 mg) was dissolved in 8.0 ml 70% formic acid. CNBr (32 mg) in 70% formic acid was added, and the reaction mixture stirred at 25°C for 20 h. At the end of the reaction an equal volume of water was added and the solution evaporated to dryness. The dried sample was dissolved in 1.0 ml 70% formic acid and diluted to 5.0 ml with 0.2 M pyridine in 30% acetic acid and applied to Sephadex G-75 column (2.5 × 200 cm) equilibrated with 0.2 M pyridine in 30% acetic acid. The column was eluted with the same buffer at a flow rate of 12 ml/h.

Results

Purification of rabbit heart aldolase

All procedures were carried out at 0—5°C unless otherwise noted.

Step 1: Extraction. Frozen rabbit heart (490 g) was thawed at room temperature, ground twice in an electric meat grinder and subsequently extracted for 20 min with 980 ml of 0.03 M KOH, 0.01 M EDTA and 0.01% β-mercaptoethanol. The suspension was centrifuged for 20 min at 13 000 × *g* and the supernatant passed through several layers of cheesecloth to remove fat particles and the residue was discarded (extract 930 ml, pH 6.8).

Step 2: Ammonium sulfate fractionation. The extract was adjusted to pH 7.5 with 1 M NH_4OH and then slowly brought to 40% saturation by the addition of 225 g of $(NH_4)_2SO_4$ during 1 h. The suspension was centrifuged for 20 min at 13 000 × *g* and the precipitate was discarded. The clear supernatant solution (960 ml) was brought to 65% saturation by the addition of 161 g of $(NH_4)_2SO_4$ over a period of 1 h. The suspension was centrifuged for 20 min at 13 000 × *g*. The supernatant solution was discarded.

The precipitate was dissolved in a minimum volume of 0.05 M Tris · HCl buffer, pH 7.5, containing 0.01 M EDTA and 0.01% β-mercaptoethanol, and clarified by centrifugation (ammonium sulfate fraction 60 ml).

Step 3: Phosphocellulose chromatography. The enzyme solution was dialyzed extensively against 0.05 M Tris · HCl buffer pH 7.5, containing 10 mM EDTA and 0.01% β-mercaptoethanol, and then applied (87 ml, 5368 mg, specific activity = 0.51) to a phosphocellulose column (2.5 × 40 cm) equilibrated with the same buffer and chromatography was carried out essentially as described by Pogell [42].

The column was first washed with the same buffer at a flow rate of 60 ml/h until no more protein was eluted. The total volume of the buffer required to wash the unabsorbed protein was 1430 ml. Aldolase was then eluted specifically from the column using the same buffer containing 1 mM Fru-1,6-P_2. Fractions (9 ml) were collected at the same flow rate and analyzed for enzyme activity and protein. The enzyme was recovered in the effluent between 100 and 145 ml after the change of buffer. These fractions were combined (phosphocellulose chromatography with substrate elution 45.3 ml). A typical phosphocellulose elution pattern is illustrated in Fig. 1.

Step 4: Crystallization. The solution (45.3 ml) was brought to 65% satura-

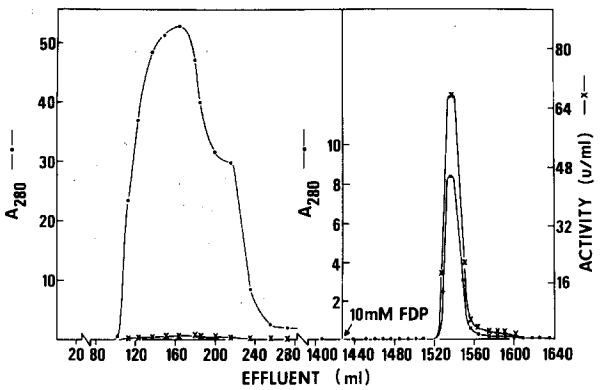

Fig. 1. Phosphocellulose elution pattern of rabbit heart aldolase. Phosphocellulose was prepared as described in Materials and Methods. The dialyzed material (87 ml; 5368 mg; specific activity = 0.51) was applied to the phosphocellulose column (2.5 × 40 cm). The first elution was carried out with 0.05 M Tris · HCl buffer, pH 7.5, containing 0.01 M EDTA and 0.01% β-mercaptoethanol, at a flow rate of 60 ml per h. When the absorbance of the effluent at 280 nm reached zero, aldolase was eluted with the same buffer containing 1 mM Fru-1,6-P_2. Fractions (9 ml) were collected at the same flow rate.

tion by the addition of 83.5 ml saturated ammonium sulfate, pH 7.5. After 10 min the suspension was centrifuged at 13 000 × *g* for 10 min. The supernatant solution contained no enzyme activity and was discarded. The precipitate was dissolved in 10 ml of 0.04 M triethanolamine buffer, pH 7.5, containing 0.01 M EDTA and the slightly turbid solution was clarified by centrifugation.

To the clarified solution (pH 7.5) saturated ammonium sulfate, pH 7.5, was added slowly while the solution was stirred until opalescence appeared. The enzyme was slowly stirred overnight at 4° C while crystallization progressed. More drops of saturated ammonium sulfate were added to the suspension which showed a distinct schlieren. The enzyme was recrystallized using the procedure described above.

A summary of the purification procedure is given in Table I. The overall purification is 80-fold and 31.8% of the original enzyme activity was recovered,

TABLE I

PURIFICATION OF RABBIT HEART ALDOLASE

Fraction	Volume (ml)	Total enzymatic activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
Extract	930	3528	25 528	0.14	100.0	1.0
Ammonium sulfate	60	2741	5 563	0.49	77.7	3.5
Phosphocellulose chromatography with substrate elution	45.3	1329	157	8.46	37.7	60.4
Crystallization	13.5	1172	113	10.13	33.2	74.1
Recrystallization	10.0	1122	100	11.12	31.8	80.1

which represented a final yield of approx. 100 mg of pure aldolase from 490 g of rabbit heart.

Purity of rabbit heart aldolase. Samples (6.5 7.9 and 13.5 mg/ml) of crystalline rabbit heart aldolase were centrifuged in a Spinco analytical ultracentrifuge Model E. The centrifugations were performed at 42 040 rev./min, at 20°C, as described in Materials and Methods. Single symmetrical boundaries were obtained in sedimentation velocity experiments for each concentration, indicating that the aldolase preparation was homogeneous.

Samples (11 and 22 μg) of crystalline rabbit heart aldolase were subjected to electrophoresis on 7.5% polyacrylamide gel supports in Tris/glycine buffer, pH 8.6, as described in Materials and Methods. Staining with aniline black showed the presence of a single protein band.

Properties of rabbit heart aldolase

Ultraviolet absorbance spectrum and extinction coefficient. The ultraviolet spectrum of crystalline rabbit heart aldolase has an absorbance maximum at 287 nm in Tris · HCl buffer, pH 7.5. The absorption ratio A_{280nm}/A_{260nm} is 1.64. A solution containing 1 mg/ml in 0.1 M NaOH gave an absorbance of 0.895 ($E^{0.1\%}_{1cm}$ = 0.895) at 280 nm (based on dry weight).

Ultracentrifugal studies. Sedimentation velocity runs were carried out with crystalline enzyme preparation which had been dialyzed at 5°C against 0.04 M triethanolamine buffer, pH 7.5, containing 0.01 M EDTA and 0.1 M KCl and samples were prepared by diluting the concentrated enzyme solution with the dialysis buffer. Values of sedimentation coefficient were corrected to viscosity and density of water at 20°C [43]. Values of $S_{20,w}$ obtained by extrapolating to zero protein concentration is 7.88 for the heart enzyme. Muscle aldolase gave almost identical values of $S_{20,w}$.

Reduction of the Schiff base intermediate with borohydride. Reaction was carried out at 0°C, with continuous monitoring of pH on a Vibret pH meter model 3920. Crystalline rabbit heart aldolase (0.44 μmol) in 0.05 M Tris buffer, pH 7.5, was mixed with 80-fold molar excess of dihydroxy[^{14}C]acetone phosphate (36 μmol). The control sample was identical except that dihydroxyacetone phosphate was omitted. The enzyme solution was adjusted to pH 6.0 with 2 M acetic acid and 36 μmol of fresh $NaBH_4$ solution was added, the pH being maintained at 6.0 with addition of 2 M acetic acid. Foaming was reduced by treating the mixture with a drop of octanol before the addition of $NaBH_4$.

Aliquots were withdrawn and assayed for enzymatic activity prior to and 5 min after the addition of $NaBH_4$. Usually over 80% inactivation was observed within a few minutes.

Identification of β-glyceryllysine. The reduced protein was precipitated by adding 0.05 ml of 3 M trichloroacetic acid to each 1.0 ml of reduced protein solution. The precipitated protein was collected by centrifugation and washed three times with 3 ml of 5% trichloroacetic acid. The protein was then washed by centrifugation with acetone and finally with ethyl ether, and dried in air. An aliquot of the labeled protein was hydrolyzed and prepared for amino acid analysis, as described in Materials and Methods.

As shown in Table II, reduction in the presence of dihydroxyacetone phosphate leads to a rapid and specific inactivation of rabbit heart aldolase, indi-

TABLE II

RABBIT HEART ALDOLASE INACTIVATION BY BOROHYDRIDE IN THE PRESENCE OF DIHYDROXYACETONE PHOSPHATE

Aldolase	Specific activity		Inactivation (%)
	Before $NaBH_4$ addition	After $NaBH_4$ addition	
In the presence of dihydroxyacetone phosphate	11	2.05	81.3
Without dihydroxyacetone phosphate	11	11	0.0

cating the presence of a Schiff base intermediate between enzyme and substrate. This reduction causes a covalent incorporation of the radioactive substrate into the protein.

When the reduced protein was hydrolyzed with 5.7 M HCl and analyzed in the short column on the amino acid analyzer, a peak corresponding to authentic β-glyceryllysine was observed. This peak was found to contain all of the radioactivity present in the hydrolyzate.

Effect of pH on enzyme activity. The crystalline rabbit heart aldolase shows a broad pH optimum, using Michaelis buffer [44], between pH 7.0 and 8.5. The rate of the cleavage reaction falls at pH 5.5 and 9.1 to give an activity 58 and 85%, respectively, of that of the pH at 7.5.

Effect of pH on the stability of rabbit heart aldolase. Crystalline rabbit heart aldolase is remarkably stable over a wide pH range. The enzyme is especially stable at high pH levels, 95% of the activity being retained after incubation at pH 10 for 15 min at 30°C in Michaelis buffer [44]. At low pH values, the pH range at which inactivation occurs is rather narrow, approx. 80% of the activity is irreversibly lost between pH 5 and 4.

Effect of temperature on enzyme activity. The activity-temperature relationship for the crystalline rabbit heart aldolase was investigated using a Gilford multiple sample absorbance recorder attached to a Beckman DU spectrophotometer. The temperature of the cuvette chamber was controlled with water circulated from a constant temperature bath. The temperature within the cells was measured before and after the absorbance change. The temperature was maintained ± 0.2°C. The solution was allowed to attain temperature equilibrium before the addition of the aldolase solution and the decrease in absorbance was recorded. The log of specific activity versus the reciprocal of temperature is linear between 10 and 25°C, but falls off at higher temperature (30 and 50°C). From the Arrhenius plot illustrated in Fig. 2, the energy of activation ($E^{\ddagger}$) from the enzymatic reaction:

Fructose-1,6-P_2 ⇌ dihydroxyacetone phosphate + D-glyceraldehyde-3-*P*

calculated between 10 and 25°C and between 30 and 50°C were estimated to be 18 and 12 kcal per mol, respectively.

Fig. 2 shows also that, for rabbit heart aldolase, a transition occurs at approx. 25°C.

Michaelis-Menten constant and the Fru-1,6-P_2 to fructose-1-P activity ratio.

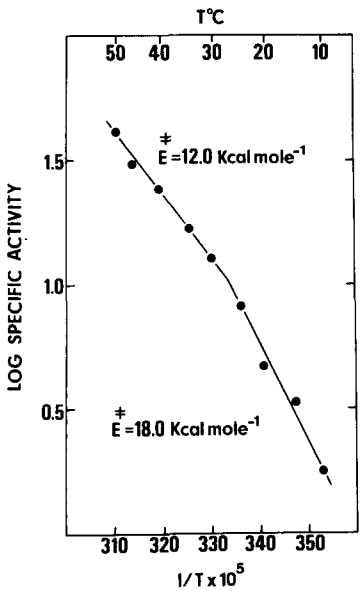

Fig. 2. Energy of activation of rabbit heart aldolase. Arrhenius plots for the cleavage reaction of rabbit heart aldolase.

The apparent Michaelis constant for fructose diphosphate is $2.0 \cdot 10^{-5}$ M. The rabbit heart aldolase is approx. 30 times as active with fructose diphosphate as with fructose 1-phosphate. Rabbit muscle aldolase gave similar results under the same conditions.

Cyanogen bromide cleavage. Fig. 3 shows the elution profile obtained when cyanogen bromide fragments of rabbit heart aldolase were passed through a column of Sephadex G-75 under the same conditions described by Lai [41] for rabbit muscle aldolase. Four peptides were obtained from rabbit heart aldolase.

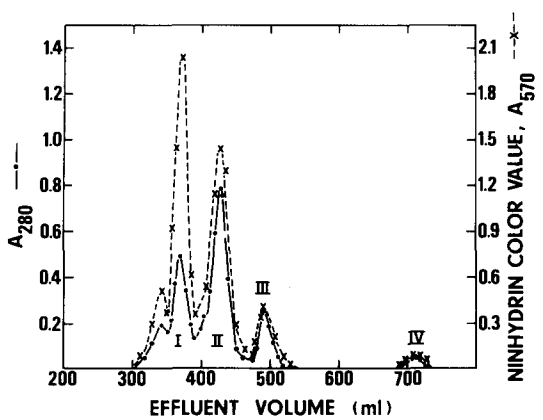

Fig. 3. Elution profiles of cyanogen bromide peptides of *S*-carboxymethylated rabbit heart aldolase on Sephadex G-75. Cleavage and separation were carried out as described in Materials and Methods. The column size was 2.5 × 200 cm and the flow rate was 12 ml/h and the buffer used was 0.2 M pyridine in 30% acetic acid. For the determination of ninhydrin color values 50-µl aliquots were used [45].

TABLE III

A COMPARISON OF AMINO ACID COMPOSITION OF RABBIT HEART AND MUSCLE ALDOLASE

Residues are expressed on the basis of molecular weight of 160 000.

Amino acid	Amino acid residues per mol	
	Heart aldolase	Muscle aldolase [a]
Lysine	104	104
Histidine	44	44
Arginine	65	60
CM-cysteine [b]	31	32
Aspartic acid	113	112
Threonine	94 [c]	92
Serine	81 [c]	84
Glutamic acid	161	160
Proline	73	76
Glycine	128	120
Alanine	174	168
Valine	82	84
Methionine	12	12
Isoleucine	74	76
Leucine	145	136
Tyrosine	45	44
Phenylalanine	28	28
Tryptophan	12 [d]	12
Total	1466	1444

[a] From Lai [41].
[b] Carboxymethylcysteine.
[c] Extrapolated to zero time.
[d] Determined spectrophotometrically by the method of Bencze and Schmid [49].

The small shoulder which appeared before peak I is probably an aggregate.

Amino acid composition. Amino acid composition of the *S*-carboxymethylated rabbit heart aldolase and a comparison with rabbit muscle aldolase is presented in Table III. Residues are expressed on the basis of molecular weight of 160 000. The muscle and the heart enzymes were found to be identical within experimental error, which shows a high similarity if not identity to the primary structure of the two enzymes.

Discussion

The purification method reported here for rabbit heart aldolase is reproducible and the recovery of enzyme is good. The enzyme has been purified 80-fold and was found to be homogeneous by electrophoretic and by ultracentrifugal analysis.

The sedimentation coefficients $S^0_{20,w}$ of rabbit heart aldolase, 7.9, and of the rabbit muscle enzyme, 8.0 [37] are similar. Like Class I aldolasses, heart enzyme forms a Schiff base intermediate in the presence of dihydroxy[^{14}C]acetone phosphate. Reduction of the enzyme-substrate complex with $NaBH_4$ leads to 81% inactivation. All the radio activity was recovered as β-glyceryllysine. This result shows that rabbit heart aldolase catalyzes the reversible cleavage of

Fru-1,6-P_2 by the formation of a Schiff base between dihydroxyacetone phosphate and the ϵ-NH_2 group of a lysine residue at the active site of the enzyme [33].

Like the muscle enzyme and other Class I aldolases [4], the heart enzyme has a broad pH optimum for Fru-1,6-P_2 cleavage and is remarkably stable over a wide pH range. The kinetic parameters K_m, and Fru-1,6-P_2 to fructose-1-P activity ratio of this enzyme are very similar to those of the rabbit muscle enzyme. The activation energies of the heart enzyme are 18 and 12 kcal/mol between 10 and 25°C and between 30 and 50°C, respectively, and are similar to those of rabbit muscle enzyme [46]. For both enzymes a transition temperature at 25°C was obtained. The curvature in the Arrhenius plots can be attributed to the existence of at least two forms of the enzyme, each being able to bind the substrate, but having different energies of activation. Such a model has been discussed by Massey et al. [47] for D-amino acid oxidase. In fact, Susor et al. [48] have demonstrated that rabbit muscle aldolase can be separated into five distinct forms when subjected to isoelectric focusing. Whether each of the forms has similar properties or whether the observed change is due to different forms dominating at different temperatures remains to be established. The general enzymatic and molecular properties of the rabbit heart muscle are identical to those of skeletal muscle aldolase and therefore is aldolase A. The elution profile and the number of peptides obtained after cyanogen bromide cleavage of rabbit heart aldolase is identical to that obtained by Lai [41] for the mucle enzyme. Four peptides were obtained for muscle and heart enzymes, which shows a homogeneous distribution of 12 methionines in four polypeptide chains each containing three methionines. Amino acid composition of rabbit heart and muscle are identical. These results point to high similarity as to the primary structure of the enzyme. On the basis of the data presented here defining the catalytic and molecular properties of rabbit heart aldolase, we conclude that the aldolase of heart and muscle of rabbit are identical, within the experimental limits, and are probably coded by the same gene.

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