Purification and Properties of the Native Form of Rabbit Liver Aldolase

EVIDENCE FOR PROTEOLYTIC MODIFICATION AFTER TISSUE EXTRACTION

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(Received 23 December 1977)

Aldolase was purified from rabbit liver by affinity-elution chromatography. By taking precautions to avoid rupture of lysosomes during the isolation procedure, a stable form of liver aldolase was obtained. The stable form of the enzyme had a specific activity with respect to fructose 1,6-bisphosphate cleavage of 20–28 $\mu\text{mol}/\text{min}$ per mg of protein and a fructose 1,6-bisphosphate/fructose 1-phosphate activity ratio of 4. It was distinguishable from rabbit muscle aldolase, as previously isolated, on the basis of its electrophoretic mobility and *N*-terminal analysis. Muscle and liver aldolases were immunologically distinct. The stable liver aldolase was degraded with a lysosomal extract to a form with catalytic properties resembling those reported for aldolase B4. It is postulated that liver aldolase prepared by previously described methods has been modified by proteolysis and does not constitute the native form of the enzyme.

Aldolase (D-fructose 1,6-bisphosphate D-glyceraldehyde 3-phosphate-lyase, EC 4.1.2.13) catalyses the following reactions.

Fructose 1,6-bisphosphate $\leftrightarrow$
glyceraldehyde 3-phosphate +
dihydroxyacetone phosphate (1)

Fructose 1-phosphate $\leftrightarrow$ glyceraldehyde +
dihydroxyacetone phosphate (2)

Aldolase exists in vertebrates as three tetrameric isoenzymes A4 (muscle type), B4 (liver type) and C4 (brain type). A–C hybrids exist in tissues, such as brain, where both A and C subunits are present (Lebherz & Rutter, 1969). The isoenzymes cleave the substrates fructose 1,6-bisphosphate and fructose 1-phosphate (eqns. 1 and 2) at different rates and can be distinguished by their characteristic activity ratios. In addition, they have different electrophoretic mobilities (Anstall *et al.*, 1966; Penhoet *et al.*, 1966; Pietruszko & Baron, 1967; Masters, 1968; Sheedy & Masters, 1969). The livers of most adult mammals contain predominantly aldolase B4 (Masters, 1968; Lebherz & Rutter, 1969). However, aldolases containing A or C subunits are present in the livers of some adult, as well as foetal or immature, animals (Sheedy & Masters, 1969).

The liver isoenzyme has been purified from a

number of mammalian species. The technique of affinity-elution chromatography from phosphocellulose has replaced other isolation methods involving ion-exchange chromatography and gel filtration (Pogell, 1962). Elution with fructose 1,6-bisphosphate concentrations of 2.5–5 mM have been used to obtain homogeneous preparations of liver aldolase (Gracy *et al.*, 1969; Gracy *et al.*, 1970; Morse & Horecker, 1968; Penhoet *et al.*, 1969). These preparations are reported to have specific activities of 1–2 $\mu\text{mol}/\text{min}$ per mg of protein with respect to fructose 1,6-bisphosphate, compared with aldolase A4, which has a specific activity of 14–18 $\mu\text{mol}/\text{min}$ per mg of protein (Penhoet *et al.*, 1969).

The properties of aldolases A4, B4 and C4 have been reviewed by Horecker *et al.* (1972). One of the major differences between the muscle and liver isoenzymes is that aldolase A4 cleaves fructose 1,6-bisphosphate with a greater efficiency than it does fructose 1-phosphate, having a fructose 1,6-bisphosphate/fructose 1-phosphate activity ratio of about 50 at a substrate concentration of 1 mM. Aldolase B4, on the other hand, is reported to cleave both substrates with equal efficiency, having a fructose 1,6-bisphosphate/fructose 1-phosphate activity ratio of 1.

Reports in the literature show that liver aldolase is characterized by a variable specific activity and is unstable on storage (Morse & Horecker, 1968; Gracy *et al.*, 1969; Penhoet *et al.*, 1969; Gracy *et al.*,

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1970; Chappel *et al.*, 1976). For these reasons, aldolase B4 has been less extensively studied than aldolase A4. An earlier report from this laboratory described the purification of two forms of aldolase from rabbit liver, one of which had a low specific activity characteristic of the liver isoenzyme and one of which had a high specific activity, but which was distinct from aldolase A4 (Chappel *et al.*, 1976). The high specific activity form of liver aldolase proved to be extremely labile and even when stored in liquid N₂ there was an almost total loss of activity towards fructose 1,6-bisphosphate. The present paper describes the isolation and properties of a stable form of aldolase from rabbit liver and presents evidence that the multiple forms previously described are the result of proteolysis.

Experimental

Materials

The substrates fructose 1,6-bisphosphate, fructose 1-phosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. NADH, NAD⁺ and NADP⁺ were from Boehringer Mannheim G.m.b.H., Mannheim, Germany. 4-morpholinepropanesulphonic acid (MOPS), Tris(hydroxymethyl)aminomethane (Tris) and phosphocellulose were from Sigma. DEAE-cellulose was the microgranular form (DE-52) from Whatman, Maidstone, Kent, England. Sephadex G-25 was from Pharmacia Fine Chemicals A.B., Uppsala, Sweden. Acrylamide and methylenebisacrylamide were obtained from E. Merck, Darmstadt, Germany. Starch for starch-gel electrophoresis was from the Electrostar Co., Madison, WI, U.S.A. Coomassie Brilliant Blue R for staining gels, phenazinemetanesulphonate and Nitro Blue Tetrazolium for staining gels for aldolase activity were from Sigma. Freund's complete adjuvant was purchased from the Commonwealth Serum Laboratories, Melbourne, Australia. Cortisol was from Ikapharm, Israel. Dimethyl suberimide hydrochloride was obtained from the Aldrich Chemical Co., Gillingham, Dorset, U.K. Rabbit muscle aldolase used as a standard in these studies was purified by Dr. R. K. Scopes.

Aldolase assay

The standard assay with fructose 1,6-bisphosphate and fructose 1-phosphate as substrates was a modification of the spectrophotometric method of Richards & Rutter (1961). Glycerophosphate dehydrogenase used in the assay was prepared from rabbit muscle by the method of Scopes (1977). Triose phosphate isomerase was a gift from Dr. R. K. Scopes. The assay was carried out in 20 mM-Tris/HCl, pH 7.5, at 25°C, with 1 mM-fructose 1,6-bisphosphate or 1 mM-fructose 1-phosphate and 50 μ M-NADH.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Electrophoresis was carried out in vertical slab gels consisting of 7% (w/v) polyacrylamide (acrylamide/bisacrylamide, 20:1) in a buffer containing 0.45 M-Tris, 0.25 M-boric acid (pH 8.6), 0.1% sodium dodecyl sulphate and 1 mM-2-mercaptoethanol. The samples, containing 2 mg of protein/ml, were heated to 100°C in 1% (w/v) sodium dodecyl sulphate and 3 mM-2-mercaptoethanol and electrophoresed for 4 h at 7 W. The gels were stained with 0.25% Coomassie Brilliant Blue in 20% (v/v) ethanol and 10% (v/v) acetic acid. Standard proteins used were phosphorylase (subunit mol. wt. 100 000), bovine serum albumin (68 000), rabbit muscle phosphoglycerate kinase (47 000), rabbit muscle glyceraldehyde 3-phosphate dehydrogenase (36 000), triose phosphate isomerase (26 500) and myoglobin (17 000). All of these were donated by Dr. R. K. Scopes.

Starch-gel electrophoresis

Starch-gel electrophoresis was carried out in vertical slabs consisting of 10% (w/v) starch in a buffer containing 4 mM-EDTA-imidazole, pH 6.8. A discontinuity, consisting of 0.5 M-MOPS-imidazole, pH 7.2, was applied in the electrode buffer. After electrophoresis, the gels were sliced horizontally and stained for aldolase activity by the method of Masters (1968).

N-Terminal analysis

N-Terminal residues were determined by using the dansyl chloride method as described by Gray (1969).

Immunochemical procedures

Antibodies against aldolase from rabbit liver were raised in chicken. Aldolase was injected intramuscularly as a 50% (v/v) emulsion in Freund's Complete Adjuvant. The first injection contained 500 μ g of aldolase. A second injection of 250 μ g was given 10 days later. Chickens were bled from the wing on day 14 after the second inoculation and serum was tested for antibodies to aldolase by immunodiffusion by the method of Ouchterlony (1958).

Immunodiffusion was carried out in 1% (w/v) Ionagar in 50 mM-sodium phosphate buffer, pH 7, containing 10 mM-KCl.

Antibodies against rabbit muscle aldolase were raised in sheep by two successive injections of 5 mg of aldolase as described above.

Protein determination

Protein was measured spectrophotometrically at 280 nm and specific activities are expressed as units (μ mol of substrate cleaved/min) per mg of protein

at 25°C. An absorption coefficient of 0.89 for a 1mg/ml solution of liver aldolase was assumed (Penhoet *et al.*, 1967). This absorption coefficient was calculated for aldolase B4. The error introduced by the use of this value for the undegraded liver enzyme was presumed to be very small.

Preparation of a lysosomal fraction

Lysosomes were isolated from guinea pig liver by the method of Beaufay (1969). Lysosomes were disrupted by freezing and thawing three times. Particulate matter was removed by centrifugation at 100000g for 10min and the supernatant fraction used as a source of proteolytic enzymes. The extract was free of aldolase activity. Liver aldolase (5mg) and muscle aldolase (2.5mg) was incubated with an equal volume of lysosomal extract for 1h at room temperature. The aldolase was then isolated from the extract by substrate elution from phosphocellulose and concentrated by ultrafiltration.

Results and Discussion

Purification of liver aldolase

Aldolase was purified by a modification of the method of Chappel *et al.* (1976). Whereas previous methods involved the disruption of tissue in a blender with high-ionic-strength buffer, it was found that gentle homogenization in conditions that minimized disruption of subcellular organelles resulted in the isolation of a stable form of aldolase with a high specific activity. In addition, it was found to be important to use liver from freshly killed animals. Liver (80–100g) was homogenized in 2vol. of a buffer containing 50mM-Tris/HCl, pH7.5, 5mM-EDTA and 0.125mg of cortisol/ml in a Potter–Elvehjem homogenizer with six strokes of the pestle. The addition of cortisol greatly decreased the release of proteolytic activity into the extract and confirmed the report of de Duve & Baudhuin (1966) that cortisol and derivatives stabilize lysosomal membranes *in vitro*. The extract was centrifuged at 700g for 10min. The pellet, containing lysosomes,

peroxisomes, mitochondria and endoplasmic reticulum (Beaufay, 1969) was discarded and all of the aldolase activity was recovered in the supernatant fraction. All steps in the purification were performed at room temperature (20–25°C) since it was found that there was no advantage in working at 4°C. Aldolase was precipitated in the 40–60% saturated $(\text{NH}_4)_2\text{SO}_4$ fraction by the addition of solid $(\text{NH}_4)_2\text{SO}_4$ and collected by centrifugation at 10000g for 10min. The precipitate was resuspended in 5–10ml of 10mM-Tris, adjusted to pH8.0 with MOPS, containing 0.2mM-EDTA (Tris/MOPS/EDTA buffer). The sample was desalted on a column (30cm $\times$ 8cm²) of Sephadex G-25, equilibrated in the Tris/MOPS/EDTA buffer, pH8.0, and treated in batches with DEAE-cellulose (25g/100g of liver) equilibrated in the same buffer. The DEAE-cellulose was removed by filtration on a Buchner funnel or by centrifugation and the resin was washed with 50ml of buffer. This step effected the removal of all the haemoglobin and other acidic proteins from the extract and constituted a major step in the purification (Table 1). All of the aldolase activity was recovered in the filtrate.

The pH of the batch-absorption filtrate was adjusted to 7.0 with 1M-MOPS and the sample was applied to a column of phosphocellulose equilibrated in Tris/MOPS/EDTA buffer, pH7.0, with a column volume of 25ml/100g of liver, column of diameter 4cm. The column was washed with 1 vol. of the equilibrating buffer and then with the same buffer containing 20mM-KCl. A small amount of protein was eluted with the buffer containing KCl. All of the aldolase was retained on the column. The aldolase was eluted from the column with 100ml of Tris/MOPS/EDTA, pH7.0, containing 20mM-KCl and 0.2mM-fructose 1,6-bisphosphate. As shown in Fig. 1, aldolase was eluted as a single peak and most of the protein applied to the column was not eluted with fructose 1,6-bisphosphate. It was essential that all of the purification steps were completed on the same day and, in general, an isolation took 4–6h.

Aldolase eluted from the phosphocellulose column

Table 1. *Summary of purification of liver aldolase*

Aldolase activity at each step in the purification was measured with fructose 1,6-bisphosphate as substrate by the standard assay procedure described in the Experimental section.

Fraction	Total protein (mg)	Total activity ($\mu\text{mol/min}$)	Specific activity ($\mu\text{mol/min per mg of protein}$)	Yield (%)	Purification (-fold)
Crude extract	375	150	0.4	100	1
14500g supernatant	274	137	0.5	91	1.25
40–60% satd. $(\text{NH}_4)_2\text{SO}_4$ fraction	100	100	1.0	67	2.5
DEAE-cellulose filtrate	50	100	2.0	67	5
Phosphocellulose eluate	3.4	96	28	64	70

Fig. 1. Elution of aldolase from phosphocellulose with fructose 1,6-bisphosphate

The filtrate from batch absorption of liver extract was eluted from phosphocellulose with 0.2M-fructose 1,6-bisphosphate, in a buffer containing 20mM-KCl and Tris/MOPS/EDTA, pH 7.0, as described in the text. A 20cm³ column was used for an 80g liver. Symbols: ●, aldolase activity; ○, A₂₈₀.

was concentrated by ultrafiltration (Amicon) and dialysed in 50mM-Tris/HCl, pH 7.5, to remove the substrate. This was necessary to prevent loss of activity of aldolase, since it has been shown that fructose 1,6-bisphosphate causes a slow irreversible inactivation of the enzyme (Woodfin, 1967). Rapid dialysis was effected by using a 100-fold excess of buffer and dialysing for 1 h with six changes. Aliquots of the aldolase were stored in liquid N₂ at a protein concentration of at least 10mg/ml.

The aldolase isolated by this method had a specific activity of 20–28 μmol/min per mg of protein and a fructose 1,6-bisphosphate/fructose 1-phosphate activity ratio of 4. Thus it was identified with the high specific activity form of liver aldolase previously reported (Chappel *et al.*, 1976) and had a specific activity higher than any species of aldolase previously reported. Under identical assay conditions, rabbit muscle aldolase had a specific activity of 9–12 μmol/min per mg of protein. A low specific activity form of aldolase was not recovered from the column, even when the column was washed with higher concentrations of fructose 1,6-bisphosphate. A 64% yield of aldolase was obtained, representing a 70-fold purification over the crude extract (Table 1). The method previously described by Chappel *et al.* (1976), and by other workers (Morse & Horecker, 1968; Penhoet *et al.*, 1969; Gracy *et al.*, 1969; Gracy *et al.*, 1970), resulted in the isolation of a low specific activity form that has properties quite distinct from the stable form described above.

Sodium dodecyl sulphate/polyacrylamide gel

electrophoresis of the purified aldolase (40 μg of protein/sample) gave a single band, corresponding to a mol. wt of 41 000. After starch-gel electrophoresis there was a single protein band that corresponded to an activity band with a cathodic mobility of 1 cm compared with an aldolase A4 mobility of 0.2 cm. Thus the low specific activity form of the liver aldolase described by other workers was absent from the present preparation. The preparation appeared to be free of contaminating protein.

Properties of liver aldolase

The molecular weight of liver aldolase was determined by using sodium dodecyl sulphate/polyacrylamide gel electrophoresis, combined with the cross-linking technique of Davies & Stark (1970). Gels were run with samples of purified muscle and liver aldolases to determine the subunit molecular weight and on muscle and liver aldolases cross-linked with dimethyl suberimide to determine the native molecular weight and subunit composition. The native molecular weight of both muscle and liver aldolases was 162 000, consisting of four subunits of mol. wt. 41 000. Despite their similar molecular weights, muscle and liver aldolase could be distinguished on starch gels, confirming that they are two distinct isoenzymes (Rajkumar *et al.*, 1966; Lebherz & Rutter, 1969; Penhoet *et al.*, 1969).

Studies on the heat stability of aldolase showed that the liver enzyme isolated in the present study is as stable to heat treatment as the muscle isoenzyme (Fig. 2). It is interesting to note that heat treatment differentially affected the activity of liver aldolase towards fructose 1,6-bisphosphate and fructose 1-phosphate. This is in disagreement with the findings of Dahlqvist & Crane (1964). The greater loss of activity with fructose 1-phosphate parallels a decreased affinity of liver aldolase towards fructose 1-phosphate when it is converted from the high-specific-activity form into the low-specific-activity form by the action of lysosomes (A. Chappel, unpublished work).

N-Terminal analysis of liver aldolase shows that the N-terminal residue is arginine, whereas the muscle enzyme has N-terminal proline (Udenfriend & Velick, 1951; Kochman *et al.*, 1968; Lai, 1968).

Antibodies against rabbit liver aldolase formed a single precipitin band with liver aldolase on immunodiffusion but did not precipitate aldolase from rabbit muscle. Similarly, antibodies against muscle aldolase did not precipitate the liver enzyme.

Lysosomal modification of aldolase

Since a comparison of the properties of liver aldolase suggested that the low-specific-activity form was a degradation product of the high-specific-activity form, experiments were performed with

lysosomal extracts to determine if one form could be converted into the other. The effects of treatment of both muscle and liver aldolases with lysosomal extracts is summarized in Table 2. Both muscle and liver aldolases were degraded by lysosomal enzymes and resulted in a marked decrease in specific activity with fructose 1,6-bisphosphate as substrate. The activity towards fructose 1-phosphate was not as markedly affected by lysosomal treatment, so that the fructose 1,6-bisphosphate/fructose 1-phosphate activity ratio of the liver enzyme was changed to a value similar to that of the liver aldolase with low specific activity described in the literature. The N-terminal residue for muscle aldolase was not changed but the N-terminal residue for aldolase

from rabbit liver was changed from arginine to a mixture of arginine, proline and serine. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of both muscle and liver aldolases degraded with lysosomal enzymes gave two bands, corresponding to molecular-weight species of the same molecular weight for both muscle and liver aldolases, but resulted in a different N-terminal residue only with liver aldolase. Since the lysosomal digestion resulted in three N-terminal residues, it may be that additional peptides were produced, but were too small to identify on sodium dodecyl sulphate/polyacrylamide gels.

Currently, three isoenzyme forms of aldolase have been described. One of the key differences between the muscle and liver forms has been discrepancy in their specific activities towards the glycolytic substrate fructose 1,6-bisphosphate. Evidence for the existence of a liver aldolase with a specific activity similar to that of the muscle enzyme has been presented by Chappel *et al.* (1976). The unstable nature of this enzyme has made it difficult to describe its properties fully and led to the suggestion that it was highly susceptible to proteolytic degradation. Whether proteolytic modification of liver aldolase occurs *in vivo*, and whether it has a role in the regulation of metabolism, is open to speculation. The evidence presented in this paper shows that a stable form of liver aldolase can be isolated and that it can be degraded to a form that has many of the characteristics of the low-specific-activity form referred to in the literature as aldolase B4. It is clear that the stable liver aldolase is distinct from the muscle form and that it represents the native form of aldolase B4.

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Fig. 2. Heat inactivation of aldolases

Samples of aldolase containing 1 mg of aldolase/ml in 20mM-Tris/MOPS, pH7.0, were incubated for 10min at temperatures ranging from 4 to 100°C. The samples were then cooled in ice, centrifuged for 5 min at 5000 g and the temperature was adjusted to room temperature. The supernatant fraction was assayed by the standard method described in the text. Symbols: ●, activity of liver aldolase with fructose 1,6-bisphosphate; ○, activity of liver aldolase with fructose 1-phosphate; ▲, activity of muscle aldolase with fructose 1,6-bisphosphate.

Table 2. Summary of properties of rabbit liver and muscle aldolases and of aldolases degraded with a lysosomal preparation

Aldolase	Specific activity with fructose 1,6-bisphosphate (μmol/min per mg of protein)	Fructose 1,6-bisphosphate/fructose 1-phosphate activity ratio at 1 mM substrate concentration	N-Terminal residue	Subunit composition
Rabbit muscle	9-12	50	Pro	4 × 41 000
Rabbit muscle degraded with lysosomes	1-2	29	Pro	41 000 29 000
Rabbit liver	20-28	4	Arg	4 × 41 000
Rabbit liver degraded with lysosomes	1-2	1	Arg/Pro/Ser	41 000 29 000

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