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ISOLATION AND CHARACTERIZATION OF MITOCHONDRIAL ALANINE AMINOTRANSFERASE FROM PORCINE TISSUE *

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Summary

Mitochondrial alanine aminotransferase L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.2) has been isolated in homogeneous form from both porcine liver and kidney cortex, but in low yield. Polyacrylamide gel electrophoresis of the purified enzyme in the presence of sodium dodecyl sulfate or 8 M urea gave a single band. An isoelectric point of 8.5 ± 0.5 and a molecular weight of 75—80 000 were obtained. The enzyme is specific for L-alanine and is inhibited by D-alanine, aminooxyacetate and cyclosterine. The K_m for pyruvate and glutamate is 0.4 mM and 32 mM, respectively. These values are similar to those determined for the cytoplasmic enzyme; however, at high concentrations, both compounds strongly inhibit the mitochondrial enzyme, an inhibition not observed with cytosolic alanine aminotransferase.

These characteristics and the fact that the mitochondrial alanine aminotransferase was inactivated by procedures effective in the preparation of the cytosolic enzyme, clearly differentiate the two proteins and further support different roles for the two alanine aminotransferases in vivo.

Introduction

It appears that, in the cells of most gluconeogenic tissues, significant amounts of two proteins with alanine aminotransferase (L-alanine-2-oxoglutarate aminotransferase, EC 2.6.2.1) activity can be found. A soluble and a

* Preliminary results have been reported [12].

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particular transamination of alanine in rat liver preparations have been observed [1—4]. Using conventional, differential centrifugation of rat liver homogenates in 0.25 M sucrose, Hopper and Segal [5] and Swick et al. [6] showed that most of the alanine aminotransferase activity was present in the cytosol, whereas only 10—20% of the total activity was recovered in the mitochondrial pellet. Takeda et al. [7] reported the separation of two alanine aminotransferase activities on DEAE-cellulose and Katunuma et al. [8] separated two such activities on calcium phosphate gels.

The cytosolic alanine aminotransferases from rat liver [9] and pig heart [10] have been purified to homogeneity and their kinetics parameters established [11,12]. The marked instability of the mitochondrial enzyme from rat liver has precluded its isolation [5,6]. More recently, using a partially purified preparation of the enzyme from porcine liver [13], we showed that the K_m value for alanine is 1.9 mM, more than one order of magnitude smaller than that reported for the cytosolic enzyme: 34 mM. The K_m for α-ketoglutarate, on the other hand, was similar for both enzymes.

These studies suggest the existence of two proteins with alanine aminotransferase activity which differ in their physicochemical characteristics and intracellular localization. We have purified the mitochondrial enzyme to homogeneity from pig liver and kidney cortex and characterized some of its properties. As a consequence, the two enzymes may be regarded as isoenzymes; unique roles have been proposed for each in gluconeogenesis [13].

Methods

Materials. Chemicals of the highest purity available were purchsed from Sigma, DEAE-cellulose from Eastman, Ampholine from LKB; Sephadex from Pharmacia; acrylamide and other chemicals for electrophoresis were obtained from Bio-Rad Labs.

Animals. Pigs (Hampshire or Chester White barrows, 70—100 kg) were used throughout and were maintained on a commercial diet. The liver or kidneys were excised immediately after death and were chilled on ice.

Assays. Because of the presence in mitochondria of a relatively high, nonspecific capacity for the oxidation of NADH, mitochondrial alanine aminotransferase activity was estimated by the two step assay [6]. After purification a direct, kinetic assay could be used [6] and gave similar results. Essentially the same direct coupled assay was used to estimate the cytosolic alanine aminotransferase, that is the activity present in the 105 000 $\times g$ supernate. Specific activity is expressed as micromoles of pyruvate formed per min per mg protein.

In order to study the reaction in the direction of alanine formation, pyruvate and glutamate at various concentrations were incubated in 0.1 M potassium phosphate buffer (pH 7.8) at 30° C for 5 min with the enzyme purified through the $(NH_4)_2SO_4$ step. After inactivation of the enzyme with trichloroacetic acid, the α-ketoglutarate formed was measured by oxidation of NADH in the presence of excess glutamate dehydrogenase [14].

Protein was determined by the method of Lowry et al. [15] with bovine serum albumin as standard.

Isolation of mitochondria. All steps in the purification of mitochondrial

alanine aminotransferase were performed at 4°C unless otherwise stated. Mitochondria were isolated from liver and kidney cortex essentially by the procedure reported previously [13] with these changes: 0.01 M Tris-HCl (pH 7.9) was used in the homogenization medium and the post-nuclear supernate was filtered throught cheese cloth before sedimentation of the mitochondria. The mitochondria were suspended in a glycerol medium [6] so that 10 ml contained the mitochondria isolated from 75 g of tissue. This preparation was stored at −70°C until used.

Solubilization. Mitochondrial alanine aminotransferase can be released completely from the particles only after disruption of the membranes. Because less than 30% of the total activity was released by the simple freezing and thawing involved in storage, this procedure by itself was inadequate. Alanine aminotransferase could be released from the mitochondria only by sonic disruption using an Ultrasonic Sonifier Cell Disruptor at 100 W.

After thawing , 10-ml aliquots of mitochondrial suspension were placed in an ice-water bath and sonicated for 30 s (15 s sonication, with 15 s intervals for cooling). Pooled samples were then centrifuged at 15 000 × *g* for 20 min.

Chromatography. 20—40 ml of the supernatant solution obtained above were placed on a column of Sephadex G-150 (2.5 × 85 cm) previously equilibrated with 0.05 M Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 20% glycerol and eluted with the same buffer at a rate of 9 ml/h; 5-ml fractions were collected. Alanine aminotransferase was retarded on this column relative to the bulk of the other proteins, including the cytosolic enzyme (Fig. 1). The fractions with the highest specific activity (fractions 35 to 45) were pooled. The high loss of enzyme activity was due to the instability of the alanine aminotransferase and to the necessity of discarding some of the enzyme-containing fractions to improve the degree of purification. This step was used, in spite of the disadvantages noted, because gel filtration through Sephadex G-150 was the most efficient method of decreasing the glycerol concentration.

The pooled fractions were applied to a DEAE-cellulose column (2.5 × 25 cm) previously equilibrated with 0.05 M Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol. The mitochondrial enzyme was eluted in the void volume of the

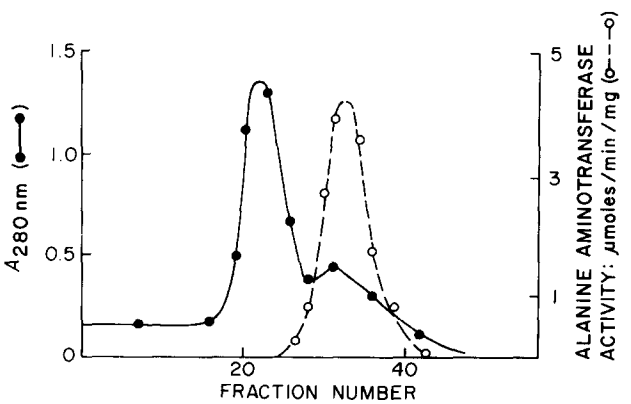

Fig. 1. Gel chromatography of mitochondrial alanine aminotransferase on Sephadex G-150. Twenty to forty ml of a 105 000 × *g* supernate obtained from sonicated mitochondria were applied onto a 2.5 × 85 cm column and eluted (9 ml/h, 5-ml fractions). ○———○, protein; ○- - - - -○, enzyme activity.

column. The use of buffers of different ionic strength and pH for the equilibration of the resin did not alter the binding of the enzyme although other proteins were differently retained.

Ammonium sulfate fractionation. Saturated $(NH_4)_2SO_4$ solution (pH 8.0) was added to the combined fractions from the DEAE chromatography step, to obtain a final concentration of 40% saturation. After stirring for 30 min, the suspension was centrifuged and the precipitate discarded. Additional $(NH_4)_2SO_2$ solution was added to bring the saturation to 65%; after stirring for 30 min the suspension was centrifuged as before. The precipitate was dissolved in a minimum amount (2—5 ml) of 0.1 M Tris-HCl (pH 8.0). The solution was deionized and equilibrated with Tris-HCl (pH 8.0), 10% glycerol with the aid of a hollow fiber Minitube (Bio-Rad Labs.). Complete equilibration was obtained in 1—2 h but resulted in a significant loss in enzyme activity.

Isoelectrofocusing. Flat bed isoelectrofocusing in granulated gel was performed as described in the LKB application note No. 198 with slight modifications. The support medium used was Sephadex G-75 superfine, suspended at 7.5% in 10% glycerol, 1% ampholines, and 0.1% L-histidine. The presence of histidine created a discontinuous pH gradient. The focusing was obtained by applying 250 V for 14—16 h and 1000 V for 1 more h and visually monitored from the migration of myoglobin and cytochrome *c*, applied as 1% solutions to one edge of the plate. Proteins were stained with Coomassie blue as described in the LKB manual. Successive scrapings of the support medium were made perpendicular to the direction of the current flow; an aliquot of each scraping was suspended in Tris-HCl 0.1 M (pH 7.8) and assayed for enzyme activity.

The isoelectric point of the mitochondrial alanine aminotransferase was also determined by preparative isoelectricfocusing in a sucrose density gradient in a 110 ml LKB 8100 column. A discontinuous gradieitn of 24 steps was prepared from 50% sucrose (containing $\frac{3}{4}$ of the total amount of carrier ampholine) and 1% sucrose (containing the remaining $\frac{1}{4}$ of the ampholites). A starting voltage of 300—400 V was applied and the temperature of the column was kept at 4°C; the focusing was complete in 48 h. The column was eluted at a flow rate of 50 ml/h and the protein profile recorded at 280 nm with an LKB 8300 Uvicord II Ultraviolet Absorptiometer. Fractions of 1.5 ml were assayed immediately for enzyme activity and then diluted with an equal volume of distilled water for pH determination.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out as described by Weber and Osborne [16]. 7.5% acrylamide was used in 0.6 × 12 cm gel tubes. The samples were run at 2 mA/tube for 6 h at room temperature. The mitochondrial alanine aminotransferase was precipitated by trichloroacetic acid, resuspended in 10% glycerol, 1% sodium dodecyl sulfate, 1% 2-mercaptoethanol in 10 mM sodium phosphate buffer (pH 7.1); the sample was heated in boiling water for 2 min, cooled and applied to the gels.

For electrophoresis in 8 M urea (pH 9.5) the enzyme, after isoelectrofocusing and precipitation with trichloroacetic acid, was dissolved in 8 M urea, 0.5% 2-mercaptoethanol, 1% sodium dodecyl sulfate in 0.017 M Tris-HCl (pH 6.8). Sodium dodecyl sulfate was added to this solution to avoid formation of protein aggregates at the top of the gel.

Molecular weight determination. SDS polyacrylamide gel electrophoresis was used to determine the molecular weight of the purified enzyme. The R_F values of standard proteins, plotted versus the log of their molecular weights, gave a straight line.

The molecular weight of the enzyme, enriched through the ammonium sulfate step, was also estimated by gel filtration through a Sephadex G-200 column (1.5 × 90 cm). The elution volume of Blue dextran gave a measure of the void volume (V_0); the elution volumes (V_e) of mitochondrial alanine aminotransferase and four reference proteins were determined. The ratios, V_e/V_0, were calculated and plotted against the logarithm of the molecular weights.

Results

Starting material. The low specific activity and the instability of mitochondrial alanine aminotransferase from rat liver precluded its use for the purification of this enzyme. Guinea pig, pig, chicken, rabbit, beef and sheep organs were analyzed for enzyme activity. Of these tissues, porcine liver and kidney cortex had the highest specific activity, and the enzyme was somewhat more stable. The purification procedure developed for pig kidney gave essentially the same results when pig liver was used. The results of the purification procedure are summarized in Table I.

Purity. Enzyme recovered from the isoelectricfocusing plate was dialyzed free of the ampholines and the protein concentration determined. The specific enzyme activity was 210—250 μmol/mg of protein. When the enzyme was focused again in a narrow pH range (7—9), only a single band was obtained. A single band was also obtained when the enzyme was analyzed by polyacrylamide disc gel electrophoresis in the presence of 1% sodium dodecyl sulfate or 8 M urea.

Molecular weight. The molecular weight of the mitochondrial alanaine aminotransferase estimated by gel filtration on a Sephadex G-200 column (1.5 × 90) and by sodium dodecyl sulfate polyacrylamide gel electrophoresis was 80 000 ± 8000 and 75 000 ± 3750, respectively.

Isoelectric point determination. The p*I* of the mitochondrial alanine aminotransferase was determined by both thin layer isoelectrofocusing in granulated

TABLE I

PURIFICATION OF MITOCHONDRIAL ALANINE AMINOTRANSFERASE FROM PIG KIDNEY CORTEX

Treatment	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Recovery (%)
1. Homogenate	50 000	9 000	0.81	100
2. Sonicated mitochondria	3 895	8 217	2.11	91
3. Sephadex G-150 (pool)	902	3 151	3.50	35
4. DEAE cellulose	404	2 082	5.15	23
5. $(NH_4)_2SO_4$ (40—65% cut, dionized)	23.28	276.8	11.89	3
6. Isoelectrofocusing	1.02	217.3	213.04	2.5

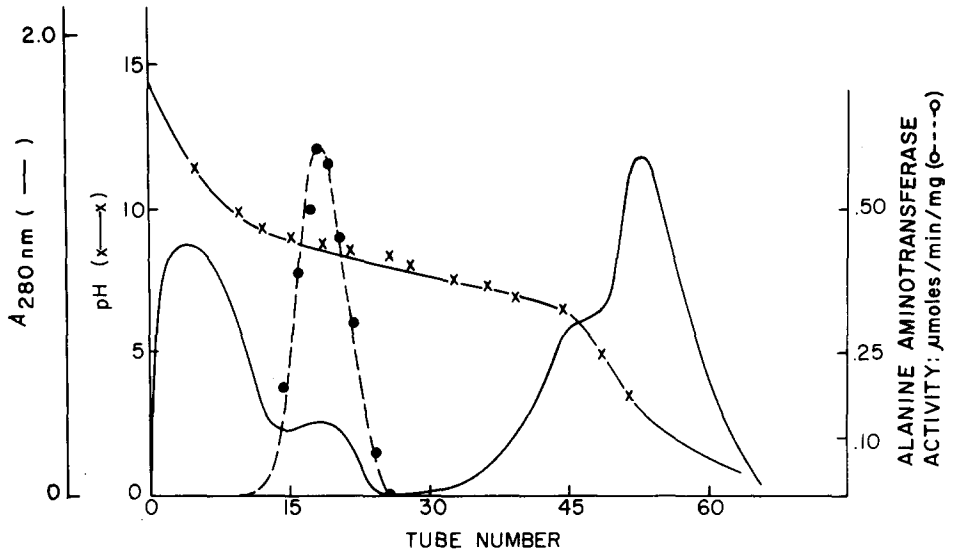

Fig. 2. Isoelectrofocusing of mitochondrial alanine aminotransferase in a sucrose density gradient. The details of the procedure are given under Methods. o———o, mitochondrial alanine aminotransferase activity; X———X, pH; ———, absorbance at 280 nm.

gel and preparative isoelectrofocusing in sucrose density gradient (Fig. 2) in the pH range of 7—9. p*I* values of 8.5 ± 0.5 were obtained by both techniques.

Stability. From previous studies and from our more recent experience it is clear that the mitochondrial alanine aminotransferase is a very labile enzyme. Swick et al. [6] reported an increased stability of the enzyme from rat liver in a medium containing 50% glycerol, alanine and cysteine. In the present study the stability of the enzyme was markedly decreased upon release from the mitochondria of porcine tissues; overnight storage at 4°C resulted in 20% loss of enzyme activity, even in the presence of 50% glycerol. The enzyme did not tolerate freezing at any stage after sonication of the isolated mitochondria.

The effect of various compounds on the stability of the enzyme was tested. Enzyme precipitated by ammonium sulfate was dissolved in the suspension medium [6] and dialyzed overnight against the same medium or one modified in varous ways. The enzyme was the most stable in K^+; substitution of Na^+ greatly increased the instability of the enzyme. 1 mM EDTA also increased the lability of alanine aminotransferase, although 1 mM Ca^{2+} was not protective and Zn^{2+}, Cu^{2+} and Ba^{2+} at the same concentration resulted in even greater losses in activity. 0.1 mM pyridoxal phosphate had no protective effect. Various sulphydryl protecting agents (0.1 mM) such as dithiothreitol, 2-mercaptoethanol and cysteine, improved the recovery of the mitochondrial enzyme slightly, just as they are protective for the cytosolic enzyme [9,11]. Among its substrates, L-alanine had a marked protective effect while α-ketoglutarate did not, and it counteracted the protective effects of the reducing agents.

Specificity. Eleven different amino acids (80 mM) were tested as substrates for the mitochondrial alanine aminotransferase in the presence of 15 mM α-ketoglutarate in an interrupted assay. The amount of glutamate formed was estimated from the reduction of NAD in the presence of excess glutamate

dehydrogenase. Only aspartate and valine resulted in a significant synthesis of glutamate; however, in neither case was the transamination activity more than 5% of that observed when alanine was the amino donor.

Inhibition. D-Alanine (80 mM), was used as substrate for the transaminase at a rate which was only 2% of that observed with L-alanine. When the two isomers were present together, D-alanine had an inhibitory effect on the transamination reaction, which was of the competitive type. Two known inhibitors of pyridoxal phosphate-dependent enzymes were also tested. Aminooxyacetate ($4 \cdot 10^{-9}$ M) inhibited the reaction by 5%; when its concentration was raised to $4 \cdot 10^{-7}$ M, an inhibition of 85% was obtained. The mitochondrial enzyme appears to be more sensitive to aminooxyacetate than the cytoplasmic one: 10^{-7} M aminooxyacetate inhibited the cytosolic enzyme by less than 50% (5). Cycloserine at concentrations of 10^{-3}, 10^{-2}, and $5 \cdot 10^{-2}$ M inhibited mitochondrial analine aminotransferase activity by 10, 52 and 100%, respectively.

pH optimum. The pH optimum of the enzyme, enriched through the 65% ammonium sulfate precipitation step, was reexamined using the following buffers: potassium phosphate 0.1 M from pH 6.0 to 9.0; 0.1 M Tris-HCl from pH 7.8 to pH 9.0 and 0.1 M glycine buffer from pH 5.0 to 11.0. The maximal activity of alanine aminotransferase was obtained betweeen pH 7.5 and pH 8.5, as observed previously [6].

We also confirmed that the activity was greater in Tris-HCl buffer than in phosphate buffer. The activity was maximal when glycine buffers were used; however, the addition of glycine to Tris-HCl (pH 7.8) did not enhance the apparent activity of the enzyme.

Kinetic characteristics. We have previously reported the kinetic parameters for the alanine aminotransferase reaction in the direction of pyruvate and glutamate formation [13]. The earlier results were confirmed with the more highly purified enzyme: the K_m values for alanine and α-ketoglutarate were 2 and 0.4 mM, respectively. No inhibitory effect was observed at the highest concentrations tested (300 mM alanine and 50 mM α-ketoglutarate). The mecha-

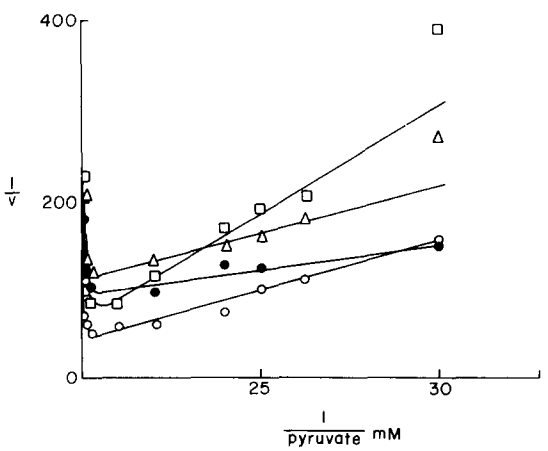

Fig. 3. Double-reciprocal plot of mitochondrial alanine aminotransferase activity with respect to pyruvate in the presence of varying concentrations of glutamate. The assays were carried out as described above. Glutamate concentration: △, 2 mM; ○, 3 mM; ●, 5 mM; □, 10 mM.

nism of the reaction is of the ping-pong type. When the reverse reaction was studied, a strong competitive substrate inhibition was observed, as shown by the double reciprocal plots (Fig. 3). Although the inhibition made it difficult to obtain an accurate determination of the kinetic parameters the data were fitted by computer to the rate equations derived by Cleland et al. [17]; the K_m values were estimated to be about 0.4 mM for pyruvate and 32 mM for glutamate. The inhibition constant for pyruvate was estimated to be about 12 mM.

Discussion

Two alanine aminotransferase isoenzymes have been found to occur in mammalian tissues. The low levels of the mitochondrial form in rat tissues has, however, failed to engender much interest in this enzyme. In porcine, ovine, and bovine liver and kidney, the majority of the alanine aminotransferase is mitochondrial while in avian species it appears to be exclusively mitochondrial [13]. Therefore its study is important to our understanding of alanine metabolism in these species.

The mitochondrial enzyme specifically transaminates alanine with almost no activity toward other amino acids; this justifies its classification as an alanine aminotransferase isoenzyme.

A number of observations suggest that the mitochondrial alanine aminotransferase is structurally different from the cytosolic isoenzyme. Steps which are efficacious in the purification of the cytosolic alanine aminotransferase completely destroy the mitochondrial enzyme [6]; the marked instability of this enzyme form has been reported repeatedly [5,6,13]. Such lability cannot be attributed to the detachment of the protein from the mitochondrial membranes; it is not membrane-bound, but is located in the same matrix compartment as glutamate dehydrogenase [13]. Mitochondrial alanine aminotransferase is still very labile after extensive purification which probably excludes the action of proteases. The molecular weight of the mitochondrial isoenzyme is 70 000 to 80 000 while that of the cytosolic form is 115 000 to 125 000 [11]. The p*I* of the mitochondrial enzyme is 8.5 ± 0.5 while that of the cytosolic alanine aminotranserase, as determined by isoelectric focusing, appears to be between pH 5.3 and 6.0 (de Rosa, G., unpublished data).

Differences in kinetic parameters suggest differences in the in vivo function of the enzymes as well. In a previous publication [13] we postulated that the entry of alanine into the gluconeogenic pathway is primarily catalysed by the mitochondrial enzyme. The hypothesis was based on the distribution of the two isoenzymes in different animal tissues and on the lower, by an order of magnitude, K_m value of the mitochondrial enzyme for alanine. Hopper and Segal [12] have reported K_m values for pyruvate and glutamate for the soluble alanine aminotransferase which are very similar to those reported here for the mitochondrial enzyme, although no indication of any inhibition by either substrate was observed. At physiological concentrations, however, glutamate exerts an inhibitory effect on the mitochondrial enzyme which is dependent on the concentration of pyruvate. Such strong inhibition by substrates is an indication

that the enzyme in vivo functions in the opposite direction [18], i.e. conversion of alanine to pyruvate.

These results indicate that in vivo, whereas the cytoplasmic alanine aminotransferase can function in both directions, the mitochondrial enzyme can only convert alanine to pyruvate. Recently Dieterle et al. [19] showed that the conversion of alanine to glucose by rat liver hepatocytes was not inhibited by α-cyano-cinnamate, and inhibitor of pyruvate transport into mitochondria. Fahien et al. [20] have shown that mitochondrial alanine aminotransferase can participate in their 'alanine dehydrogenase' system which permits the recovery of a reducing equivalent from alanine as well. These experiments lend further support to our hypothesis for the unique role of mitochondrial alanine aminotransferase in glucoenogenesis.

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