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Cytosolic L-alanine:4,5-dioxovalerate transaminase differs from the mitochondrial form

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L-Alanine:4,5-dioxovalerate transaminase was detected in the kidney cytosolic fraction with a lower specific activity than the mitochondrial enzyme. The enzyme was purified from the cytosol to homogeneity with a yield of 32%, and comparative analysis with the mitochondrial form was performed. Both forms of the enzyme have identical pH and temperature optima and also share common antigenic determinants. However, differences in their molecular properties exist. The molecular mass of the native cytoplasmic enzyme is 260 kDa, whereas that of the mitochondrial enzyme is 210 kDa. In addition, the cytoplasmic L-alanine:4,5-dioxovalerate transaminase had a homopolymeric subunit molecular mass of 67 kDa compared to a subunit molecular mass of 50 kDa for the mitochondrial L-alanine:4,5-dioxovalerate transaminase. This is the first report of two forms of L-alanine:4,5-dioxovalerate transaminase. The different responses of cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminases to hemin supplementation both *in vitro* and *in vivo* was demonstrated. Maximum inhibition of mitochondrial L-alanine:4,5-dioxovalerate transaminase activity was demonstrated with hemin injected at a dose of 1.2 mg/kg body mass, whereas the same dose of hemin stimulated the cytosolic enzyme to 150% of the control. A one-dimensional peptide map of partially digested cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase shows that the two forms of the enzymes are structurally related. Partial digestion of the cytosolic form of the enzyme with papain generated a fragment of 50 kDa which was identical to that of the undigested mitochondrial form (50 kDa). Moreover, papain digestion resulted in a threefold increase in cytosolic enzyme activity over the native enzyme, and such enhancement was comparable to the activity of the mitochondrial form of the enzyme. Therefore, we conclude that the cytosolic form of L-alanine:4,5-dioxovalerate transaminase is different from the mitochondrial enzyme.

Furthermore, immunoblot analysis indicated that the mitochondrial enzyme has antigenic similarity to the cytosolic enzyme as well as to the papain-digested cytosolic enzyme 50-kDa fragment.

It is well established that δ-aminolevulinate, the first committed precursor of heme, is synthesized by two different enzymes, δ-aminolevulinate synthetase and L-alanine:4,5-dioxovalerate transaminase. The synthesis of δ-aminolevulinate is the step at which overall regulation of heme biosynthesis occurs [1]. Varticovski et al. [2] and Noguchi and Mori [3] have purified L-alanine:4,5-dioxovalerate transaminase from bovine liver. In our laboratory the enzyme has been purified from rat liver and kidney mitochondria [4–6]. Morton et al. [7] demonstrated that respiring rat hepatocytes can incorporate radioactive 4,5-dioxovalerate into heme, thereby suggesting that L-alanine:4,5-dioxovalerate transaminase takes part in the synthesis of δ-aminolevulinate under normal physiological condition. Discovery of 4-oxo-5-hydroxyvalerate dehydrogenase [8] which synthesizes 4,5-dioxovaleric acid, further supports the concept of an additional route of δ-aminolevulinate synthesis operating in mammalian-heme biosynthesis. In light of the discovery of an alternative route of δ-aminolevulinate synthesis, it became necessary to study the regulatory role of L-alanine:4,5-dioxovalerate transaminase in heme biosynthesis. It has been shown that L-alanine:4,5-dioxovalerate transaminase is inhibited *in vitro* as well as *in vivo* by administration of hemin [6, 9]. This enzyme is induced by phenylhydrazine and it was observed that the level of enzyme in the system was inversely related to the level of the heme pool [10], thus suggesting that L-alanine:4,5-dioxovalerate transaminase is under feedback control of the intracellular heme pool.

L-Alanine:4,5-dioxovalerate transaminase from bovine liver [3], rat liver and kidney [4, 6] has been shown to be present predominantly in mitochondria. It is known that most of the mitochondrial enzymes encoded by nuclear genes are translated in the cytoplasm as larger precursors [11–15] which are subsequently processed into smaller mature forms during translocation across the mitochondrial membranes. Therefore, it is of interest to examine whether mitochondrial L-alanine:4,5-dioxovalerate transaminase is the mature product of a larger precursor synthesised in the cytosol, or a form different from mitochondrial enzyme.

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Enzymes. L-Alanine:4,5-dioxovalerate transaminase (EC 2.6.1.43); δ-aminolevulinate synthetase (EC 2.3.1.37); glutamate dehydrogenase (EC 1.4.1.3); δ-aminolevulinate dehydratase (EC 4.4.1.24); catalase (EC 1.11.1.6); papain (EC 3.4.22.2); chymotrypsin (EC 3.4.21.1).

MATERIALS AND METHODS

Materials

Male Wistar rats (150–175 g) were obtained from Haryana Agriculture University (Hissar, India). Sucrose (sucrose density grade), L-alanine, δ-aminolevulinate and hemin were from Sigma Chemical Co. (St Louis, MO); phenyl-Sepharose CL-4B, aminohexyl–Sepharose-4B, Sepharose 6B, the gel-filtration kit and molecular-mass-marker-protein kit were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). DEAE-cellulose DE-52 was purchased from Whatman (England); acrylamide and Coomassie brilliant blue R-250 were obtained from Bio-Rad (Richmond, California); 3,5-dibromolevulinic acid was bought from Porphyrin Products (Logan, Utah). Papain and chymotrypsin were obtained from SRL (India).

Methods

4,5-Dioxovalerate was prepared from 3,5-dibromolevulinic acid as described by Varticovski et al. [2].

Preparation of organelles. Organelle separation was carried out according to the procedure described by Noguchi and Mori [3] with minor modification. Rat kidney was homogenized with 4 vol. 0.25 M sucrose in 5 mM potassium phosphate buffer, pH 7.6, and centrifuged at 500 *g* for 10 min. 1.5 ml rat-kidney postnuclear fraction was loaded onto a linear sucrose density gradient (20–54%, mass/vol.) in 36 ml 5 mM potassium phosphate buffer, pH 7.6, and centrifuged at 60000 *g* for 4 h in a swinging-bucket rotor (SW 41) in a Beckman ultracentrifuge. Fractions (2 ml) were collected from the bottom of the tube. The fractions were marked as mitochondrial, cytosolic and peroxisomal by the marker enzymes glutamate dehydrogenase, δ-aminolevulinate dehydratase, and catalase, respectively. L-Alanine:4,5-dioxovalerate transaminase was assayed in all fractions.

Hemin treatment. Male Wistar rats (80–100 g) were injected with hemin. Hemin was prepared as described previously [16] and injected into the tail vein within 1 h of preparation. 30 min after injection the animals were killed by decapitation.

Enzyme assays. L-Alanine:4,5-dioxovalerate transaminase was assayed according to Varticovski et al. [2] with minor modification. The assay system used to measure the formation of δ-aminolevulinic acid contained 1 mM dioxovalerate, 20 mM L-alanine, 50 mM potassium phosphate buffer, pH 6.9, enzyme and distilled water to a final reaction volume of 1.0 ml. Incubation was carried out at 65 °C for 30 min since the optimum temperature of this enzyme was reported earlier [5, 6] to be 65 °C. The reaction was stopped by the addition of 0.2 ml 25% trichloroacetic acid. δ-Aminolevulinic acid was converted to 2-methyl-3-carbethoxy-4-(3-propionyl)pyrrole by the method of Mauzerall and Granick [17] and the Ehrlich chromophore was measured at 553 nm. Glutamate dehydrogenase was assayed as described by King [18]. δ-Aminolevulinate dehydratase and catalase were assayed by the method of Anderson and Desnick [19] and Aebi [20], respectively. One unit (1 U) L-alanine:4,5-dioxovalerate transaminase is defined as the amount of enzyme catalyzing the formation of 1 μmol δ-aminolevulinic acid · mg protein^{-1} · 30 min^{-1} at 65 °C.

Electrophoresis. The purity of the protein was checked by SDS/PAGE performed according to Laemmli [21]. Protein was assayed by Lowry's method [22].

Papain digestion of cytosolic and mitochondrial L-alanine:4,5-dioxovaleric acid transaminase. 15 μg cytosolic L-alanine:4,5-dioxovalerate transaminase was digested with 0.1, 0.5 and 1 μg papain for 10 min in 0.05 M Tris buffer, pH 8.0, containing 0.05 M cysteine and 0.02 M EDTA at 30 °C. After digestion was complete, the samples were divided into two parts. To one part, Laemmli SDS/PAGE sample buffer was added and the mixture was boiled at 100 °C. The remaining sample was used for enzyme assay.

One-dimensional peptide map. 7-μg aliquots of both the cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase were treated with 0.1 μg and 0.25 μg chymotrypsin in 0.125 M Tris buffer, pH 6.8, at 30 °C for 10 min. At the end of the incubation, the digestion was stopped by the addition of Laemmli SDS/PAGE sample buffer followed by boiling at 100 °C. The samples were electrophoresed by 15% SDS/PAGE. Peptide fragments were detected by the silver staining method [23].

Antisera preparation. Purified L-alanine:4,5-dioxovalerate transaminase (0.5–1 mg) was emulsified with 1 vol. Freund's complete adjuvant and injected subcutaneously at multiple sites on the back of a New-Zealand white rabbit. A booster injection of 200 μg was administered after 14–20 days. The rabbit was bled 10 days later and the antiserum separated. Antibody was detected by Ouchterlony double immunodiffusion [24].

Western-blot analysis. Electrotransfer of protein from a 12.5% SDS/polyacrylamide gel was according to the method of Towbin [25]. Immunodetection of the transferred protein was performed using a Promega Protblot Western-blot AP system.

RESULTS

Subcellular localization of L-alanine:4,5-dioxovalerate transaminase

A representative sedimentation in sucrose density gradient of the homogenate from rat kidney is presented in Fig. 1. The peroxisomes, mitochondria and cytosol were sedimented at a density of about 1.23, 1.21 and 1.09 g/ml, respectively. Fig. 1 shows the subcellular distribution of L-alanine:4,5-dioxovalerate transaminase and also the activity of the marker enzymes catalase, glutamate dehydrogenase and δ-aminolevulinate dehydratase in the sucrose density fractions of the postnuclear rat-kidney homogenate fraction. Kidney L-alanine:4,5-dioxovalerate transaminase was assayed at 65 °C, which was found to be the optimum temperature [6]. The L-alanine:4,5-dioxovalerate transaminase activity profile shows a major peak overlapping that of glutamate dehydrogenase and a minor peak coinciding with that of δ-aminolevulinate dehydratase, the cytoplasmic marker. Glutamate dehydrogenase activity was not detectable in the cytosolic fractions. L-Alanine:4,5-dioxovalerate transaminase activity was not detected in the cytoplasmic fraction when assayed at 37 °C.

Purification of cytosolic L-alanine:4,5-dioxovalerate transaminase

All purification procedures were carried out at 4 °C unless stated otherwise. The results of the enzyme purification are summarized in Table 1.

Fractionation of cytoplasmic extract by heat treatment. The postnuclear fraction of kidney homogenate in 0.25 M sucrose, 10 mM potassium phosphate buffer was centrifuged at 10000 *g* for 20 min and the supernatant further centrifuged at 60000 *g* for 4 h. After discarding the pellet, the supernatant

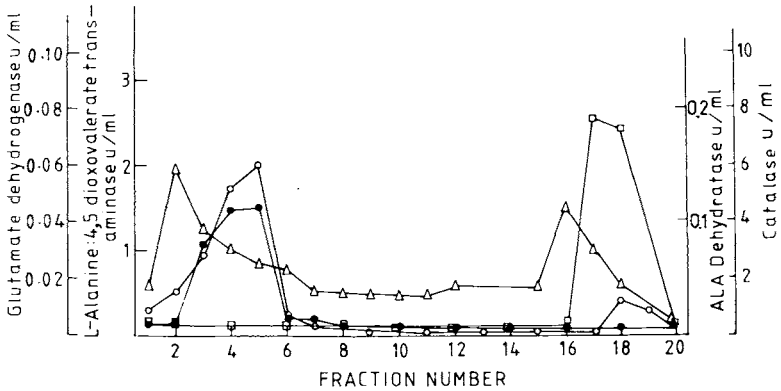

Fig. 1. *Subcellular distribution of L-alanine:4,5-dioxovalerate transaminase in rat kidney.* The kidney postnuclear supernatant was subjected to sucrose density gradient as described in Materials and Methods. Glutamate dehydrogenase (●); δ-aminolevulinate dehydratase (□); catalase (△); L-alanine:4,5-dioxovalerate transaminase (○). ALA, δ-aminolevulinate

Table 1. *Purification of L-alanine:4,5-dioxovalerate transaminase from rat kidney cytosol*

Purification step	L-Alanine:4,5-dioxovalerate transaminase				
	protein	total activity	specific activity	purification	yield
	mg	U	U/mg protein	-fold	%
Cytosol	268	6.16	0.023	1.0	100
Heat treatment	203	5.40	0.027	1.1	87.6
Phenyl-Sepharose	24	3.20	0.13	5.7	51.9
Sepharose 6B	18	3.01	0.17	7.2	49.0
Ion exchange	8	2.31	0.29	12.5	37.0
Affinity	0.3	2.0	6.67	290	32.0

was heated at 60 °C for 5 min. The precipitate was removed by centrifugation at 10000 *g* for 20 min.

Phenyl–Sepharose-CL-4B column chromatography. The supernatant obtained from the above was applied to a column of phenyl–Sepharose-CL-4B (2.9 cm × 11 cm) using 5 mM potassium phosphate buffer, pH 7.6. The enzyme did not bind to the hydrophobic support and was eluted in buffer. The fractions with high activity were pooled and concentrated using poly(ethylene glycol) 20000.

Sepharose 6B column chromatography. The concentrated sample (4 ml) was loaded on to a Sepharose 6B column (2.5 cm × 42 cm) and eluted with 5 mM potassium phosphate buffer, pH 7.6. The flow rate was 16 ml/h.

DEAE-cellulose DE-52 column chromatography. The active fractions from the previous column were pooled and loaded on to a DEAE-cellulose DE-52 column (2.75 cm × 19 cm), equilibrated with the same buffer. The enzyme was eluted with a 0–0.5 M KCl gradient, total volume 500 ml, at a flow rate of 20 ml/h. The enzyme eluted approximately in the middle of the gradient.

L-Alanine–Sepharose-4B column chromatography. The pooled fraction from DEAE-cellulose DE-52 was applied finally to an L-alanine–Sepharose-4B column (1.32 cm × 4.0 cm), which was equilibrated with 5 mM potassium phosphate buffer, pH 7.6, containing 10% glycerol. The column was washed first with 400 ml of the same buffer, then the enzyme was eluted with a 0–0.4 M KCl linear gradient of total volume 200 ml in the same buffer at a flow rate of 20 ml/h (Fig. 2). The fractions exhibiting L-alanine:4,5-dioxovalerate transaminase were pooled and concentrated using an Amicon ultrafiltration cone, dialyzed against 5 mM potassium phosphate buffer, pH 7.6, containing 10% glycerol, then stored at −20 °C.

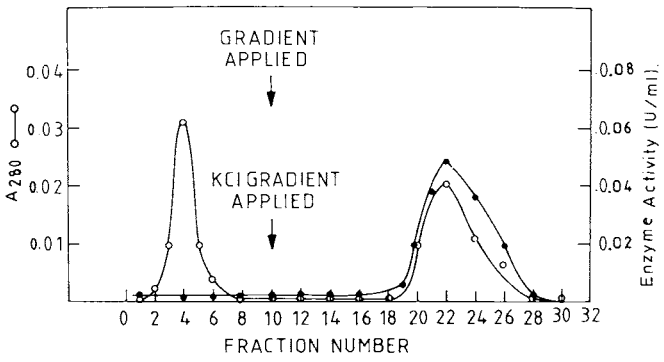

Fig. 2. *L-Alanine–Sepharose-4B affinity chromatography of rat kidney cytosolic L-alanine:4,5-dioxovalerate transaminase.* The pooled enzyme fraction was applied to the affinity column (1.32 cm × 4 cm), washed with 5 mM potassium phosphate buffer, pH 7.6 and a linear KCl gradient (0–0.4 M KCl, 200 ml) applied at a flow rate of 20 ml/h. (○) Absorbance at 280 nm; (●) L-alanine:4,5-dioxovalerate transaminase activity. Fractions of 4 ml were collected

The purity of the enzyme was checked and appeared to be homogeneous, yielding a single band in SDS/PAGE at pH 8.9 (Fig. 3).

Optimal conditions

The cytoplasmic L-alanine:4,5-dioxovalerate transaminase was shown to have a pH optimum of 6.6 and a temperature optimum of 65 °C.

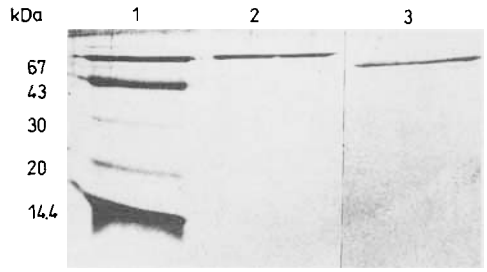

Fig. 3. *SDS/PAGE of purified rat-kidney cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase under denaturing conditions.* 10 μg of the standard marker proteins, phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and lactalbumin (14.4 kDa) and 4 μg purified samples were electrophoresed simultaneously. Calculation of the mobilities of the marker proteins as well as that of the samples are expressed relative to the bromophenol-blue dye front. The protein bands were detected by the silver staining method. Lane 1, marker proteins; lane 2, cytosolic L-alanine:4,5-dioxovalerate transaminase; lane 3, mitochondrial L-alanine:4,5-dioxovalerate transaminase

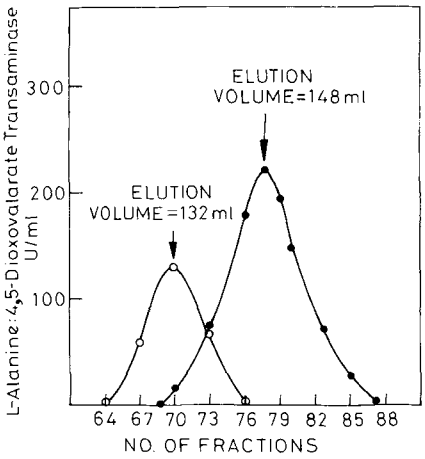

Fig. 4. *Comparison of the molecular masses of cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase on a Sepharose 6B column (2.5 cm × 42 cm).* The column was calibrated with 1 mg ferritin (440 kDa), 2 mg thyroglobulin (669 kDa), 2 mg aldolase (158 kDa) and 5 mg catalase (232 kDa). (●) Mitochondrial; (○) cytosolic

Comparison of native and subunit molecular masses of cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase

When 0.5 mg each of native cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase were run separately on a Sepharose-6B gel-filtration column, elution volumes of 132 ml and 148 ml, corresponding to molecular masses of 260 kDa and 210 kDa, respectively, were obtained for cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase (Fig. 4), indicating that they are different forms of the same enzyme.

When cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminases were subjected to denaturing SDS/PAGE the cytosolic form showed a subunit molecular mass of 67 kDa, but a subunit molecular mass of 50 kDa was obtained for mitochondrial L-alanine:4,5-dioxovalerate transaminase (Fig. 3).

Table 2. *Effect of hemin on the purified cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase*
Partially pure L-alanine:4,5-dioxovalerate transaminase, having a specific activity of 0.2 U/mg, was incubated with varying concentrations of hemin at room temperature. The reaction was started by addition of substrates and incubation at 65 °C for 30 min. The product formed was estimated as described in Materials and Methods

Hemin concentration	Specific activity of alanine:4,5-dioxovalerate transaminase	
	cytosol	mitochondria
μM	U/mg (%)	
0	0.2 (100)	1.2 (100)
10	0.2 (100)	1.0 (83)
25	0.2 (100)	0.8 (66)
50	0.15 (75)	0.5 (41)

Differences in the responses of cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase to hemin in vitro *and* in vivo

Purified cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminases were incubated *in vitro* with hemin at concentrations from 10 – 50 μM. It was observed that cytosolic and mitochondrial enzymes were inhibited by hemin to different extents. At 50 μM, the maximum concentration used, the cytosolic enzyme still retained 75% of the control activity, whereas the mitochondrial enzyme at the same concentration of hemin had only 41% of the control activity (Table 2).

Since *in vitro* incubation of the cytosolic enzyme with hemin had little effect, we further examined the possibility of a regulatory effect of hemin on the cytosolic L-alanine:4,5-dioxovalerate transaminase. Rats were administered hemin (0.8 – 2.0 mg/kg body mass) intravenously and sacrificed after 30 min. The activities of cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminases were assayed. It was observed that up to a hemin dose of 1.2 mg/kg body mass, the mitochondrial enzyme activity decreased progressively with a concomitant increase in the activity of the cytosolic enzyme to 150% of the control. However, at a higher dose of hemin (2.0 mg/kg body mass) the activities of both the cytosolic and the mitochondrial enzymes were inhibited (Fig. 5). When enzyme activity in the post nuclear fraction was assayed, there was no apparent change in the total enzyme activity. The results suggest that hemin at the dose employed (0.8 – 1.2 mg/kg body mass) interferes only with the mitochondrial L-alanine:4,5-dioxovalerate transaminase activity, while the cytosolic enzyme remained uninhibited, thus supporting the hypothesis that hemin does not inhibit the cytosolic L-alanine:4,5-dioxovalerate transaminase *in vitro* to the same extent.

One-dimensional peptide map of cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase

In order to examine the structural relationship of cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase, each of the purified enzymes was digested with 0.1 μg and 0.25 μg chymotrypsin for 10 min at 30 °C. At the end of 10 min, the digestion was stopped and the samples were run on 15% SDS/PAGE (Fig. 6). Cytosolic L-alanine:4,5-

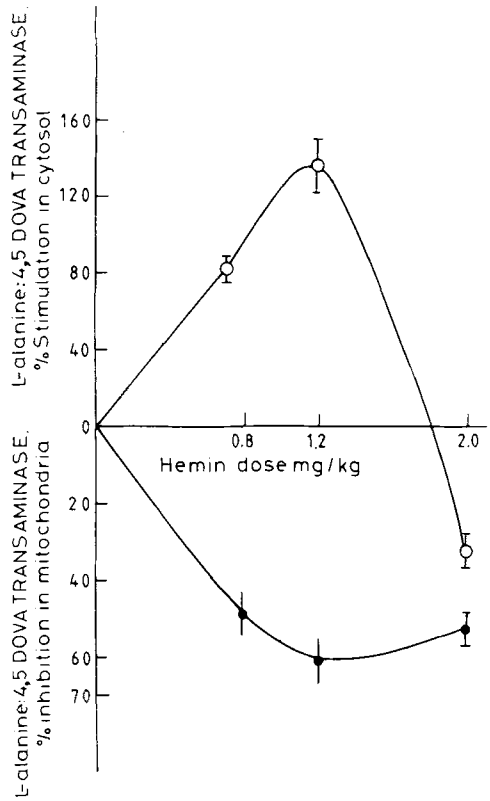

Fig. 5. *Effect of hemin treatment on rat kidney* L-*alanine:4,5-dioxovalerate transaminase.* Different doses of hemin were injected intravenously (as described in Materials and Methods) and the animals were killed 30 min after injection. L-Alanine:4,5-dioxovalerate transaminase activity is expressed as a percentage of the specific activity of the control. DOVA; dioxovalerate

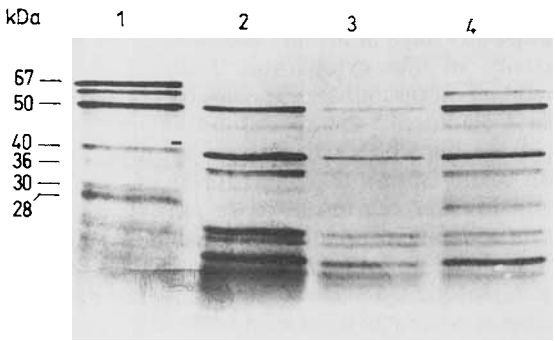

Fig. 6. *One-dimensional peptide map of purified cytosolic and mitochondrial* L-*alanine:4,5-dioxovalerate transaminases.* Pure cytosolic and mitochondrial enzyme, 7.0 μg each, were digested with 0.1 μg and 0.25 μg chymotrypsin for 10 min at 30°C. The digestion was stopped and samples were run in 15% SDS/PAGE. Lanes 1 and 4 represent cytoplasmic L-alanine:4,5-dioxovalerate transaminase digested with 0.1 μg and 0.25 μg chymotrypsin and lanes 2 and 3 represent mitochondrial L-alanine:4,5-dioxovalerate transaminase digested with 0.1 μg and 0.25 μg chymotrypsin, respectively. The protein bands were detected with silver staining

dioxovalerate transaminase, digested with 0.1 μg chymotrypsin (lane 1), yielded peptide fragments of 50, 40, 36, 30 and 28 kDa, of which the fragments (50 kDa) generated from the cytosolic enzyme were identical to the uncut mitochondrial form of the enzyme. In addition, a fragment of 40 kDa was

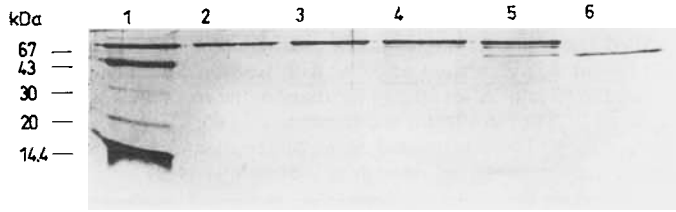

Fig. 7. *Partial digestion of cytosolic* L-*alanine:4,5-dioxovalerate transaminase with papain.* Lane 1, standard molecular mass marker proteins; lane 2, undigested cytosolic enzyme; lanes 3–5, cytosolic enzyme digested with 0.1, 0.25 and 0.5 μg chymotrypsin; lane 6, undigested mitochondrial enzyme. The bands were detected by silver staining.

observed for both cytosolic (Fig. 6, lane 1) and mitochondrial (lane 2) L-alanine:4,5-dioxovalerate transaminase digested with 0.1 μg chymotrypsin. Moreover the mitochondrial enzyme, upon digestion with 0.1 μg chymotrypsin generated a fragment 36 kDa (Fig. 6, lane 2) which was absent when the cytosolic enzyme was digested with chymotrypsin at the same concentration (lane 1). However, this fragment appeared when the cytosolic enzyme was digested with 0.25 μg chymotrypsin (Fig. 6, lane 4). On the contrary, the fragments of 30 kDa and 28 kDa obtained after digestion of the cytosolic enzyme with 0.1 μg (Fig. 6, lane 1) and 0.25 μg chymotrypsin (lane 4) were never observed as mitochondrial digestion products (lanes 2 and 3). Our observations suggests that cytosolic and mitochondrial enzymes share structural similarities while at the same time they differ to a certain extent.

Increased activity of cytosolic L-*alanine:4,5-dioxovalerate transaminase by papain digestion*

Cytosolic L-alanine:4,5-dioxovalerate transaminase (20 μg) was digested with 0.1, 0.25 and 0.5 μg papain at 37°C for 10 min. Part of each digestion mixture was subjected to 15% denaturing SDS/PAGE along with the undigested cytosolic and mitochondrial forms of the enzyme. The rest of the digestion mixture was assayed for enzyme activity. Fig. 7 shows the results of papain digestion. During digestion of cytosolic L-alanine:4,5-dioxovalerate transaminase, a fragment having the same molecular mass (50 kDa) as that of the uncut mitochondrial form (Fig. 7, lane 6) was generated progressively as the concentration of papain increased over 0.1–0.5 μg (lanes 2–4). The progressive generation of the 50 kDa band was accompanied by a gradual increase in enzyme activity (Table 3). Table 3 shows that the native cytosolic enzyme has a specific activity of 0.43 U/mg protein, which is less than that of the mitochondrial form. Digestion with papain (0.5 μg) not only generated the identical subunit of 50 kDa, but also increased the enzyme activity almost threefold. This observation shows that proteolytic hydrolysis of the cytosolic enzyme generates an enzymatically more active form.

Immunological cross-reactivity of the mitochondrial enzyme with the cytosolic form and its proteolytic fragment with an antibody raised against the mitochondrial enzyme

In order to examine the cross-reactivity of the cytosolic L-alanine:4,5-dioxovalerate transaminase and its proteolytic fragments with antiserum against the mitochondrial enzyme, cytoplasmic L-alanine:4,5-dioxovalerate transaminase (10 μg) was digested with increasing concentrations (3 μg and 6 μg)

Table 3. *Effect of papain treatment on the activity of cytosolic* L-*alanine:4,5-dioxovalerate transaminase*
Purified L-alanine:4,5-dioxovalerate transaminase (specific activity 0.43 U/mg) was incubated at 37°C with varying concentrations of papain for 10 min. After 10 min incubation, the reaction was started by the addition of substrates and incubation at 65°C for 30 min. The product formed was estimated as in Materials and Methods. The specific activity of the mitochondrial enzyme was 31.5 U/mg

Papain concentration	L-Alanine:4,5-dioxovalerate transaminase specific activity
μg	U · mg protein^{-1} · 30 min^{-1}
0	0.43
0.1	0.67
0.25	0.81
0.5	1.30

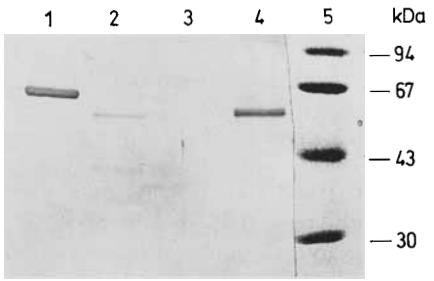

Fig. 8. *Immunoblot analysis of papain-digested cytosolic* L-*alanine:4,5-dioxovalerate transaminase using an antibody against the mitochondrial enzyme.* 10 μg cytosolic alanine:4,5-dioxovalerate transaminase was digested with papain, transferred on to nitrocellulose and the blot incubated with anti-(mitochondrial L-alanine:4,5-dioxovalerate transaminase) antibody. Lane 1, cytosolic enzyme; lane 2, cytosolic enzyme digested with 3 μg papain; lane 3, cytosolic enzyme digested with 6 μg papain; lane 4, mitochondrial L-alanine:4,5-dioxovalerate transaminase; lane 5, molecular mass marker proteins stained with 0.2% amido black

of papain for 10 min and electrophoresed simultaneously with undigested cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase in a 12.5% SDS/polyacrylamide denaturing gel. Electrophoresis was followed by electroblotting on nitrocellulose paper.

The nitrocellulose was then incubated with antibody (1:2000 dilution) raised against mitochondrial L-alanine:4,5-dioxovalerate transaminase. Fig. 8 shows that cytosolic L-alanine:4,5-dioxovalerate transaminase, as well as the fragments generated by digestion with papain, cross-react with antibody raised against the mitochondrial form of the enzyme. During partial digestion of the cytosolic L-alanine:4,5-dioxovalerate transaminase with lower concentrations of papain, it was apparent that digested fragments were equally cross-reactive with the antibody raised against the mitochondrial enzyme as well as with the 67 kDa fragment of the cytosolic enzyme. The results demonstrate an immunological relationship between the cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminase.

DISCUSSION

The present study reports the occurrence and purification of the cytoplasmic form of L-alanine:4,5-dioxovalerate transaminase in addition to our earlier report on the purification of mitochondrial L-alanine:4,5-dioxovalerate transaminase [4–6]. Since no glutamate dehydrogenase activity was detectable in the cytosolic fraction, the possibility of mitochondrial L-alanine:4,5-dioxovalerate transaminase leaching out into the cytosol is ruled out. Therefore, the enzyme activity detected may be exclusively due to a cytosolic form of L-alanine:4,5-dioxovalerate transaminase. It is important to mention here that the cytosolic enzyme has a lower specific activity than the mitochondrial enzyme. Furthermore, in this paper we described a procedure for purification of the cytosolic enzyme using L-alanine–Sepharose-4B affinity chromatography. We compared the molecular properties of the cytosolic and mitochondrial enzymes and it is evident that the cytosolic enzyme is the high-molecular-mass protein of 260 kDa. Both enzymes are homotetramers. The cytosolic enzyme has a higher subunit molecular mass (67 kDa) than the mitochondrial enzyme which has a subunit molecular mass of 50 kDa. However, they have common antigenic site(s) and their temperature and pH optima are identical. Differences in the molecular properties of L-alanine:4,5-dioxovalerate transaminase purified from the cytosol and from mitochondria are also supported by differences in the inhibitory effect of hemin *in vitro* on the activity of the enzymes. Incubation of hemin with purified enzyme has little inhibitory effect on the cytosolic enzyme. The mitochondrial form of the enzyme can be inhibited with hemin at a concentration as low as 10 μM and 60% inhibition was observed with 50 μM hemin (Table 2). In contrast, the cytosolic form of the enzyme remained fully active with hemin up to a concentration of 25 μM. However, a dose of 50 μM hemin produces only 25% inhibition.

The different nature of the mitochondrial and cytoplasmic enzymes is further elaborated by different responses to hemin injection. As reported earlier, hemin injection inhibits the activity of mitochondrial L-alanine:4,5-dioxovalerate transaminase. Inhibition (60%) of the activity of mitochondrial L-alanine:4,5-dioxovalerate transaminase is obtained with 0.8–1.2 mg/kg body mass of hemin, whereas 150% stimulation in the activity of the cytoplasmic form of L-alanine:4,5-dioxovalerate transaminase is observed. Stimulation of cytosolic L-alanine:4,5-dioxovalerate transaminase and inhibition of the mitochondrial enzyme by intravenous hemin injection clearly shows that both forms of the enzyme are different. However, our result for the *in vivo* experiment is in agreement with our *in vitro* observation that the mitochondrial form is inhibited by hemin at lower doses than the cytosolic enzyme. Cytosolic L-alanine:4,5-dioxovalerate transaminase has a lower specific activity as compared to the mitochondrial enzyme. Therefore, in order to examine whether proteolytic digestion of the enzyme generates forms of higher activity, we digested the cytosolic form with increasing concentrations of papain at 30°C for 10 min. It was observed that as the concentration of papain increases, a peptide fragment from the cytosolic enzyme with the same subunit molecular mass (50 kDa) as the native mitochondrial form, is generated. Furthermore, by assaying the activity of the papain-digested cytosolic enzyme, it was shown that the appearance of the 50 kDa fragments was accompanied by an almost threefold increase in the activity of the cytosolic L-alanine:4,5-dioxovalerate transaminase. Conversion of the cytosolic low activity form to a high activity one may be due to a change in the conformation due to papain digestion. The appearance of the 50-kDa fragment during papain digestion of the cytosolic form and its immunological cross-reactivity with antibody raised against the mitochondrial enzyme, suggest that both

mitochondrial and cytosolic L-alanine:4,5-dioxovalerate transaminases share a common fragment with a molecular mass of 50 kDa.

However, the cytosolic and mitochondrial L-alanine:4,5-dioxovalerate transaminases, digested with 0.1 μg and 0.25 μg chymotrypsin not only generate identical fragments of 50 kDa and 40 kDa, but also give rise to 30 kDa and 28 kDa fragments which are not observed as digestion products of the mitochondrial form at the concentrations of chymotrypsin used. However, a 36-kDa mitochondrial digest product (Fig. 7, lane 2), although missing from cytosolic enzyme digest with 0.1 μg chymotrypsin (Fig. 7, lane 1), appeared later upon digestion with 0.25 μg chymotrypsin (lane 4). The one-dimensional peptide map shows that although both forms share some common fragments, differences in molecular properties exist between the two forms. Our present study shows that mitochondrial and cytosolic L-alanine:4,5-dioxovalerate transaminases, although possessing some structural similarities, are basically different forms.

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REFERENCES

1. Granick, S. (1966) *J. Biol. Chem. 241*, 1359–1375.
2. Varticovski, L., Kushner, J. P. & Burnham, B. F. (1980) *J. Biol. Chem. 255*, 3742–3747.
3. Noguchi, T. & Mori, R. (1981) *J. Biol. Chem. 256*, 10335–10339.
4. Shanker, J. & Datta, K. (1983) *Biochem. Int. 7*, 23–31.
5. Shanker, J. & Datta, K. (1986) *Arch. Biochem. Biophys. 248*, 657–657.
6. Singh, N. K. & Datta, K. (1985) *Biochem. Biophys. Acta 827*, 305–309.
7. Morton, K. A., Kushner, J. P., Burnham, B. F. & Horton, W. J. (1981) *Proc. Natl Acad. Sci. USA 78*, 5325–5328.
8. Okuno, E., Minatogawa, Y. & Kido, R. (1984) *Biochem. Biophys. Res. Commun. 112*, 986–990.
9. Shanker, J. & Datta, K. (1985) *FEBS Lett. 189*, 129–132.
10. Chhabra, R., Singh, N. K. & Datta, K. (1986) *Biochem. Biophys. Res. Commun. 138*, 742–750.
11. Schatz, G. & Mason, T. L. (1974) *Annu. Rev. Biochem. 43*, 51–87.
12. Schatz, G. (1979) *FEBS Lett. 103*, 203–211.
13. Neupert, W. & Schatz, G. (1981) *Trends Biochem. Sci. 6*, 1–4.
14. Hennig, B. & Neupert, W. (1983) *Eur. J. Biochem. 121*, 203–211.
15. Hennig, B., Kochler, H. & Neupert, W. (1983) *Proc. Natl Acad. Sci. USA*, 4963–4967.
16. Yamamoto, M., Hayashi, N. & Kikuchi, G. (1981) *Arch. Biochem. Biophys. 209*, 451–459.
17. Mauzerall, D. & Granik, S. (1956) *J. Biol. Chem. 219*, 434–446.
18. King, J. (1974) in *Methods in enzymatic analysis* (Bergmeyer, H. V., ed.) vol. 2, pp. 656–659, Academic Press, New York.
19. Anderson, P. M. & Desnick, R. J. (1979) *J. Biol. Chem. 254*, 6924–6930.
20. Aebi, H. (1974) in *Methods in enzymatic analysis* (Bergmeyer, H. V., ed.) vol. 2, pp. 673–684, Academic Press, New York.
21. Laemmli, U. K. (1970) *Nature 227*, 680–685.
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
23. Merril, C. M., Goldman, D. & Sedman, S. A. (1981) *Science 211*, 1437–1438.
24. Ouchterlony, O. (1985) in *Progress in allergy* (Kellop, P., ed.) pp. 1–78, Karger, Basel.
25. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl Acad. Sci. USA 76*, 4350–4361.