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PURIFICATION AND PROPERTIES OF L-LYSINE-α-KETOGLUTARATE REDUCTASE FROM RAT LIVER MITOCHONDRIA

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Summary

L-Lysine-α-ketoglutarate reductase (N^5-(1,3-dicarboxypropyl)-L-lysine: $NADP^+$ oxidoreductase (L-lysine-forming, EC 1.5.1.8) was purified from rat liver mitochondria to a homogeneous state judged by SDS polyacrylamide gel electrophoresis, and its molecular weight was estimated as 52 000. On Sepharose 4B filtration it has a molecular weight of 230 000 and it is suggested that the active enzyme is a tetramer of subunits of similar size. The purified enzyme was clearly separated from saccharopine dehydrogenase (N^5-(1,3-dicarboxypropyl)-L-lysine:NAD^+ oxidoreductase (L-glutamate-forming, EC 1.5.1.9). The reaction of purified L-lysine-α-ketoglutarate reductase favored the forward reaction (saccharopine formation) and the rate of the reverse reaction (lysine formation) was only 3—5% that of the forward reaction. The forward reaction was specific for L-lysine, α-ketoglutarate and NADPH and followed Michaelis-Menten kinetics, whereas the dose vs. response curve of the reverse reaction was sigmoidal with saccharopine. Among the amino acids examined, ornithine, leucine and tryptophan inhibited the forward reaction competitively.

These results are different from earlier reports on human and yeast enzymes. The fact that rats fed on lysine-deficient diet do not lose weight much is discussed in relation to the properties of this enzyme.

Introduction

Lysine is a unique dietary amino acid in that a lysine-deficient diet causes only a slow decrease of body weight and does not affect the plasma and tissue concentrations of this amino acid appreciably [1—3]. Another unique feature of lysine is that it is deaminated first at its ε-amino group, with formation of saccharopine. This reaction is catalyzed by L-lysine-α-ketoglutarate reductase (N^5-(1,3-dicarboxypropyl)-L-lysine:$NADP^+$ oxidoreductase (L-lysine-forming,

EC 1.5.1.8) * [4,5]. This enzyme has only been partially purified from mammalian tissues [5—7]. It has been found in various tissues [5,7,8], but in rats, lysine is metabolized mainly in the liver [9,10]. These results prompted us to study the liver enzyme in more detail and this paper describes the purification of this enzyme to a homogeneous state from rat liver mitochondria and a comparison of its properties with those previously published on the enzymes in other mammals and yeast.

Materials and Methods

Materials. Male Wistar strain rats (approx. 200 g) fed ad libitum laboratory chow (Oriental Yeast Co., Tokyo) were used to prepare liver mitochondria. Hydroxyapatite and DEAE-cellulose were obtained from Seikagaku Kogyo, Tokyo and Sephadex G-200, QAE-Sephadex A-50, Sepharose 4B and Blue Dextran 2000 were from Pharmacia Fine Chemicals, Uppsala. Protein markers were purchased from the following sources: bovine pancreatic chymotrypsinogen A, ovalbumin and rabbit-muscle pyruvate kinase were from Sigma Chemical Co., St. Louis; pig-heart malate dehydrogenase, rabbit-muscle aldolase, beef-liver glutamate dehydrogenase, oxidized and reduced pyridine nucleotides and GTP were from Boehringer-Mannheim Co., Mannheim; sperm-whale myoglobin was from Schwarz-Mann, Orangeburg; amino acids were from Wako Pure Chemical Industries, Osaka or Tanabe Amino Acid Research Foundation, Osaka; and α-ketoglutarate was from Nakarai Chemicals, Kyoto.

Saccharopine was synthesized enzymatically by the method of Hutzler and Dancis [11] with the following modifications; the enzyme used was prepared from rat liver mitochondria, as described in this paper, and the reaction was carried out in Tris · HCl buffer (pH 8.4)/5 mM dithiothreitol. Dowex 50W × 8 and 1 × 8 ion-exchange resins were used for isolation of saccharopine. The final preparation was contaminated with 1.3% glutamate, estimated by an enzymatic method [12].

Spectrophotometric enzyme assays. Saccharopine formation (forward reaction) was measured by following the oxidation of NADPH at 340 nm. The assay system contained in a total volume of 1.0 ml: 100 μmol Tris · HCl buffer (pH 8.4), 0.2 μmol NADPH, 10 μmol α-ketoglutarate, 20 μmol 2-mercaptoethanol and enzyme. The reaction mixture was preincubated for 5 min at 37°C and the reaction was initiated by adding 40 μmol lysine. When a crude mitochondrial extract was used as the enzyme preparation, it was dialyzed beforehand, because it had high endogenous activity for NADPH oxidation.

The conversion of saccharopine to lysine (reverse reaction) was measured by following the reduction of NADP at 340 nm. The assay system contained, in a total volume of 1.0 ml: 100 μmol Tris · HCl buffer (pH 9.0), 4 μmol NADP, 1 μmol GTP, 5 μmol saccharopine, 2 μmol 2-mercaptoethanol and enzyme. The

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* All enzymes forming and degrading saccharopine were designated as saccharopine dehydrogenases by the Commission on Biochemical Nomenclature of the International Union of Biochemistry in 1972. But in this paper the classical name, L-lysine-α-ketoglutarate reductase (EC 1.5.1.8, NADP) is used for the enzyme forming saccharopine from L-lysine, α-ketoglutarate and NADPH, in view of the properties of the enzyme studied and to avoid confusion. The name saccharopine dehydrogenase (EC 1.5.1.9) is used for only the enzyme forming 2-aminoadipate semialdehyde from saccharopine and NAD.

reaction mixture was preincubated as above and the reaction was initiated by adding saccharopine. GTP was added to inhibit glutamate dehydrogenase, because the saccharopine preparation contained 1.3% glutamate as contaminant. Glutamate dehydrogenase was inhibited 95% under these conditions. GTP did not inhibit the reverse reaction. Saccharopine dehydrogenase (N^5-(1,3-dicarboxypropyl)-L-lysine:NAD^+ oxidoreductase (L-glutamate-forming, EC 1.5.1.9), also uses NADP as a cofactor [24] and, hence, spectrophotometric measurement of the reverse reaction was not possible with the crude extract. Therefore, the reverse reaction with crude extract was measured by determining the amount of hydrazone formation from α-ketoglutarate [13].

The conversion of saccharopine to α-aminoadipic semialdehyde by saccharopine dehydrogenase was measured by following the reduction of NAD at 340 nm. The reaction mixture contained, in a total volume of 1.0 ml: 100 μmol Tris · HCl buffer (pH 8.0), 3 μmol NAD, 1 μmol GTP, 1 μmol saccharopine and enzyme. The reaction was started as described above.

One unit of enzyme activity was taken as the amount forming one μmol of product per min and specific activity was expressed as units per mg protein. Protein was measured by the method of Lowry et al. [14].

Product identification. Reaction mixture similar to that described above, but on a 5-fold greater scale was incubated, deproteinized by boiling for 5 min, and centrifuged. The supernatant was concentrated in a vacuum evaporator and subjected to paper electrophoresis by the method of Hutzler and Dancis [5]. Saccharopine, lysine and glutamate were located with ninhydrin and identified by comparing their positions with those of authentic samples.

Estimation of molecular weight. A sample of 0.5 ml enzyme was applied to a Sepharose 4B column (1 × 44 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.6)/0.1 M NaCl/0.1 mM EDTA/5 mM 2-mercaptoethanol. Fractions of 0.3 ml were collected. Blue Dextran 2000 was used as a marker of the void volume. The column was calibrated by the method of Andrews [15] using the following markers: ovalbumin (M_r = 43 000), malate dehydrogenase (M_r = 70 000), hexokinase (M_r = 100 000), lactate dehydrogenase (M_r = 140 000), aldolase (M_r = 158 000), pyruvate kinase (M_r = 237 000) and glutamate dehydrogenase (M_r = 350 000).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn [16]. Protein was stained with Coomassie Brilliant Blue. Gels were calibrated with the following marker proteins: myoglobin (M_r = 17 200), chymotrypsinogen (M_r = 25 700), lactate dehydrogenase (M_r = 36 000), ovalbumin (M_r = 43 000), pyruvate kinase (M_r = 57 000) and albumin (M_r = 68 000).

Results and Discussion

Purification of L-lysine-α-ketoglutarate reductase from rat liver mitochondria. The best source of this enzyme in rats is liver mitochondria [4] and, hence, mitochondria prepared from 300 g rat liver were suspended in 250 ml of 10 mM potassium phosphate buffer (pH 7.6)/5 mM 2-mercaptoethanol/0.1 mM EDTA. All buffers used for subsequent steps contained these additives. The suspension (step I) was freeze-thawed 5 times and the supernatant (step II) was

TABLE I

PURIFICATION OF LYSINE-α-KETOGLUTARATE REDUCTASE FROM RAT LIVER MITOCHONDRIA

Step	Procedure	Total volume (ml)	Protein (mg/ml)	Specific activity (units/mg protein)	Yield (%)
I	Mitochondria	310	11.23	0.084	100
II	Extract	275	7.38	0.127	89
III	35%—55% Ammonium sulfate	34	38.13	0.198	80
IV	DEAE-cellulose	13.5	12.5	1.15	67
V	Sephadex-G-200	13.8	2.8	3.99	53
VI	Hydroxyapatite	10.5	1.24	7.35	33
VII	QAE-Sephadex	6.2	0.41	28.3	25

fractionated with $(NH_4)_2SO_4$. The precipitate formed between 35 and 55% saturation was dialyzed against 25 mM phosphate buffer (pH 7.6). The dialyzed preparation (step III) was applied to a DEAE-cellulose column (3.7 × 22 cm). The column was eluted with 2 l of a linear gradient of 25—150 mM phosphate buffer (pH 7.6). Active fractions were concentrated in an Amicon Ultrafiltration Cell, Model 202 with an XM-50 membrane. A similar method for concentrating the enzyme preparation was used in subsequent steps. The concentrated preparation (step IV) was applied to a Sephadex G-200 column (2.8 × 90 cm) and eluted with 50 mM phosphate buffer (pH 7.6). The reductase was eluted at approx. 1.6 times the void volume. The reductase fractions were concentrated and dialyzed against 5 mM phosphate buffer (pH 7.2) and the dialyzed enzyme (step V) was applied to a hydroxyapatite column (1.7 × 16 cm). The column was eluted with 300 ml of a linear gradient of 5—125 mM phosphate buffer (pH 7.2). The active fraction was concentrated and dialyzed against 100 mM Tris · HCl buffer (pH 7.6) and the dialyzed enzyme (step VI) was applied to a QAE-Sephadex column (1.8 × 18 cm). The column was eluted with 300 ml of a linear gradient of 50—200 mM NaCl in 100 mM Tris · HCl buffer (pH 7.6). The active fraction was concentrated and dialyzed against 10 mM Tris · HCl buffer (pH 7.6). The purified enzyme (step VII) was stable under these conditions at 0°C. The stabilizing effect of 2-mercaptoethanol on the human enzyme has been reported [6]. The overall purification was 300-fold with a yield of 25% in several repeated purifications. A summary of the purification procedure is shown in Table I. On SDS-polyacrylamide electrophoresis, the preparation appeared to be a homogeneous single protein (data not shown). The enzyme preparation had no detectable dehydrogenase activity for glutamate, malate, isocitrate or β-hydroxybutyrate.

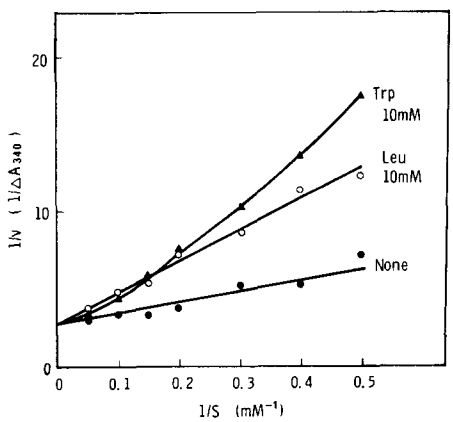
Molecular weight of L-lysine-α-ketoglutarate reductase. Gel filtration on Sephadex 4B showed that the molecular weight of the enzyme was 230 000. The enzyme of human placenta apparently exists as a complex with saccharopine dehydrogenase and the molecular weight of the complex was reported to be 480 000 [6,17]. We calculated the molecular weight of saccharopine dehydrogenase of rat liver as 43 000 (unpublished data). Thus, the sum of the molecular weights of the two enzymes is much less than that of the enzymes in

human tissue. On SDS-polyacrylamide gel electrophoresis, rat liver reductase showed a molecular weight of 52 000. Therefore, the active enzyme seems to be composed of 4 identical subunits. The molecular weight of yeast reductase was reported to be 49 000 [18], but its subunit structure is still unknown.

Substrate specificity and K_m values. The substrate specificity of the forward reaction was very strict for L-lysine, α-ketoglutarate and NADPH. Arginine, ornithine, glutamine, oxaloacetate, pyruvate and NADH did not act as substrates. It was reported that hydroxylysine and S-2-aminoethylcysteine were also found to act as substrates of the human liver reductase [7]; the K_m values of rat liver reductase for L-lysine, α-ketoglutarate and NADPH were 2.2 mM, 1.4 mM and 0.078 mM, respectively, and these values were very similar to those of the enzymes of human liver and yeast [7,19], although NADH is a cofactor for the yeast enzyme.

Inhibition of the forward reaction by ornithine, leucine and tryptophan. The forward reaction was reported to be inhibited by various amino acids [7,20]: the forward reaction of yeast reductase was strongly inhibited by ornithine, leucine, isoleucine and glutamine and that of human liver reductase by ornithine and glutamate. The forward reaction of rat liver reductase was only inhibited by ornithine, leucine and tryptophan; ornithine and leucine caused competitive inhibition with lysine, but the inhibition by tryptophan gave a slightly curved Lineweaver-Burk plot, which was reproducible (Fig. 1).

Odessey et al. [21] found that the addition of branched chain amino acids to diaphragm in vitro enhanced the release of lysine. This may be due to inhibition of muscle reductase by leucine, although the enzyme activity in diaphragm is very low [5,8]. Saccharopine also inhibited the forward reaction competitively [6,7]. This may be a cause of the stability of lysine in lysine-deficient animals. However, it is doubtful whether this inhibitory mechanism is involved in protection of lysine in vivo, because the inhibitory concentrations of these



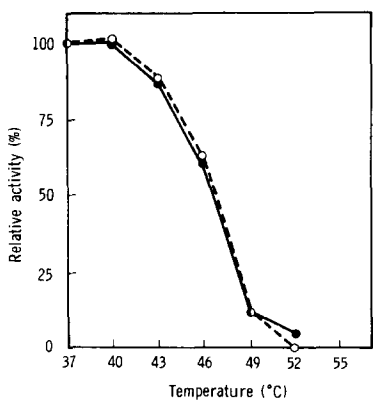


Fig. 1. Inhibition of the reductase by leucine (○) and tryptophan (▲).

Fig. 2. Heat stabilities of the forward and reverse reactions. The enzyme preparation was incubated for 5 min at the various temperatures and then the activities of the reactions in the two directions were measured. ●———●, forward and ○———○, reverse reactions.

substances are too high to be physiological and the magnitude of the inhibition is not large. Besides regulation of enzyme activity, there may also be regulation of the amount of enzyme protein, as shown in experiments in vivo: a high protein diet or administration of excess lysine induced this enzyme [8] and this change in reductase activity was reflected in a change in the rate of lysine oxidation in vivo [2,3,22]. Therefore, decrease in reductase activity may contribute to conservation of endogenous lysine in lysine-deficient animals.

Comparison of the forward (saccharopine-forming) and reverse (lysine-forming) reactions. Incubation of the enzyme with saccharopine and NADP caused detectable reduction of NADP and the product was identified as lysine, not glutamate, by paper electrophoresis. The pH optima for the forward and reverse reactions were 8.4 and 9.0, respectively. Similar differences in the pH optima of the two reactions of other enzymes have been reported [6,7,23].

The rate of the reverse reaction was about 3—5% of that of the forward reaction throughout the purification procedure and the purified enzyme separated by disc gel electrophoresis had both activities (unpublished data). The temperature sensitivities of the two reactions were similar, as shown in Fig. 2. Therefore, it was concluded that the reaction was reversible, equilibrium favoring saccharopine formation. The forward reaction followed Michaelis-Menten kinetics, but the reverse reaction gave a sigmoidal curve with increase in the concentration of saccharopine, especially with a low concentration of NADP (Fig. 3). Hill plots suggested positive cooperativity (Fig. 4).

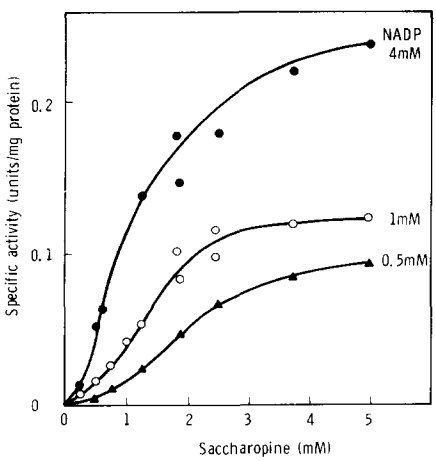


Fig. 3. Effects of the concentrations of saccharopine and NADP on the reverse reaction.

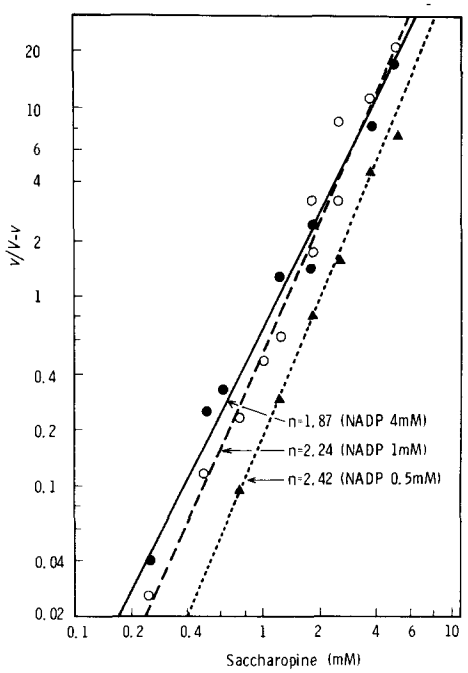


Fig. 4. Hill plots of the reverse reaction.

Fellows [23] reported that in the liver of various mammals there is another enzyme, which she named saccharopine oxidoreductase, that catalyzes lysine formation from saccharopine with NADP. She showed that the enzyme from ox liver was partially separated from lysine-α-ketoglutarate reductase by ammonium sulfate fractionation and that the oxidoreductase was more heat labile than the reductase. However, our results on the reverse reaction of rat-liver reductase were quite different: the reverse reaction in rat liver can be explained by the reversibility of the reductase. From a survey of the livers of various animals. Fellows concluded that lysine formation is very high in ox liver and lowest in rat liver [23], and the latter activity was comparable to that which we found. Thus, there does not seem to be another enzyme for catalyzing the reverse reaction in rat liver. In yeast both reactions are freely reversible [18].

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