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ISOLATION OF A BRANCHED-CHAIN α-KETO ACID DECARBOXYLASE FROM RAT LIVER

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Summary

An enzyme which catalyses oxidative decarboxylation of branched-chain α-keto acids was extracted from rat liver mitochondria with the aid of $NaClO_4$. Purification yielded a product which appeared homogenous upon electrophoresis. Some kinetic data are reported; however, the enzyme is inactive with α-ketoisovalerate. The tenacity of binding to mitochondria, specificity, and other features, suggest that the decarboxylase may be a component of an enzyme complex named α-ketoisocaproate: α-keto-β-methylvalerate dehydrogenase.

Introduction

Certain mitochondrial enzymes catalyse oxidative decarboxylation of α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate. From beef liver was obtained a purified enzyme active on the first two, but not on the third substrate [1,2]. Efforts to solubilize a similar enzyme from rat liver were unsuccessful [1,3]. Another enzyme purified from human kidney was active on α-ketoisovalerate [4], but activity towards other substrates was not reported.

These enzymes are thought to exist as multienzyme complexes with components functioning like those of the well-characterized pyruvate and α-ketoglutarate dehydrogenase systems and comprising three distinct units, namely, a decarboxylase catalysing reaction 1 below, a transacylase catalysing reactions 2

Abbreviation: DCPIP, 2,6-dichlorophenol indophenol.

and 3, and a lipoamide oxidoreductase (or dihydrolipoyl dehydrogenase), catalysing reaction 4.

$$\alpha\text{-Keto acid} + \text{thiamine-}PP \rightarrow \text{active aldehyde-thiamine-}PP + CO_2 \quad (1)$$

$$\text{Active aldehyde-thiamine-}PP + \text{lipoamide-}S_2 \rightarrow \text{acyl-S-lipoamide-SH} + \text{thiamine-}PP\,. \quad (2)$$

$$\text{Acyl-S-lipoamide-SH} + \text{CoA-SH} \rightarrow \text{acyl-S-CoA} + \text{lipoamide } (SH)_2 \quad (3)$$

$$\text{Lipoamide } (SH)_2 + NAD^+ \rightarrow \text{lipoamide-}S_2 + NADH + H^+ \quad (4)$$

The overall (or α-keto acid dehydrogenase) reaction is:

$$\alpha\text{-Keto acid} + \text{CoA-SH} + NAD^+ \rightarrow \text{acyl-S-CoA} + CO_2 + NADH + H^+ \quad (5)$$

It should be possible to assign each of the separate reactions 1—4 to a distinct enzyme of the branched-chain α-keto acid dehydrogenase system, but this has not yet been achieved. The work reported here represents progress towards that goal in that it was possible to isolate an enzyme which functions as a specific branched-chain α-keto acid decarboxylase.

Materials and Methods

Biochemicals were obtained from Sigma Chemical Co., MO, USA. All other chemicals were high grade reagents.

Enzyme assays. Decarboxylase activity (reaction 1) was measured by following reduction of DCPIP in a reaction mixture (3 ml total volume) of 144 μmol Tris-HCl (pH 7.2), 3 μmol $MgCl_2$, 60 μg DCPIP, and enzyme [4]. 100 μmol substrate then were added and the resulting absorbance change at 600 nm and 25°C was recorded. Initial velocity was determined by drawing tangents to the progress curves. Use of the 0.2 *A* scale of a Unicam SP 8000 spectrophotometer provided the necessary sensitivity.

Succinate dehydrogenase was measured similarly, by slight modifications to the procedure of Takemori and King [5]. The extinction coefficient, ϵ, for DCPIP was taken as $16.1 \cdot 10^3$.

Dehydrogenase activity (reaction 5) towards α-keto acids was measured by the method of Kanzaki et al. [6] adapted to a Turner Model 111 Fluorimeter. Increase in NADH concentration was monitored by excitation at 360 nm, emission being measured at 450 nm. The method was sensitive to a change of 0.1 nmol NADH/ml per min.

Enzyme purification. All procedures were conducted at 0—4°C and were routinely controlled by measuring decarboxylation of α-ketoisocaproate. Liver tissue from 4 male rats which had been starved 16—18 h and which averaged 200 g body weight was routinely pooled and then processed to yield mitochondria [7]. The packed mitochondria were blended into hypotonic medium (Buffer A) comprising 7.5 mM Tris-HCl, (pH 7.0), 1 mM EDTA, 0.5 mM 2-mercaptoethanol. 2 vols. Buffer A were used per g liver weight. The suspension of osmotically shocked mitochondria was centrifuged at 78 000 $\times g$ for 30 min. The resulting pellet was briefly blended in a Potter-Elvehjem homogenizer with 2.5 ml 0.8 M $NaClO_4$ and, after exactly 13 min perchlorate was

precipitated by adding a slight excess of KCl. The suspension was centrifuged at 78 000 × *g* for 30 min and the supernatant containing solubilized enzyme (10 mg protein/ml) was loaded onto a column (1.5 cm × 60 cm) of Sepharose 2B equilibrated and eluted with 30 mM Tris-HCl (pH 7.0), 1 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.76 M $(NH_4)_2SO_4$ (Buffer B) (flow rate 15 ml/h, 3-ml fractions). The fractions containing enzymic activity were pooled and brought to 80% saturation with respect to $(NH_4)_2SO_4$. Protein precipitated at 78 000 × *g* after 10 min was taken up in buffer B: glycerol (1 : 1 v/v) to give the final purified enzyme preparation.

Protein assays. Absorbance at 280 nm was used to determine relative protein concentration in fractions eluted from gel columns. Otherwise, the method of Lowry et al. [8] was used, with bovine serum albumin as a standard.

Polyacrylamide electrophoresis. This utilized a borate system with 7.5% polyacrylamide containing 6 M urea [9]. Protein bands were visualized with Amido Black.

Results and Discussion

A summary of a typical purification is given in Table I. The enzyme was eluted as a single peak from the gel filtration column (Fig. 1) and, when redissolved in the glycerol-buffer mixture, remained stable at 0—4°C for up to 12 h. Detailed studies of stability have not been undertaken.

The 5-fold purification from the stage of osmotically shocked mitochondria is additional to that achieved through subcellular fractionation of liver tissue. If the enzyme is mainly or exclusively mitochondrial as is the case with known branched-chain α-keto-acid dehydrogenase complex [3,10], isolation of this cellular compartment must have resulted in considerable enrichment. However, the specific activity at the stage of tissue homogenate or intact mitochondria could not be reliably assessed either by our assay method or by substituting ferricyanide [1,11] for DCPIP as the indicator oxidant. The 8-fold sensitivity of DCPIP compared to ferricyanide in spectrophotometric assays of this type led us to prefer the former at all stages of enzyme purification.

Our findings regarding the tenacious binding of the enzyme to rat liver mitochondria are similar to those of others [1,3] on branched-chain α-keto acid dehydrogenase. Lack of success with mechanical and sonic disruption, with organic solvents and with Triton X-100 led in the present experiments to the use

TABLE I

PURIFICATION OF DECARBOXYLASE FROM RAT LIVER

Step	Protein (mg)	Activity (units *)	Specific activity (units × 10^2/mg protein)	Recovery
1. Mitochondrial suspension (OS)	462	2.31	0.5	100
2. Supernatant after $NaClO_4$/KCl treatment **	18.3	0.10	0.55	4.0
3. Eluate from Sepharose 2B	13.8	0.35	2.54	15.0

* 1 unit = amount of enzyme catalysing decarboxylation of 1 μmol α-ketoisocaproate/min in 25°C.

** Time-dependent inhibitory effect reversed at step 3.

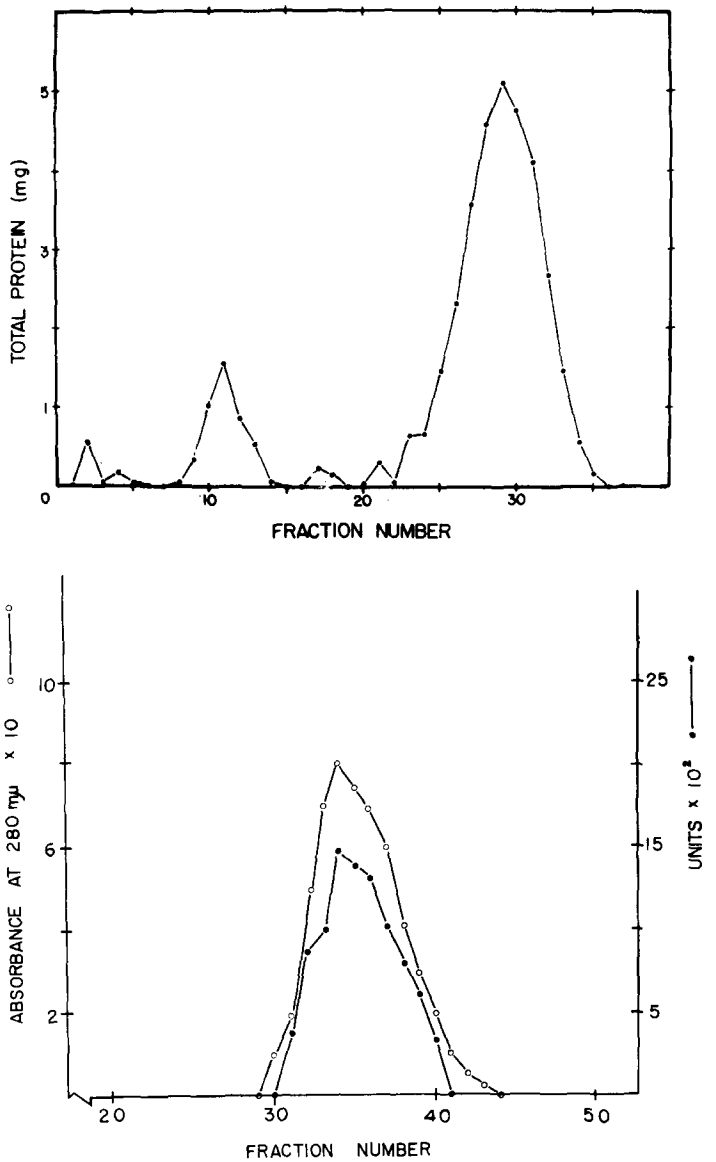

Fig. 1. (a) Elution profile of Supernatant (Step 2) from Sepharose 2B: fraction volume, 3 ml. Activity in largest peak. (b) Activity peak, decarboxylase fraction.

of the chaotropic agent $NaClO_4$ [12], but due regard had to be paid to its denaturing effects. Maximum solubilization of the decarboxylase with minimum inactivation was achieved by carefully timed exposure of lysed mitochondria to 0.8 M $NaClO_4$. Thereafter, most of the perchlorate was removed as the relatively insoluble potassium salt. Even then, it appeared that sufficient perchlorate remained dissolved together with solubilized enzyme to produce progressive inactivation of the latter. To minimize this, it was important to proceed rapidly between the perchlorate precipitation and gel filtration steps.

Chaotropes destabilize membranes and enzyme complexes and promote

solubility of membrane-bound enzymes in aqueous media, effects which are thought to involve decreased hydrophobic interactions [12].

Other workers have used perchlorate to detach succinic dehydrogenase from the inner mitochondrial membrane [13]. The less precisely known location of the multienzyme complexes with dehydrogenase activity on branched-chain α-keto acids and on α-ketoglutarate is inner membrane-matrix [10,14,15]. Therefore, special attention was given to possible contamination of the purified decarboxylase by these enzyme systems or their components. Significant activities of succinate dehydrogenase, α-ketoglutarate decarboxylase and of α-ketoisovalerate decarboxylase were present at the stage of osmotically shocked mitochondria, but such activities were not detectable in the gel eluate (Table II).

There is no firm evidence as to whether or not the purified decarboxylase was derived from a branched-chain α-keto acid dehydrogenase complex. Although overall dehydrogenase activity was indeed detected in osmotically shocked mitochondria by fluorimetric assay, no such activity remained in the purified enzyme. The addition of 0.5 units of lipoamide oxidoreductase (EC 1.6.4.3) to 0.05 units of purified decarboxylase did not restore dehydrogenase activity. A positive result would have indicated that the purified enzyme contained an associated transacylase; however, there could be additional requirements for successful reconstitution which were not met in this experiment. The question of possible physical and functional linkage of the decarboxylase to other enzymes remains open.

A single band was observed on polyacrylamide gel electrophoresis. This strongly suggests that a specific enzyme species was isolated in an electrophoretically homogenous state, since any contaminating enzymes would most probably have dissociated and separated in the system used.

Since the enzyme was only moderately retarded during gel filtration on Sepharose 2B (fractionation range $0.07-40 \cdot 10^6$ daltons) and was entirely excluded from Sephadex G-200, it is probably of relatively high molecular weight. Apart from molecular weight, however, the configuration of the molecule would be an important factor affecting such results.

Kinetic constants obtained from double reciprocal plots (α-keto acid concentration varied) were as follows: with α-ketoisocaproate and α-keto-β-methylvalerate, respectively, K_m was 2.0 and 0.87 mM; V was 0.027 and 0.010 μmol/

TABLE II

SPECIFIC ACTIVITIES (TOWARDS SUBSTRATES

Substrate	Step I (osmotic shock) $\times 10^2$	Step 3 (eluate) $\times 10^2$
α-Ketoisocaproate	0.50	2.54
α-Keto-β-methyl valerate	0.34	1.00
α-Ketoisovalerate	0.28	0
Succinate	0.92	0
α-Ketoglutarate	0.58	0
Pyruvate	0.24	0

* Specific activity = μmol substrate decarboxylated/min per mg enzyme protein.

min per mg protein. Few data are available for comparison. From measurements of decarboxylation catalysed by the intact complex isolated from beef liver [1], K_m values of 3.5 and 2.5 mM were obtained by other workers for α-ketoisocaproate and α-keto-β-methylvalerate respectively. These workers achieved a specific activity of 0.10 μmol/min per mg protein towards α-ketoisocaproate, which is four times higher than that of our enzyme. The validity of comparing enzyme data derived from different animal species is questionable, but there is evidently a qualitative species similarity in that the rat liver enzyme obtained in the present work, like that from beef liver, was inactive on α-ketoisovalerate.

Detailed characterization of the decarboxylase will be facilitated by availability of pure enzyme. In particular, advantage can be taken of the observed thiamin content (Morrison, E.Y.St.A., unpublished data) to supplement other methods of molecular weight estimation. Also, it will be of special interest to determine whether the homogeneity apparent from disc gel electrophoresis in 6 M urea results from polymeric structure comprised of identical protomers; this would contrast with the α and β subunits of the functionally analogous enzyme puruvate decarboxylase (pyruvate dehydrogenase of ref. 16).

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