Purification and Characterization of Branched Chain $\alpha$-Ketoacid Dehydrogenase from Bovine Liver Mitochondria*

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Branched chain $\alpha$-ketoacid dehydrogenase (EC 1.2.4.3(4)) was solubilized and purified from bovine liver mitochondria for the first time. Decarboxylation of $\alpha$-ketoisovalerate, $\alpha$-keto-$\beta$-methylvalerate, and $\alpha$-ketoisocaproate was catalyzed by this multienzyme complex and this activity was co-purified for each substrate. Three enzymatic functions were contained in the complex including decarboxylation of the above ketoads, transacylation of their simple acid derivatives, and reduction of NAD$^+$ as an overall reaction. Product stoichiometry of these three reactions was 1 CO$_2$;1 acyl-CoA;1 NADH. Activity depended upon the addition of thiamin pyrophosphate, CoASH, and NAD$^+$ which were dissociable cofactors. Physically, two active forms of the enzyme complex were found: a 275,000-dalton unit and a 2 x 10$^6$-dalton component. Both showed a characteristic flavin spectra and catalyzed all functions of the complex, implying that 10 small units aggregated into the larger unit. The soluble complex as visualized by electron microscopy had a diameter ranging from 12 to 24 nm corresponding to a molecular weight of 2 x 10$^6$. The size of the native membrane-bound component remains to be determined.

The discovery that branched chain $\alpha$-ketoacidemia was caused by genetically impaired branched chain $\alpha$-ketoacid dehydrogenase (EC 1.2.4.3 and 1.2.4.4) provided impetus for further biochemical definition of this presumptive mammalian multienzyme complex (1-3). More recently, branched chain $\alpha$-ketoacid dehydrogenase has been implicated as the rate-limiting step in the catabolic pathway of muscle tissue for leucine, isoleucine, and valine and therefore may control the flux of carbons and amino groups into alanine, influencing glucose production of liver, and thus control gluconeogenesis (4, 5). Reaction substrates, cofactors, and products as well as identification of the enzyme as part of the mitochondrial inner membrane suggested that branched chain $\alpha$-ketoacid dehydrogenase was a multienzyme complex similar to the pyruvate and $\alpha$-ketoglutarate dehydrogenase complexes (EC 2.1.4.1 and 1.2.4.2) which have been well characterized (6, 7). The three enzymes postulated in this complex were: 1) a deacarboxylase using thiamin pyrophosphate and Mg$^{2+}$ as cofactors, 2) a transacetylase with covalently bound lipoic acid which accepts the acyl moiety from the decarboxylase-bound thiamin pyrophosphate and transfers it to CoASH and, 3) a flavoprotein, NADH:lipoamide oxidoreductase (EC 1.6.4.3) which reoxidizes the lipoate of the transacetylase transferring the electrons to NAD$^+$. 

Despite the interest in branched chain $\alpha$-ketoacid dehydrogenase, several factors have frustrated early attempts at purification in efforts to fully characterize the complex. The enzyme was identified as a component of the mitochondrial inner membrane in human fibroblast and liver from human, rat, and bovine (3, 8-10). Removal from this membrane environment led to rapid loss of catalytic activity, a characteristic which was also true for the soluble branched chain $\alpha$-ketoacid dehydrogenase component of bacteria (11,12). A second factor making isolation difficult was that branched chain $\alpha$-ketoacid dehydrogenase in situ had low activity compared to other mitochondrial enzymes, especially pyruvate dehydrogenase and $\alpha$-ketoglutarate dehydrogenase, suggesting that it was present in low concentration. Reports of even partial purification of branched chain $\alpha$-ketoacid dehydrogenase from both mammalian and bacterial sources were therefore limited (9, 11-15).

Here we report for the first time the purification of branched chain $\alpha$-ketoacid dehydrogenase as an active, stable complex. This was attained using beef liver mitochondria which could be prepared rapidly from large quantities of fresh tissue. The enzyme was free of pyruvate dehydrogenase and $\alpha$-ketoglutarate dehydrogenase, oxidatively decarboxylated $\alpha$-ketoisovalerate, $\alpha$-keto-$\beta$-methylvalerate, and $\alpha$-ketoisocaproate, preferentially in this order, and the ratio was maintained during the co-purification.

**MATERIALS AND METHODS**

All reagents were of highest grade available and solutions were made in deionized water. 1-14C-labeled $\alpha$-ketoacids were prepared as previously described from precursor amino acids (16). $\alpha$-[1-14C]Ketoisovalerate was purchased from New England Nuclear. Calcium phosphate gel cellulose was prepared by the method of Koike and Hamada (17). Protein was determined by the method of Lowry et al. (18) or Bradford (19) using Moni-Trol from Dade, Miami, Fla., as the standard protein. Spectral analysis was performed on a Beckman 25 recording spectrophotometer or a Gilford 250 with recorder.

**Enzyme Assays—Decarboxylation of the 1-14C-labeled ketoacids** was quantitated by liquid scintillation counting of 14C in a 250-ml reaction mixture containing 0.1 mm ketoacid, 0.2 mm thiamin pyrophosphate, 0.5 mm CoASH, 0.2 mm MgCl$_2$, 1.0 mm NAD$^+$, 5 mm dithiothreitol, 100 mg of bovine serum albumin, 50 to 100 mg of enzyme protein, and 30 mm KPO$_4$, pH 7.5, as previously described (8). Reduction of NAD$^+$ was followed spectrophotometrically with time at 340 nm ($\epsilon_{\text{max}}$ = 6.22 x 10$^3$ cm$^2$ mol$^{-1}$) in a 1.0 ml reaction volume with reactants as described above except that unlabeled ketoacids served as substrate. Reactions were initiated by addition of substrate after a 7 min equilibration.

14C-labeled ketoacids were...
ductase activity was determined by following the oxidation of NADH according to the method of Ide et al. (20). Transacylase activity was determined by separation of reaction products on thin layers chromatographic plates developed in butanolic acid: H2O (6:2:3). Universally labeled α-L[14C]ketoisovalerate and α-[14C]ketoisocaproate were prepared as described for the 1-14C-labeled ketocids (16). In this way, both 14C]OAc and 14C]Acyl-CoA could be quantitated from the same reaction.

Mitochondria Isolation—Bovine liver (10 kg) was obtained fresh from the slaughterhouse. The tissue was trimmed free of fat and fiber, diced, and washed in saline (0.9% NaCl) solution until free of blood. After mincing, the tissue was homogenized in a Waring Blender with 0.32 M sucrose 1:9 (v/v) and the mitochondria isolated by differential centrifugation. The mitochondrial pellet was washed two times by resuspension in filtered sucrose followed by centrifugation at 8000 × g for 10 min. The final pellet was weighed and suspended in phosphate buffer as described below.

Electron Microscope Studies—For electron microscope observations, samples of branched chain α-ketoacid dehydrogenase containing 0.001, 0.01, or 0.1 mg of protein/ml of buffer were applied to carbon-stabilized Formvar coated 400-mesh grids by the drop method. After drying in air in a saturated atmosphere of glutaraldehyde for 10 min, grids were stained with 2% sodium phosphate>state, pH 6.8, or 0.5% uranyl acetate (21). Separate grids were also stained with osmium tetroxide. Observations were made with a Philips 400 electron microscope operating at 60 kV.

Gel Electrophoresis—A 7.5% acrylamide gel was used to separate the components of the complex. The separating gel was made 8 ml with respect to urea in a 1 M Tris-HCl buffer, pH 8.8. Application of the sample was through a 2% acrylamide stacking gel in the presence of 5 M urea and 0.02 M dithiothreitol. Electrophoresis was at 10 mA/cm. No activity was associated with the 200,000-dalton first peak and 90% of the activity was contained in the second 250,000-dalton peak.

**RESULTS**

**Solubilization and Purification of Branched Chain α-Ketoacid Dehydrogenase from Isolated Mitochondria**—The pellet of mitochondria was suspended in 30 mM KPO4, pH 7.2, (30 g/500 ml), frozen at -20°C for 12 to 18 h, and then thawed in cold running water (15 to 18°C). Solubilized protein was removed by centrifugation at 25,000 × g for 30 min. The results of these procedures are outlined in Table I. The first supernatant contained a small amount of the branched chain α-ketoacid dehydrogenase activity and most of the contaminating hemoglobin. The loss in specific activity from intact mitochondria to solubilized enzyme was a consistent finding which remains unresolved. The pellet (Pellet I) which retained most of the branched chain α-ketoacid dehydrogenase activity was suspended in a volume of deionized water equal to the first suspending volume and the freeze-thaw centrifugation procedure was repeated. A clear golden supernatant (II) resulted which contained more than 14% of the enzyme activity, the largest amount of solubilized enzyme. Additional freeze-thawing in water or buffer improved neither the yield nor the specific activity. Activity which remained in the pellet was progressively lost on subsequent days. Solubilized branched chain α-ketoacid dehydrogenase was precipitated by adjusting the solution to 50% saturation with solid ammonium sulfate and the precipitate was collected by centrifugation. A minimal amount of 50 mM KPO4, pH 7.0, was used to suspend the pellet which was then dialyzed overnight against three changes of this same buffer. This protein mixture was then adsorbed onto a calcium phosphate gel cellulose column equilibrated with 0.01 M KPO4, pH 7.0, (1000 mg of protein/100 ml, bed volume) and washed with 10 to 20 ml of 0.01 M buffer. Stepwise elution with 2 bed volumes was begun with 0.07 M Tris/glycine, pH 8.5, running buffer. Bromphenol blue (0.1%) was used to mark the tracking dye which was run off the gel to complete the run.

**Table I**

<table>
<thead>
<tr>
<th>Protein</th>
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<td>2.6</td>
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<td>4.2</td>
</tr>
<tr>
<td>Supernatant II</td>
<td>5,500</td>
<td>1.8</td>
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<td>(NH4)2SO4</td>
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<td>4.3</td>
</tr>
<tr>
<td>CaPO4 gel</td>
<td>145</td>
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</tr>
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*Specific activity, nanomoles of [14C]OAc/min/mg of protein; α-ketoisovalerate used as substrate.

**Physical Characterization**—Molecular size of the branched chain α-ketoacid dehydrogenase was estimated by gel filtration, sucrose density gradient centrifugation, and electron microscopy. The elution pattern from a Bio-Gel A-5m column can be seen in Fig. 1. Two protein peaks were eluted. Ten percent of applied branched chain α-ketoacid dehydrogenase activity was associated with the 2 × 10^6-dalton first peak and 90% of the activity was contained in the second 250,000-dalton peak.

**Summary of purification of branched chain α-ketoacid dehydrogenase from beef liver mitochondria**

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**Total activity, nanomoles of [14C]OAc/min.**

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*Fig. 1. Elution pattern and molecular weight determination of branched chain α-ketoacid dehydrogenase on Bio-Gel A-5m. A column (52 × 1 cm) was equilibrated with 50 mM KPO4, pH 7.0, and after application of enzyme and marker proteins, elution was continued with the same buffer. V0 was 13 ml with a flow rate of 3.8 ml/h; 0.72-ml fractions were collected. Inset as described in text.●, standard marker proteins; ○, Peaks I and II.*
peak (inset Fig. 1). These were the only protein peaks found. Similar data were obtained with a G-200 and Bio-Gel A-1.5m column, but on these two gels, the first protein peak eluted with V_e. The molecular weight of branched chain α-ketoacid dehydrogenase calculated by the methods of Martin and Ames (22) using a 5 to 30% linear sucrose gradient with bovine serum albumin and thyroglobulin as reference standards was 275,000 (Fig. 2).

It was possible to visualize this preparation of branched chain α-ketoacid dehydrogenase by electron microscopy after dilute solutions of the enzyme had been negatively stained with phosphotungstic acid or uranyl acetate. Regularly shaped units were apparent in several fields, the average size range being 12 to 24 nm in diameter. These units correspond to a molecular weight for the complex of approximately $2 \times 10^6$ (23). Also apparent within the larger units was a detailed substructure suggestive of multiple protein subunits making up the total complex (see Fig. 3, a to d). When grids were stained with osmium tetroxide, no lipid staining was observed, indicating a lack of membrane structure, supporting the fact that the complex has been released from the membrane and solubilized.

Gel electrophoresis in basic urea gels showed five bands. This might be expected if any components of the sample were comprised of subunits as is the case for pyruvate dehydrogenase of the pyruvate dehydrogenase complex. When pig heart lipoamide oxidoreductase was run under these conditions, two bands were evident and correspond to the two fastest moving bands of the five bands from branched chain α-ketoacid dehydrogenase (see Fig. 4).

**Catalytic Properties**—The specificity and cofactor requirements of this soluble and purified complex were studied and the results are depicted in Tables II and III. α-Ketoisovalerate, α-keto-β-methylvalerate, and α-ketoisocaproate were decar-

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Sedimentation of branched chain α-ketoacid dehydrogenase in a linear sucrose density gradient. Enzyme was layered on an 11-ml linear gradient of 6 to 30% sucrose. Tubes were spun at 14,000 rpm corresponding to an average of 190,000 $\times$ g for 20 h in an SW 41 rotor. One-milliliter fractions were collected and Lowry (18) protein and activity were determined on each fraction. Marker proteins, thyroglobulin (TG) and bovine serum albumin (BSA), were run in identical tubes in the same run.

![Fig. 3 (left)](http://www.jbc.org/)

**Fig. 3 (left).** Electron micrographs of negatively stained samples of branched chain α-ketoacid dehydrogenase. Samples were placed on grids and allowed to dry in an atmosphere saturated with glutaraldehyde before staining with sodium phosphotungstate. a, low power view illustrating the particulate nature of the sample, × 180,000, marker equals 100 nm. b to d, high power views of the large 12 to 24 nm diameter particles, × 450,000 marker equals 20 nm.

![Fig. 4 (right)](http://www.jbc.org/)

**Fig. 4 (right).** Basic urea gel electrophoresis of branched chain α-ketoacid dehydrogenase. For details see text. Arrows indicate the five protein bands visualized with fast green stain. Electrophoresis was from cathode to anode.
boxylated with relative rates in this order. This preparation of branched chain α-ketoacid dehydrogenase had activity in the nanomolar range which allowed us to follow NAD⁺ reduction spectrophotometrically at 340 nm. The ratio of [14C]O₂ released to NADH produced was 1, which would be expected for the presumed reaction since 2 electrons are produced per CO₂. In addition to the three branched chain α-ketoacids, pyruvate served as a substrate for NADH reduction, but (Y-κ-ketoisovalerate did not. Prior incubation of branched chain (Y-ketoacid dehydrogenase with either 1 mM MgC₂ or 10 mM MgCl₂ and 0.1 mM CaCl₂, conditions which stimulate either kinase or phosphatase of the pyruvate dehydrogenase complex, was without effect on the rates of conversion for any substrate (24, 25). Two further points should be noted. First, the rate of nucleotide reduction with pyruvate was below the rates observed for any of these substrates except for the indicated omissions.

Evidence presented here supports the hypothesis that a single multienzyme complex catalyzes the oxidative decarboxylation of the three branched chain α-ketoacids. Two active forms of the complex were detected, a 300,000-dalton form and a 2 million-dalton species. The smaller form showed all properties of the larger species, suggesting that aggregation of the smaller resulted in formation of the multienzyme complex. These results are consistent with lipoamide oxidoreductase being a functional part of this multienzyme complex.

The cofactors required for both CO₂ release and pyridine nucleotide reduction are shown in Table III. Both reactions showed absolute dependence on exogenous thiamin pyrophosphate, NAD⁺, and CoASH and the absence of any of these cofactors substantially reduced both the release of CO₂ and the reduction of NAD⁺. Identical patterns were found for α-ketoisovalerate, α-ketoisocaproate, and α-keto-β-methylvalerate. The variation between NADH production and CO₂ release in the absence of thiamin pyrophosphate or CoASH may reflect the level of sensitivity for the two assay methods or it may be that the decarboxylase component can function minimally independently of the other protein components.

DISCUSSION

Successful solubilization and isolation of purified branched chain α-ketoacid dehydrogenase has not been reported until now (see final paragraph). Factors such as low solubility and high lability which plagued earlier attempts at purification have been circumvented by the methods described here. Release of branched chain α-ketoacid dehydrogenase from its mitochondrial membrane rapidly decayed and prevented the membrane-bound activity could be solubilized. Lability, the second major difficulty, was minimized by maintaining a high mitochondrial protein concentration and rapidly purifying the complex by column chromatography. It is interesting to note that the enzyme activity which remained bound to the mitochondrial membrane rapidly decayed and prevented higher yields of the soluble enzyme. Whether a specific protease for this complex is present or not, as described for kidney α-ketoisocarboxylate dehydrogenase, remains speculative (96).

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proteins so that antibodies can be produced against these proteins. Such an approach will aid in identifying the mutant proteins produced and expressed in cultured skin fibroblasts from patients with branched chain \( \alpha \)-ketoacidemia. In addition, questions concerning the nuclear or mitochondrial origin of genetic control mechanisms for this enzyme complex would be explored. Only through the separation and purification of the complex components can the remaining questions be answered.

While this manuscript was in review, Pettit et al. (15) published a report of the successful purification of branched chain \( \alpha \)-ketoacid dehydrogenase from bovine kidney. Their results are similar to our findings, but lipoamide oxidoreductase did not co-purify with the kidney branched chain \( \alpha \)-ketoacid dehydrogenase.

REFERENCES

Purification and characterization of branched chain alpha-ketoacid dehydrogenase from bovine liver mitochondria.
D J Danner, S K Lemmon, J C Besharse and L J Elsas, 2nd