Purification and Characterization of Branched Chain α-Ketoacid Dehydrogenase from Bovine Liver Mitochondria*

(Received for publication, December 14, 1978)

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Branched chain α-ketoacid dehydrogenase (EC 1.2.4.3(1)) was solubilized and purified from bovine liver mitochondria for the first time. Decarboxylation of α-ketosovalerate, α-κeto-β-methylvalerate, and α-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 ketoacids, transacetylation of their simple acid derivatives, and reduction of NAD⁺ as an overall reaction. Product stoichiometry of these three reactions was 1 CO₂:1 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 × 10⁶-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 × 10⁶. The size of the native membrane-bound component remains to be determined.

The discovery that branched chain α-ketoacidemia was caused by genetically impaired branched chain α-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 α-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 glucose homeostasis (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 α-ketoacid dehydrogenase was a multienzyme complex similar to the pyruvate and α-ketoglutarate dehydrogenase complexes (EC 1.2.4.1 and 1.2.4.2) which have been well characterized (6, 7). The three enzymes postulated in this complex were: 1) a decarboxylase using thiamin pyrophosphate and Mg⁺⁺ as cofactors, 2) a transacylase 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 transacylase transferring the electrons to NAD⁺.

Despite the interest in branched chain α-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 α-ketoacid dehydrogenase component of bacteria (11, 12). A second factor making isolation difficult was that branched chain α-ketoacid dehydrogenase in situ had low activity compared to other mitochondrial enzymes, especially pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, suggesting that it was present in low concentration. Reports of even partial purification of branched chain α-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 α-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 α-ketoglutarate dehydrogenase, oxidatively decarboxylated α-ketoisovalerate, α-keto-β-methylvalerate, and α-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 α-ketoacids were prepared as previously described from precursor amino acids (16). α-[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, Fl., as the standard protein. Spectral analysis was performed on a Beckman recording spectrophotometer or a Gilford 250 with recorder.

**Enzyme Assays**—Decarboxylation of the ¹⁴C-labeled ketoacids was quantitated by liquid scintillation counting of ¹⁴CO₂ produced by a 250-µl reaction mixture containing 0.1 mm ketoacid, 0.2 mm thiamin pyrophosphate, 0.5 mm CoASH, 0.2 mm MgCl₂, 1.0 mm NAD⁺, 5 mm dithiothreitol, 100 µg of bovine serum albumin, 50 to 100 µg of enzyme protein, and 30 mm K₂PO₄ buffer, pH 7.5, as previously described (8). Reduction of NAD⁺ was followed spectrophotometrically with time at 340 nm (ɛ₉₀ = 6.22 × 10³ cm⁻¹ M⁻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. Lipoamide oxidoreduc-
ductase activity was determined by following the oxidation of NADH according to the method of Ide et al. (20). Transacetylase activity was determined by separation of reaction products on thin layers chromatographic plates developed in butanol:acetic acid:H₂O (6:2:3). Universally labeled a-[¹⁴C]ketoisovalerate and a-[¹⁴C]ketoisocaproate were prepared as described for the ¹⁴C-labeled ketocarboxylic acids (10). In this way, both ¹⁴Cl-CoA and ¹⁴C-labeled 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 (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 X 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 a-ketoacid dehydrogenase containing 0.001, 0.01, or 0.1 mg of protein/mg 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 phosphotungstate, pH 6.8, or 0.5% uranyl acetate (21). Separate grids were also stained with osmium tetroxide. Observations were made with a Phillips 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 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 8 M urea and 5 mM dithiothreitol. Electrophoresis was at 10 mA/slab during concentration and then at 34 mA for separation using a 20 ng Tris/glycine, pH 8.5, running buffer. Bromphenol blue (0.1%) was the tracking dye which was run off the gel to complete the run.

RESULTS

Solubilization and Purification of Branched Chain α-Ketoacid Dehydrogenase from Isolated Mitochondria—The pellet of mitochondria was suspended in 30 mM KPO₄, pH 7.3, (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 X 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 a-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 a-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 phosphate buffer, pH 7.0. This fraction contained pyruvate dehydrogenase and a-ketoglutarate dehydrogenase activity but not branched chain α-ketoacid dehydrogenase activity. A small amount of active branched chain α-ketoacid dehydrogenase eluted with 0.2 M phosphate buffer and the remaining activity evolved with 0.25 M buffer. High ionic strength buffer, 0.5 M, eluted all remaining protein but no active enzyme. Subsequently, a two-step elution was used starting with 0.13 M buffer to eliminate the majority of nonspecific proteins followed by 2 bed volumes of 0.25 M buffer which contained the branched chain α-ketoacid dehydrogenase. This high salt fraction represented the highest purification of the solubilized enzyme, as indicated in Table I.

Preliminary attempts with NAD-agarose and thiamin pyrophosphate-Sepharose affinity chromatography resulted in inactivation of the complex, possibly due to dissociation of the complex into its component proteins. The fraction eluted from CaPO₄ gel cellulose columns retained full activity at -70°C for more than 8 months. 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 per cent of applied branched chain α-ketoacid dehydrogenase activity was associated with the 2 x 10⁶-dalton first peak and 90% of the activity was contained in the second 250,000-dalton fraction. The activity of the enzyme as indicated in Table I. Further attempts at purification were made with molecular sieve column chromatography and sucrose density gradient centrifugation. Neither led to an increase in specific activity. Preliminary attempts with NAD-agarose and thiamin pyrophosphate-Sepharose affinity chromatography resulted in inactivation of the complex, possibly due to dissociation of the complex into its component proteins. The fraction eluted from CaPO₄ gel cellulose columns retained full activity at -70°C for more than 8 months.

TABLE I

<table>
<thead>
<tr>
<th>Protein</th>
<th>Specific activity (μmol/min/mg)</th>
<th>Total activity (µmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial homogenate</td>
<td>26,000</td>
<td>67.4</td>
</tr>
<tr>
<td>Pellet I</td>
<td>13,000</td>
<td>56.4</td>
</tr>
<tr>
<td>Supernatant II</td>
<td>5,500</td>
<td>9.9</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>2,000</td>
<td>10.9</td>
</tr>
<tr>
<td>CaPO₄ gel</td>
<td>145</td>
<td>8.4</td>
</tr>
</tbody>
</table>

1 Specific activity, nanomoles of [¹⁴C]O₂/min/mg of protein; α-ketoisovalerate used as substrate.

2 Total activity, micromoles of [¹⁴C]O₂/min.

1 S. K. Lemmon, L. J. Elsas, and D. J. Danner, manuscript in preparation.
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_o. 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).

**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).** 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, $\times 180,000$, marker equals 100 nm. b to d, high power views of the large 12 to 24 nm diameter particles, $\times 450,000$ marker equals 20 nm.

**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.
Stoichiometry and substrate specificity of branched chain α-ketoacid dehydrogenase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activitya</th>
<th>% control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Ketoisovalerate</td>
<td>10.8 ± 0.830</td>
<td>100</td>
</tr>
<tr>
<td>α-Ketocaproate</td>
<td>7.1 ± 0.589</td>
<td>100</td>
</tr>
<tr>
<td>α-Keto-β-methylvalerate</td>
<td>8.0 ± 0.570</td>
<td>7.6 ± 0.630</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>3.7</td>
<td>100</td>
</tr>
</tbody>
</table>

a Branched chain α-ketoads were present at 0.1 mM, while α-ketoglutarate and pyruvate were present at 1 mM.

Evidence presented here supports the hypothesis that a single multienzyme complex catalyzes the oxidative decarboxylation of the three branched chain α-ketoads. Two active forms of the complex were detected, a 300,000-dalton form and a 2 million-dalton species. The smaller form showed all the catalytic properties of the larger component, suggesting that aggregation of the smaller resulted in formation of the catalytic properties of the larger component, suggesting that aggregation of the smaller resulted in formation of the catalytic properties of the larger component, suggesting that aggregation of the smaller resulted in formation of the catalytic properties of the larger component.
larger. The size of the physiologically active form in the membrane remains to be determined. The theorized complex should contain a thiamin pyrophosphate-dependent decarboxylase, a CoASH-dependent transacetylase, and a flavoprotein, lipoamide oxidoreductase utilizing NAD". This is consistent with the data presented above. Thus, it appears that branched chain α-ketoacid dehydrogenase is a multienzyme complex similar to pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes but separate and specific for the branched chain α-ketoads. Lipoamide oxidoreductase, which is common to the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes, might well be common to all three complexes as suggested by these studies and supported by the findings reported by Robinson and co-workers (27, 28). They described a human patient with a defect in lipoamide oxidoreductase activity. When tissues from this patient were assayed for pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, or branched chain α-ketoacid dehydrogenase activity, no activity was detectable.

Although it is known that pyruvate dehydrogenase and α-ketoglutarate dehydrogenase do not decarboxylate the branched chain ketoacids, it is not known whether a single branched chain α-ketoacid dehydrogenase complex is multi-specific for the three branched chain ketoacids. Several lines of evidence support the idea of a single multispecific complex. First, branched chain ketoacidemia is inherited as a mono-genic trait and specifically affects this complex. No homozygous affected patient has been found with an impaired catalytic pathway for only one branched chain amino acid. Second, coordinate induction of activity for all three ketoacids by diet has been reported in rats and by supraphysiologic oral loading with thiamin in humans (9, 29). Third, with a partially purified branched chain α-ketoacid dehydrogenase from rat liver, competitive inhibition was found among the three branched chain ketoacids with a $K_i = K_{m}$ (8). Fourth, in this study, we found co-purification of all three activities and the relative ratio of activities always remained α-ketoisovalerate > α-keto-β-methylvalerate > α-ketoisocaproate (data not shown).

Based on a single report by Connelly et al. (13), a theory for separate substrate specific complexes has been proposed. In this early report, activity for α-ketoisovalerate remained particle-bound, while activity for α-ketoisocaproate:α-keto-β-methylvalerate was solubilized. Several differences should be noted between our present data and the earlier report. Connelly et al. (19) used whole liver homogenate as starting material which has a high concentration of proteolytic enzymes; they used an insensitive ferricyanide assay which measured only decarboxylase activity and the final purification was significantly less than we report here. However, the results of Connelly et al. (13) could be compatible with our current findings if we include in our complex three separate decarboxylase components which share the transacylase and oxidoreductase proteins. Selective solubilization of individual decarboxylases could have occurred under the isolation conditions described by Connelly's group (13). Only through separation of the purified complex into its component proteins can this conflict be resolved. Thus far, we have found no evidence suggesting that separate decarboxylases exist, although there are five proteins present by gel electrophoresis (see Fig. 4).

The purified branched chain α-ketoacid dehydrogenase is being further characterized and separated into its component 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 α-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 α-ketoacid dehydrogenase from bovine kidney. Their results are similar to our findings, but lipoamide oxidoreductase did not co-purify with the kidney branched chain α-ketoacid dehydrogenase.

REFERENCES

Purification and characterization of branched chain alpha-ketoacid dehydrogenase from bovine liver mitochondria.
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