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Biochemistry

Purification and characterization of branched chain α-keto acid dehydrogenase complex of bovine kidney

(multienzyme complex/subunit composition/structural organization)

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ABSTRACT **A branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex was purified to apparent homogeneity from bovine kidney mitochondria. As usually isolated, the complex ($s_{20,w}$ = 40 S) contained little, if any, dihydrolipoyl dehydrogenase. When saturated with the latter enzyme the complex had a specific activity of about 12 μmol of α-ketoisovalerate oxidized per min per mg of protein at 30° with NAD^+ as electron acceptor. In addition to α-ketoisovalerate, the complex also oxidized α-ketoisocaproate, α-keto-β-methylvalerate, α-ketobutyrate, and pyruvate. The ratios of the specific activities were 2.0:1.5:1.0:1.0:0.4, and the apparent K_m values were 40, 50, 37, 56, and 1000 μM. The complex was separated into its component enzymes. The branched chain α-keto acid dehydrogenase (6 S) consists of two different subunits with estimated molecular weights of 46,000 and 35,000. The dihydrolipoyl transacylase (20 S) contains apparently identical subunits of molecular weight about 52,000. In the electron microscope, the transacylase has the appearance of a cube, and the molecules of branched chain α-keto acid dehydrogenase appear to be distributed on the surface of the cube. In contrast to the pyruvate dehydrogenase complex of bovine kidney, the branched chain α-keto acid dehydrogenase complex apparently is not regulated by phosphorylation–dephosphorylation. Its activity, however, is subject to modulation by end-product inhibition.**

Current interest in the regulation, significance, and abnormalities (e.g., "maple syrup" urine disease) of branched chain amino acid metabolism (1) has led to renewed interest in the enzymology of branched chain α-keto acid oxidation. The branched chain α-keto acids α-ketoisovaleric, α-ketoisocaproic, and α-keto-β-methylvaleric, derived from the essential amino acids valine, leucine, and isoleucine, respectively, are oxidized by mitochondrial enzyme(s) assumed to be analogous to the multienzyme pyruvate and α-ketoglutarate dehydrogenase complexes. Each of these two complexes consists of three enzymes that, acting in sequence, catalyze overall reaction 1:

$$RCOCO_2H + CoA\text{-}SH + NAD^+ \rightarrow R\text{-}CO\text{-}S\text{-}CoA + CO_2 + NADH + H^+. \quad [1]$$

Each complex is organized about a core, consisting of dihydrolipoyl transacetylase or dihydrolipoyl transsuccinylase, to which pyruvate dehydrogenase or α-ketoglutarate dehydrogenase and dihydrolipoyl dehydrogenase are joined by noncovalent bonds (2).

Partial purification and properties of branched chain α-keto acid dehydrogenase(s) from mammalian tissues (3–6) and from bacteria (7–9) have been reported. Most of the evidence obtained with these partially purified preparations indicates that all three branched chain α-keto acids are oxidized by a single, high molecular weight dehydrogenase that is distinct from pyruvate dehydrogenase (4, 6, 9). However, the purity of these enzyme preparations is difficult to judge because their specific activities ranged from less than 0.1% to about 1.0% of specific activities reported for highly purified pyruvate and α-ketoglutarate dehydrogenase complexes. Purification of the enzyme(s) to homogeneity and analysis of the homogeneous preparation(s) are necessary to demonstrate unequivocally that a single dehydrogenase oxidizes all three branched chain α-keto acids and that this dehydrogenase is an integral part of a multienzyme complex analogous to the pyruvate and α-ketoglutarate dehydrogenase complexes.

In this communication we report the purification and some properties of an essentially homogeneous branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex from bovine kidney mitochondria. The complex coupled with added dihydrolipoyl dehydrogenase to oxidize all three branched chain α-keto acids at rates comparable to the rate of oxidation of pyruvate by the pyruvate dehydrogenase complex.

MATERIALS AND METHODS

The sodium salts of the α-keto acids were obtained from Sigma, bovine serum was from Irvine Scientific, polyethylene glycol (molecular weight, 6000) was from J. T. Baker, and Ultrogel AcA 34 was from LKB. All other chemicals were of the purest grades available commercially. Dihydrolipoyl dehydrogenase was purified from kidney mitochondria to a specific activity of about 109 μmol of NADH oxidized/min per mg of protein at 30° (10).

The initial rate of overall reaction 1 was determined by monitoring NADH formation at 340 nm and 30° with a recording spectrophotometer. The assay mixture contained, in a volume of 1.0 ml, 50 μmol of potassium phosphate buffer (pH 8.0), 0.2 μmol of thiamin pyrophosphate, 1.0 μmol of $MgCl_2$, 2.5 μmol of NAD^+, 0.13 μmol of CoA, 2.6 μmol of cysteine hydrochloride, 2.0 μmol of the sodium salt of the α-keto acid, and enzyme complex. The final pH was 7.4. To obtain maximal rates with the branched chain α-keto acid dehydrogenase it was necessary to include dihydrolipoyl dehydrogenase (10 units) in the assay mixture. The reaction was initiated by addition of enzyme complex. Initial reaction velocities are expressed as μmol of NADH formed per min.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed routinely with the discontinuous buffer system of Laemmli (11). Subunit molecular weights were estimated by the procedure of Weber and Osborn (12) in 7.5% acrylamide gels. Electrophoresis of the enzyme complex was performed on 2% agarose slab gels with 0.05 M phosphate buffer, pH 7.0. The slab gel was run at 3–4 mA for 2.3 hr.

Negatively stained specimens were prepared as described (13) and were examined in a Siemens Elmiskop 1 operating at 60 kV. The micrographs were taken at a magnification of ×100,000.

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RESULTS

Purification of Branched Chain α-Keto Acid Dehydrogenase–Dihydrolipoyl Transacylase Complex. All operations were carried out at 2°–5°. Bovine kidney mitochondria were prepared and washed twice with 0.25 M sucrose (at pH 7.0–7.4), once with deionized water, and twice with 0.02 M potassium phosphate buffer (pH 6.5) as described (14). Care was taken during the sucrose washing to remove the lysosome-enriched fluffy layer from the mitochondrial pellet. The washed mitochondrial paste was suspended in 0.02 M phosphate buffer (pH 6.5) and shell-frozen and thawed once. The thawed suspension was made 0.05 M in NaCl, 1% by volume of bovine serum was added to inhibit proteases, and the mixture was centrifuged at 14,000 rpm for 30 min in a Beckman type JA-14 rotor. The amber-colored mitochondrial extract (about 3 liters) was made 0.2 mM in thiamin pyrophosphate, and 1% by volume of bovine serum was added. The extract contained 11–15 mg of protein per ml. It was adjusted to pH 6.4 with 10% acetic acid and made 10 mM in $MgCl_2$. A 50% (wt/vol) aqueous solution of polyethylene glycol was added, with stirring, to give a final concentration of 3%. Stirring was continued for 10 min, and the precipitate was collected by centrifugation at 14,000 rpm for 10 min. The precipitate contained essentially all of the branched chain α-keto acid dehydrogenase activity as well as the pyruvate and α-ketoglutarate dehydrogenase activities.

To separate the branched chain α-keto acid dehydrogenase complex from the two other complexes, the precipitate was suspended, by means of a glass/Teflon homogenizer, in 0.05 M 2-(*N*-morpholino)propanesulfonate buffer (pH 7.0) containing 0.2 mM thiamin pyrophosphate, 1 mM $MgCl_2$, 2 mM dithiothreitol, and 1% by volume of bovine serum (buffer A). After standing for at least 1 hr, the mixture (about 300 ml) was centrifuged at 15,000 rpm for 15 min. Usually, the supernatant fluid contained the pyruvate and α-ketoglutarate dehydrogenase complexes, whereas the pellet contained most of the branched chain α-keto acid dehydrogenase activity. With some preparations it was necessary to adjust the pH of the suspension to 6.5 before centrifugation to obtain a satisfactory separation of the branched chain α-keto acid dehydrogenase complex. To extract the latter complex, the pellet was resuspended in 35 ml of buffer A, and the suspension was made 0.2 M in NaCl. After standing for at least 1 hr, the mixture was centrifuged at 18,000 rpm for 20 min, and the pellet was discarded. The supernatant fluid was diluted with buffer A to a protein concentration of 5 mg/ml. The pH was carefully lowered to 6.4 by dropwise addition, with stirring, of 10% acetic acid. Any precipitate was removed by centrifugation and discarded. The pH was lowered to 6.2 and maintained at this value for 10 min, while the mixture was stirred. The precipitate was collected by centrifugation and resuspended in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.2 mM thiamin pyrophosphate, 1 mM $MgCl_2$, 0.1 mM EDTA, 2 mM dithiothreitol, and 1 mM NAD^+ (buffer B). This suspension was allowed to stand overnight and then centrifuged at 15,000 rpm for 15 min. The pellet was discarded, and the supernatant fluid was centrifuged at 40,000 rpm for 3.5 hr. The faintly yellow pellet was redissolved in buffer B, and the solution was clarified by centrifugation at 15,000 rpm for 15 min. A summary of the purification is presented in Table 1 (procedure A). Most of the data were obtained with complex purified by procedure A.

Recently we developed a simplified procedure (Table 1, procedure B) for obtaining the branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex in higher yield, but somewhat lower specific activity, from bovine kidney mitochondrial extracts. The mitochondrial extract was made 0.2 mM in thiamin pyrophosphate and 10 mM in $MgCl_2$, and 1% by volume of bovine serum was added. The pH was carefully lowered to 6.18 by dropwise addition, with stirring, of 10% acetic acid. The light precipitate contained essentially all of the branched chain α-keto acid dehydrogenase activity but little, if any, of the pyruvate and α-ketoglutarate dehydrogenase activities. The precipitate was collected by centrifugation and resuspended in 15 ml of 0.1 M potassium phosphate buffer (pH 7.0) containing 2 mM dithiothreitol and 0.1 mM EDTA (buffer C). This suspension was allowed to stand overnight and then was centrifuged at 15,000 rpm for 15 min. The pellet was discarded, and the supernatant fluid was centrifuged at 40,000 rpm for 3.5 hr. The faintly yellow pellet was redissolved in buffer C, and the solution was clarified by centrifugation.

Table 1. Purification of branched chain α-keto acid dehydrogenase-dihydrolipoyl transacylase complex*

Fraction	Protein, mg	Specific activities KIV†	KIV:KIC:KMV‡	Recovery, %
Procedure A				
Mitochondrial extract	33,370	0.042	2.3:1.4:1.0	(100)
PEG precipitate§	911	1.6	2.0:1.4:1.0	104
Precipitate, pH 6.2	88	9.2		58
Ultracentrifuge pellet	52	11.8	2.2:1.5:1.0	44¶
Procedure B				
Mitochondrial extract	42,066	0.034		(100)
Precipitate, pH 6.18	314	4.5		99
Ultracentrifuge pellet	176	8.8		108

* From about 37.4 kg of bovine kidney.
† Micromoles of NADH formed/min per mg of protein at 30° in the presence of excess dihydrolipoyl dehydrogenase and with α-ketoisovalerate (KIV) as substrate.
‡ Ratios of specific activities obtained with α-ketoisovalerate (KIV), α-ketoisocaproate (KIC), and α-keto-β-methylvalerate (KMV) as substrate.
§ PEG, polyethylene glycol.
¶ The overall recovery varied between 40 and 60%.

Properties of Branched Chain α-Keto Acid Dehydrogenase–Dihydrolipoyl Transacylase Complex. The sedimentation velocity pattern of the highly purified complex showed a single symmetrical peak (Fig. 1*A*) with a sedimentation coefficient ($s_{20,w}$) of about 40 S. The homogeneity of the preparation was also strongly indicated by its electrophoretic pattern (Fig. 2).

The ratios of specific activities with α-ketoisovalerate, α-ketoisocaproate, and α-keto-β-methylvalerate remained essentially constant over the 250- to 300-fold purification of the complex from mitochondrial extracts (Table 1). The apparent K_m values determined from double reciprocal plots of initial velocity data were 40, 50, and 37 μM, respectively. Pyruvate and α-ketobutyrate (but not α-ketoglutarate) were also oxidized by the complex, at rates about 20 and 50%, respectively, of the rate observed with α-ketoisovalerate. The apparent K_m values were 300 mM for pyruvate and 56 μM for α-ketobutyrate.

The complex was totally dependent on CoA-SH and virtually totally dependent on thiamin pyrophosphate (95%) and on dihydrolipoyl dehydrogenase (96–99%) in catalyzing reaction 1. $NADP^+$ did not replace NAD^+. The involvement of lipoyl moieties covalently attached to the transacylase is strongly indicated by the following observations. The complex underwent an α-ketoisovalerate-dependent inactivation (about 80% in 15 min at 23°) in the presence of *N*-ethylmaleimide under conditions shown previously to result in selective modification of the lipoyl moieties in the pyruvate dehydrogenase complex (15, 16). The modified complex was also inactive with α-ketoiso-

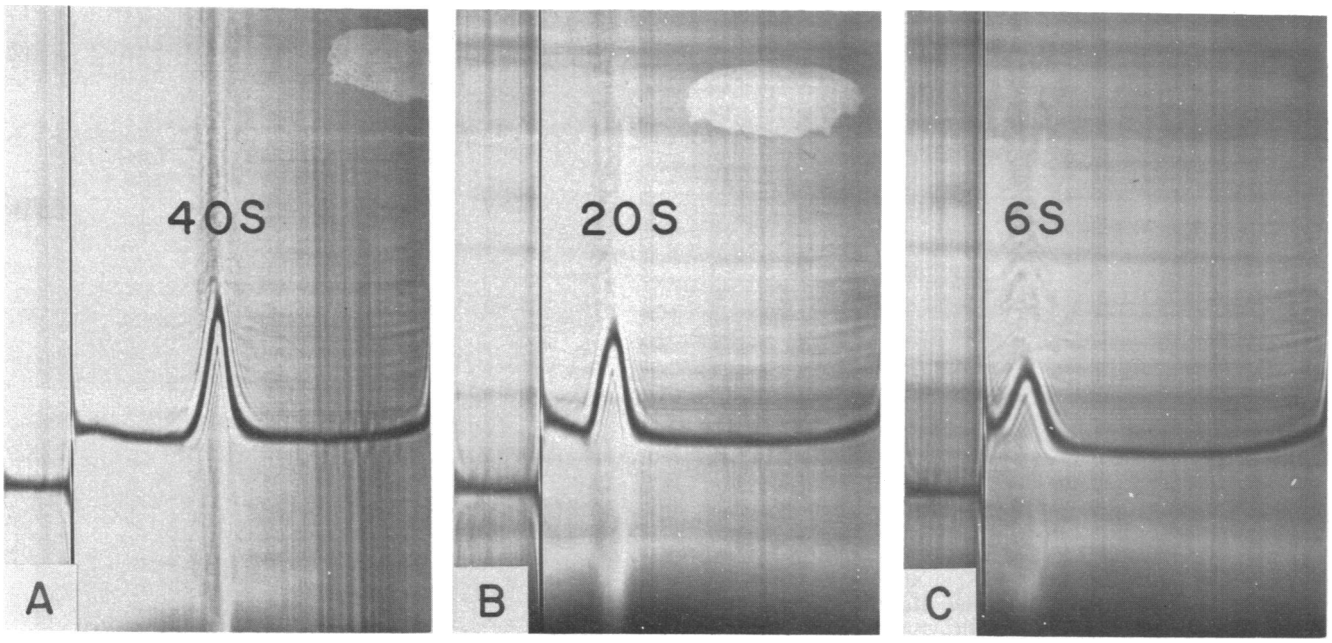

FIG. 1. Sedimentation velocity patterns obtained with the branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex (*A*) and its component enzymes, dihydrolipoyl transacylase (*B*) and branched chain α-keto acid dehydrogenase (*C*), in 0.05 M potassium phosphate buffer (pH 7.0) at 40,000 rpm and 20°. The sedimentation coefficients are corrected to water at 20°.

caproate, α-keto-β-methylvalerate, and pyruvate as substrates. This last observation provides additional evidence that a single complex catalyzes the oxidative decarboxylation of all four α-keto acids. When the complex was incubated with [2-^{14}C]-pyruvate under anaerobic conditions, [^{14}C]acetyl groups were rapidly incorporated into the transacylase (16).

Under conditions that resulted in relatively rapid phosphorylation and inactivation of the bovine kidney pyruvate dehydrogenase complex, the activity of the branched chain α-keto acid dehydrogenase complex was unaffected. Incubation of the highly purified branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex with purified pyruvate dehydrogenase kinase (14) and [γ-^{32}P]ATP for 10 min at 23° resulted in incorporation of only about 0.4 nmol of ^{32}P per mg of protein and did not affect the activity of the complex with either the branched chain α-keto acids or with pyruvate as substrate. This observation provides additional evidence that pyruvate dehydrogenase activity is inherent in the branched chain α-keto acid dehydrogenase complex and is not due to contamination with the pyruvate dehydrogenase complex. Incubation of the purified branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex with bovine heart protein kinase, adenosine 3′,5′-cyclic monophosphate, and [γ-^{32}P]ATP for 10 min at 23° also did not affect the activity of the complex, and only about 0.6 nmol of ^{32}P was incorporated per mg of protein. Crude mitochondrial extracts were also tested in the event that an unidentified kinase might be present that would interact with the branched chain α-keto acid dehydrogenase (Fig. 3).

FIG. 2. Electrophoretic pattern obtained with the branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex on 2% agarose slab gel at pH 7.0. The anode was at the bottom.

Substances that had little effect, if any, at a concentration of 0.2 mM on the activity of the branched chain α-keto acid dehydrogenase complex were valine, leucine, isoleucine, AMP, ADP, GMP, GDP, GTP, acetyl-CoA, succinyl-CoA, and β-hydroxy-β-methylglutaryl-CoA. However, isobutyryl-CoA and isovaleryl-CoA, products of the oxidation of α-ketoisovalerate and α-ketoisocaproate, respectively, inhibited the activity of the complex about 48 and 56%, respectively, at a concentration of 0.2 mM. α-Methylbutyryl-CoA, a product of α-keto-β-methylvalerate oxidation, was not available for testing. At a concentration of 0.1 mM, NADH inhibited the activity of the complex about 44%.

Subunit Composition and Organization of Branched Chain α-Keto Acid Dehydrogenase–Dihydrolipoyl Transacylase Complex. When subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, the highly purified complex gave three major bands (Fig. 4, lane A) with estimated molecular weights of about 52,000, 46,000, and 35,000. Exploratory experiments using the analytical ultracentrifuge indicated that the complex dissociated at alkaline pH and high salt concentration. The conditions worked out to

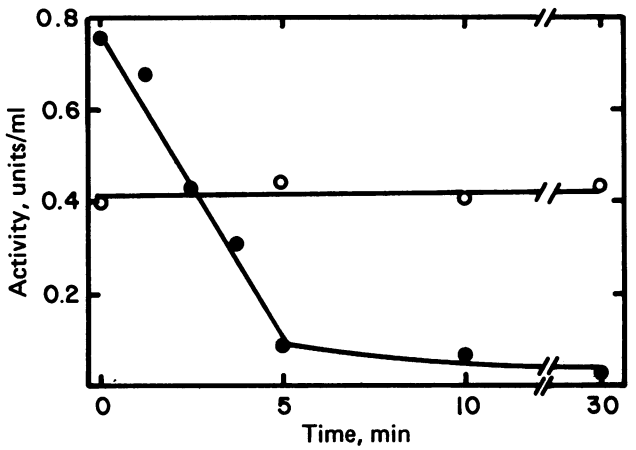

FIG. 3. Comparative effects of phosphorylation conditions on activities of the pyruvate dehydrogenase complex and the branched chain α-keto acid dehydrogenase complex. The reaction mixture contained 0.8 ml of mitochondrial extract (8 mg of protein), 20 mM phosphate buffer (pH 6.4), 0.5 mM ATP, 1.0 mM $MgCl_2$, and 50 mM NaF in a final volume of 1.0 ml. At the indicated times aliquots were assayed for NAD-reduction activity with pyruvate (●) or α-ketoisovalerate (○) as substrate. With the latter substrate, 10 units of dihydrolipoyl dehydrogenase was added to the assay mixture. In the kidney mitochondrial extracts, 40–65% of the pyruvate dehydrogenase was in the phosphorylated, inactive form.

resolve the complex were as follows. To a solution of the complex (16 mg) in 1.0 ml of 0.05 M phosphate buffer (pH 7.0) were added sufficient solid NaCl, dithiothreitol and 1 M Tris to make the solution 1 M in NaCl and 10 mM in dithiothreitol and the final pH 8.2. After standing for 30 min at 5°, the solution was chromatographed on a column (1.5 × 90 cm) of Ultrogel AcA 34 equilibrated and developed with 0.05 M Tris·HCl, pH 7.7/1 M NaCl/2 mM dithiothreitol/1 mM $MgCl_2$/0.1 mM EDTA. The dihydrolipoyl transacylase emerged at the void volume, and the branched chain α-keto acid dehydrogenase emerged at $V_e/V_0 = 1.34$. The appropriate fractions were pooled, and the two enzymes were concentrated by precipitation with solid ammonium sulfate at 25 and 35% saturation, respectively. The precipitates were dissolved in and dialyzed against buffer B. The sedimentation velocity pattern of the branched chain α-keto acid dehydrogenase showed a single peak (Fig. 1*C*) with a sedimentation coefficient ($s_{20,w}$) of about 6 S. Its dodecyl sulfate/polyacrylamide gel electrophoretic pattern showed that it consists of two different subunits (Fig. 4, lane B) with apparent molecular weights of about 46,000 and 35,000. The dihydrolipoyl transacylase had a sedimentation coefficient of about 20 S (Fig. 1*B*), and it apparently consists of a single subunit (Fig. 4, lane C) with an estimated molecular weight of about 52,000. The isolated transacylase contained trace amounts of the branched chain α-keto acid dehydrogenase.

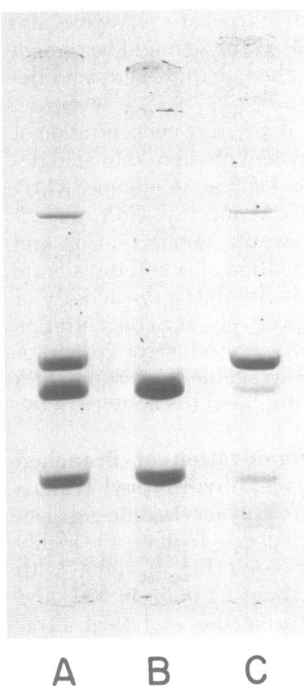

FIG. 4. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of highly purified preparations of the branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex (lane A) and its component enzymes, branched chain α-keto acid dehydrogenase (lane B) and dihydrolipoyl transacylase (lane C).

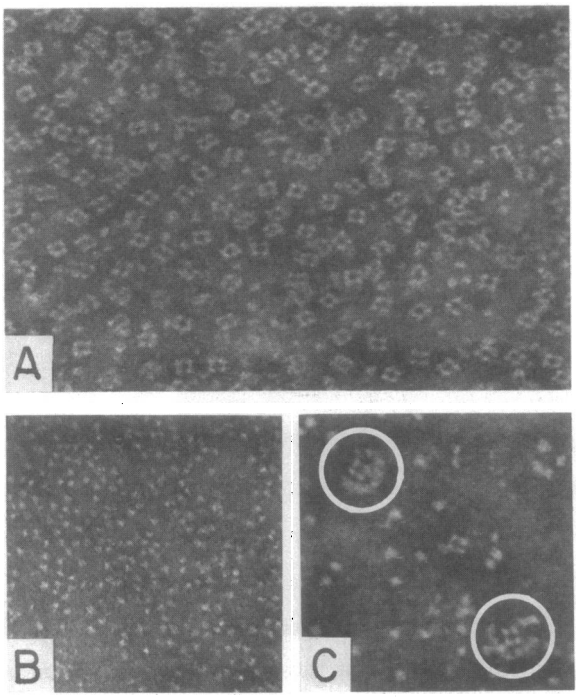

FIG. 5. Electron micrographs of the dihydrolipoyl transacylase (*A*), the branched chain α-keto acid dehydrogenase (*B*), and the branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex (C). The samples were negatively stained with methylamine tungstate. (×200,000.)

Electron micrographs of the branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex and its two component enzymes are shown in Fig. 5. The transacylase component has the appearance of a cube (Fig. 5*A*), and the molecules of the branched chain α-keto acid dehydrogenase component (Fig. 5*B*) appear to be distributed on the surface of the cube (Fig. 5*C*). The complex showed a pronounced tendency to dissociate into the dehydrogenase and the transacylase under the negative staining conditions used.

DISCUSSION

The data reported in this communication provide direct evidence that a single dehydrogenase is capable of oxidizing α-ketoisovalerate, α-ketoisocaproate, and α-keto-β-methylvalerate and that this dehydrogenase is a multienzyme complex analogous to the pyruvate and α-ketoglutarate dehydrogenase complexes.

As usually isolated from kidney mitochondria, the branched chain α-keto acid dehydrogenase–dihydrolipoyl transacylase complex is deficient in, if not completely free of, dihydrolipoyl dehydrogenase. Even in crude extracts of kidney mitochondria, which contain uncomplexed dihydrolipoyl dehydrogenase, the branched chain α-keto acid dehydrogenase activity is stimulated 2- to 3-fold by addition of dihydrolipoyl dehydrogenase.

Similar results have been obtained with the branched chain α-keto acid dehydrogenase complex of porcine liver (unpublished data). Apparently, dihydrolipoyl dehydrogenase dissociates more readily from the branched chain α-keto acid dehydrogenase complex (i.e., has a larger dissociation constant) than from the pyruvate and α-ketoglutarate dehydrogenase complexes. When saturated with dihydrolipoyl dehydrogenase, the branched chain α-keto acid dehydrogenase complex had a specific activity of about 12 μmol of α-ketoisovalerate oxidized/min per mg of protein, a value comparable to that of the pyruvate dehydrogenase complex (about 20 μmol of pyruvate oxidized/min per mg of protein). A deficiency of dihydrolipoyl dehydrogenase may account, at least in part, for the very low specific activities of partially purified preparations of branched chain α-keto acid dehydrogenase reported by other investigators (3–8).

The dihydrolipoyl transacylase core of the branched chain α-keto acid dehydrogenase complex has a cube-like appearance in the electron microscope, and it consists of subunits of molecular weight about 52,000. Its appearance is similar to that of the dihydrolipoyl transacetylase core of the *Escherichia coli* pyruvate dehydrogenase complex and the dihydrolipoyl transsuccinylase cores of the mammalian and *E. coli* α-ketoglutarate dehydrogenase complexes. (2). In view of the known subunit structure of these latter transacylases, we would expect the transacylase component of the branched chain α-keto acid dehydrogenase complex to consist of 24 identical polypeptide chains. The molecules of branched chain α-keto acid dehydrogenase appear to be distributed on the surface of the transacylase cube.

In contrast to the pyruvate dehydrogenase complex, the branched chain α-keto acid dehydrogenase complex of bovine kidney apparently is not regulated by phosphorylation–dephosphorylation. This conclusion is based on the results of experiments carried out with the highly purified complexes as well as with crude mitochondrial extracts. Under conditions that resulted in relatively rapid phosphorylation and inactivation of the pyruvate dehydrogenase complex, the branched chain α-keto acid dehydrogenase complex did not undergo phosphorylation, and its activity was unaffected. However, the activity of the bovine kidney branched chain α-keto acid dehydrogenase complex, like that of the pyruvate and α-ketoglutarate dehydrogenase complexes, is inhibited by the end products of reaction 1—i.e., branched chain acyl-CoA and NADH. An extensive analysis of end-product inhibition of the partially purified branched chain α-keto acid dehydrogenase complex of ox liver has been reported by Parker and Randle (4).

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