Catalytic and Structural Properties of the Dihydrolipoyl Transacylase Component of Bovine Branched-chain α-Keto Acid Dehydrogenase*

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Branched-chain α-keto acid dehydrogenase is a multienzyme complex consisting of three catalytic components, i.e. branched-chain α-keto acid decarboxylase (E1), dihydrolipoyl transacylase (E2), and dihydrolipoyl dehydrogenase (E3). In this report the E2 component of highly purified branched-chain α-keto acid dehydrogenase from bovine kidney and liver was characterized with an independent radiochemical assay for this component. The assay uses the model reaction: R-CO-S-CoA + Lip-SH → R-CO-S-Lip + CoA-SH, which is similar to that catalyzed by the transacylase component of the pyruvate dehydrogenase complex. In this reaction, exogenous dihydrolipoamide substitutes for the protein (E2)–bound dihydrolipoyl moiety, and [1-14C]acyl-CoA synthesized enzymatically is the acyl-CoA substrate. The thioester structure of the reaction product, S-S-CoA, was identified by mass spectrometry, its characteristic absorption at 232–245 nm and by formation of hydroxamate with hydroxylamine. Rates of the E2-catalyzed transacylation reaction with various [1-14C]acyl-CoAs are in the order of [1-14C]isobutyryl-CoA > [1-14C]isovaleryl-CoA > [1-14C]acetyl-CoA. The activity with acetyl-CoA is 15% of that with isobutyryl-CoA. The E2 activity is strongly inhibited by arsenite. Modification of the covalently bound lipooyl moiety through reductive acylation in the presence of N-ethylmaleimide is without effect on the transacylation reaction. These data, along with results of initial velocity and product inhibition suggest that the model reaction proceeds via a random Bi Bi mechanism. Limited proteolysis of purified bovine liver branched-chain α-keto acid dehydrogenase with trypsin results in complete loss of the overall activity catalyzed by the complex. Nonetheless the activity of the E2 component is not affected. The tryptic digestion cleaves E2 subunits (Mᵣ = 52,600) into a major fragment of Mᵣ = 25,700. By contrast, E1α and E1β subunits of the complex are relatively resistant to proteolysis with trypsin. The results indicate that structural properties of the E2 component of branched-chain α-keto acid dehydrogenase are similar but not identical to those of the transacylase component of the pyruvate dehydrogenase complex.

Branched-chain α-keto acid dehydrogenase catalyzes oxidative decarboxylation of the α-keto acids (Reaction 1) derived from valine, leucine, and isoleucine.

\[
\text{R-CO-COOH + CoA-SH + NAD⁺} \rightarrow \text{R-CO-S-CoA + CoA + NADH} + \text{H⁺}
\]  \hspace{1cm} (1)

The multienzyme complex presumably consists of three catalytic components: i.e. a TPP⁺-dependent branched-chain α-keto acid decarboxylase, or E1; a lipoate-containing dihydrolipoyl transacylase, or E2; and a flavoprotein of dihydrolipoyl dehydrogenase, or E3 (1). These enzyme components are believed to catalyze a coordinated sequence of reactions that lead to the overall reaction, as shown in Reaction 1, by analogy with the pyruvate dehydrogenase complex (1). The E1 and E2 components are apparently specific for branched-chain α-keto acid dehydrogenase, while the E3 component is common to branched-chain α-keto acid, pyruvate, and α-ketoglutarate dehydrogenase complexes (1). Recent studies have shown unequivocally that branched-chain α-keto acid dehydrogenase is regulated by a phosphorylation–dephosphorylation mechanism (2–6), similar to that of the pyruvate dehydrogenase complex (7).

The E2 component of branched-chain α-keto acid dehydrogenase from bovine kidney contains identical subunits with an estimated Mᵣ = 52,000 (8). Electron microscopy of E2 has the appearance of a cube to which molecules of the E1 component are distributed on the surfaces of the core (8). The E3 component is apparently only loosely associated with the complex (8). Recently the subunit structure of E2 components of pyruvate and α-ketoglutarate dehydrogenase complexes has been elucidated using limited proteolysis (9–12). The E2 (transacylase) component of bovine heart pyruvate dehydrogenase complex was shown to contain two folding domains: a compact, subunit-binding domain, and an acidic extended domain bearing the covalently bound lipooyl moiety (9, 10). The cleavage of the E2 (transacylase) subunits of the mammalian pyruvate dehydrogenase complex by trypsin (9, 10), elastase (10), papain (13), or a leupeptin-sensitive lysosomal “inactivase” (14) resulted in dissociation of the complex and consequently the loss of its overall activity. Nonetheless, the nicked E2 (transacylase) component retained its full component activity. Little is currently known concerning the subunit structure of the E2 component of branched-chain α-keto acid dehydrogenase.

Activity of branched-chain α-keto acid dehydrogenase is deficient in the human inborn error of metabolism, maple syrup urine disease (15). Genetic heterogeneity in maple syrup
urine disease has been demonstrated by partial restoration of branched-chain \(\alpha\)-keto acid dehydrogenase activity in heterozygous obtained by fusing fibroblast cultures from unrelated patients (16, 17). The presence of several components in the multienzyme complex required the development of independent component assays to determine the genetic locus affected in subjects with the disease. We have shown previously by an \(E_2\) assay that the defect in several classical maple syrup urine disease subjects resides in the \(E_2\) component of branched-chain \(\alpha\)-keto acid dehydrogenase (18, 19). A genetic defect in the \(E_3\) component also has been described, that results in multiple deficiencies in branched-chain \(\alpha\)-keto acid, pyruvate, and \(\alpha\)-ketoglutarate complex activities (20-22). \(E_2\) deficiencies of the branched-chain \(\alpha\)-keto acid and pyruvate dehydrogenase complexes were suggested in certain patients with maple syrup urine disease and in familial lactic acidosis (23, 24). However, activities of these \(E_2\) components were not directly measured in those studies.

We describe here studies of the \(E_2\) component of branched-chain \(\alpha\)-keto acid dehydrogenase using a model reaction (Reaction 2) analogous to that catalyzed by the \(E_2\) (transacetylase) component of the pyruvate dehydrogenase complex (25).

\[
R\cdot^{14}C\text{COO-S-CoA} + \text{Lip-(SH)}_2 \rightarrow R\cdot^{14}C\text{COO-S-LipSH} + \text{CoA-SH} \quad (2)
\]

In the radiochemical assay which is measured in the reversed physiological direction, exogenous dihydrolipoamide substituishes for the protein (\(E_2\))-bound lipoyl moiety and enzymatically synthesized \([1\cdot^{14}C]\)isobutryl-CoA and \([1\cdot^{14}C]\)isovaleryl-CoA are the acyl-CoA substrates. The assay is highly sensitive which allows direct measurements of the \(E_2\) activity in cell culture (18). Results presented here with highly purified bovine branched-chain \(\alpha\)-keto acid dehydrogenase preparations provide evidence for a transacylation reaction between acyl-CoA and dihydrolipoamide that is catalyzed by a specific \(E_2\) component of the complex. Moreover, limited tryptic digestion indicates that structural properties of the \(E_2\) component of branched-chain \(\alpha\)-keto acid dehydrogenase are similar but not identical to those observed in the \(E_2\) (transacetylase) component of the pyruvate dehydrogenase complex (9, 10).

**EXPERIMENTAL PROCEDURES**

**Materials**—\([1\cdot^{14}C]\)isobutryc acid (specific activity 50 mCi/mmol) and \([1\cdot^{14}C]\)isovalerate (specific activity 4.8 mCi/mmol) were obtained from Research Products International Corp. LL. \([1\cdot^{14}C]\)Valine was purchased from New England Nuclear. \([1\cdot^{14}C]\)Acetyl-CoA was obtained from P-L Biochemicals. DL-Lipoamide, CoA, NAD, pyruvate, and \(\alpha\)-ketoglutarate complex activities were determined by Lin et al. (30). The mitochondrial preparation was isolated in a Beckman LS-7000 spectrometer. The recovery of labeled thioesters was used to normalize control values.

**Enzymatic Synthesis**—The reaction mixture contained 2 units of exogenous dihydrolipoyl dehydrogenase (Reaction 1) was assayed spectrophotometrically at 2 mM a-ketoglutamate dehydrogenase complexes. The final pellet was dissolved in Buffer B containing 0.05 M KF, (pH 7.5), 0.2 mM dithioerythritol, 1 mM leupeptin, 0.1% Triton X-100. The enzyme solution was applied to a hydroxyapatite (Bio-Gel HT) column (1 x 10 cm) equilibrated with Buffer B. The column was washed with 2 bed volumes of the same buffer containing 0.14 M KF (pH 7.5). Branched-chain \(\alpha\)-keto acid dehydrogenase was eluted with Buffer B at a gradient of 0.14-0.55 M KF (pH 7.5). The fractions containing the enzyme were pooled and subjected to ultracentrifugation in a Beckman Ti-50 rotor at 2 x 10^9 x g for 4 hr. The pellet was dissolved in Buffer B (0.05 M KF, pH 7.5) by stirring overnight. The hydroxypatite column and ultrafiltration steps were repeated once to obtain a final enzyme preparation.

**Enzyme Assays**—Activity of branched-chain \(\alpha\)-keto acid dehydrogenase (Reaction 1) was assayed spectrophotometrically at 2 mM \(\alpha\)-ketosiovalerate as described previously (8), with the addition to the reaction mixture of 2 units of exogenous dihydrolipoamide dehydrogenase (\(E_3\)) component. The same reaction mixture as that for branched-chain \(\alpha\)-keto acid dehydrogenase was used for the pyruvate dehydrogenase and \(\alpha\)-ketoglutarate dehydrogenase complexes except that 2 mM pyruvate and 2 mM \(\alpha\)-ketoglutarate substituted for \(\alpha\)-ketosiovalerate, respectively. Activity of the \(E_2\) component of branched-chain \(\alpha\)-keto acid dehydrogenase was assayed according to Reaction 2. The assay mixture contained in 0.37 ml: 50 mM MOPS (pH 7.4), 2 mM dihydrolipoamide, 0.4 mM \([1\cdot^{14}C]\)isobutyryl-CoA or \([1\cdot^{14}C]\)isovaleryl-CoA (400 cpm/nmol), 12.5 \(\mu\)M leupeptin, 1.4% fetal bovine serum, and the enzyme preparation. The reaction mixture was incubated at 37°C for 20 min. The reaction was quenched with perchloric acid (4 units) and 20 \(\mu\)mol of glucose. The incubation was continued for 30-45 min. The \([1\cdot^{14}C]\)CoA was eluted from a G-25 column (1 x 50 cm) with water. No effort was made to remove ADP, AMP, P, glucose, and glucose-6-P originally present in the reaction mixture. The reaction mixture was brought to 4°C with a bath and stored at -75°C. The radioactivity of acyl-CoA was evaluated by thin-layer chromatography.

**Purification of Branched-chain \(\alpha\)-Keto Acid Dehydrogenase**—The procedure was modified from those described previously (2, 3). Mitochondria were prepared from fresh bovine kidneys or liver under hypotonic conditions as described by Linn et al. (30). The mitochondria were resuspended in Buffer A containing 0.02 mM KF, (pH 7.5), 0.2 mM TPP, 1 mM dithioerythritol, 1% fetal bovine serum, and were frozen at -75°C. To purify branched-chain \(\alpha\)-keto acid dehydrogenase, the mitochondrial suspension was thawed, and the suspension was centrifuged at 25,000 x g for 20 min. The pellet containing most of the branched-chain \(\alpha\)-keto acid dehydrogenase was reextracted with Buffer A with the addition of 5% (v/v) Triton X-100. The enzyme solution was applied to a hydroxyapatite (Bio-Gel HT) column (1 x 10 cm) equilibrated with Buffer B. The column was washed with 2 bed volumes of the same buffer containing 0.14 M KF, (pH 7.5). Branched-chain \(\alpha\)-keto acid dehydrogenase was eluted with Buffer B at a gradient of 0.14-0.55 M KF (pH 7.5). The fractions containing the enzyme were pooled and subjected to ultracentrifugation in a Beckman Ti-50 rotor at 2 x 10^9 x g for 4 hr. The pellet was dissolved in Buffer B (0.05 M KF, pH 7.5) by stirring overnight. The hydroxyapatite column and ultrafiltration steps were repeated once to obtain a final enzyme preparation.

**Polycrylamide Gel Electrophoresis**—Electrophoresis was performed in 10% slab gels with 0.1% sodium dodecyl sulfate and Tris-glycine buffer (pH 8.3) (31). The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7% (v/v) acetic acid, and destained with the same solvent. For molecular weight determination branched-chain \(\alpha\)-keto acid dehydrogenase subunits, the following proteins were used as standards: 14-kDa fructose diphosphatase (17,800), carbonic anhydrase (25,700), ovalbumin (45,000), \(\gamma\)-globulin heavy chain (62,000), and bovine serum albumin (67,000).
defined as micromole of product formed per min at 37 °C. Protein was determined by the Lowry method (32) using bovine serum albumin as standard.

Spectrophotometric Studies—To characterize the product of Reaction 2, the reaction mixture containing 0.6 mM isobutyryl-CoA or isovaleryl-CoA, 6.0 mM dihydrolipoamide, 25 mM KPi, (pH 7.4), 12.5 μM leupeptin, 5 μL of fetal calf serum and purified bovine kidney branched-chain α-keto acid dehydrogenase complex was incubated at 37 °C for 2.5 h. The reaction product was extracted with 4 mL of benzene and the solution dried under a stream of nitrogen. The residue was dissolved in ethanol and allowed to react with hydroxylamine as described by Daigo and Reed (33). The absorption spectra of acylhydroxamate was determined at 460–660 nm in a Gilford Model 2500 spectrophotometer with a reference compensator. In another study, the difference spectrum of the above ethanol solution against the solution from a CoA blank was monitored in the Gilford scanner at 220–340 nm wavelength. S-Acetyldihydrolipoamide was enzymatically synthesized enzymatically the CoA ester substrates, [l-'4C]isovaleryl-CoA and dihydrolipoamide. The use of l-isovaleryl-CoA as substrate indicates fragment ions analogous to m/z ions of dihydrolipoamide. In addition, a quasi-molecular ion (M+1) at m/z 276 for the putative S-isobutyryldihydrolipoamide was observed (Fig. 1B). Mass spectrum similar to Fig. 1B was obtained with isovaleryl-CoA as substrate, except that, in place of m/z 276, a quasi-molecular ion (M+1) at m/z 292 was observed for the putative S-isobutyryldihydrolipoamide (Fig. 1C). The S-acetyldihydrolipoamide structure of reaction products was confirmed by the formation of acylhydroxamates with hydroxyamine. Absorption spectra of acylhydroxamate show maxima at 490 and 495 nm for isovaleryl- and isobutyrylhydroxamate, respectively (data not shown). These maximal absorptions are similar to that observed in acetylhdroxamate (25). In a parallel experiment, the ultraviolet absorptions of the benzene extract of the reaction mixture were measured against the same extract of an acetyl-CoA blank. The difference spectrum showed maxima at 232 and 245 nm for isovaleryl- and isobutyryl-CoA, respectively (data not shown). These absorption maxima are characteristics of acyl thiosteres (33) and are comparable to absorbance of 236–238 nm for S-acetyldihydrolipoamide (25).

Assay for the Ez Component of Branched-chain α-Keto Acid Dehydrogenase—The above data established that purified branched-chain α-keto acid dehydrogenase catalyzes Reaction 2 with the formation of S-acetyldihydrolipoamide from isobutyryl- or isovaleryl-CoA and dihydrolipoamide. The use of 1-14C-labeled acetyl-CoA permits an assay for the Ez component of the purified complex. Fig. 2A shows that the assay with the purified kidney enzyme is linear for at least 20 min with 1-14C-labeled acetyl-CoA or [1-14C]isobutyryl-CoA as substrate. The activity with isobutyryl-CoA is slightly greater than with isovaleryl-CoA. The rate with acetyl-CoA amounts to only 15% of that with isobutyryl-CoA. Arsenite at 1.5 mM completely inhibits the reaction. By contrast, partially purified pyruvate dehydrogenase complex from bovine kidney catalyzes transacylation between [1-14C]isobutyryl-CoA and dihydrolipoamide, but without activity with [1-14C]isobutyryl or [1-14C]isovaleryl-CoA as substrate (Fig. 2B). The Ez activity of branched-chain α-keto acid dehydrogenase is also proportional to the amount of the complex added with either isobutyryl-CoA or isovaleryl-CoA as substrate (data not shown). Moreover, the rate of transacylation catalyzed by the Ez component of bovine kidney branched-chain α-keto acid dehydrogenase appeared to be...
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**Fig. 1.** Electron ionization mass spectra of dihydrolipoamide (A), isobutyryldihydrolipoamide (B) and isovalerylhydrolipoamide (C). Mass spectra were obtained as described under "Experimental Procedures" except that the ionization voltage was 15 eV for the experiment presented in B. Acylhydrolipoamides were prepared as described also under "Experimental Procedures" using purified branched-chain α-keto acid dehydrogenase from bovine liver.

**Fig. 2.** Activity of $E_2$ component as a function of incubation time. A, $E_2$ activity of branched-chain α-keto acid dehydrogenase. The assays were carried out as described under "Experimental Procedures" with 0.2 μg of purified bovine kidney branched-chain α-keto acid dehydrogenase (specific activity 4.6 units/mg of protein). Incubation times were varied as indicated. ●, [1-14C]isobutyryl-CoA (0.4 mM, 397 cpm/nmol) as the acyl-CoA substrate; ▲, [1-14C]isovaleryl-CoA (0.4 mM, 207 cpm/nmol) as substrate; ■, [1-14C]acetetyl-CoA (0.4 mM, 356 cpm/nmol) as substrate; ○, [1-14C]isobutyryl- or [1-14C]isovaleryl-CoA with 1.5 mM arsenite added. $E_2$ activity of the pyruvate dehydrogenase complex. The assays were carried out as described above except that 27 μg of partially purified pyruvate dehydrogenase complex from bovine kidney (specific activity 0.03 units/mg of protein) was the enzyme used. Symbols for [1-14C]acetetyl-CoA and [1-14C]acyl-CoA substrates are as indicated in A.

**TABLE I**

Components and overall activities of purified bovine kidney branched-chain α-keto acid dehydrogenase

| Addition | $E_1$ | $E_2$ | $E_3$ | Overall |
|----------|-------|-------|-------|---------|
| None     | 0.51  | 2.38  | 8.68  | 1.64    |
| Arsenite, 1.5 mM | 0.47  | 0     | 8.97  | 0.0     |
| $E_2$, 2 units | 0.49  | 2.32  |       | 2.53    |

Branched-chain α-keto acid dehydrogenase was partially purified from bovine kidney mitochondria through the hydroxyapatite step as described under "Experimental Procedures." Activities of $E_1$, $E_2$, and $E_3$ components and the overall reaction catalyzed by the complex (Reaction 1) were assayed as described previously (18). The concentration of [1-14C]α-ketoisovalerate (specific radioactivity 160 cpm/nmol) was 1 mM.

comparable to that of the overall reaction catalyzed by the partially purified complex (Table I). The bovine kidney branched-chain α-keto acid dehydrogenase partially purified
from the hydroxyapatite column (see “Experimental Procedures”) still retained a portion of the $E_3$ component (Table I). Addition of exogenous $E_3$ increased activity of the overall reaction but was without effect on the activity of the $E_1$ or $E_2$ component (Table I).

**Kinetics of the Transacylation Reaction**—Kinetic studies were carried out to gain insight into the mechanistic nature of the $E_3$ catalyzed reaction. Fig. 3 shows double reciprocal plots of initial velocity data from Reaction 2 at varying concentrations of $[1^{-14}C]$isovaleryl-CoA and dihydrolipoamide. The linear plots have a common intercept for all lines on the abscissa when the concentration of either isovaleryl-CoA (Fig. 3A) or dihydrolipoamide (Fig. 3B) varied. Similar results were obtained with $[1^{-14}C]$isobutyryl-CoA as the acyl-CoA substrate (data not shown). The initial velocity data indicate that the transacylation reaction proceeds via a random ordered mechanism (35) with $K_m$ values of acyl-CoA independent of the concentration of dihydrolipoamide, and vice versa. The $K_m$ values determined by common intercepts of the double reciprocal plots are 0.05, 0.10, and 2.0 mM for isovaleryl-CoA, isobutyryl-CoA, and dihydrolipoamide, respectively. By the same method, a $K_m$ value of 0.31 mM was obtained for acetyl-CoA (data not shown). Product inhibition studies show that CoA is competitive with isobutyryl-CoA (Fig. 4A) and noncompetitive with dihydrolipoamide (Fig. 4B). The results are consistent with a random Bi Bi mechanism for the transacylation reaction.

**Effect of Reductive Acylation on the Transacylation Reaction**—Since $E_3$ contains covalently bound lipoyl moieties, it seemed desirable to determine whether or not the bound prosthetic groups participate in the model reaction. We approached this question by studying the effect of reductive acylation on rates of the $E_3$-catalyzed transacylation. The protein-bound lipoyl moieties of the purified $E_2$ complex from bovine liver were reductively acylated by incubating at 4°C with 0.2 mM TPP, 1 mM MgCl$_2$, 1 mM EDTA, 0.5 mM N-ethylmaleimide, and 0.5 mM $\alpha$-ketoisovalerate in a final volume of 0.35 ml. At indicated times, the incubation was terminated and N-ethylmaleimide quenched by the addition of 10 mM mercaptoethanol. The overall (●) and $E_2$ (▲) activities in complete incubation mixtures with isovaleryl-CoA as substrate were assayed at 37°C as described under “Experimental Procedures.” As a control the overall activity in the incubation mixture without $\alpha$-ketoisovalerate (○) was also measured. The original (100%) activities for the overall and $E_2$ (transacylation) reactions were 1.74 and 2.61 units/mg of protein, respectively.
not change the overall activity, providing a control for the reductive acylation reaction. As shown also in Fig. 5, the acylated and N-ethylmaleimide-modified E₂ component retained its full activity for Reaction 2. The data suggest that in the model reaction, endogenous bound lipoyl moiety is not required.

**Limited Proteolysis of Branched-chain α-Keto Acid Dehydrogenase**—To probe the subunit structure of the E₂ component of branched-chain α-keto acid dehydrogenase, purified branched-chain α-keto acid dehydrogenase from bovine liver was subjected to limited trypsic digestion at pH 7.4, and 0 °C. The digestion was stopped by addition of the trypsin inhibitor, Na-p-tosyl-L-lysine chloromethyl ketone, and the mixtures were analyzed by sodium dodecyl sulfate-gel electrophoresis. The time course of limited trypsic digestion of the purified branched-chain α-keto acid dehydrogenase is illustrated in Fig. 6. The Coomassie Blue stained-gel showed that the E₂ subunit (Mr = 47,500 ± 1,000) was only slightly digested, while the E₁β subunit (Mr = 37,200 ± 1,700) was apparently not affected during the limited proteolysis. The cleavage of E₂ subunit was accompanied by a rapid and total loss of the overall subunit catalyzed by the complex (Fig. 7). However, the transacylation reaction (Reaction 2) catalyzed by the E₂ component was essentially unaffected by the trypsic digestion. The activity of the E₁ component, on the other hand, decreased slowly to approximately 60% of the original level within 120 min (Fig. 7). Identical conditions were employed in experiments described in Figs. 6 and 7.

**DISCUSSION**

The present study indicates that highly purified branched-chain α-keto acid dehydrogenase from bovine kidney and liver catalyzes a transacylation reaction between [1-1⁴C]acyl-CoA and dihydrolipoamide (Reaction 2), which is similar to that described for the E₂ (transacetylase) component of the pyruvate dehydrogenase complex (25). The acyl thioester structure of the transacylation product S-acyldihydrolipoamide was identified by its characteristic absorption at 235–245 nm and by formation of hydroxamate with hydroxylamine. The relatively low intensity of quasi-molecular ions of S-acyldihydrolipoamide obtained in electron ionization mass spectrometry may be due to instability of the thioester bond under electron impact. Similar results with low intensity in molecular ions were observed in chemical ionization mass spectra generated by using isobutane (data not shown). The transacylation reaction catalyzed by the E₂ component of bovine kidney branched-chain α-keto acid dehydrogenase is linear with time for 20 min (Fig. 2A). This is in contrast to the brief linearity (2 min) previously described for the transacylase component of the pyruvate dehydrogenase complex (25). Moreover, the strong inhibition of Reaction 2 by arsenite is similar to that observed with the transsuccinylase component of the α-ketoglutarate dehydrogenase complex (36).

Rates of the transacylation reaction with various [1⁴C] acyl-CoAs as substrate are in the order of [1⁴C]isobutryryl-CoA > [1⁴C]isovaleryl-CoA > [1⁴C]acetyl-CoA. Isobutyryl- and isovaleryl-CoA are physiological products from the oxidative decarboxylation (Reaction 1) of α-ketoisovalerate and α-ketoisocaproate, respectively. The E₂ activity with acetyl-CoA as substrate is consistent with the branched-chain α-keto acid dehydrogenase catalyzed-decarboxylation of pyruvate through Reaction 1 (8). By contrast, the partially purified pyruvate dehydrogenase complex from bovine kidney does not catalyze the transacylation reaction between isobutyryl- or isovaleryl-CoA and dihydrolipoamide. These data along with difference in molecular weight (Mr = 52,000 and 74,000 for the E₂ subunits of branched-chain α-keto acid and pyruvate dehydrogenase complexes, respectively) (8,9) strongly suggest that the E₂ components of these two complexes consist of different polypeptides.

The initial velocity kinetics obtained with the purified kidney branched-chain α-keto acid dehydrogenase indicates that transacylation between acyl-CoAs and exogenous dihy-
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Dihydrolipoamide proceeds via a sequential mechanism. The product inhibition data with competitive/noncompetitive patterns are consistent with a random Bi Bi rather than a completely ordered mechanism. These results suggest that the catalytic center may have two separate sites: one that binds CoA and acyl-CoAs, and another that binds dihydrolipoamide and S-acyldihydrolipoamide. The noncompetitive inhibition (Fig. 4B) presumably results from formation of an inactive ternary complex among the enzyme, CoA, and dihydrolipoamide. It should be noted that [1-14C]acyl-CoAs used as substrate in the present study may be slightly contaminated with ADP, AMP, glucose, and glucose-6-phosphate from enzymatic synthesis of the labeled thioester. However, the degree of contamination was low (<1%), and the nucleotides and sugars at 1 mM were without effect on the transacylation reaction (data not shown). Thus the kinetic data presented here demonstrate mechanistic similarities in the transacylation reaction catalyzed by the E2 components of branched-chain α-keto acid and pyruvate dehydrogenase complexes (25). The E2 components of bovine kidney branched-chain α-keto acid dehydrogenase were not separated from the complex in the present study. However, no significant differences in kinetic values were reported for the transacylase of the pyruvate dehydrogenase complex whether it was assayed as a separate subunit or in complex with the pyruvate dehydrogenase and dihydrolipoyl dehydrogenase (E3) components (25).

Since E2 of branched-chain α-keto acid dehydrogenase contains covalently bound lipoyl moieties, it appeared likely that the acyl transfer from acyl-CoA to exogenous free dihydrolipoamide is mediated through acylation of bound lipoyl moieties. The bound acyl moiety is then transferred secondarily to exogenous free dihydrolipoamide. However, modification of the E2 bound lipoyl moiety through reductive acylation in the presence of N-ethylmaleimide did not affect the transacylation reaction. The data suggest that the protein-bound lipoyl moiety does not necessarily participate in Reaction 2. Based on the latter mechanism, some degree of competition between the endogenous lipoyl moiety and exogenous dihydrolipoamide is expected, although the concentration of free dihydrolipoamide far exceeds that of the bound lipoyl moiety in the E2 assays. Direct evidence for the above conclusions must come from studies of Reaction 2 with branched-chain α-keto acid dehydrogenase devoid of E2-bound lipoyl moieties. The release of lipoyl moieties from E2 (acyltransferase) of the pyruvate dehydrogenase complex with lipomodiase (37) abolishes the overall activity without affecting any of the component activities (25, 38).

As described above, limited proteolysis has been used to determine the E2 molecular structure of α-keto acid dehydrogenase complexes (9–12). The E2 (transacetylase) activity of the pyruvate dehydrogenase complex is not affected by digestion with trypsin, although the polypeptide is cleaved into two fragments, one with catalytic activity (active domain) and the other bearing the lipoyl group (lipoyl domain) (9). The E2 subunit of the pyruvate dehydrogenase complex is more susceptible to trypsin digestion than the E2 (9, 10). In the present study, the E2-catalyzed transacylation reaction of bovine liver branched-chain α-keto acid dehydrogenase is also not affected, while the overall activity of the complex is completely lost. But in contrast to results with the pyruvate dehydrogenase complex, a single major trypsin fragment of 25,700 is observed with the E2 component of branched chain α-keto acid dehydrogenase. Fig. 6 shows that as the E2 subunit gradually disappears during trypsin digestion, the 25,700 fragment concomitantly is observed with increasing intensity. The intensity of unmodified E2 appears approximately twice that of the major trypsin fragment. Since both unmodified E2 and the putative active domain catalyze Reaction 2, the constancy of E2 activity during trypsin digestion supports the view that M, = 25,700 fragment contains the active site. The catalytically inactive lipoyl domain (9, 10) may be more susceptible to proteolysis and appears as a minor species in the trypsin digest. Moreover, the E2 subunit of branched-chain α-keto acid dehydrogenase, is relatively resistant to limited digestion with trypsin, in contrast to the sensitivity of its counterpart in the pyruvate dehydrogenase complex. The resistance to limited proteolysis can be exploited to purify the E2 component of branched-chain α-keto acid dehydrogenase.

The radiochemical E2 assay allows study of the structure and function of the E2 component in highly purified branched-chain α-keto acid dehydrogenase. The rate of the E2-catalyzed transacylation measured in the reverse direction is comparable to that of the overall reaction (Table I). The relatively high reaction rate ensures sensitivity of the E2 assay with [1-14C]acyl-CoAs as substrate. Thus the E2 assay can also be used to purify certain mutant branched-chain α-keto acid dehydrogenases. Rates of the overall reaction catalyzed by mutant branched-chain α-keto acid dehydrogenase from several maple syrup urine disease patients are too low to be readily detectable, however, the E2 activity of the complex is normal in these mutations (15). Moreover, the sensitive E2 assay permits further analysis of genetic heterogeneity in cultured cells derived from patients with different forms of maple syrup urine disease. Although the enzymatic defect in the classical maple syrup urine disease patients we studied involves the E2 component (18, 19), it is possible that in other patients the mutation may affect the E2 subunit. This possibility will be investigated among different maple syrup urine disease fibroblast strains that complement in heterokaryons produced by cell fusion (16).

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Addendum—While the present paper was in review, Hefelfinger et al. reported similar but not identical findings with limited trypsin digestion of the E2 component of purified branched-chain α-keto acid dehydrogenase from bovine liver (39).

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