Identification of Specific Subunits of Highly Purified Bovine Liver Branched-Chain Ketoacid Dehydrogenase†

S. C. Heffelfinger, E. T. Sewell, and D. J. Danner*

ABSTRACT: Branched-chain α-ketoacid dehydrogenase has been purified to homogeneity from bovine liver mitochondria. The isolated complex has a specific activity of 5–8 μmol of reduced nicotinamide adenine dinucleotide min^{-1} (mg of protein)$^{-1}$ as isolated and does not require the addition of exogenous lipoamide dehydrogenase for activity. Addition of porcine heart lipoamide dehydrogenase stimulated complex activity by no more than 20%. Four subunits copurify with the complex with molecular weights by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of 55 000, 52 000, 46 500, and 37 500. Here we show that the 52 000-dalton subunit is the lipoyl-containing transacylase component of the complex. Data are presented to support the hypothesis that the branched-chain ketoacid dehydrogenase complex is physically and catalytically similar to, but separate from, the pyruvate and α-ketoglutarate dehydrogenase complexes. The transacylase of the branched-chain ketoacid dehydrogenase complex has an exposed trypsin-sensitive region. Proteolytic action of trypsin separates a lipoyl-containing component from the remainder of the protein. Data from our laboratory presented here and elsewhere define a specific function for three of the four subunits.

Branched-chain α-ketoacid dehydrogenase (EC 1.2.4.4, BCKD), functions as a component of the mitochondrion (Danner & Elsas, 1975) and has been isolated from rabbit liver, bovine kidney, and bovine liver (Paxton & Harris, 1982; Morrison & Mullings, 1983; Pettit et al., 1978; Danner et al., 1979). On the basis of the analogous, well-characterized pyruvate dehydrogenase complex (EC 1.2.4.1, PDC), three enzymic functions are associated with the complex. E1 is a decarboxylase that utilizes the cofactor thiamin pyrophosphate (TPP) to decarboxylate the branched-chain keto acids and transfer the remaining acyl group to E2. Subsequent transfer of the acyl group to CoASH results from the action of E2, which contains a covalently bound lipoyl moiety. Reduced lipoate of E2 is reoxidized by the flavoprotein, lipoamide dehydrogenase, E3, and the reduction of nicotinamide adenine dinucleotide (NAD).

Three subunits are shown to copurify with all preparations of BCKD. Identification of function with these subunits has been suggested, but supporting data are lacking (Pettit et al., 1978). For the rabbit liver and bovine kidney preparations of BCKD the flavoprotein component must be added for assay of complex activity (Paxton & Harris, 1982; Pettit et al., 1978). No subunit with a molecular weight in the range of 55 000 is seen in these preparations. Here we report a method of isolation for BCKD from bovine liver that results in a fully active complex where E3 addition is not required and the 55 000-dalton subunit, which has been identified as E3 for BCKD (Heffelfinger et al., 1983; S. C. Heffelfinger, E. T. Sewell, and D. J. Danner, unpublished results), is present. We further report the identification of the E2 subunit. Evidence for E1 subunit identification is presented in another paper from our laboratory (S. C. Heffelfinger, unpublished results).

Materials and Methods

All reagents were reagent grade or better, and solutions were made in deionized water. Radiolabeled keto acids were prepared from 1-^{14}C-labeled amino acids as previously described (Elsas et al., 1972). Protein was determined by the method of Bradford (1976) with the mixed protein Moni-Trol (Dade, Miami, FL) as standard. Porcine heart lipoamide dehydrogenase was purchased from Sigma, St. Louis, MO. A Gilford 250 recording spectrophotometer interfaced with an Apple II+ microcomputer was used for spectral analysis.

Enzyme Assays. NADH (reduced nicotinamide adenine dinucleotide) production was followed spectrophotometrically with time at 340 nm. The 0.5-mL reaction mixture contained saturating amounts of all cofactors and substrates except where indicated. A complete reaction mixture included 0.2 mM TPP, 0.2 mM $MgCl_2$, 0.5 mM CoASH, 1.0 mM NAD, 5 mM dithiothreitol (DTT), 100 μg of bovine serum albumin, 0.1 mM keto acid, and 5–50 μg of enzyme protein in 30 mM potassium phosphate buffer, pH 7.5 at 37 °C. The reaction was initiated with keto acid.

Decarboxylation of the 1-^{14}C-labeled keto acids was quantified by liquid scintillation counting of $^{14}CO_2$ produced from a 250-μL reaction mixture containing the reactant concentrations described above (Danner et al., 1975).

Lipoamide dehydrogenase activity was determined spectrophotometrically by the oxidation of NADH in the presence of thioctic acid as in the method of Ide et al. (1976).

Mitochondria Isolation. Mitochondria were isolated from bovine liver by a modification of the procedure of Roche & Cate (1977). Reagents were as described, but centrifugation was in a Sorvall RC5B with a flow-through TZ-28 rotor. Flow rate for mitochondrial sedimentation was 120–160 mL/min. One liver routinely yielded 300–400 g wet weight of mitochondria.

Purification of BCKD. Mitochondria were thawed in cold running water, and the solution was made 50 mM with NaCl and 1% with bovine serum before centrifugation at 32000*g* for 30 min. The supernatant that contained most of the BCKD activity was made 0.2 mM TPP, 1% bovine serum albumin and 10 mM $MgCl_2$, and the pH was adjusted to 7.5 with 10 N KOH. Poly(ethylene glycol) 8000 (PEG) was added to a final concentration of 2% from a 50% (w/v) stock solution made in water. After a 10-min stirring on ice, precipitated protein was removed by centrifugation at 24000*g* for 15 min and the supernatant made 4% with PEG. The resulting pellet contained the BCKD and was suspended in 50 mM potassium phosphate, pH 7.5, 0.2 mM TPP, 2 mM DTT, 1 mM $MgCl_2$

† From the Division of Medical Genetics, Departments of Pediatrics and Biochemistry, Emory University, Atlanta, Georgia 30322. *Received June 3, 1983.* This research was supported by grants from the National Institutes of Health (HD08388) and from the Emory University Research Committee.

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Table I: Purification of Bovine Liver Branched-Chain Ketoacid Dehydrogenase[a]

fraction	vol (mL)	protein (mg/mL)	total protein (mg)	sp act. [μmol min^{-1} (mg of protein)$^{-1}$]	total act. (μmol/min)
frozen/thaw homogenate	550	24	13200	0.007	92
30K supernatant	450	16	7200	0.030	216
2-4% PEG pellet	16	17	272	0.710	193
2% PEG pellet high salt	3	14	36	2.260	82
final	1	11	11	5.500	62

[a] Specific activity measures NADH formation at 340 nm in a reaction volume of 500 μL containing 30 mM potassium phosphate, pH 7.5, 0.2 mM TPP, 0.2 mM $MgCl_2$, 0.5 mM CoASH, 1.0 mM NAD, 5 mM DTT, 100 μg of bovine serum albumin, 5–50 μg of enzyme protein, and 0.1 mM KIV to begin the reaction.

and 1% bovine serum albumin [buffer A]. With 1 M NaCl, the solution was made 0.2 M and left at 4 °C overnight. After centrifugation at 40000*g* for 20 min, the high-salt supernatant was made 2% with PEG to selectivity precipitate the BCKD protein. This pellet was suspended in buffer A and fractionated on a Sepharose 6B column (1.5 × 25 cm) with 50 mM potassium phosphate, pH 7.5, containing 0.2 mM TPP and 5 mM DTT. Three protein peaks were obtained, with BCKD activity only in the latter half of peak one. Active fractions were pooled and centrifuged at 109000*g* for 3.5 h in a Beckman TI 70.1 rotor. The pellet was suspended in 50 mM potassium phosphate, pH 7.5, containing 0.2 mM TPP, 2 mM DTT, 1 mM $MgCl_2$, 1 mM NAD, and 0.1 mM ethylenediaminetetraacetic acid (EDTA). This solution was clarified by centrifugation at 31000*g* for 10 min, and the clear supernatant, which contained all active BCKD protein, was stored in aliquots at −70 °C.

Gel Electrophoresis. Two methods of electrophoresis were used: a 10–16% gradient polyacrylamide gel and a 10% polyacrylamide gel both containing sodium dodecyl sulfate (SDS) and focused with a 3% polyacrylamide stacking gel. Reagent stocks and proportions were calculated from Laemmli's (1970) method. Running buffer for the gradient gels was 0.192 M glycine, 25 mM tris(hydroxymethyl)aminomethane (Tris), and 0.1% SDS. Running buffer for the 10% gels was either 28 mM Tris, 88 mM glycine, and 0.1% SDS, pH 8.8, or 28 mM Tris, 0.3% acetic acid, and 0.1% SDS, pH 6.5. The latter running buffer was used when acid conditions were mandatory. Sample buffer included 5 mM DTT, glycerol, 3% SDS, 62.5 mM Tris-HCl or 10 mM Tris, pH 6.5, and 0.1% acetic acid. Proteins were diluted at least 1:1 with sample buffer and electrophoresed immediately. Current was maintained at 10 mA through the stacking gel and 20 mA through the resolving gel. All gels were run at room temperature.

Labeling the Transacylase with ^{3}H-Labeled N-Ethylmaleimide (NEM). The labeling procedure required two phases. All reagents were made in 30 mM potassium phosphate, pH 7.5, the buffer for the reaction. In the first phase, 100 μg of purified liver enzyme was incubated with unlabeled NEM from 0–1 mM at 0 °C in duplicate tubes. After 30 min, the reaction was stopped with DTT at a final concentration of 10 mM. The reaction mixtures were then transferred to M_r 12 000 exclusion dialysis tubing and dialyzed 2.5 h with five changes against 30 mM potassium phosphate, pH 7.5.

In the second phase, the reagents were added directly to the fluid already in the dialysis sacks, which were hung in an empty beaker set in ice. The enzyme solution was made 0.2 mM TPP, 0.2 mM $MgCl_2$, 0.1 mM α-ketoisovalerate (KIV) or NADH, and 0.4 mM [^{3}H]NEM (692 μCi/μmol). One duplicate tube from phase one contained substrate so the reaction would proceed from E1 to E2. The other duplicate contained NADH so the reaction would run from E3 to E2. The total volume for each reaction was 50 μL. After 30 min, the reactions were made 10 mM DTT and dialyzed overnight in SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer.

Labeling the Transacylase with [U-^{14}C]-α-Ketoisocaproate ([U-^{14}C]KIC). The procedures used here are modifications of those outlined in Kresze & Ronft (1980) and Bleile et al. (1981). Each reaction contained 1 mM EDTA, 1 mM $MgCl_2$, 0.2 mM TPP, 0.6 mM NEM, and 30 μg of purified liver BCKD. These reaction mixtures were incubated 5 min at room temperature before adding [U-^{14}C]KIC to a final concentration of 13 mM (10 μCi/μmol). The reaction mixture (50 μL) was incubated another 5 min at room temperature and diluted 5-fold with cold 50 mM potassium phosphate, pH 6.5. The samples were dialyzed against two changes of phosphate buffer at 0.5 h each, diluted in SDS–PAGE sample buffer, and electrophoresed in the Tris–acetate buffer system.

Isolation of E3. BCKD was dialyzed against 50 mM potassium phosphate, pH 7.0, containing 0.5 M EDTA and 1% $(NH_4)_2SO_4$ for several hours and then absorbed onto a calcium phosphate gel cellulose column (100 mL/g of protein) (Danner et al., 1979). The protein was eluted with a linear gradient of 4–8% $(NH_4)_2SO_4$ in this phosphate buffer without EDTA. E3 activity eluted between 4.5 and 5% $(NH_4)_2SO_4$. The active fractions were pooled and passed through a Sephadex G-200 column (2.5 × 30 cm) with 50 mM potassium phosphate, pH 7.0. The flavoprotein eluted as a single peak.

Proteolytic Digestion. Enzyme was prepared for digestion by dialysis against 30 mM potassium phosphate, pH 7.2, with 0.1 mM EDTA for 11 h. Approximately 100 μg of BCKD was placed in 100 μL of 30 mM potassium phosphate, pH 7.0, at room temperature and trypsin added (0.1 μg/0.1 mg of BCKD). Aliquots of 10 μL were taken at the times indicated in the figures and diluted into 90 μL of SDS sample buffer at 50 °C.

Results

The specific activity of freshly isolated liver BCKD varied between 5 and 8 μmol of NADH min^{-1} (mg of BCKD protein)$^{-1}$ with preparations from different livers. On the basis of specific activity, this value represents an 800-fold purification over the beginning mitochondrial freeze/thaw homogenate. Purification steps are summarized in Table I. Addition of exogenous E3 to freshly isolated complex could stimulate activity no greater than 20%. However, after storage at −70 °C, complex activity decreased with time but could be restored to the original activity by the addition of 1–5 units of porcine heart lipoamide dehydrogenase. This selective loss of flavoprotein activity in the complex remains to be explained.

When the complex was resolved on SDS–PAGE in the presence of DTT, four peptides were seen. Average apparent molecular weights from density scans of several gels were 55 000, 52 000, 46 500, and 37 500 (Figure 1). On the basis of molecular-sieve chromatography on Sepharose 6B, the native complex has a molecular weight of ca. 900 000 (data not shown).

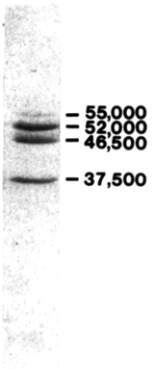

FIGURE 1: Gradient SDS–PAGE of bovine liver BCKD. Enzyme protein (30 μg) was resolved in 10–16% polyacrylamide gel with SDS and DTT. Details are under Materials and Methods.

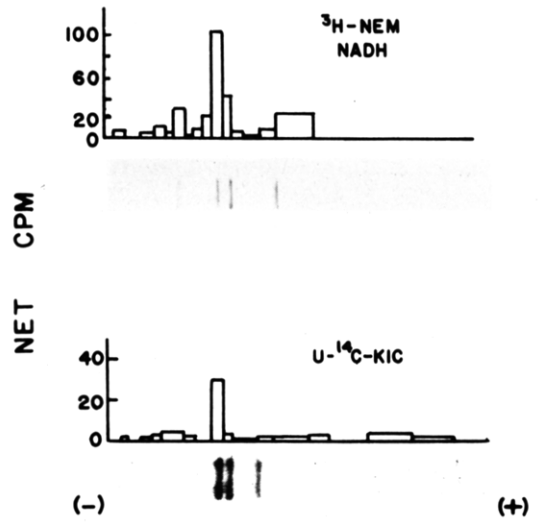

FIGURE 2: Specific labeling of E2 component of BCKD. Purified bovine liver BCKD was incubated with either [^{3}H]NEM in the presence of NADH (upper graph) or with [U-^{14}C]KIC (lower graph), electrophoresed, stained, and dried, and the gel was sliced and counted. The protein-stained gel is shown along the bottom. Each bar width indicates the size of the gel slice. Bar heights indicate net cpm.

Identification of E2 and E3 Subunits. The 55 000-dalton subunit was identified as the lipoamide dehydrogenase by isolation of this subunit from the BCKD complex. As described under Materials and Methods, active E3 was removed from the complex by selective elution after adsorption of the complex onto calcium phosphate gel cellulose. The flavoprotein migrated as a single band with a molecular weight of 55 000 on SDS–PAGE and, as shown in other studies from our laboratory, was immunologically cross-reactive with antibodies raised against porcine heart E3 (Heffelfinger et al., 1983; S. C. Heffelfinger, E. T. Sewell, and D. J. Danner, unpublished results).

To identify the presumed lipoyl-containing E2 component, the reduced thiol moiety was specifically labeled with either [^{3}H]NEM or [^{14}C]isovalerate as described under Materials and Methods. Following incubation of BCKD with the labeling group, the complex was resolved on SDS–PAGE. After being stained, the gel was dried, and the individual stained hands were cut out and counted by liquid scintillation spectroscopy. As seen in Figure 2, only the 52 000-dalton band was labeled by either radioactive isotope. Reduction of the lipoyl group of E2 could be obtained by using keto-acid substrate to drive the reaction forward or by using NADH to force the backward reaction. Labeling with [^{3}H]NEM after lipoate reduction by either method gave identical results.

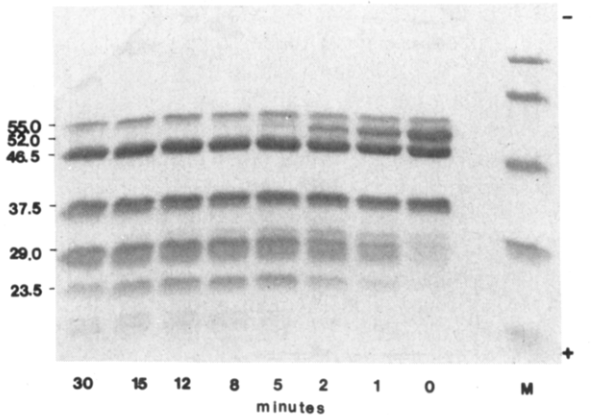

FIGURE 3: SDS–PAGE of trypsin-digested BCKD. Bovine liver BCKD was digested with trypsin (0.1 μg/0.1 mg of BCKD protein), and 10 μL aliquots were taken for electrophoresis at 0, 1, 2, 3, 5, 10, 15, and 30 min. The time points run sequentially from right to left. The 52 000-dalton band is digested with time into two bands of 29 000 and 23 500 daltons.

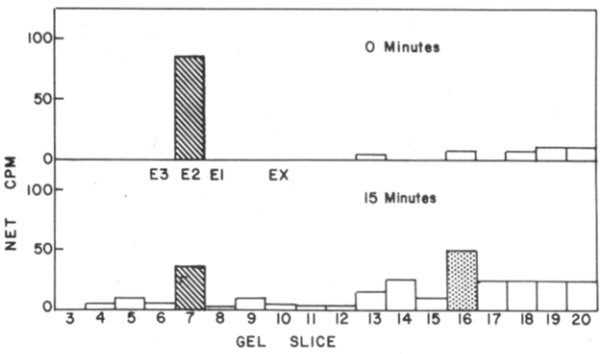

FIGURE 4: Histogram of radioactivity in E2 subunit of BCKD before and after trypsin digestion. BCKD was radiolabeled on the E2 subunit as described in Figure 2 and represented by the crosshatched bar (0 min of digestion, top half). The complex was digested with trypsin for 15 min (lower half), and the stippled bar represents the new position of the radiolabel. E3, E2, E1, and EX represent the positions of migration for the four subunits of BCKD.

The 52 000-dalton band was selectively lost by limited digestion of the complex with trypsin (Figure 3). We had previously shown that trypsin digestion led to inactivation of BCKD (Danner et al., 1980). Two major degradation products were derived from E2 with molecular weights of 29 000 and 23 500. Other degradation products were also generated and may represent intermediates in this process. When the complex was labeled with [^{14}C]isovalerate before digestion, the label moved with the loss of E2 to the 23 500-dalton product (Figure 4).

Discussion

Previously, we reported the isolation of BCKD from bovine liver mitochondria and described some catalytic characteristics of the complex (Danner et al., 1979). These results substantiated a report by Pettit et al. (1978) for the bovine kidney BCKD complex. Recently, Paxton & Harris (1982) reported the isolation of BCKD from rabbit liver. The kidney and rabbit liver enzyme are reported to have a subunit composition similar to that of the bovine liver complex reported here, on the basis of molecular weight migration patterns in polyacrylamide gels. Rabbit liver and bovine kidney BCKD, however, require the addition of lipoamide dehydrogenase for NAD-reduction activity. No 55 000-dalton peptide is reported to be present in the kidney or rabbit complex. The isolation procedure we describe here preserved the 55 000-dalton com-

ponent, and the isolated complex had 80% activity without addition of lipoamide dehydrogenase. Storage of our preparation at −70 °C for longer than 20 days led to reduction in NADH-forming activity. Original activity was restored by the addition of 1–5 units of porcine heart lipoamide dehydrogenase. This suggests a selective loss of E3 activity from the complex and requires further investigation.

In another paper, we describe the immuno-cross-reactivity of the BCKD complex with antibodies to porcine heart lipoamide dehydrogenase (Heffelfinger et al., 1983). Here we report the isolation of a 55 000-dalton subunit with the capacity to reduce thioctic acid in the presence of NADH. Although this flavoprotein functions similarly in three multienzyme complexes, the association of E3 with BCKD may be different from its association with either the PDC or the α-ketoglutarate dehydrogenase complex (KGDC), since it is affected differently by storage and is selectively lost during some BCKD isolation procedures. Our findings support the concept that the flavoprotein is common to all three mitochondrial complexes. This previously has been suggested by the reports of a human patient with an inherited defect in E3 in which all three complexes are markedly decreased in function (Robinson et al., 1977, 1980, 1981).

The 52 000-dalton band seen on SDS–PAGE has been identified as the E2 component. Unequivocal proof of the identity of E2 will require isolation and function studies on the individual protein. Pyruvate and α-ketoglutarate dehydrogenase complexes both have a transacylase core protein that contains a covalently bound lipoic acid residue as a functional component to shuttle the acyl group of the keto acid to coenzyme A. This E2 is reported to have two domains joined together by a trypsin-sensitive region. One of these domains contains the functional thiol group (Kresze & Ronft, 1980; Bleile et al., 1981). Our similar findings of two tryptic products of BCKD E2 support the similarity among the three complexes in physical structure. Unlike PDC-E2, E2 in the native BCKD complex is preferentially attacked by trypsin, suggesting that this interdomain region of BCKD is more exposed.

By combining current knowledge of the structure and function of BCKD, the following picture emerges. Four major peptides compose the active complex. A 55 000-dalton flavoprotein that functions similarly to the flavoprotein of PDC and KGDC is likely to be structurally identical for all three complexes. The 52 000-dalton subunit is the E2 transacylase that contains the functional lipoyl moiety, again similar to, but distinct from, PDC and KGDC, which have E2 components with different molecular weights (Koike & Koike, 1982). The 46 000-dalton component is probably the TPP-binding subunit of the complex (S. C. Heffelfinger, unpublished results). Only the 37 500 subunit remains to have a function assigned but has been suggested by Pettit et al. (1978) to be part of the decarboxylase component. BCKD appears to be similar if not identical in all tissues since the bovine liver, bovine kidney, and rabbit liver complexes show identical catalytic properties and physical data (current data; Pettit et al., 1978; Paxton & Harris, 1982).

Registry No. BCKD, 9082-72-8; lipoyl transacylase, 87481-50-3; lipoamide dehydrogenase, 9001-18-7.

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