Identification of Mitochondrial Branched Chain Aminotransferase and Its Isoforms in Rat Tissues*

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The tissue distribution and subcellular location of branched chain aminotransferase was analyzed using polyclonal antibodies against the enzyme purified from rat heart mitochondria (BCATₘ). Immunoreactive proteins were visualized by immunoblotting. The antisera recognized a 41-kDa protein in the 100,000 x g supernatant from a rat heart mitochondrial sonicate. The 41-kDa protein was always present in mitochondria which contained branched chain aminotransferase activity, skeletal muscle, kidney, stomach, and brain, but not in cytosolic fractions. In liver mitochondria, which have very low levels of branched chain aminotransferase activity, the 41-kDa protein was not present. However, two immunoreactive proteins of slightly higher molecular masses were identified. These proteins were located in hepatocytes. The 41-kDa protein was present in fetal liver mitochondria but not in liver mitochondria from 5-day neonates. Thus disappearance of the 41-kDa protein coincided with the developmental decline in liver branched chain aminotransferase activity. Two-dimensional immunoblots of isolated BCATₘimmunocomplexes showed that the liver immunoreactive proteins were clearly different from the heart and kidney proteins which exhibited identical immunoblots.

Investigation of BCATₘ in subcellular fractions prepared from different skeletal muscle fiber types revealed that branched chain aminotransferase is exclusively a mitochondrial enzyme in skeletal muscles. Although total detergent-extractable branched chain aminotransferase activity was largely independent of fiber type, branched chain aminotransferase activity and BCATₘ protein concentrations were highest in mitochondria prepared from white gastrocnemius followed by mixed skeletal muscles with lowest activity and protein concentration found in soleus mitochondria. These quantitative differences in mitochondrial branched chain aminotransferase activity and enzyme protein content suggest there may be differential expression of BCATₘ in different muscle fiber types.

The first step in catabolism of the essential branched chain amino acids, leucine, isoleucine, and valine, is transamination to form their respective α-keto acids, α-ketoisocaproate, α-keto-β-methylvalerate, and α-ketoisovalerate. This reaction is catalyzed by the branched chain aminotransferase (EC 2.6.1.42). Recently, the enzyme has been purified from isolated rat heart mitochondria (1). The aminotransferase appears to be a monomer and has been named BCATₘ. BCATₘ is probably the enzyme which was originally classified by its elution profile from DEAE-cellulose ion exchanger as branched chain aminotransferase Enzyme I (for review, see Ref. 2). Enzyme I is the most widely distributed form of branched chain aminotransferase in rat tissues (3, 4). Along with Enzyme I, another form, designated Enzyme II, is also a true branched chain aminotransferase (2). The form of branched chain aminotransferase that has been labeled Enzyme III is largely cytosolic and is predominant in brain, placenta, and ovary (3, 4). It also appears to be expressed in fetal and transformed tissues (3, 4). The enzyme purified from rat heart mitochondria appears to be immunologically distinct from Enzyme III, since antibodies raised against the BCATₘ did not appear to recognize the enzyme form found in brain cytosol (1). It has been suggested that these two enzyme forms may represent separate isozymes, since two independent genes for branched chain aminotransferase have been reported in mammalian cells (5).

Early investigations into the subcellular distribution of branched chain aminotransferase indicated that enzyme activity was distributed between the cytosol and mitochondrial compartments within most organs and tissues (for review, see Ref. 2). However, based on activity measurements, Hutson et al. (6) showed that in rat heart the aminotransferase is solely a mitochondrial enzyme. In a subsequent investigation of the distribution of branched chain aminotransferase activity in rat tissues, aminotransferase activity appeared to be located in the mitochondria in some, distributed between cytosol and mitochondria in other tissues, and almost undetectable in liver (7). In this study antibodies against the heart BCATₘ enzyme have been used to determine the pattern of distribution of the mitochondrial form of branched chain aminotransferase in rat tissues and subcellular fractions. The data show that BCATₘ is found in all mitochondria isolated from tissues which have branched chain aminotransferase activity and that there are multiple forms differing in charge. Finally, evidence is presented which suggests there is differential expression of BCATₘ in various skeletal muscle fiber types.

The abbreviations used are: BCATₘ, the mitochondrial branched chain aminotransferase enzyme; DIFP, diisopropylfluorophosphate; DTT, dl-dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Mops, 4-morpholinepropanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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EXPERIMENTAL PROCEDURES

Preparation of Tissue Fractions—Sprague-Dawley rats were used (Zivic-Milka). Heart mitochondria were prepared as described by LaNoe et al. (8). Skeletal muscle, liver, and kidney mitochondria were prepared as described in Refs 6 and 7) and references therein. For preparation of mitochondria from soleus and white gastrocnemius muscles, the individual muscles were taken from 15 to 20 rats and pooled for preparation of mitochondria. The remaining half muscles were used for the preparation designated mixed skeletal muscle mitochondria. Brain mitochondria were prepared using a Ficoll gradient as described by Lau and Clark (9). A crude mitochondrial fraction was prepared from stomach using differential centrifugation (7) with omission of the two wash steps. Nase was not used in preparing mitochondria from stomach. The standard isolation medium contained 225 M mannitol, 0.075 M sucrose, 0.10 mM EDTA, 5 mM Mops, pH 7.0. The protease inhibitor DIFP (1.0 mM) was added to the 600 × g supernatant before isolation of the mitochondria at 8,000 × g (heart and skeletal muscle) or to the original homogenate before differential centrifugation (other tissues). DIFP was also included in the medium during the two wash steps. The final mitochondrial pellets were resuspended in a minimal volume of isolation medium (60-80 mg mitochondrial protein/ml) containing 10 mM DIFP. Mitochondrial protein was measured by the biuret reaction in the presence of 0.125% deoxycholate using bovine serum albumin as standard.

For preparation of the mitochondria, 100,000 × g extracts, the mitochondria were diluted with deionized water to a final protein concentration of 10 mg/ml in the presence of 10 mM DIFP and 1.0 mM DTT and allowed to sit at 4°C for 15 min. The hypotonic mitochondrial suspension was then sonicated for three 5-min intervals at 50% duty cycle using a Branson sonifier (model 250). The sonication time could be reduced if the dilution suspension was from parts thawed before sonication. Subsequently, the mitochondrial sonicate was centrifuged at 100,000 × g for 1 h. DTT (1 mM) was added to the 100,000 × g supernatant before it was frozen at -20°C.

Tissue homogenates were prepared by diluting the tissue (1 g of tissue/5 ml of isolation medium) with isotonic medium containing DIFP (10 mM) and DTT (1 mM). Kidney was diluted 1 g tissue/ml of medium). Cytosol fractions were prepared as in Ref. 7. For preparation of rat brain cytosol, the brains were removed quickly and placed in the standard isolation medium containing 10 mM DIFP. The brains were blotted, weighed, diluted (1 g tissue/3 ml of medium), homogenized, and centrifuged at 14,000 × g for 10 min. The supernatant was passed through a 0.45-μm filter before immunoblotting.

Preparation of Hepatocytes—Adult rat liver hepatocytes were prepared by collagenase digestion using a modification of the method of Berry and Friend (11) as described in Spach et al. (12). Periportal and perivenous hepatocytes were prepared as described by Lindros and Penttila (13) using Gey's balanced salts made 25 mM in sodium bicarbonate. The hepatocytes were suspended in Dulbecco's minimal essential medium supplemented with 20 mM HEPES and 10 mM NaHCO3 and were washed once. The cell pellet was resuspended in mitochondrial isolation medium, washed once, resuspended in the isolation medium, and diluted 50% with H2O containing 1% Triton X-100, 1 mM DIFP, and 5 mM benzamidine. The Hepatic cell concentration was 106 cells/ml. The hypotonic cell suspension was then sonicated for three 1.0-min intervals at 50% duty cycle using a Branson sonifier (model 250), centrifuged at 14,000 × g for 30 min, and the supernatant was used for immunoblotting. The cell suspension was used for measurement of branched chain aminotransferase activity.

Fetal hepatocytes were prepared from livers obtained from 18-day fetal rats as described by Wallin and Hutson (14). Fetal hepatocytes were maintained in culture as described (14). Cultured cells were scraped off the plate and suspended in phosphate-buffered saline and used to measure branched chain aminotransferase activity. Protein was measured using the Bio-Rad Assay.

Determination of Branched Chain Aminotransferase Activity—Aminotransferase activity was measured at 37°C in 50 mM phosphate buffer, pH 7.8 (KOH), which contained 50 μM pyridoxal phosphate, 5 mM DTT, and 0.5 mg/ml bovine serum albumin as described by Hutson et al. (6). The standard assay contained 1 mM [1-14C]-α-ketoisovaleric acid and 12 mM isoleucine. When aminotransferase activity was determined in isolated mitochondria or hepatocytes, 0.4% CHAPS was included in the assay to disrupt the membranes. A unit of aminotransferase activity was defined as 1 nmol of valine formed per min at 37°C.

SDS-PAGE—SDS-PAGE was carried out according to Laemmli (15) in 10% gels. Prior to electrophoresis, all samples were boiled for 2 min in the presence of 1% SDS, with 5% β-mercaptoethanol. Low molecular weight prestained standard proteins used for molecular mass determinations were obtained from Bethesda Research Laboratories.

Sequence Analysis—The final purification step of BCATm was electrophoresis in a discontinuous nondenaturing gel which resolves the protein into two bands as described in (1). The two bands were electroeluted separately, subjected to electrophoresis in SDS-PAGE, and then transferred to an Immobilon P membrane (Millipore Corp., Bedford, MA). The single band detected by staining with Coomassie Blue dye was excised and subjected to Edman degradation in an Applied Biosystems 475A Protein/Peptide Sequencer operated by the Protein Sequence Analysis Core Laboratory at the Bowman Gray School of Medicine.

Preparation of Antiserum—Fifty μg of BCATm was used for preparation of antiserum as described in Ref. 1. Serum was stored at -20°C. Fig. 1 shows an immunoblot of purified BCATm and a heart mitochondrial 100,000 × g extract with the BCATm antiserum. The purified protein and the protein present in the crude extract appeared on the immunoblot with identical masses. Therefore, the heart mitochondrial extract was used routinely as a reference for positioning BCATm on immunoblots.

Immunoblotting—Proteins in SDS-PAGE gels were transferred to Immobilon P membranes for immunoblotting. The transfer was carried out in a Bio-Rad Trans Blott cell as described recently (16). The Immobilon P membrane was treated with 5% milk fat and reacted with antibodies as described by Reiderer et al. (16). Immunoreactive protein bands were visualized after horseradish peroxidase reduction of 4-chloro-1-naphthol (17).

A quantitative immunodot assay was performed as described in Ref. 16. Defined amounts of purified branched chain aminotransferase (in the nanogram range) and 2 μl of the mitochondrial matrix 100,000 × g supernatants were spotted in triplicate onto nitrocellulose filters and incubated with antibodies and 125I-protein A (16). Nonspecific binding was determined by using preimmune serum. These values were subtracted from values with specific antibodies to obtain counts/min for specific binding.

Two-dimensional SDS-PAGE—Two-dimensional SDS-PAGE of immunocomplexes was carried out according to a modification of the O'Farrell method (18) as described in Wallin et al. (19). The 100,000 × g supernatants from heart, kidney, and liver mitochondria (1.0 ml) were incubated with BCATm antiserum on ice for 1 h. Immunocomplexes were isolated with protein A attached to Immunobead particles (Bio-Rad) and were released from protein A in the 9 μl urine sample buffer used for isoelectric focusing.

Chemicals and Reagents—[1-14C]Valine was obtained from Amersham Corp. and later from American Radiolabeled Chemicals Inc. (St. Louis, MO). The radioactive [1-14C]-α-ketoisovaleric acid was synthesized from [1-14C]valine as described (20). [1-14C]-α-ketocaproatate and [1-14C]leucine were obtained from Amersham Corp. Nage was originally obtained from Enzyme Research Corporation (New York, NY) and later from Sigma (Sigma Type XXVII) (St. Louis, MO).
Bovine serum albumin (essentially fatty acid-free, globulin-
free) was obtained from Sigma. The Mono P chromatofocusing column
was obtained from Pharmacia LKB Biotechnology Inc. All other
reagents were reagent grade or better.

**RESULTS**

**BCAT<sub>m</sub> in Mitochondria and Cytosolic Fractions—**Immunobots of BCAT<sub>m</sub> and branched chain aminotransferase en-
zyme activity in mitochondrial extracts from heart, skeletal
muscle, stomach, brain, kidney, and liver mitochondria are
shown in Fig. 2. As observed previously (7), all of the mito-
chondria, with the exception of liver mitochondria (lane L),
had significant levels of branched chain aminotransferase
activity. The mitochondrial fraction prepared from stomach
(lane ST), a tissue with high branched chain aminotransferase
activity (4, 21), had the highest activity followed by heart
(lane H), skeletal muscle (lane M), kidney (lane K), and lower
activity was found in brain mitochondria (lane B). To verify
the low levels of BCAT<sub>m</sub> activity in liver mitochondria, these
mitochondria were tested for aminotransferase activity using
two additional substrate pairs that have been used by others
to measure liver mitochondrial branched chain aminotrans-
ferase activity (2, 22). Rates with [1-<sup>14</sup>C]-<sup>r</sup>-ketoisocaproate/Glu
and [1-<sup>14</sup>C]-<sup>r</sup>-ketoglutarate, were 1.7 and 1.2 units/mg
mitochondrial protein, respectively, and were similar to
rates observed using the standard assay, see Fig. 2.

As shown in Fig. 2, antibodies against the BCAT<sub>m</sub> protein
purified from rat heart mitochondria recognized a 41-kDa
protein in heart (lane H), skeletal muscle (lane M), stomach
(lane ST), kidney (lane K), and brain (lane B) mitochondrial
extracts, but this protein was not found in liver (lane L)
mitochondrial extracts. Even though aminotransferase activity
is 9-fold lower in brain than in heart mitochondria, a 41-
kDa protein was recognized by the BCAT<sub>m</sub> antiserum, sug-
gestive that this form of the enzyme may account for the
brain mitochondrial activity. In kidney (lane K), the BCAT<sub>m</sub>
antiserum recognized a protein of about 42.5 kDa in addition
to the 41-kDa protein. In liver (Fig. 2, lane L and Fig. 6) a
faint reaction was seen with a 42.5-kDa protein, and the
immunoblot revealed a strong reaction with a protein of about
44 kDa that was not seen in mitochondria from any of the
other tissues. Thus, the appearance of the 41-kDa protein on
immunoblots always correlated with the presence of assayable
mitochondrial branched chain aminotransferase activity.

**Distribution of BCAT<sub>m</sub> in Skeletal Muscle—**Earlier we had
reported that the distribution of branched chain aminotrans-
ferase activity between mitochondrial and cytosolic fractions
in skeletal muscle appeared to be dependent on fiber type (7).
Based on segregation of the mitochondrial marker citrate
synthase, it was calculated that branched chain aminotrans-
ferase activity was 70% cytosolic in white gastrocnemius, 40%
cytosolic in mixed skeletal muscle, and entirely mitochondrial
in soleus muscle (7). Since skeletal muscle is known to contain
only the Enzyme I form of branched chain aminotransferase
(3, 4), which we have shown to be BCAT<sub>m</sub> (1), the presence
of BCAT<sub>m</sub> antigen in skeletal muscle subcellular fractions
was investigated using immunoblotting. Immunoblots and
branched chain aminotransferase activity in mixed skeletal
muscle (lane M), isolated soleus (lane S), and white gastroc-
nemius (lane G) muscle cytosol, and Triton X-100 extracts
of the same muscle homogenates are shown in Fig. 3. When
equal amounts of protein (100 µg) were subjected to SDS-
PAGE and immunoblotting, the 41-kDa protein was found in
extracts prepared from these muscles after extraction with
Triton X-100 but was hardly detectable in the cytosol
fractions (Fig. 3). If 70 or 40% of the branched chain aminotrans-
ferase activity was cytosolic, immunoreactive protein should
have been observable in these fractions. As reported previ-
ously, total detergent extractable activity was similar in all
muscle types (7). However, Fig. 3 shows that cytosolic activi-
ties in mixed calf muscle, soleus, and white gastrocnemius
were also similar. The cytosolic activities were not corrected
for leakage of mitochondrial enzymes during preparation of
the cytosolic fraction. We have observed that leakage of

![Fig. 2. Immunoblotting of selected rat tissue mitochondrial
extracts with BCAT<sub>m</sub> antiserum. The 100,000 × g superna-
tants were prepared from the mitochondria after sonication in hypotonic
media as described under “Experimental Procedures.” Supernatants
from heart (H), mixed skeletal muscle (M), stomach (ST), brain (B),
kidney (K), and liver (L) mitochondria were subjected to SDS-PAGE.
Except for heart (35 µg of protein) and brain (60 µg of protein), 50
µg of protein was loaded on the gel. Prestained low molecular mass
standard proteins (Std) were obtained from Bio-Rad. Proteins were
transferred to an Immobilon P membrane for immunoblotting, see
“Experimental Procedures.” Mitochondrial branched chain aminotrans-
ferase activity per mg mitochondrial protein is shown below each lane.
Means and standard deviations from 3 to 18 separate
determinations are presented.](image1)

![Fig. 3. BCAT<sub>m</sub> in subcellular fractions of rat skeletal
muscles. Triton X-100 extracts (TRITON X-100) (100 µg of protein) and
100,000 × g supernatants (CYTOSOL) (100 µg of protein) pre-
pared from mixed calf muscle (M), soleus (S), and white gastroc-
nemius (G) were subjected to SDS-PAGE and immunoblotted as de-
scribed under “Experimental Procedures.” Branched chain aminotrans-
ferase activity in each fraction is shown below each lane. Mean values
(units/g of wet weight tissue) are presented and the range was
±15%.](image2)
Mitochondrial Branched Chain Aminotransferase

Mitochondrial citrate synthase usually represented from 9 to 15% of the total citrate synthase activity measured in tissue homogenates. In heart muscle, the enzyme has been shown to be solely mitochondrial (6). If not corrected for leakage, the mean cytosolic activity and total homogenate activity in heart was 345 ± 51 and 4306 ± 674 units/g tissue, respectively (mean ± standard deviation, n = 3). The low cytosolic activity and lack of significant immunoreactive protein in the cytosols prepared from different skeletal muscles suggested that branched chain aminotransferase has the same intracellular distribution in skeletal muscle as in heart muscle.

If the branched chain aminotransferase is solely mitochondrial in skeletal muscle, one would have predicted that total tissue activity would have reflected the well documented differences in soleus and white gastrocnemius mitochondria content (23). Since this was not observed, the possibility that the data could be explained by fiber-specific differences in mitochondrial aminotransferase activity and enzyme content was investigated. Mitochondria were prepared using large quantities of soleus and white gastrocnemius muscles so that reliable estimates of branched chain aminotransferase activity and mitochondrial protein could be determined. Fig. 4 shows immunoblots, mitochondrial enzyme activities, and results of quantitative immunodot analysis for BCAT_m antigen in mitochondrial preparations from soleus, white gastrocnemius, and the remaining calf muscles from the same set of animals. There are striking differences in the specific activities of BCAT_m in soleus (lane S) and white gastrocnemius (lane G) with intermediate activity found in mitochondria isolated from the remaining calf muscles (lane M). Heart mitochondrial enzyme activity and BCAT_m antigen from the same set of animals are also shown for comparison in Fig. 4 (lane H). Mean branched chain aminotransferase activity and standard deviations from five separate determinations in two additional mitochondrial preparations were 32.9 ± 2.6, 70.9 ± 4.9, and 51.5 ± 5.5 units/mg of mitochondrial protein for soleus, white gastrocnemius, and mixed skeletal muscle, respectively. Quantitative immunodot analysis of the mitochondrial extracts shown in Fig. 4 indicates that the activity differences actually reflect differences in BCAT_m enzyme content. These data provide the first evidence for quantitative differences in branched chain aminotransferase activity and BCAT_m enzyme protein in mitochondria prepared from different skeletal muscle fiber types.

Analysis of BCAT_m in Adult and Developing Liver—Oxidation of branched chain amino acids in rat liver is limited by the low aminotransferase activity (24). Mitochondria prepared from adult rat liver contained proteins recognized by the BCAT_m antiserum, but aminotransferase activity was almost at the limits of detection (see Fig. 2). These data raised the question whether or not any of the observed aminotransferase activity was actually associated with hepatocytes which are the major cell type found in liver. To answer this question, branched chain aminotransferase activity was determined in isolated hepatocytes. In addition, two hepatocyte subpopulations, isolated periporal and perivenous hepatocytes, were also examined. The data along with immunoblots of whole cell detergent extracts are shown in Fig. 5, lanes 1–3. Branched chain aminotransferase activity was low but detectable in isolated hepatocytes. All cells contained the same two immunoreactive proteins that had been observed in adult rat liver mitochondria (see Figs. 2 and 6). The data show that the two higher molecular weight proteins recognized by BCAT_m antiserum and aminotransferase activity are associated with the liver hepatocytes. Finally, no differences were seen between hepatocytes prepared from whole liver (lane 1) and the two hepatocyte subpopulations, suggesting these proteins are expressed in all hepatocytes.

Although adult rat liver aminotransferase activity is low, it is well documented that fetal rat liver has significant mitochondrial branched chain aminotransferase activity which declines right before birth (4). To determine whether or not the appearance of the 41-kDa protein coincided with the appearance and disappearance of branched chain aminotransferase activity during development, immunoreactive protein and branched chain aminotransferase activity were determined in mitochondrial (Fig. 6, lanes M) and cytosolic (Fig. 6, lanes C) fractions prepared from 18-day fetal rat liver and 5-day neonatal rat liver. These results are shown in Fig. 6 along with subcellular fractions prepared from adult rat liver. Branched chain aminotransferase activity was present in fetal liver, but activity was similar to adult liver in the 5-day neonatal rat liver. Activity was mitochondrial with little detectable activity in cytosolic fractions. The 41-kDa protein was only present in fetal liver mitochondria, although the high molecular mass proteins were found at all stages of development. Because it has been suggested that branched chain aminotransferase activity is associated with contaminating hemopoietic cells (4), activity was also measured in freshly isolated hepatocytes from 18-day fetal rats and in the
Mitochondrial Branched Chain Aminotransferase

FIG. 6. BCAT<sub>m</sub> in fetal, neonatal, and adult rat liver subcellular fractions. Mitochondrial 100,000 × g extracts (lanes M, 50 μg of protein) and cytosol fractions (lanes C, 100 μg of protein) from 18-day fetal rat (fetal liver), 5-day neonatal rat (neonatal liver), and adult rat livers (adult liver) were subjected to SDS-PAGE and immunoblotting as described under "Experimental Procedures." Lane H is a rat heart mitochondrial extract (25 pg of protein). Mean values and standard deviations (n = 3) of branched chain aminotransferase activity/mg of protein in fetal, neonatal, and adult rat liver fractions are shown below each lane.

FIG. 7. Two-dimensional immunoblotting of BCAT<sub>m</sub> antigen in mitochondrial extracts from heart, liver, and kidney. Mitochondrial 100,000 × g extracts of rat heart (A), kidney (B), and liver (C) were immunoprecipitated with anti-BCAT<sub>m</sub> antibodies and immunocomplexes isolated as described under "Experimental Procedures." Immunocomplexes were subjected to two-dimensional electrophoresis and transferred to Immobilon P membranes for immunoblotting with anti-BCAT, antiserum. A-C have the immunoreactive proteins from heart, kidney, and liver mitochondria, respectively. The arrow points to protein A released from protein A particles used for isolation of the immunocomplexes.

Evidence for Multiple Forms of BCAT<sub>m</sub>—Although the purified BCAT<sub>m</sub> appears as a single band of about 41 kDa in SDS-PAGE gels, in nondenaturing gels the enzyme appears as a doublet (1). N-terminal amino acid sequence analysis performed on each band revealed the same N-terminal sequence (VSSN), suggesting the existence of enzyme isoforms that differ in electrical charge. The appearance of several immunoreactive proteins in kidney and liver mitochondria also suggested heterogeneity among the proteins recognized by the BCAT<sub>m</sub> antiserum. To investigate these proteins further, two-dimensional immunoblotting was used as a high resolution method. The results are presented in Fig. 7. Two-dimensional immunoblotting of BCAT<sub>m</sub> revealed charge and molecular mass heterogeneity of the proteins defining the heart mitochondria 41 kDa immunoreactive band seen on the one-dimensional immunoblots of BCAT<sub>m</sub> in Fig. 1. As seen in Fig. 7A, six pools of heart BCAT<sub>m</sub> protein with different isoelectric points (pH range 6–7) were distinguished on the immunoblot. In addition, this two-dimensional system had separated BCAT<sub>m</sub> immunoreactive proteins into two pools of proteins with different molecular masses (see Fig. 7A), proteins with the higher molecular mass being more acidic than proteins with the lower molecular mass. As seen in previous figures, only a single immunoreactive protein band of 41 kDa was apparent on one-dimensional immunoblots of the rat heart enzyme. The appearance of proteins with different molecular mass after two-dimensional immunoblotting is not understood but may have resulted from exposure of the protein to the isoelectric focussing conditions (9 M urea) prior to SDS-PAGE.

Fig. 7B shows a two-dimensional immunoblot of kidney mitochondria immunoreactive proteins. This immunoblot was identical to the immunoblot of the heart proteins (compare A and B). On the other hand, a two-dimensional immunoblot of the proteins present in liver mitochondria revealed three pools of proteins with different isoelectric points (C) but with the same apparent molecular mass which was higher than the molecular mass of the immunoreactive proteins found in heart and kidney extracts. Clearly the two-dimensional immunoblot of the liver immunoreactive proteins was different from the two-dimensional blots of the heart and the kidney proteins.

Both the two-dimensional immunoblotting and the behavior of the enzyme on nondenaturing electrophoresis (1) suggested that there are at least two or more different forms of the BCAT<sub>m</sub> differing in charge and/or molecular mass. The number of charged species of BCAT<sub>m</sub> was investigated by substituting chromatofocusing on Mono P as a final purification step for heart BCAT<sub>m</sub>. At least four different peaks of BCAT<sub>m</sub> activity with pH values of 5.5, 5.4, 5.39, and 5.3 were observed, confirming the existence of multiple charged forms of the enzyme. On SDS-PAGE all fractions gave an apparent molecular mass of 41 kDa (data not shown).

DISCUSSION

The number of mammalian branched chain aminotransferases is as yet unresolved and, in light of the occurrence of hypervalinemia, there is some question as to the existence of an aminotransferase specific for valine. At least four isoforms of branched chain aminotransferase have been reported, Enzyme I, II, III, and a mitochondrial form of Enzyme I (for review, see Ref. 3). We have purified a mitochondrial form of branched chain aminotransferase, BCAT<sub>m</sub>, from rat heart and have shown that heart does not contain any cytosolic branched chain aminotransferase (1, 6).

The data presented here show that BCAT<sub>m</sub> antigen is present in all mitochondria expressing branched chain aminotransferase activity, and this conclusion is supported by the earlier work of Kadowaki and Knox (4) who found only branched chain aminotransferase activity that eluted at low salt concentrations from DEAE-cellulose (Enzyme I) in their mitochondrial fractions. The apparent molecular mass of the immunoreactive protein is about 41 kDa (range 41–42 kDa), which is somewhat lower than observed previously with the purified enzyme (1). The lower calculated molecular mass may result from differences in the Bio-Rad low molecular mass standards that were used. The tissue distribution of the BCAT<sub>m</sub> also coincides with the profile for the branched chain aminotransferase termed Enzyme I by Ichihara and co-workers (for review, see Ref. 2); however, it would appear that this enzyme is actually a mitochondrial protein rather than distributed between mitochondria and cytosol as suggested in the early studies (2–4). Originally, Kadowaki and Knox (4) had reported finding Enzyme I in the cytosol of most rat tissues. The data indicate that only two isoenzymes exist, a mitochondrial (BCAT<sub>m</sub>) and cytosolic aminotransferase (Enzyme III).

Two-dimensional immunoblotting showed that the proteins recognized in kidney mitochondria are similar to those found in heart mitochondria. On the other hand, the antigenic material in liver mitochondria is clearly distinct from the kidney and heart proteins. Whether or not these proteins
represent an inactive precursor form of the enzyme or are structurally related proteins that may turn out to be members of an as yet undefined family of proteins remains to be determined. The highest molecular mass antigen appears to be specific for liver mitochondria. Quantitative immunodot analysis of rat heart and adult liver mitochondria, using heart BCATₘ as standard, gave some indication that the liver immunoreactive protein(s) was at least as abundant as heart mitochondrial BCATₘ. 2.95 and 1.56 μg/mg mitochondrial protein in liver and heart, respectively. The liver proteins do not have a molecular mass similar to reported values for the rat liver cytosolic leucine aminotransferase (Enzyme II, 65 kDa) or the purported mitochondrial leucine (methionine) transaminase (55 kDa) purified by Ichihara and co-workers (25), although these molecular masses were determined by gel filtration not by SDS-PAGE. α-Ketoisocaprate dioxygenase (46 kDa) which was purified by Sabourin and Bieber (26) is found in liver and kidney, but it is a cytosolic enzyme. Several of the enzymes of branched chain amino acid catabolism, including isovaleryl-CoA dehydrogenase, have molecular masses between 40 and 50 kDa (27), but they are not exclusively liver proteins. Regardless of their identity, these proteins must have significant structural homology with BCATₘ.

Oxidation of branched chain amino acids in liver is limited by the low aminotransferase activity. Using a value of 10⁶ hepatic 0.2 mg of wet weight liver, values of 40-90 units/g of liver can be calculated from the data in Fig. 5. Thus, it is quite likely that parenchymal cells are the source of liver branched chain aminotransferase activity that has been reported by us and others (2, 7, 22). On the other hand, developmental changes in branched chain aminotransferase activity in rat liver coincided with the appearance and disappearance of the 41-kDa BCATₘ antigen. Kadowaki and Knox (4) concluded that the fetal liver branched chain aminotransferase activity was actually derived from the hemopoietic cells, because the pattern of disappearance of branched chain aminotransferase activity paralleled measured changes in hemopoietic cell volume. Branched chain aminotransferase activity has not been measured in hemopoietic cells, so it is not possible to calculate if all of the fetal liver activity could be accounted for by these cells. Our data suggest that some of the branched chain aminotransferase activity may actually be associated with the fetal hepatocytes. If so, the disappearance of BCATₘ in fetal liver represents a developmental change in liver gene expression as well as resulting from the loss of hemopoietic cells. Interestingly, the branched chain α-keto acid dehydrogenase enzyme complex in liver is low at birth, and enzyme levels increase during the neonatal period reaching adult levels at 21 days (28). Thus the capacity of rat liver to transaminate branched chain amino acids decreases at birth, whereas oxidative capacity increases after birth. These changes in the liver are partly responsible for the unique interorgan regulation of the catabolism of these essential amino acids in the rat.

In tissues where branched chain aminotransferase is exclusively mitochondrial, tissue activity directly reflects mitochondrial specific activity and tissue mitochondrial content. Based on activity measurements, we had concluded that the aminotransferase is exclusively mitochondrial in heart and kidney cortex (7). These conclusions are supported by the distribution of BCATₘ antigen and activity presented here and would also appear to be true for stomach, a tissue originally found by Ichihara (21) to possess high aminotransferase activity. In skeletal muscles, however, mitochondrial content varies (23), whereas total aminotransferase activity in the muscles that were examined appeared to be largely independent of muscle type (Ref. 7 and Fig. 3). Hence, when a mean specific activity for branched chain aminotransferase and citrate synthase activities in mitochondria prepared from mixed skeletal muscle preparations was used to calculate enzyme distribution, not all of the tissue activity could be accounted for by the mitochondrial fraction and was assigned to the cytosolic compartment. Nevertheless, the data shown in Figs. 3 and 4 indicate that branched chain aminotransferase activity is mitochondrial, but enzyme content varies in mitochondria in the different fiber types. In skeletal muscles such as soleus, which is composed of approximately 89% slow-twitch red fibers and 11% fast-twitch red fibers, BCATₘ specific activity is less than half that in white gastrocnemius, which consists of about 91% fast-twitch white fibers and 9% fast-twitch red fibers (23). The data raise the intriguing possibility that there is type-specific expression of BCATₘ in skeletal muscle fibers and perhaps even in mitochondrial subpopulations within individual fibers. The physiological implications of BCATₘ distribution in the different skeletal muscle fiber types is not yet known, but it is interesting to speculate that BCATₘ distribution may correlate with branched chain α-keto acid dehydrogenase enzyme complex content or activity state in the different skeletal muscle fiber types.

In summary, BCATₘ is the form of branched chain aminotransferase found in most rat tissues. The enzyme exhibits multiple forms differing slightly in charge even in fresh tissue extracts. Work is currently in progress to isolate the cDNA for BCATₘ which may provide the necessary tools to study expression of BCATₘ and identify the additional immunoreactive proteins.

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