A Novel Branched-chain Amino Acid Metabolon

PROTEIN-PROTEIN INTERACTIONS IN A SUPRAMOLECULAR COMPLEX

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The catabolic pathways of branched-chain amino acids have two common steps. The first step is deamination catalyzed by the vitamin B6-dependent branched-chain aminotransferases (BCATs) to produce branched-chain \( \alpha \)-keto acids (BCKAs). The second step is oxidative decarboxylation of the BCKAs mediated by the branched-chain \( \alpha \)-keto acid dehydrogenase enzyme complex (BCKD complex). The BCKD complex is organized around a cubic core consisting of 24 lipoate-bearing dihydrolipoamide transacylase (E2) subunits, associated with the branched-chain \( \alpha \)-keto acid decarboxylase/dehydrogenase (E1), dihydrolipoamide dehydrogenase (E3), BCKD kinase, and BCKD phosphatase. In this study, we provide evidence that human mitochondrial BCAT (hBCATm) associates with the E1 decarboxylase component of the rat or human BCKD complex with a \( K_D \) of 2.8 \( \mu \)M. NADH dissociates the complex. The E2 and E3 components do not interact with hBCATm. In the presence of hBCATm, \( k_{cat} \) values for E1-catalyzed decarboxylation of the BCKAs are enhanced 12-fold. Mutations of hBCATm proteins in the catalytically important CXXC center or E1 proteins in the phosphorylation loop residues prevent complex formation, indicating that these regions are important for the interaction between hBCATm and E1. Our results provide evidence for substrate channeling between hBCATm and BCKD complex and formation of a metabolic unit (termed branched-chain amino acid metabolon) that can be influenced by the redox state in mitochondria.

The flow of metabolites through an individual pathway and the integration of multiple metabolic pathways involve a complex interplay of different reactions and regulatory mechanisms. Until recently, the focus has been on the regulation of the individual proteins involved in these processes. The function of a protein can also be defined on the basis of its interactions with other proteins (1) and how these interactions impact on protein function and the flux of a metabolite through a pathway. The concept that metabolic enzymes associate to form supramolecular structures was developed over 50 years ago (2), and the term metabolon was introduced by Paul Srere in 1985 (3). Advantages of such a supramolecular assembly include channeling of substrates between enzymes in a pathway for efficiency, regulating the pathway flux by association and dissociation of relevant enzymes, and targeting the localization of the interacting enzymes with the appropriate intracellular structures (3). Association of mitochondrial citric acid cycle enzymes has been reported (4, 5). The goal of this study was to determine whether mitochondrial enzymes of the branched-chain amino acid (BCAA)\(^a\) catabolic pathway can associate to form a supramolecular complex.

The first step in catabolism of the essential BCAs, leucine, isoleucine, and valine, is reversible transamination catalyzed by the branched-chain aminotransferase (BCAT) isozymes to form the branched-chain \( \alpha \)-keto acids (BCKAs). Aminotransferases exhibit Ping Pong Bi Bi reaction kinetics, where \( E \)-PLP and \( E \)-PMP denote the enzyme cofactor pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate (PMP), respectively (Reactions 1 and 2).

\[
\begin{align*}
E \cdot PLP + BCAA & \rightleftharpoons E \cdot PMP + BCKA \\
E \cdot PMP + \alpha-KG & \rightleftharpoons E \cdot PLP + Glu
\end{align*}
\]

The overall reaction results in transfer of the \( \alpha \)-amino group

\(^a\)The abbreviations used are: BCAA, branched-chain amino acid; BCKA, branched-chain \( \alpha \)-keto acid; BCKD, branched-chain \( \alpha \)-keto acid dehydrogenase; BCAT, branched-chain aminotransferase; hBCATm, human mitochondrial branched-chain aminotransferase; hBCATc, human cytosolic branched-chain aminotransferase; hBCATm-Ox, oxidized hBCATm; E1, branched-chain \( \alpha \)-keto acid decarboxylase/dehydrogenase; E1p, the pyruvate dehydrogenase component of the pyruvate dehydrogenase complex; E2, dihydrolipoamide transaclyase; E3, dihydrolipoamide dehydrogenase; ITC, isothorayl calorimetry; PLP, pyridoxal 5'-phosphate; PMP, pyridoxamine 5'-phosphate; THDP, thiamine diphosphate; KIC, \( \alpha \)-ketoisocaproate; KIV, \( \alpha \)-ketoisovalerate; MVK, \( \alpha \)-keto-\( \delta \)-methylvalerate; RIV, \( \alpha \)-ketoisovalerate; \( \alpha \)-KG, \( \alpha \)-keto glutarate; DCPIP, 2,6-dichlorophenolindophenol; LBD, lipoyl-bearing domain; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; WT, wild type; lip-E2, lipoylated E2.
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from BCAA to α-ketoglutarate (α-KG) to form glutamate (Glu). In the two half-reactions shown above, the α-amino group temporarily resides on the coenzyme PLP forming PMP (6–8). The BCKA transamination products are α-ketoisocaproate (KIC), α-keto-β-methylvalerate (KMV), and α-ketovalerate (KIV). The mitochondrial isozyme (BCATm) is expressed in nearly all rats, non-human primates, and human tissues (9–12), whereas the cytosolic isozyme (BCATc) is found almost exclusively in nervous tissue (13, 14). BCATm is not found in adult rat liver (9). BCATc is the predominant isozyme in rat brain accounting for ~60–70% of total BCAT activity.

Furthermore, within a given tissue, BCATm and BCATc show cell-specific expression (14, 15). Recently, Sweatt et al. (13, 14) have shown that in the rat BCATc is expressed in peripheral nerves and selected glutamatergic and GABAergic neurons in brain. BCATm has been found in astroglia. To date, co-expression of BCATm and BCATc within the same cell type has not been observed. Outside the central nervous system, BCATm is often localized in secretory epithelial cells. With a few exceptions, BCATm co-localizes with the second enzyme in the BCAA catabolic pathway, the mitochondrial branched-chain α-keto acid dehydrogenase complex (BCKD complex).

The BCKD complex is a member of the family of highly conserved macromolecular machines also including the pyruvate dehydrogenase and α-ketoglutarate dehydrogenase complexes (16, 17). These three complexes catalyze similar multistep reactions that lead to the oxidative decarboxylation of α-keto acids giving rise to branched-chain acyl-CoAs, acetyl-CoA, or succinyl-CoA, respectively (Reaction 3).

The BCKD complex is a 4-MDa catalytic machine organized around a 24-meric core scaffold consisting of dihydrolipoamide dehydrogenase (E3) (Reaction 7),

\[
E2\text{-}[\text{lip}SH]_2 + E3\text{-}[\text{S}]_2 \rightleftharpoons E2\text{-}[\text{lip}S_2] + E3\text{-}[\text{SH}]_2
\]

REACTION 7

and NAD\(^+\) is the ultimate electron acceptor (Reaction 8) (19).

\[
E3\text{-}[\text{SH}]_2 + \text{NAD}^+ \rightleftharpoons E3\text{-}[\text{S}]_2 + \text{NADH} + \text{H}^+
\]

REACTION 8

The sum of these multistep reactions is the above overall reaction mediated by the BCKD complex (Reaction 3).

The BCKD complex is highly regulated both by short term mechanisms and by long term mechanisms (19, 22). Short term regulation is mediated by the BCKD kinase that can associate and dissociate from the complex (23–27). A specific BCKD phosphatase has also been purified, but the regulatory role of this phosphatase is not well understood (28). Moreover, identification of an inhibitor protein suggests that BCKD activity may be regulated by proteins other than the kinase and the phosphatase (29). The next step in the oxidation of BCAAs is dehydrogenation of the branched-chain acyl-CoA reaction products catalyzed by two different dehydrogenases (30). After this step the catabolic pathways diverge, and the remaining enzymatic reactions resemble β-oxidation of fatty acids.

There is considerable structural information on the mammalian BCKD complex proteins (31–35) and the BCAT isozymes (6, 7, 36). The BCAT isoforms are homodimers and belong to the fold type IV class of PLP (vitamin B\(_6\))-dependent enzymes. We have shown that human BCATm (hBCATm) contains a redox-active CXXC center that can reversibly regulate the activity (6, 7, 36–38). In this study, we demonstrate that hBCATm and the BCKD complex form a protein-protein complex (termed BCAA metabolon) and identify the E1 enzyme as the hBCATm protein partner. Furthermore, hBCATm with E1 is regulated by reduced pyridine nucleotides and is specific for the mitochondrial hBCATm isozyme. Our results provide evidence for substrate channeling between hBCATm and BCKD in this novel metabolon.

**Experimental Procedures**

Chemicals—Sequencing grade modified trypsin was purchased from Promega (Madison, WI). HEPES and potassium phosphate were obtained from Fisher. L-Leucine, L-isoleucine, L-valine, L-glutamate, all α-keto acids, dithiothreitol (DTT), β-mercaptoethanol, PLP, and isopropyl β-D-thiogalactopyranoside were purchased from Sigma. The medium used for bacterial growth contained 0.5% yeast extract, 1% polypeptide, and 0.5% NaCl and was from Fisher. The pET-28a vector was from Novagen (Madison, WI), and bacterial strain Escherichia coli BL21 (DE3) was obtained from Promega, Inc. Nickel-nitrilotri-
acetic acid resin was purchased from Qiagen (Chatsworth, CA). All other chemicals were of the highest grade commercially available.

**Animals**—Male Sprague-Dawley rats (200–300 g) were purchased from Charles River Breeding Laboratories (Raleigh, NC). Rats were housed three to four per cage and maintained with food and water in a temperature- and light-controlled environment. The animals were sacrificed under deep halothane and thiopentone anesthesia, and the livers were collected.

**Preparation of Rat Liver Mitochondrial Extracts**—The livers were removed and homogenized in isolation medium containing 225 mM mannitol, 75 mM sucrose, 0.1 mM EDTA, 5 mM MOPS, pH 7.0. Mitochondria were purified as described previously (41). The mitochondrial pellets were suspended in isolation medium at a concentration of 70–120 mg of protein/ml containing protease inhibitor mixture set III (Calbiochem). The respiratory control ratio was determined separately in the presence of magnesium by measuring the ratio of the respiratory rate in the presence of ADP (0.5 mM) to the rate measured after cessation of ADP phosphorylation with 20 mM glutamate and 1 mM malate as substrates (39). No mitochondrial preparation with a ratio less than 6 was used. Protein was determined using the Biuret assay. The mitochondria (100 mg) were allocated into separate tubes and centrifuged, and the pellets were either used immediately or stored at −80 °C. Mitochondrial pellets could be stored frozen for up to 4 weeks.

Mitochondrial proteins were extracted by gentle homogenization of the mitochondrial pellets in solubilizing buffer (SB buffer) containing 50 mM potassium phosphate, 0.2 mM EDTA, 0.75% CHAPS, 25 mM KCl, 1 mM DTT, pH 7.4, and protease inhibitor mixture set III (Calbiochem) at a mitochondrial protein concentration of 25 mg of protein/ml. The solubilized mitochondria were centrifuged at 15,000 g for 30 min. The supernatant was removed, and the sample was filtered using a 0.22-μm syringe filter.

**Protein Purification**—The overexpression and purification of the wild-type (WT) and mutant hBCAT proteins were performed according to the method of Conway et al. (37, 38). The histidine tag was removed by digestion with thrombin (100 NIH units) at 30 °C for 1 h followed by buffer exchange into the storage buffer (50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 5 mM DTT, 1 mM EDTA, 1 mM KIC, and 5 mM glucose). The mutant hBCATm proteins are as stable as WT-hBCATm. The N-terminally His 6-tagged wild-type and mutant E1 proteins were produced and subjected to exhaustive dialysis in the presence of 0.2 mM EDTA as illustrated by Wynn et al. (40). The recombinant human E2 and E3 proteins were expressed and purified as described previously (41).

**Preparation of the Human BCAT Affinity Matrix Columns**—Recombinant hBCAT proteins (1 mg/ml in 2 ml) were dialyzed against bicarbonate buffer (0.2 M NaHCO 3 and 0.5 M NaCl, pH 8.0) overnight. Two grams of activated CH-Sepharose® 4B (Sigma) were suspended in 200 ml of cold 1 mM HCl for 10–15 min. The rest of the protocol for this ligand-resin coupling was performed according to the manufacturer’s instructions. The column was stored at 4 °C equilibrated with 5 mM DTT containing phosphate-buffered saline with 0.02% sodium azide.

**Isolation of hBCATm-bound Proteins**—The hBCAT affinity columns were equilibrated with the SB buffer containing 1 mM DTT, and the filtered mitochondrial extract was passed through the column. The column was then washed with SB buffer with addition of 100 mM KCl. Bound proteins were eluted with 10 or 5 mM NADH in SB buffer without DTT. The eluted proteins were precipitated using acetone at −20 °C overnight, and the protein pellet was washed with 10% trichloroacetic acid followed by ether/ethanol (1:1 v/v). The protein pellets were dried under nitrogen, resuspended in SDS loading buffer, and analyzed by SDS-PAGE.

**Immunoblotting and BCKD Antibodies**—Proteins in the column eluate were separated by SDS-PAGE in 10% gels (42). Before electrophoresis, all samples were boiled for 2 min in the presence of 1% SDS, with 5% β-mercaptoethanol. For immunoblotting, proteins in the SDS-polyacrylamide gel were transferred to a nylon membrane (Hybond-P, GE Healthcare). The E1 and E2 antibodies against the purified subunits from the rat liver BCKD complex were gifts from Dr. Yoshiharu Shimomura (Nagoya Institute of Technology, Nagoya, Japan). The E1 antibody recognizes the E1α subunit and has weak immunoreactivity with the E1β subunit and E2. The E3 antibody was the gift of Dr. Mulchand S. Patel (State University of New York, Buffalo). Immunoblotting was performed as described by Hutson et al. (43). Immunoreactive protein bands were visualized using horseradish peroxidase-conjugated goat anti-rabbit AffinoPure IgG according to the manufacturer’s instructions (Jackson Immunoresearch).

**Protein Identification by Tandem Mass Spectrometry**—To identify the proteins in the affinity chromatographic eluate, the IP pellet containing nearly 1 mg of protein was resuspended in 50 μl of the reaction mixture (6 M guanidine HCl, 50 mM Tris-HCl, pH 8.0, and 4 mM DTT). This was followed by a standard in-solution tryptic digestion protocol following the manufacturer’s instructions, which included alkylating with iodoacetamide and enzymatic digestion using sequencing grade trypsin gold (Promega, Madison, WI). The extent of digestion was determined by SDS-PAGE; 20 μl of the digested sample was then purified on a C18-peptide trap (Michrom Bioresources, Auburn, CA) as per the manufacturer’s instructions and concentrated in a Speedvac apparatus followed by resuspension in 20 μl of 0.1% mass spectrometry grade formic acid. One-half of the sample (10 μl) was diluted 1:20, and ~4 μl (~200 ng of peptide mix) was injected onto a Thermo Surveyor high-pressure liquid chromatograph tied to a Thermo LTQ ion trap mass spectrometer with a nano-electrospray source (Thermo Electron, San Jose CA). The LC conditions and search parameters were run using conditions published previously (44). A number of significant hits were observed for each protein with Xcorr scores above 5 as reported under “Results.”

**Spectrophotometric Enzyme Assays and Kinetic Analysis**—All enzymatic assays were performed using a Beckman Coulter DU-800 spectrophotometer at 30 °C equipped with a Beckman Coulter temperature controller. The kinetics of the E1 dehydrogenase reaction (see Reaction 4) with added hBCATm (with a BCAA substrate, Reactions 9 and 10) or without hBCATm (with a BCKA substrate, Reaction 10) with purified recombi-
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nant human E1 was determined in the presence of an artificial electron acceptor 2,6-dichlorophenolindophenol (DCPIP).

\[
R\text{-CH(NH}_2\text{)-COOH} + \text{PLP-hBCATm} \implies R\text{-CO-COOH} + \text{PMP-hBCATm} \tag{REACTION 9}
\]

\[
R\text{-CO-COOH} + \text{DCPIP (oxidized)} + H_2O \rightarrow R\text{-COOH} + CO_2 + \text{DCPIP (reduced)} + H^+ \tag{REACTION 10}
\]

The assay mixture contained 100 mM potassium phosphate, pH 7.5, 2.0 mM MgCl\(_2\), 0.2 mM ThDP, and 0.1 mM DCPIP. The E1 was reconstituted with hBCATm at a 1:1 molar ratio. The rate of decarboxylation at 30 °C was measured by monitoring the reduction of the dye at 600 nm.

For the overall assay of the BCKD complex (Reaction 3), the enzymes were exchanged into phosphate buffer (30 mM potassium phosphate, pH 7.5, 50 mM KCl, 10 mM NAD\(^3\)), was monitored by formation of NADH at 340 nm.

oxidative decarboxylation of KIV) (Reactions 1 and 3) or with- reaction, either with added BCAT (deamination of valine and

various proteins, immediately prior to experiments, MgCl\(_2\) and ThDP stock solutions were added to both the reaction cell and the final concentration of 0.1 mM. Binding isotherms were calculated using the following equation:

\[
\begin{align*}
\Delta G^o &= -RT \ln K_{cat} \\
K_{cat} &= \frac{k_{cat}[S]}{[K_m + [S]]}
\end{align*}
\]

The \(K_{cat}\) and \(K_m\) values were obtained from the fitted curves and are shown in Tables 2–4. All calculations and analyses were performed using IGOR Pro software (WaveMetrics Inc., OR).

Binding Studies by Isothermal Titration Calorimetry—Human BCATs, E1, lip-LBD, E2, and E3 were dialyzed overnight against the same reservoir of 50 mM potassium phosphate buffer, pH 7.5, 50 mM KCl, 10 mM β-mercaptoethanol, 5% glycerol, and 0.2 mM EDTA. Titrations were carried out at 20 °C in a MicroCal (Northampton, MA) VP-ITC microcalorimeter. The solution of 175 \(\mu\)M hBCATm or other BCAT proteins in


denatured at 45°C (for E3 denatured in (45).

Preparation of the Oxidized Form of hBCATm—Oxidation of the hBCATm isozone was conducted by incubation with hydrogen peroxide at room temperature. A 60 \(\mu\)M enzyme solution was first exchanged into 50 mM HEPES-KOH, pH 7.5, containing 0.1 \(\mu\)M KCl and 0.1 mM EDTA using a PD-10 column (GE Healthcare). Then a 50 \(\mu\)M enzyme solution was incubated with 500 \(\mu\)M hydrogen peroxide for 2 h. The enzyme reaction mixture was exchanged into the same buffer without hydrogen peroxide.

The activity of the oxidized BCATm was 1.5 units/mg of protein compared with the reduced BCATm activity of 106 units/mg protein. Disulfide bond formation was confirmed by 5,5′-dithiobis(nitrobenzoic acid) titration (45).

RESULTS

Human hBCATm Forms a Complex with the Rat Liver Mitochondrial BCKD Complex—A strategy was developed to test whether human recombinant mitochondrial BCATm could form a stable complex with the BCKD complex and/or other mitochondrial proteins using a recombinant hBCATm affinity column to screen for potential interacting proteins. Rat liver was chosen as the mitochondrial source, because it is the only rodent tissue that does not contain BCATm. This experimental design eliminates competition with endogenous BCATm. Because chow-fed animals were used, BCKD E1 is also >90% in the active state (dephosphorylated) state (23, 46). The reduced PLP form of hBCATm was coupled to Sepharose 4B, and a detergent-solubilized liver mitochondrial extract (containing DTT to keep hBCATm in the reduced form) was passed through the column (Fig. 1). The hBCATm column was washed with the same SB buffer except that the KCl concentration was increased from 25 to 125 mM. Bound proteins were eluted from
TABLE 1
Identification of the BCKD complex enzyme components in NADH eluent by tandem mass spectrometry

| ID      | NCBI accession no. | No. of peptides identified | Highest Xcorr values |
|---------|--------------------|---------------------------|----------------------|
| E1α     | NP_036914           | 38                        | 5.6                  |
| E1β     | P35738              | 15                        | 5.9                  |
| E2      | XP_342329           | 21                        | 5.5                  |
| E3      | AAH62069            | 49                        | 5.1                  |

* Liquid chromatography/tandem mass spectrometry analysis was carried out as referenced under “Experimental Procedures.” Data were searched against the NCBI non-mammalian data base using SEQUEST using common filtering cut-off values, i.e. cross-correlation (Xcorr) values >1.8 for doubly charged ions and >2.5 for triply charged ions. In addition, ranking of preliminary score values of <5 and preliminary score values of >350 also were required for positive peptide identifications. Replicate experiments were carried out with similar results.

To determine whether any known BCAA-metabolizing enzymes were present, Western blotting was performed on proteins in the NADH fraction (Fig. 2A, lane 3) using a panel of antibodies that recognize subunits of the E1, E2, and E3 components (Fig. 2A, lanes 1, 2, and 4). The results show that all subunits of the BCKD complex components are present in the NADH fraction. The identity of the subunits of the E1, E2, and E3 components was subsequently confirmed in replicate experiments following affinity isolation of the proteins in the NADH fraction and characterization using liquid chromatography/tandem mass spectrometry (Table 1). To determine whether the effect of NADH was specific for pyridine nucleotides, the above experiments were repeated using NADPH, NAD⁺, or ATP instead of NADH. Western blots using E1 antibody (recognizes E1 subunits and E2) revealed that neither ATP nor NAD⁺ was effective in dissociating the BCKD proteins bound to the hBCATm affinity column, whereas bound proteins were partially released by NADPH (Fig. 2B). Lower concentrations of NADH (5 and 1 mM) were as effective as the 10 mM concentration used in the experiments shown in Figs. 1 and 2 (data not shown). Therefore, dissociation of the BCATm-BCKD complex shows specificity for reduced pyridine nucleotides.

BCKD complex proteins associate only with the reduced PLP form of WT-hBCATm. Human BCATm is a PLP-dependent enzyme, and the PLP cofactor is covalently attached to the Lys-202 in the active site of the enzyme (7). After transamination with BCAAs (the first half-reaction shown in Reaction 1), the PLP form of the enzyme is converted to the PMP form of the enzyme (7). A second characteristic of hBCATm is the presence of a consensus sequence CXXC motif (Cys-315 and Cys-318) which is located at the opening of the active site and 10 Å from the cofactor (6, 7, 37). The CXXC motif plays a crucial role in orienting the substrate optimally for catalysis (45). Under oxidizing conditions the CXXC center cysteines form a disulfide bond. This form of the enzyme is catalytically inactive (37, 38). To characterize further the specificity of the interaction between hBCAT and the BCKD complex proteins, affinity chromatographic experiments were performed using the PMP form of hBCATm, the hBCATm C315A/C318A double mutant, the hBCATm (C318A) single mutant, and oxidized hBCATm (hBCATm-Ox). Results for the C315A/C318A double mutant hBCATm are shown in Fig. 2C. No detectable proteins were observed in the NADH fraction on SDS-polyacryl-
amide gels or on Western blots using either E1 or E2 antibodies. Similar results were obtained for the PMP form of hBCATm and oxidized enzyme. These results indicate that the structural conformation of hBCATm is important for the interaction between hBCATm and BCKD proteins. Thus only the form of hBCATm that accepts the amino acid substrate (PLP form) in the fully reduced state associates with the BCKD complex.

The cytosolic hBCATc isozyme was also examined for its ability to bind to rat liver mitochondrial proteins. Using an hBCATc affinity column, no proteins were detected in the NADH eluent nor were any proteins eluted from the column after addition of high salt (1 or 4 M KCl) (data not shown). The lack of binding of rat liver mitochondrial proteins to hBCATc affinity column, no proteins were detected in the unchanged isotherm with E2 and E3 unequivocally demonstrate the absence of interaction of either of these two components with hBCATm and a BCAA than that with E1 and a BCKA. This increase would result in a higher $k_{cat}$ value. $K_v$ values for BCAAs and BCKAs are not directly comparable, because they are specific for their respective enzymes. Decarboxylase activity of E1, which is distinct from the overall activity of the BCKD complex, can be measured using DCPIP as an electron acceptor (Reaction 10) (47). As shown in Table 2, in the presence of valine and PLP-hBCATm there was a 5.9-fold increase in the calculated $k_{cat}$ value for E1-catalyzed decarboxylation, when compared with E1 alone with the valine-corresponding $\alpha$-keto acid KIV as substrate. When the PMP form, instead of the PLP form, of the enzyme was used with valine as substrate, decarboxylation activity was not detectable (Table 2). In the first half-reaction the $\alpha$-amino nitrogen of valine is transferred to PLP-hBCATm forming PMP-hBCATm and KIV. Under these experimental conditions, the efficient oxidative decarboxylation of the KIV product by E1 removes the $\alpha$-keto acid acceptor that can regenerate the PLP form of hBCATm. Thus, when PMP-hBCATm is incubated with E1 and valine, PMP-hBCATm likely dissociates from E1 and only a single turnover occurs.

In cells, concentrations of $\alpha$-ketoglutarate ($\alpha$-KG) are an order of magnitude higher than concentrations of BCKAs, and $\alpha$-KG appears to be the primary physiological nitrogen acceptor for the second BCAT half-reaction (48). The data...
presented in Table 2 show that over the concentration range of 0.5 μM to 20 mM, α-KG is not a substrate for the BCKD E1 enzyme, and it does not affect the E1 reaction with KIV (Table 2). Similarly, addition of α-KG does not affect overall catalysis of the BCKD complex reaction with BCKA substrates (data not shown). On the other hand, addition of α-KG, which facilitates regeneration of PLP-hBCATm by a non-BCKD substrate, enhanced the calculated \( k_{\text{cat}} \) by 12.7-fold when compared with E1 plus KIV and 2.2-fold above the rate with PLP-hBCATm and valine alone. The \( K_m \) value for valine was also decreased by ~25% in the presence of α-KG (Table 2). No detectable activity was observed with the PMP form of hBCATm and E1 in the presence of α-KG. Addition of α-KG (20 mM) with the PMP-hBCATm, valine, and the E1 reaction mixture, which allows regeneration of the PLP form of hBCATm, permits the reaction to proceed. The \( k_{\text{cat}} \) value of the valine-mediated decarboxylation was 108 min\(^{-1}\) with a \( K_m \) value of valine at 657 μM. This suggests that the generation of PLP-hBCATm results in complex formation with E1 and efficient catalysis. \( K_m \) values for valine in the coupled reaction are considerably lower than \( K_m \) values for BCAA-catalyzed transamination (38, 45, 49).

To determine whether the PLP-hBCATm has any effect on the decarboxylation of KIV by E1 in the absence of a BCAA, the DCPIP assay was carried out in the presence of E1, PLP-hBCATm, and KIV as a substrate. The calculated \( k_{\text{cat}} \) value was ~20% lower, and \( K_m \) for KIV was 40% higher in the presence of hBCATm (data not shown). Binding of PLP-hBCATm likely buries the active site of E1 and interferes with the penetration of free KIV into the E1 active site. The kinetic parameters strongly suggest that only the WT PLP-hBCATm interacts with the E1 component of BCKD complex.

Overall Catalytic Activity of the BCKD Complex Is Accelerated by Addition of the PLP Form of WT-hBCATm—Because the rate of the decarboxylation step (Reaction 4) is markedly increased in the presence of hBCATm with valine as a substrate, a question arises as to whether the rate of the overall reaction catalyzed by the BCKD complex (Reaction 3) is also enhanced under the same conditions.

To assess for overall activity, the BCKD complex was reconstituted in the presence or absence of hBCAT proteins, and α-KG was present in the assay buffer. Similar to the results shown in Table 2, addition of PLP-hBCATm resulted in ~12-fold increase in the calculated \( k_{\text{cat}} \) values for E1-catalyzed decarboxylation for all hBCAT substrates (Leu, Ile, and Val) (Table 3). Addition of BCATc, the CXXC double mutant enzyme, or oxidized hBCATm had no effect on the calculated \( k_{\text{cat}} \) for E1 decarboxylation. \( K_m \) values for BCAA substrates were again lower with the native enzyme and lower than reported values for hBCATm (45, 49). In the absence of channeling, \( K_m \) values for hBCATm alone likely reflect the relative activity of the hBCAT proteins in the transamination half-reaction.

Addition of PLP-hBCATm to the BCKD complex enhanced the overall catalytic rate of the complex by 60–80%. For all BCAA substrates, calculated \( K_m \) values are in the physiological range of observed plasma BCAA concentrations (50). Furthermore, calculated \( K_m \) values for valine and the other BCAAs are lower than reported \( K_m \) values for hBCAT-catalyzed transamination (49) and equilibrium \( K_D \) values for the first half-reaction (45). Values for valine range from 4 to 8 mM and are 3–5-fold higher than for isoleucine and leucine. Calculated \( k_{\text{cat}} \) values for valine for overall activity of the BCKD complex are 33–37% higher than for isoleucine and leucine.

As observed with the E1 decarboxylase assay, addition of hBCATc, hBCATm C315A/C318A mutant, and oxidized hBCATm did not affect the calculated \( k_{\text{cat}} \) values for the overall reaction of the BCKD complex (Table 3). Similar results were obtained with the single hBCATm C318A mutant enzyme (data not shown). The results support the hypothesis that the inter-

### TABLE 2
Addition of PLP-form of hBCATm increases the \( k_{\text{cat}} \) of the E1-catalyzed decarboxylation reaction

| Additions | Variable substrates | \( k_{\text{cat}} \) values | \( K_m \) values |
|-----------|---------------------|--------------------------|----------------|
| DCPIP assay |                      |                          |                |
| None      | KIV                 | 12 ± 1                   | 53 ± 4         |
| PLP-hBCATm | Valine              | 71 ± 3                   | 704 ± 22       |
| None      | α-KG                | ND                       | ND             |
| α-KG      | KIV                 | 11 ± 1                   | 51 ± 4         |
| α-KG + PLP-hBCATm | Valine     | 157 ± 6                 | 511 ± 8        |
| PMP-hBCATm | Valine              | ND                       | ND             |
| PMP-hBCATm | α-KG                | ND                       | ND             |
| α-KG + PMP-hBCATm | Valine     | 108 ± 17                | 657 ± 11       |

\( a \) ND indicates not detectable.

\( b \) The concentration of α-KG was fixed at 20 mM.

### TABLE 3
Effect of hBCATs on the kinetics of E1 and BCKD complex catalysis

| Addition | DCPIP assay | Overall assay |
|----------|-------------|--------------|
|         | \( k_{\text{cat}} \) | \( K_m \) | \( k_{\text{cat}} \) | \( K_m \) |
|         | min\(^{-1}\) | μM | min\(^{-1}\) | μM |
| BCKA \( a \) | 7.5 ± 1 | 7.9 ± 1 | 13 ± 1 | 36 ± 3 | 3 ± 5 | 155 ± 10 | 173 ± 13 | 201 ± 16 | 40 ± 5 | 37 ± 4 | 55 ± 3 |
| PLP-hBCATm | 91 ± 3 | 96 ± 3 | 159 ± 5 | 250 ± 10 | 233 ± 12 | 676 ± 20 | 268 ± 7 | 276 ± 10 | 367 ± 12 | 195 ± 18 | 214 ± 10 | 594 ± 12 |
| PMP-hBCATc | 7.7 ± 1 | 9.0 ± 1 | 12 ± 1 | 770 ± 21 | 690 ± 10 | 1486 ± 17 | 150 ± 12 | 165 ± 6 | 190 ± 11 | 625 ± 19 | 600 ± 16 | 1500 ± 26 |
| C315A/C318A | 7.1 ± 1 | 7.6 ± 1 | 12 ± 1 | 1600 ± 12 | 1365 ± 21 | 2510 ± 27 | 135 ± 8 | 149 ± 6 | 187 ± 5 | 926 ± 14 | 1000 ± 15 | 2300 ± 30 |
| hBCATm-Ox | 7.9 ± 1 | 8.1 ± 1 | 10 ± 2 | 12090 ± 18 | 9125 ± 36 | 41600 ± 60 | 129 ± 10 | 136 ± 12 | 180 ± 14 | 9238 ± 90 | 8953 ± 700 | 43000 ± 90 |

\( a \) The kinetic values for corresponding α-keto acids of Leu, KIC, Ile, KMV, Val, and KIV, respectively.
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FIGURE 4. Structural integrity of the phosphorylation loop of E1 is important for the hBCATm-BCKD complex interaction. A, diagram of the phosphorylation loop of the α-subunit of E1 (Tyr-286α to Gln-312α), which is located in the opening to the active site of E1. The ordered conformation of this loop is essential for the proper catalysis of E1. B, ITC titrations were performed using S292Dα E1 with WT-hBCATm. The upper panel represents the raw isotherm data versus time obtained over a series of injections of WT-hBCATm with the S292Dα E1 protein. The lower panel shows the binding isotherm plotted against the molar ratio of WT-hBCATm homodimers to the S292Dα E1 heterotetramer. No detectable binding was observed. C, same as in B, except the data are for the R287Aα E1 and WT-hBCATm. Although some initial interaction was observed, it was unstable. D, summary of the calculated thermodynamic parameters for the interaction of wild-type E1 and mutant E1 enzymes with PLP WT-hBCATm.

TABLE 4
Effect of mutations in the phosphorylation loop of the E1 subunit on BCKD activity with and without hBCATm
All conditions for the assays were same as described in the legend to Table 3. All values are the average of three independent experiments.

| Additions | E1 proteins | hBCATm | Substrate | $k_{\text{cat}}$ | $K_m$ |
|-----------|-------------|--------|-----------|--------------|------|
| Overall assay | WT | -- | KIV | 196 ± 6 | 58 ± 4 |
| | WT | + | Valine | 352 ± 10 | 610 ± 8 |
| | S292Dα | -- | KIV | 43 ± 6 | 4612 ± 27 |
| | S292Dα | + | Valine | 45 ± 6 | 4512 ± 25 |
| | R287Aα | -- | KIV | NDa | ND |
| | R287Aα | + | Valine | ND | ND |
| | D295Aα | -- | KIV | 66 ± 5 | 57 ± 4 |
| | D295Aα | + | Valine | 83 ± 9 | 4130 ± 70 |
| | R301Aα | -- | KIV | 58 ± 8 | 230 ± 8 |
| | R301Aα | + | Valine | 70 ± 6 | 4600 ± 60 |

*a ND indicates not detectable.

action between hBCATm and E1 facilitates substrate channeling between the two enzymes.

Ordered Conformation in the E1 Active Site Is Important for the Interaction with hBCATm—The phosphorylation loop in the E1 active site of the BCKD complex includes residues Y286α to E312α of the α-subunit (Fig. 4A). Residues in this loop play key roles in the regulation of overall BCKD activity by reversible phosphorylation, and the loop is very important for the interaction of E1 with the E2 component of the BCKD complex. Residues of the phosphorylation loop are located at the opening of the active site of E1, and the loop overhangs the active site cofactor ThDP. It harbors two phosphorylation sites (Ser-292α and Ser-302α). The side chains of Arg-287α, Ser-292α, Asp-295α, and Arg-301α form a hydrogen bonding network in the active site of E1 (Fig. 4A). Mutation of these key residues, phosphorylation of Ser-292α, or the absence of cofactor ThDP in E1 all trigger an ordered to disordered transition of the loop and abolish the BCKD overall activity (47). To decipher whether the loop residues of E1 are involved in the interaction with hBCATm, overall activity of the BCKD complex, E1-catalyzed decarboxylation, and ITC titration were measured in presence of WT PLP-hBCATm with a series of well characterized E1 mutants (R287Aα, S292Dα, D295Aα, and R301Aα) (31, 47).

Fig. 4B (upper panel) shows the heat of titration with E1 R297Aα and hBCATm. The unchanged ITC isotherm demonstrates the absence of interactions of E1 with hBCATm (Fig. 4B, lower panel). Fig. 4C is representative of the results with the E1 D295Aα, R301Aα mutants, and phosphorylated E1 (E1 Ser-292α-PO3). These enzymes initially showed a very weak interaction at the start of the titration, but the complexes appeared to fall apart very quickly as the reaction progressed (Fig. 4C).

Mutation at phosphorylation site S292Dα (31) results in a reduced $k_{\text{cat}}$ (18% of control value) and a 90-fold increase in the $K_m$ value for KIV of the reconstituted overall activity relative to the wild-type BCKD complex (47). With this mutant E1, addition of PLP-hBCATm had no effect on the calculated kinetic parameters of the BCKD complex (Table 4) and also did not affect E1 decarboxylase activity (data not shown). On the other hand, the BCKD complex reconstituted with the E1 R287Aα mutant was completely inactive. To understand whether PLP-hBCATm has any effect on the mutant complex, the overall activity was measured in the presence of PLP-hBCATm. The complex did not show any activity (Table 4).

In contrast, addition of hBCATm to the BCKD complex reconstituted with D295Aα E1 or R301Aα E1 showed a small, but not statistically significant, increase in $k_{\text{cat}}$. The $K_m$ value for valine with D295Aα is 6.8-fold higher than the $K_m$ value for valine with wild-type E1, and with the E1 R301Aα mutant it is 7.5-fold higher than with wild-type E1. These $K_m$ values are similar to the equilibrium $K_D$ values reported for the first half-reaction (45) and overall transamination reaction (49) and are
consistent with a lack of interaction between R301A E1 and hBCATm that was observed in the ITC experiments.

DISCUSSION

This is the first study showing that enzymes catalyzing the first two common steps in the BCAA catabolic pathway, mitochondrial BCATm and the BCKD enzyme complex (through the E1 component), can associate to form a supramolecular complex or metabolon (2) (see Fig. 4). The dissociation constant for hBCATm and E1 binding is in the micromolar range, which is indicative of a weak interaction, permitting reversible assembly/dissociation of the BCAA metabolon. The physical data (hBCATm affinity column and ITC results) show that only the PLP form of WT-hBCATm binds to the E1 component of the complex (Fig. 5). The kinetic results provide evidence that this association facilitates substrate channeling between hBCATm and E1, which increases the rate of E1-catalyzed decarboxylation as well as the overall activity of the reconstituted BCKD complex. Furthermore, results from the experiments using hBCATm and E1 mutant enzymes indicate that the kinetic effects (increase in $k_{cat}$) require a specific physical association between hBCATm and E1 and provide insight into the structural requirements for complex formation. The physical association between PLP-hBCATm and E1 serves to channel the BCKA product from the active site of hBCATm to the active site of E1 and augments the $k_{cat}$ for E1 BCKA decarboxylation (Reaction 4) —12-fold (Table 2). The smaller but significant increase in $k_{cat}$ (60–80%) for the overall reaction of the BCKD complex in the presence of PLP-hBCATm and BCAAs suggests that that the E1-catalyzed decarboxylation step (Reaction 4) is not rate-limiting in the overall reaction of the BCKD complex. Rather the results support the hypothesis that E1 catalyzed reductive acylation (Reaction 5) is rate-limiting, thus accounting for the smaller increase in overall activity in the presence of hBCATm (51, 52).

The formation of a channel between hBCATm and E1 is likely facilitated by the open conformation of both enzymes during catalysis. Mitochondrial hBCATm is a homodimeric protein containing two active sites (45). The active sites of hBCATm are located at the domain interface of each subunit. The PLP cofactor resides at the bottom of the active site. Hydrophobic residues from three hydrophobic pockets and the phosphate group of the cofactor lock the BCAA substrates in the substrate binding pocket for the transamination reaction. Unlike a number of other PLP enzymes (53), the active site of hBCATm remains open during substrate binding and catalysis (6). The residues from the second monomer interact only with the substrate and not with the PLP cofactor, and domain closure, which would block the active site, does not occur. The E1 heterotetramer also harbors two active sites, each containing a ThDP cofactor and consisting of residues from both the $\alpha$- and $\beta$-subunits (47, 54). The cofactor ThDP is located 15 Å inside from the accessible surface area of E1. The $\alpha$-subunit phosphorylation loop and hydrophobic pocket provided by the $\beta$-subunit form the active site channel. The interactions of Ser-292 with water molecules and His-291 and Arg-287 with oxygens of ThDP are important for the proper conformation of the active site channel of E1. His-146 binds the BCKA substrate through an electrostatic interaction and positions the substrate for proper nucleophilic interaction by ThDP. The E1 active site also remains open during catalysis. Several other structural features appear to influence the binding of hBCATm and E1, the structure of the CXXC center in hBCATm, and the structure of the E1 phosphorylation loop. The CXXC center is located at the opening of the active site and close to the accessible surface area of hBCATm and in the substrate channel. Oxidation of this center forms a disulfide linkage, and the protein partially loses its activity in the first half-reaction and is completely inactive in the second half-reaction (45). The results show that the reduced state of the CXXC cysteines and the net dipole charge in this center are vital for complex formation with the E1 subunit. Either oxidation or mutation of the CXXC cysteines increases the net dipole moment of the $\beta$-turn and $\beta$-sheet residues. The strong $\beta$-turn dipole destabilizes substrate as well as the cofactor conformation in the active site of oxidized hBCATm. Thus these changes in the CXXC center likely destabilize the net charges of the cofactor in the active site and subsequently interfere with the interaction with the E1 component. The form of the cofactor, PLP versus PMP, also affects the binding of E1 and hBCATm. In the PMP form of the enzyme, the cofactor is not covalently attached to Lys-202, and the free amino group of PMP has a strong positive charge. The net positive charge of the active site of PMP-hBCATm may effectively repulse with the positively charged phosphorylation loop of E1 resulting in dissociation. Thus, the integrity of this loop is also required for binding and catalysis (Table 4 and Fig. 4).

The ITC results suggest that one E1 heterotetramer of the BCKD complex associates with a single hBCATm homodimer. Based on this binding stoichiometry, each of the two E1 active sites could pair up with each of the two active sites in PLP-hBCATm to promote BCKA channeling. This mode of interaction would be facilitated by a symmetric model for E1, in which
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both active sites are equally functional and independent of each other. In support of the symmetric model, we have recently obtained evidence that both active sites in the E1 component of the human BCKD complex can operate independently without communication to the other active site (70). Each of the 12 copies of the E1 component is anchored to the individual E1/E3 binding domain of the E2 subunit that assembles into the 24-meric core scaffold of the BCKD complex. Judging from the dynamics of the flexible linker regions connecting the peripheral domains of E2 (32), we speculate that the PLP-hBCATm homodimer would have relative freedom in accessing both of the E1 active sites. Upon the conversion of a BCAA to the cognate BCKA by PLP-hBCATm, the BCKA is taken up by the conjugated E1 active site with a concomitant release of the resulting PMP-hBCAT (Fig. 5). This is followed by E1-catalyzed decarboxylation (Reaction 4) and reductive acylation (Reaction 5), the latter step being mediated by LBD binding to the same E1 active site channel. In variance with the above symmetric model, nonchemical equivalence and therefore the alternating site mechanism have recently been shown to be present in the two active sites of the related E1 heterotetramers from human and Bacillus stearothermophilus pyruvate dehydrogenase complexes (56, 57). If the symmetric model holds, these findings would suggest fundamental structural and mechanistic differences between E1 components of the α-ketoacid dehydrogenase complexes.

The apparent substrate channeling also influenced relative $k_{cat}$ and $K_m$ values for BCAA substrates when compared with hBCATm kinetic parameters for the first half-reaction in the absence of E1. Reported half-reaction equilibrium dissociation constant $K_D$ values for leucine, isoleucine, and valine are 1.6, 1.3, and 7.8 mM, and $k_{cat}$ values are 337, 331, and 290 s$^{-1}$, respectively (45). Reported $K_m$ values for hBCATm-catalyzed transamination (38) are similar to the calculated $K_D$ values for the first half-reaction. In the presence of E1, the $k_{cat}$ value for valine catalysis is faster than for leucine or isoleucine. All $K_m$ values for BCAA substrates in the BCKD-catalyzed reactions are lower than for BCATm-catalyzed transamination (Table 3), which may result from conformational changes in hBCATm and/or E1 that affect catalytic activity. If this occurs in vivo, then valine oxidation would be expected to be as rapid as isoleucine or leucine oxidation and suggests that valine is at least equivalent to isoleucine and leucine and may be the favored substrate for oxidative decarboxylation by the BCKD complex (Table 3). In addition, the BCAA metabolon may coordinate control the rate of transamination and BCKA release.

The BCAAs have unique roles in addition to their role as protein precursors. In peripheral tissues such as skeletal muscle, BCAAs are nitrogen donors (58–62) for the synthesis of alanine and glutamine thus moving nitrogen from muscle amino acid oxidation to the liver for urea synthesis. In addition, the BCAA leucine acts as an anabolic nutrient signal influencing both insulin secretion by the β-cells of the pancreas (63–65) and protein synthesis in skeletal muscle and selected other tissues (66–69).

Biological signals that affect metabolon formation permit integration of the metabolic needs of the cell with the partitioning of BCAA into the catabolic pathway. Human BCATm did not bind to phosphorylated E1 (Ser-292-P0₄) or the phosphorylation loop mutant E1 (S292D). Phosphorylation of E1 renders the complex inactive, and the aspartate mutant has impaired activity. So when E1 is inactivated by phosphorylation, BCAA transamination results in BCKA release as is observed in skeletal muscle where kinase concentrations are high and E1 is heavily phosphorylated. Similarly, in the presence of elevated NADH/NAD$^+$ ratios, the BCAA metabolon will not form and BCAA recycling or release is favored. What remains to be determined is whether BCATm affects the phosphorylation state or the rate of phosphorylation. Similarly, formation of the BCAA metabolon in tissues where leucine acts as a nutrient signal may also serve to turn off or regulate the leucine signal. Unpublished data from the BCATm knock-out mouse suggest that BCATm plays a role in leucine signaling. In conclusion, control of the metabolic flux through the degradative pathway for most indispensable amino acids usually occurs at the first step in the catabolic pathway. The BCAAs are unique in that the first step in their catabolic pathway is reversible transamination. Formation of a BCAA metabolon provides a mechanism for coordinate control of the initial step and the flux-generating step in BCAA oxidation. In addition, the tissue-specific levels of the BCKD kinase and phosphorylation state of the BCKD complex may along with metabolon formation regulate BCAA carbon loss and the site and direction of amination/deamination in accordance with individual tissue and whole body needs. This may also be important for regulating the amount of leucine in a tissue thereby providing a mechanism to regulate leucine signaling. It is clear from the BCKD kinase knock-out mouse (55) that unregulated BCAA metabolism has serious metabolic consequences. Studies aimed at identifying other interacting proteins and their role in the BCAA metabolon are in progress.

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