Overexpression and Characterization of the Human Mitochondrial and Cytosolic Branched-chain Aminotransferases*

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We have developed overexpression systems for the human branched-chain aminotransferase isoenzymes. The enzymes function as dimers and have substrate specificity comparable with the rat enzymes. The human cytosolic enzyme appears to turn over 2–5 times faster than the mitochondrial enzyme, and there may be anion and cation effects on the kinetics of both enzymes.

The two proteins demonstrate similar absorption profiles, and the far UV circular dichroism spectra show that no global structural changes occur when the proteins are converted from the pyridoxal to pyridoxamine form. On the other hand, the near UV circular dichroism spectra suggest differences in the local environment surrounding tyrosines within these proteins. Both enzymes require a reducing environment for maximal activity, but the mitochondrial enzyme can be inhibited by nickel ions in the presence of reducing agents, while the cytosolic enzyme is unaffected.

Chemical denaturation profiles of the proteins show that there are differences in structural stability. Titration of -SH groups with 5,5'-dithiobis(2-nitrobenzoic acid) suggests that no disulfide bonds are present in the mitochondrial enzyme and that at least two disulfide bonds are present in the cytosolic enzyme. Two -SH groups are titrated in the native form of the mitochondrial enzyme, leading to complete inhibition of activity, while only one -SH group is titrated in the cytosolic enzyme with no effect on activity. Although these proteins share 58% identity in primary amino acid sequence, the local environment surrounding the active site appears unique for each isoenzyme.

Branched-chain aminotransferases (BCAT)1 catalyze reversible transmission of the short chain aliphatic branched-chain amino acids, leucine, isoleucine, and valine, to their respective α-keto acids. Although transamination of branched-chain amino acids in animals and microorganisms was observed in the 1950s (for a review, see Ref. 1), it was not until 1966 that Ichihara and Koyama (2) and Taylor and Jenkins (3) each reported independently that these reactions were catalyzed by a single enzyme.

L-Leucine + α-ketoglutarate ⇌ α-Ketoisocaproate + L-glutamate
L-Valine + α-ketoglutarate ⇌ α-Ketoisovalerate + L-glutamate
L-Isoleucine + α-ketoglutarate ⇌ α-Keto-β-methylvalerate + L-glutamate

REACTIONS 1–3

Bacteria generally contain a single BCAT (4–6), but in mammals it has been established that there are two BCAT isoenzymes, a mitochondrial (BCATm) and a cytosolic (BCATc) form (7). In humans and rodents, BCATm is found in most tissues (1, 8). In contrast, BCATc is found almost exclusively in the brain (1, 9). Each rat isoenzyme has been purified (9, 10), and the cDNA sequences of the rat (11, 12) as well as the human (12, 13) BCATc and BCATm are now available. Recently, it has been reported that the mammalian BCATc gene may be regulated by the c-myc oncogene product (14) and that the BCAT may be a target for the anticonvulsant drug and nonmetabolizable leucine analog, gabapentin (15). Furthermore, in yeast, deletion of both BCAT genes results in severe growth retardation even after supplementation with branched-chain amino acids, suggesting that these enzymes perform an essential function in the cell (16). Nevertheless, the functional significance and structure of the individual eukaryotic BCAT isoenzymes is not yet known.

Another feature of the BCAT is their evolutionary relationship to the bacterial enzyme p-amino acid aminotransferase (17). Based on primary sequence comparisons, the BCAT and p-amino acid aminotransferase, which have opposite stereospecificity (L- versus p-amino acids), and another bacterial enzyme, 4-amino-4-deoxychorismate lyase, were placed in a separate folding class (fold type IV) (11, 12, 18). With a few exceptions, other known aminotransferases fall within the fold type I or L-aspartate aminotransferase superfamily. A unique feature of the fold type IV PLP-dependent enzymes is that the proton is added to or abstracted from the C4 face of the PLP cofactor (19). The crystal structure of the PMP form of p-amino acid aminotransferase has been solved by Sugio et al. (20), and the structure of the Escherichia coli BCAT in the pyridoxal form has just been reported by Okada et al. (21). The folding pattern of both enzymes is not only different from that of other known PLP-dependent enzymes but also from that of other known proteins

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∥ Other abbreviations used are: BCAT, branched-chain aminotransferase(s); BCATm, mitochondrial branched-chain aminotransferase; BCATc, cytosolic branched-chain aminotransferase; PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); KIC, α-ketoisocaproate; KIV, α-ketoisovalerate; DT, diithiothreitol; NTA, nitrolotriacetic acid; PAGE, polyacrylamide gel electrophoresis.

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The phBCATc2 plasmid was engineered by ligating the full-length brain cDNA was used in the polymerase chain reaction, and the amplification was carried out with a JASCO J-720 spectropolarimeter equipped with a variable temperature accessory. The enzymes were converted to the PLP and PMP forms by incubation with the appropriate substrate followed by dialysis. The instrument was calibrated with ammonium-cyanide/thiourea (28).

Spectrophotometric Measurements—Absorption spectra were taken with a Beckman DU 640 spectrophotometer. CD measurements were carried out with a JASCO J-720 spectropolarimeter equipped with a variable temperature accessory. The enzymes were converted to the PLP and PMP forms by incubation with the appropriate substrate followed by dialysis. The instrument was calibrated with ammonium-cyanide/thiourea (28).

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scribed by Williamson and Corkey (26). Briefly, excess α-ketoglutarate was removed from the neutralized sample by the addition of either 10 mM (BCATm) or 15 mM (BCATc) H₂O₂. Samples were incubated at room temperature for 5–15 min before an aliquot (250 µl) was added to 1 ml of assay buffer. NADH fluorescence was determined with an excitation wavelength of 340 nm using a FOCI Mark I spectrofluorimeter (Far- rand Optical Co., New York). The kinetic parameters of the amino acid reactions were determined by holding the concentration of α-ketoglutarate constant at 5 mM for BCATm and 10 mM for BCATc. For the α-keto acid/glutamate pairs, reaction rates were determined from the formation of 1–5°C-labeled branched-chain amino acid as described previously (7, 10). The glutamate concentration was kept constant at 100 mM as the high salt concentration affected the kinetic parameters. The data were fit to the following velocity equation.

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} \]  

(Eq. 1)

The \( K_m \) of α-ketoglutarate or glutamate was first estimated by varying the concentration of each branched-chain amino acid or α-keto acid at approximately 5 times \( K_m \). For each substrate pair, data were collected from six or seven concentrations of amino acid or α-keto acid. Kinetic parameters are the means and standard errors of 3–5 separate determinations from two different BCATm and BCATc preparations. In separate experiments, the apparent \( K_m \) for α-ketoglutarate and glutamate was determined by varying the concentrations of both the amino acid and α-keto acid substrates simultaneously. The data were fit to the initial velocity rate equation pertaining to a ping-pong kinetic mechanism (27) via nonlinear regression in the approach outlined by Cleland (28).

\[ v = \frac{V_{\text{max}}[A][B]}{K_m[A] + [A][B]} \]  

(Eq. 2)

In this equation, the parameters \( K_m \) and \( K_m \) represent the Michaelis constants for substrates A (amino acid) and B (α-ketoacid), respectively. Analytical Ultracentrifugation—The equilibrium sedimentation experiments were performed in the Analytical Ultracentrifugation Facility at Wake Forest University. Using a Beckman Optima XL-A analytical ultracentrifuge, BCATm and BCATc in 25 mM Tris (pH 7.5), 150 mM KCN were centrifuged at 7500, 9000, 11,000, 14,000, and 40,000 rpm. Data were gathered after 12 and 14 h at each speed. The data were examined to verify that equilibrium was established. Partial specific volumes were calculated based on the amino acid composition of each protein as described by Laue et al. (29). Data analysis was performed using Origin software provided by Beckman.

Miscellaneous Procedures—SDS-PAGE was carried out according to Laemmli (30) in 10% gels as described in Wallin et al. (10). RESULTS AND DISCUSSION Expression and Purification—The human BCATm and BCATc have been cloned (12, 13). Mature BCATm consists of 365 amino acids with a calculated molecular mass of 41,300 daltons, while BCATc consists of 385 amino acids with a calculated molecular mass of 42,800 daltons. A comparison of the amino acid sequences are found in the N-terminal portion (BCATm) identical to their respective rat enzymes. Identity is calculated molecular mass of 42,800 daltons. A comparison of the amino acid sequences are found in the N-terminal portion (BCATc, residues 1–50) of the proteins.

In initial attempts to overexpress BCATm in a variety of expression plasmids, low expression, inactive, or insoluble protein was observed. The BCATm cDNA was then cloned into the pET-28a vector, which introduced a six-histidine residue tag onto the N terminus of the protein. High levels of expression of active protein were obtained, but the majority of the protein was still found in inclusion bodies. In addition, the soluble protein would not bind to the nickel-NTA resin. This suggested that the N terminus of the BCATm fusion protein was substantially what buried, making the histidine tag inaccessible to the resin. Upon complete denaturation in 8 M urea, BCATm bound to the resin; however, active enzyme could not be recovered either by gradual refolding of the protein while it remained on the resin or by dialysis versus urea-free buffer. In 4 M urea, the tertiary or quaternary structure of the protein was altered enough to permit binding of the N-terminal histidine tag to the resin. The far UV CD spectrum of BCATm (data not shown) showed no detectable change upon increasing urea concentrations from 0 to 4 M, thus indicating that the secondary structure of BCATm remained intact. In 4 M urea, the specific activity of recombinant protein. Specific activity of purified BCATm after thrombin cleavage was 88 ± 6 units/mg of protein (n = 3). The N-terminal sequence of the recombinant protein carries the additional amino acids gshm (these additional residues are listed throughout in lowercase italic type) as verified by amino acid sequencing (see Fig. 1). SDS-PAGE of the purified recombinant BCATm protein is shown in Fig. 2A.

For BCATc, initial attempts with expression plasmids driven by the highly processive T7 RNA polymerase were problematic, because the protein was found primarily in inclusion bodies. With the lower level of expression found with the pTrcHis construct, the solubility of the recombinant protein was increased. Recently, we have expressed BCATc using the pET-28a vector/T7 expression system and removed the histidine tag. Although some protein was still found in inclusion bodies, the high yield in the pET vector/T7 system permitted purification of sufficient protein for kinetic and structural analysis. SDS-PAGE of the purified recombinant BCATc proteins is shown in Fig. 2, B and C. Unlike BCATm, recombinant BCATc readily bound to the nickel-NTA resin under native conditions, indicating that the N terminus of BCATc was exposed to the solvent, and urea addition (4 M) did not improve recovery. Thus, the behavior of the recombinant proteins on the nickel-NTA resin suggests that in the N-terminal portion of the proteins, the tertiary structure of BCATm and BCATc is different. Specific activity of BCATc, with or without the histidine tag, was 124 ± 9 units/mg of protein (n = 5). Recombinant BCATc
280 nm were determined for each protein. Values were 67,600
BCATc at 280 nm probably reflects the higher tyrosine and
zyme are essentially superimposable. The higher absorption of
respectively. In the visible range, the spectra for each isoen-
and 326 nm, indicating the pyridoxal and pyridoxamine forms,
peaks characteristic of bound cofactor are observed at 416
mM) were in 10 mM phosphate buffer, pH 7.5. Solid line, BCATc PLP
form; dashed line, BCATc PMP form; dotted line, BCATm PLP form;
dash-dot-dash line, BCATm PMP form.

Fig. 3. UV-visible spectra of human BCATc and BCATm in the
PLP and PMP forms. The proteins were converted to the PLP and
PMP forms as described under “Materials and Methods.” Proteins (0.01
nm) were in 10 mM phosphate buffer, pH 7.5. Solid line, BCATc PLP
form; dashed line, BCATc PMP form; dotted line, BCATm PLP form;
dash-dot-dash line, BCATm PMP form.

Fig. 2. SDS-PAGE of purified human BCATm and BCATc. 3–5
µg of protein were loaded onto the gels, and molecular mass standards
are indicated. Panel A shows BCATm after cleavage of the histidine tag
by thrombin (41.7 kDa). Panel B shows BCATc expressed using the
pTrcHis vector (44.3 kDa), and panel C shows BCATc expressed using
the pET-28a vector following removal of the histidine tag (43.4 kDa).

eXpressed using the pTrcHis construct (phBCATc) contained
an additional 14 amino acid residues (mgggshhhhhgmas) at the N
terminus of the recombinant protein that were contrib-
uted by the vector sequence. The protein expressed using the
pET-28a vector construct (phBCATc2) contained six additional
amino acids after thrombin cleavage. Both nucleotide sequenc-
ing of the phBCATc2 construct and N-terminal amino acid
sequences of the recombinant protein showed that the N-
terminal extension was gshmac (see Fig. 1). The N-terminal
sequence of the recombinant protein with the histidine tag
(phBCATc) was also confirmed by direct amino acid sequence
analysis. Preliminary experiments showed that the physical and
kinetic properties of the two recombinant enzymes were similar,
and both enzymes were used in subsequent experiments.

Sedimentation Equilibrium—Upon size exclusion chroma-
tography, purified rat BCATm behaved anomalously and cha-
pared to be a monomer, while rat BCATc was clearly a ho-
omodimer (9, 10). To determine unequivocally the subunit
composition of the human isoenzymes, equilibrium sedimenta-
tion experiments were performed with the recombinant pro-
teins. After the data were subjected to curve fitting analysis,
BCATm and BCATc showed average molecular weights of
81,500 ± 2510 and 83,900 ± 4800, respectively. These values
are approximately twice the calculated monomer molecular
weights of the recombinant proteins after thrombin cleavage,
41,700 (BCATm) and 43,400 (BCATc), identifying both en-
zymes as dimers in solution.

Spectral Analysis of Recombinant Human BCAT Isoen-
zymes—The absorption spectra of the recombinant enzymes at
pH 7.5 are shown in Fig. 3. In addition to the peak at 280 nm,
two peaks characteristic of bound cofactor are observed at 416
and 326 nm, indicating the pyridoxal and pyridoxamine forms,
respectively. In the visible range, the spectra for each isoen-
zyme are essentially superimposable. The higher absorption of
BCATc at 280 nm probably reflects the higher tyrosine and
tryptophan content of this protein. Extinction coefficients at
280 nm were determined for each protein. Values were 67,600
M⁻¹ cm⁻¹ per BCATm monomer and 86,300 M⁻¹ cm⁻¹ and
80,000 M⁻¹ cm⁻¹ per BCATc monomer, without and with the
histidine tag, respectively.

Fig. 4 shows the far and near UV CD spectra of the proteins.
The far UV CD spectra of both BCATc and BCATm in their
PLP and PMP forms have similar features with minima at 219
and 210 nm and a maximum at 193 nm (see Fig. 4A). α-Helical
structure is normally characterized by the presence of two
minima at 222 and 208–210 nm and a maximum at 191–193
nm (31); therefore, α-helices are present in both of these pro-
teins. β-Sheets are also present because a minimum occurs at
219 nm, which is close to the minimum (216–218 nm) normally
observed for β-forms (31). The similarity of the far UV CD
spectra of the two proteins indicates that their secondary struc-
ture content is similar. In addition, secondary structure esti-
mates calculated using the far UV CD spectra of the PLP form
of the enzymes suggested an α-helix content of 38% for BCATm
and 35% for BCATc. Predicted β-sheet was on the order of
20–24%. As shown in Fig. 4, no major change occurred in the
global structure of the proteins upon conversion of the PLP
form to the PMP form, and predicted α-helix content was simi-
lar for the PMP forms (33% for BCATm and 36% for BCATc).
The crystal structure of the PLP form of the smaller E. coli
BCAT (34 kDa) revealed a secondary structure content of 39%
α-helix and 41% β-sheet (21). The similarity of the predicted
α-helix content in the human BCAT proteins and observed
α-helix content in the bacterial enzyme structure is also con-
sistent with the hypothesis that all of these forms of the en-
zymes have the same basic folded structure.

The near UV CD spectra of the PLP form of both BCATm
and BCATc were dominated by a band at 421 nm (see Fig. 4B).
This peak resulted from the presence of the pyridoxal group in an
asymmetric environment. Despite the similarity of this peak,
there was a major difference in the spectrum of BCATc com-
pared with BCATm around 280 nm. While BCATc showed a
peak at 285 nm with a positive ellipticity, BCATm showed a
peak at 270 nm with a negative value. Upon conversion of the
PLP form to the PMP form, the peak at 421 nm disappeared for
both proteins. For BCATm, this was concomitant with the
appearance of a weaker band at 326 nm as well as a major
increase in the intensity of the band at 277 nm. When the PLP
form of BCATc was converted to the PMP form, the positive
ellipticity of the peak at 285 nm was changed to a negative
value. Since the far UV CD spectra of the PLP form of BCATm
and BCATc were very similar to their respective PMP forms
(see Fig. 4A), the conversion of PLP to PMP did not seem to
affect the global structure of the proteins. Consequently, the
changes observed around 280 nm could be assigned to an al-
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FIG. 4. Circular dichroism spectra of BCATc and BCATm in the PLP and PMP forms. Spectra from the far UV range are shown in panel A, and spectra from the near UV range are shown in panel B. Conditions were as described under “Materials and Methods.” Lines used are the same as in Fig. 3.

The activity of rat BCATm is known to be inhibited by sulfhydryl reagents (9), so the effect of DTNB on activity of the human enzymes was investigated. Spectrophotometric titration of BCATm with a 100-fold molar excess of DTNB occurred rapidly, and human BCATm activity was inhibited 90–100% after titration of both accessible -SH groups. By reducing the ratio of DTNB to enzyme to 5, the reaction could be slowed such that essentially one -SH group was titrated after 6 min. To determine the effect of this titration on BCATm activity, the protein was incubated with DTNB for 6 min in assay buffer without DTT. Then substrate was added, and activity was

 Lane A shows that a more compact structure gradually forms upon storage without a reducing agent. As shown in lane B, the addition of DTT prior to loading the sample on the gel led to the disappearance of the lower band; thus, this faster migrating band results from the formation of a disulfide bond. This is consistent with the observation that the number of titratable cysteine decreases over time in the absence of DTT.

The cytosolic isoenzyme contains 10 cysteines/monomer, yet only one -SH group was titrated in the native form of the histidine-tagged BCATc (see Table I). The phBCATc2 construct added an extra cysteine residue immediately preceding the N-terminal methionine (see Fig. 1). An additional -SH group was titrated in the native form after removal of the histidine tag, suggesting that this cysteine was accessible to DTNB. In the denatured form, 4.6 (histidine-tagged) and 6.6 (without histidine tag) -SH groups were seen, indicating that at least two disulfide bonds are present in BCATc. This conclusion is supported by the electrophoretic movement of the protein (see Fig. 5, lanes C and D). In the absence of DTT, BCATc migrated faster than when DTT was present. This increased mobility under nonreducing conditions suggests that the enzyme maintains a more compact structure due to the presence of disulfide bonds (33).

The extra -SH group titrated in this protein comes from phBCATc2 sequence that remains on the protein following removal of the histidine tag (see Fig. 1).

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DTNB Titration—Like their rat counterparts (9), human BCATm, unlike human BCATc, must be stored in a reducing environment, and all four enzymes require the addition of DTT to the assay for maximal activity, whereas the bacterial protein from Salmonella typhimurium, which contains three cysteine residues, is not sensitive to sulfhydryl reagents (32). The requirement for the presence of a reducing agent indicates a unique property of the mammalian aminotransferases, i.e. a relationship between the state of reduction of the cysteines in the proteins and activity of the enzymes. Therefore, to evaluate whether modification of sulfhydryl groups within these proteins altered their catalytic capacity, DTNB titrations were performed on both human enzymes.

Based on the primary sequence, BCATm contains six cysteine residues/monomer (see Fig. 1). As shown in Table I, two -SH groups/monomer were titrated with native enzyme. Upon denaturation, 5.7 -SH groups were titrated, indicating that no disulfide bonds were present. However, upon storage of BCATm for a few days in the absence of DTT, the number of titratable -SH groups decreased to 3.7, suggesting that a disulfide bond was gradually being formed. This process was accompanied by a decrease in the specific activity of the enzyme. Fig. 5 shows an SDS-PAGE of BCATm stored in the absence of DTT.

Table I

| Protein | Native | Denatured | Total cysteines within protein |
|---------|--------|-----------|-------------------------------|
| BCATm   | 2      | 5.7       | 6                             |
| BCATc   | With His tag (phBCATc) | 1 | 4.6 | 10 |
|         | Without His tag (phBCATc2) | 2.1 | 6.6 | 11 |

*The extra -SH group titrated in this protein comes from phBCATc sequence that remains on the protein following removal of the histidine tag (see Fig. 1).
followed for 20 min. Without DTT, the enzyme does not have maximal activity, so a sample under identical assay conditions without DTNB was used as a control. As shown in Fig. 6, BCATm activity was decreased 40% after incubation with DTNB. Therefore, it appears that labeling of one -SH group by DTNB is not enough to fully inactivate BCATm. When substrate was added along with DTNB in the spectrophotometric assay, no -SH groups were titrated. Further, after incubation with DTNB in the presence of substrate, BCATm activity was unaltered (see Fig. 6), indicating that substrate protects against labeling and inhibition. Therefore, the modified -SH group may lie in or near the active site of BCATm, and studies are now in progress to verify its location. As shown in Fig. 6, titration of the single accessible -SH group in the histidine-tagged BCATc did not affect enzyme activity. Similar results were obtained with BCATc without the histidine tag (data not shown). This suggests that the modified residue is away from the active site. Since it is known that the catalytic mechanism of transamination does not require a cysteine residue, one could speculate that the loss of activity seen with BCATm is due to steric hindrance from the introduction of the bulky DTNB molecule in the active site. Another possibility is that this effect results from a conformational change in the enzyme. Possible candidate cysteines in BCATm are the two cysteine residues that are not conserved in the BCATc sequence (Cys108 and Cys109, see Fig. 1). Ultimately, however, understanding the effects of sulfhydryl reagents on catalytic function and protein stability (see below) will require isolation of the derivatized peptides and knowledge of the crystal structure of both proteins.

The Effect of Ni^{2+} Cations on the Activity of BCATc and BCATm—Affinity chromatography using nickel-NTA resin is based on the interaction of the histidine tag in the protein with nickel cations chelated to the resin. After elution from this column, human BCATm had low specific activity, which did not increase even after the second purification step of hydrophobic interaction chromatography. However, when EDTA was added to the assay, the specific activity increased to about 75 μmol/min/mg of protein or higher. Further, the addition of Ni^{2+} cations to the assay led to 67% inactivation of the purified enzyme, while subsequent addition of EDTA restored activity completely. On the other hand, when DTT or β-mercaptoethanol was omitted from the assay, added Ni^{2+} had no inhibitory effect on BCATm activity. One possible explanation for these results is that one or more disulfide bonds are reduced by either DTT or β-mercaptoethanol, allowing interaction of Ni^{2+} with cysteine residues within the active site. This seems unlikely, because in freshly purified BCATm, titration with DTNB suggests that no disulfide bonds are present. Alternatively, one can hypothesize that empty coordinates of Ni^{2+} cations bound in or near the active site of BCATm interact with the thiol groups of DTT or β-mercaptoethanol, leading to either steric hindrance or a conformational change.

Chemical Denaturation of BCATc and BCATm—Since BCATc contains two or three disulfide bonds, one expects to see a higher stability for this protein compared with BCATm; therefore, chemical denaturation experiments were performed to compare the two proteins. Fig. 7 shows the urea denaturation of BCATc and BCATm as monitored by CD spectroscopy. Human BCATm remains partially folded up to a urea concentration of 8 M, indicating high structural stability. On the other hand, the denaturation curve of BCATc appeared as a relatively broad transition with a midpoint of 4.1 M urea. Surprisingly, this suggests that the structural stability of BCATc is lower than that of BCATm. It is generally accepted that disulfide bonds can make a substantial contribution to the stability of proteins (33); however, for BCATc, it seems that this is not the case. Moreover, the broadness of the transition indicates that unfolding of BCATc is less cooperative than BCATm. Since chemical denaturation using urea or guanidine hydrochloride is irreversible, the free energy of denaturation could not be calculated. Nonetheless, the data suggest that there are differences in the stability of the secondary structure of the two proteins.

Kinetic Parameters—The steady state kinetics of BCATm and BCATc were examined, and the calculated Michaelis-Menten parameters are summarized in Table II. Transamination of branched-chain amino acids with α-ketoglutarate exhibited kinetics similar to what has been reported for the rat enzymes. Values calculated for the $K_m$ for leucine and isoleucine were around 1.0 mM or less with a significantly higher $K_m$ observed with valine. Differences between the two isoenzymes were also
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Kinetic constants for the reactions of human branched-chain aminotransferase isoenzymes

Branched-chain aminotransferase activity was measured as described under “Materials and Methods.” The fixed substrates were BCAA and KIC. Glutamate was fixed at 100 mM. All other substrates were varied as described under “Materials and Methods.” The data were analyzed with the Michaelis-Menten equation. Means and S.E. values are from 3–5 separate determinations.

| Variable substrate | Fixed substrate | BCATm | BCATc |
|--------------------|-----------------|-------|-------|
|                    |                 | $k_{cat}$ | $K_m$ | $V_{max}$ | $k_{cat}/K_m$ | $k_{cat}$ | $K_m$ | $V_{max}$ | $k_{cat}/K_m$ |
| BCAA               |                 | [s$^{-1}$] | [mM] | [$\times 10^3$ $\mu$mol s$^{-1}$] | [s$^{-1}$] | [mM] | [$\times 10^3$ $\mu$mol s$^{-1}$] |
| Leu                | α-KG            | 105 ± 10 | 1.21 ± 0.12 | 88 | 132 ± 7 | 0.60 ± 0.04 | 220 |
| Ile                | α-KG            | 80 ± 1   | 0.60 ± 0.02 | 165 | 172 ± 9 | 0.77 ± 0.02 | 223 |
| Val                |                 | 68 ± 2   | 6.1 ± 0.11  | 11 | 122 ± 8 | 2.40 ± 0.09 | 51  |
| BCKA               |                 | 147 ± 4  | 0.50 ± 0.05  | 852 | 221 ± 3 | 0.15 ± 0.003 | 1473 |
|                    | Glu             | 244 ± 1  | 0.16 ± 0.004 | 1525 | 309 ± 11 | 0.063 ± 0.002 | 4905 |
| KIC                |                 | 476 ± 29 | 0.12 ± 0.006 | 3967 | 361 ± 5 | 0.057 ± 0.002 | 6684 |
| Glu                |                 | 426 ± 34 | 0.50 ± 0.05  | 852 | 482 ± 31 | 0.12 ± 0.005 | 4017 |
|                    | Glu             | 186 ± 9  | 0.20 ± 0.01  | 930 | 217 ± 4 | 0.07 ± 0.004 | 3100 |
|                    |                 | 121 ± 8  | 0.42 ± 0.01  | 288 | 295 ± 6 | 0.18 ± 0.01  | 1639 |

a Branched-chain amino acids.
b α-KG, α-ketoglutarate.
c Branched-chain α-keto acids.
d Assays were performed in the presence of 150 mM KCl.

The possibility that the observed differences in kinetic parameters with KIV/Glu and KIC/Ile were a result of differences in intrinsic strength was investigated by adding KCl to the α-ketoacid/Ile assay buffer (see Table II). The addition of 150 mM KCl raised the apparent $K_m$ values for KIC and KIV 2-fold or more. Smaller effects were observed on $k_{cat}$, resulting in a decrease in $k_{cat}/K_m$ for both isoenzymes. The effect of added KCl on branched-chain amino acid deamination was also examined in a single set of experiments. Increases of 40–50% in the $K_m$ values for Leu and Ile were found with BCATm. Both $K_m$ and $k_{cat}$ were increased with BCATc (data not shown). While the molecular basis for the effects of KCl on the kinetic parameters of the BCAT isoenzymes is not yet understood, there is precedence for both cation and anion effects on PLP-dependent enzymes (35–39). The structural basis for the cation stimulation of tryptophan synthase (35), dialkylglycine decarboxylase (36), tryptophanase (37), and tyrosine phenol-lyase (38) have been investigated using x-ray crystallography, and cation binding sites have been defined. In both tryptophanase (34, 35) and tyrosine phenol-lyase (36) from E. coli, cations like K$^+$ were shown to induce and stabilize active conformations of these enzymes. With aspartate aminotransferase, at alkaline pH, it was shown that chloride anions (38) as well as dicarboxylic acids (34) can act as competitive inhibitors, possibly by ion pairing with positively charged residues in the active site that serve to bind the α-carboxylate group of the substrate.

In the absence or presence of KCl, $k_{cat}$ and $k_{cat}/K_m$ values for the deamination of the branched-chain amino acids were higher for BCATc than BCATm, suggesting that BCATc turns over 2–5 times faster than BCATm. For reamination, generally $k_{cat}$ values between the two enzymes are similar; however, the markedly lower $K_m$ values for α-keto acids found with BCATc result in calculated $k_{cat}/K_m$ values that are approximately 2-fold higher for KIC and 3–4-fold higher for KIV than found with BCATm. An equilibrium constant of 1.7 originally reported by Taylor and Jenkins (3).
TABLE IV

Substrate specificity of BCATc and BCATm for selected α-keto acids

| Keto acid                  | Relative rate | BCATm | BCATc |
|----------------------------|---------------|-------|-------|
|                            | %             |       |       |
| KIC                        | 100           | 100   |       |
| KIV                        | 56 ± 1        | 56 ± 2|       |
| α-Keto-β-methylisovalerate | 39 ± 1        | 35 ± 1|       |
| α-Hydroxyisocaproate        | 0             | 1 ± 1 |       |
| α-Hydroxyisovalerate        | 0             | 1 ± 1 |       |
| α-Ketoglutarate            | 72 ± 2        | 61 ± 2|       |
| α-Ketocaprate              | 34 ± 1        | 32 ± 1|       |
| α-Ketovalerate             | 47 ± 1        | 43 ± 1|       |
| α-Ketobutyrate             | 11 ± 1<sup>a</sup> | 13 ± 1|       |
| α-Keto-γ-methylbutyrate    | 20 ± 1        | 15 ± 1<sup>a</sup> |       |
| Pyruvate                   | 0             | 3 ± 1 |       |
| Phenylpyruvate             | 0             | 1 ± 1 |       |

<sup>a</sup> n = 2.

The range of α-keto acid substrates is shown in Table IV. Rates are shown relative to KIC and [1-14C]leucine. The pattern of α-keto acids accepted by the human isoenzymes is virtually identical. As expected, the hydroxy acids of leucine (α-hydroxyisocaproate) and valine (α-hydroxyisovalerate) were not substrates (see Table IV). The straight chain α-keto acids were good substrates, with the five-carbon α-ketovalerate preferred over the six-carbon α-ketoacetate. With five carbons, α-ketovalerate has the same chain length as isoleucine and leucine but without branching. α-Ketobutyrate was transaminated poorly compared with KIV, showing that, when the carbon length was 4, branching increased affinity for the enzyme. Both α-ketoacetate and α-keto-γ-methylbutyrate have a carbon length of 6, but α-keto-γ-methylbutyrate has a sulfur atom substituted at the fifth carbon position. α-Ketocaprate was favored over the α-keto acid of methionine, suggesting that the introduction of the bulky sulfur atom decreases the affinity of the enzyme. Pyruvate and phenylpyruvate were not accepted by either human isoenzyme. This pattern of amino and α-keto acid preferences is very similar to what has been reported for the rat BCAT isoenzymes (9). Thus, although differences in apparent substrate affinity and rates of transamination between BCATm and BCATc suggest localized variations in active site architecture, overall structure appears conserved within the mammalian BCAT.

CONCLUSIONS

This paper presents the first overexpression and characterization of the human branched-chain aminotransferase isoenzymes. Our data show that the human BCAT share the basic characteristics of other known BCAT with respect to substrate specificity (1). Physical comparison of the recombinant proteins has also revealed subtle kinetic and physical differences in the two proteins with regard to stability, tertiary structure of the N-terminal regions of the proteins, and structure of the active sites. Although the structure of the E. coli protein has provided insight into the structure of the human BCAT, neither the E. coli nor the α-keto amino transferase structure appears to have sufficiently high sequence homology for use as a model in molecular replacement. Thus, understanding the molecular basis for the differences between the BCATc and BCATm pro-