Structural Determinants for Branched-chain Aminotransferase Isozyme-specific Inhibition by the Anticonvulsant Drug Gabapentin*

Received for publication, June 14, 2005, and in revised form, August 9, 2005

Masaru Goto‡, Ikuko Miyahara‡, Ken Hirotsu§, Myra Conway, Neela Yennawar§, Mohammad M. Islam‡, and Susan M. Hutson

From the ‡Department of Chemistry, Graduate School of Science, Osaka City University, Osaka 558-8585, Japan, §Department of Biochemistry and Molecular Biology, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157, and the ‡Department of Biochemistry and Molecular Biology, Althouse Lab, Penn State University, University Park, Pennsylvania 16802

This study presents the first three-dimensional structures of human cytosolic branched-chain aminotransferase (hBCATc) isozyme complexed with the neuroactive drug gabapentin, the hBCATc Michaelis complex with the substrate analog, 4-methylvalerate, and the mitochondrial isozyme (hBCATm) complexed with gabapentin. The branched-chain aminotransferases (BCAT) reversibly catalyze transamination of the essential branched-chain amino acids (leucine, isoleucine, valine) to α-ketoglutarate to form the respective branched-chain α-keto acids and glutamate. The cytosolic isozyme is the predominant BCAT found in the nervous system, and only hBCATc is inhibited by gabapentin. Pre-steady state kinetics show that 1.3 mM gabapentin can completely inhibit the binding of leucine to reduced hBCATc, whereas 65.4 mM gabapentin is required to inhibit leucine binding to hBCATm. Structural analysis shows that the bulky gabapentin is enclosed in the active-site cavity by the shift of a flexible loop that enlarges the active-site cavity. The specificity of gabapentin for the cytosolic isozyme is ascribed at least in part to the location of the interdomain loop and the relative orientation between the small and large domain which is different from these relationships in the mitochondrial isozyme. Both isozymes contain a CXXC center and form a disulfide bond under oxidizing conditions. The structure of reduced hBCATc was obtained by soaking the oxidized hBCATc crystals with dithiothreitol. The close similarity in active-site structures between cytosolic enzyme complexes in the oxidized and reduced states is consistent with the small effect of oxidation on pre-steady state kinetics of the hBCATc first half-reaction. However, these kinetic data do not explain the inactivation of hBCATm by oxidation of the CXXC center. The structural data suggest that there is a larger effect of oxidation on the interdomain loop and residues surrounding the CXXC center in hBCATm than in hBCATc.

Pyridoxal 5′-phosphate (PLP)3-dependent branched-chain aminotransferases (BCAT) reversibly catalyze the transfer of the α-amino group of the hydrophobic branched-chain amino acids (leucine, isoleucine, valine) to α-ketoglutarate to form the respective branched-chain α-keto acids and glutamate (1, 2). BCAT enzymes are found in both bacteria and higher organisms (3, 4). Mammals have a mitochondrial (BCATm) and a cytosolic (BCATc) form of the enzyme (5, 6), whereas bacteria have a single BCAT enzyme (7). Human mitochondrial BCATm (hBCATm) is expressed ubiquitously in body tissues, whereas human cytosolic BCATc (hBCATc) is found primarily in the nervous system (5, 8–10). In fact, hBCATc is the predominant isozyme in brain, accounting for 60–70% of total brain BCAT activity (8, 11, 12). In the rat nervous system BCATc is expressed in selected populations of glutamatergic and γ-aminobutyric acid (GABAergic) neurons (13), and Hutson and co-workers (14–16) have postulated the existence of a glial-neuron nitrogen shuttle for the BCATs and branched-chain amino acids involved in maintenance of neurotransmitter glutamate/γ-aminobutyric acid.

The human BCAT isozymes are 58% identical in amino acid sequence and belong along with the Escherichia coli BCAT (eBCAT) to the fold-type IV class of PLP-dependent enzymes that transfer protons on the re-face of the cofactor (17, 18). Enzymes belonging to fold-type I-III classes shuttle protons on the si-face of the planar π-system of the substrate-cofactor complex. The mammalian BCATs are homodimers with subunit molecular masses ranging from ~41,000 to 46,000 Da. The molecular mass of the hBCATc subunit is 42,800 Da; each monomer consists of 385 amino acid residues and requires one PLP as cofactor (19). The mature form of hBCATm monomer (minus its mitochondrial targeting sequence) is 365 amino acid residues (19). Structures of the fold-type IV, Bacillus sp. YM-1 d-amino acid aminotransferase (20, 21), eBCAT (22–24), hBCATm (25, 26), and E. coli 4-amino-4-deoxychorismate lyase (27) have been solved. The overall structures of these enzymes as homodimers (the dimeric unit of the hexamer in eBCAT) are similar.

Steady state kinetic analysis of the BCAT isozymes shows they have the same substrate specificity; nevertheless, there are subtle differences in catalytic efficiency with individual substrates (8, 19). Also, the steady state kcat/Km values for hBCATc are higher than for hBCATm (19). A more striking difference between hBCATc and hBCATm is the sensitivity of...
the different isoforms to inhibition by the neuroactive drug gabapentin (1-(aminomethyl)cyclohexanecarboxylic acid) (Scheme 1) (15). Gabapentin is used widely for seizure control (28–30) and is now used extensively to treat neuropathic pain, migraine headache, and several other nonepileptic conditions (31). One theory of gabapentin action, the metabolic hypothesis, suggests that gabapentin interferes with neurotransmitter glutamate synthesis via inhibition of branched-chain amino acid transamination (15, 32). It was shown by Hutson and coworkers (15) that gabapentin is a competitive inhibitor of hBCATc with a $K_I$ similar to the $K_M$ for leucine. The drug is not an effective inhibitor of hBCATm. Understanding the structural basis for the specificity of gabapentin for hBCATc and kinetic differences in the two isoforms has been hindered by the lack of structural information for both hBCATs.

Another feature of the BCATs is a consensus sequence, a CXXC motif, which is conserved in the mammalian proteins but not in lower eukaryotes or prokaryotes or in other fold-type IV PLP enzymes (25, 33). In hBCATm, the CXXC motif is in the vicinity of the phosphate group of the cofactor PLP (25). The structures of the PLP form of hBCATm and reaction intermediates show that the cysteine residues in the CXXC center, Cys-315 and Cys-318, share a hydrogen bond (25). Biochemical studies indicate that these residues can form an intrasubunit disulfide bond under oxidizing conditions and that Cys-315 is the peroxide-reactive thiol(ate) functioning as the redox sensor in the regulation of hBCATm (34). The second cysteine, Cys-318, subsequently reacts with the nascent sulfenic acid form of Cys-315, forming a disulfide bond and permitting reversible regulation by preventing over-oxidation of hBCATm and irreversible loss of enzyme activity (34). Less is known about the role of this center in hBCATc.

The crystal structures of native hBCATm and hBCATm complexed with substrates have shed light on the “cofactor-substrate-protein” interaction, the behavior of the CXXC motif, and the reaction pathway of the catalytic reaction (25, 26). Here we report the first three-dimensional structures of hBCATc complexed with gabapentin in the oxidized (hBCATc-gabapentin) and reduced states (hBCATc-gabapentin) at 1.9 and 2.4 Å of resolution, respectively, the structure of the hBCATm complexed with gabapentin in the reduced state (hBCATm-gabapentin) at 1.8 Å resolution, and hBCATc complexed with the substrate analogue, 4-methylvalerate (4MeVA) at 2.1 Å of resolution. These are the first structures of the gabapentin complexes of the mammalian BCATs. A structural comparison of these complexes provided insight into the molecular basis for the specificity of BCATc for gabapentin, which is bulkier than 4MeVA, and the differential sensitivity of the catalytic activity of the hBCAT isoforms to the redox state of the CXXC center.

**EXPERIMENTAL PROCEDURES**

**Crystallization and Data Collection**—The expression of hBCATc and the purification of the expressed enzyme have been reported (19). All crystallization trials were performed by the hanging drop vapor diffusion method at 277 K. Drops were prepared by mixing 3 μl of the protein solution (9 mg/ml, 10 mM 3-phenylpropionate (3PP)) with an equal volume of reservoir solution containing 12% (w/v) polyethylene glycol 4000 and 200 mM MgCl$_2$. Two weeks after the drop was set up for crystallization at 277 K, minute crystals of hBCATc complexed with 3PP appeared and within another month grew to chunky yellow colored crystals with the space group $P2_1 2_1 2_1$, cell dimensions of $a = 66.6$ Å, $b = 106.4$ Å, and $c = 109.9$ Å and with one dimer in the asymmetric unit. Approximately 37% of the crystal volume was occupied by the solvent (35). Because many trials for crystallization were unsuccessful without 3PP, the addition of 3PP to the protein solution was a critical factor in producing crystals. After the initial structure of hBCATc-3PP was determined, two cysteine residues, Cys-335 and Cys-338, were found to form a disulfide bond, indicating that the purified enzyme was in the oxidized state of the CXXC motif (hBCATc-ox3PP). Crystals of hBCATc-oxgabapentin and hBCATc-ox4MeVA were obtained at 293 K by soaking hBCATc-ox3PP crystals in solutions containing 10 mM gabapentin or 4MeVA, respectively, for a day before data collection. Crystals of hBCATc-gabapentin in the reduced form were obtained at 293 K by soaking hBCATc-ox3PP crystals in solutions containing 10 mM gabapentin and 2 mM DTT. Similarly, hBCATc-ox3PP crystals were soaked in solution containing 4MeVA and DTT. However, 4MeVA complex crystals were not in the reduced form but in the oxidized form.

The expression of mature hBCATm in *E. coli*, removal of the histidine tag by thrombin cleavage, and purification of the expressed enzyme have been reported (19, 36). Crystals of hBCATm were obtained using the vapor diffusion method of hanging drops at 298 K. The drop consisted of 5 μl of hBCATm solution in the PLP form and 5 μl of the reservoir. The drop was equilibrated against 1 ml of the reservoir. The protein solution contained 2.5 mg/ml hBCATm in a solution of 50 mM HEPES (pH 7.0), 20 mM DTT, and 50 mM EDTA. Yellow crystals grew readily in about 1–2 week in several conditions using the Hampton crystallization reagent kit. They were optimized in grids set up with 22–30% polyethylene glycol 1500, 100 mM HEPES (pH 6.9–7.2), and 20 mM DTT. Crystals of hBCATm-gabapentin in the reduced form were obtained at 293 K by soaking hBCATc crystals in their crystallization solutions with 70 mM gabapentin.

The x-ray diffraction data sets for hBCATc-ox3PP, hBCATc-oxgabapentin, hBCATc-gabapentin, and hBCATc-ox4MeVA were collected to 1.8, 1.9, 2.4, and 2.1 Å of resolution at 100 K on the BL6A, BL18B, NW12, and BL44B2 stations, respectively, at the Photon Factory, KEK (Tsukuba, Japan) or at the Spring-8 (Hyogo, Japan) using an x-ray beam with a wavelength of 1.0 Å and an ADSC Quantum 4R CCD camera or MarCCD165 camera. All the data were processed and scaled using the program HKL2000 (35). Data collection statistics are presented in TABLE ONE.

Diffraction data for hBCATm-gabapentin were collected to 1.8 Å of resolution at the synchrotron source in Cornell university on beam line 5.0.2 with a 2 × 2 array CCD detector using 1.071 Å wavelength radiation monochromatized with a double crystal Si (111). DENZO (37) and SCALEPACK were used to reduce the data.

**Structure Determination and Refinement**—The structure of hBCATc-ox3PP was determined with the program AmoRe (38) using the previously determined structure of unliganded hBCATm (PDB code 1EKF) in the PLP form (26) as a search model. The modeling of the polypeptide chain was performed using the program O (39). The structure was refined by simulated annealing and energy minimization with the program CNS (40). The initial structure for hBCATc-oxgabapentin was determined with AmoRe (38) using the structure of hBCATc-ox3PP as the search model. When the $R_{factor}$ value decreased below 30%, gabapentin was introduced into a peak on a simulated annealing $2F_o - F_c$ map. Water molecules were picked up on the basis of the peak heights (3.0 $\sigma$) and distance criteria (4.0 Å from protein and solvent).
Human Cytosolic Branched-chain Aminotransferase Structures

from the σ-weighted \( F_o - F_c \) map. The water molecules whose thermal factors were above the maximum thermal factor of the main chain after refinement were removed from the list. Further model building and refinement cycles resulted in an \( R_{\text{factor}} \) of 22.1% and an \( R_{\text{free}} \) of 26.8%, calculated for 63,764 reflections (TABLE ONE). During the last step of the refinement, unambiguous water molecules were added including those with a temperature factor higher than 56 Å². The maximum temperature factor of the water molecules was 62 Å².

The initial structures for hBCATc-gabapentin and hBCATc-ox4MeVA were determined with AmoRe (38) using the structure of hBCATc-ox3PP as the search model. The same refinement procedure as that used for hBCATc-oxgabapentin was applied to hBCATc-gabapentin and hBCATc-ox4MeVA. When the \( R_{\text{factor}} \) value decreased below 30%, gabapentin or 4MeVA (leucine analogue) was introduced into a peak on a simulated annealing \( 2F_o - F_c \) map. Further model building and refinement cycles for hBCATc-gabapentin resulted in an \( R_{\text{factor}} \) of 21.3% and an \( R_{\text{free}} \) of 27.2%, calculated for 28,933 reflections. Further model building and refinement cycles for hBCATc-ox4MeVA resulted in an \( R_{\text{factor}} \) of 22.8% and an \( R_{\text{free}} \) of 29.0%, calculated for 44,715 reflections. The maximum thermal factors (B-factors) of the assigned water molecules in hBCATc-gabapentin and hBCATc-ox4MeVA were 79 and 68 Å², respectively.

The molecular replacement method was used to solve the hBCATm-gabapentin structure with the earlier solved PLP form as the search probe in the program AMoRe (38), which is part of CCP4 program suite (CCP4 Collaborative Computational Project, Number 4 (1994)). The program CNS (40) was then used for all further refinements. The slow cool protocol was used to minimize model bias, and O (41, 42) was used for examination and manual adjustment of the structure during the refinement. Several cycles of positional refinement and isotropic B-factor refinement were performed after every model building cycle. The PLP was removed from the initial refinement cycles to get an unbiased view of the active site. Toward the end of the refinement \( (R_{\text{free}} 26.8\%) \), σ-weighted difference Fourier maps and \( 2F_o - F_c \) maps clearly showed the electron density for gabapentin bound to the active site. The cofactor is seen to be covalently linked to the active-site lysine, Lys-202, and gabapentin was bound as a Michaelis complex. Water molecules were automatically located using CNS and manually checked in O. A total of 341 water molecules and an acetate molecule were modeled into the structure with the average B-factors are listed in TABLE ONE. A total of 341 water molecules and an acetate molecule were modeled into the structure with the average B-factors are listed in TABLE ONE.

**Quality of the Structure**—The final model of the hBCATc-oxgabapentin comprises 358 residues (22–45, 47–193, and 199–385) for subunit 1, 356 residues (24–44, 46–193, and 199–385) for subunit 2, 2 PLPs, 2 gabapentins, and 582 water molecules. The average thermal factor of the main-chain atoms is 29.4 Å². The final model of the hBCATm-gabapentin comprises 359 residues (22–45, 47–194, and 199–385) for subunit 1, 358 residues (24–194, and 199–385) for subunit 2, 2 PLPs, 2 gabapentins, and 148 water molecules. The average thermal factor of the main-chain atoms is 24.3 Å². No interpretable electron density was observed for the residues that are not listed above.

### TABLE ONE

| Crystal         | hBCATc-oxgabapentin | hBCATc-gabapentin | hBCATc-ox 4MeVA | hBCATm-gabapentin |
|-----------------|---------------------|------------------|----------------|------------------|
| **Diffraction data** |                     |                  |                |                  |
| Resolution (Å)  | 1.9                 | 2.4              | 2.1            | 1.8              |
| No. of reflections | 63,764              | 28,933           | 44,715         | 68,169           |
| **Refinement**  |                     |                  |                |                  |
| Resolution limits (Å) | 50.0-1.9            | 50.0-2.4         | 50.0-2.1       | 25.0-1.8         |
| \( R_{\text{factor}} \) (%) | 22.1               | 21.3             | 22.8           | 20.8             |
| \( R_{\text{free}} \) (%) | 26.8               | 27.2             | 29.0           | 23.2             |
| **Deviations**  |                     |                  |                |                  |
| Bond lengths (Å) | 0.007               | 0.007            | 0.007          | 0.012            |
| Bond angles (deg) | 1.38               | 1.44             | 1.40           | 1.55             |
| Mean B factors  |                     |                  |                |                  |
| Main-chain atoms (Å²) | 29.4               | 32.0             | 24.3           | 22.6             |
| Side-chain atoms (Å²) | 32.7               | 33.3             | 27.4           | 25.1             |
| Hetero atoms (Å²) | 22.9               | 27.4             | 25.5           | 28.0             |
| Water atoms (Å²) | 40.8               | 31.2             | 30.9           | 29.1             |
| **Procheck**    |                     |                  |                |                  |
| Favorable       | 87.3                | 86.8             | 87.3           | 91.8             |
| Additional allowed | 12.6               | 12.7             | 12.4           | 7.9              |
| Generously allowed | 0.0                | 0.2              | 0.2            | 0.0              |
| Disallowed      | 0.2                 | 0.3              | 0.2            | 0.3              |

\( a \) The values in parentheses are for the highest resolution shells.

\( b \) \( R_{\text{merge}} = \frac{\sum_i (I_i - \langle I_i \rangle)}{\langle I_i \rangle} \), where \( I_i \) is the observed intensity, and \( \langle I_i \rangle \) is the average intensity for multiple measurements.

\( R_{\text{factor}} = \frac{\sum_i (I_i - F_i)}{\sum_i F_i} \), where \( I_i \) is the observed intensity, and \( F_i \) is the average intensity for multiple measurements.

\( R_{\text{free}} = \frac{\sum_i (I_i - F_i)}{\sum_i F_i} \) where \( I_i \) is the observed intensity, and \( F_i \) is the average intensity for multiple measurements.

\( f_{\text{occ}} \) was monitored with 10% of the reflection data excluded from the refinement.
Analysis of the stereochemistry with PROCHECK (43) showed that all residues except for Val-336 fall within the most favorable and additionally allowed region of the Ramachandran plot for all structures. On the basis of electron density maps, it was confirmed that Val-336 of subunit 1 in hBCATc-ox gabapentin and hBCATc-ox 4MeVA and that of both subunits in hBCATm gabapentin are in the disallowed region. Structure diagrams were drawn with the programs MOLSCRIPT (44), BOBSCRIPT (45), and PyMOL (46).

The final model of the hBCATm gabapentin comprises 363 residues (3-365) for subunit 1, 363 residues (3-365) for subunit 2, two PLPs, 1 acetate, two gabapentins, and 341 water molecules. Residues 50, 105, 315, and 316 in subunit 1 have two possible conformers, each with 0.5 occupancy. Residues 105 and 315 in subunit 2 have two possible conformers each with 0.5 occupancy. The average thermal factor of the main-chain atoms is 22.6 Å². Analysis of the stereochemistry done with PROCHECK (43) showed that 99.7% of the main chain atoms fall within the most favorable and additionally allowed region of the Ramachandran plot. Gln-316 has good electron density and is the only residue seen in the core and generously allowed regions of the Ramachandran plot.

**RESULTS AND DISCUSSION**

**Comparison of the Overall and Subunit Structure of the BCAT Isozymes—**As shown by the amino acid sequence alignment presented in Fig. 1, the human BCAT isozymes are 58% identical in amino acid sequence. The key active-site residues involved in substrate and cofactor binding are identical in the hBCATc and hBCATm structures (see Fig. 1). Thus, observed isozyme differences in kinetic properties (8, 19) and gabapentin inhibition (15) cannot be explained by mutation of active-site residues. It is more likely that the isozyme differences result from changes in the primary amino acid sequence of residues surrounding the active-site residues. Biophysical studies of the two proteins indicated that there are conformation differences in the aromatic residues in the BCAT isozyme active sites and suggested that the structure of hBCATc is more flexible than the structure of hBCATm (19).

The activity of mammalian BCAT isozymes is affected by the oxidation state of the CXXC center, with the activity of hBCATm showing greater dependence on redox state of this center than the activity of hBCATc. Oxidation of the hBCATm CXXC center to the disulfide form results in loss of enzyme activity (34). Crystals of hBCATc were formed initially only in the presence of 3PP without added DTT; hence, the crystals that formed are the oxidized form of hBCATc, with a disulfide bond between Cys-335 and Cys-338. The activity of mammalian BCAT isozymes is affected by the oxidation state of the CXXC center, with the activity of hBCATm showing greater dependence on redox state of this center than the activity of hBCATc.
Human Cytosolic Branched-chain Aminotransferase Structures

FIGURE 2. The structure of hBCATc-ox complexed with gabapentin. Each figure is viewed from the direction nearly perpendicular to the molecular 2-fold axis. The PLP (yellow) and the anticonvulsant drug gabapentin (pink) are represented by ball-and-stick models. A, view of the overall structure. The small and large domains of one subunit are represented by secondary structures drawn in green and blue, respectively. The other subunit is shown in red. B, view of the subunit structure showing secondary structure assignments. α-Helices are denoted by H1–H6, and β-strands are depicted by S1–S18. The interdomain loop and the loops from the other subunit of the dimer unit are shown in orange and red, respectively. PLP is located at the bottom of the active site with its re-face directed toward the protein inside forming a Schiff base with Lys-222. Gabapentin is bound to the si-face side of PLP and is approached by the interdomain loop. The diagram was drawn with the MOLSCRIPT (44).

resolution structure (1.8 Å) of the reduced hBCATm-gabapentin Michaelis complex was obtained.

Comparison of the oxidized hBCATc, reduced hBCATc, and hBCATm structures with each other and the other hBCATm structures (25, 26) and eBCAT dimer structures (24) shows that the overall structures of these enzymes are similar. hBCATc is a homodimer, and the Cα atoms of the two subunits are related by a non-crystallographic 2-fold axis. The homodimer and subunit structures of hBCATc-ox-gabapentin are shown with secondary structure assignments by the program DSSP in Figs. 2, A and B (47). The surface area of the subunit interface was calculated to be 2,428 Å², which amounts to about 16% of the subunit surface area (15,405 Å²). The total surface area is comparable with 16,049 Å² reported for hBCATm (25, 26). When the Cα carbon atoms (except those for the interdomain loops) of the hBCATc structures are superimposed between subunits in the different complexes, the root mean square deviation (r.m.s.d.) is 0.37 Å. When the Cα carbon atoms of the dimeric molecules are fitted between hBCATc complexes, the r.m.s.d. is 0.21 Å. The superimposition of hBCATc-gabapentin onto hBCATm-gabapentin resulted in equivalent Cα atoms with an r.m.s.d. of 0.85 Å, indicating that the overall structure of hBCATc is quite similar to that of hBCATm. At this level the subunit and overall structures of hBCATc and hBCATm are essentially the same as those of the other fold-type IV class of PLP enzymes (20, 21, 27).

The hBCATc subunit consists of a small domain (N-terminal to Ser-188), an interdomain loop (Pro-189–Pro-201), and a large domain (Val-202 to C terminus). The small and large domains are folded into an open α/β structure and a pseudo barrel structure, respectively. The large cavity is formed between the small and large domains of one subunit, and two loops from the small domain of the other subunit approach the cavity (Fig. 2). The active site is formed at the domain interface and at the subunit interface. As with other fold-type IV enzymes (20–27) the cofactor PLP is located at the bottom of the active site with its re-face toward the protein side, forming a covalent Schiff base with the side chain of the catalytic Lys-222. Gabapentin resides on the solvent side of the PLP-Lys-222 Schiff base. The long loop connecting β-strands S1 and S2, which runs on the surface of the small domain, reaches the rim of the active site and overhangs the cavity. The overhang, which is not present in eBCAT, is characteristic of hBCATc and hBCATm (25, 26).

Active Site of hBCATc-oxgabapentin—The simulated annealing omit map showed that gabapentin was bound to the active site in the place of 3PP. The structure and the hydrogen-bonding scheme of the active site are shown in Figs. 3A and 4A, respectively. The PLP cofactor forms a Schiff-base bond with the catalytic Lys-222. The O3' atom and the phosphate group of PLP are on the side of Tyr-227 and on the side of the β-turn formed at Thr-333 and Ala-334, respectively. The β-turn is followed by the redox-sensitive CXXC (Cys-335–Val-336–Val-337–Cys-338) motif. The pyridine ring of PLP is sandwiched by Leu-286 and Thr-260 from the re- and si-faces, respectively. Glu-257 forms a salt bridge with the protonated N1 atom of PLP to strengthen the electron-withdrawing effect of the pyridine ring of PLP as an electron sink, as has
been observed for the enzymes of fold-types I and IV (48–51). To fine-tune the electronic state of the cofactor, the OH group of Tyr-227 is hydrogen-bonded to $\text{O}_3$/H11032. The C4/H11032/H11005 Schiff bond is roughly coplanar with the pyridine ring of PLP with the dihedral angle of C3-C4-C4/H11032-N of 34°, indicating that the Schiff base is protonated and that an intramolecular N4/H11001–H—O3/H11032/H11002 hydrogen bond is formed. As a result, the PLP is in the ketoenamine form (19). The OH group of Tyr-90*, the guadinino group of Arg-163, the hydroxyl group Tyr-161, water molecules (W1 and W2), the main-chain C=O of Gly-97, the carboxylate of gabapentin, and the phosphate O1 atom of PLP form a hydrogen bond network and form a semicircle that encloses gabapentin on the side of the si-face of the PLP plane. The water molecule, W3, bridges Lys-99 and Gly-191 of the interdomain loop and Ala-334 of the $\beta$-turn by hydrogen bonds that align the side chain of Lys-99 and the interdomain loop in positions suitable for enzyme function.

Except for one residue (Val-336 in hBCATc is Gln in hBCATm), the residues forming the substrate binding pocket in hBCATm are conserved in hBCATc (25, 26). The template cavity of hBCATc formed at the si-face of PLP is surrounded by Phe-49, Phe-95, Tyr-161, Tyr-227, Thr-260, Thr-333, Ala-334, Tyr-90*, Leu-173*, and Val-175* and is assumed to consist of large and small sites with Phe-49, Tyr-161, and Thr-260 located at the boundary region between the large and small sites (see Fig. 5). Gabapentin, except for the aminomethyl group, nicely fits the cavity with the cyclohexane ring and the $\beta$-turn formed at Thr-333 and Ala-334 preceding the CXXC motif and water molecule (W1) and Thr-260 on the loop between $\beta$-strands S10 and S11, respectively.

**FIGURE 3. The active site in hBCATc-ox.** Each figure is viewed from the solvent side (entrance of the active site). The secondary structures of the small and the large domains of one subunit of the homodimer are drawn in green and blue, respectively. The active-site residues and PLP (yellow) are represented by the stick models. The disulfide bond of the CXXC motif is drawn in yellow. Water molecules are drawn as red circles. The loops (red) carrying Tyr-90*, Leu-173*, and Val-175* of the small domain of the other subunit and the interdomain loop (orange) carrying Tyr-193 participate in the formation of the active site. The omit electron density map is contoured at the 1.0-$\sigma$ level. A, close-up view of the active site of the hBCATc-ox-gabapentin complex. Gabapentin (pink) is enclosed in the active-site cavity with its carboxylate and aminomethyl group interacting with the $\beta$-turn formed at Thr-333 and Ala-334 preceding the CXXC motif and water molecule (W1) and Thr-260 on the loop between $\beta$-strands S10 and S11, respectively. B, close-up view of the active site of the hBCATc-ox-4MeVA complex. The active-site structure except for Thr-260 is quite similar to that of hBCATc-ox-gabapentin. 4MeVA (pink) bound to the active-site cavity is approached by Tyr-193 of the interdomain loop to be shielded in the protein inside. One of the carboxylate oxygen atoms is recognized by the $\beta$-turn and the W1 hydrogen bonded to Tyr-161 like the carboxylate of gabapentin, whereas the other oxygen interacts with Thr-260 like the aminomethyl group of gabapentin. The diagram was drawn with the program BOBSCRIPT (45).
which is bulkier than the side chain of the substrate leucine. The cyclo-
hexane ring forms van der Waals contacts with Phe-49, Phe-95, Tyr-
161, Tyr-227, Thr-260, Tyr-90*, Leu-173*, and Val-175*. In addition to
these residues, Tyr-193 of the interdomain loop approaches the cyclo-
hexane ring (Fig. 3A). The small site is occupied by the carboxylate
group of gabapentin, which forms hydrogen bonds with the main-chain
NH groups of Thr-333 and Ala-334 at the β-turn and with the water
molecule W1. The side chain of Lys-99 approaches the main-chain
carbonyl groups of the β-turn and interacts with them to polarize the
peptide bonds leading to the enhancement of the hydrogen-bond inter-
action between the carboxylate of gabapentin and the main-chain NH
groups of the β-turn (Fig. 3A). The carboxylate is, thus, recognized by
the β-turn at Thr-333 and Ala-334 with the aid of Lys-99. Similar to
Lys-99 in hBCATc, Lys-79 in the hBCATm complexes interacts with
the main chain carbonyl group of the β-turn. The disposition of gaba-
pentin, except for the aminomethyl group, in the active site is roughly
the same as that of 4MeVA or 2-methylleucine in the eBCAT complex
(22), where the cyclohexane ring and the carboxylate of gabapentin

FIGURE 4. Schematic diagram showing hydro-
gen-bond and salt-bridge interactions of the
active-site residues. Putative interactions are
shown by dotted lines if the acceptor and donor are
less than 3.5 Å apart. The hydrogen bonds associ-
ated with the phosphate group of PLP are omitted
for clarity. A, hBCATc-ox complexed with gabapentin. B, hBCATc-ox complexed with 4MeVA.
correspond to the hydrophobic side chain and the carbonylate of the substrate analog (4MeVA or 2-methylleucine), respectively. The aminomethyl group of gabapentin, which does not have its counterpart in the substrate analogue, is also located inside the template cavity at the boundary region of the large and small sites with its C-N bond projected onto the C4-C4' bond of the cofactor with respect to the cofactor plane. The amino group forms hydrogen bonds with the main-chain carbonyl group and the side-chain hydroxyl group of Thr-260. Thr-260 is, thus, the recognition site for the aminomethyl group. The side chain of Tyr-193 of the interdomain loop approaches the aminomethyl group from the solvent side. Gabapentin captured in the template cavity is almost shielded in the protein inside by the access of Tyr-193, because the accessible surface area (ASA) of gabapentin is 4 Å², indicating that hBCATc-ox-gabapentin has a closed form of the active site.

hBCATc and hBCATm are characterized by a CXXC (Cys-335–Val-336–Val-337–Cys-338 in hBCATc) consensus sequence located at the phosphate side of the cofactor PPL. In the crystals of the oxidized form of hBCATc, Cys-335 and Cys-338 form a disulfide bond with the S-S bond distance of 2.03 Å. Cys-335, Val-336, Val-337, and Cys-338 form the macrocycle at the N-terminal side of the β-turn, which acts as the recognition site for the carbonylate of gabapentin.

Active Site Structure of hBCATc-Gabapentin—An x-ray crystallographic study of hBCATm in the PLP form and of its reaction intermediates showed that the CXXC motif is in the reduced state with two cysteine residues forming a thiol-thiolate hydrogen bond, the distance of which is in the range of 3.17–3.46 Å (25, 26). In hBCATc-gabapentin, the sulfur atom of Cys-335 is at a distance of 4.7 Å from the sulfur atom of Cys-338, indicating that the CXXC motif is in a reduced state with two thiol groups. When the active-site residues, the cofactor, and gabapentin are superimposed between subunits of reduced and oxidized gabapentin complexes, the average r.m.s.d. is 0.29 Å, indicating that they are nearly identical (Fig. 6).

On the other hand, in both the oxidized and reduced hBCATc gabapentin complexes, the interdomain loop (flexible loop) shields gabapentin from solvent. The interdomain loop of the reduced enzyme is closer to the central part of the active-site cavity than in the oxidized form (resulting in the closer access of Tyr-193 to gabapentin in the reduced form than in the oxidized form) (Fig. 6). Thus, in hBCATc-gabapentin, gabapentin is less accessible to solvent than in hBCATc-ox-gabapentin. ASA values of bound gabapentin in reduced and oxidized hBCATc are 2 and 4 Å², respectively. Van der Waals interactions between Cys-335 and the main chain of the interdomain loop residues are not observed in the oxidized protein, but they are present in the reduced protein. In the reduced protein Tyr-193 can interact strongly with the cyclohexane ring of gabapentin, whereas this interaction is weaker in the oxidized hBCATc-ox-gabapentin structure. These structural differences suggest that substrate binding to the oxidized hBCATc enzyme would be weaker than binding to the reduced hBCATc.

Active Site Structure of hBCATc-ox-4MeVA—4MeVA is a substrate analog in which the α-amino group of l-leucine is replaced by a hydrogen atom. The 4MeVA is located on the si-face of PLP with one of the Ca hydrogen atoms of 4MeVA directed toward C4' of the Schiff base in PLP (Figs. 3B and 4B). The active-site structure of 4MeVA complex is quite similar to that of the oxidized gabapentin complex because the active-site residues and the cofactor except for Thr-260 are superimposed on the oxidized gabapentin complex with an r.m.s.d. of 0.21 and a 0.78 Å displacement of the Ca atom of Thr-260. The dihedral angle of C3-C4-C4'-N in the PLP-Lys-222 conjugate is ~30°, and an intramolecular N4'–H–O3' hydrogen bond is formed similarly to gabapentin complexes. The water molecules (W1, W2, and W3) located at the active site of hBCATc-ox-gabapentin are also conserved in hBCATc-ox-4MeVA (Figs. 3, A and B, and 4, A and B).

The 4MeVA bound to the template cavity is isolated from the solvent region by the access of Tyr-193 of the interdomain loop with 1 Å² ASA for 4MeVA. The binding mode of 4MeVA to the active-site cavity is roughly the same as that of gabapentin except for the aminomethyl group. The isopropyl group and the α-carboxylate of the 4MeVA complex are located at the large and small sites of the template cavity, respectively (Figs. 3B and 4B). However, the α-carboxylate of 4MeVA is significantly shifted toward Thr-260 compared with that of gabapentin. In the gabapentin complex, the β-turn preceding the CXXC motif recognizes both α-carboxylate oxygen atoms of gabapentin, and Thr-260 interacts with the aminomethyl group of gabapentin (Fig. 4A). On the other hand, in the 4MeVA complex, the β-turn interacts with one α-carboxylate oxygen atom of 4MeVA, and the side-chain OH group of Thr-260 forms a hydrogen bond with the other α-carboxylate oxygen atom (Fig. 4B). The flexible loop bearing Thr-260 plays an important role in the accommodation of not only 4MeVA (substrate leucine) but also gabapentin, which is bulkier than 4MeVA by the same template cavity. The access of gabapentin to the active site causes the short contacts between the aminomethyl group and Thr-260. Through this process, the loop connecting β-strands S10 and S11 moves to make room for the aminomethyl group, which is directly recognized by Thr-260 (Fig. 2).

Structure of hBCATm-gabapentin—The CXXC motif is in a reduced state with two thiol groups because two sulfur atoms of the motif are at a distance from 3.9 to 4.8 Å. When the Ca carbon atoms of hBCATm-gabapentin are superimposed onto those of hBCATm-isoleucine and hBCATm-valine (25), the r.m.s.d. values are 0.66 and 0.71 Å, respectively. The small domain superposition of Ca atoms between hBCATm-gabapentin and hBCATc-gabapentin reveals a good overlap (average r.m.s.d. of 0.47 Å) for the small domain but a poor overlap (average r.m.s.d. of 1.62 Å) for the large domain. The large domain fitting causes a poor overlap (average r.m.s.d. of 1.51 Å) for the small
domain but a good overlap (average r.m.s.d. of 0.53 Å) for the large domain. These results indicate that the relative orientation of the two domains is significantly different between hBCATm and hBCATc. The small domain fitting shows that the large domain of hBCATm rotates about 4.5° to widen the active site compared with the large domain of hBCATc (Fig. 7).

Unlike the situation for hBCATc, gabapentin is not an effective inhibitor of hBCATm (15). All the active-site residues of hBCATm except for Gln-316 (Val-336 in hBCATc) and the interdomain loop are conserved in hBCATc. The active-site cavity (the template cavity) has a similar shape to the template cavity in hBCATc, and Ca atoms of the conserved residues are superimposed between these two complexes with an r.m.s.d. of 0.40 Å (Fig. 8). Gabapentin is captured in the hBCATm cavity in the same manner as observed in the hBCATc-gabapentin complex. The cyclohexane ring, the α-carboxylate, and the aminomethyl group of gabapentin are recognized by the large site of the cavity, the β-turn at Thr-313 (hBCATc Thr-333), Ala-314 (hBCATc Ala-334), and Thr-240 (hBCATc Thr-260), respectively. Unlike in hBCATc-gabapentin complex, where gabapentin is shielded from solvent, the ASA of gabapentin in the hBCATm complex is 24–25 Å², indicating gabapentin is not shielded from the solvent and hBCATm is in an open conformation. hBCATm is also found in an open form in the hBCATm complexes with its amino acid substrates isoleucine or valine (25).

**Pre-steady State Kinetics of hBCAT Isozyme Inhibition by Gabapentin**—Pre-steady state inhibitory constants ($K_i$) of gabapentin binding in the active site of both reduced and oxidized forms of hBCAT isozymes were determined and compared with the pre-steady state half-reaction dissociation constants for leucine (TABLE TWO). With reduced hBCATm, 65.4 mM gabapentin was required to inhibit the binding of leucine to the enzyme; however, only 1.3 mM gabapentin was required to inhibit binding of the same concentration of leucine to reduced hBCATc. This result is consistent with the steady state kinetic data, which showed that gabapentin is a competitive inhibitor of leucine (15).

The effect of oxidation of the CXXC center on leucine and gabapentin binding was determined for both isozymes. Although our recent results show that oxidized hBCATm is inactive, the pre-steady state dissociation constants shown in TABLE TWO indicate that the first half-reaction is less efficient but not inhibited in the oxidized enzymes. Interest-
gabapentin ASA values of ~2 Å². On the other hand, the corresponding tyrosine in hBCATm is too far from gabapentin to interact with it, and gabapentin is solvent-accessible with ASA values of 24–25 Å². Similarly, the interaction of Tyr-193 with gabapentin significantly reduces ASA values of the Tyr-193 side chain. The side-chain ASA of Tyr-193 in the hBCATm side chain is 19–20 Å², whereas that in hBCATc is 72–77 Å². The specificity of hBCATc for gabapentin could be ascribed in part to the burial of hydrophobic surfaces of the gabapentin and the phenyl ring of Tyr-193 and the van der Waals interactions between Tyr-193 and gabapentin side chains and main chains are shown in reddish brown.

In conclusion, this study presents the first detailed structural information on the hBCATc isozyme and the structural basis for the inhibition of this isozyme by the neuroactive drug gabapentin and the first structure of the oxidized form of a BCAT isozyme. Differences in alignment of the small and large domains, side chain binding pockets, and PLP binding loop residues of these two enzymes can explain the differences in gabapentin binding to the two BCAT isozymes. The effect of oxidation on the BCAT isozyme leucine and gabapentin pre-steady state half-reaction kinetic constants shows a larger effect of oxidation on hBCATm than hBCATc. A further detailed kinetic analysis is in progress to determine how structural changes in the CXXC center regulate BCAT isozyme activity.

**REFERENCES**

1. Taylor, R. T., and Jenkins, W. T. (1966) *J. Biol. Chem.* 241, 4396–4405
2. Ichihara, A., and Koyama, E. (1966) *J. Biochem. (Tokyo)* 59, 160–169
3. Hutson, S. (2001) *Prog. Nucleic Acid Res. Mol. Biol.* 70, 175–206
4. Christen, P., and Metzler, D., (eds) (1985) *Transaminases*, Vol. 2, pp. 430–439, John Wiley & Sons, Inc., New York
5. Hutson, S. M. (1988) *J. Nutr.* 118, 1475–1481
6. Hutson, S. M., Fenstermacher, D., and Mahar, C. (1988) *J. Biol. Chem.* 263, 3618–3625
7. Kamitori, S., Odagaki, Y., Inoue, K., Kuramitsu, S., Kagamiyama, H., Matsuura, Y., and Higuchi, T. (1989) *J. Biochem. (Tokyo)* 105, 671–672
8. Hall, T. R., Wallin, R., Reinhardt, G. D., and Hutson, S. M. (1993) *J. Biol. Chem.* 268, 3092–3098
9. Hutson, S. M., Wallin, R., and Hall, T. R. (1992) *J. Biol. Chem.* 267, 15681–15686
10. Sweatt, A., Wood, M., Suryawan, A., Wallin, R., Willingham, M. C., and Hutson, S. M. (2004) *Am. J. Physiol.* 286, E64–E76
11. Ichihara, A. (1985) in *Transaminases* (Christen, P., and Metzler, D., eds) Vol. 2, pp. 430–438, John Wiley & Sons, Inc., New York
12. Goto, M., Shimm, H., and Ichihara, A. (1977) *Genes* 68, 663–667
13. Sweatt, A. J., Garcia-Espinosa, M. A., Wallin, R., and Hutson, S. M. (2004) *J. Comp. Neurol.* 477, 360–370
14. Lieth, E., LaNoe, K. F., Berkich, D. A., Xu, B., Ratz, M., Taylor, C., and Hutson, S. M. (2001) *J. Neurochem.* 76, 1712–1723
15. Hutson, S. M., Berkich, D., Drown, P., Xu, B., Aschner, M., and LaNoe, K. F. (1998) *J. Neurochem.* 71, 863–874
16. LaNoe, K. F., Berkich, D. A., Conway, M., Barber, A. J., Hu, L. Y., Taylor, C., and Hutson, S. (2001) *J. Neurosci. Res.* 66, 914–922
17. Yoshimura, T., Nishimura, K., Ito, J., Esaki, N., Kagamiyama, H., Manning, J., and Soda, K. (1993) *J. Am. Chem. Soc.* 115, 3897–3900

**TABLE TWO**

| PLP-enzyme | hBCATm | Gabapentin, $K_d$ | Leucine, $K_d$ | gabapentin, $K_d$ |
|------------|--------|-----------------|---------------|-----------------|
| Reduced    | 1.5 ± 0.03 | 65.4 ± 2 | 1.1 ± 0.02 | 1.3 ± 0.03 |
| Oxidized   | 13.8 ± 0.10 | 389.2 ± 9 | 3.7 ± 0.03 | 3.5 ± 0.05 |
Human Cytosolic Branched-chain Aminotransferase Structures

18. Jhee, K.-H., Yoshimura, T., Miles, E., Takeda, S., Miyahara, I., Hirotsu, K., Soda, K., Kawata, Y., and Esaki, N. (2000) J. Biochem. (Tokyo) 128, 679–686
19. Davoodi, J., Drown, P. M., Bledsoe, R. K., Wallin, R., Reinhart, G. D., and Hutson, S. M. (1998) J. Biol. Chem. 273, 4982–4989
20. Sugio, S., Petisko, G., Manning, J., Soda, K., and Ringe, D. (1995) Biochemistry 34, 9661–9669
21. Peisach, D., Chipman, D., van Opheim, P., Manning, J., and Ringe, D. (1998) Biochemistry 37, 4958–4967
22. Okada, K., Hirotsu, K., Hayashi, H., and Kagamiyama, H. (2001) Biochemistry 40, 7453–7463
23. Okada, K., Hirotsu, K., Sato, M., Hayashi, H., and Kagamiyama, H. (1997) J. Biochem. (Tokyo) 121, 637–641
24. Goto, M., Miyahara, I., Hayashi, H., Kagamiyama, H., and Hirotsu, K. (2003) Biochemistry 42, 3725–3733
25. Yennawar, N., Conway, M. E., Yennawar, H. P., Farber, G. K., and Hutson, S. M. (2002) Biochemistry 41, 11592–11601
26. Yennawar, N., Dunbar, J., Conway, M., Hutson, S., and Farber, G. (2001) Acta Crystallogr. D Biol. Crystallogr. 57, 506–515
27. Nakai, T., Mizutani, H., Miyahara, I., Hirotsu, K., Takeda, S., Jhee, K.-H., Yoshimura, T., and Esaki, N. (2000) J. Biochem. (Tokyo) 128, 29–38
28. Andrews, J., Chadwick, D., and Bates, D. (1990) Lancet 335, 1114–1117
29. Bergey, G. K., Morris, H. H., Rosenfeld, W., Blume, W. T., Penovich, P. E., Morrell, M. J., Leiderman, D. B., Crockett, J. G., LaMoreaux, L., Garofalo, E., and Pierce, M. (1997) Neurology 49, 739–745
30. McLean, M. J., Ramsey, R. E., Leppid, I., Rowan, A. J., Shellenberger, M. K., and Wallace, J. (1997) Neurology 43, 2292–2298
31. Rogawski, M., and Lescher, W. (2004) Nat. Med. 10, 685–692
32. Welty, D. F., Schielke, G. P., and Rothstein, J. D. (1995) Ann. Pharmacother. 29, 1164–1167
33. Conway, M. E., Yennawar, N., Wallin, R., Poole, L. B., and Hutson, S. M. (2002) Biochemistry 41, 9070–9078
34. Conway, M. E., Poole, L. B., and Hutson, S. M. (2004) Biochemistry 43, 7356–7364
35. Matthews, B. (1968) J. Mol. Biol. 33, 491–497
36. Conway, M., and Hutson, S. M. (2000) Methods Enzymol. 324, 355–365
37. Otwonowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307–326
38. Navaza, J. (1994) Acta Crystallogr. A 50, 157–163
39. Jones, T., Zou, J.-Y., Cowan, S., and Kjeldgaard, M. (1991) Acta Crystallogr. A 47, 110–119
40. Brunger, A., Adams, P., Clore, G., DeLano, W., Gros, P., Grosse-Kunstleve, R., Jiang, J.-S., Kuszewski, J., Nilges, M., Pannu, N., Read, R., Rice, L., Simonson, T., and Warren, G. (1998) Acta Crystallogr. D Biol. Crystallogr. 54, 905–921
41. Jones, T., and Kjeldgaard, M. (1997) Methods Enzymol. 277, 173–208
42. Kleywegt, G., and Jones, T. (1997) Methods Enzymol. 277, 208–230
43. Laskowski, R., MacArthur, M., Moss, D., and Thornton, J. (1993) J. Appl. Crystallogr. 26, 283–291
44. Kraulis, P. (1991) J. Appl. Crystallogr. 24, 946–950
45. Esnouf, R. (1997) J. Mol. Graph. Model 15, 132–134
46. DeLano, W. (2002) The PyMOL User’s Manual, DeLano, Scientific, San Carlos, CA
47. Kabush, W., and Sander, C. (1983) Biopolymers 22, 2577–2637
48. Jansonius, J. N. (1998) Curr. Opin. Struct. Biol. 8, 759–769
49. Yano, T., Kuramitsu, S., Tanase, S., Morino, Y., and Kagamiyama, H. (1992) Biochemistry 31, 5878–5887
50. Yano, T., Hinoue, Y., Chen, Y., Metzler, D. E., Miyahara, I., Hirotsu, K., and Kagamiyama, H. (1993) J. Biol. Chem. 268, 1218–1229
51. Goldberg, J., Swanson, R., Goodman, H., and Kirsch, J. F. (1991) Biochemistry 30, 305–312