Identification of a Peroxide-Sensitive Redox Switch at the CXXC Motif in the Human Mitochondrial Branched Chain Aminotransferase†

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ABSTRACT: The human mitochondrial branched chain aminotransferase isoenzyme (hBCATm) must be stored in a reducing environment to remain active. Oxidation or labeling of hBCATm with sulfhydryl reagents results in enzyme inhibition. In this study, we investigated both the structural and biochemical basis for the sensitivity of hBCATm to these reagents. In its native form, hBCATm has two reactive cysteine residues which were identified as Cys315 and Cys318 using iodinated β-(4-hydroxyphenyl)ethyl maleimide. These are located in the large domain of the homodimer, about 10 Å from the active site. The crystal structures show evidence for a thiol-thiolate hydrogen bond between Cys315 and Cys318. Under oxidizing conditions, these cysteine residues can reasonably form a disulfide bond because of the short distance between the sulfur atoms (3.09−3.46 Å), requiring only a decrease of 1.1−1.5 Å. In addition to Cys315 playing a structural role by anchoring Tyr173, which in the ketimine form increases access to the active site, our evidence indicates that these cysteine residues act as a redox switch in hBCATm. Electrospray ionization mass spectrometry analysis and UV−Vis spectroscopic studies of 5,5′-dithiobis-(2-nitrobenzoic acid) labeled hBCATm showed that during labeling, an intrasubunit disulfide bond was formed in a significant portion of the protein. Furthermore, it was established that reaction of hBCATm with H_2O_2 abolished its activity and resulted in the formation of an intrasubunit disulfide bond between Cys315 and Cys318. Addition of dithiothreitol completely reversed the oxidation and restored activity. Therefore, the results demonstrate that there is redox-linked regulation of hBCATm activity by a peroxide sensitive CXXC center. Future studies will determine if this center has an in vivo role in the regulation of branched chain amino acid metabolism.

Branched chain aminotransferases (EC 2.6.1.42) catalyze the transamination of the branched chain amino acids, leucine, isoleucine, and valine, to their respective α-keto acids, α-ketoisocaproate, α-keto-β-methylvalerate, and α-ketoisovalerate. In humans there are two isozymes, a mitochondrial (hBCATm)[1] and a cytosolic (hBCATc) form (*1*), whereas bacteria contain only a single BCAT isoform (*2*). These isozymes show tissue-specific expression. Human BCATm is found in most tissues with low levels of activity in the liver, whereas hBCATc is found mainly in the brain (*3*−*5*). Both hBCATm and hBCATc are homodimers with subunit molecular masses ranging from 41 to 46 kDa (*5*−*7*). The *Escherichia coli* (*E. coli*) BCAT enzyme consists of six identical 34 kDa subunits that are an assembly of three dimer units around a 3-fold axis (*6*, *8*).

With a few exceptions, pyridoxal phosphate (PLP) aminotransferases generally fall within the fold type I or L-aspartate aminotransferase family. However, based on primary sequence comparisons, the BCAT and D-amino acid aminotransferase (which have opposite stereospecificity, L-versus D-amino acids, respectively), and the bacterial enzyme 4-amino-4-deoxychorismate lyase (ADCL), were placed in a separate folding class (fold type IV) (*9*, *10*). A unique feature of the fold type IV PLP-dependent enzymes is that the proton is added to or abstracted from the C4′ atom of the coenzyme-imine or external aldimine intermediate on the *re* face instead of the *si* face of the PLP cofactor (*11*).

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[1] Abbreviations: hBCATm, human mitochondrial branched chain aminotransferase; hBCATc, human cytosolic branched chain aminotransferase; rBCATm, rat mitochondrial branched chain aminotransferase; rBCATc, rat cytosolic branched chain aminotransferase; sBCATm, sheep mitochondrial branched chain aminotransferase; sBCATc, sheep cytosolic branched chain aminotransferase; ADCL, bacterial enzyme 4-amino-4-deoxychorismate lyase; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; NEM, *N*-ethylmaleimide; 1,5-IAEDANS, 5-(((2-iodoacetyl)amino)ethyl) aminonaphthalene-1-sulfonic acid; CPM, 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin; PLP, pyridoxal phosphate; β-4HPEM, β-(4-hydroxyphenyl)ethyl maleimide; ^{125}I-β-4HPEM-NEM, ^{125}I-β-(4-hydroxyphenyl)ethyl maleimide with cold *N*-ethylmaleimide; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; ESI-MS, electrospray ionization mass spectrometry.

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Recently, we have found that, in addition to transamination, the human BCAT (hBCAT) catalyzes an effective β-elimination reaction (Cooper, A. J., Bruschi, S. A., Conway, M. E., and Hutson S. M., unpublished data).

A unique property of hBCAT isozymes is their sensitivity to reducing agents, whereby a loss of activity occurs unless hBCATm or hBCATc (to a lesser extent) are stored in a reducing environment (*5*, *12*, *13*). In addition, sulfhydryl reagents such as 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethyl maleimide (NEM) inhibit hBCAT isozymes. Human BCATm contains six cysteine residues (*5*). Titration of two thiol groups with DTNB leads to almost complete inhibition of activity (*5*). This relationship between the redox state of the cysteines in the protein and the activity of the enzymes has not been reported for other aminotransferases. The bacterial BCAT from *S. typhimurium*, which contains three cysteine residues, displays no sensitivity to sulfhydryl reagents (*14*). The aim of this study was (i) to identify the cysteine residues that were sensitive to the sulfhydryl specific reagents, and (ii) to address both the structural and biochemical importance of these residues that are essential in maintaining the activity of hBCATm.

As reported herein, the two reactive cysteine residues in hBCATm were identified as Cys315 and Cys318. These residues lie in a loop in the large domain of the hBCATm monomer in a CXXC motif. The crystal structures of the PLP form of hBCATm and several reaction intermediates show that Cys315 and Cys318 share a hydrogen bond (thiol−thiolate hydrogen bonding), and biochemical studies indicate that these residues can form an intrasubunit disulfide bond under oxidizing conditions. As a demonstration of the peroxide sensitivity of this redox center, data are presented showing that peroxide-induced oxidation of these residues results in complete loss of activity correlated with a decrease in thiol content, which is reversible with the reductant DTT. Cys315 and Cys318 are therefore part of a redox-active dithiol/disulfide center and may be of physiological relevance.

MATERIALS AND METHODS

Materials. Cyanogen bromide (CNBr), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), *N*-ethylmaleimide (NEM), 2,2′-dithiodipyridine, α-ketoisocaproate, α-ketoisovalerate, pyridoxal phosphate (PLP), and iodoacetamide were obtained from Sigma (St. Louis, MO). Monobromobimane, eosin-5-maleimide, *N*-(1-pyrene)maleimide, 5-(((2-iodoacetyl)amino)ethyl)aminonaphthalene-1-sulfonic acid (1,5-IAEDANS), and 7-diethylamino-3(4′-maleimidylphenyl)-4-methylcoumarin (CPM) were from Molecular Probes (Eugene, OR). Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Fisher Scientific (Suwanee, GA). Sodium ^{125}Iodine was purchased from ICN radiochemicals (Costa Mesa, CA). PD10 columns were from Pharmacia Biotech (Piscataway, NJ). IODO-GEN precoated iodination tubes and β-(4-hydroxyphenyl)ethyl maleimide (β-4HPEM) were from Pierce (Rockford, IL).

Protein Purification of hBCATm. Purification of hBCATm was carried out as described in Davoodi et al., (*5*) with the following modifications. Briefly, the hBCATm cDNA clone was ligated into the pET-28a expression vector and subsequently used to transform BL21(DE3) cells. The BL21(DE3) cells were grown and expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 4 h, 4 L of cells were harvested by centrifugation. After extraction using sonication, the protein was purified using nickel-NTA resin (Qiagen, Chatsworth, CA). The histidine tag was removed by digestion with thrombin (100 NIH units). Final purification of hBCATm was obtained after hydrophobic interaction chromatography using a HYDROPORE-PK HIC 12U column (10 × 100 mm, Varian Co. Inc). The pure hBCATm sample was then dialyzed at 4 °C into a buffer containing 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM glucose, 1 mM EDTA, 1 mM α-ketoisocaproate, 5 mM DTT. The enzyme could be stored for 2 days at 4 °C or at −20 °C for 1.5 to 2 months. The concentration of purified protein was estimated using the Schaffner and Weissmann method (*15*) or determined from the absorbance at 280 nm using the extinction coefficient of 67 600 M^{-1} cm^{-1} per monomer (*5*). The final yield of pure protein was approximately 10 mg of hBCATm/L of *E. coli*.

Branched-Chain Aminotransferase (BCAT) Assay. Human BCATm activity was measured at 37 °C in 25 mM potassium phosphate buffer, pH 7.8, and 5 mM DTT, using 1 mM α-keto[1-^{14}C]isovalerate and 12 mM isoleucine as described (*5*). A unit of enzyme activity was defined as 1 μmol of valine formed/min at 37 °C. The reaction of DTNB with hBCATm is reversed by the addition of DTT. Therefore, when measuring hBCATm inhibition of activity by DTNB, DTT was omitted from the BCAT assay.

Spectrophotometric Analysis of Thiol Groups. For titration of the solvent-accessible sulfhydryl groups in hBCATm or covalently modified hBCATm, 2 nmol of protein in 50 mM HEPES, pH 7.0, and 1 mM EDTA (buffer A) was incubated with a 100-fold excess of DTNB at room temperature for 10 min. The absorbance change at 412 nm was monitored and the concentration of free thiol groups was calculated from the liberated 2-nitro-5-thiobenzoate (thiolate) dianion (TNB) using a molar extinction coefficient of 14 150 M^{-1} cm^{-1} (*16*). To determine the total number of free thiol groups in hBCATm or NEM-labeled hBCATm, the same procedure was repeated in the presence of 6 M guanidine hydrochloride.

Chemical Modification of hBCATm with Sulfhydryl Reagents. Stock solutions of the modifying reagents were prepared in water or 5% DMSO where required. These were prepared fresh and used immediately. Ten nmoles of hBCATm was exchanged into buffer A using a PD10 column as described by the manufacturer. For the evaluation of the inhibitory effects of various sulfhydryl reagents on enzyme activity, 2 nmol of protein was reacted with a 200-fold molar excess of each thiol reagent, respectively (Table 1). Each reaction was allowed to proceed for 10 min at room temperature and stopped by the addition of a 100-fold molar excess of DTT. The % residual activity was measured using the BCAT assay. The reaction involving the reversible modifying reagent DTNB was stopped by diluting the hBCATm samples (1:50) directly into the BCAT assay buffer without DTT, and samples were assayed immediately. Control hBCATm samples were prepared without added sulfhydryl reagents. Additional labeling experiments involved the same protocol but used varied concentrations of DTNB at 1.0, 10, and 100-fold molar ratios of DTNB to hBCATm. For the subsequent NEM studies, a 20-fold molar excess of NEM was used to label 7 nmol of hBCATm. Each reaction was stopped after 10, 20, 40, 60, 240, and 600 s with a 100-fold molar excess of DTT. Excess reagents were removed

Table 1: Effects of Selected Sulfydryl Reagents on hBCATm Activity

sulfhydryl reagents	% residual activity[a]
NEM	10 ± 5
monobromobimane	9 ± 2
eosin-5-maleimide	5 ± 2
2,2′-dithiodipyridine	5 ± 1
CPM	9 ± 4
N-(1-pyrene) maleimide	6 ± 1
iodoacetamide	61 ± 1
IAEDANS	61 ± 8
DTNB	15 ± 5

[a] Results reported as mean ± standard deviation expressed as % residual activity compared with appropriate controls. hBCATm in buffer containing 50 mM HEPES, pH 7.0, with 1 mM EDTA, was incubated with a 200-fold molar excess of each inhibitor for 10 min as described in Materials and Methods. The reaction involving the reversible modifying reagent DTNB was stopped by diluting hBCATm samples (1:50) directly into the hBCATm assay buffer omitting DTT and assayed immediately. All other BCAT transaminase reactions were assayed in the presence of 5 mM DTT.

using PD10 gel filtration. Both the thiol content and BCAT activity were measured as described above.

The effect of branched chain amino acid or α-keto acid substrates on NEM or DTNB-linked inhibition was determined by incubating the enzyme for 5 min with a saturating concentration of each respective substrate (10-fold over the K_m) followed by addition of either a 10-fold molar excess of NEM or a 100-fold molar excess of DTNB. After 5 min of incubation, aliquots were removed and assayed for BCAT activity.

Oxidation of hBCATm with H_2O_2. Human BCATm was exchanged into buffer A. Seven nmoles of hBCATm per aliquot were incubated with increasing equivalents of H_2O_2 (1, 2, 4, 8, 16, and 32). The samples were incubated for 24 h at 4 °C after which aliquots were taken and the BCAT activity and thiol content were measured as described above. The remainder of the fraction was reacted with a 100-fold molar excess of DTT and allowed to incubate for 18 h at 4 °C where the activity was again measured as described above. Control samples were incubated under the same sample conditions without the addition of H_2O_2.

Iodination of hBCATm with β-4HPEM. Iodination of β-4HPEM (structure available in the Pierce catalog) with Na^{125}I was carried out as described by Wallin et al. (*17*). The ^{125}I-labeled β-4HPEM was diluted with unlabeled NEM (^{125}I-β-4HPEM-NEM) to a final concentration of 10 mM and specific radioactivity of 1.4×10^7 dpm/nmol. A 10-fold molar excess of the stock solution was added to 27 nmol of hBCATm and allowed to react at room temperature for 20 min. Unreacted ^{125}I-β-4HPEM-NEM was quenched by adding 10 μL of 100 mM glutathione, and excess reagents were removed by gel filtration.

Cyanogen Bromide (CNBr) Digestion of hBCATm. The lyophilized ^{125}I-β-4HPEM-NEM labeled hBCATm (3 nmol) was resuspended in 200 μL of 70% formic acid. Cyanogen bromide (100 mM) was added, and the reaction was allowed to proceed in the dark at room temperature overnight. CNBr peptide fragments were separated in a 16.5% Tricine SDS−PAGE system (*18*), and radioactive bands were detected using a Phosphorimager 445 SI (Molecular Dynamics). The radioactive bands were excised from the gel and subjected to N-terminal amino acid sequencing using automated Edman degradation. This was carried out on an Applied Biosystems 492 protein sequenator (Protein Core laboratory of Comprehensive Cancer Center of Wake Forest University School of Medicine, Winston-Salem, NC).

Mass Spectrometry Analysis of hBCATm (Both Unlabeled and Labeled with DTNB). The protein samples were run on a Micromass Quattro II triple quadropole mass spectrometer (Micromass, Manchester, England) fitted with an electrospray source. Flow injection analysis was used with the carrier solvent consisting of 50:50 acetonitrile/water with 1% formic acid. Approximately 10% acetonitrile was added to the protein samples, which were in 10 mM ammonium bicarbonate, and 5−10 μL (1 nmol of hBCATm) of the sample solution was injected. Each sample required 8 to 20 scans (3 s scans for 1 min), and the data were processed using MassLynx Version 2.0 and the Maximum Entropy software supplied with the program to generate spectra on the absolute molecular weight scale. The Quattro II mass spectrometer was purchased in part with funds from National Science Foundation BIR-9414018 and updated with funds from the North Carolina Biotechnology Center (9903IDG-1002) and Winston-Salem Foundation. Part of the operating costs for Analytical Chemistry Laboratory came from National Cancer Institute Center Grant CA12107 awarded to the Comprehensive Cancer Center of Wake Forest University.

Crystallographic Studies. Coincident with these biochemical studies of hBCATm, X-ray crystallography was used to analyze the location and structural implications of Cys315 and Cys318 in both the PLP (*7*) and ketimine forms of hBCATm.[2] Crystals of hBCATm were obtained using the vapor diffusion method of hanging drops at room temperature. The ketimine intermediate was obtained by soaking the crystals in the substrate isoleucine and monitoring the color change from yellow (bound PLP) to clear. Data for the isoleucine soaked crystals were collected to 1.95 Å resolution at the synchrotron source in Advanced Light Source, 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). Details of the crystallization conditions are given in Yennawar et al.[2]

RESULTS AND DISCUSSION

Chemical Modification of hBCATm with Sulfhydryl Reagents. The sensitivity of the hBCAT isozymes to inhibition by sulfhydryl reagents and the requirement for the presence of a reducing agent for maximal enzyme activity suggested that the state of reduction of cysteine residues in the protein influences catalytic function (*5*, *12*, *13*). On the basis of the deduced amino acid sequence, hBCATm contains six cysteine residues per monomer (*5*). With native enzyme, DTNB titration of purified recombinant hBCATm indicated that loss of enzyme activity occurred when approximately two thiol groups per monomer were titrated (*5*). To identify the reactive cysteine residues, hBCATm was labeled with DTNB and digested with endoproteinase Lys C, and the peptides in the digest were separated by reverse-phase HPLC (Vydac, 218TP). However, the reactive cysteine residues could not be identified because the DTNB label appeared to be lost during digestion and peptide separation.

[2] Yennawar, N., Conway, M. E., Yennawar, H., Farber, G. K., and Hutson, S. M. (2002) *Biochemistry*, in press.

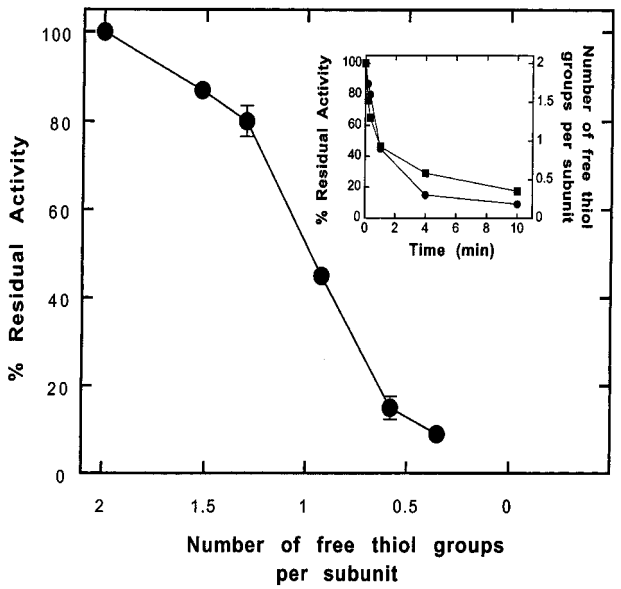


FIGURE 1: Inactivation of hBCATm with a 20-fold molar excess of NEM. hBCATm was exchanged into buffer containing 50 mM HEPES, pH 7.0, and 1 mM EDTA. Seven nmole aliquots of protein were incubated with a 20-fold molar excess of NEM. Each reaction was stopped with a 100-fold molar excess of DTT at times ranging from 10 s to 10 min. Labeled hBCATm was isolated from excess reagents using a PD10 column equilibrated in 50 mM HEPES, pH 7.0, and 1 mM EDTA. Samples were then diluted and assayed for enzyme activity as described in Materials and Methods and expressed as % residual activity with respect to control. The number of thiol groups titrated in each sample was also assessed by DTNB titrations. The inset illustrates the time-dependent disappearance of reactive thiol groups (●) and loss of activity (■) on incubation with DTNB.

Therefore, to identify other inhibitory sulfhydryl reagents (to replace DTNB), a panel of irreversible sulfhydryl reagents was screened for their effect on hBCATm activity, and the results were compared to those with DTNB (Table 1). NEM and monobromobimane (hydrophobic), eosin-5-maleimide and 2,2′-dithiodipyridine (large and hydrophilic), CPM (moderately hydrophobic), and *N*-(1-pyrene) maleimide (hydrophobic) all inhibited hBCATm activity by greater than 90%, suggesting that the reactive cysteine residues are readily accessible. Neither iodoacetamide nor IAEDANS, both of which contain iodine, were good inhibitors. Since these reagents act via molecular displacement of the iodine, the results suggest that there is some steric hindrance that limits the effectiveness of these reagents at one or both of the reactive cysteine residues.

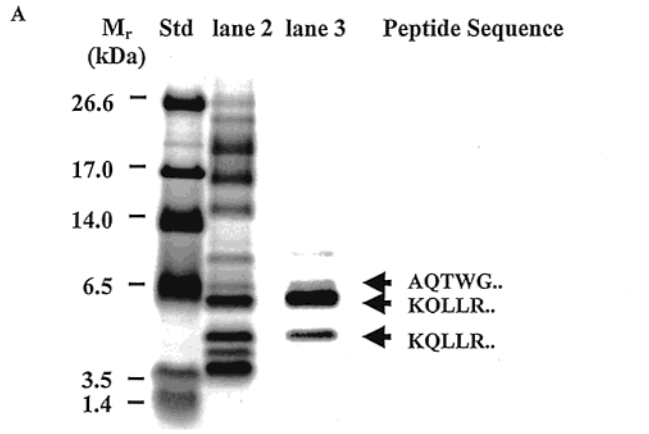
NEM, an irreversible modifier of thiol groups, was used to address the question of which of the six cysteine residues were reactive. The extent of reaction of NEM with hBCATm was characterized by subsequent thiol determination (using DTNB), and the % residual activity of the NEM labeled hBCATm protein was measured. Figure 1 demonstrates that incubation of hBCATm with NEM results in a loss of activity correlated with a loss in the number of free thiol groups titrated. Approximately one thiol group reacts very rapidly (within the first minute) with 56% loss in activity. The titration of the second group occurs at a slower rate (over 10 min) with a further 35% loss in activity (Figure 1, inset). Therefore, NEM modifies approximately two reactive thiol groups per monomer resulting in nearly full inhibition of enzyme activity. From the data, it appears that one of the thiol groups is more reactive than the other and has a slightly greater impact on the activity of the enzyme. For complete inactivation, it is necessary to label both of these groups. The similar effects of DTNB and NEM treatments on the enzyme suggest that both reagents modify the same sulfhydryl groups.

To understand the inactivation mechanism, the protective effects of the substrates leucine and α-ketoisocaproate on NEM and DTNB-linked inactivation of hBCATm were studied. The inactivation of hBCATm by NEM was completely unprotected by substrates, whereas inactivation of enzyme by DTNB was partially protected (10−15%). These results suggest that the cysteine residues are located near but not in the active site.

Identification of the Reactive Cysteine Residues in hBCATm. To identify which cysteine residues were modified by DTNB and NEM, hBCATm protein was labeled with ^{125}I-β-4HPEM-NEM and chemically cleaved with CNBr. Separation of the peptide fragments on a 16% tricine gel revealed two major radiolabeled bands with molecular masses of approximately 5400 and 4700 Da and a more faint band at 6500 Da (lane 3 Figure 2A). The theoretical cleavage map is shown in Figure 2B. From the theoretical map, peptide M10 contains Cys315 and Cys318 and has a calculated molecular mass of 4790 Da. If both Cys315 and Cys318 reacted with ^{125}I-β-4HPEM-NEM, the molecular mass of the M10 fragment would increase by ∼600 Da, resulting in a peptide with a predicted molecular mass of 5400 Da. This peptide corresponds to the heaviest labeled band in Figure 2A. The other four cysteine residues would be located on fragment M7, which has a predicted molecular mass of 14 700 Da. While a band greater than 14 kDa was observed on the Coomassie stained gel, no corresponding high molecular weight radioactive peptide was found (lanes 2 and 3 of Figure 2A, respectively). N-terminal amino acid sequencing of the 5400 and 4700 Da radiolabeled peptides revealed the same amino acid sequence: Lys−Gln−Leu−Leu−Arg−Ala−Leu−Glu−Glu−Gly−Arg−Val−Arg−Glu for both of the peptide fragments. The lower second band may be due to partial labeling and/or nonspecific cleavage after the cysteine residues. In addition, the third, fainter band (6500 Da) was sequenced (Ala−Glu−Thr−Try−Gly−Glu−Phe−Arg−Val−Glu−Arg−Thr) and was found to correspond to the N-terminal sequence of the predicted peptide fragment from the cleavage site before that of M10. This peptide thus corresponds to the combined M9−M10 peptide without cleavage at Met293, and should also contain Cys315 and Cys318. In summary, these results confirm that the reactive cysteine residues in hBCATm are Cys315 and Cys318.

Localization of Reactive Cysteine Residues in the hBCATm Structure. Alignment of the amino acid sequence of hBCATm with 37 eukaryotic and bacterial BCAT, five D-AAT, and six ADCL sequences showed that the amino acid sequence containing Cys315 and Cys318 shows considerable conservation of sequence in this region among all the fold type IV proteins. The alignments for 11 representative BCAT sequences are shown in Table 2. The residues highlighted by an asterisk are conserved in nearly all the fold type IV protein sequences or >90% of the BCAT sequences. The two cysteine residues forming a CXVC motif are found only in the mammalian BCAT proteins.

The three-dimensional structure of the PLP form of hBCATm in two crystal forms (PDB codes 1EKP, 1EKF)



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1    ASSSFKAADL  QLEMTQKPHK  KPGPGEPLVF  GKTFTDHMLM
41   VEWNDKGWGQ  PRIQPFQNLT  LHPASSSLHY  SLQLFEGMKA

81   FKGKDQQVRL  FRPWLNMDRM  LRSAMRLCLP  SFDKLELLEC   M7
121  IRRLIEVDKD  WVPDAAGTSL  YVRPVLIGNE  PSLGVSQPRR

161  ALLFVILCPV  GAYFPGGSVT  PVSLLADPAF  IRAWVGGVGN
201  YKLGGNYGPT  VLVQQEALKR  GCEQVLWLYG  PDHQLTEVGT

241  MNIFVYWTHE  DGVLELVTPP  LNGVILPGVV  RQSLLDMAQT
281  WGEFRVVERT  ITMKQLLRAL  EEGRVREVFG  SGTACQVCPV   M10

321  HRILYKDRNL  HIPTMENGPE  LILRFQKELK  EIQYGIRAHE
361  WMFPV
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FIGURE 2: Identification of the reactive cysteine residues in hBCATm using ^{125}I-β-4HPEM-NEM (A) and predicted CNBr cleavage sites (B). Human BCATm was labeled with ^{125}I-β-4HPEM-NEM and chemically cleaved with CNBr overnight in the dark. The labeled samples were pretreated with 5% β-mercaptoethanol and incubated at 37 °C for 30 min. The fragments were separated on a 16.5% Tricine-SDS−PAGE system (panel A). Lane 1 contains low-range protein standards from BioRad with the indicated M_r values. Lane 2 contains 125 μg of hBCATm protein treated with CNBr and stained with Coomassie blue. The gel was then submitted to phosphor imaging to visualize the radiolabeled peptides (lane 3). The arrows indicate major radioactive peptides that were subjected to N-terminal amino acid sequencing. Shown in panel B are the predicted sites of cleavage for CNBr after methionine residues (shown in bold letters). The six cysteine residues of hBCATm are double underlined, and four are in the peptide designated M7 (shown in italics). The peptide designated M10 (underlined), contains the reactive residues, Cys315 and Cys318, which were identified using iodinated ^{125}I-β-4HPEM-NEM.

(*7*) shows that each monomer of the hBCATm homodimer is composed of a small domain (residues 1−170) and a large domain (residues 182−365) connected by an interdomain loop (residues 171−181). Cys315 and Cys318 are located in the large domain. This domain is built of four α-helices and a ten-stranded antiparallel β-pleated sheet (*7*). Cys315 is located between the β-strands comprised of residues 305−311 and 317−324, with Cys318 in the beginning of the second strand. Although shorter in length, equivalent antiparallel β-strands are found in the known fold type IV protein structures including *E. coli* ADCL (β13 and β14) (*6*), *Bacillus subtilis* D-AAT (β13 and β14) (*19*), and hBCATm (β14 and β15) (*7*).

In both the orthorhombic and monoclinic crystal structures of the PLP form of hBCATm, Cys315 and Cys318 are about 10 Å from the active site and do not appear to interact directly with any residues involved in PLP or substrate binding. However, the cysteine residues are close to residues in the active site which are structurally important. The crystal

Table 2: Alignment of the amino acid sequence of hBCAT with eukaryotic and prokaryotic organisms[a]

BCAT PROTEINS	SEQUENCE ALIGNMENTS
hBCATc	RVREMFSSGTA**CVVC**PVSDILYK
hBCATm	RVREVFGSGTA**CQVC**PVHRILYK
rBCATc	RVKEMFGSGTA**CVVC**PVASILYE
rBCATm	RVREVFGSGTA**CQVC**PVHQILYE
sBCATc	RVREMFSSGTA**CVVC**PVSTILYK
sBCATm	RVREVFGSGTA**CQVC**PVHQILYQ
C.elegans 1	RLYEMFGSGTACVVSPVGKILYH
C.elegans 2	RVHEFFVSGTAANVGPVSEIVYC
A.thaliana	DADEVFCTGTAVVVAPVGTITYQ
E.coli	LADEVFMSGTAAEITPVRSVDGI
M.tuber	EITEVFACGTAAVITPVARVRHG
	* * *** **

[a] The CXXC region is highlighted in bold and is conserved only in mammalian BCAT proteins. The residues highlighted by an asterisk are conserved in nearly all the fold type IV protein sequences. Accession numbers and sources of the putative BCAT proteins shown are hBCATc (*5*); hBCATm (human, U21551); rBCATc (rat, U35774); rBCATm (rat, U68417); sBCATc (sheep, AF184916); sBCATm (sheep, AF050173); *Caenorhabditis elegans* 1 (P54688); *Caenorhabditis elegans* 2 (NM075613); *Arabidopsis thaliana* (AJ271731); *Escherichia coli* (NC000913); *Mycobacterium tuberculosis* (AE007072).

structures show that Cys315 and Cys318 also share a hydrogen bond, which under oxidizing conditions can reasonably form a disulfide bond with little structural rearrangement. The bond lengths (e.g., 3.09 and 3.46 Å in monomers A and B, respectively, of the orthorhombic PLP form) and angles (e.g., in monomers A and B, the angle at C^{β} 315- S^{γ} 315-S^{γ} 318 is 122.8° and 122.7°, respectively, and the angle at C^{β} 318- S^{γ} 318-S^{γ} 315 is 65.9° and 63.2°, respectively, in the orthorhombic PLP form) are consistent with the presence of thiol−thiolate hydrogen bonds in both monomers, which given the geometry suggests that Cys318 S^{γ} is the ionized form and the Cys315 is the thiol hydrogen bond donor. The proximity of the two sulfur atoms in monomer A is in fact very close; similar close hydrogen bonds (3.0−3.1 Å) between thiol and thiolate sulfurs have been seen in redox-active disulfide centers of both reduced thioredoxin (*20*, *21*) and the N-terminal domain of AhpF, a flavin-containing disulfide reductase (*22*). The interaction seen between Cys315 and Cys318 affects the geometry of the preceding β-turn between residues 312 and 314. The peptide dipole vectors at these residues in this loop are close to parallel to each other such that the positive ends of the dipoles are stabilizing the negative charge on the phosphate (Figure 3A).[2] Since the carboxylate of the bound substrate (isoleucine) is on the same side as the phosphate of the PLP in the BCAT enzymes, additional stabilization of the negative charges in this region is beneficial for enzyme activity.[2] As shown in Figure 3B, the dipole vector is perturbed in the Tris-inhibited hBCATm structure (PDB code 1EKV), and the Cys315 and Cys318 hydrogen bond is disrupted.[2]

Two additional crystal structures, of hBCATm in the pyridoxamine phosphate form and with isoleucine as the ketimine intermediate, were also solved (the PDB code for the pyridoxamine phosphate form is 1KTA and that for the ketimine is 1KT8) and give insight into the roles the cysteine residues may play.[2] These structures suggest that modification or oxidation of Cys315 and/or Cys318 could be expected to interfere with hBCATm activity. As shown in Figure 4A, the cysteine residues are at the entrance to the active site and do not interact with other residues prior to substrate

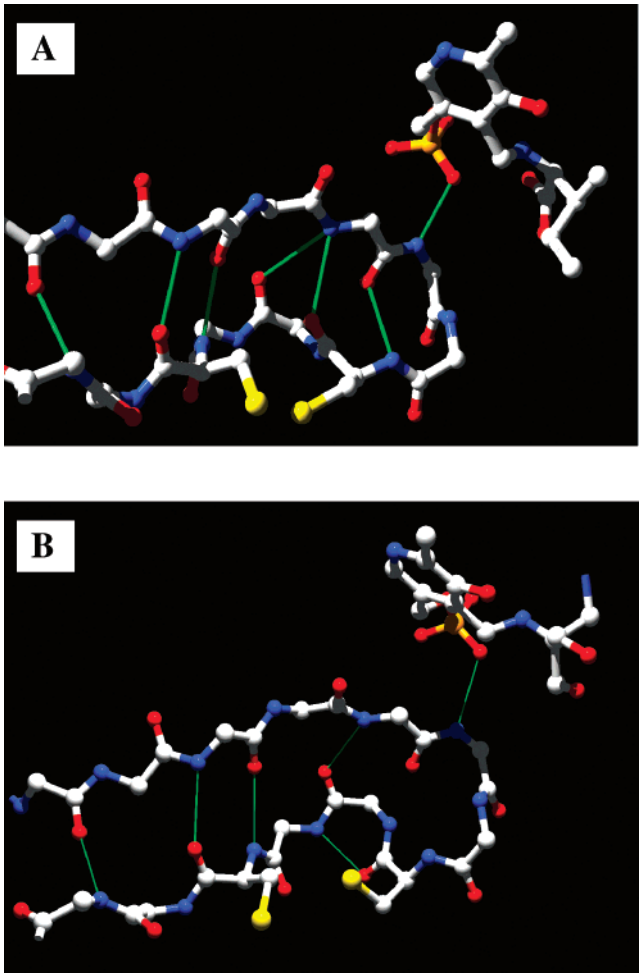


FIGURE 3: The location of Cys315 and Cys318 in relation to the peptide dipole interaction with the phosphate of the cofactor in the ketimine (A) and Tris-inhibited (B) forms of monomer B. Cys315 and Cys318 participate in a thiol−thiolate hydrogen bond with the average distance between the sulfurs ranging from 3.09 to 3.46 Å in the different crystal forms. (A) The peptide dipole vectors in the β-turn including residues 312−314 are parallel to each other such that their partial positively charged end faces the negatively charged phosphates. (B) In the Tris-inhibited structure (PDB code 1EKV), the thiol−thiolate hydrogen bond is disrupted and consequently the dipole alignment in the β-turn is perturbed.

binding. The positioning of the cysteine residues of hBCATm, external to the active site, explains why incubation of the enzyme with either branched chain amino acids or the respective keto acids provided little or no protection against inhibition by DTNB and NEM. In the ketimine form of hBCATm, the Cys315 and Cys318 bond distances suggest that the thiol thiolate bonds are retained in both monomers (3.07 Å for monomer A and 3.20 Å in monomer B) with shortening of the hydrogen bond distance in monomer B (Figure 4B). The interdomain loop (residues 171−181) is mostly disordered in monomer A and ordered in monomer B in the various forms of the enzyme. The ring atoms of Tyr173 in monomer B move, increasing the opening into the active site from about 9 Å in the pyridoxal phosphate form of the enzyme to 11.5 Å in the ketimine and the pyridoxamine phosphate forms. The Tyr173 side chain moves closer to S^{γ} atom of Cys315 (3.9 Å in monomer B of the PLP form versus 3.2 Å in monomer B of the ketimine form) and may be stabilized by this interaction (Figure 4B). Therefore, domain closure is not observed during catalysis.[2]

Cysteine solvent accessibility calculations for Cys315 and Cys318 using the program AREAIMOL, which is part of the CCP4 program suite (*23*), suggest that in monomer B of the PLP form Cys315 (4.0 Å^2) and Cys318 (3.0 Å^2) have similar solvent accessibility, whereas in monomer A Cys315 (30 Å^2) is slightly more solvent exposed than Cys318 (9.0 Å^2). In general in the other structures Cys315 is slightly more solvent exposed than Cys318. Cys315 may therefore be more exposed for reaction with the inhibitors, although Cys318 is likely to be the more nucleophilic of the two residues if in fact it is the thiolate. Interestingly, the structural and functional properties of these cysteinyl residues in hBCATm appear to differ from those in typical CXXC centers. Generally, the cysteine residue of a CXXC center closest to the N-terminus of a protein is the nucleophilic thiolate anion with the lower pK_a, and this residue lies just prior to an α-helix for charge stabilization by the helix dipole (*24*, *25*).

Several models could explain the effects of DTNB or NEM labeling on hBCATm activity. First, the derivatized cysteine residues could partially block access of the substrate to the active site either directly or through steric hindrance affecting the motion of Tyr173 during movement of the interdomain loop. Second, a change in the geometry of the residues at the active site could occur upon addition of the bulky TNB or NEM moieties or upon disulfide bond formation. Third, the peptide dipole vectors of Gly312, Thr313, and Ala314 align to form a net positive dipole moment in the region of the pyridoxal phosphate oxygens (Figure 3A) as mentioned above. The formation of the dipole aids PLP in its role as an electron-withdrawing moiety, which is important for the enzyme chemistry. The side-chains of Cys315 and Ser311 seem to provide the anchor to stabilize this backbone structure. DTNB and NEM might therefore also disrupt the formation of a net dipole, removing this favorable environment. In addition, Thr313 (conserved in BCAT proteins) hydrogen bonds to the phosphate of PLP and to the carboxylate of the substrate as seen in the ketimine structure of hBCATm. Binding of DTNB or NEM may also perturb this interaction affecting the activity of the enzyme.

Evidence for Redox Cycling and Impact on Activity. The X-ray crystallographic results show that Cys315 and Cys318 share a hydrogen bond. The close proximity of these two cysteine residues raises the possibility that they may be capable of disulfide formation. The CXXC motif is common to redox-active disulfide centers, and disulfide formation in hBCATm would be consistent with the apparent loss of TNB from DTNB-treated hBCATm as shown in the peptide mapping experiments.

On reaction of hBCATm with a 100-fold excess of DTNB for 10 min, 1.8−2.0 equivalents of *p*-nitrothiobenzoate (TNB) are released from the native enzyme. Approximately six equivalents of TNB (5.7 ± 0.4) are released from the guanadinium denatured enzyme, suggesting that none of the six cysteine residues per molecule of enzyme is involved in intra- or intermolecular disulfide bridges. However, upon storage of hBCATm for several days in the absence of DTT, the total number of titratable SH groups decreases to 3.7, suggesting that one or more disulfide bonds are gradually being formed (*5*). This process was accompanied by a decrease in activity.

Evidence for a redox-active CXXC center was obtained by further analysis after titrating hBCATm with DTNB. Ellmans reagent, DTNB, modifies thiol groups through a

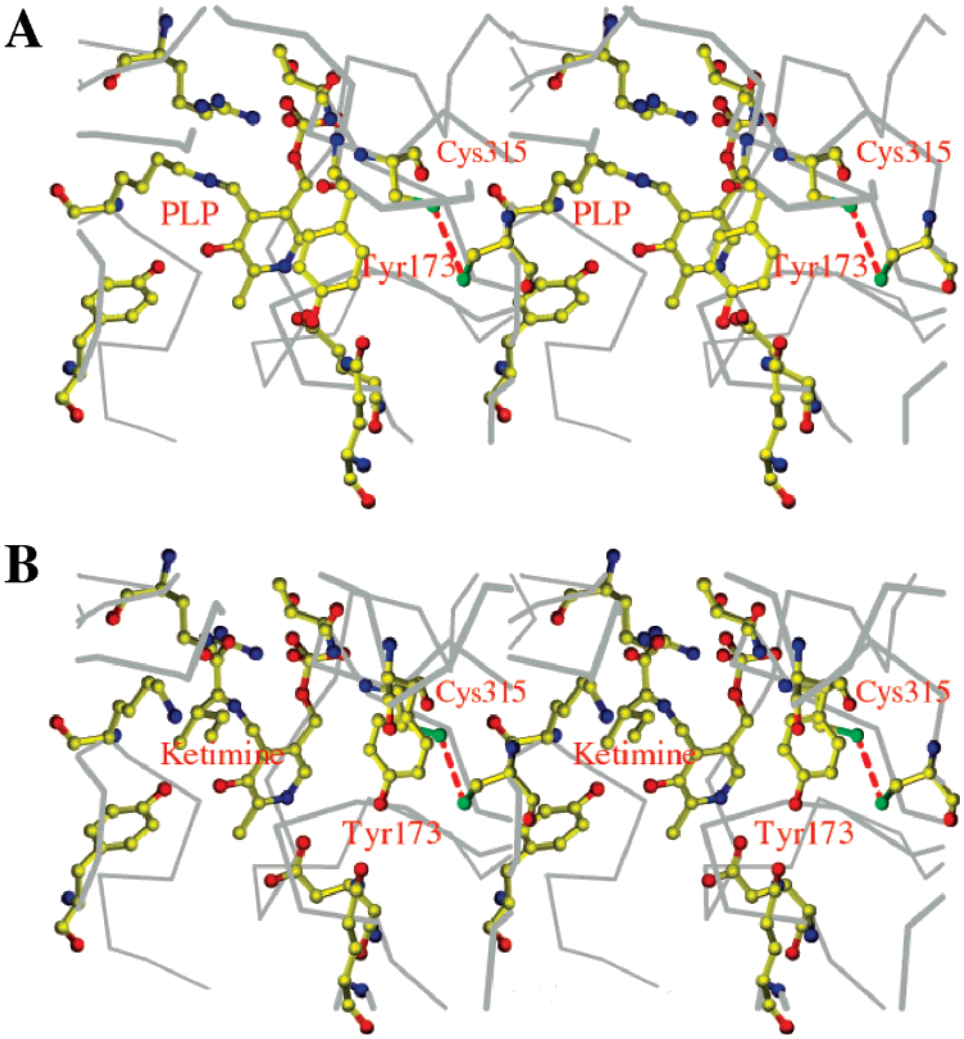
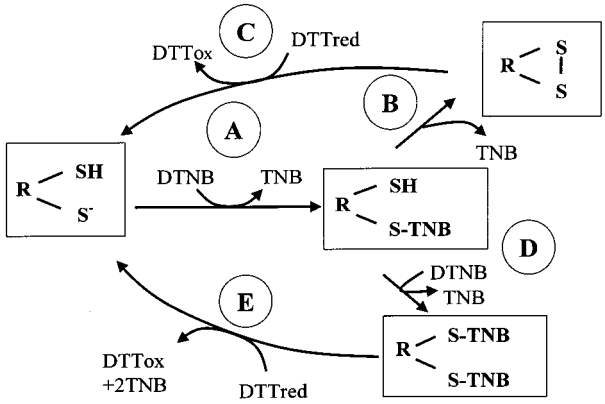


FIGURE 4: Stereoview of Cys315 and Cys318 near the active site of hBCATm in both the PLP crystal form (without substrate, panel A) and in the ketimine form (with isoleucine, panel B). The backbone of hBCATm is shown in gray. Sulfur, oxygen, and nitrogen groups are shown in green, red, and blue, respectively. The residues in the active site are (clockwise) Cys315 (upper right), Cys318, Gln224, Glu237, Tyr207, Lys202, Arg99, Thr313, Tyr173. Without substrate (A), the cysteine residues are not interacting with other residues in the substrate-binding pocket. In the ketimine form (B) (with isoleucine as the substrate), however, the ring atoms of Tyr173 move closer to the S^{γ} atom of Cys315, widening the access channel to the active site. In addition, a new hydrogen bond between the hydroxyl of Tyr173 and the side chain oxygen of Gln224 is formed.

thiol-disulfide interchange reaction by forming a mixed disulfide with the cysteinyl residue and releasing a free TNB anion (Scheme 1, A and D). On formation of a TNB-conjugated cysteine within many CXXC motifs, the thiol group of the other cysteine can readily react to form an intrasubunit disulfide bond (another thiol/disulfide interchange reaction), and the TNB of the mixed disulfide is released (Scheme 1B). The net result is the reduction of one DTNB molecule to form two free TNB anions and the oxidation of the protein dithiol to the disulfide (Scheme 1B). Where hBCATm was labeled with a 100-fold molar excess of DTNB for 15 min, the stoichiometry of TNB released was estimated to be between 1.9 and 2.1 per subunit (n = 4). The unreacted DTNB was removed, and a 100-fold excess of DTT was added to quantitatively release any TNB remaining bound to cysteinyl residues. The stoichiometry of 0.4−0.6 TNB molecules released per subunit (n = 4) on DTT treatment was significantly lower than the 2.0 released during the initial reaction with DTNB, strongly suggesting the formation of 0.7−0.8 disulfide bonds per subunit on DTNB treatment of hBCATm (Scheme 1, C versus E). To obtain additional evidence that the process depicted in Scheme 1B can occur, hBCATm was incubated with limiting amounts of DTNB. Approximately two TNB molecules were released per molecule of DTNB indicating a preference for pathway B over pathway D under these conditions.

Scheme 1



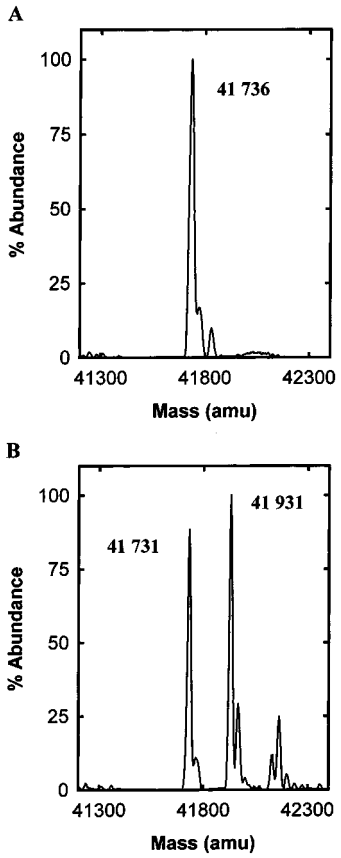


FIGURE 5: Electrospray mass spectrometry analysis of hBCATm samples in the absence (A) or presence (B) of DTNB treatment. Human BCATm (control) and the DTNB-labeled protein were dialyzed in 10 mM ammonium bicarbonate. The samples were then prepared for mass spectrometry analysis as described in Materials and Methods. Shown are the transformed data which represent the relative abundance of masses for control hBCATm (A) and hBCATm incubated with DTNB (B). The major peaks shown in A (41 736) and B (41 731 and 41 931) were accompanied by minor peaks at higher molecular weights in each case (41 784 and 41 836 in panel A, and 41 760 and 41 968 amu in panel B), which may represent adventitious oxidation on prolonged incubation (addition of two oxygen atoms). Two other minor species in panel B likely represent protein labeled with two TNB moieties (42 124) and a corresponding oxidized species (42 164 amu).

Electrospray ionization mass spectrometry (ESI-MS) of the DTNB-labeled protein showed that there was a mixture of species in the sample. Figure 5A shows the protein in its reduced state with a molecular mass at 41 736 ± 5 (the predicted molecular mass based on the amino acid sequence is 41 731 (*5*)). The two minor peaks (41 784 and 41 836) may represent some oxidation of hBCATm as it was dialyzed without DTT overnight. Figure 5B representing the DTNB-treated hBCATm shows two main peaks, one with a molecular mass of 41 731 ± 5 and a second peak with a molecular mass of 41 931 ± 5. Given that one TNB moiety represents 198 amu, the peak at approximately 41 931 amu corresponds to hBCATm with one covalently attached TNB. On the basis of the titration results, the 41 731 amu species should represent oxidized hBCATm with one disulfide bond (theoretically a loss of 2 amu). Minor peaks are also observed at 41 760, 41 968, 42 124, and 42 164 amu. The peak at 42 124 is consistent with the addition of two TNB molecules. Mass shifts of about 32 Da for each of the main peaks in the spectrum suggest partial oxidation of the protein with the addition of two oxygen atoms. In summary, the mass spectrometry data, in conjunction with the titration studies, indicate that DTNB-labeled hBCATm is predominantly a mixture of singly TNB-labeled protein and an oxidized form containing a disulfide bond.

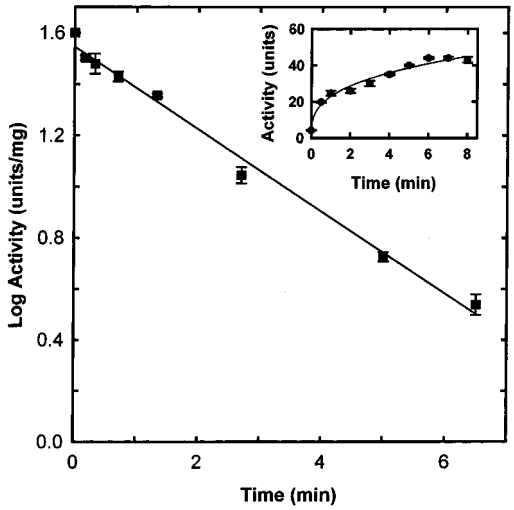


FIGURE 6: Log of residual activity of hBCATm incubated with a 10-fold molar excess of DTNB. The reaction was carried out in 50 mM HEPES, pH 7.0, with 1 mM EDTA, in a total volume of 1.0 mL at room temperature. Aliquots were taken at the given time points and assayed for activity as described in Materials and Methods. The sample labeled for 10 min was then treated with 100-fold molar excess of DTT, and aliquots were removed to determine the activity of hBCATm (inset). Data are represented as a mean ± SEM ($n = 6$).

To assess the functional effect of TNB labeling and/or disulfide formation, hBCATm was incubated with a 10-fold molar excess of DTNB, and activity was measured over time (Figure 6). A decrease in activity over time was observed, and after 6.5 min, only 7.5% of the initial activity remained. The loss in activity of hBCATm by DTNB labeling is reversed by the addition of DTT (Figure 6 inset). Under conditions where a degree of labeling similar to that of the sample analyzed by ESI-MS (Figure 5) was observed (approximately 2 TNB molecules released per subunit), the enzyme was essentially completely inactivated. Thus, the species observed in Figure 5B represent inactive forms of hBCATm, and the 41 731 amu species is likely to represent oxidized hBCATm with an intramolecular disulfide bond.

To avoid the generation of a mixed population of TNB-labeled and disulfide-containing hBCATm and to assess the capacity of H_2O_2 to oxidize the putative redox-active CXXC in hBCATm, H_2O_2 was used in place of DTNB as the oxidant. Cellular H_2O_2 can oxidize methionine and cysteine residues in proteins to methionine sulfoxide and cysteine sulfenic acid (or disulfide), respectively, each of which can be readily reduced back to the unmodified amino acid by various cellular reductants. In mammalian cells, certain extracellular stimuli including cytokines and growth factors induce a transient increase in the intracellular concentration of H_2O_2 (*26*). Direct inhibitory effects of H_2O_2-mediated oxidation are observed in some proteins, e.g., protein tyrosine phosphatases and papain, containing essential, activated (i.e., low pK_a) cysteinyl residues at their active sites (*27*). This phenomenon, designated redox signaling, could apply to other transcriptional regulators and cysteine-containing enzymes such as hBCATm.

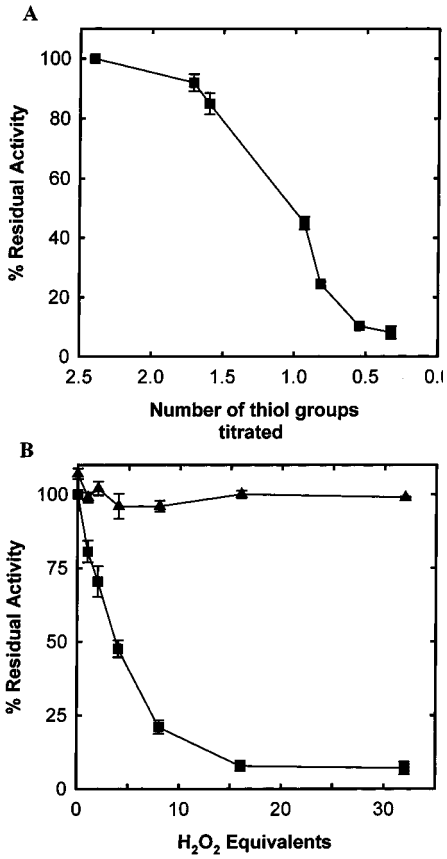


FIGURE 7: The effect of H_2O_2 on both the activity and thiol content of hBCATm. The reactions were carried out in 50 mM HEPES, pH 7.0, and 1 mM EDTA at 4 °C for 18−24 h. As shown in panel A, residual activity was determined and compared with the number of titratable thiol groups ($n = 3$). In panel B, samples were treated with H_2O_2 as in panel A (■), and then reincubated with 100-fold excess of DTT (and reanalyzed for activity) (▲) ($n = 5$).

Treatment of hBCATm with 1−32 equiv of H_2O_2 results in a "dose dependent" decrease in titratable thiols and a corresponding, nearly complete loss in activity (Figure 7A). Following incubation of hBCATm with H_2O_2, addition of excess DTT resulted in complete recovery of the activity as compared with untreated controls (Figure 7B). Reduced thioredoxin from *E. coli* was also partly able to restore the activity of H_2O_2 treated hBCATm (data not shown). These results, together with the NEM and DTNB labeling experiments described above, implicate Cys315 and Cys318 as a redox-active CXXC center that can regulate hBCATm activity. Moreover, inhibitory disulfide bond formation that occurs on addition of H_2O_2, provides the basis for future studies to determine if this regulatory feature is of physiological importance. Thus, hBCATm may be added to the growing list of enzymes in which a redox-sensitive switch allows for modulation of its activity in response to the redox status of the cell.

CONCLUSION

In summary, we have shown that hBCATm contains a redox-active CXXC center. These two cysteinyl residues lie within an otherwise conserved region of the sequence, but are found only in the mammalian BCAT proteins. The reactive cysteine residues of hBCATm were identified as Cys315 and Cys318 and are responsible for the requirement of hBCATm for reducing agents to maintain maximal activity. In this study, Cys315 and Cys318 have been shown to comprise a regulatory redox-active CXXC center, where the activity of hBCATm can be controlled by the oxidant H_2O_2 and the reductant DTT. In addition, Cys315 and Cys318 form a thiol−thiolate hydrogen bond in several forms of the enzyme, and Cys315 plays a structural role by anchoring Tyr173 during catalysis. In conclusion, the data presented herein show that the cysteine residues Cys315 and Cys318 are key residues in the control of hBCATm activity through a CXXC redox center.

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