Roles for Cysteine Residues in the Regulatory CXXC Motif of Human Mitochondrial Branched Chain Aminotransferase Enzyme†

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ABSTRACT: The redox-active dithiol/disulfide C315-Xaa-Xaa-C318 center has been implicated in the regulation of the human mitochondrial branched chain aminotransferase isozyme (hBCATm) [Conway, M. E., Yennawar, N., Wallin, R., Poole, L. B., and Hutson, S. M. (2002) *Biochemistry 41*, 9070−9078]. To explore further the mechanistic details of this CXXC center, mutants of the Cys residues at positions 315 and 318 of hBCATm were individually and in combination converted to alanine or serine by site-directed mutagenesis (C315A, C315S, C318A, C318S, C315/318A, and C315/318S). The effects of these mutations on cofactor absorbance, secondary structures, steady-state kinetics, and sensitivity toward hydrogen peroxide (H_2O_2) treatment were examined. Neither the UV−visible spectroscopic studies nor the circular dichroism data showed any major perturbations in the structure of the mutants. Kinetic analyses of the CXXC mutant proteins indicated primarily a modest reduction in k_{cat} with no significant change in K_m. The largest effect on the steady-state kinetics was observed with the C315 single mutants, in which substitution of the thiol group resulted in a reduced k_{cat} (to 26−33% of that of wild-type hBCATm). Moreover, the C315 single mutants lost their sensitivity to oxidation by H_2O_2. The kinetic parameters of the C318 mutants were largely unaffected by the substitutions, and as with wild-type hBCATm, reaction of the C318A mutant protein with H_2O_2 resulted in the complete loss of activity. In the case of oxidized C318A, this loss was largely irreversible on incubation with dithiothreitol. Mass spectrometry and dimedone modification results revealed overoxidation of the thiol group at position 315 to sulfonic acid occurring via a sulfenic acid intermediate in the H_2O_2-treated C318A enzyme. Thus, C315 appears to be the sensor for redox regulation of BCAT activity, whereas C318 acts as the "resolving cysteine", allowing for reversible formation of a disulfide bond.

Transamination of the branched chain amino acids (BCAA),[1] leucine, isoleucine, and valine, to their respective α-keto acids, α-ketoisocaproate, α-keto-β-methylvalerate, and α-ketoisovalerate, respectively, is catalyzed by pyridoxal phosphate (PLP)-dependent branched chain aminotransferases (BCATs) (EC 2.6.1.42). There are two BCAT isozymes in humans, a mitochondrial (hBCATm) form found in most tissues and a cytosolic (hBCATc) form which is localized primarily in the central nervous system (for a review, see ref *1*). Furthermore, two alternatively spliced isoforms of hBCATm have been reported recently. One contains a 12-amino acid deletion that starts immediately following C315. This isoform binds to and acts as a corepressor of thyroid hormone receptor β1 (*2*). The second isoform contains a 100-amino acid deletion that starts shortly after the N-terminal methionine. Thus, it lacks the mitochondrial targeting sequence, and is found in the cytosol of placenta and most human tissues (*3*).

The BCATs are classified in the fold type IV class of PLP-dependent enzymes (*4*−*7*). The members of this class include the BCAT enzymes as well as bacterial D-amino acid aminotransferase (which has stereospecificity that is the opposite of the BCAT, D-amino acids vs L-amino acids) (*5*), and bacterial 4-amino-4-deoxychorismate lyase (*8*). A unique feature of this group of three 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 (*9*).

A distinctive feature of hBCATm and hBCATc proteins is a CXXC motif located ∼10 Å from the active site (*7*). Multiple-sequence alignments of the BCAT proteins indicate that this CXXC motif is conserved in mammalian proteins but not in lower eukaryotes or prokaryotes or in other fold

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[1] Abbreviations: BCAA, branched chain amino acids; BCAT, branched chain aminotransferase; hBCATm, human mitochondrial branched chain aminotransferase; hBCATc, human cytosolic branched chain aminotransferase; WT, wild-type; CXXC mutant proteins, C315A, C318A, C315/318A, C315S, C318S, and C315/318S; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; PLP, pyridoxal phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; H_2O_2, hydrogen peroxide; ESI-MS, electrospray ionization mass spectrometry; TNB, 2-nitro-5-thiobenzoate; NBD chloride, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; NBD, 4-nitrobenz-2-oxa-1,3-diazole.

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type IV PLP enzymes (*10*). X-ray crystallographic results indicate that the short distance between the sulfur atoms (3.09−3.46 Å) of the two CXXC cysteines is compatible with disulfide bond formation under oxidizing conditions (*7*). Recently, it was shown that modification of C315 and C318 with thiol specific reagents or oxidation of the thiols to a disulfide bond results in the loss of hBCATm activity (*10*). Moreover, addition of dithiothreitol (DTT) completely reversed the oxidation and restored activity, suggesting that this peroxide-sensitive CXXC center is involved in the redox-linked regulation of hBCATm activity (*10*).

To further understand the regulation of hBCATm by this redox-active CXXC motif, either C315, C318, or both were mutated to alanine or serine using site-directed mutagenesis. As reported herein, the results strongly suggest that C315 is the peroxide-reactive thiol(ate) functioning as the redox sensor in the regulation of hBCATm. The second cysteine, C318, subsequently reacts with the nascent sulfenic acid form of C315, forming a disulfide bond and permitting reversible regulation by preventing overoxidation of hBCATm.

EXPERIMENTAL PROCEDURES

Materials. DTT, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dimedone, α-ketoisocaproate, α-ketoisovalerate, and PLP were obtained from Sigma (St. Louis, MO). Hydrogen peroxide (H_2O_2) (30%) was purchased from Fisher Scientific (Suwanee, GA). The PD10 columns and the Mono-Q HR 5/5 (1 mL) anion-exchange column were from Amersham Pharmacia Biotech (Piscataway, NJ). Apollo ultrafiltration devices (7 mL) were from Orbital Biosciences (Topsfield, MA). The QuikChange site-directed mutagenesis kit was from Stratagene Corp. (La Jolla, CA). The QIAprep spin miniprep kit and the MinEluate gel extraction kit were from Qiagen (Valencia, CA). Oligonucleotides were synthesized by MWG Biotech (High Point, NC). Purified human thrombin was obtained from Enzyme Research Laboratories (South Bend, IN).

Site-Directed Mutagenesis of Wild-Type (WT) hBCATm. The hBCATm cDNA clone previously ligated into the pET-28a expression vector (*11*) was used as the plasmid DNA template for oligonucleotide-directed mutagenesis in this study. To substitute C315 and C318 with alanine or serine in either position or both, synthetic oligonucleotides and their exact complements containing the desired mutations (C315A, C318A, C315/318A, C315S, C318S, C315/318S, and C108S) were designed. The following sense primers were used: 5′-TCG GGC ACC GCT GCG CAG GTC TGC CCA-3′ for C315A, 5′-GCT TGC CAG GTC GCG CCA GTG CAC CGA-3′ for C318A, 5′-TCG GGC ACC GCT TCC CAG GTC TGC CCA-3′ for C315S, 5′-GCT TGC CAG GTC TCC CCA GTG CAC CGA-3′ for C318S, 5′-GGC ACC GCT GCG CAG GTC GCG CCA GTG CAC CGA-3′ for C315/318A, 5′-GGC ACC GCT TCC CAG GTC TCC CCA GTG CAC CGA-3′ for C315/318S, and 5′-GCC ATG CGC CTG TCC CTG CCG AGT TTC-3′ for C108S. The mutagenesis method followed that recommended with the QuikChange kit. Briefly, the PCR was carried out in the MiniCycler PCR machine (MJ Research, Inc.) using the purified double-stranded hBCATm plasmid DNA, and each mutagenic primer pair, for 16 cycles. The PCR product was incubated with *Dpn*I endonuclease, which digests the parental methylated DNA template. Plasmid DNA was purified by agarose gel electrophoresis and extracted using the MinEluate gel extraction kit from Qiagen. The mutated plasmid was transformed into the Epicurian Coli XL1-Blue supercompetent cells, and positive mutant colonies were selected by kanamycin resistance. Colonies were subcultured, and the plasmid DNA was isolated using the QIAprep Spin Miniprep Kit. The desired mutation and fidelity of PCR amplification were confirmed by DNA sequence analysis using the ABI 377 DNA sequenator in the DNA Sequencing Core Laboratory of the Comprehensive Cancer Center of the Wake Forest University School of Medicine. The WT hBCATm and CXXC mutant plasmids were transformed into BL21(DE3) cells as described by Davoodi et al. (*11*).

Expression and Purification of WT hBCATm and Mutant Proteins. Purification of WT and CXXC mutant hBCATm proteins was carried out as previously described by Conway and Hutson (*12*), with the following modifications. Briefly, hBCATm was extracted from pelleted bacteria using sonication. The histidine-tagged fusion protein was purified using nickel−NTA resin (Qiagen, Chatsworth, CA), followed by digestion with thrombin (100 NIH units) to remove the affinity tag. Purified WT or mutant proteins were obtained after anion-exchange chromatography using the Mono-Q HR 5/5 anion-exchange column (Pharmacia), omitting the hydrophobic interaction chromatography step used by Davoodi et al. (*11*). The proteins were selectively eluted using a sodium chloride gradient from 0 to 0.5 M in 10 mM potassium phosphate (pH 8.0) over the course of 20 min at a flow rate of 1.0 mL/min. The purified proteins were 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, and approximately 15% glycerol. The WT hBCATm enzyme could be stored at 4 °C for 2 days or at −20 °C for 1.5−2 months. The CXXC mutant proteins were stable at −20 °C for 6 months. The concentration of the purified protein was estimated using the method of Schaffner and Weissmann (*13*) or determined from the absorbance at 280 nm using the extinction coefficient of 67 600 M^{-1} cm^{-1} per monomer (*11*). The final yield of the pure protein was approximately 10 mg of purified protein/L of *Escherichia coli*.

BCAT Assay and Steady-State Kinetics. The standard assay for BCAT activity was performed at 37 °C by assessing the formation of [1-^{14}C]valine from α-keto[1-^{14}C]isovalerate as described previously (*11*). The standard assay solution (0.5 mL) contained 25 mM potassium phosphate buffer (pH 7.8), 5 mM DTT, 1 mM α-keto[1-^{14}C]isovalerate, and 12 mM isoleucine at 37 °C as described previously (*11*, *12*). A unit of enzyme activity was defined as 1 μmol of valine formed per minute under standard conditions. All assays were performed in duplicate or triplicate. Specific activities of WT BCATm and the mutant enzymes for different amino acids (including the branched chain amino acids, glutamate, tyrosine, tryptophan, phenylalanine, aspartate, threonine, aspartate, norleucine, and norvaline) were compared using 1 mM α-keto[1-^{14}C]isovalerate and an amino acid concentration of 10 mM in the standard assay buffer. For steady-state kinetic determinations, reaction rates with the amino acid/α-keto[1-^{14}C]isovalerate pairs were determined holding the concentration of α-ketoisovalerate constant at 2 mM, which was saturating for the BCAA and glutamate. The concentrations of the amino acids isoleucine (0−2 mM), leucine (0−2 mM), valine (0−30 mM), and glutamate (0−40 mM) were varied. Stock solutions of the dicarboxylic acid amino acid

glutamate were neutralized with KOH. Data were collected for eight or ten concentrations of each amino acid. K_m and V_{max} values were calculated from the respective Lineweaver−Burk plots.

Spectrophotometric Measurements. Absorption spectra were obtained with a Beckman DU640 spectrophotometer at a protein concentration of 1 mg/mL for all proteins. Circular dichroism (CD) measurements were determined with a JASCO J-720 spectropolarimeter equipped with a variable-temperature accessory. WT hBCATm and the CXXC mutant proteins were dialyzed into 10 mM potassium phosphate buffer (pH 7.5) containing either 12 mM leucine or 6 mM isoleucine. Excess substrate was then removed by further dialysis in 10 mM potassium phosphate buffer (pH 7.5). CD spectra in the near-UV region were measured in a 1 cm quartz cylindrical cuvette at a protein concentration of 1 mg/mL. In the far-UV region, CD spectra were acquired using a protein concentration of 0.5 mg/mL in 10 mM potassium phosphate buffer and a cuvette with a path length of 0.05 cm.

Spectrophotometric Analysis of Thiol Groups. For titration of the solvent-accessible thiol groups in reduced or oxidized hBCATm proteins, 10 nmol of protein was exchanged into buffer containing 50 mM HEPES (pH 7.0) and 1 mM EDTA (buffer A) using a PD10 column (Amersham Biosciences) as described by the manufacturer (*12*). Two nanomoles of protein were then 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} (*14*).

H_2O_2 Sensitivity of WT hBCATm and the CXXC Mutant Proteins. Proteins were first exchanged into buffer A, and then 7 nmol of protein per aliquot was incubated with varying amounts of H_2O_2 for 18−24 h at 4 °C, after which aliquots were removed for BCAT activity and thiol content measurements (see above). The remainder of the fraction was incubated with a 100-fold molar excess of DTT for 18 h at 4 °C, after which the activity was again measured as described above. Control samples were incubated under the same conditions without the addition of H_2O_2.

Mass Spectrometry Analysis of WT and CXXC Mutant hBCATm Proteins. The amino acid substitution(s) in each of the mutated proteins was confirmed by mass spectrometry (*10*) on a Micromass Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, England) fitted with an electrospray source. Sample pretreatment followed the method previously described by Conway et al. (*10*).

Electrospray ionization mass spectrometry (ESI-MS) analysis of H_2O_2- and/or dimedone-treated proteins was also employed for sulfenic acid identification and assessment of oxidation rates. Each mutant or WT protein was initially exchanged into buffer A using a PD-10 column and then concentrated using a 7 mL Apollo ultrafiltration device with a 30 kDa cutoff (Orbital Biosciences, Topsfield, MA). Samples (4.5 nmol each) were brought to 210 μL total with additional HEPES buffer, 10 μL of dimethyl sulfoxide, or dimedone in dimethyl sulfoxide (final dimedone concentration of 5 mM), and H_2O_2 at 0, 1, 1.5, or 2 mM. Following incubation at 25 °C for up to 23 h, catalase (5 μL of a 1 mg/mL stock solution) was added followed by incubation for a further 20 min, and then 15 μL samples were frozen on dry ice and stored at −80 °C until they were assayed (within 24 h). The remainder of each sample was exchanged into 10 mM ammonium bicarbonate buffer using four rounds of dilution (to 6.5 mL) and reconcentration (to ∼50 μL) with the Apollo ultrafiltration devices. Particulate matter was removed by centrifugation, and samples were analyzed by ESI-MS after addition of acetonitrile to a final concentration of 50% and formic acid to 1%.

Table 1: Physical Characterization of the CXXC Mutant Proteins Compared with WT hBCATm

protein	predicted molecular mass (amu)	observed molecular mass (amu)[a]	moles of thiol groups per mole of protein[b]
WT hBCATm	41 732	41 730 ± 2	2.44 ± 0.03
C315A	41 702	41 700 ± 3	0.77 ± 0.03
C315S	41 717	41 716 ± 3	1.05 ± 0.03
C318A	41 702	41 700 ± 2	1.11 ± 0.02
C318S	41 717	41 717 ± 3	1.13 ± 0.02
C315/318A	41 672	41 670 ± 4	0.44 ± 0.02
C315/318S	41 702	41 700 ± 2	0.43 ± 0.01

[a] Results from mass spectrometry are represented as the mean atomic mass units ± standard errors of the mean. Flow injection analysis was used with the carrier solvent consisting of a 50:50 acetonitrile/water mixture 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 WT hBCATm or CXXC mutant protein) of the sample solution was injected. Each sample required 8−20 scans (3 s scans for 1 min), and the data were processed using MassLynx version 3.5 and the Maximum Entropy software supplied with the program to generate spectra on the absolute molecular weight scale. [b] The moles of thiol groups per mole of protein are reported as the means ± standard errors of the mean from four to six determinations. This was estimated by titrating 2 nmol of protein with a 100-fold excess of DTNB and measuring the increase in absorbance at 412 nm over the course of 10 min. The moles of thiol group per mole of protein were determined using the molar extinction coefficient 14 150 M^{-1} cm^{-1} for TNB.

RESULTS

Expression and Purification of the CXXC Mutant Proteins. Nucleotide sequence analysis showed that the mutations had been introduced in all of the mutant plasmid DNAs and that the sequences of the coding regions were correct. Wild-type hBCATm and CXXC mutant proteins were overexpressed in *E. coli* strain BL21(DE3) and yielded similar amounts of purified proteins (8−10 mg/L). There were no changes required for the purification of the CXXC mutant proteins compared to the protocol used for WT hBCATm. The purified enzymes migrated as single bands with an approximate molecular mass of 42 kDa on SDS−PAGE, and the purity of each protein was judged to be >98% (data not shown). ESI-MS showed that the observed molecular mass of the respective mutant enzymes corresponded to the predicted molecular masses (Table 1).

A previous study indicated that C315 and C318 represent the two most reactive thiols in reduced hBCATm (*10*). To evaluate whether mutagenesis of one or both of the cysteine residues in the CXXC motif resulted in the concomitant loss of the predicted number of free thiols in the mutant proteins, DTNB titrations were performed on all proteins. As shown in Table 1 and in agreement with previous reports (*10*, *11*), approximately two thiol groups per monomer were titrated in reduced, native WT hBCATm. For the C315A, C318A, C315S, and C318S hBCATm mutants, approximately one

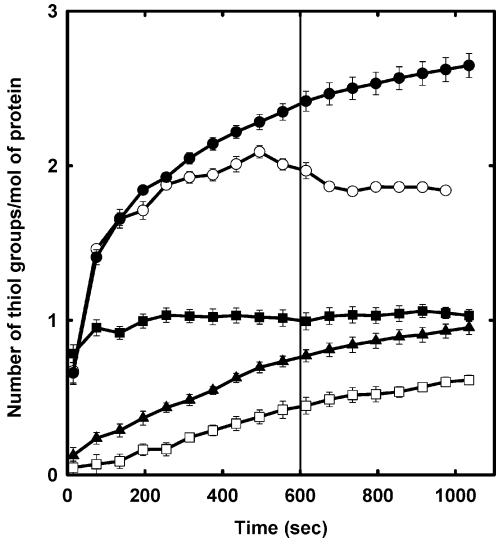


FIGURE 1: Titration of WT hBCATm and the CXXC mutant proteins with DTNB. Two micromoles of protein was incubated with a 100-fold excess of DTNB, and the increase in absorbance was measured at 412 nm over the course of 20 min: WT hBCATm (●), C108S (○), C318S (■), C315S (▲), and C315,318S (□). The line at 600 s indicates the point at which values were taken for Table 1.

thiol reacted with DTNB within 10 min, whereas with the C315/318A and C315/318S mutants, <0.6 thiol group per subunit was detected.

Figure 1 shows the number of free thiols titrated with DTNB versus time for the wild type and the serine mutants of hBCATm [similar results were obtained for the alanine mutants (data not shown)]. As shown, titration of the C315 mutant proteins with DTNB occurred at a slower rate than titration of the C318 mutant proteins. These results suggest that the pK_a of the thiol group at position 315 is lower than that of the thiol group at position 318 and/or that this thiol group is more accessible to DTNB modification. Also, as was observed in the double mutants and less so in WT hBCATm, a third thiol group appeared to react slowly with DTNB. Solvent accessibility information calculated from X-ray crystallographic data (*7*) suggested C108 as the potential third reactive thiol group. Mutation of this residue to serine had little effect on the BCAT activity of the protein (87 ± 3 units/mg of protein), which was approximately 90% of that of WT hBCATm (95 ± 2 units/mg of protein). On the other hand, the DTNB titration curve for C108S was similar to that of WT hBCATm, except that it reached a plateau at a value of two thiol groups, with no additional reaction after 8 min (Figure 1). These results strongly suggest that C108 is the thiol responsible for the slow, steady increase in the level of TNB formation observed with the WT and mutant hBCATm proteins.

Spectroscopic Analysis of WT and Mutant Proteins. The UV−visible spectral characteristics of WT and mutant hBCATm proteins were very similar in both the PLP and PMP forms. As seen for WT hBCATm (*11*), the CXXC mutants exhibit absorption spectra for the enzyme-bound PLP cofactor with maxima at 426 nm, an absorbance band which is characteristic of the Schiff base between Lys202 and PLP (internal aldimine). Upon addition of the leucine substrate, the spectrum changes from a maximum at 426 nm to one at 325 nm, representing the PMP form of the proteins (data not shown).

Circular dichroism spectroscopy was used to determine if introduction of mutations at the CXXC center affected hBCATm secondary structure. The mutant spectra have similar shapes and intensities relative to those of WT hBCATm, indicating that the mutations do not cause any major changes in secondary structure (data not shown).

Comparison of the Activities of WT hBCATm and the CXXC Mutant Proteins. To assess the functional effects of the CXXC mutations, the activity of the respective mutant proteins was measured first using the standard BCAT assay. All single and double mutations resulted in active enzymes; however, the specific activities of all mutants lacking a thiol group at C315 were significantly lower than that of WT hBCATm in the standard BCAT assay. The activity of WT hBCATm was 95 ± 2 units/mg of protein. Mutations at C318 had no effect or resulted in a relatively modest decrease of <10% in enzyme activity (94 ± 1 units/mg of protein for C318A and 87 ± 2 units/mg of protein for C318S). The specific activities of C315A and C315S were 57 ± 1 and 58 ± 1 units/mg of protein, respectively, which represents a 40% reduction relative to that of WT hBCATm. However, when both C315 and C318 were changed to either alanine or serine, the result was a marked decline in activity, corresponding to losses of 62 and 72% of activity for C315/318A (38 ± 1 units/mg of protein) and C315/318S (28 ± 2 units/mg of protein), respectively, compared to WT hBCATm.

Steady-State Kinetic Parameters of WT hBCATm and Its CXXC Mutants. The steady-state kinetic profiles of WT hBCATm and CXXC mutant proteins were examined with the three BCAA and glutamate using α-ketoisovalerate as the fixed substrate (Table 2 and Figure 2). Previously, it was shown that the K_m values for WT hBCATm were similar using isoleucine or leucine with α-ketoisovalerate as the amino group acceptor, and significantly higher K_m values were observed for valine and glutamate (*11*). Also, calculated k_{cat} values suggested that the enzyme turns over slightly faster with isoleucine than with leucine, followed by valine and glutamate. In addition, the k_{cat}/K_m values exhibited a preference of WT hBCATm for isoleucine.

The single mutations at C318 resulted in relatively small effects on the kinetic parameters for branched chain amino acids (values within 20−30% of WT hBCATm values). An exception was the C318S enzyme, for which the apparent K_m value for leucine was 56% higher than the value with WT hBCATm (Table 2). As with the other CXXC mutant proteins, apparent K_m values of C318 mutants for glutamate were higher than values observed with WT hBCATm (75−117%; see Table 2). Mutating C318 of hBCATm to Ala or Ser resulted in measured k_{cat} values for the BCAA that were similiar to or higher than that observed with WT hBCATm. There was a moderate increase in the k_{cat} with the dicarboxylic acid substrate glutamate (40−60%) compared to that of WT hBCATm. Although both K_m and k_{cat} increased with glutamate as the substrate, the k_{cat}/K_m values for glutamate were 30% lower than the WT values (Figure 2). The k_{cat}/K_m values for C318 mutants were within 20−30%, for BCAA, of WT values (Figure 2).

Mutation of the thiol group at position C315, with retention of the thiol group at position C318, had a larger effect on the kinetic parameters than the substitution of the thiol group at position C318. For all substrates, there was a significant decrease in the k_{cat} values of the C315 mutants, to values

Table 2: Kinetic Constants for Branched Chain Amino Acid Substrates of WT and Mutant hBCATm Enzymes[a]

	isoleucine		leucine		valine		glutamate	
enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)
WT hBCATm	230 ± 1	0.62 ± 0.03	194 ± 0	0.80 ± 0.02	186 ± 0	4.80 ± 0.25	139 ± 1	12 ± 0
C315A	60 ± 2	0.50 ± 0.00	51 ± 2	0.70 ± 0.01	50 ± 0	3.67 ± 0.19	47 ± 2	25 ± 1
C315S	75 ± 6	0.52 ± 0.01	52 ± 4	0.77 ± 0.05	60 ± 2	3.20 ± 0.00	54 ± 2	26 ± 1
C318A	264 ± 1	0.80 ± 0.07	204 ± 2	0.80 ± 0.03	209 ± 11	3.85 ± 0.27	201 ± 10	26 ± 1
C318S	245 ± 11	0.77 ± 0.00	250 ± 10	1.25 ± 0.05	196 ± 10	4.27 ± 0.36	225 ± 6	21 ± 3
C315/318A	48 ± 5	0.38 ± 0.04	50 ± 5	1.60 ± 0.11	42 ± 2	2.63 ± 0.10	61 ± 6	18 ± 2
C315/318S	58 ± 3	0.37 ± 0.02	30 ± 1	1.20 ± 0.00	53 ± 3	6.30 ± 0.26	56 ± 9	22 ± 1

[a] BCAT activity was measured as described in Experimental Procedures. The fixed substrate, α-ketoisovalerate, was held at a concentration of 2 mM. V_{max} (used to calculate k_{cat}) and K_m values were obtained from Lineweaver−Burk plots. Results are reported as means ± standard errors of the mean ($N = 3$).

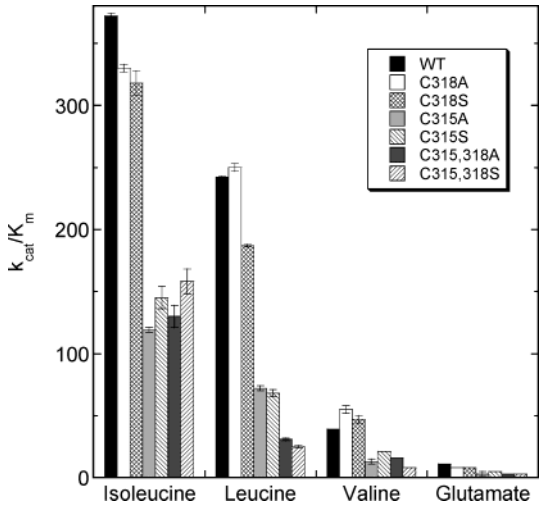


FIGURE 2: Comparison of the catalytic efficiencies (k_{cat}/K_m) of WT hBCATm and the CXXC mutant proteins with amino acid substrates. The values from Table 2 were used to calculate k_{cat}/K_m.

approximately 26−39% of that of the k_{cat} of WT hBCATm (Table 2). In general, K_m values were less affected than k_{cat} values. However, as observed with the single C318 mutations, the K_m values for glutamate were approximately double those observed with the WT enzyme. The k_{cat}/K_m values for all substrates were correspondingly lower than that of the WT enzyme (Figure 2).

The two double mutants, C315/318A and C315/318S, had k_{cat} values that were similar to values observed with the single C315 mutant enzymes. Changes in K_m values were modest but larger than those observed with the single mutants. For example, observed K_m values for isoleucine were 40% lower whereas K_m values for leucine were higher (50−100%) than WT values (Table 2). Overall, k_{cat}/K_m values were significantly lower than WT values and similar to values obtained with the single C315 mutants (Figure 2).

To determine whether mutations at the CXXC center resulted in changes in substrate specificity of hBCATm for amino acids other than the BCAA and glutamate, the amino acid preference of the individual mutants was compared with that of WT hBCATm. In these studies, concentrations of 10 mM of the various amino acids were used and the α-ketoisovalerate concentration was kept constant at 1 mM. In agreement with previous results (*11*) and the kinetic data in Table 2, isoleucine was the preferred substrate for WT hBCATm followed by leucine and then valine. Norleucine and norvaline were transaminated by WT hBCATm with norvaline (39% of the leucine control) preferred over norleucine (22% of the leucine control) (data not shown). Whereas the substrate preference for BCAA of all the CXXC mutant enzymes was similar to that of WT hBCATm, generally they showed a clearer preference for norleucine (64−84% of the leucine control) over norvaline (5−26% of the leucine control) with the ratio of activity with norleucine versus norvaline ranging from 12.8 in the C315A mutant to 2.6 in the C318S mutant compared to 1.8 with WT BCATm. Neither WT hBCATm nor the CXXC mutants showed significant transaminase activity with aromatic amino acids (tyrosine, tryptophan, and phenylalanine), aspartate (one carbon shorter than glutamate), threonine, or serine.

Effects of Hydrogen Peroxide on WT and CXXC Mutants of hBCATm. Previously, it was demonstrated that the activity of WT hBCATm is abolished upon reaction with H_2O_2, and that loss of enzyme activity correlated with the formation of an intrasubunit disulfide bond between C315 and C318 (*10*). To assess the mechanism by which this oxidation occurs, each of the CXXC mutant proteins was treated with H_2O_2 as described in Experimental Procedures. Both BCAT activity and thiol content were measured after 18 h at 4 °C with or without H_2O_2 treatment (Table 3). Under these conditions, H_2O_2 treatment of WT hBCATm resulted in a 95% loss of enzyme activity and a loss of approximately two thiol groups. The inhibition of activity was reversed by addition of DTT (to 94% of the control value). Treatment with H_2O_2 had comparable effects on the thiol content and BCAT activity of C108S as observed with WT hBCATm, resulting in a 95% loss in BCAT activity and loss of two thiol groups.

As described above, the C315A and C315S mutant proteins have 40% less activity than WT hBCATm in the standard assay. The activity and thiol content of the C315 mutants were not affected significantly by H_2O_2, indicating that the C315 mutants have lost their sensitivity to oxidation (Table 3). On the other hand, the activity of both C318A and C318S was inhibited by 95 and 98%, respectively, after incubation with H_2O_2, and the loss of activity correlated with the loss of approximately one thiol group. Furthermore, unlike that in WT hBCATm, the loss in activity was only partially reversible with DTT (to approximately 30% of that of the untreated control enzyme). Neither the activity nor the thiol content of the double mutants, C315/318A and C315/318S, was affected by H_2O_2.

To investigate the difference in the sensitivity of the C315 and C318 mutants toward oxidation by H_2O_2, the effects of a wide range of H_2O_2 concentrations on activity of these mutant proteins were compared (Figure 3). Under similar conditions, less H_2O_2 was required to inactivate C318A,

Table 3: Effect of Hydrogen Peroxide on WT and Mutant hBCATm Enzymes[a]

	BCAT activity (units/mg)[b]			no. of thiol groups per subunit[c]	
enzyme	without H_2O_2	with H_2O_2	addition of DTT to H_2O_2-treated protein[d]	without H_2O_2	with H_2O_2
WT hBCATm	90 ± 2	5 ± 0	85 ± 2	2.36 ± 0.03	0.80 ± 0.02
C315A	57 ± 1	66 ± 3	60 ± 2	0.82 ± 0.03	0.73 ± 0.02
C315S	58 ± 1	55 ± 3	64 ± 2	1.05 ± 0.03	0.78 ± 0.04
C318A	94 ± 1	5 ± 0	29 ± 2	1.11 ± 0.02	0.30 ± 0.04
C318S	87 ± 2	2 ± 0	26 ± 0	1.13 ± 0.02	0.23 ± 0.02
C315/318A	38 ± 1	43 ± 1	37 ± 1	0.59 ± 0.02	0.40 ± 0.01
C315/318S	28 ± 3	31 ± 2	28 ± 2	0.60 ± 0.08	0.61 ± 0.02
C108S	87 ± 3	6 ± 3	86 ± 3	2.04 ± 0.08	0.03 ± 0.00

[a] WT and mutant hBCATm proteins were treated with 64 equiv of H_2O_2 for 18−24 h at 4 °C as described in Experimental Procedures. [b] Activity measurements following H_2O_2 treatment did not include DTT. Results are reported as means ± standard errors. [c] The number of thiol groups per subunit of protein is reported as the mean ± standard error ($N = 3$), determined before and after the addition of H_2O_2 by adding a 100-fold excess of DTNB to 2 nmol of protein and measuring the increase in absorbance at 412 nm after 10 min. [d] H_2O_2-treated proteins were subsequently treated with a 100-fold molar excess of DTT and allowed to incubate overnight for 18 h prior to measurement of BCAT activity.

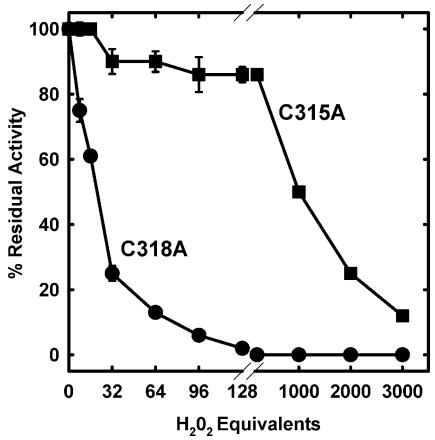
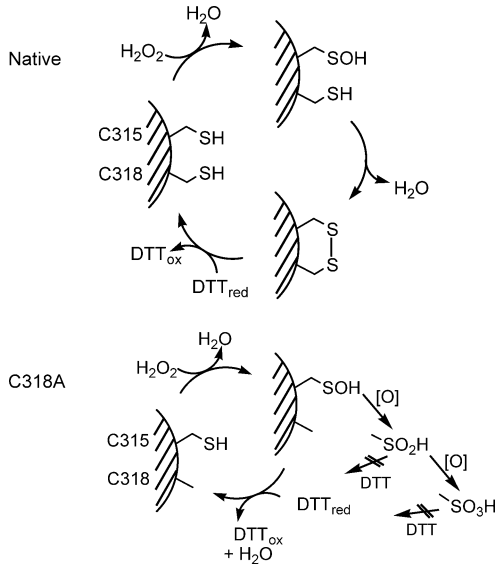


FIGURE 3: Effect of H_2O_2 on the activity of C315A, C318A, and WT hBCATm proteins. Branched chain aminotransferase activity was measured as described in Experimental Procedures following incubation with H_2O_2 in 50 mM HEPES (pH 7.0) and 1 mM EDTA at 4 °C for 18−24 h. C315A (■) and C318A (●) were treated with H_2O_2 prior to determination of BCAT activity. Data are means ± standard errors of the mean ($N = 3$).

Scheme 1



whereas inactivation of C315A required considerably higher concentrations of H_2O_2 (Figure 3). Unlike the case with WT hBCATm, loss of BCAT activity of the peroxide-treated C318A (and C318S) mutant was largely irreversible with DTT addition (Table 3). The inability to recover activity in the C318 mutants likely results from overoxidation of the cysteine residue at position 315 to sulfinic or sulfonic acids due to the lack of the proximal C318 thiol group (see below).

Inactivation, Oxidation, and Trapping of the Sulfenic Acid Intermediate in C318A hBCATm. In wild-type hBCATm, putative sulfenic acid formation by H_2O_2-mediated oxidation of a single cysteine within the CXXC motif is likely followed rapidly by disulfide bond formation with the proximal thiol; the data described above support C315 as the H_2O_2-reactive thiol. In the mutant lacking C318, disulfide bond formation cannot occur, and the sulfenic acid may therefore persist long enough to allow for its trapping (Scheme 1). Unless specifically stabilized within protein microdomains, sulfenic acids also exhibit a propensity toward further oxidation to sulfinic and sulfonic acids, particularly in the presence of excess H_2O_2 (*15*). Methods used to definitively demonstrate sulfenic acid formation have employed trapping agents that either react only with the sulfenic acid but not with the thiol form of cysteine (TNB and dimedone) or react with both but give adducts with distinct spectral and mass signatures for each product (7-chloro-4-nitrobenz-2-oxa-1,3-diazole, abbreviated NBD chloride) (*16*).

Preliminary ESI-MS experiments for assessing oxidation via oxygen mass addition indicated a progressive increase in the mass of the C318A hBCATm mutant upon incubation with inactivating amounts of H_2O_2, with the apparent incorporation of up to three oxygen atoms into the protein (data not shown). Addition of TNB (which condenses with sulfenic acids to form disulfide bonds, thereby decreasing the A_{412}) at various time points did result in a decrease in its absorbance, but control reactions in the absence of protein suggested that H_2O_2 alone was responsible for most or all of the TNB absorbance decrease. NBD chloride did not react with even the thiol (i.e., reduced) form of the C318A mutant; no indication of adduct formation was obtained for either H_2O_2-treated or untreated proteins. Although denaturation of the protein would have exposed the cysteine thiol or putative sulfenic acid for modification, the other six cysteinyl residues would also have formed NBD adducts, and formation of even a stoichiometric amount of the NBD adduct with sulfenic acid would have been difficult to discern (at 347 nm) due to the large 420 nm peak of the thiol adducts (*17*).

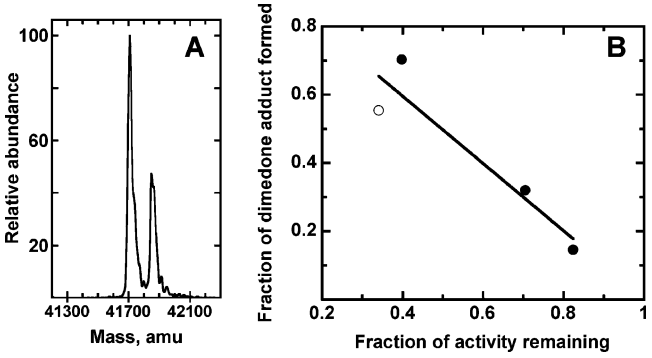


FIGURE 4: Dimedone adduct formation and activity loss for the H_2O_2-treated C318A mutant of hBCATm. Samples (4.5 nmol each) were incubated with 1.5 (○) or 2 mM H_2O_2 (●) and 5 mM dimedone at 25 °C prior to addition of catalase, then assayed for BCAT activity, and analyzed by electrospray ionization mass spectrometry as described in Experimental Procedures. Panel A shows the transformed mass spectrometry data for the sample after treatment for 427 min, with peaks of maximal abundance at 41 708 and 41 848 amu for the free and dimedone-adducted proteins, respectively. Panel B illustrates the correlation between the fraction of dimedone adduct formed and the BCAT activity of the C318A samples from two independent experiments, after treatment for 224, 427, 1180, and 1375 min (in order of the increasing level of adduct formation; $r = 0.936$ and slope $= -0.987$).

Dimedone used as a trapping agent for protein sulfenic acids has the disadvantage of being detected only by ESI-MS and the expected increase of 140 amu upon adduct formation. A major advantage to using this reagent is, however, that dimedone reacts with only the sulfenic acid form of cysteine residues and can therefore be present during the incubation of the thiol-containing protein with H_2O_2 (*17*). Therefore, dimedone was added to each sample of C318A prior to addition of 1 or 2 mM H_2O_2; controls lacking dimedone, H_2O_2, or both were also included. After incubation at 25 °C for approximately 4, 7, and 23 h, catalase was added, aliquots were taken for activity measurements, and the remainder was exchanged into ammonium bicarbonate buffer for ESI-MS analysis.

Pseudo-first-order oxidation rates as assessed by inactivation were directly proportional to the amount of H_2O_2 added. Using this method, a second-order rate of ~0.4 M^{-1} min^{-1} for H_2O_2-mediated inactivation of C318A at 25 °C was determined in the presence of dimedone; WT inactivation rates measured in parallel experiments were similar (data not shown). In the absence of dimedone, this rate was ~1.3 M^{-1} min^{-1}, suggesting that there may be some background reactivity between dimedone and H_2O_2. For samples incubated with H_2O_2 in the presence of dimedone, the larger mass adduct was clearly observed (Figure 4A). Indeed, adduct formation correlated well with enzyme inactivation (Figure 4B), suggesting that, under these conditions, dimedone trapped most or all of the nascent sulfenic acid intermediate before it could be further oxidized. In control samples lacking dimedone or H_2O_2 treatment, none of this adduct was observed (not shown). Furthermore, neither WT nor C315 mutants (C315A and C315S) treated under conditions where 56% of the C318A mutant was converted to the dimedone adduct gave any indication of adduct formation by ESI-MS. In the case of the C315 mutants, the remaining thiol is not sensitive toward H_2O_2 oxidation. The WT enzyme is partially inactivated under these conditions; however, the inactivation is reversible, and disulfide bond formation is apparently more rapid than the reaction of the sulfenic acid intermediate with dimedone. Over the course of the incubation with H_2O_2 and dimedone, the mass of the C318A mutant increases gradually for both the dimedone-lacking peak and the dimedone adduct peak, from a starting value of 41 696.4 ± 1.5 (and 41 836 amu for the adduct) to values ~32−43 amu larger than the "parental" peaks. The mass difference between the two peaks (with and without dimedone adducted), however, remains relatively constant, at 140−150 amu. This suggests oxidation of at least one additional residue within the protein, tentatively identified as C108 on the basis of experiments described above (Figure 1).

DISCUSSION

The reactivity of the CXXC mutant proteins toward H_2O_2 has provided insight into the function of each of the reactive cysteine residues in the redox-linked regulation of hBCATm. In general, the effect of site-directed mutagenesis of the two targeted cysteine residues, particularly when C315 was replaced, was a decrease in catalytic efficiency and a modest increase in K_m only for the dicarboxylic acid substrate amino acid glutamate. Therefore, the most important factor influencing hBCATm kinetic parameters in the mutant enzymes was the site of the mutation rather than the nature of the amino acid being substituted (alanine or serine). Particularly striking is the loss of H_2O_2 sensitivity observed upon replacement of C315, but not C318, with serine or alanine. As the effect on catalysis by mutation of C318 is moderate, it appears that this residue serves no obligatory role in catalysis of BCAA or glutamate transamination. However, the role of C318 was clarified on H_2O_2 treatment of C318A and C318S (preserving only C315 of the CXXC motif), where it was observed that oxidative inactivation of these mutant proteins was largely irreversible with DTT. This protein differed, therefore, from WT hBCATm, where reversible oxidation was observed (Table 3). These results support our proposal that C315 is the sensor for H_2O_2, whereas C318 acts to prevent overoxidation of WT hBCATm.

Previously, bond distances and angles in the X-ray crystallographic structures of reduced hBCATm were reported to be consistent with the conclusion that C318 was the thiolate anion of the thiol/thiolate pair (*7*). However, results from the studies of CXXC mutants suggest the opposite based on DTNB reactivity (Figure 1) and oxidation studies (Figure 3). These results suggest that the thiol group at position 315 may have a lower pK_a than the thiol group at position 318, accounting for the greater reactivity of the C318 mutants with DTNB and H_2O_2. The differences in reactivity of C315 and C318 could not be readily explained by solvent accessibility calculations, because both thiols exhibit similar solvent accessibility in the crystal structures (*7*). However, there are differences in the environment of the cysteines in the protein. The interdomain loop shields C315 from the solvent. In the structures of WT hBCATm in different crystal forms, the interdomain loop adopts significantly different conformations and has high thermal factors in the X-ray refinement (Figure 5) (*7*). This indicates that the loop is flexible and in solution could be exposing C315, allowing it to react. C318 is surrounded by more rigid parts of the protein and is participating in van der Waals interactions with the side chains of residues V182 and M241

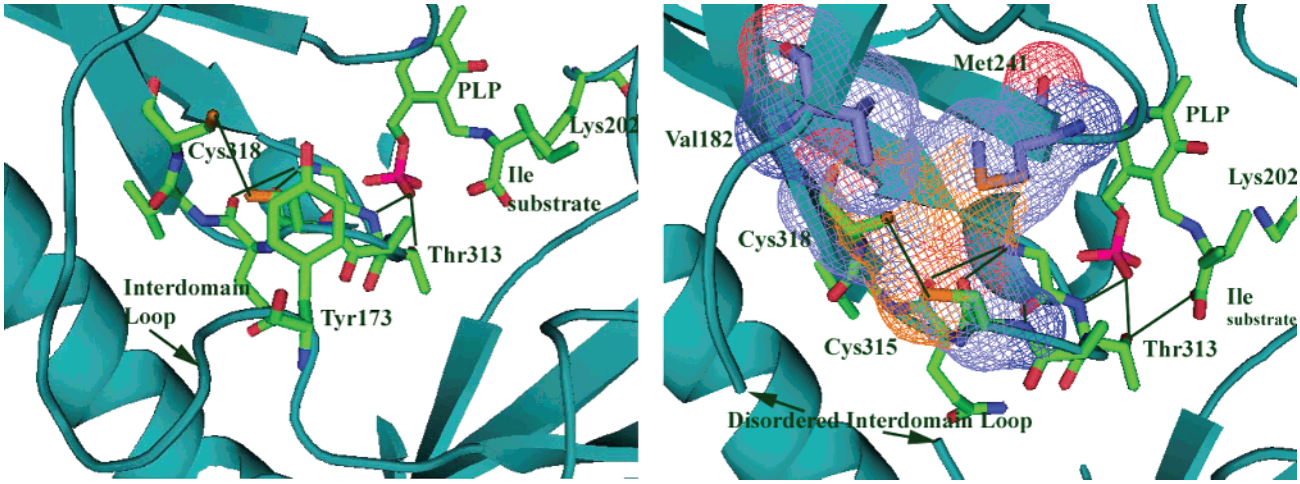


FIGURE 5: View down the likely path taken by the substrate to reach the active site of monomer A (right) and monomer B (left) of the isoleucine ketimine intermediate structure (PDB entry 1KT8). The hydrogen bond interactions are represented with black lines. The substrate and the PLP cofactor are stabilized by favorable interactions with the residues in the β-turn preceding the cysteines of interest, C315 and C318. C315 could become solvent exposed by the movement of Y173 of the flexible interdomain loop. The right panel illustrates that C318 is partially shielded from the solvent by its van der Waals interactions with V182 and M241. This figure was generated using Pymol (*29*).

which partially shield it from the solvent (Figure 5, right panel).

Previously reported X-ray crystallographic data from the study of the structure of substrate-bound hBCATm also provide support for the interaction of the side chain of Y173 with C315 (*7*). This interaction may stabilize the protein during formation of the ketimine intermediate of WT hBCATm, suggesting a possible structural role for C315. Subsequent analysis of the X-ray crystallographic structures of the C315A and C315/318A mutants (N. Yennawar, M. E. Conway, and S. M. Hutson, unpublished data) suggests that replacement of C315 with alanine precludes this interaction with Y173. Although serine can participate in an O−H···π hydrogen bond, in the Ser mutant structure the serine side chain reorients in a new low-energy conformer and does not interact with either Y173 or C318.

Perhaps surprising is our finding that the conversion of C315 to a sulfinic or sulfonic acid has the same inactivating effect as the generation of a disulfide bond between the two cysteines of the CXXC motif. In both cases, the thiol group at C315 is removed, although our mutagenesis studies indicate that just the lack of a thiol group at position 315 does not necessarily result in the complete loss of activity. The inactivating effects of the two different types of oxidation products at C315 may also involve different mechanisms, perhaps combining both loss of a thiol group at this position and structural perturbations unique to each oxidized species. Additional insights may be gained with the future determination of crystal structures for the oxidized forms of both wild-type and C315A hBCATm.

In general, the cysteine residue of a redox-active CXXC center closest to the N-terminus of a protein is the nucleophilic thiolate anion with the lower pK_a, although these motifs are typically found at the N-terminal end of α-helical structures (*18, 19*); in hBCATm, C315 lies between two β-strands and C318 begins the second strand (*10*). Nonetheless, our biochemical results are consistent with what has been observed with other CXXC-containing enzymes and support the conclusion that C315 is the redox sensor of the thiol/thiolate pair.

Cysteine sulfenic acids are characterized as unstable transient reaction intermediates (*20*), but can be stabilized in the microenvironments of proteins (*15*). These intermediates have long been considered to be involved in thiol oxidations that yield disulfides and higher-sulfur oxyacids (*21*), and are increasingly appreciated for the role they can play in redox regulation of enzymes and transcription factors (*22*). Thus, as shown in Scheme 1 (Native), oxidation of WT hBCATm with H_2O_2 results in the formation of a disulfide bond, likely via intermediate sulfenic acid formation at C315, which is reversible upon the addition of thiol-containing compounds. On the other hand, loss in BCAT activity observed after oxidation of C318A with H_2O_2 was largely irreversible upon addition of excess DTT, likely due to the formation of sulfinic and/or sulfonic acids via the sulfenic acid intermediate (Scheme 1, C318A). Progressive oxidation was shown in mass spectrometric studies of H_2O_2-treated mutant proteins, where sequential oxidation of the thiol group at position 315 to sulfenic, sulfinic, and perhaps sulfonic acid resulted in increases in molecular mass. The formation of the putative sulfonic acid via a sulfenic acid intermediate correlated with the inability of the oxidized C318A mutant to completely recover BCAT activity. This hypothesis is supported by the detection of the sulfenic acid intermediate using dimedone. Dimedone is a nucleophilic reagent specific for sulfenic acids, and it is unreactive toward thiol groups (*23*). Although it lacks any distinguishing spectral features, its incorporation may be assessed by an increase in molecular mass of 140 amu as detected by ESI-MS. Indeed, generation of the dimedone adduct of the H_2O_2-treated C318A mutant (Figure 4A) correlated well with the irreversible loss of BCAT activity (Figure 4B). On the other hand, WT hBCATm inactivated by H_2O_2 treatment formed no adduct with dimedone, indicating that disulfide bond formation following sulfenic acid generation is rapid relative to the dimedone reaction in the WT enzyme. Thus, we conclude that C318 functions to prevent overoxidation of C315 in WT hBCATm, permitting reversible, redox-sensitive regulation of the enzyme.

New information is emerging that suggests BCATm may be a multifunctional protein. Recently, we have found that hBCATm associates with the second enzyme in the catabolic pathway, the branched chain α-keto acid dehydrogenase enzyme complex, and that this association can be regulated

by NADH (*24*). This interaction may depend on the structural integrity of the CXXC center. We have also found that, in addition to transamination, the native human BCAT (hBCAT) catalyzes an effective β-elimination reaction (*25*). Furthermore, the discovery of two additional alternatively spliced forms of hBCATm increases the likelihood that hBCATm is a multifunctional enzyme, particularly as one of these forms binds to and acts as a corepressor of one of the thyroid hormone receptors (*2*, *3*). This latter form does not contain C318. One may speculate that the oxidation state of C315 influences the binding properties of hBCATm and its ability to act as a corepressor, similar perhaps to the redox control of OxyR-mediated transcriptional activation (*26*). Another potential advantage of the redox regulation of hBCATm may involve the preservation of leucine under conditions of oxidative stress. This branched chain amino acid substrate of BCAT is an anabolic nutrient signal and an essential amino acid. Understanding the biochemical basis for this redox regulation is important, because it may lead to a better understanding of the metabolism of these essential nutrients and to the identification of additional functions for the mammalian BCAT family (BCATm and BCATc) as has been described for a number of other metabolic enzymes (*27*, *28*).

In summary, the effects of H_2O_2 on BCAT activity, thiol content, and sulfenic acid generation as studied using hBCATm CXXC mutants strongly suggest that C315 is the peroxide-sensitive thiol(ate) which is initially oxidized in WT hBCATm. Furthermore, the thiol group at position C318 functions to prevent overoxidation of C315 beyond the sulfenic acid state by forming a more stable, readily reducible disulfide bond. Thus, this CXXC motif interacts as a unit in the control of the activity of hBCATm under the influence of the redox state of the cell.

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