Regulatory Control of Human Cytosolic Branched-Chain Aminotransferase by Oxidation and S-Glutathionylation and Its Interactions with Redox Sensitive Neuronal Proteins[†]

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ABSTRACT: Redox regulation of proteins through oxidation and S-thiolation are important regulatory processes, acting in both a protective and adaptive role in the cell. In the current study, we investigated the sensitivity of the neuronal human cytosolic branched-chain aminotransferase (hBCATc) protein to oxidation and S-thiolation, with particular attention focused on functionality and modulation of its CXXC motif. Thiol specific reagents showed significant redox cycling between the reactive thiols and the TNB anion, and using NEM, four of the six reactive thiols are critical to the functionality of hBCATc. Site-directed mutagenesis studies supported these findings where a reduced *k*cat (ranging from 50−70% of hBCATc) for C335S, C338S, C335/8S, and C221S, respectively, followed by a modest effect on C242S was observed. However, only the thiols of the CXXC motif (C335 and C338) were directly involved in the reversible redox regulation of hBCATc through oxidation (with a loss of 40−45% BCAT activity on air oxidation alone). Concurrent with these findings, under air oxidation, the X-ray crystallography structure of hBCATc showed a disulphide bond between C335 and C338. Further oxidation of the other four thiols was not evident until levels of hydrogen peroxide were elevated. S-thiolation experiments of hBCATc exposed to GSH provided evidence for significant recycling between GSH and the thiols of hBCATc, which implied that under reducing conditions GSH was operating as a thiol donor with minimal S-glutathionylation. Western blot analysis of WT hBCATc and mutant proteins showed that as the ratio of GSH:GSSG decreased significant S-glutathionylation occurred (with a further loss of 20% BCAT activity), preferentially at the thiols of the CXXC motif, suggesting a shift in function toward a more protective role for GSH. Furthermore, the extent of S-glutathionylation increased in response to oxidative stress induced by hydrogen peroxide potentially through a C335 sulfenic acid intermediate. Deglutathionylation of hBCATc-SSG using the GSH/glutaredoxin system provides evidence that this protein may play an important role in cellular redox regulation. Moreover, redox associations between hBCATc and several neuronal proteins were identified using targeted proteomics. Thus, our data provides strong evidence that the reactive thiol groups, in particular the thiols of the CXXC motif, play an integral role in redox regulation and that hBCATc has redox mediated associations with several neuronal proteins involved in G-protein cell signaling, indicating a novel role for hBCATc in cellular redox control.

Proteins with reactive cysteine residues are integral to the control of redox status within the cell and are fast becoming the focus of the field in redox cell signaling (*1*). The biological significance of these redox interactions is diverse in nature, but modification of thiol groups is central to each mechanism. Biological roles range in diversity from redox regulation of the bacterial transcriptional activator, OxyR of *E. coli* (*2*) and the mammalian protein tyrosine phosphatases (*3*), to their direct involvement in catalytic mechanisms, as described for the thioredoxins (*4*) and peroxiredoxins (*5*). Redox interactions in the cell linked with oxidative and nitrosylated stress are characteristically associated with the transient intermediate of cysteine oxidation, sulfenic acid (Cys-SOH), which is of considerable biological importance (*6, 7*). Because of its chemically reactive nature, Cys-SOH has the potential to generate other forms of reversibly (RSSR) or irreversibly (sulfinic-Cys-SO_2^- and sulfonic-Cys-SO_3^-) modified cysteine groups (*6*), although recently the sulfinic moiety formed in peroxiredoxins has been shown to be reduced by sulfiredoxin (*8*). Progression of the oxidation of thiol groups in redox-sensitive proteins to these irreversible

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forms can lead to complete inactivation of key metabolic enzymes resulting in cellular damage (*9*). Cellular reductants such as the thioredoxin system (*10*), the glutaredoxin (Grx[1]) system (*10, 11*) and/or reduced glutathione (GSH) can reduce these disulphide bonds in a thiol−disulphide exchange forming a mixed disulphide or stable disulphide bonded intermediates with Cys-SOH (S-glutathionylation), ultimately recycling them back to their thiol state (*12*). Reversible reactions in biological systems provide a potential point for regulation and control and may protect proteins against irreversible oxidation or themselves modulate protein function (reviewed in Poole (*6*)).

The branched chain aminotransferases (BCATs), undergo thiol modifications under oxidizing conditions (*13, 14*). The BCATs (EC 2.6.1.42) belong to the fold-type IV class of pyridoxal 5′-phosphate (PLP)-dependent enzymes (*15*) and catalyze the transfer of the α-amino group of the branched chain amino acids (BCAA) (isoleucine, leucine, and valine) to α-ketoglutarate forming glutamate and the respective branched chain α-keto acids (*15*). In mammals, there are two isoforms (*16*). The mitochondrial isoform, hBCATm, is found in most tissues, and the cytosolic isoform, hBCATc, is found in the nervous system (*17, 18*). Using site-directed mutagenesis and electron spray ionization mass spectrometry analysis (ESI-MS), we showed that C315 of the redox-active dithiol/disulfide CXXC center in mitochondrial hBCATm acts as a redox sensor via sulfenic acid formation, with the thiol group at position C318 permitting reversible oxidation (*14, 19*). X-ray crystallography studies and kinetic analysis demonstrated that the predominant effect of oxidation was on the second half-reaction rather than the first half-reaction, where disruption of the CXXC center results in altered substrate orientation and an unprotonated PMP amino group, thus rendering the enzyme catalytically inactive (*19*). Recently, using rat liver homogenate evidence for substrate channelling between the BCAT proteins and the branched-chain α-keto acid dehydrogenase enzyme complex, which can be influenced by changes in redox state, have been reported (*20*). Here, the reduced state of the CXXC motif was shown to be necessary for the binding of BCATm to the E1 subunit of the branched-chain keto acid dehydrogenase complex. Thus, the CXXC center has a role in BCAA metabolon formation.

Although the BCAT proteins possess 58% sequence homology, they show tissue specific expression with the hBCATc isozyme localized almost exclusively in neurons (*17*). Unlike hBCATm, the crystal structure of hBCATc showed that it has an additional four solvent accessible cysteine residues as well as its CXXC motif (*21*). In the current study, we investigated the role of these thiol groups with respect to functionality and redox modification. Further investigations probed the effect of altering the ratio of cellular reductants and their reducing systems under oxidative stress on hBCATc S-glutathionylation. Finally, we provide the first evidence that the CXXC motif of hBCATc is integral in the association of several neuronal proteins and that these associations are disrupted under oxidative stress.

[1] Abbreviations: BCAT, branched-chain aminotransferase; hBCATm, human mitochondrial branched-chain aminotransferase; hBCATc, human cytosolic branched-chain aminotransferase; BCAA, branched-chain amino acids; WT, wild-type; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethyl-maleimide; DTT, dithiothreitol; PLP, pyridoxal 5′-phosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; H_2O_2, hydrogen peroxide; PVDF, polyvinylidene difluoride; ESI-MS, electrospray ionization mass spectrometry; TNB, 2-nitro-5-thiobenzoate; Q-TOF MS, quadrupole time-of-flight mass spectrometry; GSH, glutathione; GSSG, glutathione disulphide; Grx, glutaredoxin; Cys-SOH, cysteine sulfenic acid, BCKD complex; branched-chain α-keto dehydrogenase complex.

EXPERIMENTAL PROCEDURES

Materials. Dithiothreitol (DTT), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *n*-ethyl maleimide (NEM), dimedone, α-ketoisocaproate, α-ketoisovalerate, PLP, GSH, glutathione disulphide (GSSG), Grx, glutathione reductase (GR), CH-sepaharose, and NADH were obtained from Sigma (Gillingham, Dorset). Hydrogen peroxide (H_2O_2) (30%) was purchased from Fisher Scientific (Loughborough, Leicestershire). The PD10 columns and the Mono Q HR 5/5 (1 mL) anion-exchange column were from Amersham Pharmacia Biotech (Chalf-ont St. Giles, Bucks). Apollo ultrafiltration devices (7 mL) were from Orbital Biosciences (Topsfield, MA). The QuikChange site-directed mutagenesis kit was from Stratagene Corporation (La Jolla, CA). The QIAprep spin miniprep kit and the MiniEluate 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 (Southbend, IN). Radioactive [1-^{14}C]valine was from American Radiolabeled Chemicals Inc. (St, Louis, MO).

Site-Directed Mutagenesis of Wild-Type (WT) hBCATc. The thiol groups of the reactive cysteine residues of hBCATc were mutated to serine, using synthetic oligonucleotides and their exact complements containing the desired mutations (C221S, C235S, C242S, C293S, C335S, C338S, and C335/8S). The following sense primers were used: C221S (5′-GCC TGG AAA GGT GGA ACT GGG GAC TCC AAG ATG GGA GGG-3′), C235S (5′-GGC TCA TCT CTT TTT GCC CAA TCT GAA GCA GTA GAT AAT GGG-3′), C242S (5′-GCC CAA TGT GAA GCA GTA GAT AAT GGG TCT CAG CAG GTC CTG TGG-3′), C293S (5′- CAA GGA GTG ACA AGG CGG TCC ATT CTG GAC CTG GCA CAT CAG TGG-3′), C335S (5′-GGC TCT GGT ACA GCC TCT GTT GTT TGC CCA-3), C338S (5′-GGT ACA GCC TGT GTT GTT AGC CCA GTT TCT GAT ATA CTG-3′), and C335/8S (5′-GGT ACA GCC TCT GTT GTT AGC CCA GTT TCT-3′). Mutagenesis was conducted using the Quick-Change kit protocol as described in Conway et al. (*14*). The desired mutation and fidelity of PCR amplification was confirmed by DNA sequence analysis using the ABI 377 DNA sequenator in the DNA Sequencing Core Laboratory of Comprehensive Cancer Center of Wake Forest University School of Medicine. The WT hBCATc and thiol mutant plasmids were transformed into BL21(DE3) cells as described in Davoodi et al. (*22*).

Overexpression and Purification of WT hBCATc and Mutant Proteins. Purification of WT and mutant hBCATc proteins was carried out as previously described in Conway et al. (*21*). Briefly, after the histidine-tagged hBCATc fusion protein was extracted and purified using nickel-NTA resin (Qiagen, Chatsworth, CA), thrombin (100 NIH units per extraction) was used to remove the affinity tag. The final purification step for WT hBCATc and mutant proteins was anion-exchange chromatography using the Mono-Q HR 5/5

anion-exchange column (GE Healthcare). The purified proteins were dialyzed at 4 °C into a buffer containing 25 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM glucose, 1 mM EDTA, 1 mM α-ketoisocaproate, 5 mM DTT, and ~15% glycerol. The concentration of purified protein was determined using the Schaffner and Weissmann (*23*) method or calculated from the absorbance at 280 nm using the extinction coefficient of 86,300 M^{-1} cm^{-1} per monomer for hBCATc (*22*). The final yield was approximately 10 mg of purified protein/L of *E. coli*. There were no changes required for the purification of the mutant proteins. Electrospray ionization mass spectrometry showed that the observed molecular mass of the respective mutant enzymes corresponded to the predicted molecular masses, and the purity of each protein was determined to be >98%.

Branched-Chain Aminotransferase Activity Assay and Kinetic Studies. The activity of hBCATc and the mutant enzymes was assayed at 37 °C by measuring the formation of [1-^{14}C]valine from α-keto[1-^{14}C]isovalerate as described previously (*24*). Enzyme activity was measured in 25 mM potassium phosphate buffer at pH 7.8 containing 5 mM DTT, 1 mM α-keto[1-^{14}C]isovalerate, and 12 mM isoleucine (*24*). A unit of enzyme activity was defined as 1 μmol of valine formed per min. All assays were performed in triplicate.

Catalytic rate constants for hBCATc and the mutant proteins with the amino acid/α-keto[1-^{14}C]isovalerate pairs were determined as previously described (*14*). The concentrations of the amino acids were isoleucine (0−2 mM), leucine (0−2 mM), valine (0−30 mM), and glutamate (0−40 mM). Stock solutions of the dicarboxylic acid glutamate were neutralized with KOH. Data were collected for 8−10 different concentrations of each amino acid. The k_{cat} and k_{cat}/K_m values were calculated from the respective Lineweaver−Burk plots.

Spectrophotometric Analysis of Thiol Groups. Solvent accessible thiol groups in hBCATc were assayed by DTNB titration. Ten nmol of protein were exchanged into buffer containing 50 mM HEPES at pH 7.2 and 1 mM EDTA (buffer A) using a PD10 column (Amersham Biosciences). Titration of 2 nmol of protein with a 100-fold excess of DTNB was carried out at room temperature for 15 min. The absorbance change at 412 nm was monitored and the concentration of free thiol groups calculated from the liberated 2-nitro-5-thiobenzoate (thiolate) dianion (TNB) using a molar extinction coefficient of 13,600 M^{-1} cm^{-1} (*21*).

Chemical Modification of hBCATc with Thiol Specific Reagents. For measuring the effect of DTNB on hBCATc activity, a 10-fold molar excess of DTNB in buffer A was used, and aliquots were removed at different time points and assayed for activity using the standard BCAT assay. The remaining sample was incubated with a 100-fold molar excess of DTT for 2 h at room temperature, after which the activity was again measured as described above. Control samples were incubated under the same conditions without the addition of DTNB.

To evaluate the inhibitory effects of the irreversible reagent, NEM on hBCATc activity, 7 nmol of protein was reacted with a 10-fold molar excess of NEM for 10 min at room temperature with constant stirring. The reaction was terminated by adding a 100-fold molar excess of DTT. Subsequently, activity of the modified protein was measured using the standard BCAT assay. The % residual activity remaining after modification was calculated relative to control WT hBCATc. To calculate the number of free thiols available, the NEM-labeled protein was exchanged into buffer A and titrated with DTNB, as described above. Also, an aliquot of NEM-labeled hBCATc was analyzed by a Quadrupole Time-Of-Flight Mass Spectrometer (Q-TOF MS) as described below.

H_2O_2 Sensitivity of WT hBCATc and the Mutant Proteins. Wild-type hBCATc and the mutant proteins, C221S, C235S, C242S, C293S, C335S, C338S, and C335/8S (6 nmol) exchanged into buffer A were incubated with increasing concentrations of H_2O_2, (0.1−2 mM) for 30 min at 25 °C. Aliquots were removed for BCAT activity measurement (assayed without DTT). Control samples were incubated under the same conditions without H_2O_2. Aliquots of these samples were treated with a 100-fold excess of DTT for 2 h at room temperature.

Trapping of Cysteine Sulfenic Acid. Wild-type hBCATc was exchanged into buffer A containing dimedone using a PD10 column. Four nmol of protein was incubated with 5 mM dimedone for 20 min at room temperature. Dimedone was omitted from control samples that contained only the dimethyl sulfoxide. In addition, a sample containing dimedone alone and no peroxide was also included. Peroxide was added, and the samples were incubated for 30 min at 25 °C, before the addition of catalase (5 μL of a 1 mg/mL solution). Samples were dialyzed into 10 mM ammonium bicarbonate overnight with several buffer changes. Following dialysis, protein samples were concentrated using a 7 mL Apollo ultrafiltration device with a cutoff of 30 kDa (Orbital Biosciences, Topsfield, MA). Samples were analyzed by Q-TOF MS after the addition of acetonitrile to a final concentration of 50% in 1% formic acid. In addition, BCAT activity was measured after dimedone treatment as described above.

Mass Spectrometry Analysis of WT and Mutant hBCATc Proteins. The amino acid substitution(s) in each of the mutated proteins was confirmed by mass spectrometry on a Micromass Quattro II triple quadropole mass spectrometer (Micromass, Manchester, England) fitted with an electrospray source [purchased with funding from the National Science Foundation (BIR-9414018), the North Carolina Biotechnology Center (9903IDG-1002), and the Winston-Salem Foundation] as previously described (*14*).

For Q-TOF MS analysis, acetonitrile and formic acid (final concentration 50% and 1%, respectively) were added to protein samples in 10 mM ammonium bicarbonate before injection into the Q-TOF MS (Waters, Manchester). Each single scan required 20−30 scans (1s scans for 1−2 min), and the data were processed using MassLynx Version 4.0 and the Maximum Entropy software supplied with the program to generate spectra on the absolute molecular weight scale. Sample analysis was carried out using the Q-TOF MS from the Bristol, Research and Genomics Institute, University of the West of England, U.K.

S-Glutathionylation of hBCAT Proteins. S-glutathionylation of WT hBCATc and the mutant proteins, C335S, C338S and C335/8S, was investigated using decreasing ratios of GSH:GSSG (i.e., 10 mM GSH only; 7.5 mM GSH and 1.25 mM GSSG; 5 mM GSH and 2.5 mM GSSG; 2.5 mM GSH and 3.75 mM GSSG; and 5 mM GSSG only, respectively). After buffer exchange, 1 μg of protein was incubated with

the above ratios of GSH:GSSG for 30 min at 25 °C. Also, aliquots were removed to measure BCAT activity (without DTT in the assay). S-Glutathionylated proteins were identified using Western blot analysis. Control samples did not contain any GSH or GSSG. To quantify the extent of S-glutathionylation, 4 nmol of protein were incubated with 10 mM GSH or 5 mM GSSG for 30 min at 25 °C. The number of GSH adducts present in the GSH and GSSG fraction was assessed using Q-TOF MS analysis ± dialysis.

Formation of mixed disulphide bonds with GSH (S-glutathionylation) under oxidative conditions was carried out in buffer A using 4 μM enzyme incubated with increasing H_2O_2 concentrations (50−500 μM) in the presence of 10 mM GSH at 25 °C for 30 min. In the controls, GSH was omitted. As a negative control in each experiment, peroxide and GSH were not incubated with hBCATc. The S-glutathionylated proteins were detected by Western blotting using a mouse monoclonal anti-GSH conjugate primary antibody (Virogen, MA).

Detection of S-Glutathionylated hBCAT Proteins by Western Blot Analysis. One microgram of each hBCAT sample was subjected to electrophoresis using 12% SDS−PAGE at 180 V, then proteins were transferred for 1 h 15 min at 125 V at 8 °C to hybond nitrocellulose ECL membranes (Amersham), and the membranes were blocked overnight (15−17 h) at 4 °C in 2 mM Tris, 0.2 M NaCl, and 0.1% Tween-20, (TBST buffer) containing 5% casein. Membranes were washed three times (30 min each) with TBST buffer before incubation with TBST (plus 5% BSA) containing mouse monoclonal anti-GSH primary antibody (1:2000 dilution) for 1 h at room temperature. After washing, the membranes were incubated with rabbit antimouse IgG HRP conjugate secondary antibody (1:1000 dilution) (Dako) for 1 h. The membranes were incubated with ECL substrate (Pierce) and exposed to ECL hyperfilm (Amersham) before staining the gel with GelCode Blue (Pierce).

Reduction of S-Glutathionylated hBCATc Using the Grx System. Reduction of S-glutathionylated hBCATc was conducted in 100 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM GSH, 100 μg/mL BSA, 100 μM NADPH, 0.14 Units/mL glutathione reductase, and 50 nM Grx. Protein concentrations varied from 1−4 μM of S-glutathionylated hBCAT. Protein deglutathionylation was measured continuously at 340 nm, and Grx activity was calculated using the molar extinction coefficient of 6,200 M^{-1} cm^{-1} for NADPH. The amount of GSH released from hBCATc by Grx was determined from the concentrations of hBCATc in the assay and the amount NADPH oxidized.

BCAT Affinity Column Preparation. Two milligrams of purified WT hBCATc were dialyzed overnight against bicarbonate buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl, pH 8.0). Two grams of cyanogen activated Sepharose 4B (Sigma) was suspended in 200 mL of cold 1 mM HCl for 10−15 min, and the column prepared according to the manufacturer's instructions. The resin was then mixed with the protein in 0.2 M $NaHCO_3$, sealed, and gently mixed overnight at 4 °C followed by 3 washes with 0.2 M $NaHCO_3$ at pH 8.0. The resin was added to 4 mL of 0.2 M $NaHCO_3$ at pH 8.0 with 1 M ethanolamine, and mixed gently for 1 h at 4 °C, then washed with buffer A (0.2 M $NaHCO_3$ at pH 8.0 containing 0.5 M NaCl), buffer B (2 M urea in distilled H_2O), buffer C (0.1 M sodium acetate and 0.5 M NaCl at pH 4.0), and finally with PBS. The column was stored at 4 °C in PBS adjusted to contain 0.2% sodium azide and 5 mM DTT until use.

Redox Binding of Neuronal Proteins to hBCATc. The IMR32 human neuronal cell lines were propagated in 10% v/v heat inactivated fetal calf serum (Invitrogen), 4 mM L-glutamine, and 6% v/v penicillin/streptomycin supplemented RPMI 1640 (Sigma), at 37 °C, 5% CO_2, and maintained between 50−80% confluence in 75 cm^2 cell culture flasks (Corning). Cytosolic proteins from 50−80% confluent flasks were extracted on ice in 1 mL of 50 mM Tris buffer (pH 7.4) containing, 25 mM KCL, 1 mM DTT, and 1 mM EDTA 1% v/v protease inhibitor cocktail (Amersham), with 2 repeat sonications for 15 s at 1 min intervals followed by centrifugation at 15,000*g* at 4 °C for 45 min. The protein content of the supernatant was quantified using the amido black assay as previously described (*23*).

Final amounts of 0.8 mg of cytosolic extract was loaded onto an hBCATc affinity column pre-equilibrated with cytosolic extraction buffer, followed by a 3 column volume wash with, extraction buffer (without DTT), then buffer B (50 mM Tris, pH 7.4, 100 mM KCL, 10 mM CHAPS, and 1 mM EDTA). Bound proteins were eluted from the column in 2 mL of buffer B adjusted to contain 10 mM NADH, followed by 3 column volumes of Buffer B. Control experiments were carried out substituting NAD^+ for NADH. In a separate experiment, cells were treated with 500 μM peroxide before affinity chromatography. The NADH peak fractions were pooled and precipitated in 3 volumes of chilled acetone, incubated for 1 h at −20 °C, before centrifugation at 15,000*g*, at 4 °C for 10 min. The pellet was air-dried on ice for around 15 min before resuspension in 30−40 μL buffer (0.06 M Tris at pH 6.8, 2% SDS, 10% glycerol, and 0.025% bromophenol blue). Protein content was quantified using the amido black assay, before being resolved by SDS−PAGE (12% resolving gel).

Protein bands were excised from the Coomassie stained gel, alkylated in the dark in 0.1 mM NH_4HCO_3 and 5 mM iodoacetamide, and digested with 0.25 mg of trypsin (Promega) in 0.1 mM NH_4HCO_3 for 16 h at 37 °C. The peptides were extracted in 10 μL of 50% v/v acetonitrile, 5% v/v formic acid with 10 min water bath sonication at 37 °C and subjected to Q-TOF MS MS.

RESULTS

Chemical Modification of hBCATc with Thiol Specific Reagents. To investigate the reactivity of the thiols of the CXXC motif and the four other solvent accessible thiol groups identified in the crystal structure of hBCATc (*21*), the cysteine residues were reacted with sulfydryl specific reagents.

Titration of WT hBCATc with DTNB revealed a time-dependent reaction of a total of 6 ± 0.4 thiol groups per monomer (Figure 1A, closed circles). Two thiol groups reacted within the first 10 s followed by the reaction of approximately 2 additional thiol groups within the first minute (Figure 1A inset, closed circles). Reaction of the final 2 thiol groups with DTNB occurred by 10 min. Mutating cysteine residues to serine in the CXXC region yielded a total of 3 ± 0.5 thiol groups per monomer, and a slower rate of reaction with DTNB compared with that of WT

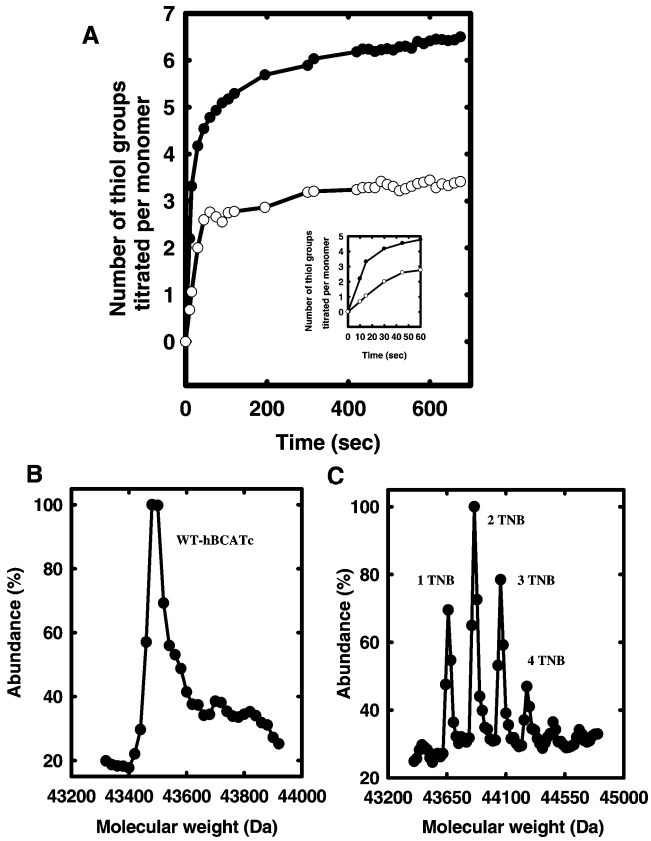


FIGURE 1: DTNB titration of WT and mutant BCAT (C335/8S) proteins. DTNB titration and Q-TOF MS were performed as described in Experimental Procedures. Panel A shows DTNB titration of hBCATc (●) and the C335/8S mutant protein (○) with a 100-fold excess of DTNB. Inset shows initial 60 s. Panels B and C show the transformed Q-TOF MS analysis data, which represent the relative abundance of masses for control WT hBCATc (B) and WT hBCATc labeled with 100-fold excess DTNB (C). The major peak shown in (B) corresponds to 43,480 amu, and (C) shows 4 peaks of, 43,678, 43,876, 44,074, and 44,272 amu, respectively. These peaks represent 1, 2, 3, and 4 TNB moieties, respectively.

hBCATc (Figure 1A inset, open circles), which implies that C335 and C338 are the most reactive cysteine residues. On the basis of DTNB titration of WT hBCATc, labeling of 6 thiol groups resulted in a loss of 60−70% of BCAT activity compared to that of the control enzyme incubated without DTNB and assayed without DTT. The majority of BCAT activity was recovered after incubation with DTT (89% ± 1% SEM, $n = 3$). The results appeared consistent with the structural data, which suggested there are 6 solvent accessible cysteine residues (*21*). However, when excess DTNB was removed and the protein was treated with 100-fold excess of DTT to release bound TNB, only 1 ± 0.4 equivalents of TNB was released per subunit as opposed to the expected 6 TNB anions. Quadrupole TOF MS analysis of the DTNB-treated proteins showed that there was a mixture of species. Figure 1B shows the WT-hBCATc without DTNB showing a molecular mass of 43,480 amu. The DTNB-treated protein showed four peaks with a major peak at 43,876 (Figure 2C). The molecular masses were 43,678, 43,876, 44,074, and 44,272 amu, corresponding to one, two, three, and four TNB (198 amu) anions, respectively. Thus, the average number of TNB anions detected was 2 TNB molecules, as opposed to the expected 6 observed from the spectral analysis, implicating significant redox cycling of the TNB anion.

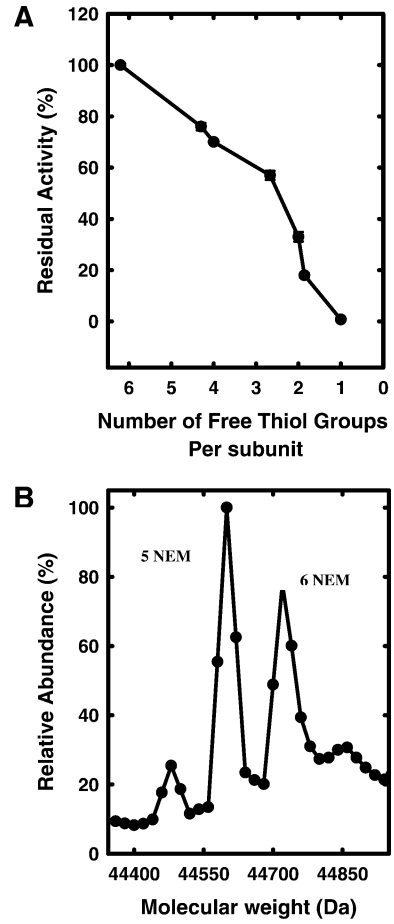


FIGURE 2: Inhibition of hBCATc activity with the irreversible thiol specific reagent NEM. Panel A: Seven nmol of hBCATc in buffer A was labeled with a 10-fold molar excess of NEM for 10 min at room temperature. The number of free thiol groups remaining was calculated by DTNB titration and plotted versus the % residual BCAT activity. BCAT activity was measured using the standard BCAT assay with DTT as described in Experimental Procedures. BCAT activity is presented as percent of control BCAT incubated without NEM. Data are the means ± standard errors of the mean ($n = 3$). Panel B shows the transformed Q-TOF MS analysis data, corresponding to the relative abundance of masses for hBCATc labled with NEM. Two main peaks are noted, which represent hBCATc with 5 and 6 NEM moieties, respectively.

An independent evaluation of cysteine labeling was determined using the irreversible thiol specific reagent NEM (Figure 2A). Free thiols were estimated using DTNB after the removal of excess NEM. Labeling of two thiol groups with NEM correlated with a decrease in activity of 33% ± 2%, and a further loss in activity to 80% ± 3% was observed when two additional thiol groups were labeled (Figure 2A). Loss of an additional thiol group as monitored by DTNB titration resulted in the complete inhibition of enzyme activity. Labeling of 5−6 thiol groups was confirmed using Q-TOF MS (Figure 2B). Also, denaturation of the NEM labeled protein with guanidine chloride resulted in the titration of 4 thiols groups compared with that of 10 thiol groups titrated in denatured WT-hBCATc. The results suggest that all six thiol groups are accessible to thiol specific reagents and that at least 4 thiol groups must be in the reduced state to maintain a fully active enzyme.

Steady-State Kinetic Parameters of WT hBCATc and the Cysteine Mutants. The NEM results suggest that mutation of at least 4 cysteine residues will have functional effects

Table 1: Kinetic Constants for the Reactions of WT hBCATc and the Cysteine Mutant Proteins C335S, C338S, C335/8S, C221S, C235S, C242S, and C293S

WT hBCATc and mutant proteins	isoleucine		leucine		valine		glutamate	
	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^3$ M^{-1} s^{-1})
WT hBCATc	1075 ± 1	3359 ± 2	898 ± 1	2494 ± 1	1236 ± 1	618 ± 1	1075 ± 1	108 ± 1
C335S	341 ± 2	1364 ± 2	253 ± 2	505 ± 2	170 ± 1	89 ± 2	141 ± 2	43 ± 2
C338S	529 ± 6	2116 ± 9	430 ± 4	1652 ± 3	597 ± 2	597 ± 1	741 ± 2	62 ± 1
C335/338S	419 ± 1	1496 ± 3	213 ± 2	474 ± 3	292 ± 11	132 ± 3	208 ± 10	31 ± 1
C221S	503 ± 11	1118 ± 10	286 ± 10	1059 ± 1	443 ± 10	201 ± 3	403 ± 6	31 ± 1
C235S	779 ± 5	3895 ± 9	756 ± 5	3151 ± 1	838 ± 2	1397 ± 1	1157 ± 6	126 ± 1
C242S	870 ± 3	2071 ± 10	651 ± 1	1302 ± 1	688 ± 3	313 ± 1	517 ± 9	40 ± 1
C293S	843 ± 3	2107 ± 10	803 ± 1	2294 ± 1	1111 ± 3	579 ± 1	1035 ± 9	103 ± 1

on hBCATc activity. Therefore, cysteine (C) to serine (S) mutants of each of the 6 cysteine residues were prepared using site-directed mutagenesis as was the double mutant with both C335 and C338 substituted with serine. The steady-state kinetic profiles of WT hBCATc and hBCATc cysteine mutants were determined with the three BCAAs and glutamate using α-ketoisovalerate as the fixed substrate (Table 1). Mutation of the thiol group at the N-terminal hBCATc CXXC center cysteine (C335), with retention of the thiol group at position C338, had a larger effect (37% further loss in overall steady state kinetics) on the kinetic parameters than the substitution of the thiol group at position C338 alone (Table 1). However, mutation of the C-terminal cysteine (C338) and the double mutant significantly affected the kinetic constants. For all amino acid substrates, there was a significant decrease (70% to 80%) in k_{cat} with the C335S mutant compared to the values for WT hBCATc (Table 1). In general, K_m values were less affected than k_{cat} values, with the exception of glutamate, where the K_m value for glutamate with the C335S mutant enzyme was approximately 3-fold lower than that observed with WT enzyme. The k_{cat}/K_m values for all substrates were correspondingly lower than that of the WT enzyme (Table 1). The hBCATc C338S mutant enzyme exhibited k_{cat} values for the BCAAs that were 50% lower than that observed with WT hBCATc. With the exception of glutamate, where the K_m value was not changed and valine where the K_m value was 50% lower than the value for WT hBCATc, K_m values for isoleucine and leucine were largely unaffected for the C338S mutant. The k_{cat}/K_m value for C338S was within 30 to 40% for the BCAAs relative to the WT hBCATc values. Because both k_{cat} and K_m were changed, the resultant k_{cat}/K_m value for valine was similar to that of WT hBCATc. The double mutant C335/8S had k_{cat} values that were similar to values observed with the single C335 mutant enzymes. K_m values were largely unchanged compared to those of WT hBCATc. Overall, k_{cat}/K_m values were significantly lower than WT values and similar to values obtained with the single C335 mutants (Table 1).

The other non-CXXC cysteine single mutant enzymes exhibited reduced k_{cat} values compared to those of the WT enzyme. Changes were most apparent for the C221S mutant followed by the C242S mutant (Table 1). The C235S mutant enzyme exhibited k_{cat} values that were within 20 to 30% of WT hBCATc values, but interestingly also had lower K_m values, hence higher calculated k_{cat}/K_m values than those of the WT enzyme. The C293S mutant and WT hBCATc had similar kinetic constants with valine, leucine, and glutamate as substrates but resembled the C242S mutant with isoleucine. Therefore, site-directed mutagenesis studies of hBCATc showed that mutation of the thiols of the CXXC motif and the C221S mutant had significant effects on the catalytic efficiency of these enzymes. Relative to the k_{cat} of WT hBCATc, the k_{cat} of the other cysteine residues were largely unaffected, with a modest change to the turnover of C242S and C293S.

Reactivity of the hBCATc Thiols to Hydrogen Peroxide. To establish sensitivity of the solvent accessible thiols to oxidation, WT hBCATc and the mutant proteins were treated with increasing concentrations of H_2O_2. Reactivity of C335/8S with DTNB demonstrated that the most reactive thiol groups were C335 and C338 as shown by a 2-fold decrease in DTNB titration with this mutant (Figure 1A).

Air oxidation of hBCATc results in a loss of 40−45% BCAT activity when assayed in the absence of DTT. Additional oxidation using hydrogen peroxide of WT hBCATc and the mutants C221S, C293S, C242S, and C235S did not correlate with a loss in activity until concentrations of hydrogen peroxide exceeded 5 mM (oxidation of C221S has been included as an example in Figure 3A). However, substituting a thiol group at position C338 with serine in hBCATc resulted in increased peroxide sensitivity with a 60% loss of hBCAT activity observed (Figure 3A, closed circles). The activity lost with this oxidation was partially recovered upon addition with DTT (45%), which correlated with a recovery of 3 titratable thiol groups (data not shown). This oxidized protein migrated to a higher molecular weight band, indicating dimerization of the protein (Figure 3B), which dissociated when treated with DTT to its monomeric form (data not shown). In the C335S mutant where the activity was only 60% of the control, the remaining activity was also affected on treatment with peroxide (Figure 3, open triangles). When both thiols were mutated, ≤ 30% of the BCAT activity remained, and there was no further effect by peroxide at these concentrations.

Dimedone, a sulfenic acid specific reagent was used to study oxidative modification of the CXXC thiols. Quadrupole TOF MS analysis of peroxide treated WT-hBCATc revealed a sulfenic acid intermediate shown by an increase in 140 amu, which is equivalent to one dimedone molecule (Figure 4). Formation of this alkylated product correlated with a loss of 45% of BCAT activity. Thus, oxidation results in the reversible inhibition of this protein through a sulfenic acid intermediate, resulting in the formation of a disulphide bond between C335 and C338. Further oxidation did not result in

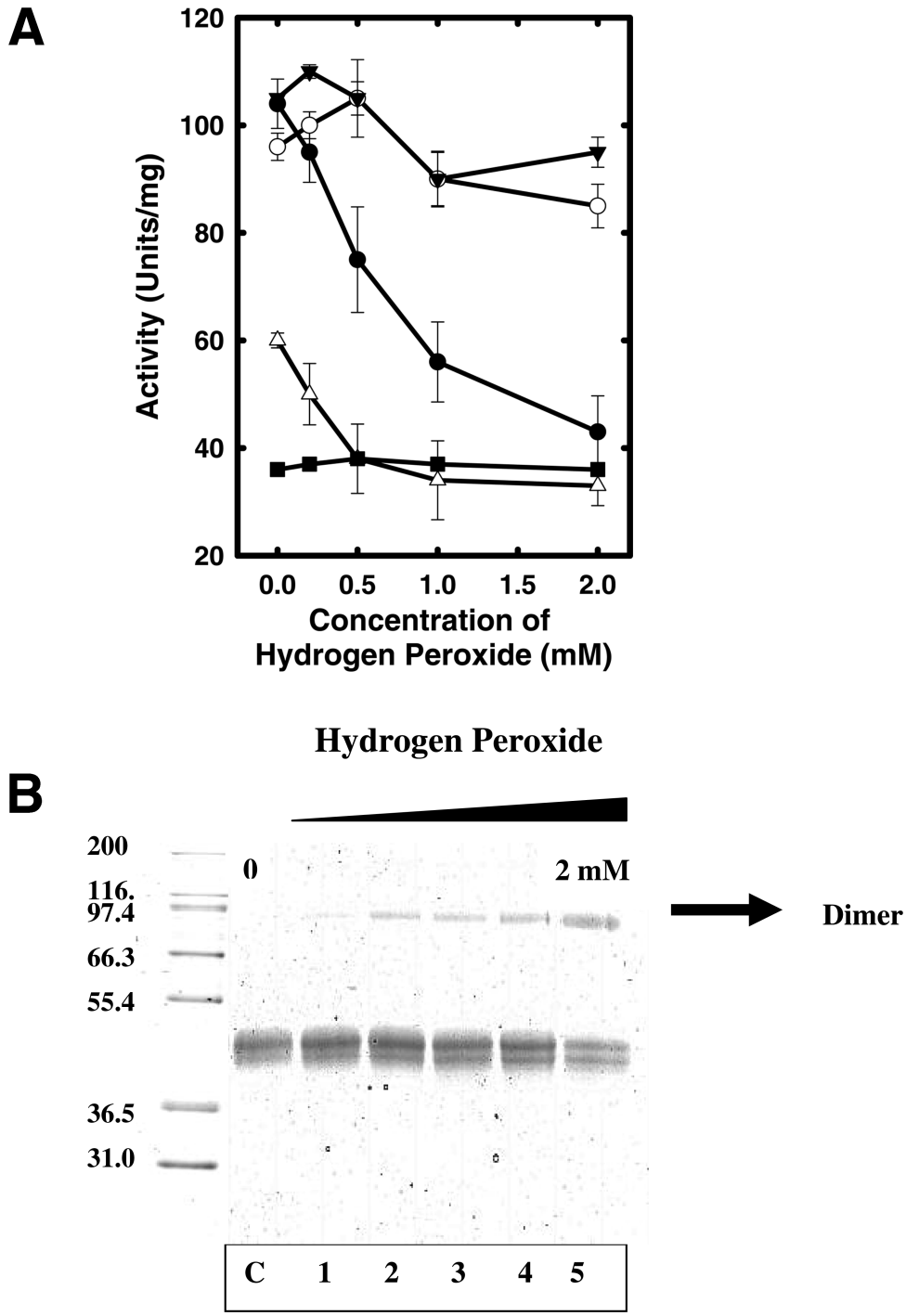


FIGURE 3: Effect of hydrogen peroxide on WT hBCATc and cysteine mutant enzymes. Panel A: BCAT activity was measured as described in Experimental Procedures following incubation with increasing concentrations of H_2O_2 (0.1−2 mM) in 50 mM HEPES (pH 7.2) and 1 mM EDTA at 25 °C for 30 min. Symbols used are WT hBCATc (○), C219S mutant (▼), C338S mutant (●), C335S mutant (△), and C335/338S mutant (■). Data are the means ± standard errors of the mean ($n = 3$). Panel B: Migration of the peroxide treated C338S mutant on 12% SDS−PAGE. Conditions were (from left) standards; control C338S without peroxide; lane 1,100 μM peroxide; lane 2, 200 μM peroxide; lane 3, 400 μM peroxide; lane 4, 800 μM peroxide; and lane 5 2.0 mM peroxide.

thiol modification of the other reactive cysteines in WT-hBCATc until higher concentrations of peroxide were utilized.

S-Glutathionylation of WT hBCATc and the Reactive Cysteine Mutants with GSH and GSSG. Sulphenic acids are transient reactive intermediates that can readily react with other free thiol/thiolates, forming disulphide bonds either intra/intermolecularly or externally with other thiol containing proteins or small molecules. One of these reactions, termed S-glutathionylation, occurs between protein sulfenic acids and GSH or between protein thiols and GSSG. To determine if hBCATc formed mixed disulphide S-glutathionylated products, and to establish which of the reactive thiols was involved in this mechanism, WT hBCATc and the mutant proteins were incubated with decreasing ratios of GSH: GSSG.

As the ratio of GSSG:GSH increased, the intensity of the band containing S-glutathionylated WT hBCATc also increased (Figure 5A, lanes 1−6), which correlated with a gradual loss of 25% BCAT activity, when assayed in the

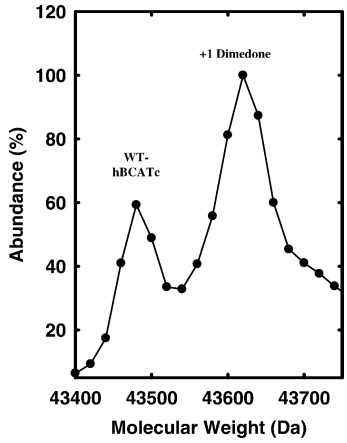


FIGURE 4: Identification of the sulfenic acid intermediate in WT hBCATc. Briefly, protein samples (4 nmol) were incubated in the presence of 5 mM dimedone, for 20 min at room temperature, which was subsequently treated with peroxide and incubated for 30 min at 25 °C. The reaction was stopped with catalase (5 μL of a 1 mg/mL solution) and samples prepared for Q-TOF MS analysis as described in Experimental Procedures. Presented are the transformed mass spectrometry data for the sample after treatment, with peaks of maximal abundance at 43,480 and 43,620 amu, respectively.

absence of DTT (WT hBCATc, 120 IU compared to 90 IU). Under the same conditions, the mutant proteins showed varying degrees of S-glutathionylation (Figure 5B−D). The C335S mutant protein and the double mutant protein did not show evidence of measurable S-glutathionylation until the redox environment switched completely to GSSG (Figure 5B and D, respectively). However, S-glutathionylation was observed at a 2:1 ratio of GSH/GSSG for the C338S mutant (Figure 5C, lane 4), which correlated with a loss of 15% BCAT activity (100 to 85 IU). These findings indicate that GSH incorporation occurred preferentially at C335 followed by C338 and that it is not until the redox environment shifted to a completely oxidizing state that the other thiols became S-glutathionylated.

Quadrupole TOF MS analysis was used to quantify the extent of S-glutationylation in WT-hBCATc incubated with GSH or GSSG. Incubation with GSH alone showed a major peak at 43,480 amu and a minor peak relative to control hBCATc (Figure 6A compared with 6B) with a molecular weight of 43,786 amu corresponding to an increase in 306 amu. Removal of excess GSH using PD10 gel filtration showed only one peak identified with a molecular mass of 43,480 amu, the same as that reported for control hBCATc (data not shown for brevity). When WT hBCATc was incubated with GSSG, 2 main peaks were identified with molecular masses of 44,090, and 44,700 amu, respectively. As the molecular weight of GSH is 306 Da, these masses corresponded to hBCATc with 2 and 4 glutathione adducts, respectively. The predominant species in this sample was hBCATc with 4 GSH adducts (44,700 amu). Thus, on exposure to GSH or GSSG the number of glutathione adducts increases from 1 with GSH to 4 with GSSG.

Under oxidizing conditions hydrogen peroxide mediated an increase in S-glutathionylation of WT hBCATc as observed by the faster migration of the protein, resulting in a doublet on a 12% SDS−PAGE (Figure 7A, lanes 6−10). Western blot analysis using anti-GSH confirmed that this modification was due to S-glutathionylation (Figure 7B, lanes 6−10). Thus, when levels of hydrogen peroxide increased, the degree of association of hBCATc with GSH also significantly increased.

Protein Deglutathionylation Using the Physiological Reducing System Grx. Protein deglutathionylation can be catalyzed by a number of different reducing systems. To investigate the ability of the Grx system (Scheme 1) to catalyze the release of GSH adducts from S-glutathionylated WT-hBCATc, release of GSH was measured by observing the loss in absorbance of NADPH at 340 nm. The oxidation of GSH to GSSG by the oxidized active site of Grx requires two consecutive reactions. Here, the catalysis of the S-glutathionylated hBCATc is mediated by reduced Grx, facilitated by GSH. This is coupled with glutathione reductase where loss in absorbance is as a result of oxidation of NADPH to $NADP^+$. The rate of formation of $NADP^+$ directly correlated with the amount of S-glutathionylated protein ($r = 0.99$), implicating that this reaction is reversible using the Grx system (Figure 8). The number of GSH adducts released was calculated to be 1.85 ± 0.182.

Redox Association of Neuronal Proteins with the CXXC Motif of WT hBCATc. To examine the physiological potential of hBCATc, we investigated whether the CXXC motif had redox associations with proteins extracted from neuronal cells. Neuronal proteins extracted from IMR32 cells were reacted with WT hBCATc bound to an affinity matrix, where bound proteins were selectively eluted with NADH (Figure 9A, peak (ii), closed circles). When NAD^+ was used in place of NADH, the proteins were not eluted (data not shown for brevity). Treatment of the neuronal cells with hydrogen peroxide resulted in a loss of association between these proteins and hBCATc (Figure 9A, open circles). Several of these proteins were identified using Q-TOF MS MS analysis. Peptide mass finger printing using MASCOT identified the following proteins: β-tubulin, septin 4, kalirin RhoGEF, sodium channel type 10 α subunit, and myosin 6.

DISCUSSION

Reactive thiol groups are central to the redox control of many metabolic proteins and are now recognized as key players in preserving the redox status of the cell (*1*). Alteration to this redox status, where the balance is tipped to a more oxidizing environment has been implicated in the pathogenesis of many diseases (reviewed in Droge (*25*)). Here, using thiol specific reagents, oxidation and S-glutathionylation, the role of the 6 solvent accessible cysteine residues in the redox regulation and S-thiolation of hBCATc was determined. Furthermore, redox associations between hBCATc and proteins extracted from neuronal cells were investigated to explore the cellular role of these reactive thiols.

Our findings demonstrate that although 4 thiols groups are critical in maintaining full functionality of the protein only the thiols of the CXXC motif have a direct role in the redox regulation of hBCATc. S-thiolation of hBCATc under oxidizing conditions, followed by deglutathionylation via the GSH/Grx repair pathway, demonstrated that the thiols of the CXXC motif are targets for the physiologically important reducing systems of the cell. This implies that these reducing mechanisms may be using hBCATc as a redox sink conserving GSH or that the protein itself is regulated during oxidative stress via S-glutathionylation. Moreover, redox associations

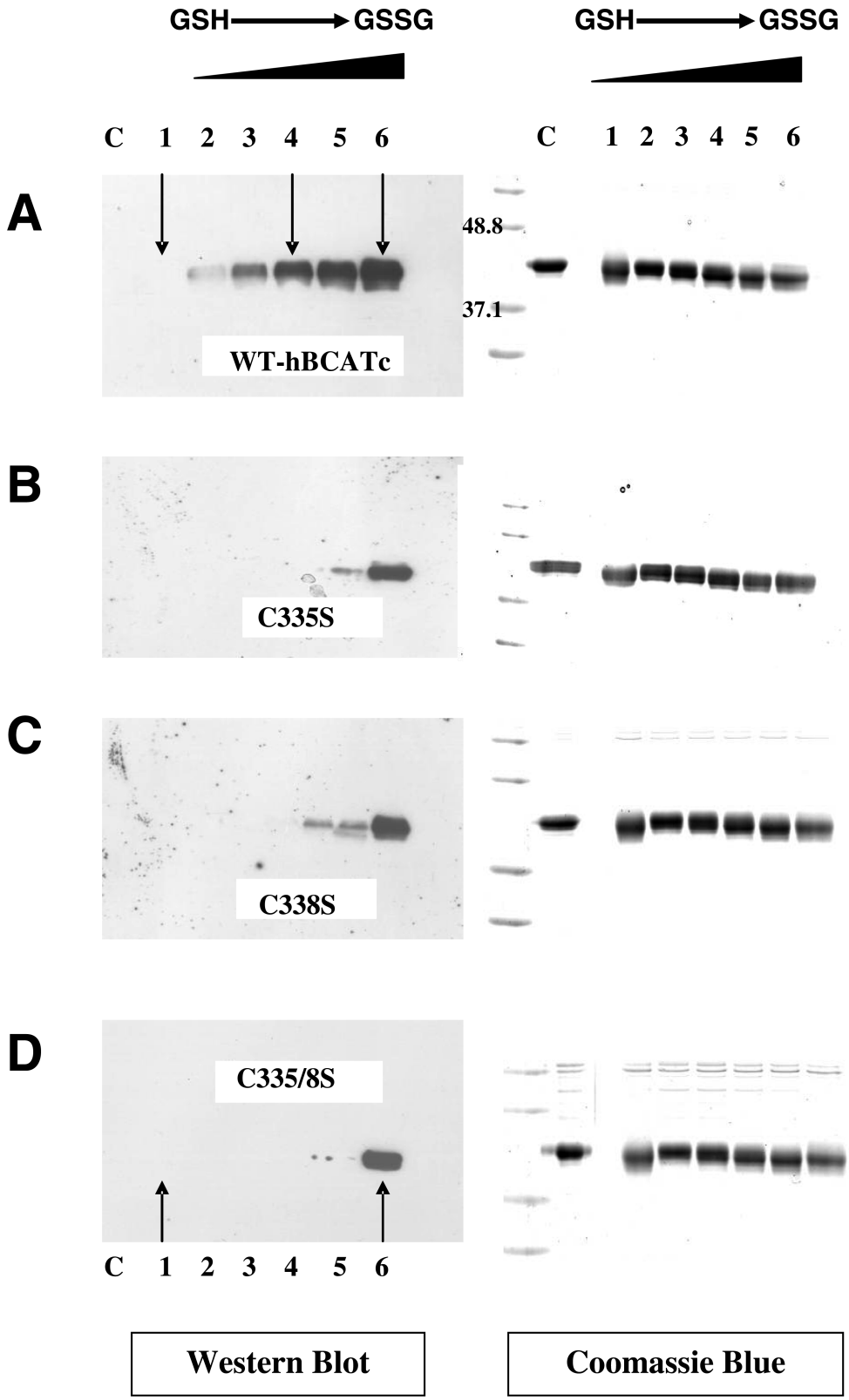


FIGURE 5: S-Glutathionylation of WT-hBCATc and the BCATc mutant proteins, C335S, C338S, and C335/8S. Protein samples were exchanged into buffer containing 50 mM HEPES, pH 7.2, and 1 mM EDTA and S-glutathionylation performed as described in Experimental Procedures at varying ratios of GSH:GSSG at 25 °C. S-Glutathionylated proteins were identified using Western blot analysis (left column). The modified proteins were identified using an anti-GSH IgG2a mouse monoclonal (see Experimental Procedures). Conditions were control + β-mercaptoethanol; lane 1, no additions; lane 2, 10 mM GSH; lane 3, 7.5 mM GSH and 1.25 mM GSSG; lane 4, 5 mM GSH and 2.5 mM GSSG; lane 5, 2.5 mM GSH and 3.75 mM GSSG; and lane 6, 5 mM GSSG. Adjacent to the Western blots (right column) is the corresponding Coomassie stain of these gels, which demonstrates equal loading.

with proteins extracted from neuronal cells offer intriguing links between phosphorylation, G-protein cell signaling, and redox regulation.

Sole reactive cysteine residues (e.g., C3635 in the ryanodine receptor) or cysteines in motifs conserved throughout evolution, such as the CXXC motif (e.g., present in thiore-

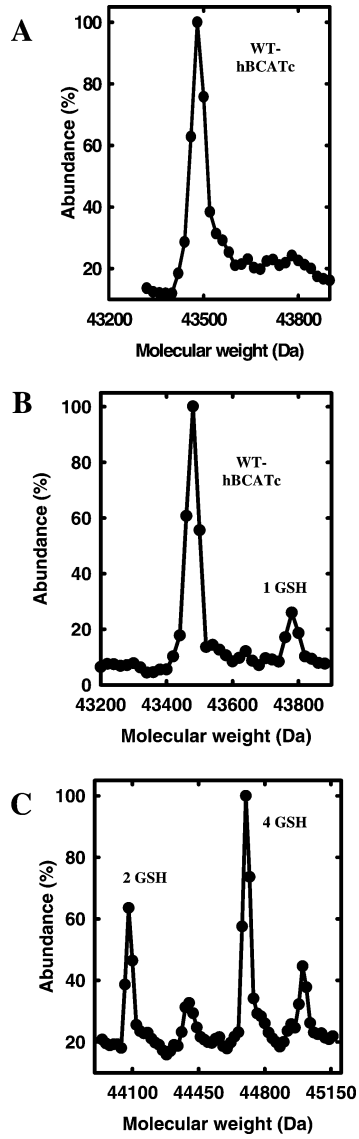


FIGURE 6: Evidence for GSH redox cycling using Q-TOF MS analysis of WT-hBCATc. The hBCATc protein was exchanged into buffer A and incubated with 10 mM GSH or 5 mM GSSG, respectively, for 30 min at 25 °C, which were subjected to Q-TOF MS analysis as described in Experimental Procedures. Panel A: The major peak corresponds to control WT hBCATc, 43,480 amu. Panel B: This reflects an aliquot taken after incubation with GSH prior to gel permeation. Two peaks are shown, the first corresponding to WT hBCATc, and the second with a molecular weight of 43,786 amu corresponds to hBCATc with 1 GSH adduct. Panel C: This is an aliquot of protein taken after incubation with 5 mM GSSG after PD 10 separation, showing two main peaks of molecular weights 44,090, and 44,700 amu, respectively. These values correspond to hBCATc with 2 and 4 GSH adducts, respectively.

doxins and glutaredoxins), serve as sites for redox control of proteins through reversible thiol disulphide exchange (*6, 26*). Here, evidence for redox cycling in hBCATc was demonstrated using the thiol specific reagent DTNB analyzed by Q TOF MS (Figure 1C). Despite labeling of 6 thiol groups as monitored by the release of TNB, an average of only 2 thiol groups were labeled per monomer. Because C335 and C338 readily form an intrasubunit disulfide bond (*21*), the DTNB modification can also result in oxidation to generate a disulfide bond through a thiol-disulfide interchange reaction. Here one TNB-mixed disulfide is formed with the most reactive cysteinyl residue (C335), and then the second cysteine (C338) attacks the mixed disulfide, releasing the TNB anion and forming the disulfide bond between cysteines (C335−C338). This reaction results in the release of 2 TNB molecules, but without incorporating TNB into the protein. These findings indicate that inactivity associated with DTNB labeling was a result of both disulphide bond formation between C335 and C338, and the addition of TNB anions. Reversibility of this inactivity using DTT presented the first evidence of redox regulation of this isoform.

As NEM is an irreversible thiol reagent, redox cycling is prevented, and thus, an accurate correlation between the loss of thiols and BCAT activity could be demonstrated. These studies showed that at least 4 thiol groups of hBCATc were required to maintain maximum BCAT activity, with the remaining 2 thiols having a minimal role (Figure 2A). Steady state kinetic analysis of the cysteine to serine mutant proteins supported these studies, where mutations at C335S, C338S, C335/8S, and C221S resulted in a profound decrease in catalytic efficiency, with a modest effect observed for C242S. Minimal effects on the kinetic parameters of C293S and C235S imply that these thiol residues are less important to the functionality of the protein than the other reactive cysteines. Thus, in contrast to the mitochondrial isoform hBCATm, where only the thiol groups of the CXXC motif are important mechanistically (*13*), at least 4 of the solvent accessible thiols groups (C335, C338, C221, and C242) are integral in preserving the catalytic efficiency of hBCATc. Therefore, the potency of thiolate anions is dependent on their accessibility, their pK_a, and location within a protein as observed for the BCAT proteins (*21*). Other groups have supported the select reactivity of thiolate groups often in a very thiol rich environment, for example, of the 100 cysteine residues in the ryanodine receptor type 1, only a discrete subset of cysteines were shown to be redox sensitive (*26*). Interestingly, not all proteins with CXXC motifs have redox functionality, for example, redox regulation of the PLP dependent enzyme, cystathione β-synthase was shown to be through its heme group rather than its CXXC motif as originally expected (*27*).

Mutation and oxidation studies of the thiols of the CXXC motif confirm that C335 is the redox sensor, potentially through a sulfenic acid intermediate conserving aspects of redox regulation between isoforms (*14*). However, increased sensitivity of the mutant, C338S, to oxidation demonstrated that C335 now formed an alternative intersubunit disulphide bond with additional oxidation of the other accessible thiols, resulting in an inactive protein. As the recovery in BCAT activity for oxidized C338S was only partial (correlating with the recovery of three titratable thiol groups), it seems likely that the two remaining cysteine residues are oxidized to higher oxidation states such as sulfenic or sulfonic acid. Therefore, rather than C335 becoming overoxidized to sulfonic acid as occurs with its equivalent C315 in the C318S mutant protein of hBCATm (*14*), its oxidation is reversible through the formation of an intersubunit disulphide bond. These studies indicate that C335 is the most reactive thiolate and forms a reversible disulphide bond with C338 on air oxidation alone, and the role of C338 is to prevent unfavorable dimerization of hBCATc. Redox control through disulphide bond formation has also been reported for several proteins including GAPDH (*28*), creatine kinase (*29*), κB kinase (*30*), and β-Actin (*31*). However, it appears that only

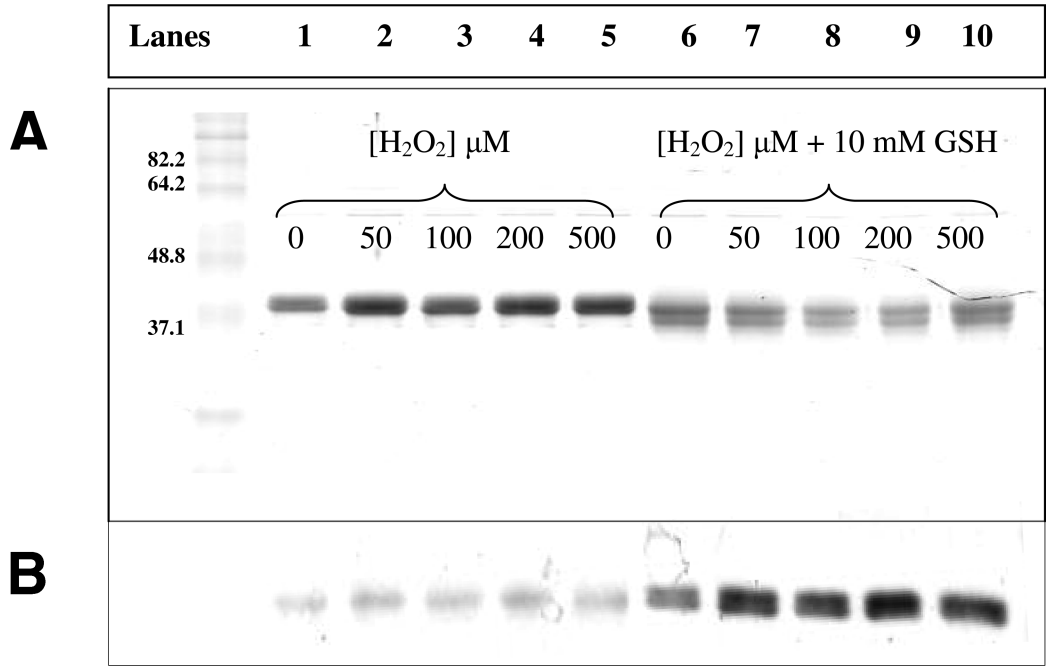
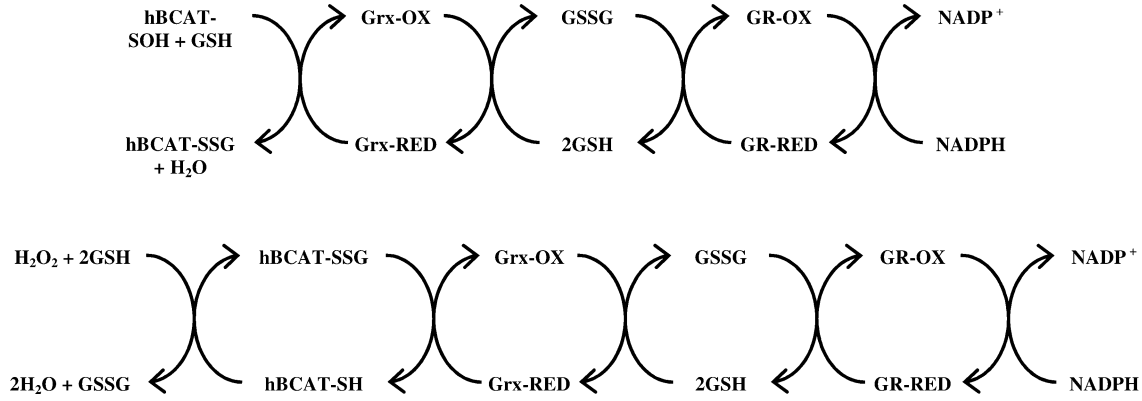


FIGURE 7: S-Glutathionylation of WT hBCATc induced by oxidation. Panel A: Twenty nmol of protein was exchanged into buffer A. Four nmol of enzyme was incubated with increasing H_2O_2 concentrations (50−500 μM) in the presence of 10 mM GSH at 25 °C for 30 min. Panel A shows the Coomassie stain of hBCATc with peroxide ± GSH. Conditions: standards; lanes 1−5 show hBCATc with increasing peroxide (0−500 μM) without GSH; and lanes 6−8 show hBCATc incubated with GSH and peroxide (0−500 μM). Panel B: The *S*-glutathionylated proteins of the above incubations were detected by Western blot analysis using an anti-GSH IgG2a mouse monoclonal as described in Experimental Procedures.

Scheme 1



proteins involved in the reducing systems of the cell, such as the glutaredoxins and thioredoxins, are regulated through a CXXC motif (*1*). Although the redox sensitivity of other proteins with CXXC motifs have yet to be characterized (such as Kalirin, NP-001019831), redox regulation of hBCATc is unlike that observed for enzymes involved in energy metabolism or the cytoskeletal framework but more like the reducing systems of the cell.

Although both NEM labeling and steady state kinetic analysis of at least two of the four thiol (C221, C293, C235, and C242) groups demonstrated the importance of these residues in maintaining functional activity, oxidation of these groups resulting in complete inactivation was only observed when the concentration of peroxide increased to pathophysiological levels. However, studies investigating the redox regulation of β-Actin and tublin, respectively, used concentrations between 5−20 mM peroxide to induce an altered response (*31*). Thus, although the concentrations of peroxide to induce oxidation of the other thiols are considered to be physiologically high, on the basis of the concentrations used in the above studies, there may be precedence for its importance.

Physiologically, the precise role of protein S-glutathionylation is unknown, but it has been suggested as a mechanism for cells to store GSH during oxidative stress or to prevent permanent loss of protein function as a consequence of the irreversible oxidation of critical thiolates to cysteine sulfinic and sulfonic acids (*12*). Here, following gel permeation WT hBCATc exists in a partially oxidized state, and as a consequence, only minimal S-glutathionylation was observed with GSH (Figure 6B). As this adduct was subsequently lost after gel permeation (due to a loss in the reducing environment) these results suggest that like redox cycling shown between hBCATc and the TNB anion, C338 forms an intrasubunit disulphide bond with C335 releasing GSH. However, under oxidizing conditions (+GSSG), loss of the GSH adduct was not observed because of significant S-glutathionylation of the

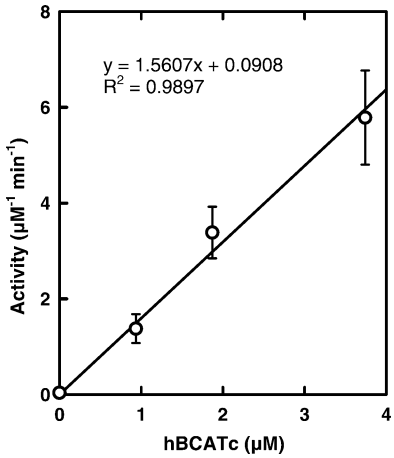


FIGURE 8: Reduction of S-glutathionylated hBCATc using the Grx system. Reduction of S-glutathionylated hBCATc was conducted as described in Experimental Procedures. The reaction was started by the substrate, i.e., by adding protein concentrations from 1−4 μM of S-glutathionylated hBCAT, respectively. The amount of GSH removed by Grx was determined spectrophometrically following the loss of absorbance of NADPH as described in Experimental Procedures.

thiol groups (Figure 6C). Thus, as the environment shifted from a GSH rich to a GSSG rich environment, the extent of S-glutathionylation increased, preferentially through C335. Here, these novel results suggest that under reducing conditions the role of GSH is primarily as a reductant, whereas S-glutathionylation was moreso a feature of oxidative stress. This was confirmed when S-glutathionylation of hBCATc was induced through peroxide treatment (Figure 7). Redox sensitive proteins such as GAPDH (*32*), α/β Actin (*33*), β-tubulin (*34*), κB kinase (*30*), creatinine kinase (*29*), and protein kinase C (*35*) are susceptible to S-glutathionylation via thiol disulphide exchange, resulting in altered activity. As such, these proteins have been used as biomarkers of redox state (*12, 36*) and as tools to decipher mechanisms by which redox control is mediated (*31, 34, 37*). Here, partial loss in activity is seen as S-glutathionylation increases in particular as the environment becomes more oxidizing. Widespread S-glutathionylation occurs in the nervous system (*38*), where hBCATc is expressed and localized in neurons (primarily glutamatergic and GABAergic) (*17*). Thus, under reducing conditions, the thiols of hBCATc are in constant flux with GSH, and during oxidative stress when the ratio of GSSG increases relative to GSH, which is seen in many neurodegenerative disorders (*39*), S-glutathiolylation increases, potentially protecting the protein or using hBCATc as a GSH reservoir until the oxidative insult is removed, that is, via deglutathionylation.

Deglutathionylation of protein thiols is catalyzed by the glutatredoxin system (*40*). This system involves the coordinated control of GSH, Grx, and glutathione reductase (using NADPH as the reducing equivalent) in the maintenance of the reducing capacity of the cell. The GSH/Grx reducing/repair system (*34*) operates in a series of thiol exchange reactions, resulting in the degluathionylation of the modified protein and the release of GSH (Scheme 1). Here, we demonstrated for the first time the ability of the Grx system to catalyze the deglutathionylation of hBCATc-SSG (Scheme 1, Figure 9), which indicates a potential site for reversible regulation in the cell. This GSH/Grx system has also been implicated in the repair of oxidatively damaged proteins such as GAPDH and phosphofructokinase (*41*). Recently, S-glutathionylation of tubulin and the neuron specific microtubule-associated proteins were linked to a role in the repair of damaged cytoskeletal proteins, and tubulin may function as a cellular redox buffer (*34*). Thus, as hBCAT-S-SG is a substrate for the GSH/Grx repair system, this offers a site for redox regulation, and we can speculate that hBCATc may play a similar role to that suggested for tubulin (*34*).

Oxidative stress, S-gluathionylation, and the GSH/Grx repair system have been intrinsically linked with the pathogenesis of several neurodegenerative diseases such as Parkinson's and Alzheimer's disease (*25*). All enzymes of the Grx system have been shown to be upregulated in neuronal cells in response to oxidative stress (reviewed in Dringen (*42*)), and this correlates with widespread S-glutathionylation (*38*). Using proteomic analysis, four key proteins associated with mild cognitive impairment, a precursor of Alzheimer's disease, were identified (*43*). One of the proteins identified was glutamine synthetase, which is involved in the glutamate/glutamine cycle. This cycle interacts with the BCAT cycle, which supplies nitrogen for *de novo* glutamate synthesis (*44*). Although it is not known whether oxidative damage of BCATs contributes to this neurological disease process, expression of hBCATc is induced in surviving neurons of the substantia nigra in a neonatal model of neuronal cell death (*45*). Thus, an understanding of how redox changes and S-glutathionylation occurs *in vivo* may help to understand the role of BCATs in health and disease. The functional significance of these interactions described in this article remains to be fully established.

Having established the role of the reactive cysteines *in vitro*, we investigated the redox association between BCAT and neuronal cellular proteins under conditions of oxidative stress. Under reducing conditions, using extracts from the neuronal cells IMR32 we found that hBCATc binds specifically to several proteins, including septin 4, kalirin rho GEF, β-tubulin, myosin-6, and the sodium channel type 10 α subunit. Interestingly, these proteins have either known reactive cysteines or CXXC motifs (*46–50*). They also have phosphorylation sites and/or are directly involved or controlled by G protein cell signaling, which is known to be modulated by peroxide. Under oxidative conditions, these proteins did not bind to the BCAT column, indicating that the reactive thiols of hBCATc are integral to these redox associations. Recent work in our group showed the association between the mitochondrial hBCAT protein and the E1 subunit of the branched-chain α-keto dehydrogenase (BCKD) complex demonstrating substrate channelling between proteins and the formation of a metabolic unit. We showed that either mutation of the thiols of the CXXC motif of hBCATm or phosphorylation of the E1 subunit prevented complex formation (*20*). Thus, as these neuronal cell signaling proteins are linked through redox interactions with hBCATc, which has several phosphorylation sites, this protein may also be regulated by phosphorylation.

In conclusion, this study has clearly demonstrated that hBCATc has at least 4 thiols that are central to the functionality of the protein, and our findings clearly show an important novel mechanism for redox regulation. The

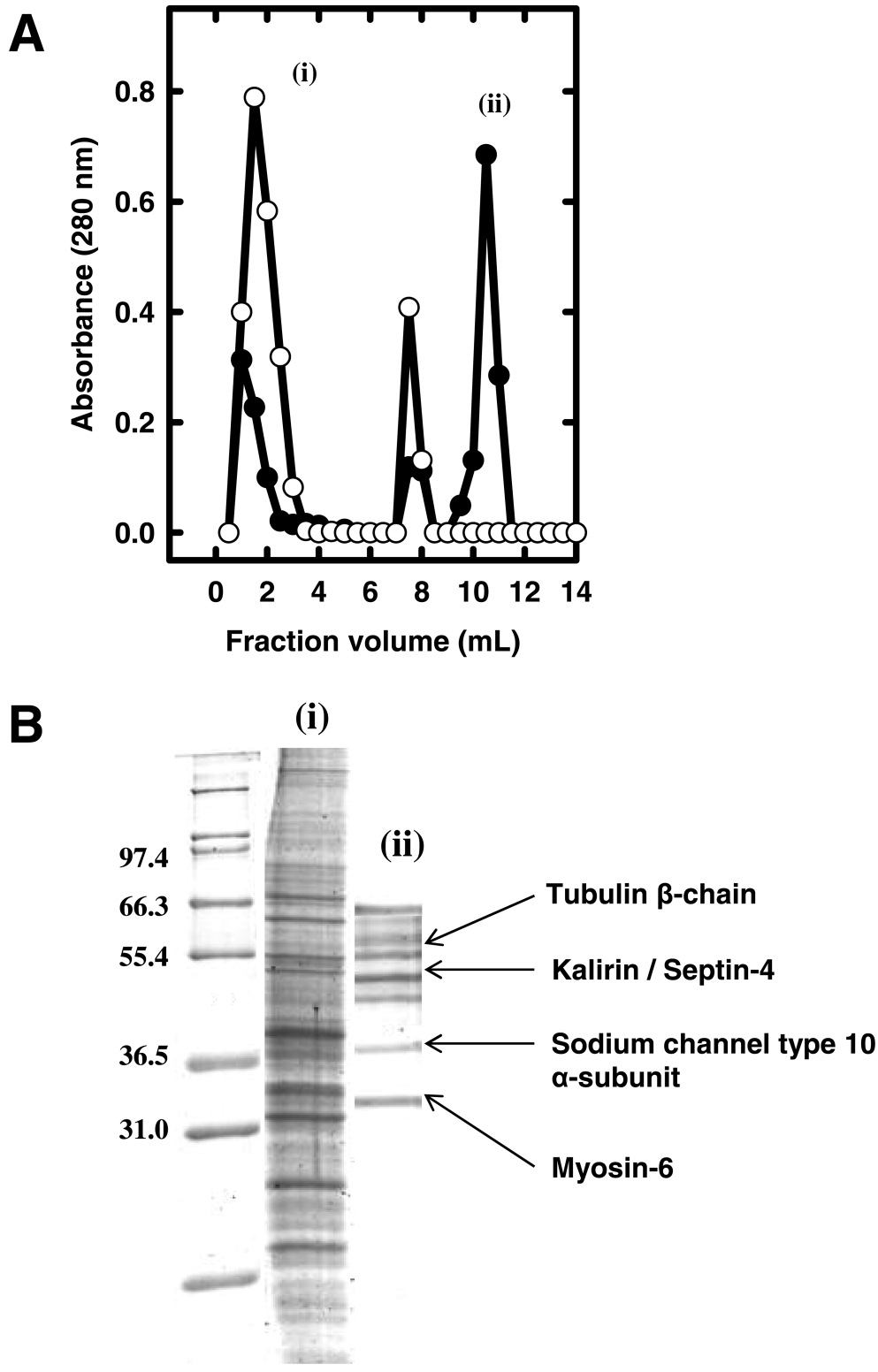


FIGURE 9: Identification of several redox sensitive proteins extracted from the neuronal cells IMR32, associated with WT hBCATc. Panel A: Proteins extracted from neuronal cells were applied to a WT hBCATc sepharose affinity column (●). Following washing (peak (i)), proteins bound to the affinity column were eluted with buffer containing 10 mM NADH (peak (ii)). Treatment of cells with hydrogen peroxide resulted in the loss of binding (○). Panel B: Fractions from peak (ii) were pooled and resolved using 12% SDS−PAGE. Using Q-TOF MS MS and peptide mass finger printing using MASCOT, the following proteins β-tubulin, septin 4, kalirin RhoGEF, sodium channel subunit 3, and myosin 6 were identified.

redox cycling of cysteine residues and the potential to form a mixture of thiol species dependent upon the cellular redox status suggests that hBCATc represents a new protein regulated by S-glutathiolation, which was confirmed by its interaction with the physiologically important Grx reducing system. Finally, the redox associations of hBCATc with neuronal proteins imply that this enzyme may play a pivotal role in regulating and/or mediating the degree of S-thiolation with specific neuronal proteins involved in G protein cell signaling.

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