Conformation-dependent inactivation of human betaine-homocysteine S-methyltransferase by hydrogen peroxide *in vitro*

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Betaine-homocysteine S-methyltransferase (BHMT) transfers a methyl group from betaine to Hcy to form DMG (dimethylglycine) and Met. The reaction is ordered Bi Bi; Hcy is the first substrate to bind and Met is the last product off. Using intrinsic tryptophan fluorescence [Castro, Gratson, Evans, Jiracek, Collinsova, Ludwig and Garrow (2004) *Biochemistry* **43**, 5341–5351], it was shown that BHMT exists in three steady-state conformations: enzyme alone, enzyme plus occupancy at the first substrate-binding site (Hcy or Met), or enzyme plus occupancy at both substrate-binding sites (Hcy plus betaine, or Hcy plus DMG). Betaine or DMG alone do not bind to the enzyme, indicating that the conformational change associated with Hcy binding creates the betaine-binding site. CBHcy [S-(δ -carboxybutyl)-D,L-homocysteine] is a bisubstrate analogue that causes BHMT to adopt the same conformation as the ternary complexes. We report that BHMT is susceptible to conformation-dependent oxidative inactivation. Two oxidants, MMTS (methyl methanethiosulphonate) and hydrogen peroxide (H_2O_2), cause a loss of the enzyme's catalytic Zn (Zn^{2+} ion) and a correlative

loss of activity. Addition of 2-mercaptoethanol and exogenous Zn after MMTS treatment restores activity, but oxidation due to H_2O_2 is irreversible. CD and glutaraldehyde cross-linking indicate that H_2O_2 treatment causes small perturbations in secondary structure but no change in quaternary structure. Oxidation is attenuated when both binding sites are occupied by CBHcy, but Met alone has no effect. Partial digestion of ligand-free BHMT with trypsin produces two large peptides, excising a seven-residue peptide within loop L2. CBHcy but not Met binding slows down proteolysis by trypsin. These findings suggest that L2 is involved in the conformational change associated with occupancy at the betaine-binding site and that this conformational change and/or occupancy at both ligand-binding sites protect the enzyme from oxidative inactivation.

Key words: betaine-homocysteine S-methyltransferase (BHMT), conformational change, hydrogen peroxide (H_2O_2), Met, oxidative stress, proteolysis, zinc.

INTRODUCTION

Betaine-homocysteine S-methyltransferase (BHMT; EC 2.1.1.5) belongs to a family of enzymes that methylate thiols or selenols (Pfam 02574). A unique feature of Pfam 02574 members is that each contains a novel metal-binding motif that includes three Cys residues required for binding Zn (Zn^{2+} ion) [1–3]. The role of Zn in these enzymes is to deprotonate their thiol or selenol substrates, thereby activating them for nucleophilic attack on their respective methyl donor substrates [4]. The BHMT reaction catalyses a methyl transfer from betaine (Bet) to Hcy to form DMG (dimethylglycine) and Met. The reaction mechanism is ordered Bi Bi; Hcy is the first substrate to bind and Met is the last product off [5,6]. In humans, BHMT is primarily expressed in hepatocytes and the proximal tubules of the kidney cortex [7].

The crystal structures of human BHMT [2] and rat BHMT [8] have been solved at varying degrees of resolution. Much of the rat BHMT structure was solved using diffraction data obtained from rat BHMT crystals in combination with molecular replacement techniques that used the co-ordinates of the human monomer as the model. Human (406 residues) and rat (407 residues) BHMT share 92% amino acid identity and, as expected, their structures are nearly superimposable. Recombinant BHMT is a homotetramer of 222 point symmetry that is best characterized as a dimer of dimers (Figure 1). The first three quarters of monomer sequence (10–318) encode a $(\beta/\alpha)_8$ barrel that contains residues required for Zn binding (Cys²¹⁷, Cys²⁹⁹ and Cys³⁰⁰) and most of the residues

required for ligand binding. The remainder of the amino acid sequence (319–406) encodes residues that have a dominant role in oligomerization. Important features of this region include the 'dimerization arm' (319–371) followed by a flexible linker and a C-terminal α -helix (381–407). We designate the C-terminal α -helix as ' α H' to reflect that it is a non-barrel secondary structure, consistent with the nomenclature first used to describe BHMT structure [2]. The dimerization arm of one monomer makes important contacts with loops L1 (38–52) and L7 (248–276) of the other monomer within the dimer. These loops have a significant role in shaping the active-site cavity. A smaller region within the dimerization arm, referred to as the 'hook' region (354–369), encodes residues that participate in dimer–dimer contacts. Other prominent dimer–dimer contacts are mediated by α H [8]. The two α H helices from each dimer are closely packed against the dimerization arms (316–349) of its partner dimer, resulting in the formation of the tetramer (Figure 1C). We showed that a BHMT truncation mutant lacking residues beyond the dimerization arm is active, but does not accumulate in *Escherichia coli* [9], suggesting that post-dimerization arm residues including α H have a role in stabilizing BHMT. In this same report, we showed that His³³⁸ of one monomer contributes to Bet binding at the active site of the other monomer, indicating that the complete active site is formed upon dimerization. The active site of BHMT, including residues known to be betaine-binding determinants, is shown in Figure 2.

Intrinsic tryptophan fluorescence has been used to describe three different steady-state conformations of BHMT [5]. When

Abbreviations used: apoBHMT, BHMT without zinc bound; Bet, betaine; BHMT, betaine-homocysteine S-methyltransferase; CBHcy, S-(δ -carboxybutyl)-D,L-homocysteine; DMG, dimethylglycine; DTT, dithiothreitol; 2ME, 2-mercaptoethanol; MMTS, methyl methanethiosulphonate; PAR, 4-(2-pyridylazo)-resorcinol; TCEP, tris(2-carboxyethyl)phosphine; Zn, Zn^{2+} ion.

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Figure 1 Gross features of BHMT structure

Shown is rat BHMT (PDB accession code 1UMY). The majority of the BHMT molecule is a $(\beta/\alpha)_8$ barrel (red). The dimerization arm (green) and terminal α -helix (blue) are critical for oligomerization. Loop L2, a barrel substructure (pink), is located near the entrance to the active-site Cys residues (golden) containing the catalytic Zn (black). The partner dimers are shown in lighter shades of the same colours. (A) BHMT monomer. (B) BHMT dimer. (C) BHMT tetramer. (D) BHMT primary structure. The positions of Cys residues that co-ordinate Zn are noted. Part of the crystal structure has not been resolved, including residues 82–90 in L2. The image was created using Jmol version 10 (<http://jmol.sourceforge.net/>).

Figure 2 Human BHMT dimer in complex with CBHcy

Shown is the active site of human BHMT with CBHcy bound (PDB accession code 1LT8). The entrance to the active-site is at the upper left corner. In red are Zn-binding Cys residues that are susceptible to oxidation, as well as other active-site residues that could be modified by exposure to H_2O_2 , including Trp⁴⁴, Tyr⁷⁷, Tyr¹⁶⁰ and His²¹⁸ of chain A (light grey), and His³³⁸ of chain B (dark blue). The image was created with iMol version 0.30 (<http://www.pirx.com/iMol/index.html>).

excited at 295 nm, ligand-free enzyme has a fluorescence emission maximum at 334 nm. Enzyme saturated with Hcy or Met results in a 4 nm red shift ($\lambda_{\max} = 338$ nm), which is associated with a very small increase in fluorescence intensity. When the BHMT–Hcy binary complex is converted into the ternary complex by the addition of saturating levels of Bet (catalytic ternary complex) or DMG (abortive ternary complex), there is an additional 4 nm red shift ($\lambda_{\max} = 342$ nm) and a very large increase (60 %) in its fluorescence intensity. In the presence of saturating levels of CBHcy [S-(δ -carboxybutyl)-D,L-homocysteine], a BHMT bisubstrate analogue, the complete 8 nm red shift and large increase in fluorescence intensity happen in concert, mimicking the ternary complexes. By individually replacing each Trp residue within BHMT to either a Phe or Ala residue, we established that there is movement of the dimerization arm upon binding of each substrate [5]. Comparison of the rat and human BHMT structures indicates that at least part of this movement includes a repositioning of His³³⁸ as a Bet-binding determinant.

Further comparison of the crystal structures of ligand-free rat BHMT with human BHMT complexed with CBHcy provides additional evidence of conformational changes upon ligand binding. One interesting difference is that loop L2 (74–97), which is partially disordered in both structures, is extended away from the active site in the ligand-free rat enzyme, but bends in towards the active site in the structure of the human enzyme complexed with CBHcy. Gonzalez et al. [8] proposed that, in the ligand-free enzyme, L2 is open to allow ligands access to the active site, but upon the formation of the ternary complex, L2 closes over the active-site portal. Part of this movement includes Phe⁷⁶

and Tyr⁷⁷, which move into place as Bet-binding determinants. Gonzalez et al. [8] also proposed that these residues may be involved in orientating Hcy into the active site, and in turn trigger a conformational change that completes the creation of the Bet-binding site. By monitoring ligand binding by intrinsic fluorescence, we proved that the Bet-binding site is not present until Hcy binds [5]. The Y77F BHMT mutant was unable to undergo the first 4 nm red shift when presented with high levels of Hcy, adding biochemical evidence for a role of Tyr⁷⁷ in Hcy recognition. Other important structural differences between these two enzyme conformations include the location of Tyr¹⁶⁰, which in the ligand-free enzyme is the fourth ligand to Zn, but in the CBHcy-complexed enzyme has moved to become a Bet-binding determinant.

In addition to correlating the biochemical properties of BHMT with its structure, we are interested in the role BHMT has in the regulation of sulphur amino acid metabolism. Hcy resides at a key regulatory locus between re-methylation to Met and trans-sulphuration to Cys. There is emerging evidence that sulphur amino acid metabolism is regulated, in part, by the intracellular thiol redox balance [10–12]. Met synthase is inactivated by oxidative stress in *E. coli* [13]. Approximately half of the Cys found in glutathione is derived from Met, and the availability of Cys limits glutathione synthesis [14]. An increase in flux through trans-sulphuration and decrease in flux through re-methylation during oxidative stress has also recently been demonstrated in porcine lens tissue [15].

If the metabolic preference is for Hcy to be converted into Cys rather than Met during oxidative stress, then slowing down flux through the BHMT reaction might be an additional mechanism to achieve higher intracellular glutathione levels. In the present study, we evaluated the sensitivity of BHMT to oxidative inactivation by a thiol-specific modifier, MMTS (methyl methane-thiosulphonate), and H₂O₂. We also evaluated whether activity could be rescued by the introduction of a thiol reducing agent and an exogenous Zn and whether oxidative inactivation of BHMT depends upon the conformation of the enzyme.

EXPERIMENTAL

Reagents

MMTS, L-Met, catalase, trypsin, trypsin/chymotrypsin inhibitor and PAR [4-(2-pyridylazo)-resorcinol] were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.). TCEP [tris(2-carboxy-ethyl)phosphine] was purchased from Pierce (Rockford, IL, U.S.A.). CBHcy was a gift from Jiri Jiracek (Academy of Sciences, Czech Republic). All other reagents were of the highest purity commercially available.

BHMT purification and assay

All experiments used recombinant human BHMT purified according to the method described by Breksa and Garrow [3]. Protein was concentrated using Centrprep 10 kDa molecular mass cut-off filters (Millipore, Billerica, MA, U.S.A.) and then dialysed against a buffer containing 20 mM Tris (pH 8) and 100 μ M TCEP. When an experiment required different buffer conditions, the enzyme was either diluted or changed by dialysis. Enzyme purity was verified by SDS/PAGE (12.5 % polyacrylamide) and visualized by Coomassie Blue staining. Enzyme concentration was measured using the Bio-Rad (Hercules, CA, U.S.A.) protein assay, and activity was determined using radiolabelled Bet as described previously [16].

CD and glutaraldehyde cross-linking of BHMT

Far-UV CD spectra were recorded using a Jasco J-720 spectropolarimeter at room temperature (25 °C). Protein samples (0.5 mg/ml final concentration) were in Tris buffer (pH 8) containing 25 μ M TCEP. Spectra were obtained for BHMT alone, BHMT in the presence of 1 mM Met and BHMT in the presence of 50 μ M CBHcy. The spectrum of each ligand in the buffer was subtracted from the corresponding protein sample. Using these saturating ligand concentrations, BHMT was then exposed for 2 h to 10 mM H₂O₂ and their spectra were obtained again. Five spectra were taken and averaged for each treatment.

Before and after treatment for 1 h with 50 mM H₂O₂, BHMT (0.9 mg/ml) was incubated with or without 0.01 or 0.05 % glutaraldehyde in 50 mM Tris (pH 7.5) buffer containing 5 mM DTT (dithiothreitol) for 30 min at room temperature. The protein samples were then mixed with sample loading buffer [50 mM Tris, pH 6.8, 2 % (w/v) SDS, 0.1 % (w/v) Bromophenol Blue, 10 % (v/v) glycerol and 100 mM 2ME (2-mercaptoethanol)] and boiled for 5 min. Cross-linked adducts were separated using SDS/PAGE (10 % polyacrylamide) and visualized by Coomassie Blue staining. The details of this procedure were published recently [9].

Concurrent measurement of Zn and BHMT activity during oxidation

The loss of Zn from the active site of BHMT following exposure to 2 mM MMTS or 10 mM H₂O₂ was monitored continuously for 2 h at room temperature using PAR. PAR increases in absorbance at 500 nm as it chelates free Zn [4,17]. For this assay, a working solution of 0.2 mM PAR in 100 mM Tris (pH 8) buffer containing 800 mM NaCl was combined with an equal volume of 2 mg/ml BHMT. While monitoring Zn loss, aliquots were taken at various time points for parallel measurement of BHMT activity and kept on ice. MMTS reaction samples were quenched at each time point with 2 mM 2ME and 2.5 mM EDTA, whereas H₂O₂ reaction samples were quenched with 2 mM 2ME, 2.5 mM EDTA and 0.04 mg/ml catalase prior to initiating the BHMT assay.

The effect of pre-exposure of BHMT to saturating levels of Met (1 mM) or CBHcy (50 μ M) on MMTS- or H₂O₂-induced loss of Zn from the enzyme was evaluated using the method described above to determine if the conformational state of the enzyme changes its susceptibility to oxidation.

Post-oxidation rescue of BHMT activity

Aliquots of BHMT were treated with water (control), 2 mM MMTS or 10 mM H₂O₂ for 2 h. Then, samples were injected into 10 kDa molecular mass cut-off Slide-A-Lyzer cassettes (Pierce) and dialysed against six changes of buffer (4 litres) containing 20 mM Tris (pH 8), 5 mM 2ME and 2.5 mM EDTA to remove all traces of oxidant and unincorporated Zn. After dialysis, half of each sample was adjusted to 500 μ M zinc chloride to provide the metal to the active site of any apoBHMT (BHMT without zinc bound) competent to bind it. All samples were then assayed for BHMT activity.

Partial trypsin digestion of BHMT in the absence and presence of ligands

Purified BHMT (2 mg/ml) in 10 mM Tris buffer (pH 8.0) containing 1 mM DTT was prewarmed to 37 °C. Digestions were performed in the absence or presence of 1 mM Met or 50 μ M CBHcy. After removing an untreated baseline sample, trypsin (500 ng/ μ l) was added to each sample to a final concentration of 40 ng/ μ l protease. At various time points, aliquots containing 10 μ g of BHMT were mixed with 1 μ g of trypsin/chymotrypsin

Figure 3 CD of BHMT is slightly perturbed by H_2O_2

CD spectra of BHMT (0.5 mg/ml) were obtained in the absence or presence of saturating concentrations of ligands, both before and after oxidation for 2 h with 10 mM H_2O_2 . The spectra of the binary (BHMT + 1 mM Met) and ternary mimic (BHMT + 50 μM CBHcy) complexes are distinctly different from ligand-free BHMT. After oxidation, the spectra are shifted (grey lines). The change is smallest when BHMT is in the ternary complex mimic.

inhibitor to quench proteolysis. Then, 5 μg of each sample was subjected to SDS/PAGE (15% polyacrylamide) and the BHMT protein and derived peptide fragments were visualized by Coomassie Blue staining. Using a duplicate gel, peptides were blotted on to nitrocellulose, visualized by Ponceau staining, excised from the membrane and sequenced at the University of Illinois' Biotechnology Center.

RESULTS AND DISCUSSION

CD spectra and glutaraldehyde cross-linking of BHMT

The CD spectrum of BHMT changes in the 210–230 nm range when presented with saturating concentrations of Met or CBHcy, as shown in Figure 3. These CD spectra results are consistent with our earlier study that used the intrinsic fluorescence of BHMT to describe three different steady-state enzyme conformations [5]. Overall, the change in the CD spectrum of BHMT in the presence or absence of Met, and in particular CBHcy, is remarkable. This is in contrast with the relatively moderate change in BHMT structure upon binding CBHcy, as viewed by comparing the ligand-free rat enzyme with the human enzyme in complex with CBHcy. Since the CD spectrum of a protein is a summation of all the ϕ and ψ angles that make up that protein, it must be that the additive effect of many minute structural changes when BHMT binds one of these ligands collectively causes the significant changes observed in its CD spectrum.

When enzyme was pretreated with 10 mM H_2O_2 in the absence or presence of these ligands, small changes in the CD spectrum were observed, indicating that oxidation of BHMT is accompanied by small changes in secondary structure. The smallest shift in CD spectrum following H_2O_2 treatment occurred in the presence of CBHcy. These results suggest that the conformation of BHMT when complexed with CBHcy affords greater protection against oxidation by H_2O_2 .

Recently, our laboratory showed that oligomerization is required for BHMT activity [9]. When BHMT is cross-linked with 0.01 or 0.05% glutaraldehyde, a signature banding pattern on SDS/polyacrylamide gels is observed consistent with the formation of dimers, trimers and a high molecular mass complex. H_2O_2 did not change the cross-linking profile of BHMT (Figure 4), indicating that the quaternary structure of BHMT is not disrupted by exposure to H_2O_2 .

Figure 4 The oligomerization state of BHMT is not disrupted following treatment with H_2O_2

BHMT oligomerization was assessed using glutaraldehyde cross-linking. BHMT (0.9 mg/ml) was treated with either water or 50 mM H_2O_2 for 2 h and then incubated with 0, 0.01 or 0.05% glutaraldehyde for 30 min at room temperature. Adducts were then separated by SDS/PAGE (10% polyacrylamide) and visualized by Coomassie Blue staining. Lane 1, BHMT; lane 2, BHMT + 0.01% glutaraldehyde; lane 3, BHMT + 0.05% glutaraldehyde; lane 4, H_2O_2 -treated BHMT; lane 5, H_2O_2 -treated BHMT + 0.01% glutaraldehyde; lane 6, H_2O_2 -treated BHMT + 0.05% glutaraldehyde. H_2O_2 had no effect on the formation of dimers (90 kDa), trimers (135 kDa) or higher molecular mass (HMW) complexes.

Figure 5 BHMT activity is quantitatively correlated to its Zn content during oxidative inactivation with MMTS or H_2O_2

BHMT (2 mg/ml) was combined with an equal volume of 0.2 mM PAR. Samples were then oxidized with either 2 mM MMTS (A) or 10 mM H_2O_2 (B), and Zn loss (●) was monitored over 2 h. Aliquots were taken from the cuvette and quenched at discrete time points to assay BHMT activity (○). BHMT activity directly correlates to the Zn content of the enzyme.

Oxidative loss of Zn correlates with loss of activity

Time-course data show that the activity of BHMT is correlated to its Zn content. MMTS (2 mM) or H_2O_2 (10 mM) cause BHMT to lose nearly all of its Zn and activity by 2 h (Figure 5). Rate constants for MMTS-induced activity loss (0.05 min^{-1}) and Zn loss (0.07 min^{-1}) are similar. Loss is slower with H_2O_2 but the rate constants are also similar (0.02 min^{-1} for activity loss and 0.03 min^{-1} for Zn loss). The reaction between BHMT and H_2O_2 was studied further and displays pseudo-first-order kinetics with respect to H_2O_2 concentration (results not shown).

MMTS is a thiol-specific modifier that oxidatively adds a thiomethyl group to Cys residues within proteins (Cys-S-S-CH_3). This modification is reversible by addition of a disulphide reducing agent. We previously used this procedure to make apoBHMT and establish that Zn is required for catalysis [1]. BHMT has eight Cys residues. Three of these residues (Cys²¹⁷, Cys²⁹⁹ and Cys³⁰⁰) provide thiolates for Zn binding. We previously made a mutant form of BHMT that has the five Cys residues not involved in Zn binding changed to Ala, and this mutant retains near-normal activity [2]. This five-Cys mutant loses Zn at the same rate as

Figure 6 BHMT inactivation by MMTS is reversible, but H₂O₂-induced inactivation is not

BHMT (2 mg/ml) was incubated for 2 h with 2 mM MMTS or 10 mM H₂O₂ and then dialysed against a buffer containing 10 mM EDTA to remove free Zn. A portion of each sample was then further dialysed in a buffer containing 10 mM 2ME and 1 mM zinc chloride. Enzyme activity was fully recovered after MMTS treatment, but H₂O₂-induced loss of activity was irreversible. Results shown are the means $\pm$ S.D. Letters indicate significance using Student's *t* test with $P < 0.0001$.

wild-type enzyme when treated with MMTS or H₂O₂ (results not shown). Therefore it is unlikely that the Cys residues outside of the active site have a role in the oxidative inactivation of BHMT.

We have not characterized the amino acid modifications caused by H₂O₂ treatment. Cys and Met residues are easily oxidized by H₂O₂ [18–20]. Several other residues, including His, Trp and Tyr, are susceptible to oxidation by H₂O₂ in the presence of redox-active metal ions [21–24]. It is possible that H₂O₂ is causing oxidation of many residues in BHMT in our system because our buffers were not pretreated with chelating agents. Aside from the Cys residues required for Zn binding, it is unknown what other oxidative modifications may affect enzyme activity. Specifically, His²¹⁸ functions as a second-sphere ligand to Zn through its interactions with Cys²¹⁷ and Trp⁴⁴; Tyr¹⁶⁰ and His³³⁸ are also within the active site, shown in Figure 2. It is possible that oxidation of one or more of these residues could reduce or abolish catalytic activity.

Tertiary butyl hydroperoxide is a useful reagent for investigating the oxidation of Met residues located on the surface of proteins because the bulky *t*-butyl group prevents this peroxide from entering the interior of proteins [25]. When BHMT was incubated with up to 78 mM *t*-butyl hydroperoxide, no appreciable amount of Zn or activity was lost, indicating that oxidation of surface Met residues is not involved in the oxidative inactivation of BHMT.

Reversibility of BHMT inactivation

We previously showed that apoBHMT could be made replete with Zn and completely regain activity by the addition of exogenous zinc chloride [1]. To test whether H₂O₂ inactivation was reversible, we treated enzyme with 2 mM MMTS or 10 mM H₂O₂ for 2 h and then dialysed the enzyme solutions against a buffer containing 2ME and EDTA. As depicted in Figure 6, upon the addition 500 μ M zinc chloride, the MMTS-treated enzyme regained full activity, consistent with our previous finding [1]. The H₂O₂-inactivated enzyme was unable to regain any activity. It is likely that H₂O₂ oxidizes one or more of the active-site Cys residues to

Figure 7 CBHcy protects BHMT from oxidation-induced Zn loss

BHMT (2 mg/ml) was combined with an equal volume of 0.2 mM PAR in the presence or absence of ligands. Samples were then oxidized with either 2 mM MMTS (left panel) or 10 mM H₂O₂ (right panel) and Zn loss was monitored over 2 h. Incubation with 50 μ M CBHcy (dotted line) slowed down the rate of Zn loss, whereas 1 mM Met (broken line) had no effect.

Figure 8 CBHcy Met protects BHMT from trypsin digestion

BHMT (2 mg/ml) was treated with 0.5 μ g/ml trypsin at 37°C in the absence of ligands (A) or in the presence of Met (B) or CBHcy (C). After 0–16 h, samples were quenched with 1 μ g of trypsin/chymotrypsin inhibitor and then separated by SDS/PAGE (15% polyacrylamide). Masses of full-length and digestion products are indicated in kDa. Incubation with 50 μ M CBHcy slowed down the rate of degradation, whereas 1 mM Met had no effect.

a sulphinate or sulphonate, modifications that cannot be reversed *in vitro* by the addition of 2ME, thereby precluding the reincorporation of a Zn atom capable of rescuing catalytic activity.

CBHcy protects BHMT from oxidation and proteolysis

CD and intrinsic fluorescence studies show that BHMT undergoes conformational changes upon ligand binding [2,5,8]. Figure 7 demonstrates ligand-specific protection against H₂O₂- and MMTS-induced loss of Zn from BHMT. CBHcy (50 μ M) attenuates the loss of Zn caused by both MMTS and H₂O₂ by 75%, whereas 1 mM Met has no protective effect. When CBHcy binds to BHMT, the conformation adopted by the enzyme mimics the BHMT–Hcy–Bet and BHMT–Hcy–DMG ternary complexes [5]. In the presence of Met, BHMT adopts the same conformation as the BHMT–Hcy binary complex [5]. We chose not to use Hcy in these studies because both MMTS and H₂O₂ would react with this substrate, confounding the interpretation of our results.

When BHMT is partially digested with trypsin, the enzyme is cleaved over time into two major fragments of approx. 10 and 35 kDa (Figure 8A). BHMT activity decreases at a rate corresponding to proteolytic cleavage (results not shown). When the

enzyme is in the presence of saturating levels of Met or CBHcy, the former offers no protection from cleavage (Figure 8B), whereas the latter clearly slows down digestion (Figure 8C). These results parallel those obtained in the oxidation experiments, where the enzyme is protected against oxidation when it adopts the conformation associated with the ternary complex, as mimicked by BHMT–CBHcy, but not in the BHMT–Met binary complex. N-terminal sequencing of the tryptic fragments reveals that the 10 kDa peptide is composed of a single N-terminus: K⁷AKK-AGILERL¹⁷, which is produced as a result of cleavage after Lys⁶. The 35 kDa band contains two overlapping peptides, G⁸⁷NYVLEKISG⁹⁶ and I⁹⁴SGQEVNEAA¹⁰³, which are produced as a result of cleavage after Arg⁸⁶ and Lys⁹³. We are unable to assign any structural significance to the Lys⁶ cleavage since this residue and those preceding it have not been resolved in any crystal structure to date. Arg⁸⁶ and Lys⁹³ are part of a large substructure, loop L2, composed of residues 74–97. The sensitivity of BHMT to cleavage within L2 is an interesting outcome. L2 is not entirely resolved in either the human or rat BHMT structures but what portions of L2 have been resolved have been shown to be in different positions relative to the active site [2,8], which is confirmed by the effect of ligand binding in these trypsin experiments.

Combined, the effects of Met and CBHcy on enzyme oxidation and trypsin digestion indicate that the enzyme is protected from oxidation and digestion only when both substrate-binding sites of BHMT are occupied. In addition, these findings suggest that L2 is involved in the conformational change associated with occupancy at the Bet-binding site and that this conformational change and/or occupancy at both ligand-binding sites protect the enzyme from oxidative inactivation.

Biological implications

We have not investigated whether BHMT can be inactivated by H₂O₂ *in vivo* or whether the cell's antioxidant defence mechanisms can shield BHMT from this pro-oxidant. Since BHMT is approx. 1% of the soluble protein in liver [7], it seems unlikely that it could be immune to oxidation by H₂O₂ *in vivo*, particularly in pathophysiological states where H₂O₂ production in liver is greatly enhanced, such as septic shock. It is possible that inactivation of BHMT under extreme conditions is of benefit to animals since it could result in bolstering the production of Cys from Hcy, which in turn could enhance glutathione synthesis. These possibilities warrant further investigation.

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