S-Glutathionylation Enhances Human Cystathionine β-Synthase Activity Under Oxidative Stress Conditions

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Abstract

**Aims:** Cystathionine β-synthase (CBS) catalyzes the first and rate-limiting step in the two-step trans-sulfuration pathway that converts homocysteine to cysteine. It is also one of three major enzymes responsible for the biogenesis of H2S, a signaling molecule. We have previously demonstrated that CBS is activated in cells challenged by oxidative stress, but the underlying molecular mechanism of this regulation has remained unclear. **Results:** Here, we demonstrate that S-glutathionylation of CBS enhances its activity *2-fold in vitro*. Loss of this post-translational modification in the presence of dithiothreitol results in reversal to basal activity. Cys346 was identified as the site for S-glutathionylation by a combination of mass spectrometric, mutagenesis, and activity analyses. To test the physiological relevance of S-glutathionylation-dependent regulation of CBS, HEK293 cells were oxidatively challenged with peroxide, which is known to enhance the trans-sulfuration flux. Under these conditions, CBS glutathionylation levels increased and were correlated with a *3-fold increase in CBS activity*. **Innovation:** Collectively, our results reveal a novel post-translational modification of CBS, that is, glutathionylation, which functions as an allosteric activator under oxidative stress conditions permitting enhanced synthesis of both cysteine and H2S. **Conclusions:** Our study elucidates a molecular mechanism for increased cysteine and therefore glutathione synthesis via glutathionylation of CBS. They also demonstrate the potential for increased H2S production under oxidative stress conditions, particularly in tissues where CBS is a major source of H2S. *Antioxid. Redox Signal.* 22, 350–361.

Introduction

Cystathionine β-synthase (CBS) is a pyridoxal 5'-phosphate (PLP)-dependent hemeprotein (2, 33) which catalyzes the first steps in the trans-sulfuration pathway that converts homocysteine to cysteine, the limiting reagent in the synthesis of the antioxidant, glutathione (GSH). Alternatively, CBS can catalyze the condensation of cysteine and homocysteine or the condensation of two moles of cysteine to produce hydrogen sulfide (H2S) (6, 45), a signaling molecule with cytoprotective effects (19–21, 24). Mutations in CBS represent the single most common cause of severe hereditary hyperhomocysteinemia and lead to a number of complications affecting the cardiovascular, ocular, skeletal, and central nervous systems (26).

Human CBS is a modular protein comprising three functional domains (50). The N-terminal domain binds the heme cofactor, which functions as an allosteric regulator (44), inhibiting CBS activity in response to binding of CO or NO (3, 47) or during reduction of nitrite (14). Nitration of tryptophan

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residues in CBS by peroxynitrite results in alterations in the heme pocket, leading to loss of cysteinate coordination by the heme ligand Cys52 and concomitant inactivation of CBS (5). While a structural role for the heme in stabilizing the CBS structure has been invoked (29), homologous CBSs from lower organisms, for example, yeast, are stable without this cofactor (18). The central catalytic domain of CBS contains the PLP cofactor essential for catalysis and a CXXC motif, whose function, if any, has yet to be determined (30, 48). The C-terminal regulatory domain contains a tandem repeat of “CBS domains” that bind S-adenosylmethionine (AdoMet), an allosteric activator (11, 39). The regulatory domain can be cleaved to yield a truncated dimeric enzyme, which is more active than the full-length enzyme but unresponsive to AdoMet (23).

The trans-sulfuration pathway is a quantitatively significant route for cysteine, the limiting substrate for GSH synthesis (36). GSH is an abundant antioxidant in mammalian cells and plays an important role in intracellular redox homeostasis and in cellular defense against xenobiotic stress (27). It is, therefore, not surprising that a number of enzymes in the sulfur metabolic nexus display sensitivity to redox changes. Indeed, metabolic labeling studies have revealed that flux through the trans-sulfuration pathway is increased in response to oxidative stress (36). Thus, a threefold increase in the level of cystathionine formation catalyzed by CBS was observed when HepG2 cells were exposed to 200 μM tert-butyl hydroperoxide (tBuOOH).

Since CBS is the committing enzyme in the trans-sulfuration pathway, we focused on it as a locus for potential regulation in response to exogenous oxidants. However, since no changes were seen in CBS protein levels in oxidatively challenged cells (36), we hypothesized that the activity of CBS might be regulated in response to oxidative stress via reversible post-translational modification of one or more cysteine residues. Human CBS has 11 cysteine residues, including two in the CXXC motif, which have been observed in the oxidized disulfide and reduced dithiol states (30, 48) and were the initial focus of our study to elucidate how the activity of CBS is upregulated under oxidative conditions. However, as reported here, our studies led us to the unexpected discovery that the activity of CBS is regulated via glutathionylation, which occurs at a cysteine residue that does not reside within the CXXC motif. We demonstrate that glutathionylation of CBS results in an ~2-fold increase in enzyme activity in vitro. Furthermore, glutathionylation of CBS is also observed in cultured cells subjected to oxidative stress and is correlated with an ~3-fold increase in CBS activity. These results suggest a model for an auto-corrective cellular response to GSH pools compromised under oxidative stress conditions via glutathionylation of CBS, which increases flux through the trans-sulfuration pathway, thereby increasing availability of cysteine, the limiting reagent for GSH synthesis.

Results

CBS is S-glutathionylated in vitro

To test whether CBS can be glutathionylated, the protein was initially treated with dithiothreitol (DTT) to reduce all accessible cysteines to thiols. After removal of DTT, we then determined the effect of different concentrations of diamide on glutathionylation of CBS. For this, the GSH concentration was fixed at 1 mM and the diamide concentrations were

![FIG. 1. S-glutathionylation of CBS in vitro and its effect on activity.](image-url)
varied from 0 to 1 mM. The minimum diamide concentration that was required for glutathionylation detectable by Western blot analysis was 0.7 mM (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars). Hence, in subsequent experiments, CBS was oxidized with 1 mM diamide and then treated with an excess of GSH (1.5 mM) to oxidize exposed cysteines and to subsequently glutathionylate one or more susceptible thiols. Formation of S-glutathionylated CBS was detected by Western blot analysis using an anti-SSG antibody (Fig. 1a, top panel). Two bands cross-reacted with the anti-SSG antibody and corresponded to the CBS monomer and dimer with molecular masses of ~63 and ~130 kDa, respectively (lane 4). Addition of DTT to the glutathionylated sample resulted in loss of the cross-reacting bands, which is consistent with reduction of the mixed disulfide bond between GSH and CBS (lane 5). Diamide treatment of CBS led to intersubunit cross-linking and in Western blots using CBS antibody (Fig. 1a, lower panel), bands corresponding to the monomeric, dimeric, and multimeric (>250 kDa) species were observed (lanes 3 and 4), which were resolved into the monomeric species only on DTT treatment (lane 5). S-glutathionylation was not observed in control samples in which CBS was treated with diamide or GSH alone (top panel, lanes 2 and 3).

Proportion of CBS that is glutathionylated

Native CBS has 11 cysteine residues, and the number of accessible thiols was determined using Ellman’s reagent (5,5′-dithiobis-2-nitrobenzoic acid [DTNB]). A total of 4.7 ± 0.3 cysteines were modified per CBS monomer and the number decreased to 3.5 ± 0.25 after diamine (1 mM) + GSH (1.5 mM) treatment. This result indicated that one of the five accessible cysteines in the untreated sample was oxidized and/or modified after diamine + GSH treatment. Previously, two cysteines, Cys15 and Cys431 in the heme and regulatory domains, respectively were reported to be solvent accessible in CBS, using the DTNB assay (12). The basis for the discrepancy between these two data sets is not known but could result from differences in handling of proteins samples given the propensity of full-length CBS to aggregate (41). To determine what proportion of the protein was glutathionylated, untreated and diamine-oxidized CBS were treated with biotinylated glutathione ethyl ester (BioGEE). Approximately 80% ± 4% of the protein was retained on the streptavidin-agarose resin corresponding to the proportion of protein that was glutathionylated.

FIG. 2. Effect of GSSG on CBS. (a) S-glutathionylation was carried out by treating CBS [0.5 mg/ml with 1 mM DA/1.5 mM GSH (lane 2), 1 mM GSSG (lane 3), 2 mM GSSG (lane 4), or 5 mM GSSG (lane 5) followed by nonreducing SDS-PAGE and Western blot analysis with anti-SSG antibody (top) or anti-CBS antibody (bottom). (b) Effect of GSSG on CBS activity. CBS (0.5 mg/ml) was incubated with the indicated concentrations of GSSG for 1 h at room temperature, and then the samples were dialyzed to remove GSSG. CBS activity was measured using the methylcysteine assay. The graph represents the treated compared with untreated controls and is the average ± SD (n = 3). GSSG, glutathione disulfide.
Glutathionylation increases CBS activity

We next investigated the effect of glutathionylation on CBS activity. The standard in vitro assay for CBS is not suitable for measuring the activity of glutathionylated CBS, as homocysteine is used as a substrate and can reduce the mixed disulfide between GSH and CBS. Hence, we used a modified assay in which S-methylcysteine is employed as a substrate. The product of this reaction is methanethiol, which is detected by UV-visible absorption or fluorescence spectroscopy after treatment with DTNB or Thiostar, respectively. Glutathionylation increased the activity of CBS to 226% ± 15% compared with untreated control samples (Fig. 1b). Interestingly, glutathionylated CBS was not further activated in the presence of 1 mM AdoMet, an allosteric activator. Unexpectedly, diamide alone (1 mM) also increased the activity of CBS to 149% ± 2% of the control value. Addition of DTT (10 mM) partially reversed the activity of glutathionylated CBS to 157% ± 12% of the control value. However, the basal activity level was not completely recovered.

We next determined whether the physiologically relevant oxidants glutathione disulfide (GSSG) or H₂O₂ activate CBS. Under oxidative stress conditions, the GSSG pool size increases and this can promote protein glutathionylation. Treatment of reduced CBS with 1–5 mM GSSG showed increasing glutathionylation of the CBS monomer but not of the cross-linked dimer as observed with diamide + GSH (Fig. 2a). However, CBS treated with GSSG did not show a detectable change in activity even in the presence of 5 mM GSSG (Fig. 2b), suggesting that the proportion of glutathionylation with this reagent was significantly lower than that...
observed with GSH. This was confirmed by thiol quantitation of CBS treated with 2 mM GSSG, which revealed that 4.8±0.35 cysteines were reduced and accessible for modification by DTNB, which is identical to the value obtained in the untreated control (4.7±0.3). Hence, the high sensitivity of the Western blot method enables detection of the very low proportion of CBS that is glutathionylated in the presence of GSSG.

In contrast to GSSG treatment, the activity of CBS increased when treated with different concentrations of H₂O₂ (10–200 μM) for 30 min and then exposed to GSH, peaking at 25 μM H₂O₂, where the activity was 159%±11% of the control value (Fig. 3). Beyond this concentration of H₂O₂, the activity of CBS decreased. Unlike diamide treatment, which only results in formation of disulfide bonds, treatment with H₂O₂ can generate sulfenic, sulfinic, and sulfonic acids in addition to disulfides. Of these, the overoxidized sulfenic and sulfonic acids cannot be modified by GSH. Hence, the proportion of glutathionylated CBS is expected to be lower in H₂O₂ versus diamide-treated samples. To test this possibility, the extent of BioGEE modification of H₂O₂-oxidized CBS samples was determined after affinity purification using a streptavidin column. In comparison to the 80%±4% modification observed in diamide-treated samples, 64%±3% of CBS was glutathionylated in H₂O₂-treated samples. Treatment of CBS with 25 μM H₂O₂ alone did not affect its activity and in the presence of 10 mM DTT, basal CBS activity was fully recovered (Fig. 3).

CBS is glutathionylated at Cys346

The site(s) of glutathionylation on CBS were identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS) after tryptic digestion of glutathionylated CBS (generated in the presence of diamide + GSH) (Fig. 4 and Supplementary Fig. S2). Before trypsin digestion, CBS was denatured and the free thiol groups were blocked with iodoacetamide. The experiment was repeated thrice, and MS analysis identified a single modified peptide (MLIAQEGLLCGGSAGSTVAVVK) and Cys346 as the site of glutathionylation (Fig. 4). The difference in the monoisotopic masses of the triply charged glutathionylated (827.41 Da) and control iodoacetamide-derivatized (744.73 Da) peptides is 248.04 Da (3·82.68 Da), which corresponds to the expected mass difference between GSH (306.09 Da) and iodoacetamide (58.05 Da) modification of the cysteine residue. The modified M337-K359 peptide had an observed molecular mass of 2479.21 Da in excellent agreement with a mass of 2479.23 Da calculated for the presence of a single glutathione-Cys346 mixed disulfide.

Mutation of Cys346 leads to loss of glutathionylation

To validate Cys346 as the site of glutathiolation in CBS, we generated the Cys346Ser mutant. The purity of the mutant CBS was judged to be >90% by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

**FIG. 5.** Mutation of Cys346 abolishes glutathionylation of CBS. (a) Wild-type and Cys346Ser CBS were treated with 1 mM DA and 1.5 mM GSH for 30 min at room temperature, subjected to nonreducing SDS-PAGE followed by immunoblotting with anti-SSG antibody (top) or anti-CBS antibody (bottom). The CBS monomer (63 kDa) and a dimeric species were detected with anti-SSG antibody in the DA+GSH-treated wild type but not in Cys346Ser CBS. In contrast, anti-CBS antibody was observed in both the monomeric and cross-linked dimeric species in samples that were oxidized with DA. Wild-type and Cys346Ser CBS were treated with ±1 mM DA and 1.5 mM GSH for 30 min at room temperature (b) or with ±25 μM H₂O₂ for 30 min followed by 125 μM GSH (c). The samples were then diazylzed, and CBS activity was measured using the methylcysteine assay as described in Figure 1 legend. *p<0.05 or **p<0.01 versus controls. N.S., not significant.
and comparable to that of the wild-type protein (not shown). However, the yield of the purified Cys346Ser mutant (~1.0 mg/L of culture) was ~7-fold lower than that of wild-type CBS. The activities of the wild-type and mutant Cys346Ser CBS were assessed in 100 mM HEPES buffer pH 7.4, and in the presence or absence of 1 mM AdoMet using the standard radioactive and lead acetate assays as described under the “Materials and Methods” section. The basal activity of Cys346Ser CBS was ~1.3-fold lower than wild-type CBS in both assays.

We next determined the effect of the Cys346Ser mutation on glutathionylation of CBS by Western blot analysis. In contrast to wild-type CBS, which exhibited strong signal intensity due to glutathionylation, this modification was not observed with the Cys346Ser mutant (Fig. 5a). Unlike wild-type CBS, no change in Cys346Ser CBS activity was observed before and after treatment with diamide + GSH (Fig. 5b). Similarly, pretreatment with 25 μM H2O2 followed by GSH did not affect the activity of the mutant CBS (Fig. 5c). Collectively, these results are consistent with Cys346 being the site of glutathionylation, which is correlated with increased CBS activity.

In addition to Cys346, we also mutated the residues in the CXXC motif to generate the double mutant, Cys272Ala/Cys275Ser. Similar to wild-type CBS, the activity of the double mutant was increased by treatment with diamide + GSH (data not shown). These data rule out the cysteines in the CXXC motif as sites for glutathionylation and are consistent with the mass spectrometric data. Exposure to 200 μM H2O2 (200 μM) results in a gradual decline in the CBS activity for 10, 30, and 60 min. Prolonged exposure to 200 μM H2O2 for 10, 30, and 60 min decreased CBS activity using methylcysteine in the presence of the CSE activator, propargylglycine (1) was added to the reaction mixture. Formation of methanethiol was measured by gas chromatography coupled to a sulfur chemiluminescence detector as described under the “Materials and Methods” section. The data are representative of at least three independent experiments. *p < 0.05 or **p < 0.01 versus control. BioGEE, biotinylated glutathione ethyl ester; CSE, cystathionine-γ-lyase; PPG, propargylglycine.

**FIG. 6.** Oxidative stress increases CBS S-glutathionylation and activity in HEK293 cells. (a) HEK293 cells were preincubated ± 250 μM BioGEE for 1 h and subsequently exposed to 200 μM H2O2 for 10, 30, and 60 min. Biotin-bound proteins were extracted using streptavidin affinity resin and eluted with 10 mM DTT. The eluents (top) and whole cell extracts (10 μg protein/lane) were subjected to nonreducing SDS-PAGE and probed with anti-CBS antibody. (b) Cell lysates from HEK293 cells exposed to 200 μM H2O2 for 10, 30, and 60 min were used to assay for CBS activity using methylcysteine in the presence of the CSE inhibitor, 2.5 mM PPG. CH3SH production was measured by gas chromatography coupled to a sulfur chemiluminescence detector as described under the “Materials and Methods” section. The data are representative of at least three independent experiments. *p < 0.05 or **p < 0.01 versus control. BioGEE, biotinylated glutathione ethyl ester; CSE, cystathionine-γ-lyase; PPG, propargylglycine.

To assess the physiological relevance of CBS glutathionylation, we examined whether this modification is observed in cells and is responsive to oxidative stress conditions induced by H2O2 treatment. For this, HEK293 cells were incubated with 250 μM BioGEE for 1 h, followed by 10, 30, and 60 min exposure to H2O2 (200 μM) as described in the “Materials and Methods” section. While 100 μM H2O2 is unlikely to be physiologically relevant, such concentrations have been previously used and are necessary to observe detectable changes in metabolite pools (36). Incorporation of BioGEE into CBS enables it to be pulled down by streptavidin-agarose beads, followed by detection of the modification by Western blot analysis using CBS antibodies. Exposure to 200 μM H2O2 induced a significant increase in glutathionylated CBS within 10 min (Fig. 6a). Prolonged exposure to H2O2 resulted in a gradual decline in the CBS glutathionylation level. After 60 min of H2O2 exposure, the level of glutathionylated CBS was similar to that seen in untreated cells (Fig. 6a, lanes 2 and 5). In the absence of BioGEE treatment, glutathionylation of CBS could not be detected as expected (lane 1). The dimeric form of CBS seen under in vitro conditions using the strong oxidant, diamide, was not seen in the cell culture experiments (data not shown). These results indicate that a low level of CBS glutathionylation exists under steady-state conditions and that it increases transiently and rapidly after an oxidative challenge.

The activity of CBS was also measured in HEK293 cell lysates from control and H2O2-treated cultures. Since both CBS and cystathionine-γ-lyase (CSE) can, in principle, utilize methylcysteine to produce methanethiol, a suicide inactivator of CSE, propargylglycine (PPG) (1) was added to the reaction mixture. The rate-limiting enzyme in the GSH synthesis pathway is γ-glutamylcysteine ligase (Fig. 7). The trans-sulfuration

H2O2 induces S-glutathionylation of CBS in cultured cells and enhances CBS activity

Discussion

GSH is an abundant peptide antioxidant that is maintained at concentrations varying from ~1 to 10 mM depending on the cell type. Its synthesis is limited by cysteine, and the rate-limiting enzyme in the GSH synthesis pathway is γ-glutamylcysteine ligase (Fig. 7).
pathway is an important determinant of intracellular cysteine concentration and accounts for ~40%–50% of the cysteine that is tied up in the GSH pool in some cell types (36, 54). An important role of GSH is the removal of H₂O₂ catalyzed by GSH peroxidase. The product of the reaction is GSSG, which builds up initially under oxidative stress conditions, thus diminishing the GSH pool size (Fig. 7). The cellular autoregressive response for normalizing GSH levels under these conditions includes upregulation of c-glutamylcysteine ligase (42, 43, 56) and as we had previously shown, increased trans-sulfuration flux (36). While regulation of c-glutamylcysteine ligase occurs at the transcriptional level (34, 35, 37), the mechanism of increased trans-sulfuration flux was not known. Upregulation of the trans-sulfuration flux is expected to involve CBS, which catalyzes the rate-limiting step in the two-step pathway. Since Western blot analysis had revealed that CBS protein levels were unchanged in cell lysates from oxidatively challenged cells versus untreated controls, a post-translational mechanism for upregulation of the trans-sulfuration flux was implicated (36). In this study, we report that S-glutathionylation of CBS represents a mechanism for enhancing CBS activity under oxidative stress conditions.

S-glutathionylation is a reversible post-translational modification of proteins, which is important in redox signaling and metabolism, cytoskeletal assembly, and ion homeostasis (8, 9, 32, 51). Glutathionylation frequently inhibits enzyme activity by targeting an active site cysteine; for example, in protein tyrosine phosphatase 1B (38) and caspase 3 (17) or a cysteine that impedes access to the active site, for instance, in methionine synthase (16). In contrast, CBS is glutathionylated at Cys346 and this modification activates the enzyme. The mechanism of allosteric activation of CBS by glutathionylation warrants further elucidation. Allosteric modulation by glutathionylation has also been described in thioredoxin, where modification of a cysteine residue that is not in the catalytic CXXC motif results in inhibition of its oxidoreductase activity (4, 31).

Both surface exposure and perturbation of the thiol pKa to lower values are important predictors of the glutathionylation propensity of cysteines in protein structures (46). Typically, electrostatic stabilization of the thiolate anion with positively charged residues in the vicinity enhances the reactivity of cysteines. However, exceptions to these generalizations are known to exist, for example, in cyclophilin A, where the site of glutathionylation is not the cysteine whose side chain exhibits the greatest accessible surface area (15). The structure of full-length human CBS (10) reveals that Cys346 is located near the dimer interface in a hydrophobic pocket and is not particularly well exposed (Fig. 8). Since glutathionylation is only detected at Cys346 (Fig. 4) and lost on its mutagenesis to serine (Fig. 5), protein dynamics could allow access to this reactive cysteine residue. Alternatively, in the cell, glutathionylation of CBS might be catalyzed, for...
example, by glutaredoxin, which could increase exposure of Cys346 in the inter-protein complex. The relatively buried position of Cys346 might explain why GSSG, which is twice the size of GSH, is unable to modify it efficiently to impact activity under in vitro conditions (Fig. 2). In cells exposed to H2O2, glutathionylation of CBS increased transiently before returning to steady-state levels (Fig. 6).

Since glutathionylation of CBS is efficient with GSH but not with GSSG, it implies that the thiol of Cys346 should first be oxidized, for example, to a sulfenic acid before being attacked by GSH (Fig. 7b). Hence, Cys346 could function as a redox sensor with its proportion increasing with increasing concentrations of reactive oxygen species under conditions of cellular oxidative stress, which, in turn, sets up S-glutathionylation of CBS. In cells exposed to oxidative challenge, glutathionylation of CBS increased transiently before returning to low steady-state levels and was correlated with an ~3-fold increase in CBS activity in cell lysates (Fig. 6). It is not known whether or not deglutathionylation of CBS is catalyzed, and this issue needs to be investigated.

The expected consequence of CBS glutathionylation is increased synthesis of cysteine, the product of the canonical trans-sulfuration pathway. This model is supported by the previously observed increase in the trans-sulfuration flux as evidenced by increased synthesis of cystathionine (and subsequently GSH) in cells challenged with H2O2 (36). The combined effect of upregulation of the rate-limiting enzymes in the trans-sulfuration and GSH synthesis pathways via glutathionylation of CBS and increased synthesis of γ-glutamylcysteine ligase (42, 43, 56), respectively, is restoration of the GSH pool size, which is initially depleted under oxidative stress conditions.

A second predicted consequence of CBS glutathionylation is the increased production of H2S. In this study, we have employed S-methylcysteine a substrate analog of cysteine, to monitor the effect of glutathionylation on CBS activity. The product of this reaction is methanethiol, an H2S surrogate. Our study demonstrates that glutathionylation increases methanethiol production by CBS, indicating that H2S production is predicted to increase both as a consequence of enhanced CBS activity and greater availability of the H2S substrate, cysteine. H2S is a signaling molecule that is generated by at least three enzymes, CBS, CSE, and mercaptopuruvate sulfurtransferase, whose relative contributions vary in a tissue-specific manner. In tissues such as the brain, where CBS is dominant, enhanced H2S production might be a significant outcome of oxidative stress-induced glutathionylation. H2S has been shown to protect against oxidative stress in brain cells (25, 52) and in HUVECs (55).

In summary, we report a new post-translational modification of CBS that provides a molecular explanation for the old observation that trans-sulfuration flux is increased under oxidative stress conditions (36). The study also demonstrates the potential to increase H2S production under oxidizing conditions via S-glutathionylation of CBS. However, the physiological effects of enhanced H2S remain to be fully elucidated.

Materials and Methods

Materials

[1-14C]-Serine (164 mCi/mmol) was purchased from Perkin Elmer. Anti-glutathione (anti-SSG) antibody was from Virogen. Thiostar® (thiol fluorescent detection reagent) was purchased from Arbor Assays. BioGEE and streptavidin-agarose were purchased from Invitrogen. All other chemicals were purchased from Sigma or Fisher.

Protein purification

The pGEX4T-1-C346S and pGEX4T-1-C272A/C275S plasmid constructs for expressing mutant CBS were generated by site-directed mutagenesis using the Quikchange II XL site-directed mutagenesis kit (Agilent) with the following mutagenic primers and the corresponding antisense primers containing the opposite sequence:

CBS346Ser: 5’-GCAAGAGGGGCTGCTAGCGGTG-GAGTGCCTGGCAG-3’

Cys272Ala: 5’-CAGGAAGCTGAAGGAGAAGGCTCC-TGGGTCAGGATCATTCG-3’

Cys275Ser: 5’-GAAGAGAGAGCTCTGGAAGCAG-GACTTGGGTGGAG-3’

Mutation was confirmed by nucleotide sequence determination at the DNA Sequencing Core Facility (University of Michigan, Ann Arbor).

Wild-type and mutant CBSs were expressed and purified as previously described (49) with the exception that the Cys346Ser mutant was induced in the presence of 3% ethanol to promote correct protein folding (28). In brief, cells obtained from a 6-L culture were resuspended in 500 ml of 1× phosphate-buffered saline (PBS); supplemented with 10 mM β-mercaptoethanol, 10 mg PLP, 50 mg of lysozyme, and a protease inhibitor cocktail tablet (from Roche Diagnostics). The cells were lysed by sonication, and the supernatant was obtained by centrifugation at 15,000 g for 30 min. The supernatant was loaded onto a Glutathione Sepharose 4B column, which was pre-equilibrated with 1× PBS. The column was washed with 1× PBS, and glutathione S-transferase (GST)-fused CBS was eluted with 50 mM Tris, pH 8.0 in the presence of 10 mM GSH. The GST-fused CBS was cleaved with thrombin at 4°C at a final concentration of 2 U/mg protein, and the GST tag was removed on a Q sepharose column (2.5 × 12 cm) equilibrated with 50 mM Tris buffer, pH 8.0, and then washed with the same buffer. The protein was eluted with a 500 ml gradient ranging from 0 to 500 mM NaCl in 50 mM Tris-HCl, pH 8.0. The fractions of interest were dialyzed, concentrated, and stored at −80°C.

S-glutathionylation of CBS in vitro

Purified CBS (0.5 mg/ml) protein was treated with 1 mM diamide and 1.5 mM GSH in Buffer A (50 mM Tris-HCl, pH 8.0) at room temperature for 30 min. After this reaction, where indicated, 10 mM DTT was added for 30 min at room temperature to induce deglutathionylation and the samples were analyzed using Western blotting. For S-glutathionylation by disulfide exchange with GSSG, recombinant CBS (0.5 mg/ml) protein was incubated for 120 min with 1–5 mM GSSG at room temperature in Buffer A. For S-glutathionylation with H2O2, purified CBS (0.5 mg/ml) protein was treated with 10–200 μM H2O2 at room temperature for 30 min and then, 50–1000 μM GSH (i.e., five equivalents relative to H2O2) was added to the reaction mixture for 30 min.
Determination of the fraction of S-glutathionylated CBS

Titration of free thiol groups with DTNB. Purified wild-type CBS was treated with 10 mM DTT for 30 min at room temperature, and then DTT was removed using a concentrator and washed with 50 mM Tris·HCl, pH 8.0 with six buffer exchanges. For the preparation of S-glutathionylated proteins, CBS was treated with 1 mM diamide and 1.5 mM GSH in Buffer A at room temperature for 30 min, followed by ultrafiltration using a 1 ml spin column.

To examine the number of free thiol groups in CBS, the unmodified and modified CBS (5 μM) was titrated with 0.5 mM DTNB. Control samples contained the same amount of DTNB in the same buffer but were devoid of CBS protein. The final mixtures (200 μl) were incubated for 10 min at room temperature, and the samples were analyzed spectrophotometrically at 412 nm using an extinction coefficient of 13,600 M⁻¹ cm⁻¹ (40).

Separation of S-glutathionylated CBS using streptavidin-agarose resin. Purified CBS was treated with 1 mM diamide and 1.5 mM BioGEE at room temperature for 30 min, after which excess diamide and BioGEE were completely removed using ultrafiltration tubes and 1 × PBS buffer. Then, 100 μl CBS (0.5 mg/ml) was added to 100 μl streptavidin-agarose resin pre-equilibrated with 1 × PBS buffer and incubated with gentle rotation for 30 min at room temperature. The streptavidin-agarose resin bound glutathionylated CBS and was centrifuged at 1000 g for 5 min. The protein concentration of the supernatant was measured using the Bradford reagent to estimate the proportion of S-glutathionylated CBS. Control reactions in which CBS (0.5 mg/ml) was not treated with diamide and BioGEE were prepared in parallel.

For S-glutathionylation with H₂O₂, purified CBS (0.5 mg/ml) protein was treated with 25 μM H₂O₂ at room temperature for 30 min and then 125 μM BioGEE was added to the reaction mixture for 30 min. Excess H₂O₂ and BioGEE were removed using ultrafiltration tubes and washed with 1 × PBS buffer, and the samples were processed as described earlier. Control reactions in which CBS (0.5 mg/ml) was not treated with H₂O₂ and BioGEE were prepared in parallel.

CBS assay

Enzyme activity was measured using [¹⁴C]-serine as previously described (49). Briefly, the 200 μl assay mixture contained 100 mM HEPES, pH 7.4, 0.25 mM PLP, 0.5 mg/ml bovine serum albumin, 30 mM [¹⁴C]-serine (~40,000 dpm), and 20 mM l-homocysteine. The reaction mixture was incubated at 37°C for 5 min, and the reaction was started by the addition of 5.0 μg of purified CBS. After 30 min, the reaction was terminated by the addition of 200 μl of 10% trifluoroacetic acid. AdoMet (1 mM final concentration) was added to the reaction mixture during the preincubation when indicated. One unit of CBS catalyzes the formation of 1 μmol of cystathionine in 1 h at 37°C under standard assay conditions.

H₂S production assays

H₂S generation using l-cysteine and l-homocysteine as substrates was measured as previously described (7). The reaction mixture containing 100 mM HEPES buffer, pH 7.4, 0.4 mM lead nitrate, 25 mM l-homocysteine, and CBS was preincubated at 37°C for 3 min and the reaction was started by the addition of 25 mM l-cysteine. For testing the effect of AdoMet, 1 mM AdoMet (final concentration) was added to the reaction mixture along with cysteine. An extinction coefficient of 5500 M⁻¹ cm⁻¹ at 390 nm was used for estimation of the lead sulfide concentration.

Activity assay for S-glutathionylated CBS in vitro

The activity of S-glutathionylated CBS was measured using methylcysteine as substrate. Before the start of the assay, S-glutathionylated CBS was centrifuged using a Centricon concentrator to change the buffer using a 1 ml spin column. The reaction mixture containing 100 mM HEPES buffer, pH 7.4, 10 mM methylcysteine, and 50 μmol thiostar thiol fluorescent detection reagent was added to a 96-well plate, and the reaction was started by the addition of 15 μg of CBS. The 96-well plate was placed in a multifunctional microplate reader, and the kinetics of the enzyme-catalyzed reaction was studied. An excitation wavelength of 400 nm and an emission wavelength of 515 nm were set for measuring the production of the thiol adduct between CH₃SH and Thiostar. The activity of unmodified wild-type CBS was set at 100%. Alternatively, DTNB was used in the reaction mixture to detect formation of methanethiol using an extinction coefficient of 13,600 M⁻¹ cm⁻¹ (40).

Western blot analysis

Proteins were blotted onto a polyvinylidenedifluoride membrane, incubated with anti-CBS or anti-SSG primary antibody at 4°C overnight, and then reacted with horseradish-peroxidase-conjugated secondary antibodies for 1 h. Signals were visualized by enhanced chemiluminescence reagents.

LC-MS/MS analysis

A 50 μl solution of CBS (100 μM) was reacted with 2 mM diamide and 3 mM GSH for 30 min at room temperature. Untreated CBS was used as a control. The samples were dialyzed against 200 mM Tris-HCl buffer (pH 8.0) and then denatured using 6 M guanidine hydrochloride. At the end of the experiments, the free thiol groups of samples were derivatized with iodoacetamide at a final concentration of 10 mM. The samples were concentrated using a 1 ml Centricon columns, diluted 10-fold with ammonium bicarbonate buffer pH 8.0, and then digested using a protein:trypsin ratio of 20:1 at 37°C for 12 h. The digested samples were acidified with 10% formic and directly used for LC-MS/MS analyses.

All analyses were carried out using an Agilent 1200 Series HPLC, ACE 5 C18-300 column (2.1 × 100 mm) at a flow rate of 0.1 ml/min, and the injection volume was 6 μl. Mobile phase A and B consisted of 0.1% formic acid in H₂O and 0.1% formic acid in acetonitrile, respectively. Separation of peptides was achieved using the following gradient: t = 0 min: 5% B; t = 1 min: 5% B; t = 2 min: 5% B; t = 10 min: 15% B; t = 38 min: 40% B; t = 43 min: 100% B; t = 44 min: 100% B; t = 45 min: 5% B; and t = 60 min: 5% B (column re-equilibration). A high-performance liquid chromatography (HPLC) system was directly coupled to a BrukerToSInteraction 70 Hybrid FTMS instrument equipped with an electrospray...
ionization source. The system was controlled by HyStarv.3.4.8.0 software (BrukerDaltonics). MS data were collected with a resolving power of 110,000 (at m/z 400) in a positive mode using the following conditions: a capillary voltage of 4500 V and an end plate offset of −500 V. The dry temperature was set at 180°C. Dry gas flow was maintained at 4 L/min. Acquisition range was 344–2200 m/z with 0.4 s ion accumulation time. For MS/MS analysis, automated MS/MS spectra were acquired during the run in the data-dependent acquisition MS/MS boost mode with the selection of the most abundant precursor ion and CID fragmentation. Data were deconvoluted and a peak list created by DataAnalysis v.4.0 software (BrukerDaltonics). MS/MS search was performed against the amino-acid sequence of the CBS protein with a mass tolerance of 30 ppm for both the precursor ions and the fragment ions using BioTools v.3.2 software (BrukerDaltonics).

**Cell culture and preparation of cell lysates**

Human embryonic kidney cells, HEK293, were grown in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were grown at 37°C in an atmosphere of 5% CO₂ and 95% air and grown to 80%–90% confluence in six-well plates. To generate whole-cell extracts, cells were rinsed thrice with ice-cold PBS buffer and then lysed in RIPA buffer (50 mM Tris-Cl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and a protease inhibitor cocktail tablet/50 ml). After centrifugation at 12,000 g for 10 min, the supernatant was collected and the protein concentration was determined by the Bradford method.

**Streptavidin pull-down assay**

HEK293 cells were preincubated with 250 µM BioGEE, a membrane-permeable analogue of GSH (13) for 1 h, and subsequently exposed to 200 µM H₂O₂ for 10, 30, and 60 min. Biotin-bound proteins were extracted using streptavidin affinity resin and eluted with 10 mM DTT. The eluent was subjected to nonreducing SDS-PAGE and probed with an antibody that was specific to CBS. Cells were incubated without BioGEE and served as a control. An equal amount of total whole cell extract (10 µg) at the designated time point was then subjected to nonreducing SDS-PAGE and probed with antibodies that were specific to CBS.

Streptavidin-agarose beads were used to pull down the biotinylated GSH-conjugated proteins. Briefly, before the immobilization, the beads were washed thrice with RIPA buffer to remove preservatives. Equal amounts of protein samples from HEK293 cells treated with H₂O₂ for different time periods were then mixed with 20 µl of beads, and the mixture was incubated at room temperature with gentle rotation for 30 min. The bead-protein complex was separated, and the pellet containing labeled proteins was re-suspended in RIPA buffer, followed by three washes with RIPA buffer. Finally, proteins bound to streptavidin via a mixed disulfide bond were eluted from the beads by incubation for 30 min with 50 mM Tris-Cl pH 7.4 containing 0.1% SDS and 10 mM DTT. Proteins in the eluent were detected by Western blotting.

**Activity assay of S-glutathionylated CBS in cell lysates**

HEK293 cells treated with 200 µM H₂O₂ for different time periods were washed thrice with ice-cold PBS buffer and then scraped from 100 mm plates. The harvested cells were transferred to 1.5 ml centrifuge tubes and centrifuged for 5 min. The supernatant was carefully discarded, and the pellet was weighed. HEPES buffer (100 mM, pH 7.4) was added to the tube (15 µl buffer/1 mg cell pellet), and the cell suspension was frozen and thawed thrice using an acetone/dry ice bath. HEK293 cells that were not treated with H₂O₂ served as a control.

The reaction mixtures (500 µl) containing 100 mM HEPES buffer pH 7.4, cell lysate (400 µl), 10 mM methylcysteine, and 2.5 mM PPG, an inhibitor of CSE, were prepared in polypropylene syringes, as previously described (22). Syringes were sealed with plungers and made anaerobic by flushing the headspace with nitrogen five times using a three-way stopcock, and then left under nitrogen in a final total volume of 5 ml. Syringes were placed in an incubator at 37°C with gentle shaking (75 rpm) for 20 min. Control reactions in which buffer replaced the cell lysate were prepared in parallel. Aliquots of 0.1–0.3 ml from the gas phase of the reaction syringes were collected using gas-tight syringes inserted through a septum attached to the stopcock, and injected in an HP 6890 gas chromatograph (Hewlett Packard) that was equipped with a DB-1 column (30 m × 0.53 mm × 1.0 µm) as previously described (53). Helium was used as the carrier gas at a flow rate of 1 ml/min, and a temperature gradient ranging from 30°C to 110°C over a 20 min period was used. Methanethiol was detected using a 355 sulfur chemiluminescence detector (Agilent). A methanethiol standard was obtained from Cryogenic Gases. An internal standard of dimethyl sulfide was added to the reaction mixtures.

**Statistical analysis**

Data from experiments were expressed as mean ± SD. Student's t-test was used, where appropriate, for comparing the mean values between control and tested groups. Statistical significance was considered at p < 0.05.

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**Author Disclosure Statement**

No competing financial interests exist.

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**Abbreviations Used**

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<th>Abbreviation</th>
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<tr>
<td>AdoMet</td>
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<td>BioGEE</td>
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<td>CBS</td>
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