# Allosteric control of human cystathionine β-synthase activity by a redox active disulfide bond

Received for publication, September 26, 2017, and in revised form, December 27, 2017 Published, Papers in Press, January 3, 2018, DOI 10.1074/jbc.RA117.000103

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Edited by F. Peter Guengerich

**Cystathionine β-synthase (CBS) is the central enzyme in the trans-sulfuration pathway that converts homocysteine to cysteine. It is also one of the three major enzymes involved in the biogenesis of $H_2S$. CBS is a complex protein with a modular three-domain architecture, the central domain of which contains a $^{272}CXXC^{275}$ motif whose function has yet to be determined. In the present study, we demonstrated that the CXXC motif exists in oxidized and reduced states in the recombinant enzyme by mass spectroscopic analysis and a thiol labeling assay. The activity of reduced CBS is ~2–3-fold greater than that of the oxidized enzyme, and substitution of either cysteine in CXXC motif leads to a loss of redox sensitivity. The $Cys^{272}$–$Cys^{275}$ disulfide bond in CBS has a midpoint potential of −314 mV at pH 7.4. Additionally, the CXXC motif also exists in oxidized and reduced states in HEK293 cells under oxidative and reductive conditions, and stressing these cells with DTT results in more reduced enzyme and a concomitant increase in $H_2S$ production in live HEK293 cells as determined using a $H_2S$ fluorescent probe. By contrast, incubation of cells with aminooxyacetic acid, an inhibitor of CBS and cystathionine γ-lyase, eliminated the increase of $H_2S$ production after the cells were exposed to DTT. These findings indicate that CBS is post-translationally regulated by a redox-active disulfide bond in the CXXC motif. The results also demonstrate that CBS-derived $H_2S$ production is increased in cells under reductive stress conditions.**

Cystathionine β-synthase (CBS)[3] is a pyridoxal 5′-phosphate (PLP)-dependent enzyme that plays a key role in the metabolism of sulfur-containing amino acids (1–3). CBS, the first enzyme of the trans-sulfuration pathway, catalyzes the condensation of serine and homocysteine to produce cystathionine, which is subsequently converted to cysteine, the limiting substrate in the synthesis of the antioxidant glutathione (4, 5). Alternatively, CBS, which is one of three key enzymes involved in the production of $H_2S$, can catalyze the condensation of homocysteine and cysteine or condensation of two molecules of cysteine to produce $H_2S$, a recently recognized endogenous gasotransmitter that mediates diverse biological functions (6–9). Mutations in the *cbs* gene represent the single most common cause of homocystinuria, which leads to a number of complications affecting the cardiovascular, ocular, skeletal, and central nervous systems (10).

Human CBS is a modular protein composed of three functional domains that is intricately regulated (11, 12). The N-terminal domain binds the heme cofactor, which seems to function as a redox sensor (13) that inhibits CBS activity by means of binding of CO or NO (14–17) or during reduction of nitrite (18, 19). Nitration of tryptophan residues in CBS results in alterations of the heme pocket, which lead to the loss of cysteinate coordination by the ligand $Cys^{52}$ and concomitant inactivation of CBS (20). Additionally, a structural role for the heme in the proper folding of CBS has been described (21). The C-terminal domain contains a tandem of the so-called CBS domain, which forms a regulatory domain that binds *S*-adenosylmethionine, an allosteric activator (22–24). Cleavage of the regulatory domain from full-length CBS yields a truncated dimeric enzyme that is more active than full-length CBS but is unresponsive to *S*-adenosylmethionine (25). The central domain contains the PLP cofactor, which is essential for catalysis, and a $^{272}CXXC^{275}$ motif, the function of which has yet to be determined (26, 27).

The quantitative significance of the trans-sulfuration pathway for provision of intracellular cysteine, which is required for glutathione synthesis and $H_2S$, links the metabolism of homocysteine directly to cellular redox homeostasis (28). Therefore, it is not surprising that a number of enzymes in the sulfur metabolic pathway display sensitivity to redox changes. Because CBS is the limiting enzyme in the trans-sulfuration pathway, we focused on evaluating its activity with respect to regulation in response to the ambient redox state. Indeed, our previous report used *S*-methylcysteine, a substrate analog of cysteine, to show that *S*-glutathionylation at $Cys^{346}$ enhances human CBS activity under oxidative stress conditions (29). Furthermore, in addition to heme, human CBS has a second putative redox-

This work was supported by Grant 31471086 from the National Natural Science Foundation of China, Grant 2017GY-143 from the Key Science and Technology Program of Shaanxi Province, China, Grant G2017KY0001 from the Fundamental Research Funds for the Central Universities of China, and Grant Z2017233 from the Seed Foundation of Innovation and Creation for Graduate Students in Northwestern Polytechnical University. The authors declare that they have no conflicts of interest with the contents of this article.

This article contains supporting text and Figs. S1–S3.

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[3] The abbreviations used are: used are: CBS, cystathionine β-synthase; PLP, pyridoxal 5′-phosphate; AOAA, aminooxyacetic acid; $MM(PEG)_{24}$, methyl-$PEG_{24}$-maleimide; GSSG-EE, oxidized glutathione ethyl ester; CSE, cystathionine γ-lyase; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; CPM, 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin; GST, glutathione *S*-transferase.

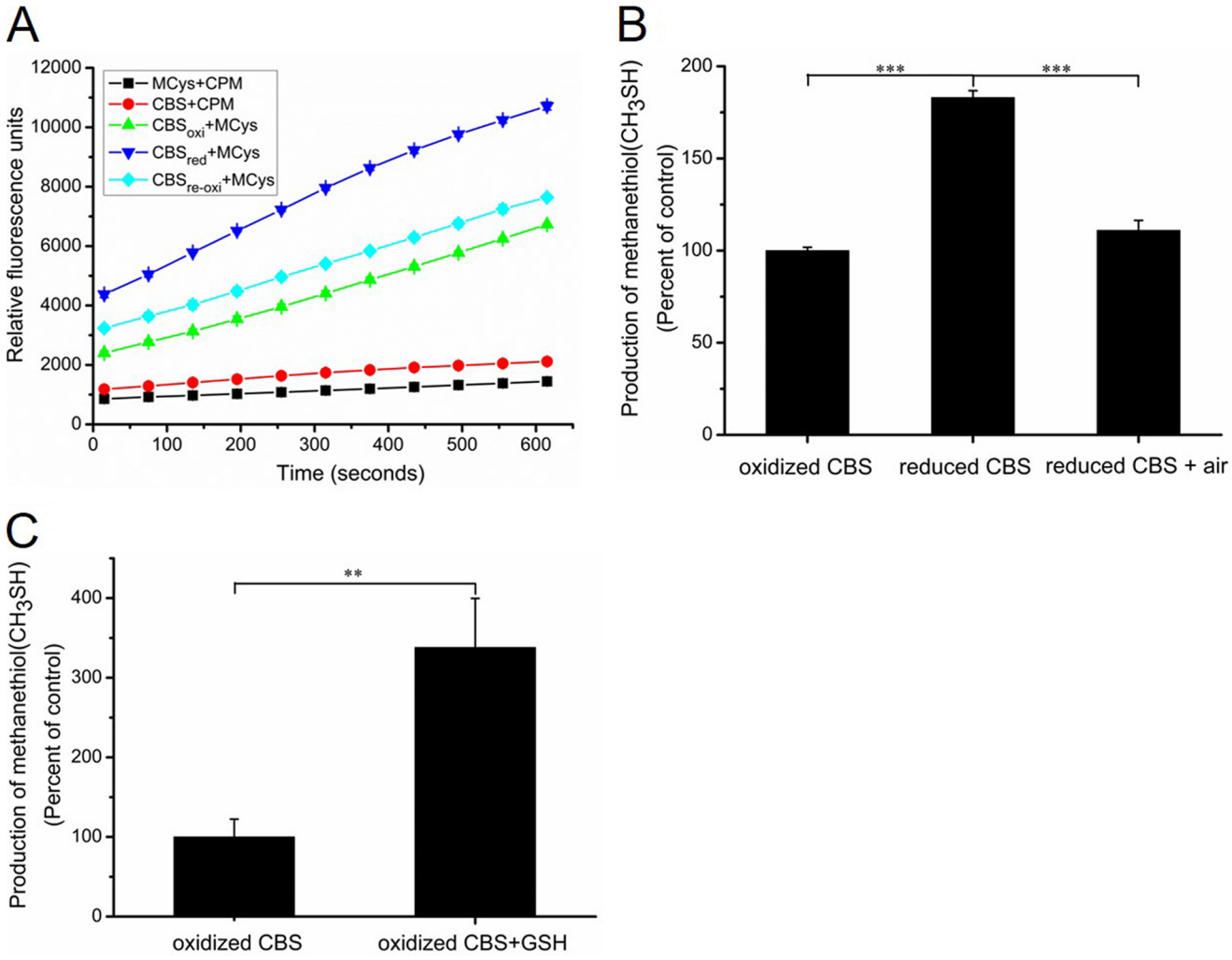


**Figure 1. The redox state of CBS affects its activity *in vitro*.** *A*, recombinant wildtype CBS (1 mg/ml) was incubated with 10 mM DTT for 1 h at 4 °C, and the samples were then ultrafiltered to remove DTT. CBS activity was measured using *S*-methylcysteine (10 mM) as a substrate. The *data points* and *error bars* are the means ± S.D. of three independent experiments. *MCys*, *S*-methylcysteine; *CBS$_{oxi}$*, no treatment; *CBS$_{red}$*, DTT treatment; *CBS$_{reoxi}$*, CBS$_{red}$ was reoxidized by exposure to air. *B*, methanethiol ($CH_3SH$) production catalyzed by CBS was determined, and the initial reaction rates were calculated according to the data from Fig. 1*A*. The graph represents the relative activity of samples compared with the oxidized CBS and shows the means ± S.D. ($n = 3$). ***, $p < 0.001$. The specific activity of the oxidized CBS in the *S*-methylcysteine assay (using DTNB detection) was 0.45 ± 0.04 $\mu$mol methanethiol $h^{-1}$ $mg^{-1}$. *C*, recombinant CBS protein (20 $\mu$g) was treated with 20 mM GSH for 2 h at 4 °C. The activity was measured using *S*-methylcysteine (10 mM) as a substrate in the presence of 20 mM GSH. The $CH_3SH$ production was measured by gas chromatography. The graph represents the relative activity of sample compared with untreated CBS (oxidized CBS) and the means ± S.D. ($n = 3$). **, $p < 0.01$.

active center, a C*XX*C motif that is surface-exposed in full-length CBS that lacks the loop of residues 516–525 (26). However, because high concentrations of homocysteine and cysteine are used as substrates in the canonical CBS activity assay, which would reduce the disulfide of the C*XX*C motif, the activity of oxidized CBS cannot be assessed using this assay.

We hypothesized that redox changes in the C*XX*C motif in CBS might regulate enzyme activity under various redox conditions and that the C*XX*C motif would be a redox sensor. Here, we found that the C*XX*C motif exists in oxidized and reduced states in recombinant CBS and in HEK293 cells. The activity of reduced CBS increased by ~2–3-fold compared with oxidized CBS *in vitro* and resulted in a concomitant increase in CBS-derived $H_2S$ in response to reductive stress in HEK293 cells. Additionally, the midpoint potential of the $Cys^{272}$–$Cys^{275}$ disulfide bond in CBS was determined. These observations indicate that CBS activity is allosterically regulated by a redox-active disulfide bond.

## Results

### *Reduced CBS has a higher activity than oxidized CBS*

Previous reports showed that the C*XX*C motif in CBS exists in oxidized disulfide and reduced dithiol states in the structure of the truncated catalytic core enzyme that lacks the C-terminal regulatory domain (27). To investigate whether the activities of the reduced and oxidized forms of wildtype CBS differ, a modified assay in which *S*-methylcysteine served as the substrate was developed. The production of methanethiol ($CH_3SH$) can be monitored by fluorescence spectroscopy in the presence of a fluorescent thiol probe (Fig. 1*A*). The relative activity of the reduced wildtype CBS was 1.8-fold higher than that of the oxidized CBS. When the reduced CBS was reoxidized by exposure to air, the activity of reoxidized CBS was restored to a level similar to that of the wildtype CBS (Fig. 1, *A* and *B*). Additionally, human CBS contains 11 cysteine residues, one of which, $Cys^{52}$, coordinates the heme

axially (10). The number of thiols in CBS protein was determined using a 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) assay. To fully expose the cysteine residues of the precipitated CBS proteins, 1% (w/v) SDS was used according to a previous report (37). A total of 9.8 ± 0.22 cysteines were modified per reduced CBS monomer, and the number decreased to 7.6 ± 0.12 when the reduced CBS were reoxidized by exposing them to air. This result indicated that the C*XX*C motif in reduced dithiol state can be oxidized to the disulfide state by exposure to air.

To avoid partial oxidization of reduced CBS after the removal of DTT, the activity of CBS that had been pretreated with 20 mM GSH was measured using gas chromatography in reaction mixtures that contained 20 mM GSH. The activity of the reduced CBS was ~3-fold higher than that of the oxidized CBS (Fig. 1*C*). These results showed that CBS activity was regulated under the different redox conditions, and reduced CBS can readily be reoxidized by exposing it to air in a buffer lacking reductants.

### *The CXXC motif in CBS exists in disulfide and thiol states under oxidative and reductive conditions*

The vicinal cysteine residues of the C*XX*C motif are in the reduced state in the crystal structure of the modified CBS that lacks the loop from residues 516–525 (26). Although the vicinal cysteine residues of the C*XX*C motif can be oxidized to form a disulfide bond, which is observed in the crystal structure of the truncated catalytic core CBS (27), it is not known whether the oxidized disulfide bond of the C*XX*C motif exists in wildtype CBS. Purified wildtype CBS was treated with or without 10 mM DTT, and the resulting samples were analyzed using LC-MS/MS with a 99.8% of sequence coverage. The results showed that a unique peptide, K<u>C</u>PG<u>C</u>RIIGVDPE, contained a disulfide bond, which demonstrated that the oxidized form of the C*XX*C motif was detected. The redox state of the $Cys^{272}$–$Cys^{275}$ disulfide bond in purified CBS, and its susceptibility to reduction was determined. The unpaired $Cys^{272}$ and $Cys^{275}$ cysteines but not the $Cys^{272}$–$Cys^{275}$ disulfide-bond cysteines were alkylated with iodoacetamide. The monoisotopic mass $[M+2H]^{2+}$ of the disulfide bond peptide was 692.8389 Da (observed nominal mass = 1384.6711 Da; expected nominal mass = 1384.6712 Da) (Fig. 2*A* and Fig. S1), and the monoisotopic mass $[M+2H]^{2+}$ of the unpaired $Cys^{272}$–$Cys^{275}$ peptide that was alkylated with iodoacetamide was 750.8683 Da (observed nominal mass = 1500.7297 Da; expected nominal mass = 1500.7297 Da) (Fig. 2*B* and Fig. S2). Additionally, according to the peak area from the ion chromatogram, the relative ion abundance of the peptide containing $Cys^{272}$–$Cys^{275}$ disulfide bond in the reduced CBS was ~6% of that in the oxidized CBS, which suggested that more than 90% of the disulfide bond in the C*XX*C motif was reduced after DTT treatment of the oxidized CBS (Fig. 2*A*).

In addition to the mass spectrometry, methyl-$PEG_{24}$-maleimide ($MM[PEG]_{24}$) was used to label the thiols of the oxidized and reduced CBS. The difference between the molecular masses of the labeled oxidized and reduced forms of CBS was ~3 kDa (Fig. 2*C*), which suggested that a single disulfide bond is formed in the purified CBS. Collectively, these results indicate that a reversible redox change in the vicinal cysteines involving a dithiol-disulfide reaction exists in wildtype CBS protein under different redox conditions.

### *The redox state of CXXC motif regulates CBS activity*

Because the $Cys^{272}$–$Cys^{275}$ was the unique disulfide bond detected in the oxidized CBS, we speculated that redox state of the C*XX*C motif would regulate CBS activity. The C272A, C275A, and C272A/C275A (double mutant) forms of the enzyme were purified, and the purity of each mutant protein was judged to be >90% by SDS-PAGE analysis. The yield of both purified C272A and C275A proteins (~3 mg/liter culture) was ~3-fold lower than the wildtype CBS (~10 mg/liter culture), and the C272A/C275A protein was obtained in very low yield (~0.5 mg/liter culture). The activities of the wildtype and mutant proteins were assessed in 100 mM Hepes buffer (pH 7.4) using the methylene blue assay. The basal activities of C272A and C275A mutant enzymes were 25.5 ± 0.86 and 25.9 ± 0.30 $\mu$mol $mg^{-1}$ $h^{-1}$, respectively, and were ~1.2-fold higher than the wildtype CBS (21.6 ± 0.39 $\mu$mol $mg^{-1}$ $h^{-1}$). However, the activity of the C272A/C275A enzyme (10.9 ± 0.38 $\mu$mol $mg^{-1}$ $h^{-1}$) was ~2-fold lower than the wildtype CBS (Fig. 3*A*).

Previous studies indicated that the heme content in CBS protein affects the specific activity of enzyme (39). Therefore, the heme concentrations of wildtype and mutant CBS proteins were determined using the pyridine hemochrome assay (32, 33). The C272A, C275A, and C272A/C275A mutant enzymes have 0.92 ± 0.04, 1.14 ± 0.08, and 1.06 ± 0.01 hemes per CBS monomer, respectively, and are similar to that of wildtype CBS (0.98 ± 0.01 hemes per monomer). These results indicated that the lower activity of C272A/C275A double mutant may result from the conformational changes in CBS, not from the low heme content.

We next determined the effect of these mutations in the C*XX*C motif on the activity of CBS. Unlike wildtype CBS, no changes in the activity of these three mutants were observed before and after treatment with DTT (Fig. 3*B*). Collectively, these results were consistent with $Cys^{272}$–$Cys^{275}$ being the unique disulfide bond in purified CBS, with its redox state regulating CBS activity.

### *Redox potential of the $Cys^{272}$–$Cys^{275}$ disulfide bond*

The midpoint potential of the $Cys^{272}$–$Cys^{275}$ disulfide bond was determined using DTT as the reductant. Because $Cys^{272}$–$Cys^{275}$ is the unique disulfide bond observed in purified wildtype CBS (Fig. 2), the DTNB assay was employed to determine the number of free thiol groups in the CBS protein, which was preincubated in solutions that were prepared by mixing different ratios of reduced dithiothreitol ($DTT_{red}$) and oxidized dithiothreitol ($DTT_{oxi}$, trans-4,5-dihydroxy-1,2-dithiane; Sigma) followed by denaturation. The calculated midpoint potential for the CBS disulfide bond was −314 ± 2.4 mV (95% confidence interval: −320 to −308 mV) (Fig. 4), which is similar to the cytosolic glutathione redox potential ($E_{GSH}$) of −300 to −320 mV in unstressed cells (40, 41).

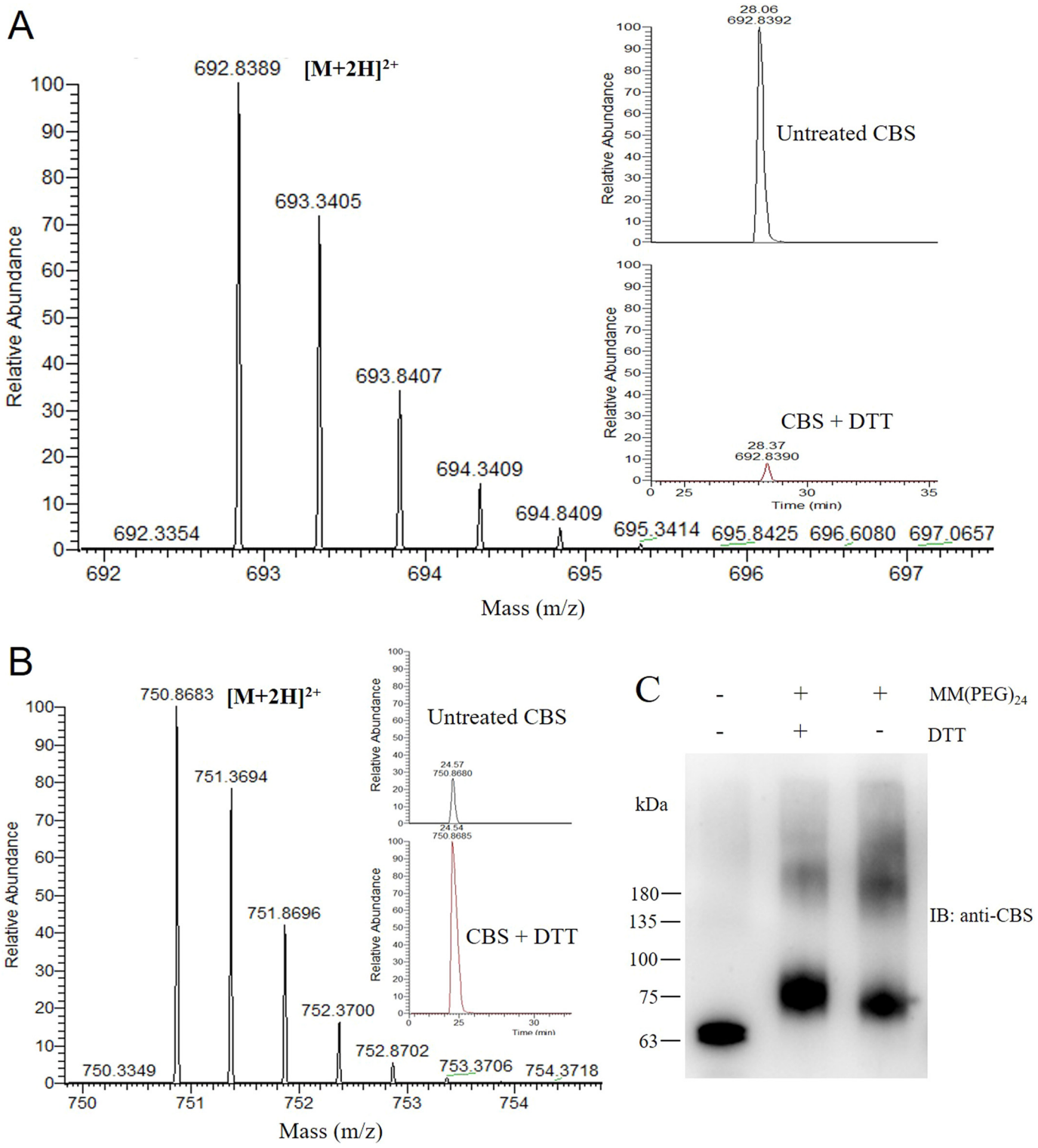


**Figure 2. The $^{272}CXXC^{275}$ motif in CBS protein exists in disulfide and thiol states under oxidative and reductive conditions.** *A*, recombinant CBS protein was analyzed by LC-MS/MS. The mass spectrum of the KCPGCRIIGVDPE peptide that contained the oxidized form of the $^{272}CXXC^{275}$ motif is shown. The monoisotopic mass $[M+2H]^{2+}$ of this peptide was 692.8389 Da (observed nominal mass = 1384.6711 Da; expected nominal mass = 1384.6712 Da). The chromatographic peaks of the oxidized peptide from untreated CBS and reduced CBS are shown in the *inset*. *B*, recombinant CBS was treated with 10 mM DTT for 1 h and then was analyzed by LC-MS/MS. The mass spectrum of the KCPGCRIIGVDPE peptide showing the reduced form of $^{272}CXXC^{275}$ motif is shown. The monoisotopic mass $[M+2H]^{2+}$ of this iodoacetamide-derivatized peptide was 750.8683 Da (observed nominal mass = 1500.7297 Da; expected nominal mass = 1500.7297 Da). The chromatographic peaks of the reduced peptide from the untreated CBS and reduced CBS are shown in the *inset*. *C*, the recombinant CBS was incubated without or with 10 mM DTT for 1 h and then precipitated with trichloroacetic acid. The precipitated CBS was labeled with the methyl-PEG-maleimide reagent $MM(PEG)_{24}$. The labeled samples were resolved on SDS-PAGE and immunoblotted for CBS. The molecular size markers in kDa are indicated at the *left*.

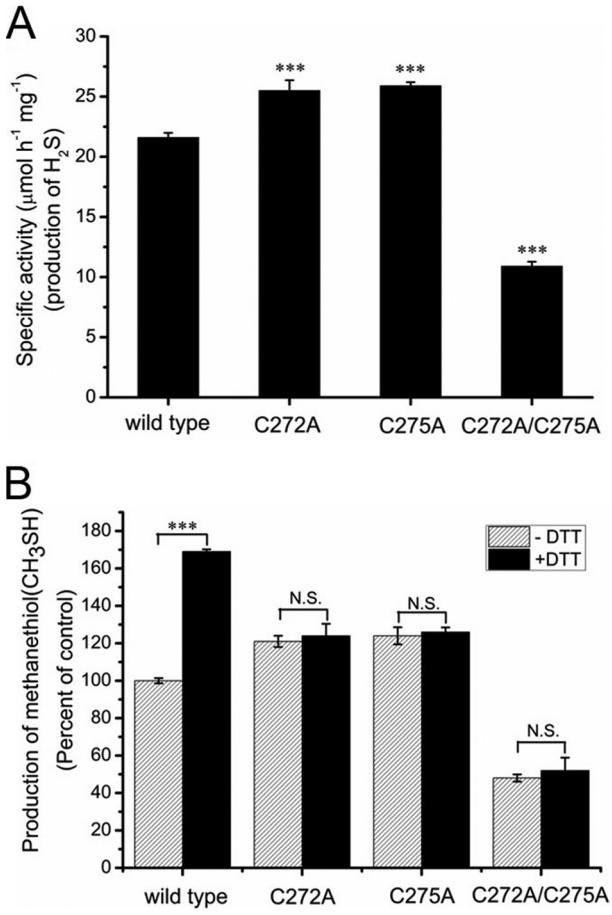


**Figure 3. Redox state of $^{272}$*CXXC*$^{275}$ motif regulates CBS activity.** *A*, the specific activities of wildtype CBS and of the CBS variants C272A, C275A, and C272A/C275A (double mutant) were measured using the methylene blue assay. The *data points* and *error bars* indicate the means ± S.D. ($n = 4$). ***, $p < 0.001$ *versus* wildtype CBS. *B*, the wildtype CBS protein and CBS variants were incubated without or with 10 mM DTT. The activities of these samples were determined using *S*-methylcysteine as the substrate in a 200-μl reaction solution containing 30 μM CPM fluorescent probe and 100 mM Hepes, pH 7.4. The *data points* and *error bars* show the means ± S.D. of three independent experiments. ***, $p < 0.001$ *versus* control; *N.S.*, not significant.

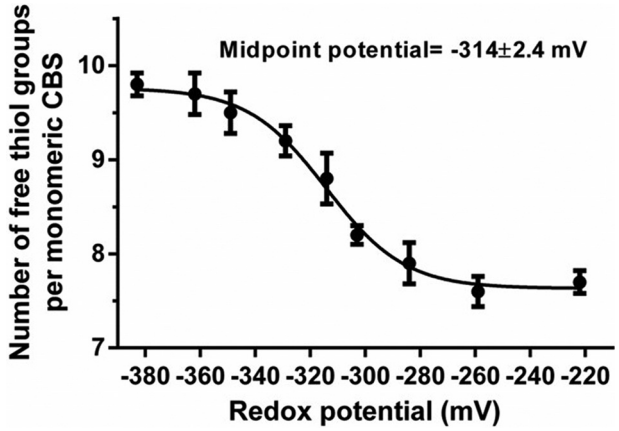


**Figure 4. Redox potential of the Cys$^{272}$–Cys$^{275}$ disulfide bond in CBS.** Recombinant CBS (100 μg) protein was preincubated in solutions containing various concentrations of reduced dithiothreitol ($DTT_{red}$) and oxidized dithiothreitol ($DTT_{oxi}$, *trans*-4,5-dihydroxy-1,2-dithiane), pH 7.4, for 1 h at 37 °C and then precipitated with trichloroacetic acid. Precipitated CBS was dissolved with 100 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS. The number of free thiol groups was determined by titration with 0.5 mM DTNB. The *data points* and *error bars* show the means ± S.D. ($n = 3$).

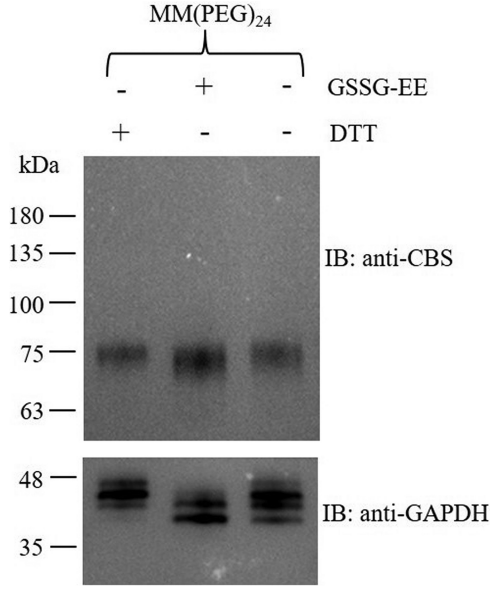


**Figure 5. CBS exists in oxidized and reduced states in HEK293 cells under oxidative and reductive conditions.** HEK293 cells were exposed to 0.5 mM DTT or 0.5 mM oxidized GSSG-EE for 10 min followed by washing three times with ice-cold PBS buffer. Then the cells were treated with 15% (v/v) trichloroacetic acid at 4 °C for 30 min before being alkylated with 10 mM $MM(PEG)_{24}$. The samples were resolved on SDS-PAGE and immunoblotted (*IB*) for CBS. The molecular size markers in kDa are indicated at the *left*.

### *CBS exists in oxidized and reduced states in HEK293 cells under oxidative and reductive conditions*

A thiol labeling assay was employed to determine whether the C*XX*C motif in CBS exists as the oxidized or reduced form respectively in HEK293 cells. Briefly, the cells were incubated with DTT or oxidized glutathione ethyl ester (GSSG-EE) to induce reductive stress or oxidative stress, respectively. Subsequently, the cells were treated with TCA to avoid artificial thiol-disulfide modifications, and then the free thiol groups in proteins were labeled with $MM[PEG]_{24}$, which has a molecular mass of 1240 Da. As shown in Fig. 5, the difference between the average molecular masses of the labeled CBS under reductive and oxidative stress was ~2–3 kDa, which suggested that a single disulfide bond is formed. These results indicated that the C*XX*C motif in CBS protein exists in reduced and oxidized states in mammalian cells and that it can be influenced by relevant cellular redox stresses. Unexpectedly, glyceraldehyde-3-phosphate dehydrogenase, which was used as a loading control, also exists in reduced and oxidized states in cells (Fig. 5). Additionally, the cytotoxicity of 0.5 mM DTT was assessed by using the cell counting kit-8 assay (supporting Experimental procedures). The results showed that DTT treatment for 24 h did not significantly affect the viability of HEK293 cells (Fig. S3).

### *Reductive stress enhances CBS-derived $H_2S$ production in cultured cells*

The activity of purified CBS was regulated as a function of the different redox states (Fig. 1). It was therefore anticipated that cleavage of the Cys$^{272}$–Cys$^{275}$ disulfide bond would increase the catalytic activity of the enzyme to concomitantly enhance CBS-derived $H_2S$ production. This was tested using fluorescent images of the endogenous $H_2S$ production in live HEK293 cells

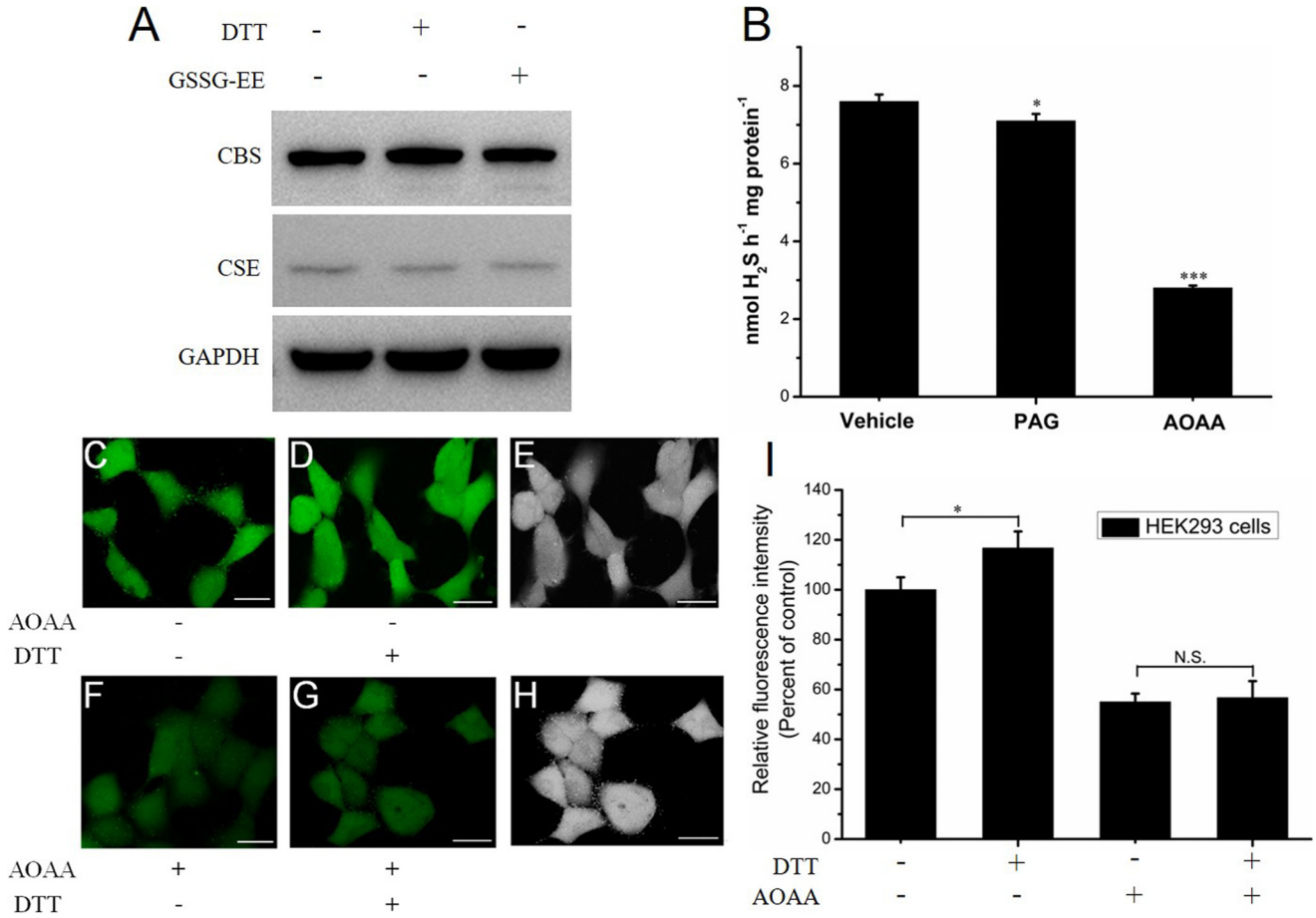


**Figure 6. Increasing activity of CBS in its reduced state increases the $H_2S$ signaling in live HEK293 cells.** *A*, HEK293 cells were exposed to 0.5 mM DTT or 0.5 mM oxidized GSSG-EE for 10 min. The cell lysates (50 $\mu$g) were resolved on SDS-PAGE and immunoblotted for CBS. *B*, $H_2S$ production was measured in HEK293 cells using the methylene blue method. The inhibitors 1 mM AOAA and 2 mM DL-propargylglycine (*PAG*) were used to inhibit CBS and CSE, respectively. The data are presented as the means ± S.D. ($n = 3$). *, $p < 0.05$; ***, $p < 0.001$ *versus* vehicle. *C*, HEK293 cells were incubated with 2 $\mu$M SF7-AM for 30 min at 37 °C, washed, and then imaged using confocal microscopy. *D*, the same dishes of cells shown in *C* were incubated with 0.5 mM DTT for 30 min at 37 °C and then imaged. *E*, brightfield images of the same dishes of cells shown in *D*. *Scale bar*, 25 $\mu$m. *F*, HEK293 cells were incubated with 0.5 mM AOAA for 24 h to inhibit CBS activity and were then washed. Subsequently, the cells were incubated with 2 $\mu$M SF7-AM for 30 min at 37 °C, washed, and then imaged. *G*, The same dishes of cells shown in *F* were incubated with 0.5 mM DTT for 30 min at 37 °C and then imaged. *H*, brightfield images of the same dishes of cells shown in *G*. *Scale bar*, 25 $\mu$m. *I*, quantification of the confocal fluorescence images of $H_2S$ signaling in HEK293 cells, with data from *C*, *D*, *F*, and *G* for comparison. The graph represents the relative fluorescence intensity compared with that of non-treated cells (*C*) and shows the means ± S.D. ($n = 3$). *, $p < 0.05$; *N.S.*, not significant.

exposed to 0.5 mM DTT. Compared with the very low level of expression of cystathionine γ-lyase (CSE), CBS is highly expressed in HEK293 cells as indicated by Western blot analysis (Fig. 6*A*). These results are consistent with a previous report (42). Moreover, the expression levels of CBS and CSE were not significantly changed when cells were incubated with DTT or GSSG-EE for 10 min. We also determined the activities of CBS and CSE in cell lysates using the methylene blue assay. As shown in Fig. 6*B*, compared with the $H_2S$-producing activity of the control group (7.6 ± 0.18 nmol $h^{-1}$ $mg^{-1}$), the activity of the cell lysate in the presence of 1 mM AOAA or 2 mM DL-propargylglycine was 2.8 ± 0.06 or 7.1 ± 0.18 nmol $h^{-1}$ $mg^{-1}$, respectively. Therefore, the amount of CSE-derived $H_2S$ was 0.5 nmol $h^{-1}$ $mg^{-1}$, and CBS-derived $H_2S$ was 4.3 nmol $h^{-1}$ $mg^{-1}$. The results indicated that the $H_2S$-producing activity of CBS was 8.6 times higher than that of CSE, which suggested that CBS is the major enzyme that catalyzes the biosynthesis of $H_2S$ in the cytoplasm of HEK293 cells.

Initial studies reported relatively high hydrogen sulfide concentrations of 30 to >100 $\mu$M in mammalian tissues (43–46), and these values are much higher than those determined in this study. Actually, the reevaluated endogenous $H_2S$ concentrations are orders of magnitude lower than those previously measured, with recent studies suggesting a concentration range of 15 to 120 nM in mouse liver and brain tissues (47, 48).

We next investigated the effect of reductive stress conditions on the CBS-derived $H_2S$ production using the $H_2S$ fluorescent probe SF7-AM in live HEK293 cells. Live cells were incubated with SF7-AM and imaged before and after treatment with DTT. The cells displayed a clear increase in the intracellular fluorescence compared with vehicle control (Fig. 6, *C–E* and *I*). To validate the contribution of CBS to the enhancement of $H_2S$ signaling, the cells were incubated with AOAA, an inhibitor of CBS and CSE, and then were imaged before and after treatment with DTT. The results showed that intracellular fluorescence was not significantly changed (Fig. 6, *F–H* and *I*), which indicated that reductive stress caused the increase in CBS activity in mammalian cells and the concomitant enhancement of the intracellular $H_2S$ production.

## Discussion

A previous study indicated that under the conditions of the *in vitro* assay, the heme group, rather than the C*XX*C motif in CBS, is the redox sensor (27). However, because the high concentration of homocysteine and cysteine in the reaction mixture would reduce the C*XX*C disulfide, the role of the C*XX*C motif in redox regulation of CBS cannot be assessed in this assay. In addition to the C*XX*C motif, the heme in CBS has been proposed to be a redox sensor (49). Binding of exogenous ligands like NO and CO to the heme in a ferrous form of CBS is correlated with loss of enzyme activity (14–17). However, because of the low heme redox potential (−350 mV) of wildtype CBS (49), the existence of a ferrous form of CBS *in vivo* under physiological conditions remains an open question.

To address these gaps in our understanding of the redox regulation of CBS, we have employed *S*-methylcysteine to determine the activities of both oxidized and reduced CBS. Indeed, the dithiol form of the C*XX*C motif in CBS is associated with a more active form of the enzyme (Fig. 1). Additionally, reduced CBS is readily oxidized by exposing it to air in the absence of reductants, as evidenced by determination of thiols using DTNB assay. The activity of reduced CBS is 1.8-fold higher than oxidized CBS as indicated by the fluorescent thiol assay, whereas reduced CBS has a ~3-fold increase in activity as shown by the gas chromatographic assay (Fig. 1*C*). The basis for the discrepancy between these two data sets could be the result of reoxidization of the partially reduced CBS in the fluorescent thiol assay, which lacks reductants.

The cysteine residue is characterized by five angles, and the different angles can permit 20 different possible combinations of disulfide bond. These disulfide bonds can be classified into three main types: the spiral, hook, and staple bonds (50). Based on this classification standard, the $Cys^{272}$–$Cys^{275}$ disulfide bond in CBS is classified as a −/+RHHook catalytic bond of the type observed in oxidoreductases (*e.g.* thioredoxin) and isomerases (*e.g.* protein disulfide isomerase) rather than an allosteric disulfide bond, which has the −RHStaple configuration. However, the distance between the C*XX*C motif and the active site is ~20 Å, and it appears unlikely that these cysteines play a catalytic role. Additionally, mutation of the cysteines in the C*XX*C motif results in retention of enzyme activity (Fig. 3*A*), which excludes an essential role for these cysteines in catalysis. Moreover, in the present study, we have provided clear evidence that the C*XX*C motif in CBS exists in both oxidized disulfide bond and reduced dithiol states (Fig. 2), and changes in redox state of C*XX*C motif regulate the activity of CBS (Fig. 1).

The surface exposure of the C*XX*C motif observed in the structure of the modified human full-length CBS is probably important for access to the bond by the cellular GSH/GSSG molecules (26). The midpoint potential of the CBS disulfide bond is −314 mV (Fig. 4) at pH 7.4. In mammalian cells, the cytosolic glutathione redox potential ($E_{GSH}$) of unstressed cells is typically found in the range between −300 to −320 mV (40, 41), which is similar to the midpoint potential of the CBS disulfide bond. Therefore, the C*XX*C motif in CBS is likely to exist as an equivalent mixture of the oxidized and reduced states, which was confirmed by thiol-labeling assays in HEK293 cells (Fig. 5). This midpoint potential (−314 mV) of the CBS disulfide bond allows CBS activity to be fine-tuned in response to stress *in vivo*.

Under reductive stress conditions, an increase in the proportion of the reduced C*XX*C motif in CBS is primarily responsible for an increase in CBS activity, thus leading to the enhancement of $H_2S$ production (Fig. 6). These results seem at first glance to be contradictory to our previous observations that under oxidative stress *S*-glutathionylation of CBS at $Cys^{346}$ increases its activity (29). However, because glutathionylation of CBS at $Cys^{346}$ is efficient with GSH but not with GSSG, it implies that the thiol of $Cys^{346}$ should first be oxidized to a sulfenic acid by increasing concentrations of reactive oxygen species before being attacked by GSH under conditions of cellular oxidative stress (29). Actually, a high level of reactive oxygen species does not necessarily indicate a more oxidized intracellular redox state. It has been shown that in tumors, high levels of reactive oxygen species are often counterbalanced with high levels of reductants such as GSH, NADPH, and vitamin C (51–53). Therefore, CBS activity would be coordinately regulated by both thiol-disulfide exchange reactions and reactions with partially reactive oxygen species at the level of the C*XX*C motif and $Cys^{346}$.

Conformational changes are the hallmarks of protein dynamics and often intimately related to protein functions (54). Changes in the redox state of the C*XX*C motif, which can be mediated by thiol-disulfide exchange reactions, will probably lead to the conformational changes of CBS and concomitant influence of CBS activity. Indeed, mutagenesis of cysteine residues in C*XX*C motif significantly affected the specific activity of CBS (Fig. 3*A*). Because the crystal structure of full-length oxidized CBS is not available, the structural basis for regulating the CBS activity by the redox state of the C*XX*C motif is not known, and this issue needs to be investigated.

The present study provided evidence that CBS activity is regulated by the redox state (thiol or disulfide) of the C*XX*C motif. As a result, reduced CBS has higher $H_2S$-producing activity in cells exposed to reductive challenge (Fig. 6). A recent study demonstrated that the intracellular redox state, which was measured using a redox-sensitive organic reporter molecules assembled on gold nanoshells, undergoes reductive changes associated with the induction of hypoxia based on the use of surface-enhanced Raman spectroscopy nanosensors (55). Hypoxia is a feature of solid tumors and generally occurs at distances greater than 100 $\mu$m from functional blood vessels (56). The increase in reductive stress under hypoxic conditions is predicted to increase production of CBS-derived $H_2S$.

In summary, our findings imply that CBS activity is controlled by the redox state (thiol or disulfide) of the C*XX*C motif in the presence of different oxidants or reductants in solution. This may cause the C*XX*C motif to act as a redox sensor. This study also demonstrates that there is an increase in $H_2S$ production under reductive conditions via reduction of the C*XX*C motif in CBS. However, the physiological effects of enhanced $H_2S$ under reductive stress conditions remain to be fully elucidated.

## Experimental procedures

### *Materials*

MM[PEG]$_{24}$ was purchased from Thermo Fisher Scientific (Waltham, MA). The anti-cystathionine β-synthase (anti-CBS) antibody was obtained from ABclonal (Wuhan, China). The anti-cystathionine γ-lyase (anti-CSE) antibody was purchased from Abcam (Cambridge, MA). The fluorescent thiol probe 7-diethylamino-3-(4-maleimidophenyl)-4-methylcoumarin (CPM) and hydrogen sulfide probe (SF7-AM) were obtained from Sigma–Aldrich. Methanethiol ($CH_3SH$) and dimethyl sulfide were purchased from ANPEL Laboratory Technologies Inc. (Shanghai, China). GSSG-EE was synthesized by Ontores Biotechnologies (Hangzhou, China). The cell counting kit-8 was purchased from Dojindo (Kumamoto, Japan). Unless otherwise specified, all chemicals were used as received.

### *Protein expression and purification*

Mutagenesis of CBS was performed using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). The mutations were verified by DNA sequencing (Sangon, Shanghai, China). The expression and purification of the wildtype and variant versions of human CBS were performed as described previously for the wildtype proteins (30). Generally, cell pellets obtained from a 2-liter culture were resuspended in 200 ml of PBS supplemented with 20 mg of lysozyme, 5 mg of PLP, and a protease inhibitor tablet (Roche Applied Science). The cells were lysed by sonication, and the supernatant was obtained by centrifugation at 12,000 rpm for 30 min. Subsequently, the supernatant was loaded on a GSTrap FF column (GE Healthcare) that had been equilibrated with PBS. The column was subsequently washed with at least 20 column volumes of PBS, and glutathione *S*-transferase (GST)-fused CBS was eluted with 20 mM GSH in 50 mM Tris (pH 8.0). The GST-fused CBS was cleaved with thrombin at a final concentration of 5 units/mg protein at 4 °C, and the GST tag was removed using a Q Sepharose FF column (GE Healthcare) that had been equilibrated with 50 mM Tris buffer (pH 8.0). The CBS protein was eluted with a 200-ml linear gradient of 0 to 500 mM NaCl in 50 mM Tris-HCl (pH 8.0). Fractions containing the pure CBS protein were pooled and desalted, and the aliquoted enzyme was stored at −80 °C. The purities of all protein preparations were judged to be >90% by SDS-PAGE analysis.

### *$H_2S$ production assays*

The $H_2S$ measurements were performed using the methylene blue assay according to Asimakopoulou *et al.* (31) with some modifications. First, $H_2S$ production in the reaction is trapped by $Pb(NO_3)_2$ to produce PbS. Subsequently, in acid solution PbS reacts with *N*,*N*-dimethyl-*p*-phenylenediamine-sulfate to produce methylene blue in the presence of $FeCl_3$. The concentration of methylene blue is determined from the absorbance at 670 nm. In the case of the CBS enzyme, each test consisted of a 200-μl reaction mixture containing 2.5 μg of the purified CBS, 5 mM cysteine, 5 mM homocysteine, and 100 mM Hepes buffer (pH 7.4). For the cell lysate, HEK293 cells were washed three times with ice-cold PBS buffer and then scraped from 100-mm plates. The cells were harvested by centrifugation for 5 min, and the supernatant was carefully discarded. Next, 500 μl of Tris-HCl buffer (50 mM, pH 8.0) was added to the tube (500 μl buffer/1 plate cells), and the cell suspension was frozen and thawed three times using a liquid nitrogen/water bath. The reaction mixtures (200 μl) containing 180 μl of the cell lysate, 5 mM cysteine, and 5 mM homocysteine were prepared. The inhibitors aminooxyacetic acid (1 mM) or DL-propargylglycine (2 mM) were added to the reaction 10 min before the cysteine was added to the solution. After 30 min of incubation at 37 °C, the reaction was terminated by adding 1% (w/v) $Pb(NO_3)_2$ to trap the $H_2S$ followed by 15% (v/v) TCA to precipitate the proteins. Subsequently, *N*,*N*-dimethyl-*p*-phenylenediamine-sulfate (dissolved in 7.2 M HCl) was added to the reaction mixture immediately followed by addition of $FeCl_3$ in 1.2 M HCl. The reaction mixtures were centrifuged at 12,000 rpm for 10 min to remove precipitation. The absorbance of the supernatant was measured at 670 nm. The reaction mixture without the substrates was used as the negative control. The $H_2S$ content was calculated against a calibration curve of standard $H_2S$ solutions.

### *Determination of heme content*

Heme concentration was determined by the pyridine hemochrome assay as described previously (32, 33). The reaction mixture (200 μl) contained purified CBS (20 μM), 100 μl of 40% (v/v) pyridine, and 500 μM potassium ferricyanide (dissolved in 0.2 M NaOH). The reaction was initiated by the addition of 2 μl of 0.5 M sodium dithionite (dissolved in 0.5 M NaOH) and incubated for 5 min at room temperature. Heme concentration was determined from the absorbance at 557 nm using the extinction coefficient of 23.98 $mM^{-1}$ $cm^{-1}$ for the pyridine hemochromogen.

### *Measurement of CBS activity using a fluorescent probe*

Purified wildtype CBS (1 mg/ml) was treated with 10 mM DTT for 1 h at 4 °C, and then DTT was removed by ultrafiltration using a 1-ml spin column. As a result, the DTT concentration was diluted ~1000-fold by washing with the nitrogen-bubbled 100 mM Hepes (pH 7.4) buffer. The reduced CBS solutions were reoxidized by exposing them to air, pipetting up and down 6–8 times, and incubating for 2 h at 4 °C. The activities of reduced CBS (DTT treatment), oxidized CBS (no treatment), and reoxidized CBS were determined using *S*-methylcysteine as a substrate according to our previous report with some modifications (29). CBS can catalyze the decomposition of the *S*-methylcysteine, a thiol-free substrate analog of cysteine, to produce methanethiol ($CH_3SH$), which can be continuously monitored with the fluorescent thiol probe CPM. The reaction mixture (200 μl) containing 10 mM *S*-methylcysteine, 30 μM CPM thiol fluorescent detection reagent, and 100 mM Hepes buffer (pH 7.4) was added to a 96-well plate, and the reaction was started by the addition of 5 μg of CBS. The 96-well plate was placed in a multifunctional microplate reader. The fluorescence of the mixture was monitored at 460 nm ($\lambda_{ex}$ = 400 nm) for 10 min at 37 °C. The activity of oxidized CBS was set as 100%. Alternatively, DTNB was used in the reaction mixture to detect production of methanethiol using an extinction coeffi-

cient of 13,600 $M^{-1}$ $cm^{-1}$ at 412 nm for 2-nitro-5-thiobenzoic acid anion (29, 34).

### Measurement of CBS activity using a gas chromatographic assay

CBS activity that leads to production of methanethiol ($CH_3SH$) was also determined using a gas chromatographic assay according to our previous report with some modifications (29). Recombinant CBS protein (20 $\mu$g) was preincubated in a 100 mM Hepes buffer (1 ml) containing 20 mM GSH for 2 h at 4 °C in a sealed bottle (20 ml), and the reaction was initiated by the addition of 10 mM *S*-methylcysteine. After incubation at 37 °C with gentle shaking for 10 min, 0.5-ml aliquots from the gas phase of the sealed bottles were collected using gas-tight syringes and injected in a GC 9860 gas chromatograph (Shanghai Qiyang Information Technology Co., Ltd., Shanghai, China) that was equipped with a SE-30 column (30 m × 0.53 mm × 1.0 $\mu$m). $CH_3SH$ production was detected using a pulsed flame photometric detector. A methanethiol standard was obtained from ANPEL Laboratory Technologies Inc. (Shanghai, China). An internal standard of dimethyl sulfide was added to the reaction mixtures. The activity of the untreated CBS protein was defined as 100%. The peak area of $CH_3SH$ was normalized to the peak area of an internal standard.

### Mass spectrometry

A solution of CBS (50 $\mu$l at 5 mg/ml) was treated with 10 mM DTT for 1 h at room temperature. Untreated CBS was used as a control. The samples were ultrafiltered and buffer-exchanged using a 1-ml spin column with 20 mM phosphate buffer (pH 7.8) and then denatured using 6 M guanidine hydrochloride. Subsequently, the free thiol groups of the samples were derivatized with iodoacetamide at a final concentration of 25 mM. The samples were concentrated, and the buffer was exchanged using a 1-ml spin column with 20 mM phosphate buffer (pH 7.8) and then digested using a protein:endoproteinase Glu-C ratio of 20:1 at 37 °C for 16–18 h. The digested samples were acidified with 10% (v/v) formic acid and directly used for LC-MS/MS analyses. All analyses were performed using a Thermo EASY-nLC1000 Nano HPLC system with a Thermo EASY column SC200 (150 $\mu$m × 100 mm, RP-C18) at a flow rate of 0.3 $\mu$l/min. The mobile phases A and B consisted of 0.1% (v/v) formic acid in $H_2O$ and 0.1% (v/v) formic acid in 84% (v/v) acetonitrile, respectively. The peptides were eluted using the following gradient: $t$ = 0–50 min: 0–60% B; $t$ = 51–53 min: 60–100% B; and $t$ = 54–60 min: 100% B. A HPLC system was directly coupled to a Q Exactive hybrid quadrupole Orbitrap mass spectrometer (Thermo Finnigan). Positive ions were generated by electrospray, and the Q Exactive spectrometer was operated in data-dependent acquisition mode.

A survey scan from $m/z$ 300–1800 was acquired in the Q Exactive spectrometer (resolution = 70,000 at $m/z$ 200, with an accumulation target value of 3,000,000 ions) with lockmass enabled. Up to 10 of the most abundant ions were sequentially isolated and fragmented within the linear ion trap using collisionally induced dissociation with an activation $q$ of 0.25. The $m/z$ ratios selected for MS/MS were dynamically excluded for 10 s. The MS data were searched against the database from the National Center for Biotechnology and Information. The search parameters were: precursor tolerance, 10 ppm; and product ion tolerances, ±0.1 Da. Methionine oxidation, deamidation of glutamine and asparagines, and carbamidomethylation of cysteine were selected as variable modifications. The relative ion abundance of the peptides containing the oxidized or reduced C*XX*C motif was calculated according to the peak areas from the ion chromatogram. The sequence coverage of wildtype CBS protein obtained by MS analysis was 99.8%.

### Labeling of unpaired cysteine thiols in CBS

Recombinant wildtype CBS 100 $\mu$l (1 mg/ml) was treated with or without 10 mM DTT for 1 h at 4 °C and then was precipitated with 15% (v/v) trichloroacetic acid at 4 °C for 30 min. The precipitated protein was washed three times with cold acetone and then redissolved in 100 $\mu$l of phosphate buffer (100 mM) containing 1% (w/v) SDS, pH 7.0. Subsequently, the methyl-PEG-maleimide reagent, $MM[PEG]_{24}$, was added to a final concentration of 10 mM. After 16 h of incubation at 25 °C, the labeled samples were resolved on SDS-PAGE, protein-transferred to a PVDF membrane, and blotted with anti-CBS and goat anti-rabbit peroxidase antibodies. The blots were visualized using chemiluminescence (Tanon, Shanghai, China). The molecular mass of $MM(PEG)_{24}$ is 1240 Da.

HEK293 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum and a 1% (v/v) penicillin-streptomycin solution at 37 °C and 5% (v/v) $CO_2$. After the cells had reached ~80% confluence in a 10-cm plate, DTT or oxidized glutathione ethyl ester (GSSG-EE) was added to a final concentration of 0.5 mM. After 10 min of incubation, the cells were washed three times with ice-cold PBS supplemented with 0.5 mM DTT or GSSG-EE and then incubated with a final concentration of 15% (v/v) TCA for 30 min at 4 °C to avoid artificial thiol-disulfide modifications (35). The pellets obtained by centrifugation at 12,000 rpm for 10 min were redissolved in 100 $\mu$l of phosphate buffer (100 mM) containing 1% (w/v) SDS, pH 7.0. Subsequently, $MM(PEG)_{24}$ was added to a final concentration of 10 mM. After 16 h of incubation at 25 °C, the labeled samples were resolved on SDS-PAGE and immunoblotted for CBS.

### Redox potential of the CBS disulfide bond

DTNB assay was employed to determine the redox potential of the CBS disulfide bond. Briefly, redox buffers were prepared in a 100 mM Hepes buffer containing various concentrations of reduced dithiothreitol ($DTT_{red}$) and oxidized dithiothreitol ($DTT_{oxi}$, *trans*-4,5-dihydroxy-1,2-dithiane; Sigma), pH 7.4. The total dithiothreitol concentration in the redox buffers was 10 mM, and all solutions were deoxygenated by bubbling with nitrogen for 30 min. The $DTT_{oxi}/DTT_{red}$ redox potential was calculated according to the Nernst equation,

$$E_h = E_0 + \frac{RT}{nF} \times \ln\left(\frac{[DTT_{oxi}]}{[DTT_{red}]^2}\right) \quad \text{(Eq. 1)}$$

where $E_0 = -352$ mV at pH 7.4, $R$ is the gas constant, $T$ is the absolute temperature, and $F$ is Faraday's constant ($n$ = 2), and $[DTT_{oxi}]$ and $[DTT_{red}]$ are molar concentrations of oxidized and reduced DTT, respectively (36). Subsequently, recombi-

nant wildtype CBS (100 $\mu$g) was preincubated in solutions (500 $\mu$l) with various concentrations of $DTT_{oxi}$ and $DTT_{red}$ for 1 h at 37 °C and then precipitated with 15% (v/v) trichloroacetic acid at 4 °C for 30 min. The precipitated protein was washed three times with cold acetone and then was dissolved in 200 $\mu$l of 100 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS. To fully expose the cysteine residues of the precipitated CBS proteins, 1% (w/v) SDS was used in the DTNB assay according to a previous report (37). Control samples contained the same amount of DTNB in the same buffer, but the CBS protein was omitted. The final mixture was incubated with 0.5 mM DTNB for 10 min at room temperature, and the samples were analyzed spectrophotometrically at 412 nm using an extinction coefficient of 13,600 $M^{-1}$ $cm^{-1}$ for 2-nitro-5-thiobenzoic acid anion (29). The number of free thiol groups of CBS protein was determined, and the midpoint potential of the CBS disulfide bond was calculated.

### *Confocal images of endogenous $H_2S$ production in live HEK293 cells*

Confocal fluorescence imaging studies were performed with a Leica SP8 laser scanning confocal microscopy with a 63× oil objective. The hydrogen sulfide fluorescent probe SF7-AM was excited using a 488-nm Argon laser, and the emissions between 500 and 650 nm were collected using a detector (38). The cells were imaged at 37 °C with 5% (v/v) $CO_2$ during the course of the experiment. All imaging experiments were carried out in 35-mm glass-bottomed dishes (NEST, Wuxi, China). HEK293 cells were incubated with 2 $\mu$M SF7-AM for 30 min at 37 °C with 5% (v/v) $CO_2$. The medium was exchanged, and images were acquired for cells in three different fields. For DTT stimulation, 10 $\mu$l of 50 mM DTT was added directly to dishes for a final concentration of 0.5 mM. After 30 min of incubation at 37 °C with 5% (v/v) $CO_2$, the same dishes of cells were imaged. For the inhibitor experiments, 10 $\mu$l of 50 mM AOAA was added to dishes to a final concentration of 0.5 mM. After 24 h of incubation, the cells were incubated with 2 $\mu$M SF7-AM for 30 min. Then the medium was exchanged, and images were acquired for the cells in three different fields. Subsequently, 0.5 mM DTT was added to the same dishes for an additional 30 min of incubation at 37 °C, and the cells were imaged. Image analysis was performed using ImageJ (National Institute of Health). The images were quantified using the mean pixel intensity after setting a common threshold for all images.

*Author contributions*—W. N. conceptualization; W. N. resources; W. N., J. W., and M. W. data curation; W. N. funding acquisition; W. N., J. W., J. Q., M. W., P. W., F. C., and S. Y. investigation; W. N. methodology; W. N. writing-original draft.

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