Allosteric control of human cystathionine beta synthase activity by a redox active disulfide bond

Running Title: Allosteric control of CBS activity

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Abstract

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 H2S. CBS is a complex protein with a modular three-domain architecture, the central domain of which contains a C272XXC275 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- to 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 Cys272-Cys275 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 human embryonic kidney 293 (HEK293) cells under oxidative and reductive conditions, and stressing these cells with dithiothreitol (DTT) results in more reduced enzyme and a concomitant increase in H2S production in live HEK293 cells as determined using a H2S fluorescent probe. By contrast, incubation of cells with aminooxyacetic acid (AOAA), an inhibitor of CBS and cystathionine γ-lyase (CSE), eliminates the increase of H2S 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 H2S production is increased in cells under reductive stress conditions.

Introduction

Cystathionine β-synthase (CBS) 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 hydrogen sulfide (H2S), can catalyze the condensation of homocysteine and cysteine or condensation of two molecules of cysteine to produce H2S, 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 Cys52 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 (SAM), 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 SAM (25). The central domain contains the PLP cofactor, which is essential for catalysis, and a C272XXC 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 H2S, 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. Since 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 Cys346 enhances human CBS activity under oxidative stress conditions (29). Furthermore, in addition to heme, human CBS has a second putative redox-active center, a CXXC 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 CXXC motif, the activity of oxidized CBS cannot be assessed using this assay.

We hypothesized that redox changes in the CXXC motif in CBS might regulate enzyme activity under various redox conditions and that the CXXC motif would be a redox sensor. Here, we found that the CXXC motif exists in oxidized and reduced states in recombinant CBS and in human embryonic kidney 293 (HEK293) cells. The activity of reduced CBS increased by ~2 to 3 fold compared to oxidized CBS in vitro and resulted in a concomitant increase in CBS-derived H2S in response to reductive stress in HEK293 cells. Additionally, the midpoint potential of the Cys272-Cys275 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 CXXC 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 wild-type CBS differ, a modified assay in which S-methylcysteine served as the substrate was developed. The production of methanethiol (CH3SH) can be monitored by fluorescence spectroscopy in the presence of a fluorescent thiol probe (Fig. 1A). The relative activity of the reduced wild-type 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 wild-type CBS (Fig. 1, A and B). Additionally, human CBS contains 11 cysteine residues, one of which Cys52 coordinates the heme axially (10). The number of thiols in CBS protein was determined using DTNB assay. In order 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 re-oxidized by exposing them to air. This result indicated that the CXXC 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 dithiothreitol (DTT), the activity of CBS that had been pretreated with 20 mM glutathione (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. 1C). 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 CXXC 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 CXXC 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 CXXC motif exists in wild-type CBS. Purified wild-type CBS was treated with or without 10 mM DTT, and the resulting samples were analyzed using LC-MS/MS (liquid chromatography-tandem mass spectrometry) with a 99.8% of sequence coverage. The results showed that a unique peptide, KCPGCRIGVDPE, contained a disulfide bond, which demonstrated that the oxidized form of the CXXC motif was detected. The redox state of the Cys272-Cys275 disulfide bond in purified CBS and its susceptibility to reduction was determined. The unpaired Cys272 and Cys275 cysteines but not the Cys272-Cys275 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. 2A, and supplemental Fig. S1), and The monoisotopic mass [M+2H]2+ of the unpaired Cys272-Cys275 peptide that was alkylated with iodoacetamide was 750.8683 Da (observed nominal mass = 1500.7297 Da; expected nominal mass = 1500.7297 Da) (Fig. 2B, and supplemental Fig. S2). Additionally, to the peak area from the ion chromatogram, the relative ion abundance of the peptide containing Cys272-Cys275 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 CXXC motif was reduced after DTT treatment of the oxidized CBS (Fig. 2A).

In addition to the mass spectrometry, methyl-PEG24-maleimide (MM[PEG]24) was used to label the thiols of the oxidized and reduced CBS. The difference between the molecular weights of the labeled oxidized and reduced forms of CBS was ~3 kDa (Fig. 2C), 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 wild-type CBS protein under different redox conditions.

The redox state of CXXC motif regulates CBS activity—Because the Cys272-Cys275 was the unique disulfide bond detected in the oxidized CBS, we speculated that redox state of the CXXC 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/l culture) was ~3-fold lower than the wild-type CBS (~10 mg/l culture), and the C272A/C275A protein was obtained in very low yield (~0.5 mg/l culture). The activities of the wild-type 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 μmol mg⁻¹ h⁻¹ and 25.9 ± 0.30 μmol mg⁻¹ h⁻¹, respectively, and were ~1.2-fold higher than the wild-type CBS (21.6 ± 0.39 μmol mg⁻¹ h⁻¹). However, the activity of the C272A/C275A enzyme (10.9 ± 0.38 μmol mg⁻¹ h⁻¹) was ~2-fold lower than the wild-type CBS (Fig. 3A).

Previous studies indicated that the heme content in CBS protein affects the specific activity of enzyme (39). Therefore, the heme concentrations of wild-type 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 wild-type 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 CXXC motif on the activity of CBS. Unlike wild-type CBS, no changes in the activity of these three mutants were observed before and after treatment with DTT (Fig. 3B). Collectively, these results were consistent with the Cys272-Cys275 being the unique disulfide bond in purified CBS, with its redox state regulating CBS activity.
Redox potential of the Cys\textsuperscript{272}-Cys\textsuperscript{275} disulfide bond— The midpoint potential of the Cys\textsuperscript{272}-Cys\textsuperscript{275} disulfide bond was determined using DTT as the reductant. Since Cys\textsuperscript{272}-Cys\textsuperscript{275} is the unique disulfide bond observed in purified wild-type 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\textsubscript{red}) and oxidized dithiothreitol (DTT\textsubscript{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\textsubscript{GSH}) of -300 to -320 mV in unstressed cells (40, 41).

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 CXXC motif in CBS exists as the oxidized or reduced form respectively in HEK293 cells. Briefly, 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 trichloroacetic acid (TCA) to avoid artificial thiol-disulfide modifications, and then the free thiol groups in proteins were labeled with methyl-PEG-maleimide (MM[PEG]\textsubscript{34}), which has a molecular weight of 1240 Da. As shown in Fig. 5, the difference between the average molecular weights of the labeled CBS under reductive and oxidative stress was ~2 to 3 kDa, which suggested that a single disulfide bond is formed. These results indicated that the CXXC motif in CBS protein exists in reduced and oxidized states in mammalian cells and that this thiol-disulfide exchange reaction is influenced by relevant cellular redox stresses. Unexpectedly, gyceraldehyde 3-phosphate dehydrogenase (GAPDH), 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 CCK-8 assay. The results showed that DTT treatment for 24 h did not significantly affect the viability of HEK293 cells (supplemental Fig. S3).

Reductive stress enhances CBS-derived H\textsubscript{2}S 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\textsuperscript{272}-Cys\textsuperscript{275} disulfide bond would increase the catalytic activity of the enzyme to concomitantly enhance CBS-derived H\textsubscript{2}S production. This was tested using fluorescent images of the endogenous H\textsubscript{2}S production in live HEK293 cells exposed to 0.5 mM DTT. Compared with the very low level of expression of cystathionine \(\gamma\)-lyase (CSE), CBS is highly expressed in HEK293 cells as indicated by western blot analysis (Fig. 6A). 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. 6B, compared with the H\textsubscript{2}S-producing activity of the control group (7.6 ± 0.18 nmol h\textsuperscript{-1} mg\textsuperscript{-1}), the activity of the cell lysate in the presence of 1 mM AOAA or 2 mM DL-propargylglycine (PAG) was 2.8 ± 0.06 nmol h\textsuperscript{-1} mg\textsuperscript{-1} or 7.1 ± 0.18 nmol h\textsuperscript{-1} mg\textsuperscript{-1}, respectively. Therefore, the amount of CSE-derived H\textsubscript{2}S was 0.5 nmol h\textsuperscript{-1} mg\textsuperscript{-1}, and CBS-derived H\textsubscript{2}S was 4.3 nmol h\textsuperscript{-1} mg\textsuperscript{-1}. The results indicated that the H\textsubscript{2}S-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\textsubscript{2}S in the cytoplasm of HEK293 cells.

Initial studies reported relatively high hydrogen sulfide concentrations of 30 \(\mu\)M 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\textsubscript{2}S concentrations are orders of magnitude lower than those previously measured, with recent studies suggesting a concentration range of 15 nM 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\textsubscript{2}S production using the H\textsubscript{2}S 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\textsubscript{2}S 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$_2$S production.

**Discussion**

A previous study indicated that under the conditions of the in vitro assay, the heme group, rather than the CXXC motif in CBS, is the redox sensor (27). However, since the high concentration of homocysteine and cysteine in the reaction mixture would reduce the CXXC disulfide, the role of the CXXC motif in redox regulation of CBS cannot be assessed in this assay. In addition to the CXXC 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, due to the low heme redox potential (-350 mV) of wild-type 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 $\delta$-methylcysteine to determine the activities of both oxidized and reduced CBS. Indeed, the dithiol form of the CXXC 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. 1C). The basis for the discrepancy between these two data sets could be the result of reoxidation 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 -/+$\delta$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 -RHS Staple configuration. However, the distance between the CXXC 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 CXXC motif results in retention of enzyme activity (Fig. 3A), which excludes an essential role for these cysteines in catalysis. Moreover, in the present study, we have provided clear evidence that the CXXC motif in CBS exists in both oxidized disulfide bond and reduced dithiol states (Fig. 2), and changes in redox state of CXXC motif regulate the activity of CBS (Fig. 1).

The surface exposure of the CXXC 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 ($E_{\text{GSH}}$) 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 CXXC 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 CXXC motif in CBS is primarily responsible for an increase in CBS activity, thus leading to the enhancement of H$_2$S 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 Cys346 increases its activity (29). However, since glutathionylation of CBS at Cys346 is efficient with GSH but not with GSSG, it implies that the thiol of Cys346 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 counter-balanced 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 CXXC motif and Cys346.

Conformational changes are the hallmarks of protein dynamics and often intimately related to protein functions (54). Changes in the redox state of the CXXC 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 CXXC motif significantly affected the specific activity of CBS (Fig. 3A). 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 CXXC 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 CXXC motif. As a result, reduced CBS has higher H2S-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 μm from functional blood vessels (56). The increase in reductive stress under hypoxic conditions is predicted to increase production of CBS-derived H2S.

In summary, our findings imply that CBS activity is controlled by the redox state (thiol or disulfide) of the CXXC motif in the presence of different oxidants or reductants in solution. This may cause the CXXC motif to act as a redox sensor. This study also demonstrates that there is an increase in H2S production under reductive conditions via reduction of the CXXC motif in CBS. However, the physiological effects of enhanced H2S under reductive stress conditions remain to be fully elucidated.

**Experimental procedures**

**Materials**—Methyl-PEG24-maleimide (MM[PEG]24) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). 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, USA). The fluorescent thiol probe CPM (7-Diethylamino-3-(4-maleimidophenyl)-4-methyl coumarin) and hydrogen sulfide probe (SF7-AM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanethiol (CH3SH) and dimethyl sulfide was purchased from ANPEL Laboratory Technologies Inc. (Shanghai, China). Oxidized glutathione ethyl ester (GSSG-EE) was synthesized by Ontores Biotechnologies (Hangzhou, China). The cell counting kit-8 (CCK-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 Quickchange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA). The mutations were verified by DNA sequencing (Sangon, Shanghai, China). The expression and purification of the wild-type and variant versions of human CBS were performed as described previously for the wild-type proteins (30). Generally, cell pellets obtained from a 2-liter culture were resuspended in 200 ml of phosphate-buffered saline (PBS) supplemented with 20 mg of lysozyme, 5 mg of PLP, and a protease inhibitor tablet (Roche, Basel, Switzerland). 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, USA) 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 glutathione (GSH) in 50 mM Tris, pH 8.0. The GST-fused CBS was cleaved with thrombin at a final concentration of 5 U/mg protein at 4 °C, and the GST tag was removed using a Q sepharose FF column (GE Healthcare, USA) 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 (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) analysis.

**H₂S production assays**—The H₂S measurements were performed using the methylene blue assay according to Asimakopoulou with some modifications (31). First, H₂S production in the reaction is trapped by Pb(NO₃)₂ to produce lead sulfide (PbS). Subsequently, in acid solution PbS reacts with N,N-dimethyl-p-phenylenediamine-sulfate to produce methylene blue in the presence of FeCl₃. 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 cell lysate, 5 mM cysteine, 5 mM homocysteine and 100 mM Hepes buffer (pH 7.4, was added to a 96 well plate containing 2 μl of wild-type CBS (20 μM), 100 μM 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 hemechromogen.

**Measurement of CBS activity using a fluorescent probe**—Purified wild-type CBS (1 mg/ml) was treated with 10 mM dithiothreitol (DTT) for 1 h at 4 °C, and then DTT was removed by ultrafiltration using a 1 µl spin column. As a result, the DTT concentration was diluted approximately ~1000-fold by washing with the nitrogen-bubbled 100 mM Hapes (pH 7.4) buffer. The reduced CBS solutions were re-oxidized 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 re-oxidized 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 (CH₃SH), 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 (λᵮₑₓ = 400 nm) for 10 min at 37 °C. The activity of oxidized CBS was set as 100%. Alternatively, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) was used in the reaction mixture to detect production of methanethiol using an extinction coefficient of 13,600 M⁻¹ cm⁻¹ at 412 nm for 2-nitro-5-thiobenzoic acid anion (TNB⁻²) (29, 34).

**Measurement of CBS activity using a gas chromatographic assay**
CBS activity that leads to production of methanethiol (CH₃SH) was also determined using a gas chromatographic assay according to our previous report with some modifications (29). Recombinant CBS protein (20 μg) was preincubated in a 100 mM Hepes buffer (1 ml) containing 20 mM glutathione (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 μm). CH₃SH 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 CH₃SH was normalized to the peak area of an internal standard.

Mass spectrometry—A solution of CBS (50 μl 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 liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses. All analyses were performed using a Thermo EASY-nLC1000 Nano HPLC system with a Thermo EASY column SC200 (150 μm×100 mm, RP-C18) at a flow rate of 0.3 μl/min. The mobile phases A and B consisted of 0.1% (v/v) formic acid in H₂O 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; t= 54-60 min: 100% B. A high-performance liquid chromatography (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 CXXC motif was calculated according to the peak areas from the ion chromatogram. The sequence coverage of wild-type CBS protein obtained by MS analysis was 99.8%.

Labeling of unpaired cysteine thiols in CBS—Recombinant wild-type CBS 100 μl (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 μl phosphate buffer (100 mM) containing 1% (w/v) SDS, pH 7.0. Subsequently, the methyl-PEG-maleimide reagent, (MM[PEG]₃₄), 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 (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), 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 weight of MM(PEG)₃₄ is 1240 Da.
Human embryonic kidney 293 (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) CO₂. After the cells had reached ~80% confluence in a 10-cm plate, dithiothreitol (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 12000 rpm for 10 min were redissolved in 100 μl phosphate buffer (100 mM) containing 1% (w/v) SDS, pH 7.0. Subsequently, MM(PEG)₁₂₄ 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 de-oxygenated by bubbling with nitrogen for 30 min. The DTT_oxi/DTT_red redox potential was calculated according to the Nernst equation (Equation 1).

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

Where \(E_0 = -352 \text{ mV at pH } 7.4\), \(R\) 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, recombinant wild-type CBS (100 μg) was preincubated in solutions (500 μl) 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 μl of 100 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS. In order 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 (TNB⁻) (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 H₂S 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) CO₂ during the course of the experiment. All imaging experiments were carried out in 35 mm glass-bottom dishes (NEST, Wuxi, China). HEK293 cells were incubated with 2 μM SF7-AM for 30 min at 37 °C with 5% (v/v) CO₂. The medium was exchanged, and images were acquired for cells in three different fields. For DTN stimulation, 10 μl 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) CO₂, the same dishes of cells were imaged. For the inhibitor experiments, 10 μl 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 μM 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 incubation at 37 °C, and the cells were imaged. Image analysis was performed using Image J (National Institute of Health). The images were quantified using the mean pixel intensity after setting a common threshold for all images.
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The abbreviations used are: CBS, cystathionine β-synthase; HEK293 cells, human embryonic kidney 293 cells; PLP, pyridoxal 5′-phosphate; DTT, dithiothreitol; AOAA, aminooxyacetic acid; DL-propargylglycine (PAG); SAM, S-adenosylmethionine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MM(PEG)24, methyl-PEG24-maleimide; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GSH, glutathione; GSGG, glutathione disulfide; GSSG-EE, oxidized glutathione ethyl ester; TCA, trichloroacetic acid; CSE, cystathionine γ-lyase; NADPH, nicotinamide adenine dinucleotide phosphate; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid CPM, 7-Diethylamino-3-((4-maleimidophenyl)-4-methylcoumarin;
Figure 1. The redox state of CBS affects its activity in vitro. A, Recombinant wild-type 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 errors are the means ± SD of three independent experiments. MCys: S-methylcysteine; CBSoxi (no treatment); CBSred (DTT treatment); CBSredoxi (CBSred was re-oxidized by exposure to air). B, Methanethiol (CH$_3$SH) production catalyzed by CBS was determined and the initial reaction rates were calculated according to the data from Figure 1A. The graph represents the relative activity of samples compared with the oxidized CBS and shows the means ± SD (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 μmol methanethiol h$^{-1}$ mg$^{-1}$. C, Recombinant CBS protein (20 μg) was treated with 20 mM glutathione (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$_3$SH production was measured by gas chromatography. The graph represents the relative activity of sample compared with untreated CBS (oxidized CBS) and the means ± SD (n=3), **p<0.01.
Figure 2. The CXX 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 CXX 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 CXX 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)²⁺. 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 C\textsuperscript{272}XXC\textsuperscript{275} motif regulates CBS activity. A, The specific activities of wild-type CBS and of the CBS variants C\textsuperscript{272}A, C\textsuperscript{275}A and C\textsuperscript{272}A/C\textsuperscript{275}A (double mutant) were measured using the methylene blue assay. The data points and error bars indicate the means ± SD (n=4). ***p<0.001 versus wild-type CBS. B, The wild-type 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 errors show the means ± SD of three independent experiments. ***p<0.001 versus control. N.S., not significant.
Figure 4. Redox potential of the Cys²⁷²-Cys²⁷⁵ 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 errors show the means ± SD (n=3).
Figure 5. CBS exists in oxidized and reduced states in HEK293 cells under oxidative and reductive conditions. Human embryonic kidney 293 (HEK293) cells were exposed to 0.5 mM DTT or 0.5 mM oxidized glutathione ethyl ester (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 methyl-PEG_{24}-maleimide, MM(PEG)_{24}. The samples were resolved on SDS-PAGE and immunoblotted for CBS. The molecular size markers in kDa are indicated at the left.
Figure 6. Increasing activity of CBS in its reduced state increases the H₂S signaling in live HEK293 cells.

A, HEK293 cells were exposed to 0.5 mM DTT or 0.5 mM oxidized glutathione ethyl ester (GSSG-EE) for 10 min. The cell lysates (50 μg) were resolved on SDS-PAGE and immunoblotted for CBS. B, H₂S production was measured in HEK293 cells using the methylene blue method. The inhibitors 1 mM aminoxyacetic acid (AOAA) and 2 mM PAG (DL-propargylglycine) were used to inhibit CBS and CSE, respectively. The data are presented as the means ± SD (n=3). *p<0.05, **p<0.001 versus vehicle. C, HEK293 cells were incubated with 2 μM 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 μm). 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 μM 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 μm). I, Quantification of the confocal fluorescence images of H₂S 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 ± SD (n=3). *p<0.05, N.S., not significant.
Allosteric control of human cystathionine beta synthase activity by a redox active disulfide bond
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