A Novel Enhancing Mechanism for Hydrogen Sulfide-producing Activity of Cystathionine β-Synthase*

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H₂S is produced from cysteine by cystathionine β-synthase (CBS) in the brain and functions as a neuromodulator. Although the production of H₂S is regulated by Ca²⁺ and calmodulin in response to neuronal excitation, little is known about the molecular mechanism for the regulation in CBS activity. Here we show that four cysteine residues of CBS are involved in the regulation of its activity in the presence of Ca²⁺ and calmodulin. Sodium nitroprusside (SNP), a modifying agent for cysteine residues, enhances CBS activity, whereas N-ethylmaleimide, an alkylating agent for cysteine residues, completely abolished the effect of SNP. Site-directed mutagenesis of the 13 cysteine residues of CBS identified four cysteine residues that are involved in the regulation of CBS activity by SNP, and two of the four residues are involved in the regulation of the basal CBS activity. The enhancement of CBS activity by SNP is independent of nitric oxide production. In the presence of Staphylococcus aureus α-hemolysin, which permeabilizes the cell membrane, exogenously applied SNP enhances the activity of CBS in intact cells. The present study demonstrates a novel mechanism for the regulation of CBS activity and provides a possible therapeutic application of SNP for the diseases in which CBS activity is deficient.

Relatively high endogenous levels of H₂S, which is well known toxic gas, have been found in the brains of rats, humans and bovine (1–3), suggesting that H₂S may have a physiological function. Endogenous H₂S in the brain is produced from L-cysteine by the pyridoxal 5'-phosphate-dependent enzyme, cystathionine β-synthase (CBS) (4–7). CBS is expressed in the brain, and a CBS activator, S-adenosylmethionine (AdoMet), enhances H₂S production (6). These observations, together with our recent finding that endogenous H₂S is under detectable levels in the brains of CBS knock-out mice, indicate that CBS is a major H₂S-producing enzyme in the brain (7).

The production of H₂S is regulated by a Ca²⁺- and calmodulin-mediated pathway that is activated in response to neuronal excitation (7). Physiological concentrations of H₂S specifically potentiate the activity of the N-methyl-D-aspartate (NMDA) receptor, and hippocampal long term potentiation is altered in CBS knock-out mice (6, 7). H₂S can regulate the release of corticotropin-releasing hormone from the hypothalamus (8). In addition to the function in the brain H₂S relaxes smooth muscle in synergy with nitric oxide (NO) by activating ATP-dependent potassium channels (9, 10). Based upon these observations it has been proposed that H₂S may function as a neuromodulator, as well as a smooth muscle relaxant (6, 9).

Sodium nitroprusside (SNP), which substitute serine for cysteine, were introduced by PCR-based site-directed mutagenesis (QuickChange™ site-directed mutagenesis kit; Stratagene) using rat CBS cDNA inserted in pME18s expression vector (DNAX Research Institute, Inc.) as a template. The sequence of each mutant was confirmed by sequencing. The carboxy-terminal truncation mutant of CBS (1–396) was described previously (7).

EXPERIMENTAL PROCEDURES

Materials—SNP, SNAP, 3-morpholinosydnonimine (SIN-1), potassium ferricyanide, sodium ferrocyanide, and NEM were purchased from Sigma. All the other chemicals were from Wako (Japan). Rats (ICR; Sprague-Dawley) were purchased from Clea (Japan).

Site-directed Mutagenesis of CBS—Thirteen single point mutations, which substitute serine for cysteine, were introduced by PCR-based site-directed mutagenesis (QuickChange™ site-directed mutagenesis kit; Stratagene) using rat CBS cDNA inserted in pME18s expression vector (DNAX Research Institute, Inc.) as a template. The sequence of each mutant was confirmed by sequencing. The carboxy-terminal truncation mutant of CBS (1–396) was described previously (7).

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FIG. 1. SNP enhances the H2S-producing activity of CBS. A, a comparison of the chemical structures of SNP (a), ferricyanide (b), ferrocyanide (c), SIN-1 (d), and SNAP (e). B, SNP enhances H2S production. Thirty-three μg of brain extracts were incubated with or without (−) 300 μM SIN-1, SNAP, ferricyanide, or ferrocyanide in the presence (+) or absence (−) of 0.6 mM Ca2+ and 9.6 μM calmodulin at 37 °C for 30 min. C, SNP enhances the CBS activity in a dose-dependent manner. Thirty-three μg of brain extracts were incubated with varying concentrations of SNP (○), SIN-1 (□), or SNAP (■) in the presence of Ca2+ and calmodulin. H2S produced from l-cysteine was measured by gas chromatography. All data represent the mean ± S.E. of three experiments.

Transient Expression of CBS Mutants—COS-7 cells were transfected into 100-mm dishes 12 h before transfection. Immediately before the transfection, Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum was changed to serum-reduced medium (OPTI-MEM; Invitrogen), and cells were transfected with 10 μg of plasmid DNAs using 60 μg of LipofectAMINE reagent (Invitrogen) in 6.4 ml of medium for 5 h. After 48 h the cells were washed with phosphate-buffered saline and lysed at 4 °C for 30 min with 1 ml of lysis buffer (150 mM NaCl, 20 mM Tris/HCl (pH 7.5), 1 mM EDTA, 1 mM EGTA, 1% buffered saline and lysed at 4 °C for 30 min. The reaction was terminated by the addition of a mixture of water-saturated phenol and 9.6 μM calmodulin. The assay for the H2S-producing activity of CBS, even though they release NO, does not have any effect on the H2S-producing activity of CBS, even though they release NO (data not shown) (Fig. 1B). In contrast, the enhancing effect of SNP on CBS activity in the presence of Ca2+ and calmodulin was examined in the standard non-reducing conditions (7). In the presence of Ca2+ and calmodulin, 300 μM SNP increases the production of H2S 3-fold (410 ± 42 pmol H2S/mg protein/min, n = 3) (Fig. 1B). In contrast, the enhancing effect of SNP is not observed in the absence of Ca2+ and calmodulin. The NO donors, SIN-1 and SNAP, do not have any effect on the H2S-producing activity of CBS, even though they release NO (data not shown) (Fig. 1B). Compounds related structurally to SNP, ferricyanide, and ferrocyanide, also have no effect on CBS activity (Fig. 1, A and B). These observations suggest that only SNP enhances H2S-producing activity of CBS and that this effect of SNP is independent of NO.
The dose-response curve of SNP was examined by measuring the production of H$_2$S. The enhancing effect of SNP on H$_2$S production is increased in a dose-dependent manner and reaches the maximum level at 300 μM (Fig. 1C). The enhancing effect is decreased at concentrations greater than 300 μM. Neither SIN-1 nor SNAP has any effect on CBS activity in the range of concentrations tested (Fig. 1C).

The enzymatic activity of CBS has two metabolic outcomes (2, 18). In addition to the production of H$_2$S from cysteine, CBS catalyzes the reaction with substrate homocysteine to produce cystathionine. To examine whether SNP also enhances the cystathionine-producing activity of CBS, the effect of SNP on the production of cystathionine was measured in the presence or absence of Ca$^{2+}$ and calmodulin. 300 μM SNP enhances the production of cystathionine 4-fold more greatly in the presence of Ca$^{2+}$ and calmodulin (1400 ± 70 pmol cystathionine/mg protein/min, n = 3) than in the absence of Ca$^{2+}$ and calmodulin (260 ± 10 pmol cystathionine/mg protein/min, n = 3) (Fig. 2A).

In the presence of Ca$^{2+}$ and calmodulin 300 μM SNP enhances the production of cystathionine 2.4-fold more greatly than that without SNP (590 ± 98 pmol cystathionine/mg protein/min, n = 3). SIN-1, SNAP, ferricyanide, or ferrocyanide did not enhance the production of cystathionine (Fig. 2A). The dose-response curve of SNP was also examined by measuring the production of cystathionine. The enhancing effect of SNP on cystathionine production is increased in a dose-dependent manner up to 800 μM (Fig. 2B). Neither SIN-1 nor SNAP has any effect on CBS activity in the range of concentrations tested (Fig. 2B). These observations indicate that SNP enhances both the H$_2$S- and cystathionine-producing activities of CBS, although the concentrations for the maximal responses are different between both pathways.

**SNP Interacts with Cysteine Residues of CBS to Enhance Its Activity**—Because the enhancement of CBS activity by SNP requires Ca$^{2+}$ and calmodulin, there are two possible targets for SNP. One possibility is that SNP interacts with calmodulin and modifies the CBS activity. Alternatively, SNP interacts with CBS and enhances its activity. To address this problem, the effect of SNP on a mutant form of CBS (1–396), which lacks the calmodulin binding domain but is constitutively active without calmodulin, was examined (7). The activity of the mutant CBS (1–396) in the absence of Ca$^{2+}$ and calmodulin was enhanced further by the application of 300 μM SNP (8565 + 1245 pmol H$_2$S/mg protein/min, n = 3) to a level similar to that of the wild-type CBS in the presence of Ca$^{2+}$ and calmodulin (8227 ± 50 pmol H$_2$S/mg protein/min, n = 3) (Fig. 3). These observations indicate that SNP does not interact with calmodulin to enhance CBS activity.

Because SNP reacts with the thiol groups of sulfur-containing amino acids, especially cysteine (14), it is possible that the enhancement of CBS activity by SNP is induced by modifying the cysteine residues of CBS. To examine this possibility, the effect of NEM, which alkylates thiol of cysteine residues, on the enhancement of CBS activity by SNP was investigated by measuring the production of H$_2$S in the presence of Ca$^{2+}$ and calmodulin. The enhancement of CBS activity by SNP (368.4 ± 13.7 pmol H$_2$S/mg protein/min, n = 3) was suppressed by NEM in a dose-dependent manner, and 1 mM NEM completely suppressed the effect of SNP (119.7 ± 2.5 pmol H$_2$S/mg protein/min, n = 3) (Fig. 4, A and B). The basal CBS activity, however, was not changed by NEM (Fig. 4A). These observations suggest that NEM masks cysteine residues to hinder SNP from interacting with cysteine residues to suppress the enhancing effect of SNP on CBS activity.

**Identification of Cysteine Residues Modified by SNP**—To identify cysteine residues of CBS that interact with SNP, the effect of SNP on 13 CBS mutants was examined. Each of the 13 cysteine residues of CBS was replaced with serine residue by site-directed mutagenesis. The mutants were expressed in COS-7 cells, and the expression levels of each mutant were examined by Western blot analysis (Fig. 4C). There were no significant differences in the expression levels of each mutant, and endogenous CBS in COS-7 cells was under the detectable level (Fig. 4C). The enhancement of CBS activity by SNP was then examined by measuring the amounts of H$_2$S produced by each CBS mutant. CBS activity enhanced by SNP was suppressed in C49S, C162S, C367S, and C476S mutants by 37, 33, 39% of the wild-type CBS, respectively (Fig. 4C). All the mutants, however, show similar basal H$_2$S-producing activity to the wild-type CBS in the absence of SNP (Fig. 4C). These observations suggest that at least four cysteine residues in CBS are involved in the modification by SNP to enhance CBS activity.

**SNP Enhances CBS Activity in Intact Cells in the Presence of α-Hemolysin—Loss of CBS activity causes homocystinuria (18). Pyridoxine (vitamin B6) has been used for therapeutic trials of homocystinuria and has improved some cases, but the remaining patients are not pyridoxine-responsive (21, 22). Because
SNP enhances CBS activity, SNP can possibly be used for therapeutic purposes. As an NO donor SNP can be applied outside of the cells, but as a cysteine residue-modifying agent SNP must enter into the cells to interact with CBS. To examine the effect of exogenously applied SNP on CBS activity in intact cells, H2S production was measured by gas chromatography. All data represent the mean ± S.E. of three experiments.

**DISCUSSION**

The present study demonstrates that SNP enhances the H2S-producing activity of CBS by modifying cysteine residues of CBS via an NO-independent mechanism. This effect of SNP requires Ca2+ and calmodulin, but SNP does not interact directly with calmodulin (Fig. 3). We proposed recently a possible mechanism for the potentiation of the H2S-producing activity of CBS by Ca2+ and calmodulin that is similar to that proposed for the potentiation of CBS by AdoMet (7). In this model the carboxy-terminal domain covers the catalytic domain of CBS in the absence of Ca2+ and calmodulin in the basal state. When Ca2+ and calmodulin binds to the calmodulin binding consensus sequence, the catalytic domain is exposed by opening of the carboxy-terminal domain, and CBS becomes active (7). Taking this model into account, it is possible that SNP may not be able to access cysteine residues in the wild-type CBS in the absence of Ca2+ and calmodulin. This model is also supported by our observation that the CBS mutant (1–396), which lacks the carboxy-terminal domain to hinder the access of SNP, is activated further by SNP (Fig. 3).

Under reducing conditions NO interacts with the heme group, and the CBS activity is reduced. However, the inhibition...
of CBS activity by NO is not observed in standard non-reducing conditions that were used in the present study (see also Refs. 23 and 25). In addition to our observations, there are several examples in which SNP exerts its effects independently of NO. The release of CGRP and substance P from dorsal horn is evoked by SNP (17). The release is also induced by photoinactivated SNP, as well as ferricyanide, which is structurally similar to SNP except that it lacks the coordinated NO, suggesting that its three-dimensional structure is necessary to evoke the release of CGRP and substance P. The binding of MK-801, an agonist of NMDA receptors, to NMDA receptors is inhibited by SNP and ferrocyanide but not by other NO donors such as SNAP and S-nitroso-L-glutathione (16). In contrast to the above data, the enhancing effect of SNP on CBS activity is not induced by ferricyanide or ferrocyanide (Fig. 1). These observations suggest that the coordinated NO of SNP may bind to the cysteine residues of CBS, and the remaining part of SNP may change the three-dimensional structure of CBS and modify CBS activity. Although both NEM and SNP modify cysteine residues, the specificity and the effect of modification is apparently different. NEM alkylates all cysteine residues, whereas SNP specifically modifies in a functional manner only four cysteine residues of CBS, Cys-49, Cys-162, Cys-367, and Cys-476 (Fig. 4). SNP enhances CBS activity, whereas NEM does not have any effect on basal CBS activity.

Of the four cysteine residues modified by SNP, Cys-162 and Cys-367 in rat CBS correspond to Cys-165 and Cys-370 in human CBS, respectively, and both cysteine residues are changed to tyrosine by the mutations found in homocystinuria patients (20). Mutants Cys-49 and Cys-476 have a reduced basal cystathionine-producing activity similar to the wild-type CBS, whereas mutants Cys-162 and Cys-367 have a much lower basal CBS activity (Fig. 5). The human C52S mutant shows a reduced basal cystathionine-producing activity (27), whereas the corresponding rat CBS has an H2S-producing activity similar to the wild-type CBS for H2S production (Fig. 4C). This discrepancy may be because the present study was performed in the presence of Ca2+ and calmodulin, and H2S production was assayed rather than cystathionine production. In addition, to measure the amount of H2S, the enzyme assay was performed in anaerobic conditions in which the CBS activity is enhanced relative to the aerobic conditions (23).

The CBS activity is reduced severely in homocystinuria (21). Although the two mutants at Cys-162 and Cys-367 do not respond to SNP, approximately 50 other mutants have the intact cysteine residues and can respond to SNP (20). This observation suggests that there may be an endogenous substance that interacts with Cys-162 or Cys-367 to regulate CBS activity and that both CBS mutants found in homocystinuria patients can not function properly because of a lack of the interaction with the possible endogenous regulator. Enhancement of CBS activity in vitro requires 300 μM SNP, whereas in intact cells the maximal effect is obtained at 1 μM (see Fig. 1C and Fig. 5A). The difference of the potency of SNP between in vitro and in intact cells may be because the in vitro experiments was performed in the presence of 1 mM free cysteine, which compete with cysteine residues of CBS, whereas there is much less endogenous cysteine in intact cells. Alternatively, there may be a synergy between SNP and the endogenous regulator to enhance the CBS activity in intact cells. The requirement of the much lower dosage in vivo, however, may be an advantage for therapeutic applications.

Another possible example of the disease in which the CBS activity may be deficient is Alzheimer’s disease (AD). Plasma levels of homocysteine, which is a substrate for CBS to produce cystathionine, is high in AD patients (28), and the level of AdoMet, a CBS activator, is low in AD brains (29, 30). AdoMet improves cognitive decline in patients with AD (31). Despite these observations little attention has been paid to CBS activity in AD. We have shown recently that brain H2S levels and CBS activities are decreased severely in AD patients (30). The present observations may provide a new approach for the therapy for these diseases in which CBS activity is deficient.

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A Novel Enhancing Mechanism for Hydrogen Sulfide-producing Activity of Cystathionine β-Synthase
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Additions and Corrections

Vol. 277 (2002) 42680–42685

A novel enhancing mechanism for hydrogen sulfide-producing activity of cystathionine \(\beta\)-synthase.

Ko Eto and Hideo Kimura

Because some of the data in this manuscript are not correct, we would like to retract this paper. Our recent measurements show that the dependency of cystathionine \(\beta\)-synthase on calmodulin is not critical. The errors were probably caused by the contamination by sulfur-containing substances in calmodulin and by the instability of the preparations used for gas chromatography.

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Analysis of the effect of bulk at \(N^2\)-alkylguanine DNA adducts on catalytic efficiency and fidelity of the processive DNA polymerases bacteriophage T7 exonuclease and HIV-1 reverse transcriptase.

Jeong-Yun Choi and F. Peter Guengerich

Page 19223, Tables III and IV: Units in the headings within these tables should be changed from \(\mu\)M to nM.

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Transcription coactivator PBP, the peroxisome proliferator-activated receptor (PPAR)-binding protein, is required for PPAR\(\alpha\)-regulated gene expression in liver.

Yuzhi Jia, Chao Qi, Papreddy Kashireddy, Sailesh Surapureddi, Yi-Jun Zhu, M. Sambasiva Rao, Derek Le Roith, Pierre Chambon, Frank J. Gonzalez, and Janardan K. Reddy

Dr. Kashireddy’s name was misspelled. The correct spelling is shown above.

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