Production of the Neuromodulator H₂S by Cystathionine β-Synthase via the Condensation of Cysteine and Homocysteine*

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Hydrogen sulfide (H₂S) has been observed in relatively high concentrations in the mammalian brain and has been shown to act as a neuromodulator. However, there is confusion in the literature regarding the actual source of H₂S production. Reactions catalyzed by the cystathionine β-synthase enzyme (CBS) are one possible source for the production of H₂S. Here we show that the CBS enzyme can efficiently produce H₂S via a β-replacement reaction in which cysteine is condensed with homocysteine to form cystathionine and H₂S. The production of H₂S by this reaction is at least 50 times more efficient than that produced by hydrolysis of cysteine alone via β-elimination. Kinetic studies demonstrate that the $K_m$ and $K_{cat}$ for cysteine is 3-fold higher and 2-fold lower, respectively, than that for serine. Consistent with these data, in vitro reconstitution studies show that at physiologically relevant concentrations of serine, homocysteine, and cysteine, about 5% of the cystathionine formed is from cysteine. We also show that AdoMet stimulates this H₂S producing reaction but that there is no evidence for stimulation by calcium and calmodulin as reported previously. In summary, these results confirm the ability of CBS to produce H₂S, but show in contrast to prior reports that the major mechanism is via β-replacement and not cysteine hydrolysis. In addition, these studies provide a biochemical explanation for the previously inexplicable homocysteine-lowering effects of N-acetylcysteine treatments in humans.

Recently, there has been increased interest in endogenously produced hydrogen sulfide (H₂S) as a physiologically important molecule. Relatively high concentrations of H₂S have been observed in the brains of rats, humans, and cows (1–3). At physiological concentrations it has been shown that H₂S enhances N-methyl-D-aspartate receptor-mediated response and can modify long term potentiation (4–6). H₂S also inhibits smooth muscle cell proliferation via the mitogen-activated protein kinase pathway and protects neurons against oxidative stress (7). H₂S (see Fig. 2, lane 1). Into this strain was transformed a plasmid expressing either wild-type human CBS or a truncated human CBS (amino acids 1–409) (16). Total yeast extracts were made as described previously (17).

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How is endogenous H₂S produced? Potential sources are alternative reactions catalyzed by the enzyme cystathionine β-synthase (CBS) (6). The normal cellular function of CBS is to catalyze the condensation of serine with homocysteine to form cystathionine and water, a key reaction in the transsulfuration pathway. CBS uses pyridoxal phosphate (PLP) as a co-factor and is a member of the β-family or fold type II of PLP containing enzymes. Enzymes in this family characteristically have the ability to catalyze β-replacement and β-elimination reactions from a variety of different substrates (9).

There are two potential mechanisms through which CBS could produce H₂S. First, CBS could catalyze the production of H₂S from cysteine by a β-elimination or an α,β-elimination reaction (Fig. 1, Alternate Reactions 3 and 4, respectively). This type of reaction has been reported to occur with CBS isolated from mouse brain and from CBS present in rat liver and kidney extracts (6, 10). An alternative source for H₂S production would involve a β-replacement reaction. Using this mechanism CBS can produce H₂S from the reaction of l-cysteine and 2-mercaptoethanol to form S-hydroxyethyl-l-cysteine and H₂S (Fig. 1, Alternate Reaction 2) (11). While this reaction would not be expected to occur in vivo, a similar β-replacement reaction could occur by the condensation of homocysteine with cysteine (Fig. 1, Alternate Reaction 1). This potential reaction is interesting because it would also be an alternative method for metabolizing homocysteine. Elevated plasma homocysteine levels have been linked to a variety of human diseases, including heart attack, stroke, Alzheimer disease, and osteoporosis (12–15). Since CBS is a key regulator of homocysteine, it is possible that this alternative reaction may have clinical relevance.

In this paper we report the characterization of the biochemical and kinetic properties of human CBS in catalyzing various H₂S-producing reactions. We find that human CBS can efficiently catalyze the formation of H₂S via the condensation of homocysteine with cysteine and that this reaction is likely to occur in vivo.

MATERIALS AND METHODS

CBS Expression Systems—Two expression systems were used to produce human CBS. For the yeast system we used a yeast strain (WY218) that was deleted for endogenous yeast CBS (CYS4) and deleted for yeast O-acetylseryl/0-acetylhomo serine sulfhydrylase. Extracts from WY218 exhibit no CBS activity and have no ability to form H₂S (see Fig. 2, lane 1). Into this strain was transformed a plasmid expressing either wild-type human CBS or a truncated human CBS (amino acids 1–409) (16). Total yeast extracts were made as described previously (17).

1 The abbreviations used are: CBS, cystathionine β-synthase enzyme; PLP, pyridoxal phosphate; PBS, phosphate-buffered saline; GST, glutathione S-transferase; Bicine, N,N-bis(2-hydroxyethyl)glycine; AdoMet, S-adenosylmethionine; aa, amino acids; CGL, cystathionine γ-lyase.
H$_2$S Production by Human CBS in Saccharomyces cerevisiae—To identify whether human CBS had H$_2$S-forming activity, we measured H$_2$S formation using a gel activity assay. This method, yeast extracts are separated on native gels, exposed to various substrates, and then assessed for H$_2$S formation in situ (see “Materials and Methods”). We examined H$_2$S formation from either 10 mM l-cysteine alone, 10 mM l-cysteine with 10 mM l-homocysteine, or 10 mM l-cysteine and 10 mM 2-mercaptoethanol. The yeast strain we used (WY218) was deleted for endogenous yeast CBS and contained either a control plasmid, a plasmid expressing wild-type human CBS (aa 1–551), or one expressing a truncated human CBS (aa 1–409) lacking the C-terminal regulatory domain. The truncated form of CBS has been shown to be hyperactive and not responsive to allosteric regulation by AdoMet (16). As shown in Fig. 2, both the full-length and truncated form of the enzyme have significant H$_2$S forming ability when cysteine is combined with either homocysteine or β-mercaptoethanol. However, neither full-length nor truncated CBS has significant H$_2$S forming ability when only cysteine is present. These results show that human CBS is much more active at producing H$_2$S by a β-replacement reaction then by a β-elimination reaction.

AdoMet is an allosteric effector of CBS that stimulates CBS activity by relieving the inhibition of the C-terminal domain (17). As expected, we found that addition of AdoMet stimulated H$_2$S formation from the wild-type enzyme (Fig. 2) but not the truncated enzyme. This result suggests that the regulation of the H$_2$S forming β-replacement reactions is similar to that of the canonical reaction.

We also examined the H$_2$S forming ability of recombinant human CBS purified from E. coli. In this experiment, reactions were carried out in solution, and lead sulfate accumulation was determined using a spectrophotometer. As shown in Fig. 2B, the purified enzyme produces significant levels of H$_2$S when either 10 mM L-homocysteine or 10 mM β-mercaptoethanol were combined with 10 mM l-cysteine. However, when 10 mM l-cysteine was incubated in the absence of a co-substrate we saw no detectable levels of H$_2$S formation. These results confirm that CBS produces H$_2$S through condensation of cysteine with homocysteine (or some other substrate) via β-replacement.
either cysteine or serine in the formation of cystathionine in an

cysteine usage were measured using an amino acid analyzer
1 h at 37 °C. Subsequently, cystathionine formation and serine/

were added to 200 ng of purified recombinant CBS and incubated for

truncated human CBS (aa 1–409). The

extract (WY218 extract); and

shows an identically loaded native PAGE gel developed under different

were developed in reactions containing 0.4 mM

2-ME)

were developed using 2-mercaptoethanol (alternate reaction 1), the middle gels were developed using cysteine

(S-adenosylmethionine, while the bottom three panels

were developed in reactions containing 0.4 mM S-adenosylmethionine. The gels on the left were developed using cysteine + homocysteine (alternate reaction 1), the middle gels were developed using 2-mercaptoethanol (2-ME) + homocysteine (alternate reaction 2), and the gels on the right were developed only in the presence of cysteine (alternate reactions 3 and 4). The molecular mass markers at the top left are indicated: ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa). Markers are shown after staining with Coomassie Blue. B, 200 ng of purified recombinant CBS was assessed for H2S production as described under “Materials and Methods.” All reactions were done in triplicate, and standard deviation is as shown. The units for enzyme activity are nmol of H2S produced per mg of protein per minute. There was no detectable H2S produced in the reaction containing cysteine alone.

rather than through cysteine hydrolysis and an elimination reaction.

Kinetic Studies of Purified Human CBS—We next compared the relative efficiency of utilization of each of these substrates. We determined the $K_m$ and $V_{max}$ for both L-serine and L-cysteine under conditions in which L-homocysteine was present at 10 mM in the reaction mixture. We found that the $K_m$ of cysteine compared with serine was 3.5-fold higher (Table I) and the $V_{max}$ 2.3-fold reduced. The ratio of $K_{cat}/K_m$ is about 8-fold decreased, suggesting that under physiological conditions serine would be utilized in preference to cysteine.

Competition Studies—We next examined the utilization of either cysteine or serine in the formation of cystathionine in an

in vitro competition assay. Either serine, cysteine, or both were added to 200 ng of purified recombinant CBS and incubated for

1 h at 37 °C. Subsequently, cystathionine formation and serine/
cysteine usage were measured using an amino acid analyzer
(Fig. 3). At high concentrations (10 mM) of serine, cysteine, and
homocysteine, cysteine was used to make 44% of the cystathionine, while serine was used to make 56%. As we decreased the concentration of substrates to 1 mM, the amount of cystathionine formed from cysteine decreased to 35%. At the lowest concentration tested (0.1 mM) only 23% of the cystathionine came from cysteine. These data are consistent with the kinetic data (a 3-fold difference in $K_m$) and show that the ratio of cysteine to serine used in cystathionine formation depends greatly on the concentration of the substrates present in a tissue.

In examination of the literature we found widely varying references for the concentrations of cysteine, serine, and homocysteine in mammalian livers (20, 21). Therefore, we determined the concentrations of these amino acids in mouse liver ourselves. Mouse livers were weighed, extracted, and then analyzed for free amino acid content using an amino acid analyzer. This allowed us to determine the free amino acid content per milligram of liver. To estimate how much water was in the tissue, we determined the weight of mouse livers before and after desiccation. The difference in weight indicates the amount of water in the sample, and this number was then used as the denominator in our calculations. Using this procedure, we estimated that in mouse liver the concentrations of serine, cysteine, and homocysteine are 0.72, 0.47, and 0.58 mM, respectively. In an in vitro reaction using these concentrations of substrates, we determined that about 5% of the cystathionine formed in a mouse liver was derived from cysteine (Fig. 3, right-hand panel).

No Effects of Calcium and Calmodulin on H2S-producing
Enzyme Activity—It has been previously reported that CBS contains a calmodulin (CaM) binding motif and that the hydrolysis of cysteine is regulated by calcium and CaM (6). We failed to see any effect of CaM and or calcium addition on CBS activity from mouse liver extract, purified recombinant human CBS produced in E. col, or from yeast extracts expressing human CBS (data not shown). Since the experiments reported by Kimura were done on mouse brain, we also examined mouse brain extracts for stimulation by Ca2+ and CaM. Again, we failed to see any stimulation of CBS activity in the presence of Ca2+/CaM (data not shown). These studies show that CBS is not regulated by Ca2+/calmodulin.

DISCUSSION

The goal of this work was to clarify the role that CBS may have in the endogenous production of H2S in vivo. Work in the field of molecular neurology has clearly established that H2S can modulate neuronal signals by modulating signaling of the N-methyl-D-asparate receptor (4–6). However, there has existed confusion as to the source of H2S in vivo. Most of the literature on endogenous H2S suggests that H2S is formed primarily from the hydrolysis of cysteine by the action of cystathionine β-synthase. The data presented here do support the idea that H2S is produced by CBS. However, the mechanism for its production is not by the hydrolysis of cysteine but rather by a β-replacement reaction utilizing homocysteine and cysteine. This reaction is essentially identical to the endogenous β-replacement reaction involving homocysteine and serine, except that cysteine is substituted for serine, resulting in the formation of H2S instead of H2O. This reaction, like the canonical reaction, is stimulated by addition of AdoMet. Unlike previous investigators, we did not observed any evidence for the stimulation of CBS by Ca2+/calmodulin (6).

Although our data are in conflict with some recent work, it is actually quite consistent with work from the 1960s examining H2S formation from partially purified serine sulphydrylase derived from chicken liver. Braunstein and colleagues (22)
thervemore, we failed to observe any detectable H2S formation in the extracts of mouse livers of animals deleted for endogenous CBS. The evidence that CGL can produce H2S comes from work in which propargylglycine was shown to inhibit H2S production in crude liver extracts (10). One possible explanation for this apparent contradiction is that in the context of a crude rat liver extract the propargylglycine may be affecting other enzymes, resulting in decreased production or increased metabolism of H2S.

Our data suggest that the production of H2S from cysteine and homocysteine does occur in vivo. We found that wild-type human CBS has a $K_m$ value for l-cysteine of 6 mM, about 3-fold higher than the $K_m$ for serine. We also found that in three substrate reactions (cysteine, homocysteine, and serine) a small but significant portion of the cystathionine produced came from cysteine. When the substrates were added at physiologic concentrations (as determined in mouse liver) we found that about 5% of the cystathionine produced came from cysteine. This is consistent with the observation that H2S levels are at least an order of magnitude less than cysteine, homocysteine, or S-adenosylmethionine levels in the brain (25).

Our findings may also have clinical relevance. H2S levels have been shown to be severely decreased in the brains of Alzheimer disease patients and in the aorta of hypertensive rats (25). If these low levels of H2S are found to be pathogenic in human disease, it may be possible to increase H2S and lower homocysteine levels by increasing the concentration of cysteine in tissues. In fact, it has been shown that pharmacologic doses of N-acetylcysteine can lower plasma homocysteine levels in humans (26–28). The likely reason for this effect is that N-acetylcysteine is converted to cysteine inside cells, thereby increasing the concentration of cysteine and thus driving the conversion of homocysteine to cystathionine. This treatment would also be expected to increase the production of H2S.

In summary, the findings presented here help clarify the potential role of CBS in the production of H2S and the reduction of homocysteine and support the view that CBS may play a key role in neurobiology.

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