H₂S Biogenesis by Human Cystathionine γ-Lyase Leads to the Novel Sulfur Metabolites Lanthionine and Homolanthionine and Is Responsive to the Grade of Hyperhomocysteinemia

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Taurai Chiku, Dominique Padovan, Weidong Zhu, Sangita Singh, Victor Vititsky, and Ruma Banerjee

From the Department of Biological Chemistry, University of Michigan Medical Center, Ann Arbor, Michigan 48109-0606 and the Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68588-0664

Although there is a growing recognition of the significance of hydrogen sulfide (H₂S) as a biological signaling molecule involved in vascular and nervous system functions, its biogenesis and regulation are poorly understood. It is widely assumed that desulfhydration of cysteine is the major source of H₂S in mammals and is catalyzed by the transsulfuration pathway enzymes, cystathionine β-synthase and cystathionine γ-lyase (CSE). In this study, we demonstrate that the profligacy of human CSE results in a variety of reactions that generate H₂S from cysteine and homocysteine. The γ-replacement reaction, which condenses two molecules of homocysteine, yields H₂S and a novel biomarker, homolanthionine, which has been reported in urine of homocystinuric patients, whereas a β-replacement reaction, which condenses two molecules of cysteine, generates lanthionine. Kinetic simulations at physiologically relevant concentrations of cysteine and homocysteine, reveal that the α,β-elimination of cystine accounts for ~70% of H₂S generation. However, the relative importance of homocysteine-derived H₂S increases progressively with the grade of hyperhomocysteinemia, and under conditions of severely elevated homocysteine (200 μM), the α,γ-elimination and γ-replacement reactions of homocysteine together are predicted to account for ~90% of H₂S generation by CSE. Excessive H₂S production in hyperhomocysteinemia may contribute to the associated cardiovascular pathology.

A recent in vivo study has demonstrated the efficacy of H₂S in attenuating myocardial ischemia-reperfusion injury by protecting mitochondrial function (7). The role of H₂S in inflammation is suggested by several studies (8–11); however, the underlying mechanism is unknown. Remarkably, H₂S can also induce a state of suspended animation in mice by decreasing the metabolic rate and the core body temperature presumably by inhibiting cytochrome c oxidase in the respiratory chain (12).

Endogenous H₂S is presumed to be generated primarily by desulfhydration of cysteine catalyzed by the two pyridoxal phosphate (PLP)-dependent enzymes in the transsulfuration pathway: cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE) (13, 14). In fact, it is widely assumed, based on the reported absence of CSE in the brain (15) and of H₂S in the brain of CBS knock-out mice (16), that CBS is the primary source of H₂S in this organ, whereas CSE plays the equivalent role in the peripheral vasculature (3). However, recent studies have demonstrated that CSE is both present and active in the brain (17, 18) and that H₂S is in fact detected in the brains of transgenic mice lacking CBS (19). The major role of CSE in H₂S biogenesis in the peripheral system has been convincingly demonstrated in CSE knock-out mice, which exhibit significantly reduced H₂S levels in the serum and lower H₂S production rates in aorta and heart (20). The CSE knock-out mice exhibit hypertension and reduced endothelium-dependent vasorelaxation.

CSE belongs to the γ-family of PLP-dependent enzymes and catalyzes α,γ-elimination of cystathionine to give cysteine, α-ketobutyrate, and ammonia (Fig. 1, reaction 1) (21). In principle, a variety of CSE-catalyzed reactions leading to H₂S formation can be considered, including cysteine-dependent β-reactions (Fig. 1, reactions 2, 3, and 6) and homocysteine-dependent γ-reactions (reactions 4 and 5). An alternative route to H₂S synthesis from cysteine catalyzed by CSE has been proposed to involve β-elimination of cysteine, leading to the intermediate formation of thiocysteine (reaction 7), which decomposes to H₂S in a nonenzymatic reaction with other thiols (13, 22, 23). However, the significance of cysteine as a source of H₂S, in the reducing intracellular environment is uncertain.

In this study, we have elucidated the kinetics of H₂S biosynthesis from cysteine and homocysteine catalyzed by recombinant human CSE. The kinetic data have been utilized to simu-

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1 These authors contributed equally to this work.

2 To whom correspondence should be addressed: 3320B MSRB III, 1150 W. Medical Center Dr., University of Michigan, Ann Arbor, MI 48109-0606. Tel.: 734-615-5238; E-mail: rbanerje@umich.edu.

3 The abbreviations used are: PLP, pyridoxal 5′-phosphate; CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; DTNB, dithiobisnitrobenzene; HPLC, high pressure liquid chromatography; MS, mass spectrometry; HCYs, homocysteine.
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late the rate of $H_2S$ production by CSE at physiologically relevant concentrations of substrates and at three concentrations of homocysteine, to mimic normal, mild, and severe hyperhomocysteinemia, and the simulated data have been validated experimentally. The simulations predict that the relative contribution of homocysteine versus cysteine to $H_2S$ biogenesis by CSE increases with the grade of hyperhomocysteinemia. Our studies have led to the identification of two novel sulfur metabolites generated as byproducts of $H_2S$ synthesis by CSE, lanthionine and homolanthionine. The latter could serve as a biomarker for $H_2S$ production under hyperhomocysteinemic conditions.

EXPERIMENTAL PROCEDURES

Purification of Human CSE

Recombinant human CSE (polymorphic variant S403) was expressed in the Escherichia coli strain BL21(DE3) using an expression plasmid generously provided by Dr. Marcus Wahl (Max Planck Institute, Martinsried, Germany). The protein was purified as described previously (24) with the following modification. After the Superdex S-200 (Sigma) size exclusion column, the active fractions were pooled, concentrated, and dialyzed against 100 mM Hepes buffer, pH 7.4, before being stored at $-80^\circ C$. The concentration of CSE was determined using the Bradford reagent (Bio-Rad) with bovine serum albumin as a standard.

Enzyme Activity Assays

The following assays were employed to assess CSE activity. In all assays, the concentration of the variable substrate ranged from $0.2 \times K_{m1}$ to $30 \times K_{m1}$. One unit of activity is defined as the amount of enzyme needed to form 1 $\mu$mol of product min$^{-1}$.

Detection of Cysteine—The DTNB assay was used to measure cysteine produced by CSE from cystathionine, as described previously (25). Briefly, 970 $\mu$L of Hepes buffer (100 mM, pH 7.4) containing various amounts of a diastereomeric mix of cystathionine was mixed with 10 $\mu$L of 0.1 $M$ DTNB (in ethanol) and incubated at 37 °C for 3 min. PLP was omitted from the reaction mixture, since its addition consistently leads to a slight inhibition of the enzymatic activity. Enzyme (20 $\mu$L of 1 mg/ml protein) was added to initiate the reaction, and an increase in absorption at 412 nm due to formation of the nitrobenzene thiolate anion was monitored for 1 min in a Cary100 UV-visible spectrophotometer thermostatted at 37 °C. Control experiments lacking CSE or substrate yielded the background rates for the reaction of DTNB with the free thiols of CSE or the impurities contained in cystathionine (~90% purity) and were subtracted from the enzyme assay data. A molar extinction coefficient of 13,600 $M^{-1} cm^{-1}$ was used to estimate the concentration of cysteine generated.

Detection of $H_2S$—$H_2S$ generation was measured in one of two ways. For in-gel assays, $H_2S$ production was assayed by reaction with lead acetate using a modification of a previously described method (26, 27). Purified CSE (40 $\mu$g/lane) was loaded into wells of a native 4–15% gradient Tris-glycine gel (Bio-Rad). Immediately after gel electrophoresis (at 4 °C), the gel was cut between the lanes, and the strips were soaked for 6 h at room temperature in 40 ml of the reaction mixture (100 mM Hepes buffer (pH 7.4), 0.4 mM lead acetate, and substrates: reaction 1 (30 mM L-homocysteine), reaction 2 (10 mM L-cysteine, 30 mM L-homocysteine), or reaction 3 (10 mM L-cysteine)). Bands producing $H_2S$ developed a dark brown color that was analyzed using the Gel Doc 2000 gel documentation system (Bio-Rad).

Production of $H_2S$ by CSE from different substrates was measured in a spectrophotometric assay in which the reaction of $H_2S$ with lead acetate to form lead sulfide was monitored continuously by the increase in absorption at 390 nm. After the reaction mixture (980 $\mu$L) containing 100 mM Hepes buffer (pH 7.4), 0.4 mM lead acetate, and varying concentrations of substrate (homocysteine, cysteine, or both) was preincubated at 37 °C for 4 min, 20 $\mu$g of CSE was added to the assay mixture to initiate the reaction, which was monitored at 37 °C for 3 min. Lead acetate (0.4 mM) did not inhibit CSE, as determined in the DTNB assay described above. The molar extinction coefficient for lead sulfide under these con-
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H$_2$S Production at Physiologically Relevant Concentrations of Substrate—H$_2$S formation was detected using the lead acetate assay described above with the following exception. The reaction mixture (1-ml final volume) contained 100 mM Hepes buffer (pH 7.4), 0.4 mM lead acetate, 5 µM cystathionine, 100 µM cysteine, and either 10, 40, or 200 µM homocysteine. Following incubation at 37°C for 4 min, the reaction was initiated by the addition of 100 µg of CSE (corresponding to 2.2 µM active sites), and the reaction was monitored at 390 nm for 3 min. The higher concentration of protein was necessary for monitoring the slow reaction rates at these low substrate concentrations. We note that the total substrate concentration for H$_2$S generation (i.e. cysteine and homocysteine) varied from 50- to 140-fold excess over the concentration of active sites, and ~2-10 turnovers were completed during the 3-min time course of the assay.

Mass Spectrometric (MS) Analysis of Reaction Products

For the qualitative analysis of other products in H$_2$S generation reactions, a Q TRAP$^{TM}$ mass spectrometer (Applied Biosystems) equipped with a Turbo ion spray source operated in the positive ion mode was employed. Data acquisition was conducted using Analyst software (Applied Biosystems) with a built-in information-dependent acquisition scan function. The supernatant from the assay mixture obtained after protein precipitation by trichloroacetic acid was injected into the mass spectrometer. Control reaction mixtures from which CSE was omitted were run separately.

Analysis of Kinetic Data

Cystathionine, the substrate for CSE, is a condensation product of two amino acids, serine and homocysteine. The active site pocket therefore has binding determinants for two amino acids. In the H$_2$S-generating reactions catalyzed by CSE (reactions 2–6), either one (reaction 2 and 4) or both (reactions 3, 5, and 6) amino acid binding pockets are occupied. We refer to the kinetic parameters associated with the single substrate reaction (i.e. ignoring H$_2$O) as $K_{m1}$ and $V_{max1}$. The parameters $K_{m2}$ and $V_{max2}$ then refer to substrate binding at the second site and the reaction velocity of the bimolecular reaction involving two amino acids, respectively.

Cysteine Production from Cystathionine—The $K_{m}$ and $V_{max}$ values for reaction 1 were determined directly from Michaelis-Menten kinetic analysis using the DTNB assay described above and Equation 1.

$$V_{cysteine} = \frac{V_{max}[CST]}{K_{m(CST)} + [CST]}$$  (Eq. 1)

We note that commercially available cystathionine is a mixture of diastereomers of which only one, the L, L-isomer, is expected to serve as substrate for CSE. Hence, the substrate concentration was divided by a factor of 4 to obtain the value for $K_{m}$ that is reported.

Pyrurate or $\alpha$-Ketobutyrate Generation—The $K_{m}$ and $V_{max}$ values for CSE-catalyzed pyruvate (reaction 2) or $\alpha$-ketobutyrate (reaction 4) production from cysteine or homocysteine,
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respectively, were determined using the α-ketoacid assay described above and Equations 2 and 3.

\[ v_{\text{pyruvate}} = \frac{V_{\text{max}}[\text{Cys}]}{K_m(\text{Cys}) + [\text{Cys}]^{1 + \frac{[\text{Cys}]}{K_i}}} \]  
(Eq. 2)

\[ v_{\alpha-\text{keto} \text{butyrate}} = \frac{V_{\text{max}}[\text{HCys}]}{K_m(\text{HCys}) + [\text{HCys}]^{1 + \frac{[\text{HCys}]}{K_i}}} \]  
(Eq. 3)

To account for the observed substrate inhibition, an inhibition constant (i.e. the \(K_i\) term) was included in Equations 2 and 3.

\[ v_{\text{Cys}5} = \frac{V_{\text{max}}[\text{Cys}]}{K_m(\text{Cys}) + [\text{Cys}]^{1 + \frac{[\text{Cys}]}{K_i}}} \]  
(Eq. 4)

\[ v_{\text{HCys5}} = \frac{V_{\text{max}}[\text{HCys}]}{K_m(\text{HCys}) + [\text{HCys}]^{1 + \frac{[\text{HCys}]}{K_i}}} \]  
(Eq. 5)

\[ v_{\text{HCys5}} = \frac{V_{\text{max}}[\text{Cys}][\text{HCys}]}{K_m(\text{Cys}) + [\text{Cys}]^{1 + \frac{[\text{Cys}]}{K_i}}} \]  
(Eq. 6)

Binding of the second mole of cysteine or homocysteine in reactions 3 and 5, respectively, will affect the \(V_{\text{max}}\) values for H₂S formation in the unimolecular reactions 2 and 4. To account for this, an inhibition term where \(K_i = K_m(\text{Cys}) + [\text{Cys}]^{1 + \frac{[\text{Cys}]}{K_i}}\) was introduced as shown in Equations 4–6.

Equations 4–6 describe a random sequential, ordered sequential, and ping-pong mechanism, respectively. A Hill coefficient was included in these equations to account for cooperativity of binding for the second substrate, which was indicated by the kinetic data and our fitting attempts. Equations 4–6, as written, describe \(v_{\text{HCys5}}\) from cysteine (i.e. reactions 2 + 3) for the alternative mechanisms.

For analysis of \(v_{\text{HCys5}}\) from homocysteine (i.e. reactions 4 + 5), Equations 4–6 were also employed, making the corresponding substitutions (i.e. [Cys] for [HCys], etc.). Unlike cysteine, the dependence of the reaction velocity for H₂S generation on homocysteine concentration did not show two well separated phases. Hence, the values for \(K_m(\text{HCys})\) \(V_{\text{max}}\), and \(K_i\) for α-ketoacid production (obtained from Equation 3) were used as input parameters in Equations 4–6. The quality of fits obtained for the ordered sequential mechanism was significantly worse than for the other two mechanisms (Tables S1 and S2). In contrast, the quality of fits for the ping-pong versus the random sequential mechanism was indistinguishable.

Cystathionine Production from Cysteine Plus Homocysteine—The \(K_m\) and \(V_{\text{max}}\) values for reaction 6 were determined using the HPLC assay for cystathionine formation, as described above, and Equation 7.

\[ v_{\text{Cys7}} = \frac{V_{\text{max}}[\text{Cys}][\text{HCys}]}{[\text{Cys}][\text{HCys}] + K_{\text{m1}[\text{Cys}]}(1 + \frac{[\text{HCys}]}{K_{\text{m1}[\text{HCys}]}}) + [\text{Cys}][\text{Km2}[\text{Cys}]](1 + \frac{[\text{HCys}]}{K_{\text{m2}[\text{Cys}]}})} \]  
(Eq. 7)

In principle, reaction 6 can follow either a binary or ternary mechanism with either cysteine or homocysteine binding first. However, a reasonable fit was only obtained for the ping-pong mechanism where cysteine binds first (Table S3). Equation 7 describes a ping-pong mechanism, in which competitive inhibition terms for the binding of the first and second substrates were included, since the simultaneous presence of both substrates leads to competition at each binding site by the other substrate (\(K_i(\text{HCys}) = K_m(\text{HCys})\) and \(K_{\text{m1}[\text{Cys}]} = K_{\text{m2}[\text{Cys}]}\)).

\(H_2S\) Production from Cysteine and Homocysteine—Next, the goodness of the kinetic parameters obtained for reactions 2–6 was assessed by fitting the experimental data for \(H_2S\) formation obtained in the presence of 10 mM cysteine and varying concentrations of homocysteine. In this set of experiments, the observed rate of \(H_2S\) production represents the sum of reactions 2–6, as described by Equation 8.

\[ v_{\text{HCys5}} = v_2 + v_3 + v_4 + v_5 + v_6 \]  
(Eq. 8)

The values of \(v_2,v_6\) corresponding to the reaction velocities for 2–6 were computed using Equations 9–13 for the ping-pong mechanism,

\[ v_2 = \frac{V_{\text{max}}[\text{Cys}]}{K_{\text{m1}[\text{Cys}]} + [\text{Cys}] + \frac{1}{K_i(\text{Cys})}} \]  
(Eq. 9)

\[ v_3 = \frac{V_{\text{max}}[\text{Cys}][\text{HCys}]}{[\text{Cys}][\text{HCys}] + K_{\text{m1}[\text{Cys}]}(1 + \frac{[\text{HCys}]}{K_{\text{m1}[\text{HCys}]}}) + [\text{Cys}][\text{Km2}[\text{Cys}]](1 + \frac{[\text{HCys}]}{K_{\text{m2}[\text{Cys}]}})} \]  
(Eq. 10)

\[ v_4 = \frac{V_{\text{max}}[\text{HCys}]}{K_{\text{m1}[\text{HCys}]} + [\text{HCys}] + \frac{1}{K_i(\text{HCys})}} \]  
(Eq. 11)

\[ v_5 = \frac{V_{\text{max}}[\text{HCys}][\text{HCys}]}{[\text{HCys}][\text{HCys}] + K_{\text{m1}[\text{HCys}]}(1 + \frac{[\text{HCys}]}{K_{\text{m1}[\text{HCys}]}}) + [\text{HCys}][\text{Km2}[\text{HCys}]](1 + \frac{[\text{HCys}]}{K_{\text{m2}[\text{HCys}]}})} \]  
(Eq. 12)
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the kinetic parameters were obtained as described above. A term for competitive inhibition ($K_m(1 + [I] / K_i)$) was introduced in Equations 9–13, since the simultaneous presence of both substrates leads to competition for each binding site by the other substrate.

Determination of the Rates of H₂S Production at Physiological Substrate Concentrations—The contributions of the various CSE-catalyzed reactions to total H₂S production were computed at normal, medium, and high homocysteine concentrations (10, 40, and 200 μM, respectively), using Equations 9–13. The following steady concentrations of substrates were used: [homocysteine] = 10, 40, or 200 μM; [cysteine] = 100 μM; and [cystathionine] = 5 μM. The $K_i$ for cystathionine was ignored, since the concentration of cystathionine used to simulate physiological conditions is low (5 μM), whereas the $K_i$ for cystathionine for H₂S production is relatively high (0.78 ± 0.1 μM). For instance, the inclusion of the $K_i$ term for cystathionine affected the value for $v_2$ by <2%. The values for $v_1$ corresponding to reaction 1, at varying homocysteine concentrations, were computed using Equation 14,

$$v_1 = \frac{V_{max} [\text{CST}]}{K_{m(CST)}(1 + [\text{Cys}] / K_{m1(\text{Cys})} + [\text{HCys}] / K_{m1(\text{HCys})}) + [\text{CST}]}$$

where cysteine and homocysteine act as competitive inhibitors for binding of cystathionine. $K_i(\text{Cys})$ and $K_i(\text{HCys})$ were assumed to be equal to $K_{m1(\text{Cys})}$ and $K_{m1(\text{HCys})}$, respectively.

The resulting reaction rates ($v_1 - v_6$) for reactions 1–6 were then used to calculate the turnover numbers (i.e. $v/[E]$) for each reaction at the substrate concentrations described above and are expressed per mole of CSE active site.

RESULTS

Purification and Biophysical Characterization of CSE—Purification of recombinant human CSE was accomplished in three chromatographic steps, and the purified protein was judged to be ~95% pure by gel electrophoresis (Fig. 2, inset). The typical yield was ~20 mg of pure protein/liter of culture. The specific activity of as-purified CSE is 3.1 ± 0.1 units/mg in the DTNB assay with cystathionine as substrate and is similar to the value published previously (2.5 units/mg) (25). As expected, the absorption spectrum of purified CSE is typical of a PLP-dependent enzyme with a maximum at 428 nm due to PLP.

H₂S Production by Human CSE—The ability of human CSE to generate H₂S was first assessed by an in-gel activity assay. For this experiment, native gel strips containing equal amounts of purified...
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**TABLE 1**  
Kinetic parameters for reactions catalyzed by CSE  
All values are the average of at least three independent experiments ± S.D.

| Reaction Number | Reaction Description | $V_{max}$ | $K_m$ | $K_i$ | $n$ | $k_{cat}$ | $k_{cat}/K_m$ | Units | Conditions |
|-----------------|----------------------|-----------|-------|-------|-----|----------|--------------|-------|------------|
| 1               | γ-Elimination of cystathionine | 3.1 ± 0.1 | 0.28 ± 0.03 | 3.2 ± 0.2 | 3.2 | 2.3 | 8.2 | units/mg | | |
| 2               | Pyruvate generation | 0.42 ± 0.07 | 3.7 ± 1.1 | 32.0 ± 9.5 | 0.31 | 0.88 | 0.08 | units/mg | | |
| 3               | H2S generation from Cys | 0.6 ± 0.1 | 1.7 ± 0.7 | 33 ± 8 | 0.47 | 0.27 | 0.1 | units/mg | | |
| 4               | α-Ketobutyrate generation | 1.2 ± 0.3 | 33 ± 8 | 3.0 ± 1.0 | 0.85 | 0.026 | 0.026 | units/mg | | |
| 5               | H2S generation from HCys | 1.2 ± 0.3 | 2.7 ± 1.4 | 14.5 ± 6.8 | 0.76 | 1.0 | 0.1 | units/mg | | |
| 6               | Cystathionine generation | 6.6 ± 0.47 | 5.9 ± 1.2 | 1.8 ± 0.6 | 4.9 | 0.83 | 0.83 | units/mg | | |

CSE were exposed to the following conditions (30 mM homocysteine, 30 mM homocysteine plus 10 mM L-cysteine, or 10 mM L-cysteine). H2S generation was revealed by the appearance of a dark lead sulfide-containing band on the gel. As shown in Fig. 3 (inset), the most intense bands were seen in the presence of homocysteine (lane a). When both homocysteine and cysteine were present in the reaction mixture, lower H2S production was observed (lane b), whereas cysteine alone supported the lowest level of H2S generation (lane c). These results indicate that at saturating concentrations, homocysteine rather than cysteine is the more effective substrate for H2S generation by CSE. We note that CSE migrates as higher order oligomers.

The kinetics of H2S generation by CSE were further characterized using a continuous spectrophotometric assay. The specific activities under $V_{max}$ conditions for H2S formation are 6.6 ± 0.5 units/mg from homocysteine and 1.2 ± 0.3 units/mg from cysteine (Table 1). As also seen in Fig. 3, the rate of H2S formation from homocysteine is higher than from cysteine or from homocysteine plus cysteine. The decrease in the initial velocity of H2S formation when both substrates are present in comparison with the rate observed with homocysteine alone results from the occupancy of a portion of the enzyme active sites by the slower substrate, cysteine. This has the net effect of decreased total H2S flux generation. Conversely, the apparent activation of H2S production when both substrates are present in comparison with cysteine alone results from the fraction of the enzyme that is catalyzing homocysteine-dependent H2S production, which occurs at a faster rate than from cysteine. Propargylglycine, a suicide inhibitor of CSE (32), completely blocked H2S formation (not shown). Unlike rat CSE that reportedly uses cysteine (Fig. 1, reaction 7) rather than cysteine to generate H2S (23), H2S formation from cysteine was not observed with human CSE (data not shown).

**Product Analysis of H2S-producing Reactions**—To distinguish between the multiple routes for H2S generation by CSE (Fig. 1, reactions 2–6), reaction products were analyzed by mass spectrometry, HPLC, and UV-visible absorption spectroscopy for detection of the keto acids, pyruvate and α-ketobutyrate (28). In the presence of cysteine, pyruvate and a novel metabolite, lanthionine ($m/z = 209$; Fig. 4), were observed, consistent with an α,β-elimination reaction (reaction 2) and a β-replacement reaction (reaction 3). In the presence of homocysteine, cysteine, and homocystine, the major products were homocystathionine ($m/z = 241$), cystathionine ($m/z = 237$), lanthionine ($m/z = 209$), and homocystathionine ($m/z = 241$) (Fig. 4). These results are consistent with the known reactions of CSE and the proposed roles of cystathionine as a thiol metabolite and a precursor of H2S.

![FIGURE 4. Product analysis by MS of the CSE-catalyzed reactions in the presence of homocysteine plus cysteine (A), homocysteine alone (B), or cysteine alone (C). Parent ions with $m/z$ values of 122 (cysteine), 136 (homocysteine), 209 (lanthionine), 223 (cystathionine), 237 (homolanthionine), 241 (cysteine), and 269 (homocysteine) are seen.](image-url)
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Effect of Nitric Oxide (NO) on $H_2S$-producing Activity of CSE—Previously, it has been reported that the NO donor, sodium nitroprusside, increases the endogenous levels of $H_2S$ in vascular tissues (6). The mechanism of this increase was proposed to involve either an NO-induced increase in CSE activity or NO-dependent up-regulation of CSE expression (6). However, we observed no effect of sodium nitroprusside on $H_2S$ production by CSE (data not shown), indicating that the effect of NO is not at the level of CSE activity.

Kinetics of $H_2S$ Generation by CSE—Product analyses provided direct evidence for five of the six possible CSE-dependent $H_2S$-generating reactions described in Fig. 1 (i.e. reactions 2–6). The kinetics of pyruvate (reaction 2) and $\alpha$-ketobutyrate (reaction 4) formation from cysteine and homocysteine, respectively, and the kinetics of $H_2S$ formation from the same substrates (i.e. reactions 2 + 3 or reactions 4 + 5) are shown in Fig. 5. The kinetic data were fitted to alternative mechanisms (i.e. binary versus ternary), and the data are presented in Tables S1 and S2. The values of the kinetic parameters obtained from fits to the ping-pong mechanism allowed deconvolution of the $K_m$ and $V_{max}$ values associated with each of the four reactions (Table 1). The dependence of the rate of $H_2S$ formation on cysteine concentration is markedly biphasic (Fig. 5B). CSE exhibits a considerably higher affinity for cysteine binding to site 1 ($3.7 \pm 1.1$ mM) than to site 2 ($33 \pm 8$ mM), and cooperativity for binding of the second mole of cysteine was seen ($n = 3 \pm 1$).

Deconvolution of the two phases contributing to the rate of $H_2S$ formation from homocysteine (Fig. 5D) reveals that the $K_m$ for site 1 is 2-fold lower than for site 2 ($2.7 \pm 1.4$ and $5.9 \pm 1.2$ mM, respectively). The kinetics of reaction 6 (i.e. the condensation of homocysteine and cysteine) were monitored by the rate of cystathionine formation. The kinetic data could only be fit
this reaction with those of the side reactions leading to H$_2$S generation (Table 1). Under $V_{\text{max}}$ conditions, the most efficient H$_2$S-generating reaction (i.e. $\gamma$-replacement of homocysteine (reaction 5)) exhibits a $k_{\text{cat}}/K_m$ value that is $\sim$10-fold lower than that for the $\alpha,\gamma$-elimination of cystathionine. Furthermore, the $K_m$ for cystathionine (0.28 ± 0.03 mM) is significantly lower than for homocysteine.

In the cell, the substrate concentrations are low compared with their $K_m$ values (i.e. $[S] \ll K_m$). Under these conditions, most of the enzyme active sites are unoccupied, and the partitioning of CSE into the various H$_2$S-generating reactions is governed by the rate of each reaction (i.e. $v = V_{\text{max}}[S]/K_m$). This is distinct from the situation under in vitro steady-state assays conducted at high concentrations of substrate, where the $k_{\text{cat}}/K_m$ ratio determines the enzyme specificity for competing substrates. Thus, in the cell, substrate availability will play a crucial role in determining the partitioning of CSE between competing reaction paths, and regulatory mechanisms are likely to exist that lead to enhanced or diminished production of H$_2$S and to the diversion of CSE from its role in the transsulfuration pathway.

Using the kinetic parameters described in Table 1, we simulated the relative contributions of each of the reactions to total H$_2$S production at three concentrations of homocysteine, representing normal (10 $\mu$M) versus moderate (40 $\mu$M) and severe (200 $\mu$M) hyperhomocysteinemia (Tables 2 and 3 and Fig. 6, B and C). According to our simulations, under normal conditions, $\alpha,\beta$-elimination of cysteine (reaction 2) is predicted to be the major source of CSE-derived H$_2$S, accounting for $\sim$70% of the total (Table 3 and Fig. 6, B and C). The $\alpha,\gamma$-elimination of homocysteine (reaction 4) is the next significant contributor ($\sim$29%), whereas the $\beta$- and $\gamma$-replacement reactions (reactions 3, 5, and 6) are of negligible importance. The balance between the reaction shifts, however, with increasing concentrations of homocysteine such that the $\alpha,\gamma$-elimination of homocysteine (reaction 4) becomes a significant source of H$_2$S at moderate and the principal source of H$_2$S at severely elevated homocysteine concentrations (Fig 6, B and C). The condensation reaction between 2 mol of cysteine (reaction 3) is a minor contributor to the net H$_2$S pool. Since the rate
of reaction 5 has a square dependence on the concentration of homocysteine, it exhibits the greatest sensitivity to increasing homocysteine concentrations, changing ~230-fold between 10 and 200 μM homocysteine (Table 3). Homocysteine elimination could therefore be a useful biomarker for H₂S production at high homocysteine concentrations. Generation of H₂S by the γ-replacement of homocysteine (reaction 5) accounts for ~13% of total H₂S generation by CSE under conditions of severe hyperhomocysteinemia. Cystathionine formation (reaction 6) is also predicted to rise with increasing homocysteine (Table 3), but it is unlikely to build up, since it is an efficient substrate for CSE. Under conditions of cystinuria, lanthionine production via reaction 3 would be expected to increase.

Comparison of Experimental versus Simulated Kinetic Data at Physiological Concentrations of Substrates—To test the validity of the simulations described above, the kinetics of the CSE-catalyzed production of H₂S at substrate concentrations chosen to mimic their physiological levels were determined. At low homocysteine concentrations (10 μM), 70% of H₂S is predicted to result from the α,β-elimination of cysteine (v/[E] = 0.0081 s⁻¹) and 29% from the α,γ-elimination of homocysteine (v/[E] = 0.00335 s⁻¹) (Table 2). The experimentally observed turnover number for H₂S formation under these conditions was 0.012 ± 0.001 s⁻¹ and similar to the calculated value of 0.0115 s⁻¹. As the concentration of homocysteine increases, the net rate of H₂S production is expected to increase. In addition, the proportion of H₂S that is derived from homocysteine increases from 29 to 63 to 90% as homocysteine increases from 10 to 40 to 200 μM, respectively (Table 3). In contrast, the rate of H₂S production from cysteine is virtually unchanged, whereas the proportion of cysteine-derived H₂S decreases from 70 to 37 to 10%. The value of v/[E] for H₂S generation is predicted to increase to 0.0217 and 0.0781 s⁻¹ at 40 and 200 μM homocysteine, respectively. The experimentally observed v/[E] values for H₂S formation under these conditions were found to be 0.015 ± 0.007 s⁻¹ (40 μM homocysteine) and 0.06 ± 0.001 s⁻¹ (200 μM homocysteine). The excellent correspondence between the experimentally measured and predicted turnover numbers for H₂S production support the validity of the simulations reported here.

**DISCUSSION**

The nonenzymatic liberation of H₂S from organic polysulfides in garlic bulbs has been reported recently and provides a mechanistic explanation for the vasorelaxation of garlic (33). However, despite the growing interest in H₂S biology and the therapeutic potential of H₂S-releasing compounds (2), surprisingly little is known about the enzymatic production of this gas and how it may be influenced by changes in sulfur amino acid levels in disease states.

Since the enzymes in the transsulfuration pathway, CBS and CSE, catalyze elimination/addition reactions at the β- and γ-positions of sulfur-containing amino acids, respectively, they are logical candidates for the generation of H₂S. However, conflicting reports in the literature ascribe the generation of H₂S by CBS and CSE to different substrates. For example, cysteine was proposed to be the preferred substrate for H₂S by CBS (23), whereas the β-replacement of cysteine by homocysteine is reported to be the preferred route for H₂S generation by CBS (27). In this study, we have investigated the various reactions catalyzed by CSE that result in H₂S biogenesis and, as side products, the novel amino acids, lanthionine and homolanthionine. The multitude of H₂S-generating reactions (Scheme 1) and the relatively high Kₘ values for homocysteine and cysteine exhibited by CSE versus the intracellular concentrations of these amino acids makes kinetic analysis complex and necessitates the use of simulations to deconvolute the contributions of different substrates to the overall H₂S pool.

The reaction catalyzed by CSE in the transsulfuration pathway involves elimination at the γ-carbon of cystathionine. We find that the catalytic efficiency (kₐcat/Kₘ) of the canonical cysteine elimination reaction from cystathionine is ~20- and ~30-fold higher for H₂S elimination from homocysteine and cysteine, respectively (Table 1). At physiologically relevant concentrations of homocysteine (10 μM), cysteine (100 μM), and cystathionine (5 μM), the turnover number for cystathionine cleavage (0.039 s⁻¹) is still 5-fold greater than for cysteine.

**TABLE 3**

The relative contributions of H₂S-generating reactions at varying concentrations of homocysteine as predicted from kinetic data analyses

| Substrate | Reaction number | 10 μM HCys | 40 μM HCys | 200 μM HCys |
|-----------|----------------|------------|------------|-------------|
| Cys       | 2              | 70.5       | 36.9       | 9.6         |
| Cys + Cys | 3              | 2.5 × 10⁻⁴ | 1.3 × 10⁻⁴ | 3.5 × 10⁻⁵ |
| HCys      | 4              | 29.1       | 60.6       | 77.7        |
| HCys + HCys | 5          | 0.36       | 2.4        | 12.5        |
| HCys + Cys | 6            | 0.015      | 0.07       | 0.26        |

*The reaction numbers correspond to those shown in Fig. 1.
°One unit corresponds to 1 μmol of product formed min⁻¹. The Kₘ and V_max values were determined as described under “Experimental Procedures” and reported in Table 1.
°°In reactions involving two substrates, the order of the Kₘ values reflects the substrate order in the first column.
°°°The values for the turnover numbers at varying concentrations of homocysteine (5 μM) and cysteine (100 μM) were obtained as described under “Experimental Procedures” considering a ping-pong mechanism for the bimolecular reaction and the Hill coefficients (n) reported in Table 1.
°°°°Fold change refers to the change in v/[E] with respect to normal conditions (i.e. 10 μM homocysteine, which is assigned a value of 1 for each reaction).
Biogenesis of $H_2S$ from Cysteine and Homocysteine

Scheme 1. Postulated reaction mechanisms for CSE-catalyzed reactions with cysteine and homocysteine.
Biogenesis of H$_2$S from Cysteine and Homocysteine

cleavage (0.008 s$^{-1}$) and $\sim$12-fold higher than for homocysteine cleavage (0.0034 s$^{-1}$) (Table 2). Under these conditions, the $\alpha,\beta$-elimination of cysteine catalyzed by CSE (Fig. 1, reaction 2) is the major source of H$_2$S, accounting for $\sim$70% of its production, whereas the $\alpha,\gamma$-elimination of homocysteine (reaction 4) accounts for $\sim$29% (Table 3 and Fig. 6).

The normal range for plasma total homocysteine concentration in humans is $\sim$6–15 $\mu$M and is $\sim$25-fold lower than the concentration of total cysteine ($\sim$250 $\mu$M) (34). In patients with hyperhomocysteinemia, plasma homocysteine levels can range from 20–50 $\mu$M (in moderate hyperhomocysteinemia) to $\geq$100 $\mu$M (severe hyperhomocysteinemia) (35). Since CSE catalyzes homocysteine-dependent production of H$_2$S, we have simulated the effect of moderate (40 $\mu$M) and severe (200 $\mu$M) hyperhomocysteinemic conditions, which boost the ratio of homocysteine/cysteine, on H$_2$S biogenesis. As expected, H$_2$S derived from homocysteine-dependent reactions increased proportionately with the grade of hyperhomocysteinemia (Table 3 and Fig. 6). The reaction displaying the greatest sensitivity to homocysteine concentrations was the condensation of 2 mol of homocysteine to give homolanthionine catalyzed by CSE (Fig. 1, reaction 5), which increased $\sim$230-fold at severely elevated homocysteine concentrations (Table 2). Under these conditions, homocysteine rather than cysteine becomes the preferred source for CSE-derived H$_2$S.

The sensitivity of the CSE-catalyzed $\gamma$-replacement reaction to homocysteine suggests that homolanthionine, which is expected to be more stable than H$_2$S, could be a suitable biomarker for this reaction. Indeed, homolanthionine was reported in urine samples from homocystinuric patients nearly 4 decades ago, when it was proposed to be derived from homocysteine or homoserine metabolism (36). The first report of biologically derived homolanthionine dates back to 1963 in a mutant strain of $E. coli$ (37), and the accumulation of this compound was later reported in other organisms (38, 39). In Corynebacterium glutamicum, homolanthionine is an intermediate in a novel pathway for isoleucine synthesis (40). Homolanthionine formation by both rat and human liver CSE by the condensation of homocysteine and homoserine has been reported (41). Our study reveals that homolanthionine is generated by the condensation of two homocysteine molecules in a reaction catalyzed by CSE, thus linking the origin of homolanthionine to homocysteine metabolism and CSE. Homolanthionine could potentially be catabolized by reversal of the CSE reaction, and the metabolic fate of this compound needs to be evaluated.

Our results suggest that under hyperhomocysteinemic conditions, H$_2$S production may be enhanced and could contribute to the associated cardiovascular pathology. In an in vivo model for myocardial ischemia-reperfusion, a U-shaped H$_2$S dose dependence curve was observed, with the cardioprotective effect of H$_2$S decreasing at higher concentrations (7). In a rat model of stroke, administration of high NaHS levels increased infarct volume (42), and H$_2$S was found to be proinflammatory in a mouse endotoxic shock model (8). Mutations in CBS are the most common cause of severe hyperhomocysteinemia in comparison with defects elsewhere in the pathway (e.g. methionine synthase, methylenetetrahydrofolate reductase, and methionine synthase reductase). In homocystinuric individuals with CBS deficiency, CSE may be the major source of H$_2$S. Our studies suggest that inhibition of CSE in hyperhomocysteinemic individuals could be a useful strategy for attenuating the attendant cardiovascular pathology seen with this disease.

In conclusion, our study reveals the relative importance of cysteine- versus homocysteine-dependent reactions to H$_2$S biogenesis catalyzed by CSE. Since these CSE-dependent routes for H$_2$S generation represent side reactions relative to its role in the transsulfuration pathway, it is critically important to understand how these enzymatic reactions are regulated so that the same catalyst can serve dual roles. In some cell types, such as vascular endothelial cells, the transsulfuration pathway is not intact, since CBS is reported to be absent (47). Since H$_2$S is a signaling molecule, it is to be expected that its generation is regulated, and Ca$^{2+}$-calmodulin has been reported to activate CSE (20). Under hyperhomocysteinemic conditions, H$_2$S homeostasis may be dysregulated and could contribute to the cardiovascular etiology associated with this disease.

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