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The Quantitative Significance of the Transsulfuration Enzymes for H_2S Production in Murine Tissues

Omer Kabil, Victor Vitvitsky, Peter Xie, and Ruma Banerjee

Abstract

The enzymes of the transsulfuration pathway, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), are important for the endogenous production of hydrogen sulfide (H_2S), a gaseous signaling molecule. The relative contributions of CBS and CSE to H_2S generation in different tissues are not known. In this study, we report quantification of CBS and CSE in murine liver and kidney and their contribution to H_2S generation in these tissues and in brain at saturating substrate concentrations. We show that CBS protein levels are significantly lower than those of CSE; 60-fold and 20-fold in liver and kidney, respectively. Each enzyme is more abundant in liver compared with kidney, twofold and sixfold for CBS and CSE, respectively. At high substrate concentrations (20 m*M* each cysteine and homocysteine), the capacity for liver H_2S production is approximately equal for CBS and CSE, whereas in kidney and brain, CBS constitutes the major source of H_2S, accounting for $\sim$80% and $\sim$95%, respectively, of the total output. At physiologically relevant concentrations of substrate, and adjusting for the differences in CBS versus CSE levels, we estimate that CBS accounts for only 3% of H_2S production by the transsulfuration pathway enzymes in liver. *Antioxid. Redox Signal.* 15, 363–372.

Recognized previously as a poison for aerobic organisms, H_2S has recently emerged as a member of the family of gaseous signaling molecules and appears to modulate a variety of cellular processes (6, 12, 19, 22). H_2S induces opening of ATP-sensitive potassium channels and relaxation of vascular smooth muscle cells (41), regulates the sensitivity of *N*-methyl-D-aspartate receptor, and increases synaptic transmission in hippocampal neurons (1), reduces myocardial ischemia/reperfusion injury (8), inhibits cell proliferation (39), reduces mitochondrial respiration, and induces a suspended animation–like metabolic state (4). Despite the varied physiological effects attributed to H_2S, little clarity exists on H_2S levels and production capacity in tissues, and H_2S concentrations varying over a 10^5-fold range have been reported in biologic samples [reviewed in (28)]. Furthermore, mechanisms that regulate H_2S production and clearance are not known. The primary sources of endogenously produced H_2S are two enzymes in the transsulfuration pathway, CBS and CSE, which catalyze H_2S biogenesis via condensation of cysteine and homocysteine and desulfhydration of cysteine, respectively (33). Although the combined actions of 3-mercaptopyruvate sulfurtransferase and cysteine aminotransferase, involved in the cysteine catabolic pathway are proposed to contribute to H_2S production (32), these reactions can lead to H_2S release only in the presence of a reductant (16, 26) (Fig. 1A). The metabolic removal of H_2S occurs in the mitochondrion where H_2S is oxidized to thiosulfate (14) and the electrons are transferred to the electron-transport chain to generate energy (5, 13). Thiosulfate is further metabolized to sulfate, a major secreted end product of H_2S metabolism (3).

The canonic role of the transsulfuration pathway is to clear homocysteine, a toxic intermediate in the methionine cycle, and to divert it to cysteine synthesis (Fig. 1A) (43). CBS is a heme protein that catalyzes the PLP-dependent β-replacement of serine by homocysteine to form cystathionine (Fig. 1A) (18). The second enzyme in the transsulfuration pathway, CSE, which also is PLP dependent, catalyzes the α,γ-elimination of cystathionine to form cysteine and α-ketobutyrate and ammonia. CBS also catalyzes an alternative β-replacement of cysteine by homocysteine (Fig. 1B, reaction iii), at an approximately fourfold faster rate than the corresponding one between serine and homocysteine, owing in part to the better leaving-group potential of H_2S versus H_2O. In addition, the lack of reaction stringency allows CBS to catalyze H_2S production via other reactions, including β-replacement of cysteine by water, to give serine (Fig. 1B, reaction i), and β-replacement of cysteine by a second mole of cysteine (reaction ii), albeit less efficiently (33). Detailed kinetic analyses suggest that among these reactions, β-replacement of cysteine by homocysteine is primarily responsible for H_2S production by CBS under both maximal velocity and physiologic substrate concentrations (33).

Biological Chemistry Department, University of Michigan Medical Center, Ann Arbor, Michigan.

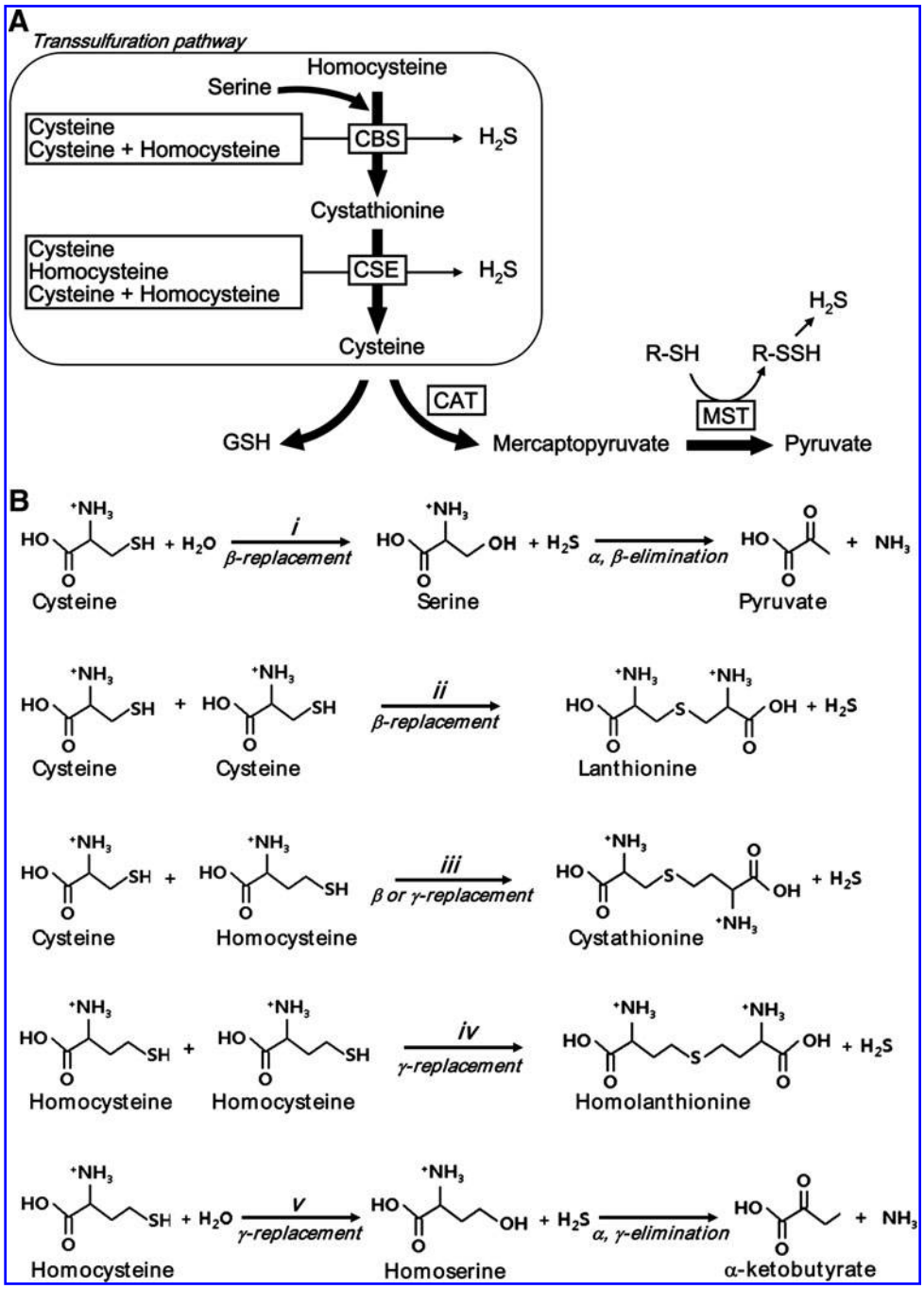

FIG. 1. Schematic of H_2S-generation (A) and H_2S-generating reactions catalyzed by CBS and CSE (B) in the transsulfuration pathway (*black arrows*). GSH, glutathione; CAT, cysteine aminotransferase; MST, 3-mercaptopyruvate sulfurtransferase.

CSE is even more versatile in terms of its reactivity because it can work at both the β-and γ-carbons of substrates generating H_2S via a multitude of reactions from cysteine and homocysteine [*i.e.*, α,β-elimination of cysteine to give pyruvate (Fig. 1B, reaction i,), β-replacement of cysteine by a second cysteine (reaction ii), α,γ-elimination of homocysteine to give α-ketoglutarate (reaction v), γ-replacement of homocysteine by cysteine (reaction iii), or γ-replacement of homocysteine by a second mole of homocysteine (reaction iv) (7)]. Under maximal-velocity conditions, the γ-replacement of homocysteine by a second mole of homocysteine (reaction iv) constitutes the most efficient H_2S-producing reaction. However, kinetic simulations at physiologically relevant substrate concentrations indicate that the α,β-elimination of cysteine (reaction i) is the preferred route for H_2S generation by CSE (7). H_2S production by CSE is sensitive to homocysteine concentrations, and under conditions of hyperhomocysteinemia, the H_2S-generating activity of CSE shifts toward condensation of two moles of homocysteine. The product of this reaction, homolanthionine, has been detected in urine samples of homocystinuria patients (30).

The relative contributions of CBS and CSE to total H_2S production in different tissues are not known. Simulations based on detailed *in vitro* kinetic analyses predict that if (a) CBS and CSE are present at equimolar concentrations, and (b) CBS is fully activated by its allosteric regulator *S*-adenosylmethionine (AdoMet), then CBS will be the primary source of H_2S at physiologically relevant substrate concentrations. Under such conditions, the ratio of CBS to CSE-derived H_2S is simulated to be ~7:3 (33). However, tissue-specific differences in the relative CBS and CSE expression levels would result in one or the other enzyme being the more prominent source of H_2S generation, leading to significant deviations from the computed ratio. For instance, in brain, the CBS is assumed to be the major contributor to H_2S generation because of its high expression level relative to CSE (1). This is consistent with the observation that in the CSE knockout mouse, brain H_2S levels were unchanged. In contrast, serum H_2S levels and the rate of H_2S production in aorta and heart were significantly reduced, suggesting that CSE is the primary source of H_2S generation in peripheral tissues (40).

Although the activities of CBS and CSE in the transsulfuration pathway have been reported in different tissues from various species, the relative quantities of these proteins in each tissue are not known. Kidney and brain exhibit twofold to threefold and fivefold to 25-fold lower CBS activity, respectively, compared with liver (9, 25, 37). Similarly, kidney and brain exhibit about threefold and ≥500-fold lower CSE activity, respectively, than does liver (9, 25). However, direct comparison of CBS and CSE activities between tissues cannot be made from the reported data because of differences in substrate concentrations used for activity measurements.

In this study, we used quantitative Western blot analysis to determine the relative protein expression levels of CBS and CSE in murine liver and kidney and demonstrate that it is tissue dependent. We also determined the H_2S production rate by murine liver, kidney, and brain in the presence and absence of a CSE inhibitor, propargylglycine, and CBS activator, AdoMet, which allows dissection of the CSE contribution to the total H_2S pool. This study interrogates, for the first time, the relative contributions of the preferred H_2S-generating reactions catalyzed by CBS and CSE (in the presence of cysteine and homocysteine) versus previous studies that have focused on the desulfhydration of cysteine, which overestimates the contribution of CSE.

Materials and Methods

Animals and tissue collection

Male Balb/c mice (7 to 10 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were killed in a CO_2 atmosphere, and liver, kidney, and brain were collected immediately, frozen in liquid nitrogen, and stored at −80°C until further use. All procedures for animal handling were performed in accordance with the protocols approved by the University's Committee on Use and Care of Animals.

Antibody production and purification

Polyclonal anti-human CBS and anti-human CSE chicken antibodies were developed in Aves Labs, Inc. (OR) against the respective recombinant human proteins purified in our laboratory, as described previously (36, 42). The proteins, which were >95% pure, were separated on denaturing 10% polyacrylamide gels, and the CBS and CSE bands were excised and used as antigens. Total IgY from each hen was purified by using either CBS or CSE affinity columns prepared with the Actigel-ALD kit (Sterogene), according to the vendor's protocol.

Western blot analysis

Frozen tissues were powdered in liquid nitrogen by using a porcelain mortar and pestle, and the powder was suspended in lysis buffer (100 m*M* sodium phosphate, pH 7.4, containing 0.2% Triton X-100, 1 m*M* β-mercaptoethanol, and 1% protease inhibitor cocktail (Sigma), 25 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml aprotinin), and incubated on ice for 30 min followed by centrifugation at 14,000 *g* for 10 min at 4°C. The protein concentration in tissue extracts was determined by using the Bradford assay and bovine serum albumin as a standard. Proteins were resolved on 10% sodium dodecylsulfate (SDS)-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Blots were probed with polyclonal anti-CBS or anti-CSE chicken antibodies. Secondary anti-chicken antibodies conjugated with horseradish peroxidase (Aves) were used (1:50,000 dilution), and signals were visualized by using the chemiluminescent peroxidase substrate kit SuperSignal West Dura (Thermo Scientific).

Quantification of liver and kidney CBS and CSE levels with Western blot analysis

Recombinant human CBS was purified as described previously (15). Dr. Taurai Chiku (University of Michigan) kindly provided the recombinant human CSE used in this study. For quantitative Western blot analysis, each gel contained four lanes in which purified recombinant CBS or CSE of known amount was loaded, along with varying amounts of tissue samples, to generate a standard curve from each blot. The Western blot band density of the purified recombinant proteins was used to generate the standard curve, and the protein concentration was precisely determined by total amino acid analysis (Protein Structure Core Facility, University of Nebraska Medical Center). Western blot signals were quantified by using Image J software. The signal intensities of individual tissue samples were quantified by using the coefficient obtained from the standard curve generated by using purified proteins.

H_2S-production assays

Frozen mouse tissue was disrupted by using a glass homogenizer in 100 m*M* HEPES, pH 7.4, to obtain a tissue concentration of 200 mg/ml. Reactions for H_2S production were prepared in polypropylene syringes, as described previously (11). Tissue homogenate (425 μl), homocysteine, and/or cysteine were mixed in 5-ml syringe barrels in a total reaction volume of 500 μl. For CSE inhibition experiments, tissue extracts were preincubated with 3 m*M* propargylglycine (PPG) for 3 min at room temperature before the addition of substrates. For CBS activation experiments, AdoMet was added to a final concentration of 500 μ*M*. Syringes were sealed with plungers and made anaerobic by flushing the headspace with

nitrogen 5 times by using a three-way stopcock, and then left in nitrogen in a final total volume (aqueous + gas) of 5 ml. Syringes were placed in an incubator at 37°C with gentle shaking (75 rpm) for 20 min. Control reactions, in which buffer replaced tissue homogenate, were prepared in parallel. Aliquots of 0.1 to 0.3 ml from the gas phase of the reaction syringes were collected by using gas-tight syringes through a septum attached to the stopcock, and injected in an HP 6890 gas chromatograph (GC) (Hewlett Packard) equipped with a DB-1 column (30 m × 0.53 mm × 1.0 μm). The carrier gas (helium) flow rate was 1 ml/min, and a temperature gradient ranging from 30°C to 110°C over a 20-min period was used. For samples with a high concentration of H_2S, a 10-fold dilution with nitrogen was used to prevent column overloading. H_2S was detected by using a 355 sulfur chemiluminescence detector (Agilent).

The H_2S standard was obtained from Cryogenic Gases (Detroit, MI) and had a stock concentration of 40 ppm (1.785 μM) in nitrogen. Various volumes of H_2S were injected into the GC to generate a standard curve. For the quantification of H_2S in samples, 0.1 to 0.3 ml of the gas phase from the reaction mixture was injected into the GC. The amount of H_2S in the injected volume was calculated from the peak areas by using the calibration coefficient obtained from the standard curve. Accounting for the reaction volume gives the total amount of H_2S in the gas phase of the reaction syringe. Dissolved H_2S was calculated by multiplying the concentration of H_2S in the gas phase by 1.6, the ratio between the concentration of H_2S in the gas and liquid phases (11). The HS^- ion concentration in the liquid phase was calculated for pH 7.4 (the pH of the reaction mixture) by using a pK_a of 6.8 for ionization of H_2S. The sum of H_2S in both phases produced in blank reactions lacking tissue was subtracted from that of reactions containing tissue. The resulting H_2S concentration was then used to obtain the specific activity for H_2S production by dividing the net H_2S produced by the incubation time and by the amount of tissue. Specific activity was expressed as nanomoles per minute per milligram.

Results

Quantification of CBS and CSE protein levels

CBS and CSE protein levels in mouse liver (Fig. 2A and B) and kidney (Fig. 3A and B) tissues were estimated by quantitative Western blot analysis, as described under Methods. Quantification of the Western blot data revealed that CBS is twofold higher, and CSE is sixfold higher in liver than in kidney (Figs. 2C and 3C). In both tissues, CSE was significantly more abundant than CBS; ~60-fold higher in liver and 20-fold higher in kidney. The amount of CBS protein was estimated to be 0.13 ± 0.025 ng/μg of total protein in liver lysate and 0.055 ± 0.026 ng/μg of total protein in kidney lysate. For CSE, liver and kidney protein amounts were estimated to be 8.0 ± 0.4 and 1.26 ± 0.04 ng/μg of total protein, respectively. Although CBS and CSE antibodies highlighted protein bands in brain samples, they exhibited aberrant migration (Fig. 4A and B). With CBS, in addition to a band of the expected size, we also observed a band that was larger by ~20 to 30 kDa and one significantly smaller band (~35 kDa). With CSE, we detected a single band, also of higher molecular mass (~55 kDa). We noted that a higher than expected molecular mass for CSE was previously reported, although not discussed (31). Although the higher-molecular-mass bands could represent modified forms of the protein [*e.g.*, both CBS and CSE can be sumoylated (2,17)], in the absence of further validation of the identities of the highlighted bands, the brain Western data were not processed for quantitative analysis.

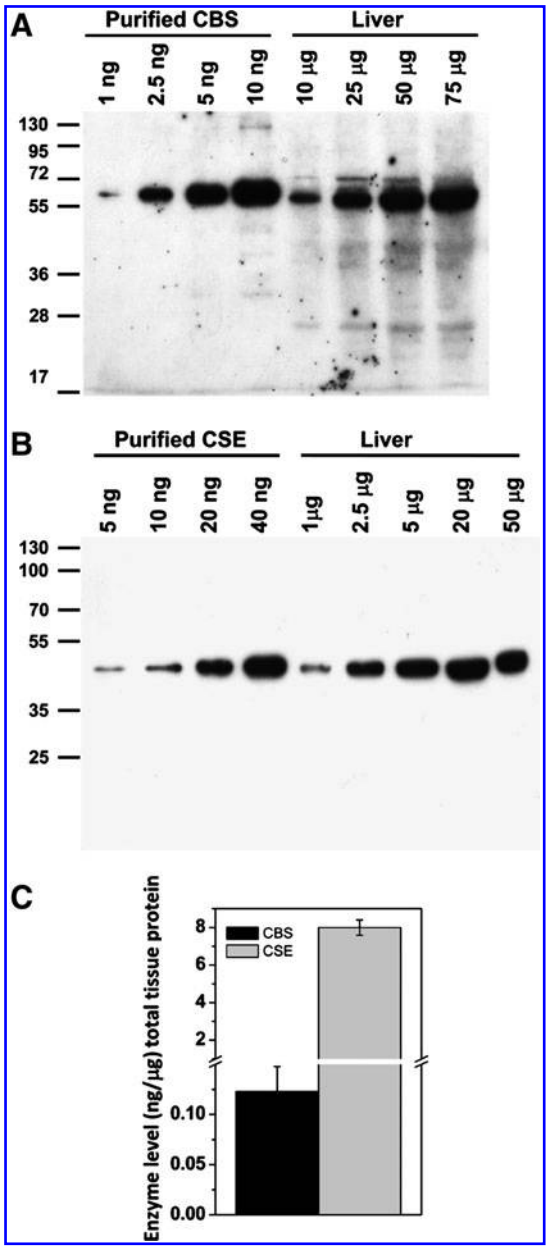

FIG. 2. Quantitative Western blot analysis of transsulfuration pathway enzymes in liver. Representative Western blot in which the indicated amounts of liver extract were separated in parallel with known amounts of CBS **(A)** or CSE **(B)**. Quantitation of CBS and CSE protein levels in liver **(C)**.

H_2S production by liver

H_2S was monitored by using a sulfur chemiluminescence detector coupled to a gas chromatographer, as described

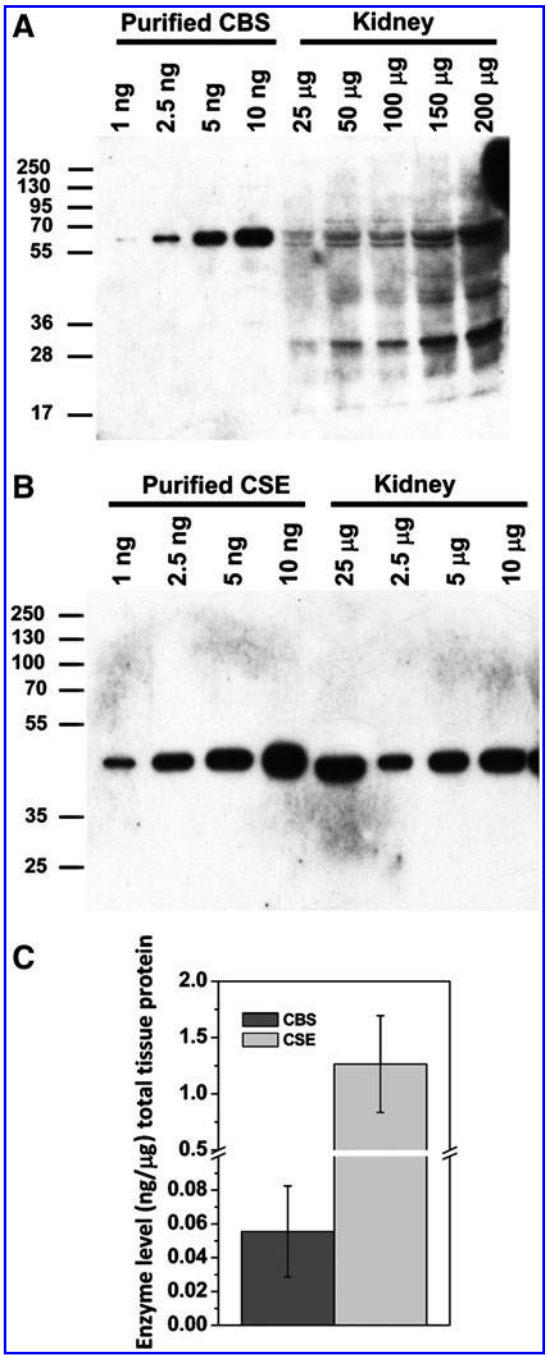

FIG. 3. Quantitative Western blot analysis of the transsulfuration pathway enzymes in kidney. Representative Western blot in which the indicated amounts of kidney extract were separated in parallel with known amounts of CBS **(A)** or CSE **(B)**. Quantitation of CBS and CSE protein levels in kidney **(C)**.

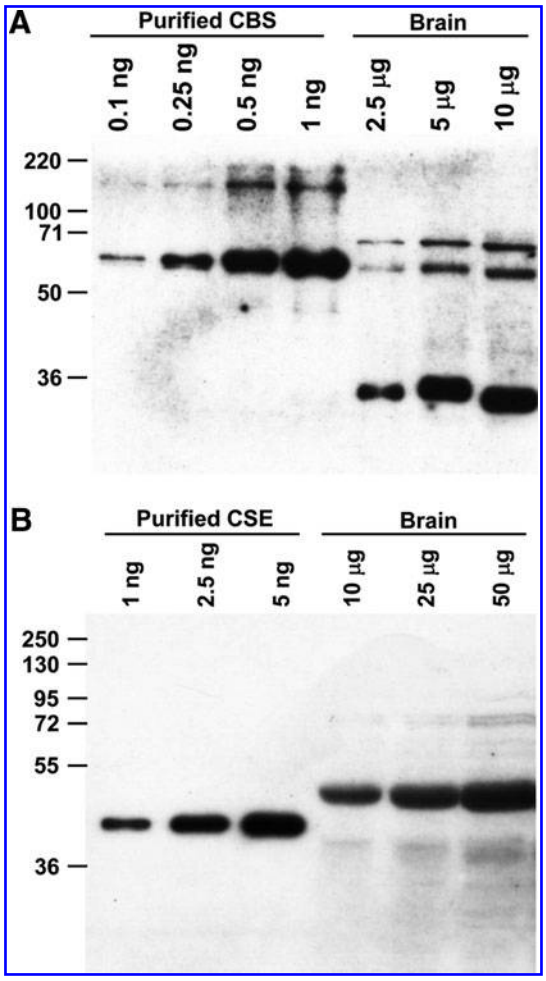

FIG. 4. Western blot analysis of CBS (A) and CSE (B) in brain. Representative Western blot for CBS contains, from left to right, 0.1, 0.25, 0.5, and 1 ng of purified CBS per lane and 10, 20, and 40 μg of total brain protein per lane. Representative Western blot for CSE contains, from left to right, 2.5, 5, and 10 ng of purified CSE per lane and 25, 50, and 100 μg of total brain protein per lane.

previously (11). Because volatile sulfur-containing organic compounds, such as carbonyl sulfide, methanethiol, and dimethylsulfide, can be produced during H_2S metabolism (21), chromatographic conditions were used that yielded excellent baseline separation of these compounds from H_2S, which eluted early at 1.1 min (Fig. 5). However, none of the compounds other than H_2S was observed in our samples. The detection limit for H_2S under our experimental conditions is $\sim$4 n*M*. The rate of H_2S production was determined in tissue homogenates in the presence of various concentrations of the substrates, cysteine and homocysteine (Fig. 6A and Table 1). The linear dependence of the assay on time ($\leq$20 min), on substrate (0.5 to 15 m*M*), and on tissue protein concentration, was established before measuring the rate of tissue H_2S generation. The background rate of desulfhydration of cysteine and homocysteine was measured for every substrate concentration used and subtracted from the experimental value obtained in the presence of tissue extract. In the presence of saturating concentrations of cysteine and homocysteine (20 m*M* each), the rate of liver H_2S production was 1,191 $\pm$ 133 nmole/min/g tissue at 37°C (Table 1). Addition of the irreversible inhibitor of CSE, propargylglycine, reduced the rate of H_2S production by about twofold. When the H_2S rate was measured in the presence of either substrate alone, it was about threefold lower: 407 $\pm$ 67 nmole/min/g tissue in the presence of 20 m*M* cysteine and 377 $\pm$ 36 nmole/min/g tissue in the

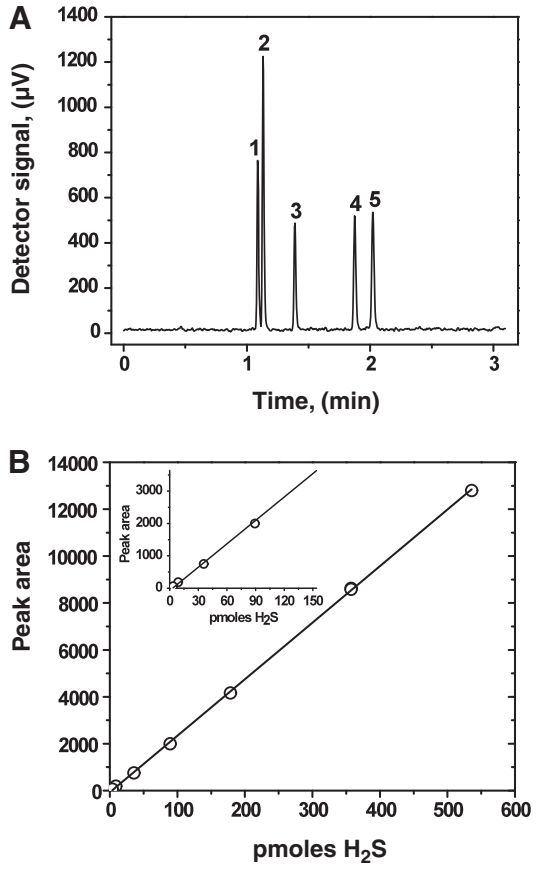

FIG. 5. Gas chromatogram showing separation of sulfur compounds (A) and standard curve for H_2S generated by injecting different amounts of H_2S into the GC, as described under Methods (B). (A) The following standards were injected in a total volume of 200 μl: 1-H_2S (2.7 ppm), 2-COS (5 ppm), 3-CH_3SH (5 ppm), 4-CH_3CH_2SH (5 ppm), and 5-CH_3SCH_3 (5 ppm), respectively, in nitrogen. The standards were obtained from Cryogenic Gases (Detroit, MI).

presence of 20 m*M* homocysteine. Propargylglycine inhibited H_2S production >90% when cysteine alone was used as substrate and almost 100% when homocysteine alone was used. As expected, lower rates of H_2S production were observed as the concentrations of cysteine and homocysteine were decreased. H_2S production increased about twofold in the presence of AdoMet and propargylglycine when either cysteine or cysteine+homocysteine was used as a substrate (Fig. 6B).

H_2S production in kidney

In kidney, the rate of H_2S generation in the presence of homocysteine and cysteine was 196.0±3.6 nmole/min/g tissue, which is sixfold lower than that in liver (Fig. 6C). H_2S generation from cysteine or homocysteine alone was reduced approximately threefold (57±8 and 60±12 nmole/min/g tissue, respectively). Addition of propargylglycine to kidney extract reduced H_2S production by 15% in the presence of both substrates, and 40% when homocysteine was used alone. Propargylglycine had no effect on H_2S generation from cysteine alone.

H_2S production in brain

The rate of H_2S generation in brain homogenate was significantly lower compared with liver and kidney (15.3± 2.2 nmole/min/g tissue in the presence of cysteine and homocysteine) (Fig. 6D). When either cysteine or homocysteine was used alone, the rate of H_2S generation decreased by fourfold and 23-fold, respectively (3.8±0.6 and 0.66± 0.02 nmole/min/g tissue, respectively). Propargylglycine did not reduce the rate of H_2S production in brain extract when either cysteine or cysteine+homocysteine was used as substrates. In contrast, the rate of H_2S production was inhibited by about twofold in the presence of homocysteine alone.

Discussion

Although the number of reports on the varied physiologic effects of H_2S continues to increase (4, 8, 24, 29), large gaps remain in our understanding of fundamental aspects of H_2S metabolism. The balance between H_2S generation by the transsulfuration enzymes and clearance via a mitochondrial catabolic pathway determines the steady-state tissue concentrations of H_2S, which appear to be very low (11, 28). The combined activities of cysteine aminotransferase and mercaptopyruvate sulfur transferase generate sulfane sulfur, which, in the presence of a reductant, can liberate H_2S (16). The physiologic relevance of this two-enzyme system in the cysteine catabolic pathway in the presence of a coupled oxidoreductase, to H_2S production remains to be established. Assessment of the relative capacities for H_2S production by CBS and CSE requires information about their prevalence in different tissues and about their relative abundance. However, reports on the relative levels of CBS and CSE and their immunohistochemical localization are confounded by nonquantitative Western blot data and by the use of commercially available antibodies that exhibit cross-reactivity with other proteins. *In vitro* kinetic analyses of the two major H_2S generators, CBS and CSE, have led to simulations of the relative contributions of each enzyme to H_2S production at physiologically relevant substrate concentrations, assuming equimolar concentrations of each enzyme (7, 33). However, for the simulations to have tissue relevance, the relative concentrations of CBS and CSE in the tissue of interest is necessary. In this study, we quantified CBS and CSE protein levels in liver and kidney and analyzed their contributions to H_2S generation in liver, kidney, and brain. Our results show that significant tissue-specific differences exist in the relative and absolute levels of CBS and CSE and that these differences are mirrored by differences in the H_2S production capacity of these tissues.

Quantitative Western blot analysis reveals that CBS levels are about twofold higher in liver than in kidney, which is in excellent agreement with the two- to threefold higher liver CBS activity compared with kidney homogenates (9, 25, 37). Similarly, the observed sixfold higher CSE in liver versus kidney is in good agreement with the approximately threefold difference in CSE activity between these tissues (9, 25). As discussed earlier, Western analyses detected the presence of either multiple bands (for CBS) or a single high-molecular band (with CSE) and were not subjected to quantitative analysis because their identities of these bands were not confirmed. A limitation of our study is that we used anti-

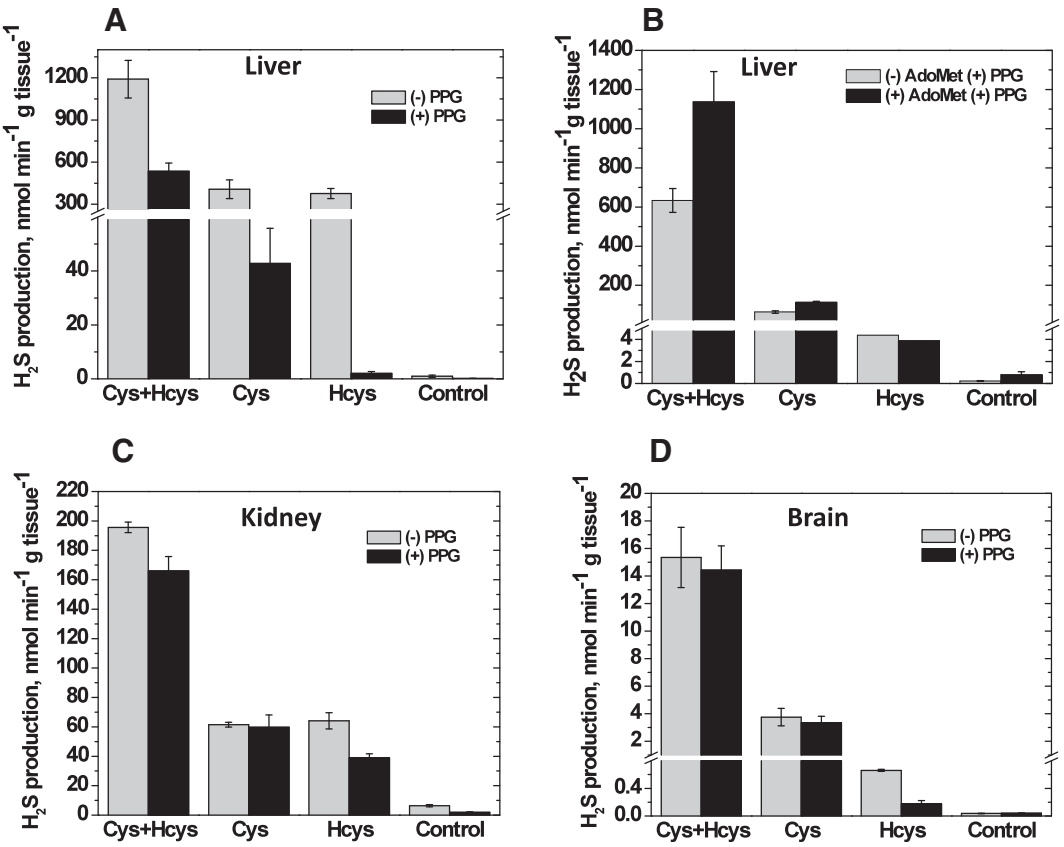

FIG. 6. H_2S generation by murine tissues in the presence of different substrates. H_2S production rates were measured in liver **(A, B)**, kidney **(C)**, and brain **(D)** extract ± 3 m*M* propargylglycine, as described under Methods in the presence of either 20 m*M* cysteine + 20 m*M* homocysteine, 20 m*M* cysteine, or 20 m*M* homocysteine. When the effect of AdoMet (500 μM) was examined, all reaction mixtures contained 3 m*M* propargylglycine to inhibit CSE. The control reaction mixture lacked tissue. The data represent the mean ± SD of three independent experiments.

bodies generated against human CBS and CSE to detect and quantitate the corresponding murine proteins. Human and mouse CBS and CSE show 80% and 84% sequence identities, respectively. Because the antibodies were raised against denatured proteins and are polyclonal, we do not expect differences in reactivity to be large enough to negate our results.

Most studies evaluating H_2S production in tissues use cysteine as the sole substrate (11, 23, 27). However, as expected from detailed *in vitro* kinetic analyses with purified human CBS and CSE (7, 33), and confirmed in this study (Table 1), the sole use of cysteine leads to a significant underestimation of the H_2S-generation rate and biases the data toward

Table 1. H_2S Production Rates by Murine Tissues

	Liver	*Kidney*	*Brain*	*Tissue-free blank*
Substrate (mM)	*Nmol/min/g tissue*	*Nmol/min/g tissue*	*Nmol/min/g tissue*	*Nmol/min/ml*
Cys + Hcy				
20	1,191 ± 133	196.0 ± 3.6	15.3 ± 2.2	0.024 ± 0.004
Cys				
20	407 ± 67	57 ± 8	3.8 ± 0.6	0.002 ± 0.00014
10	294 ± 89	33 ± 2	2.7 ± 0.2	0.0014 ± 0.00013
5	182 ± 17	23.0 ± 1.7	1.4 ± 0.1	–
1	38 ± 6	10.9 ± 0.8	0.35 ± 0.02	–
Hcy				
20	377 ± 36	60 ± 12	0.66 ± 0.02	0.024 ± 0.002
10	202 ± 35	43 ± 6	0.47 ± 0.1	0.01 ± 0.003
5	79.4 ± 0.1	29 ± 5	0.39 ± 0.02	0.006 ± 0.002
1	9 ± 2	14 ± 3	0.08 ± 0.03	0.0022 ± 0.0004

Anaerobic incubation conditions were used in the assays, and the values are the mean ± SD of three experiments. The tissue-free blank values for H_2S production were subtracted from the values obtained from the corresponding tissue-containing samples before conversion of the rates into units of nanomoles per minute per gram of tissue, as described under Methods.

CSE-dependent H_2S production, particularly in liver. Furthermore, hydroxylamine is commonly used as a "specific" CBS inhibitor (1, 23) although this compound has been historically used to release the cofactor from PLP enzymes (34). Because CBS and CSE are both PLP-dependent enzymes, the use of hydroxylamine in an assay mixture inactivates both enzymes. Given these limitations with earlier estimations, we determined tissue H_2S production rates in the presence of one or both substrates, cysteine and homocysteine, and in the presence or absence of the CSE-specific inhibitor, propargylglycine.

H_2S is redox active (16), and its catabolism is oxygen dependent (5). Under aerobic conditions, steady-state H_2S levels reflect the difference between production and oxidation rates and are negligible (11). Hence, our studies were conducted under anaerobic conditions that inhibit sulfide oxidation. The rate of H_2S production by liver homogenates in the presence of 20 m*M* each of homocysteine and cysteine were sixfold and 79-fold higher compared with the rates in kidney and brain, respectively. When cysteine is used, either alone or in combination with homocysteine, H_2S is generated by the activities of both CSE and CBS, and deconvoluting the contributions of the individual enzymes requires activity measurements in the presence of the CSE inhibitor, propargylglycine, or in the presence of homocysteine, which, by itself, is a substrate only for CSE (33). Because propargylglycine inhibits CSE- but not CBS-dependent H_2S production, the ~50% reduction in liver H_2S generation from cysteine and homocysteine observed in the presence of propargylglycine indicates that CBS and CSE contribute approximately equally to H_2S production under saturating substrate concentrations. H_2S production by CBS increased about twofold in the presence of AdoMet when cysteine or cysteine + homocysteine was used as a substrate. When either cysteine or homocysteine alone was used as substrate, an ~65% lower rate of H_2S production was observed, which was inhibited by 92% and 99.7%, respectively, by propargylglycine. The almost complete inhibition by propargylglycine of H_2S production from either substrate alone indicates that CSE is the major catalyst for H_2S formation under these conditions and is consistent with the lack of measureable AdoMet stimulation under these conditions (Fig. 6A and B). It also confirms the *in vitro* steady-state kinetic data (33), which predict that the primary reaction catalyzed by CBS under conditions of substrate saturation is the β-replacement of cysteine by homocysteine (versus the alternative reactions, β-replacement of cysteine by water and the condensation of two moles of cysteine) (33).

For liver, assuming equimolar protein concentrations and by using physiologically relevant concentrations of substrate, the relative contributions to H_2S production of CBS/CSE was estimated to range from 1:3 to 7:3, depending on the magnitude of allosteric activation of CBS by AdoMet (33). The observed contribution of hepatic CBS to H_2S production from cysteine alone (~8%) is in good agreement with the value of 2% estimated from the 60-fold lower CBS versus CSE protein levels and their comparable catalytic efficiencies for cysteine-dependent H_2S production [k_{cat}/K_m for CBS and CSE are 0.08 mM/s (7) and 0.07 mM/s (33), respectively].

However, under cellular conditions, CBS and CSE are not equally abundant. Applying the results from quantitative Western blot analyses obtained in this study (Figs. 2C and 3C; for example, CSE is 60-fold more abundant than CBS in liver), we estimate that CBS accounts for only 3% of hepatic H_2S generation capacity (Fig. 7). This value is expected to be about twofold lower in the complete absence of AdoMet activation. Furthermore, as homocysteine concentrations increase to levels seen in mild or severe forms of homocystinuria (20), the relative contribution of CBS is predicted to diminish even further. Thus, our analysis predicts that in liver, CSE accounts for 97% to 99% of the H_2S output over a homocysteine concentration range of 10 to 200 μM.

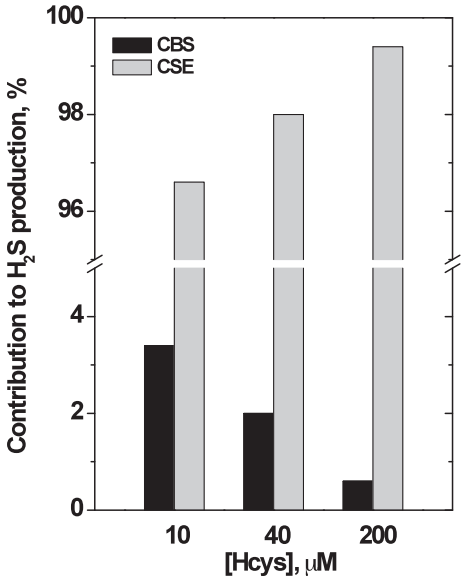

FIG. 7. Simulated contributions of fully activated CBS and CSE to liver H_2S production at physiologically relevant substrate concentrations and varying grades of hyperhomocysteinemia. The contributions of the transsulfuration enzymes were estimated based on previously reported simulations (33) and by taking into account the 1:60 ratio for CBS to CSE in mouse liver, as determined in this study (Fig. 1). The substrate concentrations used for simulation were 560 μM serine, 100 μM cysteine, and varying homocysteine [10 μM (normal), 40 μM (mild hyperhomocysteinemia), and 200 μM (severe hyperhomocysteinemia)].

The picture for the relative H_2S generation potential under saturating substrate concentrations in kidney is quite different from that in liver. Kidney H_2S production was relatively insensitive to propargylglycine, being reduced only 15% when cysteine and homocysteine were simultaneously used as substrates (Fig. 6C). This is consistent with CBS being the major source of H_2S in kidney. In the presence of either cysteine or homocysteine alone, H_2S production was reduced about threefold in comparison to the rate observed in the presence of both substrates. Interestingly, whereas propargylglycine did not inhibit cysteine-dependent production of H_2S, it inhibited the homocysteine-dependent H_2S production rate by 1.6-fold. Renal cysteine concentration is high, ~1 m*M* (35), which is ~10-fold higher than that in liver (60–180 μM), and in brain (50–120 μM) (35, 37). Hence, the residual H_2S production from homocysteine in the presence of propargylglyine, observed in kidney but not in liver samples, could be derived from the CBS-catalyzed β-replacement of cysteine present in kidney extract, by homocysteine. The greater relative importance of CBS for H_2S production in kidney versus liver can be explained at least in part by differences in the relative expression levels of the transsulfura-

tion enzymes in the two tissues. Thus, CSE is 60-fold higher than CBS in liver but only 20-fold higher in kidney, resulting in a threefold higher relative abundance of CBS in kidney than in liver. Our results are consistent with an earlier study on the protective effect of H_2S on renal ischemia/reperfusion injury that concluded that CBS is primarily responsible for H_2S generation in kidney (38).

Enzyme assays in rat tissue extracts estimate that CBS is about fivefold less active (10), and CSE, ~500-fold less active (25) in brain than in liver. Based on these data, and assuming a correspondence between protein level and enzyme activity, CBS is predicted to be about twofold more abundant than CSE in rat brain, although for reasons discussed earlier, this could not be assessed in the current study. The relative lack of sensitivity of H_2S production to propargylglycine, when cysteine + homocysteine or cysteine alone was used as substrate, indicates that the CBS is primary source of H_2S in brain (Fig. 6D). H_2S production in the presence of homocysteine accounted for only 4% of the rate observed in the presence of cysteine + homocysteine and was largely inhibited by propargylglycine, supporting the conclusion that CSE is not a significant source of brain H_2S, even at saturating substrate concentrations. This is consistent with brain H_2S levels being unchanged in CSE-knockout mice, whereas aorta and heart H_2S levels were decreased ~80% (40).

In summary, we demonstrate that significant differences exist between tissues in the relative contributions of CBS versus CSE and in their net H_2S-production capacity. The use of multiple assay methods in the presence and absence of AdoMet, the allosteric activator of CBS, and properglyglycine, a CSE inhibitor, allows evaluation of the individual contributions of the two enzymes to H_2S generation. Extrapolation of H_2S output by CBS versus CSE in tissues requires quantitative information on their protein levels and detailed kinetic information on the H_2S-generating reactions at physiologically relevant substrate concentrations. We demonstrate how adjusting for protein levels of CBS and CSE results in a very different picture of their relative contributions to liver H_2S generation at physiologically relevant concentrations of cysteine and homocysteine.

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Author Disclosure Statement

No competing financial interest exists.

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Address correspondence to:
Prof. Ruma Banerjee
Department of Biological Chemistry
University of Michigan Medical School
1150 W. Medical Center Dr.
3320B MSRB III
Ann Arbor, MI 48109-0600

E-mail: rbanerje@umich.edu

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Abbreviations Used

3MST = 3-mercaptopyruvate sulfurtransferase
AdoMet = *S*-adenosylmethionine
CAT = cysteine aminotransferase
CBS = cystathionine β-synthase
CSE = cystathionine γ-lyase
GC = gas chromatography
GSH = glutathione
PLP = pyridoxal-5'-phosphate
PPG = propargylglycine

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