Biochemical and Biophysical Research Communications 431 (2013) 131–135

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hydrogen sulfide is produced by cystathionine γ-lyase at the steady-state low intracellular Ca^{2+} concentrations

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ARTICLE INFO

Article history:
Received 20 December 2012
Available online 10 January 2013

Keywords:
Hydrogen sulfide
Cystathionine γ-lyase
Calcium
Pyridoxal 5′-phosphate

ABSTRACT

Hydrogen sulfide (H_2S) is recognized as a physiologic mediator produced in a variety of tissues. It is produced by three enzymes, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3MST). However, the regulation of H_2S production by CSE has not well been understood. Here we show that H_2S producing activity of CSE is regulated by intracellular Ca^{2+} concentrations. In the presence of pyridoxal 5′-phosphate (PLP) CSE efficiently produces H_2S at the steady-state low Ca^{2+} concentrations but is suppressed at high Ca^{2+} concentrations. In the absence of PLP H_2S production maintains the suppressed levels at high Ca^{2+} concentrations and decreased further at low Ca^{2+} concentrations. These observations suggest that CSE produces H_2S at the steady-state in cells and that the production is suppressed when the intracellular Ca^{2+} concentrations are increased.

1. Introduction

H_2S facilitates the induction of hippocampal long-term potentiation (LTP), a synaptic model of memory formation, by enhancing the activity of *N*-methyl D-aspartate (NMDA) receptors and induces Ca^{2+} waves in astrocytes, which, in turn, modulate synaptic activity [1,2]. H_2S relaxes smooth muscle in various tissues including vasculature, regulates insulin release, and facilitates angiogenesis [3–8]. In addition, H_2S was found to have a cytoprotective effect on neurons against oxidative stress [9,10]. This finding led to the identification of its protective effect on various tissues against several insults including ischemia-reperfusion injury [11–13].

H_2S is synthesized by three enzymes: cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE), and 3-mercaptopyruvate sulfurtransferase (3MST) along with cysteine aminotransferase (CAT) or D-amino acid oxidase (DAO) [1,3,13–18]. H_2S production by CBS is enhanced by *S*-adenosyl methionine, and the 3MST/CAT pathway is regulated by Ca^{2+} [1,12]. Although the activity of CSE was reported to be regulated by Ca^{2+}/calmodulin, it was estimated in the presence of 1–2 mM Ca^{2+} that are too high as intracellular Ca^{2+} concentrations [19]. Because the intracellular Ca^{2+} concentrations are between 100 nM and 3 μM, it is necessary to re-evaluate the regulation of CSE activity by Ca^{2+}. The present study shows that CSE produces H_2S at the steady-state low Ca^{2+} concentrations and that the production is suppressed by increased Ca^{2+}. We also found that calmodulin is not involved in the regulation of CSE activity.

2. Materials and methods

2.1. Chemicals

β-Chloro-L-alanine hydrochloride (β-CA), peroxidase (POD), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pyridoxal 5′-phosphate (PLP), pyruvate oxidase (POP), thiamine pyrophosphate (TPP) and Tween 20 were purchased from Sigma Aldrich (St. Louis, MO, USA). Ammonium sulfate, calcium chloride ($CaCl_2$), *N*-(carboxymethyl-aminocarbonyl)-4,4′-bis(dimethylamino)-diphenylamine sodium salt (leuco dye; DA-64), citric acid monohydrate, L-cysteine hydrochloride monohydrate, cystine, disodium hydrogenphosphate 12-water (Na_2HPO_4 $12H_2O$), 4,4′-Dithiopyridine (4-PDS), dithiothreitol (DTT), ethanol, magnesium chloride ($MgCl_2$), sodium dihydrogenphosphate dihydrate (NaH_2PO_4 $2H_2O$), sodium hydroxide (NaOH), sodium sulfide nonahydrate (Na_2S $9H_2O$) and sucrose were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Ethylenediaminetetraacetic acid (EDTA) and 3-(*N*-morpholino) propane sulfonic acid (MOPS) were purchased from dojindo (Kumamoto, Japan).

2.2. Purification of CSE

All animal procedures were approved by the National Institute of Neuroscience Animal Care and Use Committee.

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http://dx.doi.org/10.1016/j.bbrc.2013.01.010

CSE was prepared according to the previous procedure [20]. Liver was obtained from 8-week-old male Wistar rats starved 24 h (Japan Clea, Tokyo, Japan). Rats were anesthetized with pentobarbital (5 mg/100 g body weight), and liver was perfused with PBS through portal vein. Twenty gram liver was homogenized in 50 ml buffer containing 0.25 M sucrose, 1 mM DTT and 2 mM EDTA with a Potter type homogenizer and a Teflon pestle (800 rpm, 10 strokes) on ice. Homogenates were centrifuged at 5000*g* for 20 min at 4 °C (M-201 IVD, Sakuma, Tokyo, Japan), and the supernatant was centrifuged at 105,000*g* for 60 min at 4 °C with an angle headed rotor (Beckman type 41) by an ultracentrifuge (Optima XL-100 K; Beckman coulter, Brea, CA, USA).

Solid ammonium sulfate was gradually added to the supernatant stirring with a magnetic stirrer until the final concentration reached 50%. After 2 h stirring, the solution was centrifuged at 10,000*g* for 60 min at 4 °C. Solid ammonium sulfate was added to the supernatant to make the final concentration of 75% and stirred overnight at 4 °C. The solution was centrifuged at 10,000*g* for 60 min at 4 °C, and the precipitates were dissolved in 40 ml of 100 mM sodium phosphate buffer (pH7.5) containing 1 mM DTT, 1 mM EDTA and 20 μM PLP (buffer A), then 36 ml of cold ethanol (−20 °C) was gradually added by stirring at −5 °C until the ethanol concentration reached 47.4%. After stirring for 25 min, the sample was centrifuged at 10,000*g* for 60 min at 4 °C. After removing ethanol by blowing nitrogen gas, the precipitates were dissolved in 5 ml buffer A, and homogenized with a Potter type homogenizer and a Teflon pestle (300 rpm, 10 strokes). The homogenates were centrifuged at 10,000*g* for 60 min at 4 °C. The supernatant was collected, while the precipitates were dissolved again in 5 ml of buffer A and repeated the above procedure. The resultant supernatant was pooled with the previous supernatant, and buffer A was added to reach 40 ml.

Thirty-six ml of cold ethanol (−20 °C) was added to the 40 ml solution with stirring at −5 °C. After 25 min stirring, the sample was centrifuged at 10,000*g* for 60 min at 4 °C. The precipitates were dissolved in 2 ml 10 mM phosphate buffer (pH 7.5: buffer B) containing 1 mM DTT and 20 μM PLP. The procedure was repeated, and supernatants were collected. The supernatants were dialyzed with 2000 ml buffer B overnight and centrifuged at 10,000*g* for 60 min at 4 °C.

The supernatant was applied to hydroxyapatite column and washed with 2 volumes of 50 mM phosphate buffer (pH 7.5) containing 1 mM DTT and 20 μM PLP then 2 volumes of 100 mM phosphate buffer (pH7.5) containing 1 mM DTT and 20 μM PLP. The column was eluted with 300 mM phosphate buffer (pH 7.5) containing 1 mM DTT and 20 μM PLP to collect 2 ml each fraction, of which the CSE activity was evaluated.

Ammonium sulfate was gradually added to the active CSE fractions with stirring to reach 2 M. The resultant solution was applied to phenylsepharose column and washed with 2 volumes of 50 mM phosphate buffer (pH 7.5) containing 0.7 M ammonium sulfate, 1 mM DTT and 20 μM PLP, the eluent was obtained with 50 mM phosphate buffer (pH 7.5) containing 0.3 M ammonium sulfate, 1 mM DTT and 20 μM PLP. The active fraction was obtained as CSE fraction.

2.3. CSE activity assay

The activity of CSE was assayed by the previous procedure [20]. Ten microlitres of sample and 15 μl of 100 mM β-CA were added to 85 μl of 100 mM Tris–phosphate (pH 8.0) containing 2.35 mM EDTA and 35 μM PLP, and the mixture was incubated at 37 °C for 15 min. The reaction was terminated by adding 150 μl of 6 mM 4-PDS. After 5 min, 52 μl of the resulting solution was mixed with 140 μl of color-producing reagent containing 0.1 M PIPES (pH 6.4), 0.823 mM TPP, 6.857 mM Mg^{2+}, 0.274 U/ml POD, 0.274 U/ml DA-64, and 10.94 U/ml of POP. The reaction mixture was then incubated at 37 °C for 10 min, and the absorbance was measured at 727 nm using a Bio-Rad Benchmark Plus microplate spectrophotometer (Bio-Rad).

2.4. H_2S production assay

The enzymatic production of H_2S was performed as described [16]. The substrate was added to 100 μl of purified CSE in a 15 ml centrifugation tube and incubated at 37 °C. The reaction was terminated by adding 200 μl of 1 M sodium citrate buffer (pH 6.0). The mixtures were incubated at 37 °C for 10 min with shaking on a rotary shaker NR-3 (TAITEC, Saitama, Japan) to facilitate a release of H_2S gas from the aqueous phase. Two microlitres of approximate 14.5 ml of head-space gas was applied to a gas chromatograph (GC-2014; Shimadzu, Kyoto, Japan) equipped with a flame photometric detector and a data processor C-R8A Chomatopac (Shimadzu). The standard curve for H_2S was prepared by using Na_2S, a source of H_2S, as described [21].

2.5. Spectroscopic methods

The spectral changes of purified CSE were recorded after the incubation with 2 mM cysteine for 16 min at 37 °C with a spectrophotometer DU730 (Beckman Coulter, Brea, CA, USA).

2.6. Western blot analysis

The samples were separated by SDS–PAGE with a 15% polyacrylamide gel (DRC, Tokyo, Japan) and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Benford, MA). After blocking with a blocking solution containing 3% skim milk (Becton Dickinson, Sparks, MD) and 0.1% Tween 20 in PBS (PBST) for 1 h, the membrane was incubated with either rabbit anti-CSE polyclonal antibody (1:5000) [22], rabbit anti-CBS polyclonal antibody (1:3000) [23] or rabbit anti-rat 3MST antibody (1:3000) [24] at 4 °C overnight. For the detection of cysteine aminotransferase (CAT), after blocking with Blocking Reagent (Roch Diagnostics GmbH, Mannheim Germany), the membrane was incubated with either sheep anti-aspartate aminotransferase (AST) (cytosolic CAT) polyclonal antibody (1:30,000) (Rockland, Gilvertsville, PA, USA) or sheep anti-GOT2 (mitochondrial CAT) polyclonal antibody (1:20,000) (Lifespan biosciences, Victoria, BC, Canada) at 4 °C overnight. The membrane was washed with PBST and incubated with a horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000; GE Healthcare Bio-Science) or anti-sheep IgG (1:10,000; Rockland) in PBST for 1 h at room temperature. The horseradish peroxidase was visualized using Millipore Immobilon Western Chemiluminescent substrate (Milipore). The chemiluminescence was captured on Amersham Hyperfilm™ ECL (GE Healthcare Bio-Science).

2.7. Staticics

All statistical analyses of the data were performed using Microsoft Excel 2004 for Mac (Microsoft, Redmond, WA, USA) with the add-in software Statcel2 (OMS, Saitama, Japan). Differences between two groups were analyzed with Student's *t* test.

3. Results and discussion

3.1. Kinetic properties of cystathionine γ-lyase (CSE)

Although the kinetic properties of CSE in H_2S production were previously reported using recombinant CSE expressed in *Escherichia coli.* [14,25,26], it was necessary to determine the H_2S pro-

ducing activity of purified CSE from mammalian tissues. We confirmed that purified rat liver CSE, which was not contaminated with other H_2S-producing enzymes such as 3MST, CBS, and cytosolic- and mitochondrial-CAT, produced H_2S from cysteine in a concentration-dependent manner (Fig. 1A and B).

The Lineweaver–Burk plot analysis showed that Mikaelis constant (K_m) value of CSE for cysteine was 2.56 ± 0.11 mM, and V_{max} value of cysteine for H_2S production was 51.6 ± 5.1 nmol min^{-1} - mg^{-1} (Fig. 1C and D). The present K_m value falls within the range of the previously estimated values with recombinant human CSE (1.7–3.5 mM) [14,25,26].

Because CSE has a higher affinity to cystine than cysteine, we examined the kinetic properties of CSE with cystine as a substrate. CSE produced H_2S from cystine in a concentration dependent manner (Fig. 1E). The K_m value of CSE for cystine was 0.19 ± 0.02 mM, and V_{max} value of cystine for H_2S production was 20.1 ± 2.5 nmol min^{-1} mg^{-1} (Fig. 1F). The present K_m value is approximately twice as great as that previously reported rat and cat liver CSE for cystine, 0.07 mM and 0.09 mM, respectively [27,28]. The discrepancy may be caused by the assays used; we determined it with H_2S production, while they did it with pyruvate-production. These observations suggest that CSE has an affinity to cystine approximately 11 times as high as that to cysteine. However, cystine is mainly localized to the extracellular fluid, while cysteine is a dominant form in cytosol. Therefore, cysteine must be a substrate for CSE in cells rather than cystine.

3.2. H_2S producing activity of CSE is suppressed by Ca^{2+}

Although it was reported that Ca^{2+}/calmodulin regulates H_2S producing activity of CSE, the activity was examined in the presence of 1–2 mM Ca^{2+} in the previous study [19]. The intracellular concentration of Ca^{2+} is approximately 100 nM in the steady-state and is increased to 2–3 μM in the activated cells [29]. Therefore, it is necessary to re-evaluate the effect of Ca^{2+} and calmodulin on CSE activity. Because CSE is a PLP-dependent enzyme, we examined the effect of Ca^{2+} on CSE activity in the presence of PLP [30–32]. H_2S was efficiently produced at 0–100 nM Ca^{2+}, but the production

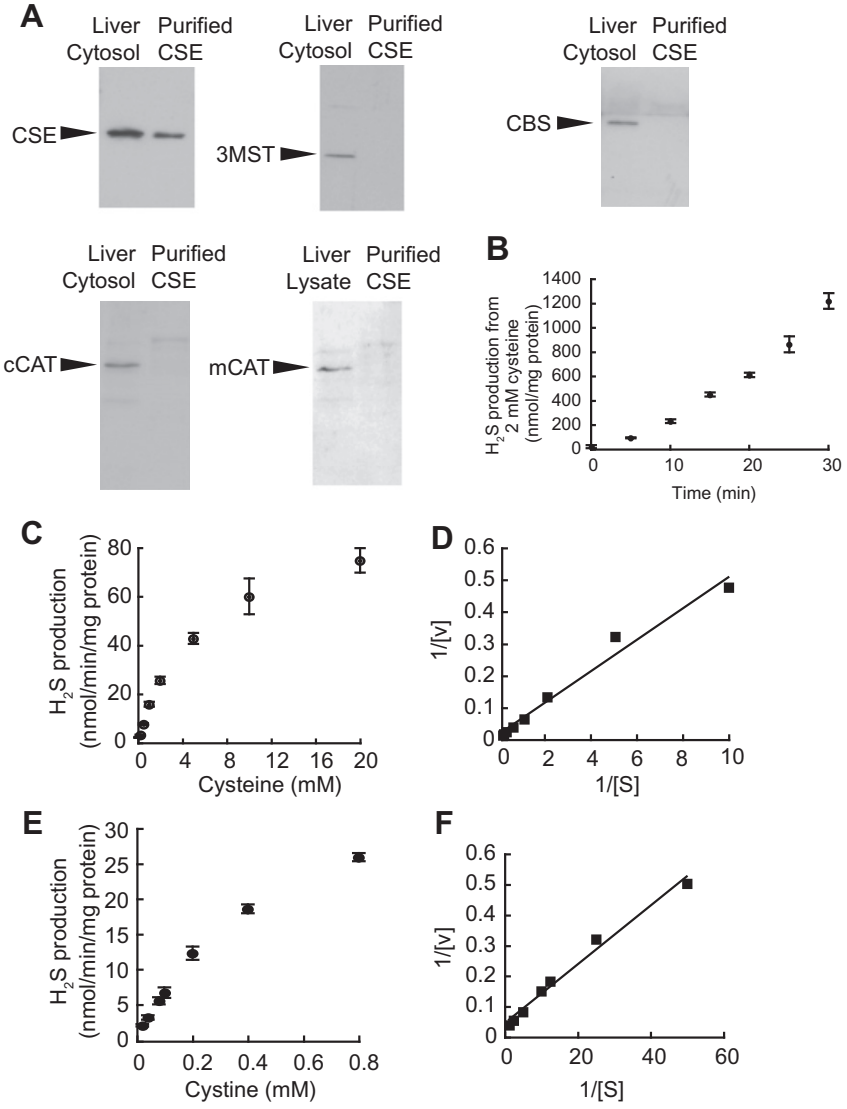

Fig. 1. The kinetic properties of purified rat liver CSE. (A) Western blot analysis of purified CSE with antibodies against CSE, 3MST, CBS, cytosolic and mitochondrial CAT. Two point five μg of liver cytosolic fraction was applied for all left lanes except for detection of mCAT for which 2.5 μg whole liver lysates were applied. Twenty five ng purified CSE was applied for the detection of CSE, and 2.5 μg of purified CSE was applied for the detection of 3MST, CBS and cytosolic CAT (cCAT) and mitochondrial CAT (mCAT) to each right lane. (B) H_2S production measured with purified CSE in the presence of 2 mM cysteine. (C–F) Kinetic properties of CSE in H_2S production from cysteine (C and D) and cystine (E and F). Purified rat liver CSE was mixed with cysteine (C) or cystine (E), and the amount of produced H_2S was measured. The data were analyzed by Lineweaver–Burk plot analysis (D and F). All data are represented as the mean ± S.E.M of three experiments.

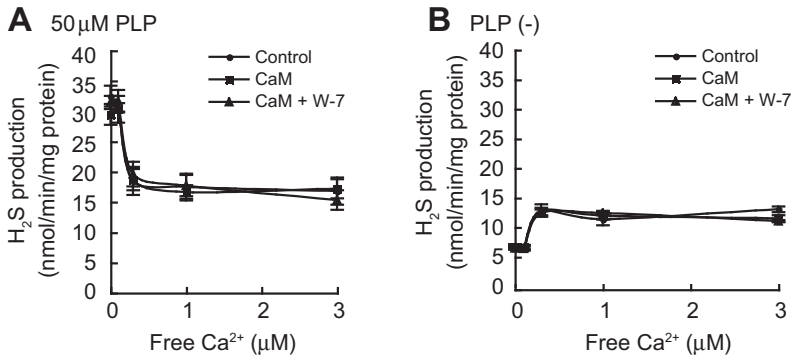

Fig. 2. H_2S production by CSE is suppressed by Ca^{2+} in the presence of PLP. (A) Ca^{2+}-dependency of H_2S production in the presence of PLP. H_2S production by purified rat liver CSE in the presence of 50 μM PLP and 2 mM cysteine was measured. (B) H_2S production by CSE in the absence of PLP. Note that H_2S production shows a similar level at Ca^{2+} concentrations greater than 300 nM irrespective of PLP. The production was not changed even in the presence or absence of 1 μM calmodulin or 100 μM W-7, a calmodulin inhibitor. All results are indicated as means ± SEM of at least three experiments.

was decreased at 300 nM Ca^{2+}. The state of suppression was maintained up to 3 μM Ca^{2+} (Fig. 2A). The involvement of calmodulin in H_2S production was also examined. Neither calmodulin nor a calmodulin specific inhibitor, W-7, has any effect on the production of H_2S (Fig. 2A). These observations suggest that H_2S is produced at a steady-state in cells and that the production is suppressed when the intracellular Ca^{2+} concentrations are increased.

PLP is essential for the metabolism of amino acids, and PLP deficiency leads to a lot of physical and psychological disorders [33,34]. We also examined the effect of Ca^{2+} on CSE activity in the absence of PLP. At Ca^{2+} concentrations between 300 nM and 3 μM, H_2S production in the absence of PLP was maintained at the same level with that in the presence of PLP (Fig. 2). However, H_2S production was decreased at 0–100 nM Ca^{2+} in the absence of PLP. Either calmodulin or W-7 did not have any effect on the production of H_2S even in the absence of PLP (Fig. 2B). These observations suggest that H_2S production by CSE at the steady state low Ca^{2+} concentrations greatly depends on PLP.

PLP, which is in Schiff-base linkage with CSE, dissociates from CSE to form a new linkage with a substrate cysteine and produces H_2S. CSE linked with PLP exhibits a characteristic internal aldimine absorbance peak at 427 nm, while a peak disappears in the presence of cysteine [25]. Because it is possible that Ca^{2+} facilitates the linkage formation between cysteine and PLP, we examined the effect of Ca^{2+} on the dissociation of PLP from CSE in the presence of cysteine. The amplitude of 427 nm peak was decreased in the absence of Ca^{2+}, while the peak remained in the presence of 3 μM Ca^{2+} (Fig. 3). These observations suggest that the steady state low Ca^{2+} concentrations facilitate the linkage formation between cysteine and PLP, resulting in enhancing H_2S production, while the linkage formation is suppressed when the Ca^{2+} concentrations are increased, leading to the suppression of H_2S production.

In conclusion, CSE produces H_2S at the steady-state low Ca^{2+} concentrations in cells and that the production is suppressed when the intracellular Ca^{2+} concentrations are increased. Calmodulin is not involved in H_2S production by CSE.

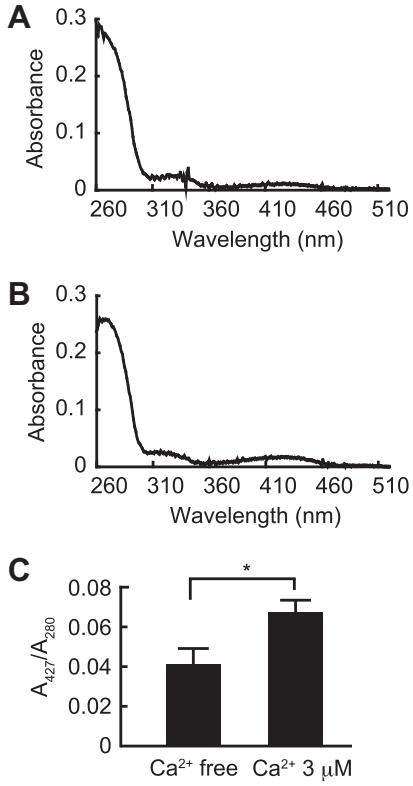

Fig. 3. PLP dissociates from CSE at low Ca^{2+} concentrations to produce H_2S. (A) PLP dissociates from CSE in the absence of Ca^{2+}. After 16 min of incubation with 2 mM cysteine, a characteristic internal aldimine absorbance peak at 427 nm was disappeared. (B) PLP maintains its linkage to CSE in the presence of 3 μM Ca^{2+}. (C) The A_{427}/A_{280} ratios in the presence or absence of Ca^{2+}. All results are indicated as means ± SEM of at least three experiments $P < 0.05$.

Acknowledgments

This work was supported by a grant from National Institute of Neuroscience and by JSPS KAKENHI Grant Number 23659089 from Grant-in-Aid for Challenging Exploratory Research to H.K., by JSPS KAKENHI Grant Number 23790316 from Grant-in-Aid for Young Scientists (B) to Y.M. and by JSPS KAKENHI Grant Number 23700434 from Grant-in-Aid for Young Scientists (B) to N.S.

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