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Assay Methods for H₂S Biogenesis and Catabolism Enzymes

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Abstract

H₂S is produced from sulfur-containing amino acids, cysteine and homocysteine, or a catabolite, 3-mercaptopyruvate, by three known enzymes: cystathionine β -synthase, γ -cystathionase, and 3-mercaptopyruvate sulfurtransferase. Of these, the first two enzymes reside in the cytoplasm and comprise the transsulfuration pathway, while the third enzyme is found both in the cytoplasm and in the mitochondrion. The following mitochondrial enzymes oxidize H₂S: sulfide quinone oxidoreductase, sulfur dioxygenase, rhodanese, and sulfite oxidase. The products of the sulfide oxidation pathway are thiosulfate and sulfate. Assays for enzymes involved in the production and oxidative clearance of sulfide to thiosulfate are described in this chapter.

1. INTRODUCTION

The steady-state intracellular concentrations of H₂S are the product of its enzymatic generation and clearance rates (Kabil, Motl, & Banerjee, 2014; Vitvitsky, Kabil, & Banerjee, 2012). H₂S is synthesized by at least three known enzymes: cystathionine β -synthase (CBS), γ -cystathionase (CSE), and mercaptopyruvate sulfurtransferase (MST) (Kabil & Banerjee, 2014). Of these, the first two constitute the cytoplasmic transsulfuration pathway, while the third is found in the cytoplasm and in mitochondria and is part of the cysteine catabolic pathway (Fig. 1). The contribution of each of these enzymes to net H₂S production is dictated by its presence and relative tissue concentration, which varies in a cell-specific manner (Chiku et al., 2009; Kabil, Vitvitsky, Xie, & Banerjee, 2011; Singh, Padovani, Leslie, Chiku, & Banerjee, 2009; Yadav, Yamada, Chiku, Koutmos, & Banerjee, 2013). The enzymes in the mitochondrial sulfide oxidation pathway convert H₂S to thiosulfate and sulfate, which are subsequently eliminated. The component enzymes include sulfide quinone oxidoreductase (SQR), sulfur dioxygenase (also known as ETHE1 or persulfide dioxygenase), rhodanese (or thiol sulfurtransferase), and sulfite oxidase (Fig. 1). Of these, SQR is anchored in the inner mitochondrial membrane, rhodanese and sulfur dioxygenase are in the mitochondrial matrix, and sulfite oxidase is in the intermitochondrial membrane space. The overlapping substrate specificities combined with the substrate ambiguity of CBS and CSE have confounded assessment of their contributions to H₂S production in cell and tissue samples. Similarly, multiple acceptors of the persulfide moiety generated in the SQR-, rhodanese-, and MST-catalyzed reactions complicate their enzymatic

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assays and interpretation of the organizational logic of the sulfide oxidation pathway (Hildebrandt & Grieshaber, 2008; Jackson, Melideo, & Jorns, 2012; Libiad, Yadav, Vitvitsky, Martinov, & Banerjee, 2014). Methods for assaying the individual enzymes are discussed in this chapter.

2. ASSAYS FOR H₂S BIOGENESIS

CBS and CSE catalyze the successive conversion of homocysteine to cystathionine and then cysteine in the transsulfuration pathway (Eqs. 1 and 2). The substrate ambiguity of each enzyme supports multiple H₂S-generating reactions in which the amino acids cysteine and homocysteine are utilized as the sulfur source. CBS catalyzes the β -replacement of cysteine by homocysteine (Eq. 3), cysteine by water (Eq. 4) or cysteine by a second mole of cysteine (Eq. 5) generating H₂S and the corresponding products. CSE catalyzes the α,β -cleavage of cysteine (Eq. 6), the α,γ -cleavage of homocysteine (Eq. 7), and the γ -replacement of homocysteine by a second mole of homocysteine (Eq. 8) generating H₂S. In addition, CSE, like CBS, catalyzes the condensation of two moles of cysteine (Eq. 5) and of cysteine and homocysteine (Eq. 3), producing H₂S:

Under $V_{\max}$ conditions, the k_{cat} for the CBS-catalyzed reactions decrease in the following order: (3) [61 $\times$] $\gg$ (4) [1.6 $\times$] > (5) [1 $\times$] where the round and square brackets denote the reaction number and the relative rate constants with the slowest reaction designated as 1 $\times$. Under $V_{\max}$ conditions, the k_{cat} for the CSE-catalyzed H₂S-generating reactions show the following order: (6) [270,000 $\times$] > (7) [111,500 $\times$] $\gg$ (8) [1400 $\times$] $\gg$ (3) [50 $\times$] $\gg$ (5) [1 $\times$].

MST is a sulfurtransferase that transfers the sulfur from mercaptopyruvate to an active site cysteine to form a persulfide intermediate, MST-SSH (Eq. 9). A variety of small molecules

in addition to thioredoxin can accept the persulfide group and in the presence of a reductant, H₂S is released (Eq. 10):

2.1. Assays for CBS and CSE

2.1.1 H₂S formation from cysteine or cysteine+homocysteine

Reagents

- 200 mM HEPES buffer, pH 7.4
- 400 mM D,L-homocysteine (pH adjusted to 7.4 with 10 M NaOH)
- 1 M L-cysteine
- 40 mM lead acetate
- 2.0 mg ml⁻¹ purified human CBS or purified human CSE in 100 mM HEPES buffer, pH 7.4
- Deionized water

Method: In a polystyrene cuvette, add 500 µl HEPES buffer, 125 µl homocysteine, and 25 µl of cysteine or only 25 µl cysteine, 10 µl lead acetate, and water to bring the reaction volume to 990 µl. Place the cuvette in a spectrophotometer for 5 min at 37 °C, maintained using a circulating water bath. Initiate the reaction with 10 µl of CBS or 10 µl of CSE and monitor the increase in absorbance at 390 nm due to formation of lead sulfide. From the slope of the absorbance change, calculate the specific activity of CBS or CSE using a molar extinction coefficient of 5500 M⁻¹ cm⁻¹ for lead sulfide (Singh et al., 2009).

2.1.2 Methanethiol formation from methylcysteine

Reagents

- 200 mM HEPES buffer, pH 7.4
- 500 mM methylcysteine
- 4.0 mg ml⁻¹ purified human CBS or purified human CSE in 100 mM HEPES, pH 7.4
- 50 mM DTNB (dithiobisnitrobenzoic acid)
- Deionized water

Method: In a polystyrene cuvette, add 500 µl HEPES buffer, 20 µl methylcysteine, 10 µl DTNB, and water to bring the reaction volume to 990 µl. Place the cuvette in a spectrophotometer connected to a water bath maintained at 37 °C for 5 min. Add 10 µl CBS or CSE and monitor the increase in absorbance at 412 nm due to generation of the

nitrobenzene thiolate anion (which forms due to the reaction of the methanethiol product with DTNB). From the slope of the absorbance change, calculate the specific activity of CBS or CSE using a molar extinction coefficient of $13,600\text{ M}^{-1}\text{ cm}^{-1}$.

2.1.3 Assessing H₂S production by CBS versus CSE in tissue samples

Reagents

- 300 mM propargylglycine
- 200 mM HEPES buffer, pH 7.4
- 400 mM D,L-homocysteine
- 400 mM L-cysteine
- 40 mM lead acetate
- 100 mg ml⁻¹ tissue extract in 100 mM HEPES buffer, pH 7.4
- Deionized water

Method: Using a glass homogenizer, disrupt frozen tissue in 200 mM HEPES, pH 7.4, to obtain a concentration of 100 mg tissue wet weight ml⁻¹. Incubate on ice for 20 min. In a polystyrene cuvette, add 815 µl HEPES buffer, 10 µl lead acetate, and 100 µl tissue extract (total volume 925 µl). Place the cuvette in a cuvette holder for 5 min, at 37 °C maintained using a circulating water bath. Initiate the reaction by adding a mixture of 50 µl homocysteine and 25 µl cysteine (to obtain 10 mM final concentration each of the L-form of the amino acid) and monitor the increase in absorbance at 390 nm due to formation of lead sulfide. From the slope of the absorbance change, calculate the specific activity using a molar extinction coefficient of $5500\text{ M}^{-1}\text{ cm}^{-1}$ for lead sulfide.

To determine the contribution of CBS activity only, preincubate 100 µl tissue extract with 10 µl of propargylglycine, a suicide inactivator of CSE (Abeles & Walsh, 1973), for 5 min on ice before using it in the above reaction. The total H₂S production rate, contributed by CBS and CSE, is obtained from the reaction rate in the absence of propargylglycine. Calculate the contribution of CBS to total H₂S production from the assay in the presence of propargylglycine. Subtract the contribution of CBS from the total H₂S production rate to determine the contribution of CSE.

2.2. Assays for MST

2.2.1 MST assay using small molecule acceptors

Reagents

- 400 mM HEPES buffer, pH 7.4
- 30 mM 3-mercaptopyruvate
- 400 mM D,L-homocysteine
- 400 mM dihydrolipoic acid, pH adjusted to ~7.4 by using 10 M NaOH
- 400 mM glutathione

- 1 M L-cysteine
- 10 mg ml⁻¹ bovine serum albumin
- 40 mM lead acetate
- 1.0 mg ml⁻¹ purified MST in 100 mM Tris, pH 8.0
- Deionized water

Method: In a polystyrene cuvette, add 500 µl HEPES buffer, 10 µl 2-mercaptopyruvate, 10 µl bovine serum albumin, 10 µl lead acetate, and 50 µl dihydrolipoic acid, or 100 µl homocysteine, or 25 µl cysteine or 125 µl GSH. Adjust the reaction volume to 990 µl using deionized water and place the cuvette in spectrophotometer with a water-jacketed cuvette holder maintained at 37 °C. Add 10 µl of MST and monitor the increase in absorbance at 390 nm due to formation of lead sulfide. From the linear portion of the reaction curve, calculate the specific activity of MST using a molar extinction coefficient of 5500 M⁻¹ cm⁻¹ for lead sulfide (Yadav et al., 2013).

2.2.2 MST assay using thioredoxin

Reagents

- 400 mM HEPES buffer, pH 7.4
- 300 mM sodium mercaptopyruvate
- 1 mM purified human thioredoxin
- 180 µM purified human thioredoxin reductase
- 20 mM NADPH
- 40 mM lead acetate
- 10 mg ml⁻¹ bovine serum albumin
- 1.0 mg ml⁻¹ purified human MST in 100 mM Tris, pH 8.0
- Deionized water

Method: In a polystyrene cuvette, add 500 µl of HEPES buffer, 10 µl 3-mercaptopyruvate, 20 µl thioredoxin, 20 µl thioredoxin reductase, 10 µl NADPH, 10 µl bovine serum albumin, and 10 µl of lead acetate. Adjust the volume of the reaction mixture to 990 µl with deionized water and place the cuvette for 5 min in a spectrophotometer with a waterjacketed cuvette holder maintained at 37 °C. Add 10 µl of purified MST and monitor the increase in absorbance at 390 nm due to formation of lead sulfide. From the linear portion the reaction curve, calculate the specific activity of MST using a molar extinction coefficient of 5500 M⁻¹ cm⁻¹ for lead sulfide (Yadav et al., 2013).

3. ASSAYS FOR ENZYMES INVOLVED IN H₂S CATABOLISM

The mitochondrial sulfide oxidation pathway converts sulfide to thiosulfate and sulfate, which are eliminated. SQR catalyzes the first step in the pathway, which involves a two-

electron oxidation of sulfide to persulfide and proceeds via an enzyme-bound sulfane sulfur intermediate. The physiologically relevant sulfide acceptor of the reaction is controversial, and sulfide, thiols (Eq. 11), and sulfite all function as acceptors in the *in vitro* assay. The electrons are transferred from SQR, a flavoprotein, to coenzyme Q (Eq. 12) and thence, to the electron transfer chain:

Sulfur dioxygenase catalyzes the oxygen-dependent four-electron oxidation of glutathione persulfide (GSSH) to sulfite (Eq. 13). The enzyme belongs to the family of 3His-1Asp mononuclear iron oxygenases (Kabil & Banerjee, 2012).

Rhodanese or thiosulfate sulfurtransferase catalyzes a transsulfuration reaction using a variety of sulfur donors and acceptors. The common method for assaying rhodanese was described by Sörbo (1955) and monitors the transfer of the sulfane sulfur from thiosulfate to cyanide and yields sulfite and thiocyanate (Eq. 14). Other sulfur donors in the rhodanese-catalyzed reaction include thiosulfonates and GSSH (Eq. 15), while compounds such as sulfite (Eq. 15), glutathione, cysteine, and homocysteine can serve as sulfur acceptors:

3.1. Assay for SQR

Reagents

- 200 mM sodium phosphate buffer, pH 7.4, containing 0.06% 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine
- 15 mM sodium sulfide
- 100 mM sodium sulfite
- 40 mM CoQ₁ in DMSO
- 10 mg ml⁻¹ bovine serum albumin
- 400 mM glutathione
- 1 M L-cysteine
- 400 mM D,L-homocysteine, pH adjusted to ~7.4 by using 10 M NaOH

- 0.01 mg ml⁻¹ purified SQR in 100 mM Tris, pH 8.0, containing 0.03% 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine
- Deionized water

Method: In a polystyrene cuvette, add 500 µl of sodiumphosphate buffer, 1.5 µl CoQ₁, and 10 µl bovine serum albumin and mix by pipetting. Add 10 µl of sulfite or 125 µl glutathione or 50 µl cysteine or 250µl homocysteine (Libiad et al., 2014). Adjust the reaction volume to 985 µl using deionized water. Incubate the reaction mixture in a water-jacketed cuvette holder maintained at 25 °C for 3 min. Add 5 µl of SQR and 10 µl of sulfide simultaneously using two pipettes and monitor the decrease in absorbance at 278 nm due to reduction of CoQ₁. From the slope of the reaction progress, calculate the specific activity using the molar extinction coefficient ($\epsilon_{\text{ox-red}} = 12,000 \text{ M}^{-1} \text{ cm}^{-1}$) for reduction of CoQ₁ at 278 nm (Jackson et al., 2012).

3.2. Assay for sulfur dioxygenase (or persulfide dioxygenase or ETHE1)

3.2.1 Preparation of GSSH

Reagents

- 350 mM sodium phosphate, pH 7.4
- Solid sodium sulfide
- 50 mM GSSG dissolved in anaerobic 350 mM sodium phosphate buffer, pH 7.4

Method: In an anaerobic glass vial, add 5 ml GSSG and add 240 mg of solid sodium sulfide (to obtain a final concentration of 200 mM). Seal the reaction vial to prevent loss of H₂S, remove the vial from the anaerobic chamber and incubate at 37 °C for 20 min. Measure the concentration of the GSSH product using the cold cyanolysis method as described (Wood, 1987).

3.2.2 Oxygen consumption assay

Reagents

- 100 mM sodium phosphate, pH 7.4
- 50 mM GSSH
- 0.5–2 µg sulfur dioxygenase in 50 mM Tris, pH 8.0, containing 0.5 M NaCl
- Deionized water

Method: In a Gilson-type chamber containing a Clark oxygen electrode and a magnetic stir bar washed with deionized water, add 1.5 ml of sodium phosphate. Cap the chamber to prevent additional O₂ from dissolving into the buffer and inject 0.5–2 µg of sulfur dioxygenase. Initiate the reaction by addition of 30 µl GSSH. Record oxygen consumption using a Kipp and Zonen BD single channel chart recorder. From the linear portion of the curve, calculate the rate of oxygen consumption. The concentration of dissolved O₂ at room temperature is ~280 µM. The rate of oxygen consumption is reported in units of µmol O₂ consumed min⁻¹ mg of enzyme⁻¹.

3.3. Assays for rhodanese

3.3.1 Assay for thiocyanate formation by rhodanese

Reagents

- 1 M sodium thiosulfate
- 1 M potassium cyanide
- 15% (w/v) formaldehyde solution
- Ferric nitrate nonahydrate solution (6.6 g of $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ dissolved in 3.4 ml nitric acid. The final volume is adjusted to 50 ml).
- 300 mM HEPES buffer, pH 7.4, containing 150 mM NaCl.

Method: In a polystyrene cuvette add 12.5 μl of sodium thiosulfate, 12.5 μl of potassium cyanide, and 225 μl of HEPES buffer at 25 °C. The reaction is initiated by addition of 0.5 μg of rhodanese. After 5 min, the reaction is terminated by addition of 250 μl of 15% (w/v) formaldehyde and the reaction mixture is centrifuged for 5 min at $10,000 \times g$ to remove the protein. Addition of 0.5 ml of ferric nitrate to the supernatant results in the development of a red color due to ferric thiocyanate formation, which is monitored at 460 nm. A control reaction lacking rhodanese is run in parallel. The amount of thiocyanate formed is determined using a standard curve generated with thiocyanate ranging from 0.1 to 5 μmol . One unit of enzyme activity catalyzes the formation of 1 μmol of thiocyanate min^{-1} at 25 °C. The specific activity is expressed as units mg protein^{-1} .

3.3.2 Assay for thiosulfate production by rhodanese

Reagents

- 1 M sodium thiosulfate
- 100 mM sodium sulfite
- 50 mM GSSH (see Section 3.2.1 for preparation)
- 100 mM monobromobimane dissolved in DMSO
- 100% methanol
- 100% acetic acid
- 0.2 mM sodium citrate, pH 2.0
- 100 mM HEPES buffer, pH 7.4, containing 150 mM NaCl

Method: The assay mixture is made by mixing 2 μl of sodium sulfite, 8 μl GSSH in 200 μl of HEPES buffer, and 1 μg of rhodanese. The reaction is initiated by addition of 1 μg of rhodanese and incubated for 5 min at 25 °C followed by derivatization of samples with 2 μl of monobromobimane. Incubation is continued for 10 min prior to acidification with 100 μl of sodium citrate. A control reaction lacking rhodanese was prepared in parallel. The derivatized samples are centrifuged at $10,000 \times g$ for 10 min at 4 °C and 50 μl of the supernatant is injected onto a C8 reverse phase HPLC column (4.6×150 mm, 3 μm packing,

Phenomenex) pre-equilibrated with 80% solvent A (10% methanol and 0.25% acetic acid) and 20% of solvent B (90% methanol and 0.25% acetic acid). The sample is eluted using the following gradient: solvent B: 20% from 0 to 10 min, 20–40% from 10 to 25 min, 40–90% from 25 to 30 min, 90–100% from 30 to 32 min, 100% from 32 to 35 min, 100–20% from 35 to 37 min, and 20% from 37 to 40 min. The flow rate is 0.75 ml min⁻¹. The biman adduct of thiosulfate elutes at ~22 min under these conditions and is detected by excitation at 340 nm and emission at 450 nm. The concentration of thiosulfate is determined using thiosulfate standards of known concentration. One unit of enzyme activity catalyzes the formation of 1 μ mol of thiosulfate min⁻¹ at 25 °C. Specific activity is expressed as units mg⁻¹ protein.

3.3.3 Assay for H₂S production by rhodanese

Reagents

- 1 M sodium thiosulfate
- 200 mM glutathione
- 200 mM cysteine
- 200 mM homocysteine
- 100 mM lead acetate
- 100 mM HEPES buffer, pH 7.4, containing 150 mM NaCl

Method: Prepare the reaction mixture in a polystyrene cuvette by adding 1.5 μ l of thiosulfate, 50 μ l glutathione (or 50 μ l cysteine or 100 μ l homocysteine), 2 μ l lead acetate, and 350 μ l HEPES buffer in a final volume of 500 μ l. The cuvette is placed in a spectrophotometer maintained at 37 °C for 4 min. The reaction is initiated by addition of 1–10 μ g of rhodanese. The increase in absorbance at 390 nm due to formation of lead sulfide is monitored. The specific activity is calculated using a molar extinction coefficient of 5500 M⁻¹ cm⁻¹ for lead sulfide (Singh et al., 2009).

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Figure 1.

Scheme showing enzymes involved in H₂S biogenesis and oxidation. For simplicity, H₂S production by MST is shown in the mitochondrion only although the enzyme can be present in the cytoplasm and mitochondrion. CBS, CSE, CAT, MST, and SQR denote cystathionine β-synthase, γ-cystathionase, cysteine aminotransferase, mercaptopyruvate sulfur transferase, and sulfide quinone oxidoreductase, respectively. Electrons from the mitochondrial sulfide oxidation pathway are transferred via ubiquinone to complex III in the electron transfer chain.