The Crosstalk between Hydrogen Sulfide and Nitric Oxide Signaling Pathways

Jing Yuan Rebecca Wang

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The Crosstalk between H$_2$S and NO Signaling Pathways

By

Jing Yuan Rebecca Wang

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

2012

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“The Cross-Talk Between H₂S and NO Signaling Pathways”

by

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DECLARATION OF CO-AUTHORSHIP/PREVIOUS PUBLICATION

I hereby declare that this thesis incorporates material that is result of a joint research with Adam Faccenda under the supervision of Professor Bulent Mutus. The collaboration is covered in Chapter 2,3 and 4 of the thesis.

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ABSTRACT

Hydrogen sulfide (H$_2$S) produced in the reverse transsulfuration pathway is a significant signaling molecule. The two known pyridoxal-5'-phosphate (PLP)-dependent enzymes that catalyze H$_2$S generation in mammals are cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). The interaction between H$_2$S/CSE and NO/NOS systems, which have regulatory roles, has been recently discovered in mice. To study the effects of signaling molecules NO$_x$ on human recombinant cystathionine γ-lyase, recombinant CSE was incubated in the presence of low or high oxygen contents with NO donors diethylamine NONOate (DEANO) and S-Nitrosoglutathione (GSNO). The enzyme was then tested for H$_2$S production upon incubation with the CSE substrate, L-cysteine. A novel, real-time H$_2$S detection method using H$_2$S-permeable polydimethylsiloxane (PDMS) and thiol-reactive probe, Ellman's reagent, was utilized for enzyme activity measurement. These experiments reveal that the catalytic efficiency, $K_{cat}/K_M$, of substrate was found to be significantly affected by NO incubation. NO incubation decreased the enzyme activity by lowering the binding affinity of the substrate to the enzyme. At 2 ppm or 16 ppm oxygen concentration, NO released from NO donor was proposed to directly modify protein residues such as cysteine, tryptophan and tyrosine. In addition, inhibition of NO production in RAW 264.7 macrophage can induce H$_2$S production. These studies indicate a potential regulatory role of NO in H$_2$S production by either directly reacting with H$_2$S or modulating H$_2$S-producing pathways.
DEDICATION

I dedicate my dissertation work to my family – a special gratitude to my loving parents.
ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor, Dr. Mutus for his continuous support throughout my research and graduate studies. His patience, motivation, immense knowledge and bright ideas have helped me build a firm background in scientific research and laboratory skills. His guidance has helped me at all time, both in my research and in the writing of this thesis.

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<tr>
<td>3MST</td>
<td>3-Mercaptopyruvate sulfurtrnasferase</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CAT</td>
<td>Cysteine aminotransferase</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon Monoxide</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathionine β-synthase</td>
</tr>
<tr>
<td>CSE</td>
<td>Cystathionine γ-lyase</td>
</tr>
<tr>
<td>CysNO</td>
<td>S-nitrosocysteine</td>
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<tr>
<td>DEA/NO</td>
<td>Diethylamine NONOate</td>
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<td>DTNB</td>
<td>5,5'-dithio-bis-2-nitrobenzoic acid</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSNO</td>
<td>S-nitrosoglutathione</td>
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<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HS$^-$</td>
<td>Hydrosulfide Anion</td>
</tr>
<tr>
<td>H$_2$S</td>
<td>Hydrogen Sulfide</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>L-NAME</td>
<td>L-N$^G$-Nitroarginine methyl ester</td>
</tr>
<tr>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MB</td>
<td>Methylene Blue</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
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<td>Peroxynitrite</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
</tr>
<tr>
<td>PLP</td>
<td>Pyridoxal-5’-phosphate</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>RSNO</td>
<td>S-nitrosothiols</td>
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CHAPTER 1

GENERAL INTRODUCTION OF H₂S

1.1 General Introduction of H₂S

1.1.1 History of Hydrogen Sulfide and Existing H₂S in Physiological Environment

H₂S is known as a toxic gas with an odor resembling that of rotten eggs. It is a major component in the origin of life and it continues to be critically important for life on earth (1). Naturally occurring H₂S is often formed from the breakdown of the organic compounds by bacteria in the absence of oxygen. The first record observation of H₂S was in 1713. An Italian physician described in his book, Disease of workers, that workers who were exposed to an unknown acid had painful inflammation around their eyes. Later around 1750, a Swedish chemist accidentally mixed potassium ferrous sulfide with a mineral acid, forming a malodorous gas. This was the first occasion H₂S had been synthesized. In the 20th century, investigations on the biological effects of H₂S began (2).

In the past H₂S was most recognized for its toxicity which has been found to be achieved via its inhibitory interaction with hemoglobin and cytochrome c oxidase. To effect inhibition, the HS⁻ anion binds to ferric heme in cytochrome c oxidase. The blockage of mitochondrial cytochrome c oxidase by H₂S is believed to be the mechanism involved in lethal sulfide poisonings. This inhibition results in decreased oxidative phosphorylation, which will in turn lower the metabolic rate and body temperature, resulting in hypothermia (2). In contrast, physiological H₂S concentrations actually facilitate mitochondrial consumption and ATP production (3). The American conference
of Industrial Hygienists has set up a standard for the exposure limitation, called a threshold limit value (TLV). In the workplace, the H$_2$S TLV is 10 ppm in air for 8 h a day, five days a week over a working lifetime (2).

1.1.2 Physical properties and solubility of H$_2$S

Similar to other gaseous molecules, H$_2$S is lipophilic and freely penetrates plasma membranes. This is a very important property because it determines the traveling route and the extent of H$_2$S action. H$_2$S is twice as soluble in lipid membrane as in water. This high membrane permeability coefficient of H$_2$S displays a low barrier to intercellular transport.

In a physiological environment, H$_2$S is usually referred to as the total free sulfide pool of H$_2$S, HS$^-$ and S$^-$. H$_2$S is a weak acid with pK$_{a1}$ 6.9 and pK$_{a2}$ 11.96, and will dissociate to HS$^-$ and S$^-$ (Scheme 1) (4). At a pH of 7.4, the ratio of HS$^-$:H$_2$S is 1:3. At physiological pH, H$_2$S which is in the form of hydrosulfide accumulates near its source, due to the low diffusion rate resisted by lipid membrane (5). Free H$_2$S (H$_2$S + HS$^-$) concentration in humans varies. The concentration of total H$_2$S is estimated around 100 pM in blood, and 15 nM in tissue (6).

\[
pK_{a1} = 6.75 \quad pK_{a2} = 11.96
\]

\[
\begin{align*}
H_2S & \rightleftharpoons H^+ + HS^- \\
& \rightleftharpoons H^+ + S^{2-}
\end{align*}
\]

Scheme 1: H$_2$S dissociation in aqueous solution
1.1.3 H2S as a gasotransmitter

Recently, H2S has been recognized as a common component of mammalian tissues and cells; an interesting finding considering the longstanding reputation of H2S as a highly toxic compound. It may be important for homeostatic control and functions similar to other toxic gases including nitric oxide (NO) and carbon monoxide (CO) that also function as gasotransmitters. After careful consideration, H2S was deemed to fulfill all of the criteria that define a gasotransmitter (7). The term gasotransmitter refers to a gaseous molecule that is synthesized and released in a biological system and has a signal transduction function. The following are identification criteria for gasotransmitters. It must

1. be a gas
2. be endogenously and enzymatically generated in a regulated manner
3. cause, upon exogenous application, well-defined physiological effects at relevant concentrations that mimic the effect of the endogenously produced H2S on tissue activity
4. act on specific cellular targets
5. use a specific mechanism of inactivation (8).

According to the definition, H2S is a small gaseous molecule that is able to cross the lipid membrane. Since H2S is highly permeable in lipids and can penetrate cellular membranes, it is a good signaling molecule (9). It is enzymatically synthesized and its endogenous metabolism is tightly regulated. The cellular effects of H2S do not need second messenger or specific cognate membrane receptors, but it triggers defined signaling cascades directly (3).
Specific cellular targets of H$_2$S: Several reported receptors are found to be tissue- and species-dependent. For example, H$_2$S activates glutamate receptors during repetitive nerve stimulation via cAMP-dependent pathways, by opening several ion channels. (8,10,11).

As a result, H$_2$S has been identified as the third signaling gasotransmitter after NO and CO. Now, researchers are trying to determine the regulatory functions of H$_2$S by comparing it to the functions of NO and CO, as well as exploring the interactions between these three gasotransmitters (12,13).

1.1.4 H$_2$S in the biological system

A number of organisms have been shown to have the ability to produce and utilize H$_2$S (13). In animals, the highest production rate of H$_2$S was found in the brain, cardiovascular system, liver and kidney (14). The two known existing enzymes that can catalyze H$_2$S production in mammals are cystathionine β-synthase (CBS; EC 4.2.1.22) and cystathionine γ-lyase (CSE; EC 4.4.1.1). These two enzymes are involved in the H$_2$S production in the transsulfuration pathway. A third source of H$_2$S production is through the enzyme pair, 3-mercaptopryruvate sulfurtransferase and cysteine aminotransferase, through the catalysis transfer of sulfur to an unknown acceptor. This third source of H$_2$S generation is also known as cysteine catabolism (4). This H$_2$S production pathway is regulated by Ca$^{2+}$ and facilitated by thioredoxin and dihydrolipoic acid (15). The relative contribution of the third source of H$_2$S to signaling events are largely unknown (12). A minor component of H$_2$S in biological environment is produced nonenzymatically from
the reduction of elemental sulfur to H₂S using reducing equivalents obtained from the oxidation of glucose (16).

CBS is essentially responsible for the H₂S production in the central nervous system. In comparison, CSE chiefly generates H₂S outside of the cardiovascular system (13). MST is expressed in kidney, liver, cardiac tissue and in the brain (17). CBS and CSE are expressed in the cytoplasm, but have also been detected in the nucleus (4).

1.1.5 Transsulfuration and Reverse Transsulfuration of H₂S Production

Transsulfuration and reverse transsulfuration are the two alternate pathways for interconverting methionine and cysteine (18) (Figure 1.1). The reverse transsulfuration pathway normally takes place in mammals. This pathway involved in two major enzymes: CBS and CSE (19). Transsulfuration has the following steps. Serine and homocysteine formed from the methionine pathway would be catalyzed by CBS. The cystathionine produced by CBS will subsequently catalyzed by CSE to form cysteine (3). The resulting cysteine further undergoes cysteine catabolism to produce pyruvate and NH₃. In addition, CBS and CSE catalyze several reactions to produce H₂S, and other side products such as lanthionine, α-ketobutyrate, pyruvate as well as cystathionine. CBS participates in four major reactions which involve the β-carbon of cysteine (20). The chief route responsible for H₂S production is the condensation of homocysteine and cysteine. H₂S cannot be formed by homocysteine alone catalyzed by CBS (12). CSE catalyzes six H₂S-producing reactions which include α,β–elimination, β–replacement, α,γ–elimination, β or γ–replacement using cysteine and homocysteine (12). The major substrate responsible for H₂S production by CSE is cysteine (4).
The transsulfuration pathway is the reverse pathway of reverse transsulfuration. The \( \text{H}_2\text{S} \) biosynthesis pathways occur in prokaryotes, fungi, and plants. Cystathionine \( \gamma \)-synthase (CGS) and cystathionine \( \beta \)-lyase (CBL) are the two enzymes involved in this pathway (3).

![Transsulfuration Pathway Diagram](image)

**Figure 1.1 Transsulfuration pathway.** The top part depicts the transsulfuration pathway. CBS and CSE are involved in this pathway. The red arrows indicate the major \( \text{H}_2\text{S} \) production reaction for CBS and CSE (4).
1.1.6 H$_2$S Catabolism

Labeling with $^{35}$S sulfide was used as the earliest method to track the route taken by H$_2$S once it is produced. This labeling allowed the biochemists to determine that metabolized H$_2$S is located in mitochondria (4). Currently, there are two known H$_2$S catabolism pathways. The most common pathway is the oxidation of H$_2$S in mitochondria to produce persulfide, sulfite, thiolsulfate and sulfate. Some of these converting reactions are enzyme-dependent and some are not (4,14).

1.1.7 H$_2$S Measurement

With increasing knowledge of H$_2$S, an accurate and reliable method of measuring H$_2$S is an essential factor to determine its critical biological activities (21). H$_2$S exists in several ionic states associated with different environments. The stability is also influenced by different inorganic and organic compounds present in biological environments. These determinants make it difficult to make accurate measurements of H$_2$S. Several methods for measuring free H$_2$S are based on its derivatization to generate products for measurement by UV/Vis and fluorescence spectroscopy, or ion-specific electrodes (22-24). Many existing methods have been criticized for their overestimation of the concentration of H$_2$S in biological systems. Most of these methods rely on conversion of all sulfide species to H$_2$S or S$^{2-}$ under acidic or alkaline conditions and their indirect detection by trapping with metal ions to form stable, insoluble complexes. One of the most widely used methods is the methylene blue (MB) assay, where H$_2$S is reacted with $N,N$-dimethyl-$p$-phenylenediamine and ferric chloride in a strong acid solution. The product methylene blue can be quantified with spetrophotometric analysis.
The disadvantages of the methylene blue assay are that the wavelength of the blue color formed by sulfide can be interfered with other coloured compounds, and artificial acidification would shift the equilibrium to favour the gas phase of H₂S, and therefore underestimate the actual sulfide concentration due to loss of H₂S to the atmosphere (8). A recently published assay using fluorimetric, reverse-phase (RP)-HPLC has gone a long way in eliminating the interferences encountered with the methylene blue assay (21). Although this method solves the detection limitation and interference issue, it is not able to provide continuous measurement. Among all these methods, the putatively most accurate and reliable method is gas chromatography. Even though this method has a high reputation for accuracy, the method of collecting minute volumes of the gas samples from biological fluids results in large sample losses due to the rapid degradation and high liability of H₂S (8). Additionally, some other methods fail to report the sensitivity of the assay and/or controls for background interference (4). For example, some methods use cysteine, the substrate of the enzymes to stimulate H₂S generation, but the additional cysteine was not subtracted from the result. In summary, there is an urgent need for the accurate and specific detection of H₂S. The ideal H₂S detector should be able to measure H₂S continuously without contamination from other biological thiols, and the detection limit should be sensitive at biological concentration (25). Most importantly, the sensor should be cheap and readily available.
Scheme 2: Sulfide reacts with $N,N$-dimethyl-$p$-phenylenediamine forming a blue chromophore

1.2 Sources of $H_2S$ in biological systems

1.2.1 Enzymes that catalyze the formation of $H_2S$: CSE

The PLP–dependence of CSE is conserved in various organisms. There are two isoforms of human CSE. The internal deletion of 132 base pairs from the long isoform forms the short isoform (26). Naturally occurring mutations were also discovered for CSE, which are T67I and Q240E (27). These two types of CSE mutations are found associated with cystathioninuria. Both mutations reduce the enzymatic activities in comparison to the wild-type enzyme, and therefore lead to the accumulation of cystathionine. The reduction in activity is attributed to the lower affinity of PLP binding (3). A common single nucleotide polymorphism resulting from a replacement of serine at position 403 with isoleucine has also been discovered. The natural variant is ethnicity-dependent and it is thus more obvious in an ethnically diverse country, such as Canada (27). Determination of the CSE crystal structure has revealed the active site of CSE as well as the CSE protein folding (13). With the binding of PLP, human CSE
predominately exists as a tetramer in solution, where each subunit is 44.5 kDa. The
tetramers are asymmetric in CSE-PLP complex structures (13). Without the binding of
the PLP cofactor, the two dimers are symmetrically related with each asymmetric unit
(26). Each individual monomer is assembled by hydrogen bonds and hydrophobic
interactions to form a tetramer (28). Each CSE monomer contains two domains: a larger
PLP-binding domain and a smaller domain. The active site (PLP binding site) forms a
strong hydrogen bond and π–stacking interactions with the prosthetic group PLP (29).
Observations show that without the binding of PLP, the enzyme can exist in multiple
conformational states. However, the CSE-PLP tetramer is more stable compared to CSE
without the binding of PLP (27). Visually, PLP bound CSE has a yellow colour (13).

The most common inhibitor of CSE is DL-propargylglycine (PAG) and β-cyano-L-
alanine (β-CNA), where PAG is an irreversible inhibitor and β-CNA is a reversible
inhibitor (8,30). In detail, the inhibition mechanism of CSE by propargylglycine (PAG) is
described as the steps of deprotonation of progargyl glycine. Then, the β-position of the
alkyne is deprotonated to generate an activated alkene. Finally, the vinyl ether is formed
by nucleophilic attack (27). As a result of these reactions, the inhibitor occupies the side
chain of CSE. PAG inhibition essentially blocks the accessibility of the substrate to the
active site via steric hindrance (13).

1.2.2 Enzymes that catalyze the formation of H₂S: CBS

CBS is a homotetramer, composed of four identical subunits. Each subunit has a
molecular weight of ~63 kDa (31). Human CBS is a dual cofactor-dependent enzyme.
The cofactors are PLP and heme. Only highly developed animals, such as mammals,
contain the second cofactor, heme. The heme group is found to reversibly regulate the
activity of the enzyme and is redox active (32). It also plays a catalytic role in homocysteine-dependent \( \text{H}_2\text{S} \) production (32). In recombinant CBS, there are two estimated hemes per tetramer (33). The location of the PLP-binding site is confirmed at the N-terminus of the protein (33). In addition to the two cofactors, the enzyme binds to S-adenosylhomocysteine (AdoMet) at the C-terminal of the enzyme (34). AdoMet is a V-type allosteric effector. The influence of AdoMet is to increase the \( V_{\text{max}} \), but has no effect on the \( K_M \) of the reaction (32). The biological mechanism of AdoMet binding on CBS is, however unknown. The C-terminus of CBS is responsible for the structural organization, or, the formation of the tetramer; it also regulates activity of the enzyme (32). Human CBS has two repeated CBS domains, which named after the CBS enzyme (35). Deletion of the CBS domain was found to increase enzyme activity, and therefore the two repeating CBS domains have an inhibitory role on the enzymatic function (36). The CBS domain is also proposed to act as an energy sensor for the cellular energy status (30). Two commonly used CBS inhibitors are hydroxylamine and aminoxyacetate (9). Human CBS can also be inhibited by homocysteine (24).

1.2.3 MST and CAT

The structure of MST (EC 2.8.1.2) is described to have two domains which are connected by a linker region. The cofactor of MST is zinc (37). Another enzyme that has been reported to be involved in cysteine catabolism is CAT (EC 2.6.1.3) (38). CAT also uses PLP as a cofactor (3). The 2- and 3-mercaptoproprionic acids are uncompetitive and noncompetitive inhibitors of MST (8). Hydrogen peroxide and tetrathionate inhibit MST by impeding the catalytic cysteine residue (8).
1.2.4 Inhibitors of CSE, CBS as well as MST and CAT

Most of these inhibitors mentioned above are general inhibitors rather than specific inhibitors to CBS, CSE or MST. For instance, hydroxylamine is a general PLP-dependent enzyme inhibitor rather than specific inhibitor for H₂S enzyme (1). It inhibits the enzyme by releasing the cofactor: PLP. Currently, there are no entirely specific CSE or CBS inhibitors available. Therefore the experimental results, which use the non-specific inhibitors for one of these enzymes, should be explained carefully with the consideration of other influences of using non-specific inhibitors as the treatment (11). The exploration of the enzyme structure is essential for searching for more CSE/CBS-selective inhibitors.

1.2.5 Cloning of CBS and CSE

In the reverse transsulfuration pathway, CBS catalyzes the first step of the pathway (24), and CSE catalyzes the second step in the pathway. Due to the importance of these two enzymes in the H₂S generating mechanism, research on purification and usage of these enzymes have become popular. However, the practice of purifying CBS from mammalian tissues is a complicated procedure, due to its susceptibility to proteolysis and the tendency to form precipitates (31,39). Therefore, using recombinant CBS is a more convenient method to study the enzyme. In addition, CSE purifications from different species, such as rats streptomyces, and tissues have been described (15). Nonetheless, purification of the enzyme from tissue does not provide a good source for obtaining the enzyme.

For the purpose of extracting the specific protein from the bacterial cell lysate, an affinity tag is normally employed. Tag-fused protein allows for the separation of the
protein from crude biological sources using an affinity column. After purification, the tags can be cleaved using chemical agents or enzymes. The most widely used tags are glutathione-S-transferase (GST) and 6-His tags. GST-fused protein can be purified using a column with a high affinity for glutathione. The drawbacks of using GST are the fact that GST will leave 11- or 23-residue extensions after the cleavage of the tag (24). In contrast, a 6-His tag adds 6 histidine residues to the end of the protein, which can then be bound to the affinity media through metal ions (40). The most widely used is the nickel column. The protein can be eluted using imidazole buffer. The advantage of using this tag is that the removal of the 6-His tag is not required (24). Here, the efficient purifications were achieved by obtaining both CBS and CSE. In this purification, his tags were added to both terminals of CBS and CSE.

1.3 Functions and related Diseases

1.3.1 Over-production and under-production of H₂S related diseases and functions of H₂S enzymes in Pathology

H₂S plays important roles in various organ systems including the cardiovascular system, nervous system, endocrine system, immune system, respiratory system, reproductive system, and urinary system (3). Lower levels of H₂S have been implicated in the occurrence of Alzheimer’s disease and diabetes in mammals (3,9,41). For instance, Alzheimer’s patients, reportedly have attenuated levels of H₂S compared with the healthy
people (42). In contrast, high levels of H$_2$S have been implicated in Down syndrome (25,43) and cardiovascular disease (33). Specifically, the CBS gene in humans is located on chromosome 21, which is the chromosome that is a trisomy in Down syndrome sufferers. Therefore, the overproduction of H$_2$S is postulated to have a relationship with Down syndrome (14).

In the CSE/H$_2$S pathway, Kabil and Banerjee (4) found cystathionase deficiency in CSE knockout mice (4). This symptom named cystathioninuria, which lead to an excess of cystathionine in the urine. Cystathioninuria has also been found associated with natural CSE mutations (34). In hyperhomocysteinemic conditions, CSE-related H$_2$S generation may be enhanced and it may explain that H$_2$S/CSE is involved in cardiovascular pathology (44). Symptoms such as atherosclerosis, myocardial infarction, hypertension and shock are linked to the CSE/H$_2$S cardiovascular diseases (14,45,46). Defects in CBS gene cause an inherited disorder which can lead to homocystinuria (47) which affects major organ systems including cardiovascular, ocular, skeletal and the central nervous system (4,48). The homocysteine build up has been implicated in coronary and cerebrovascular atherosclerosis (49). Mercapto-lactate-cysteine disulfiduria was found related to the deficiency of MST/H$_2$S pathway (50). Since defects of all three enzymes involved the accumulation of homocysteine, increased conversion of homocysteine to H$_2$S by CBS, CSE and 3MST gene therapy could potentially improve these symptoms (28).
1.3.2 H₂S functions as an Anti-Oxidant

H₂S functions as an antioxidant in two ways: (1) directly scavenging the reactive oxygen species (ROS); (2) reducing glutathione disulfide (51). For instance, elevated production of ROS has been found at the location of inflammation (11). In this situation, H₂S can be used as an agent to facilitate the elimination of ROS. Work has been done by supplying homocysteine to check if the stimulated H₂S synthesis facilitates the antioxidant ability. The results show that high percentages of H₂S induce ROS and reactive nitrogen species (RNS) production, but low levels of H₂S react with H₂O₂, ONOO⁻, O₂⁻ (52). In another study, it is proposed that the efficiency of H₂S as an antioxidant can be improved by combining with certain agents, such as GSH, SOD, L-NAME or vitamin C (53). This combination hints at a new route for designing novel antioxidant and therapeutic drugs. In summary, low H₂S may play a beneficial role by eliminating ROS and RNS.

1.3.3 H₂S and Inflammation

Inflammation is defined as a protective physiological response to local injury and tissue damage of vascular tissues (54). There are five clinical conditions including redness, heat, swelling, pain and loss of function (11) to describe inflammation. The known gas mediators NO and CO have been shown to regulate the inflammatory in the biological systems (17). Recently, it has been proposed that H₂S has a role in the inflammatory process (55,56). During inflammation, the surrounding tissues become mildly acidic (11). According to Scheme 1, the locally produced sulfide in the inflammation environment would shift the equilibrium towards the formation of H₂S.
Therefore, H₂S rather than other forms of sulfide would likely be found at the site of inflammation.

H₂S is believed to have two contradicting roles in inflammation. It acts as both a pro- and anti-inflammatory molecule (9). Li et al. reported that the physiological concentration of H₂S has anti-inflammatory effects, while higher concentrations of H₂S can produce pro-inflammatory effects (10). The H₂S inflammatory role was also studied in different systems. In the gastrointestinal tract, the H₂S regulating role functions by activating K_ATP channels in order to promote the inflammation response (57). The similar H₂S function was observed in pancreas (7), but the actual mechanisms are largely unknown. In conclusion, H₂S pathway is a possible route for targeting the inflammation treatment. However, much work needs to be done for understanding the mechanisms of the contradictory roles of H₂S in inflammation.

1.3.4 H₂S and Signal Transduction

H₂S plays several functions in signal transduction mechanisms. It was first discovered as a vasorelaxant and a cardioprotective agent (12). Unlike NO, which activates the soluble guanylate cyclase/cGMP pathway, H₂S-induced vasorelaxation is dependent on ATP-regulated potassium channels in vascular smooth muscle cells (14,58). In the brain, H₂S also can be utilized as a neuromodulator, or to control inflammation, regulate insulin release, modulate angiogenesis, and act as a cytoprotectant (9,10,15,24). In the gastrointestinal system, H₂S may regulate the secretion of bile (59). The reducing ability mentioned above makes H₂S good candidate as a redox cell signal (50). As with inflammation, the mechanisms by which H₂S mediates signal transduction are largely unknown.
1.4 Nitric Oxide

1.4.1 General Introduction of NO

NO, an inorganic molecule plays important regulatory and pathological roles in almost all tissues and systems (14). It was the first molecule that was recognized as a gasotransmitter, which involves transmitting signal information between cells in various tissues. Several important functions of NO are that it serves as a vasodilator (60), an inhibitor of various cellular functions (53,61-67), and also functions as a neurotransmitter (30). It is biologically synthesized from L-arginine (68) under the catalytic reaction of NO synthase (NOS). There are three isoforms of NOS: nNOS, iNOS and eNOS. nNOS converts L-arginine to NO and citrulline in the nervous system; eNOS generates NO in the vascular system; iNOS is ubiquitously expressed in the body, but most concentrated in the cellular immune response, such as macrophages. Calcium-calmodulin activates nNOS and eNOS (30).

1.4.2 Lipopolysaccharide

Lipopolysaccharide (LPS) is a complex glycolipid, which is a major component of the outer leaflet of the membrane of Gram-negative bacteria. It protects bacteria from host defenses and mediates direct interactions with host cell receptors and antibiotics. As an endotoxin, it results in host tissue damage (69). LPS induces iNOS expression as well as the production of NO (41) and other pro-inflammatory cytokines (70).

1.4.3 L-NG-Nitroarginine methyl ester

L-NG-Nitroarginine methyl ester (L-NAME) (Figure 1.2) has been widely used as a competitive inhibitor of NOS (53). The inhibition is due to structural similarities between L-NAME and L-arginine (Figure 1.2), the natural substrate of NOS (71), and
therefore it blocks the substrate binding site. When L-NAME binds to NOS, the ability NOS of converting L-arginine to NO is decreasing.

\[
\text{O}_2\text{N-NH}_3\text{CH}_2\text{COCH}_3\cdot\text{HCl}
\]

(A)

\[
\text{H}_2\text{N-CH}_2\text{CH}_2\text{NH}_2\text{CH}_2\text{COOH}
\]

(B)

Figure 1.2 Structures of NOS inhibitor L-NAME hydrochloride (A) and arginine (B). Structure of L-NAME is similar to arginine.

1.4.4 NO donors: GSNO and DEANO

NO donors have been chemically synthesized recently and they are grouped based on the structure of the NO-releasing moiety. The most widely used NO donors are diazeniumdiolates, such as diethylamine NONOate (DEA/NO) (71). They are true NO–donors, which release a predictable amount of NO (Scheme 3(B)). The second type is small molecular weight S-nitrosothiols which donate NO\(^+\) rather than NO. S-
nitrosocysteine and S-nitrosoglutathione (GSNO) both belong to this class. The third type of NO donor is nitrite (Scheme 3(A)). Nitrite releases NO$^+$ in a reductive environment. The fourth type of NO donor is oxadiazole-$N$-oxide (72), which is a thiol dependent heterocyclic compounds considered as NO releasing prodrugs (73).

\[ \text{A} \quad \text{O}_- \text{N}^\cdot \text{N}^\cdot \text{O} \xrightarrow{\text{H}^+} \text{RH} + 2\text{NO}^\cdot \]

\[ \text{B} \quad 2 \text{R}^- \text{S}^- \text{N}=\text{O} \xrightarrow{} \text{RSSR} + 2\text{NO}^\cdot \]

Scheme 3: (A) Mechanism of NO release from DEA/NO. (B) The reaction of GSNO releases NO.

1.4.5 NO and H$_2$S Interaction – The “Cross Talk” between NO and H$_2$S

A plethora of recent evidence shows that the two signaling molecules, NO and H$_2$S interact with each other. In the brain, NO donors reportedly modify CBS, causing an increase in its activity (14). In human recombinant CBS, NO can bind to the heme group which leads to a lowered enzyme activity (48). In the rat aorta, NO donors increase CSE-dependent H$_2$S biogenesis in a cGMP-dependent manner. Furthermore, pre-incubating NO donors increases CSE mRNA and protein levels in smooth muscle cells, which will ultimately result in increased H$_2$S production (8,14,54,61). Additionally, LPS-induced NO production is associated with increased plasma H$_2$S concentration and H$_2$S synthesizing activity in human cells and tissues (10).
1.4.6 S-Nitrosylation

Protein S-nitrosylation refers to the reaction of a cysteine thiol group with NO in the presence of an electron acceptor, oxygen, to form a covalent S-NO bond. In mammalian tissues, the S-nitrosothiol (RSNO) concentration varies from nM to μM. Endogenously, S-nitrosylation occurs between the sulfur atom from cysteine or homocysteine and NO. The mechanism of an S-nitrosylation is an electrophilic attack of a NO\(^+\) equivalent to sulfur and is followed by deprotonation of thiol group. It is also possible that the NO\(^-\) equivalents attack the electropositive cysteine groups (74). Both of the mechanisms are believed plausible. The S-nitrosylation reaction is believed to be a third order reaction, the mechanism of which is shown in Scheme 4. The synthesis process, transportation, activation and catabolism of RSNO are thought to be regulated (75). Evidence illustrates that there is a specific enzyme that catalyzes this reaction and only specific thiols from a protein can be targeted to form RSNO. For example, ceruloplasmin, a copper-carrying protein in the blood, catalyzes low molecular weight RSNO, such as GSNO. Hemoglobin can also serve as an enzyme that catalyzes RSNO formation (74,76). Recently, the smallest S-nitrosothiol, HSNO, resulting from the reaction of H\(_2\)S with NO has been identified and proposed to regulate the physiological effects of both NO and H\(_2\)S (77).

S-nitrosylation results in special physiological or pathophysiological activity by modifying protein function. Some proteins’ activity is increased by s-nitrosylation modification, such as thioredoxin, while others may be inhibited, such as methionine adenosyl transferase. In some extreme cases, S-nitrosylation abolishes the catalytic activity of a protein, such as GAPDH (30). In most cases, nitrosylation inhibits enzyme
and receptor activity. This supports the idea that cysteine thiols which are nitrosylated are critical for enzymatic activity.

One of the widely used methods in identifying RSNO is to use the biotin switch assay. In the biotin switch assay, free thiols in the proteins are blocked by a sulfhydryl-reactive compound, methyl methanethiosulfonate. Then, thiols are exposed to ascorbate and labeled with biotin, which are then coupled to streptavidin. Finally, the S-nitrosylated proteins are separated by gel electrophoresis (30). Sometimes, the cell permeable thiol-alkylating agent, iodoacetamide is used to react with thiol proteins and small molecules, glutathionine, cysteine and H₂S to block thiols (78). One of the new methods is to use gold nanoparticles to isolate thiol-containing peptides to identify SNO (79).

\[
\begin{align*}
2\text{NO} + \text{O}_2 & \rightarrow 2\text{NO}_2 \\
\text{NO}_2 + \text{NO} & \rightarrow \text{N}_2\text{O}_3 = \text{NO} + \text{NO}_2^- \\
^+\text{NO} + \text{NO}_2^- + \text{SH} & \rightarrow \text{RSNO} + \text{HNO}_2
\end{align*}
\]

**Scheme 4: Proposed NO S-nitrosylation mechanism**

### 1.5 RAW 264.7 Macrophages

Macrophages are a heterogeneous population of mononuclear phagocytes found in the body. These cells are vital for the immunity in response to microorganisms and inflammation (80). RAW 264.7 cells are a macrophage-like cell line derived from mice. This cell line has the ability to synthesize both NO and H₂S. The literature states that the H₂S-production rate in RAW 264.7 cell is 0.04882 nmol·S⁻¹·mg·protein⁻¹ (70).
Macrophages are the major cellular targets for LPS. Both NO and H$_2$S production rates can be enhanced by LPS treatment (70). Since the Western blots do not show the expression of CBS, the source of H$_2$S from RAW 264.7 is likely entirely from CSE alone (80). One of the beneficial functions of using this cell line is that it can survive in higher than physiological H$_2$S concentrations for a long period of time compared with other cell lines (68). In view of these properties, RAW 264.7 cells are ideal for studying the interplay between NO and H$_2$S signaling in a cellular setting.

1.6 Summary and Purpose of the study

As discussed above, information regarding H$_2$S is still not fully understood. The first issue required to assess is the lack of a reliable assay for H$_2$S. An accurate and specific detection method would allow us to kinetically characterize enzymatic catalysis as well as assess the actual H$_2$S concentrations under controlled conditions. In addition, many of the biological effects that have been found for H$_2$S are controversial. The main reason for the contentious results is due to many of the studies being carried out with indirect measurement of H$_2$S using thiosulfate or sulfhemoglobin. Indirect measurement could cause the overestimation or underestimation of the actual H$_2$S concentration. Thus, a proper method of H$_2$S assay is required.

Therefore, this study is aimed at finding an accurate and continuous method for the measurement for H$_2$S. Here a simple method based on the selective permeability of
polydimethylsiloxane (PDMS) to free H$_2$S was developed. The new H$_2$S assay was then employed to test the effect of NO and NO donors on the H$_2$S-producing enzymes CSE and CBS as well as studying the effects of NO on the cellular production of H$_2$S.
CHAPTER 2
MATERIALS AND METHODS

2.1 Materials

2.1.1 Materials

Recombinant human CSE (polymorphic variant S403) pET22b plasmid was generously provided by Dr. Ruma Banerjee (University of Michigan Medical Center, Michigan, USA). Recombinant Zebrafish CBS was obtained from Addgene. Tryptone, and yeast extract were purchased from Bio Basic Inc. Potassium phosphate dibasic, potassium phosphate monobasic, Tris-HCl, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were obtained from Fisher Scientific. EDTA, Lysozyme, DNase I, phenylmethanesulfonylfluoride, Isopropyl β-D-1-thiogalactopyranoside, Triton X100, His-Select Nickel Affinity Gel, Kanamycin, chloramphenicol, imidazole, 5,5′-Dithiobis-(2-nitrobenzoate), cysteine hydrochloride, lipopolysaccharide, No-nitro-L-arginine methyl ester hydrochloride, DL-propargylglycine, glutathione, sodium nitrite, diethylamine NONOate, homocysteine and pyridoxal-5′-phosphate were all purchased from Sigma Aldrich. Polydimethylsiloxane (PDMS) Sylgard 184 Silicone Elastomer Kit was purchased from Dow Corning (Cat#3097358-1004). Sodium sulfide nonahydrate was purchased from MP Biomedicals. RAW 264.7 macrophages and Dulbecco’s Modified Eagle Medium were obtained from ATCC. Fetal Bovine Serum was purchased from Invitrogen. Penicillin and Streptomycin were purchased from GIBCO. Zebar™ spin desalting columns were obtained from Thermo Scientific.
2.1.2 Equipment

-Amicon centrifugal filter tubes; EMD Millipore, Billerica, MA, USA

-Centrifuge Jouan BR4i; Thermo Electron Corporation, Asheville, NC, USA

-C25 Incubator Shaker; New Brunswick Scientific, Enfield, CT, USA

-Model 100 Sonic Dismembrator; Fisher Scientific, Ottawa, ON, Canada

-Agilent 8543 UV-VIS Spectrophotometer, Agilent Technologies, Inc. Mississauga, ON, Canada

-Mettler AJ Balance; Mettler, Toledo, Mississauga, ON, Canada

-Orion Model 420A pH Meter, Thermo Electron Corp., Burlington, ON, Canada

-Type 37900 Culture Incubator, PGC Scientifics, Palm desert, CA, USA

-Wallac 1420 Victor 3 Fluorescent Plate Reader, Perkin Elmer, Woodbridge, ON Canada

-Haemocytometer, Reichert Co, Buffalo, NY, USA

-SDS-PAGE gel apparatus, Bio-rad, Mississauga, ON, Canada
2.2 Methods

2.2.1 Purification of CBS and CSE

CSE was purified according to the method of Steegborn et al. (81), but with some modifications. Human Histidine-tagged cystathionine gamma-lyase was expressed in *E. coli* XL1B using an expression plasmid generously provided by Dr. Ruma Banerjee (The cloning of the CSE and CBS gene was done by Dr. Sirinart Ananvoranich.). Cells were grown in 1 L of Terrific Broth using kanamycin and chloramphenicol as the antibiotics until the OD$_{600}$ reading was between 0.4 and 0.6. Expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside at 37 °C, and the cells were further incubated for 4 h. Cells were then collected by centrifugation at 6000 rpm for 30 min at 4 °C. The cell pellet was resuspended in 20 mL of lysis buffer which contained 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 100 μg/mL lysozyme, 50 μg/mL DNase I, 2 mM PMSF (in isopropanol), and 1% Triton x100. The resuspended cells were then incubated on ice for 30 min and sonicated using the tip of a Sonic Dismembrator at level 6 for 8 pulses, with 20 s per pulse. The lysate was then centrifuged at 10 000 rpm for 30 min at 4 °C. The supernatant was collected and added to a His-Select Ni-Affinity column (Sigma-Aldrich) equilibrated with 10 mM imidazole in 50 mM Tris-HCl pH 8.0. Elution was achieved using 250 mM imidazole in 50 mM Tris-HCl pH 8.0. The collected fractions were pooled together and dialyzed overnight against 100 mM HEPES buffer at pH 7.4. After dialysis, the enzyme was concentrated by centrifugation at 4 °C for 1 h at 3500 rpm in an Amicon centrifugal filter tube. After several spins, ~1mL of the protein was left in the column. The concentrated CSE was obtained and stored at ~80 °C. Protein concentration was determined using the BCA protein assay with bovine serum albumin as
the standard. The enzyme purity was then assessed by SDS-PAGE with coomassie blue staining. Enzyme activity was determined by monitoring the reaction of DTNB with cysteine and H₂S produced from the catalytic consumption of cystathionine by CSE at 405 nm in plate reader. CBS was purified and concentrated using the same procedures.

2.2.2 Fabrication of the PDMS Wells

The curing agent was mixed thoroughly using the volumetric ratio of 0.08 to monomer to make PDMS. The mixture was degassed using the vacuum of lyophilizer. Both parts of the molds were sprayed with a silicone-based lubricant and then the female part was filled with ~4mL of the degassed PDMS mixture. The top portion of the mold was clamped into the male part using a 2 in. metal C-clamp. The assembly was incubated at 70 °C for at least 2 h. The assembly was allowed to cool down and was carefully pried apart. The solid PDMS polymer was removed from the male portion and was rinsed with distilled water, followed by drying at room temperature (Figure 2.1).
Supplementary Information

Figure 2.1 Representative diagrams of the PDMS well assembly mold. The male portion (A) was fabricated from Teflon and the female portion (B) was made from aluminum. Both parts were sprayed with a silicone-based lubricant. The aluminum part was filled with ~4mL of PDMS and the Teflon was placed on top of it. The assembled mold was clamped and baked at ~70 °C for at least 2 h. After the mold was cooled, the PDMS wells (C) were removed from the mold and rinsed with distilled water.

2.2.3 H$_2$S measurement using cuvette- or plate-based method

The buffer used in this study was 0.1 M sodium phosphate (pH 7.4) with 2 mM EDTA and all solutions were degassed by stirring and sonicating under vacuum for 1 h, followed by purging with argon while stirring for 2 h. Two methods were developed to measure H$_2$S production, which are using cuvette or using the plate. In the cuvette-based assay (Figure 2.2 A), H$_2$S measurement was done by using the thiol probe DTNB and spectrophotometer equipped with a thermostat. This measurement was set up by placing
the H$_2$S sources in the PDMS well, which was floating on 1.6 mL of 50 μM DTNB in a cuvette containing a stirring bar. For this method, H$_2$S concentrations were calculated using the extinction coefficient of the NTB$^{2-}$ anion ($\varepsilon_{412} = 14$ 150M$^{-1}$ cm$^{-1}$). In the plate-based procedure (Figure 2.2 B), 140 μL of the H$_2$S source was placed into the wells of 96-well plate. PDMS wells, which contained DTNB (100 μL, 50 μM) were then placed into the 96-well plate overtop the H$_2$S source. The PDMS wells were inserted in to the plate within 20 s between the sample addition and acquisition. Absorbance at 405 nm was measured using a plate reader. For the plate-based procedure, H$_2$S concentrations were calculated using a standard curve with Na$_2$S and the appropriate acid dissociation constants (Henderson – Hasselbalch). The sample volume required for each method was comparable: 20 – 100 μL for the cuvette-based procedure and 140 ± 20 μL for the plate-based procedure.
Figure 2.2 Schemes of the two experimental setups representing the cuvette- and plate-based PDMS H$_2$S assay. (A) In the cuvette based method, DTNB was placed in the cuvette with a stirring bar. The PDMS containing the H$_2$S source floated on top. Absorbance at 412 was measured using a UV/Vis spectrophotometer, and free H$_2$S concentration was determined using the extinction coefficient for NTB$^{2-}$. (B) In the plate-based procedure, 140 μL of the H$_2$S source was placed into the wells of 96-well plate. PDMS wells that contained DTNB were then placed into the 96-well plate over top of the H$_2$S sources. The PDMS wells were inserted into the plate within 20 s between the sample addition and addition. Absorbance at 405 nm was taken using a plate reader.

2.2.4 Measurement of H$_2$S Production by Purified CSE and CBS by the Cuvette-Based Method

All solutions were prepared in 0.1 M sodium phosphate buffer (pH 7.4) with 2 mM EDTA and all of the measurements were taken at 37 °C. H$_2$S produced by CBS and CSE on different substrates was examined by measuring the absorbance of NTB$^{2-}$, which results from the cleavage of the disulfide bond of DTNB by H$_2$S. PLP with a final of 50 μM was premixed with 1.4 μM CSE. 160 mM stock solutions of each substrate were
made and diluted to various concentrations ranging from 2 to 60 mM in phosphate buffer. The substrate was added into PDMS wells before measurement, which was floating in a cuvette which contained 1.6 mL of 50 μM DTNB and a stir bar. The floating PDMS cup was checked not to block the light path of the spectrophotometer. The cuvette was placed into the spectrophotometer, thermostated at 37 °C and stirred at 175 rpm, and was allowed to thermally equilibrate for 2 min before acquisition. The measurement at 412 nm was done at 20 s intervals for a total of 1000 s. After the background measurement of DTNB was stable after 100 s, the mixture of PLP and CSE was added to the PDMS cup with a final volume of 80 μL containing 5 μg of purified CSE. Each sample rate was obtained by calculating the linear slope after the slope for the 100 s prior to adding CSE. The extinction coefficient used for NTB$^{2-}$ was 14 150 M$^{-1}$ cm$^{-1}$. The detection of the H$_2$S produced by CBS used the same procedure.

2.2.5 Synthesis of S-nitrosoglutathione

GSNO was synthesized by dissolving glutathione in 0.1 M HCl in a beaker. In another beaker, an equimolar amount of sodium nitrite was dissolved in H$_2$O. Dissolved sodium nitrite was then added to the acidic glutathione solution while stirring vigorously. After the solution turned red, it was kept stirring for another 5 min in the dark. Then, the pH of the solution was adjusted to ~7.0 by adding 95% NaOH. The solution was lyophilized to obtain the GSNO.

2.2.6 Mammalian cell culture and treatment

RAW264.7 macrophages were kept in a humidified incubator at 37°C which was maintained in a 5% CO$_2$ environment. Dulbecco’s modified Eagle medium was mixed with 10% heat-inactivated fetal bovine serum (100 U/mL), penicillin and streptomycin.
(100 μg/mL) before using it. The treatments were added when cells were at 70-80% confluency. LPS stock solution was prepared by dissolving it in DMSO and then it was diluted with DMEM to a final concentration of 4 mg/mL. 2.5 μL of the stock solution was added to 10mL of media of the cell culture to a final concentration of 1 μg/mL and incubated at 37°C with 5% CO₂ for 12h. Stocks of L-NAME were prepared in DMSO at a concentration of 160mM and added to the cells at a final concentration of 4 mM. Samples treated with both LPS and L-NAME were treated first with LPS. The cells were then washed twice with media and incubated for another 12 h with or without L-NAME.

2.2.7 Effects of DEA/NO on CSE activity

All of the solutions were made using 100 mM phosphate buffer with 0.2 mM EDTA at pH 8.0. All measurements were taken at 37 °C. After purification, an aliquot of CSE was transferred to speta-sealed vial and was purged with either 2 ppm or 16 ppm oxygen with moisture for 40 min on ice. The following steps were done in dark to prevent the photodecomposition of S-nitrosylated proteins. DEA/NO was dissolved in phosphate buffer and added to CSE with a final concentration of 1 μM. The mixture was covered with aluminum foil and agitated for 90 min at 4 °C. Then, the solution containing CSE was buffer exchanged using the Zeba™ spin desalting column with phosphate buffer to eliminate excess DEA/NO. The buffer exchanged CSE concentration was measured using the BCA protein assay. PLP at a final concentration of 50 μM was premixed with 1.4 μM CSE in buffer and the mixture was separated into aliquots, and each fraction was frozen in the dark before use. The influence of NO on CSE was assayed using the same cuvette-based PDMS method as described above. 160 mM of cysteine solution was diluted with buffer in different concentrations and incubated for 2
min at 37 °C. The substrate in buffer was added to the PDMS wells before each measurement. The PDMS wells were then placed in to a cuvette which contained 1.6 mL of 50 μM DTNB and a stir bar. The measurement at 412 nm was done at 20 s intervals for a total of 1000 s. After 120 s, the background was stable, treated CSE was added. The extinction coefficient used for NTB$^2$ was 14 150 M$^{-1}$ cm$^{-1}$.

### 2.2.8 Effects of GSNO on CSE

Effects of GSNO on CSE were assessed using the cuvette-based PDMS method as described for the DEA/NO effects on CSE. All of the solutions were made using 100 mM phosphate buffer with 0.2 mM EDTA at pH 8.0 and all measurement was taken at 37 °C. The exception is that GSNO is a fast NO$^+$ donor, hence it was incubated with CSE in dark in 10 min before the buffer exchange. In addition, various of GSNO concentrations (0.5 μM, 1 μM, 10 μM, 20 μM, 40 μM, 50 μM) were incubated with CSE after incubation with either 2 ppm or 16 ppm oxygen.

### 2.2.9 Statistical analysis

All data are expressed as the mean ± the standard error of the mean. The kinetic parameters for initial rate of CSE and CBS producing H$_2$S, $K_M$, $V_{max}$, and $K_{cat}$ results were calculated using the fitted curve of Michaelis-Menten equation.

For the DEA/NO and GSNO effect on CSE under 2 ppm or 16 ppm oxygen, $K_M$, $V_{max}$, and $K_{cat}/K_M$ of CSE production of H$_2$S using cysteine were determined. Data were fitted using the Michaelis-Menten equation. The comparison between the controls (without NO donor) and the treated (with NO donor) were compared using the student’s t-test. A $p$-value <0.05 was considered significant (82).
CHAPTER 3

RESULTS

3.1 Purification of CBS and CSE

CSE purification: Recombinant His-tagged human CSE was purified by a combination of Ni-affinity chromatography (Sigma-Aldrich) and gel filtration chromatography (Sigma-Aldrich). All fractions were tested for cysteine/H₂S production, using cystathionine as the substrate. The fractions with activity were pooled and concentrated. From the purification Table 3.1, the enzyme was purified 2.45-fold with the Ni column and appeared as the band with a molecular weight of ~45 kDa (expected molecular weight ~47.7 kDa) in SDS-PAGE (Figure 3.1 lane 2). This Ni-column eluate was further purified by gel filtration (G-25) (Figure 3.1 lanes 3 and 4). Gel filtration activity was increased 1.35-fold. The typical yield of CSE was ~0.95 mg of protein/liter of culture (3.31-fold increase in specific activity with respect to crude-Table 3.1).

CBS purification: Recombinant His-tagged zebrafish CBS was purified by a combination of Ni-affinity chromatography (Sigma-Adrich) and gel filtration chromatography (Sigma-Adrich). All fractions were tested for Cysteine/H₂S production, with cystathionine as the substrate. The fractions with activity were pooled and concentrated. From the purification Table 3.2, the enzyme was purified 1.59-fold with the Ni column and appeared as the band with molecular weight of ~70 kDa (expected molecular weight ~68 kDa) in SDS-PAGE (Figure 3.2 lane 2, 3 and 4). This Ni-column eluate was further purified by gel filtration (G-25) (Figure 3.2 lane 5). Gel filtration activity was increased activity by 1.91-fold. The yield of CBS was ~0.8 mg of
protein/liter of culture (3.04-fold increase in specific activity with respect to crude-Table 3.2).

<table>
<thead>
<tr>
<th>Purification Step of CSE</th>
<th>Volume</th>
<th>Protein Concentration</th>
<th>Protein mass</th>
<th>Activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble cell extract</td>
<td>25 mL</td>
<td>1.59 mg/mL</td>
<td>39.75 mg</td>
<td>46.88 units</td>
<td>1.18 units/mg</td>
</tr>
<tr>
<td>Ni-Selective Chromatography</td>
<td>24 mL</td>
<td>0.38 mg/mL</td>
<td>9.12 mg</td>
<td>26.4 units</td>
<td>2.89 units/mg</td>
</tr>
<tr>
<td>Purified enzyme</td>
<td>2.45 mL</td>
<td>0.73 mg/mL</td>
<td>0.80 mg</td>
<td>3.12 units</td>
<td>3.91 units/mg</td>
</tr>
</tbody>
</table>

Table 3.1 Purification table of his-tagged human recombinant CSE expressed in *E. coli*
<table>
<thead>
<tr>
<th>Purification Step of CBS</th>
<th>Volume</th>
<th>Protein Concentration</th>
<th>Protein mass</th>
<th>Activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mL</td>
<td>mg/mL</td>
<td>mg</td>
<td>units</td>
<td>Units/mg</td>
</tr>
<tr>
<td>Soluble cell extract</td>
<td>25</td>
<td>2.6</td>
<td>65</td>
<td>144.95</td>
<td>2.23</td>
</tr>
<tr>
<td>Ni-Selective Chromatography</td>
<td>20</td>
<td>0.59</td>
<td>92.04</td>
<td>68.64</td>
<td>3.54</td>
</tr>
<tr>
<td>Purified enzyme</td>
<td>2.7</td>
<td>0.7</td>
<td>1.9</td>
<td>12.86</td>
<td>6.77</td>
</tr>
</tbody>
</table>

Table 3.2 Purification table of his-tagged human recombinant CBS expressed in *E. coli*
Figure 3.1 SDS-PAGE of CSE at different purification stages. Lane 1: protein ladder. Lane 2: CSE after Ni-affinity chromatography. Lane 3 and Lane 4: CSE after gel filtration chromatography.

Figure 3.2 SDS-PAGE of CBS at different purification stages. Lane 1: protein ladder. Lane 2, Lane 3 and Lane 4: CBS after Ni-affinity chromatography. Lane 5: CBS after gel filtration chromatography.
3.2 The standard curve of H$_2$S obtained using PDMS wells

The standard curve (Figure 3.3) of H$_2$S was obtained using the PDMS-permeability method for quantifying H$_2$S. The production curve was measured using the cuvette-based procedure at pH 7.4, 37 °C. The concentration of solution sulfide generated by free H$_2$S diffusion across the PDMS membrane was calculated using the extinction coefficient for the NTB$^{2-}$ anion. The permeability rate-based standard curve was linear through ~2 orders of magnitude with a detection limit of 13.5 ± 5.3 ppb (m) (parts per billion (by mole))/min (or 0.75 ± 0.29 μM/min in 1.6 mL of buffer) free H$_2$S as solution sulfide. Rate was calculated within 2 min of sulfide addition to the PDMS well. The measurement was stopped at 3 min and this data was used to generate a standard curve in the physiologically relevant region of sulfide concentrations. The detection limit estimated from the end point titration is 9.2 ± 1.9ppb (m) (or 0.51 ± 0.10 μM in 1.6 mL of buffer) free H$_2$S as sulfide in solution.
Figure 3.3 H$_2$S released by H$_2$S donor Na$_2$S measured using DTNB and the cuvette-based method. (A) Diffusion rate of H$_2$S across PDMS-membrane produced by various concentrations of Na$_2$S. (B) Standard curve calculated from the initial rates of (A).
3.3 Kinetics of \( \text{H}_2\text{S} \) production by CBS and CSE using the PDMS method

In the interest of validating the cuvette-based method, we continuously monitored \( \text{H}_2\text{S} \) release from recombinant CSE and CBS as a function of substrate concentration using cysteine, homocysteine or cystathionine. As can be seen from the representative kinetic plots of \( v_o \) versus [Cys], and [HCys] of CSE and CBS (Figure 3.4), the data displayed saturation kinetics. The \( v_o \) vs. [substrate] data was fit to the Michaelis-Menten equation. The steady state kinetic parameters (\( K_M \), \( V_{\text{max}} \), and \( k_{\text{cat}} \)) were estimated from the fitted curve (Table 3.3 and Table 3.4). The \( K_M \) values of CSE catalytic reaction using cysteine and homocysteine were compared with the literature values obtained using the lead acetate attraction method. The \( K_M \) determined for cysteine 3.98 ± 2.44 mM, which is comparable to the literature value 2.75 mM. The \( K_M \) for homocysteine was 7.87 ± 4.75, which is ~3 times that of the literature value. The \( K_M \) values of CBS for cysteine and homocysteine are 9.39 ± 1.35 and 7.21 ± 1.18, respectively.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( K_M ) (mM)</th>
<th>( V_{\text{max}} ) (nmol H(_2\text{S})·min(^{-1}))</th>
<th>( k_{\text{cat}} ) (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>3.98 ± 2.44 (2.75)</td>
<td>0.37 ± 0.01</td>
<td>3.25 ± 0.14</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>7.87 ± 4.75 (2.7 ± 0.85)</td>
<td>1.25 ± 0.28</td>
<td>10.94 ± 2.48</td>
</tr>
</tbody>
</table>

Table 3.3 Kinetic parameters of CSE catalysis. The result was determined using PDMS cuvette-based method. The \( K_M \) values in brackets are literature values obtained using Pb-precipitation \( \text{H}_2\text{S} \) assay.
Table 3.4 Kinetic parameters of CBS catalysis. Results were determined using the PDMS cuvette-based method.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M$ (mM)</th>
<th>$V_{max}$ (nmol H$_2$S·min$^{-1}$)</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>9.39 ± 1.35</td>
<td>0.24 ± 0.02</td>
<td>2.98 ± 0.31</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>7.21 ± 1.18</td>
<td>0.25 ± 0.004</td>
<td>0.57 ± 0.04</td>
</tr>
</tbody>
</table>

Figure 3.4 Representative data fitted to the Michaelis-Menten equation. (A), and (B) are the initial rates ($v_o$) of CSE-catalyzed H$_2$S formation as a function of [HCys], and [Cys], respectively. (C) and (D) are the initial rates ($v_o$) of CBS-catalyzed H$_2$S formation as a function of [HCys] and [Cys], respectively.
3.4 The use of the PDMS method to study the effect of Nitric Oxide on CSE activity

In these experiments, CSE was exposed to the NO donor, DEA/NO, for 90 min, in the presence of 2 ppm or 16 ppm oxygen. The enzyme solution was passed through a desalting column (Thermo Scientific) to remove any NO\textsubscript{x} ions that might have formed during the incubation period. A control CSE fraction was identically treated with varying oxygen concentrations, but without DEA/NO. A constant amount of control and NO-exposed CSE (~1.4 \textmu M) were exposed to varying amounts of Cys. The amount of H\textsubscript{2}S produced was continuously measured by the PDMS method (Faccenda, Wang et al. 2012). Representative plots and kinetic parameters are shown in Figure 3.5. The K\textsubscript{M} (binding affinity) values of control were compared with the literature values. In the 2 ppm oxygen control, the K\textsubscript{M} value for cysteine was 7.27 mM, which is ~2.6-fold higher than the literature value (2.75 mM). In 16 ppm oxygen control, the K\textsubscript{M} value for cysteine is 4.42 mM, which is ~1.6-fold higher than the literature value (2.75 mM). The differences in K\textsubscript{M} values are likely due to the different methods used. The literature values used lead acetate-based method to monitor the formation of lead sulfide from H\textsubscript{2}S. The heavy metal ion and protein interactions may cause artificial catalytic effects, which may result in a lower K\textsubscript{M} value.

The kinetic parameters of DEA/NO-treated CSE were then compared with control CSE values. At 2 ppm oxygen environment using cysteine, control and DEA/NO-treated kinetic parameters were compared. K\textsubscript{M} showed a ~2.4-fold increase (Figure 3.5 (B)), and V\textsubscript{max} of CSE was found to increased of 1.3-fold after treatment with DEA/NO (Figure 3.5 (C)). Therefore, K\textsubscript{cat}/K\textsubscript{M} decreased ~1.7-fold (Figure 3.5 (D)). With DEA/NO incubation
under 16 ppm oxygen using cysteine, $K_M$ increased ~1.5-fold (Figure 3.5 (F)) and $V_{\text{max}}$ decreased ~2.3-fold (Figure 3.5 (G)), which attenuated the $K_{\text{cat}}/K_M$ of CSE ~3-fold (Figure 3.5 (H)). It is interesting that the increase in $K_M$ at 2 ppm (~2.4-fold) is higher than the increase in $K_M$ at 16 ppm oxygen (~1.5-fold), which indicates different reaction mechanisms of NO with thiol in low oxygen versus high oxygen environments.
Figure 3.5 DEA/NO-treated CSE under 2 ppm or 16 ppm oxygen. The enzyme solution was measured for \( \text{H}_2\text{S} \) kinetics using the PDMS cuvette-based method. Reactions were maintained at 37 °C and the CSE concentration in each reaction is \(~1.4 \ \mu\text{M} \). (A) Representative plot of the initial rate (\( v_0 \)) of CSE (purged with 2 ppm oxygen and incubated with or without 0.1 \( \mu\text{M} \) DEA/NO) catalyzed \( \text{H}_2\text{S} \) formation as a function of [Cys]. (B) The binding affinity substrate concentration of maximum velocity (\( V_{\text{max}} \)) of control and NO donor treated CSE with 2 ppm oxygen with Cys. (D) The substrate concentration of catalytic efficiency (\( K_{\text{cat}}/K_M \)) of control and NO donor treated CSE with 2 ppm oxygen with Cys. (E) Representative plot of the initial rate (\( v_0 \)) of CSE (purged with 16 ppm oxygen and incubated with or without 0.1 \( \mu\text{M} \) DEA/NO) catalyzed \( \text{H}_2\text{S} \) formation as a function of [Cys]. (F) The binding affinity (\( K_M \)) of control and NO donor treated CSE with 16 ppm oxygen with Cys. (G) The substrate concentration of maximum velocity (\( V_{\text{max}} \)) of control and NO donor treated CSE with 16 ppm oxygen with Cys. (H) The substrate concentration of catalytic efficiency (\( K_{\text{cat}}/K_M \)) of control and NO donor treated CSE with 16 ppm oxygen with Cys. Errors bars represented standard deviation (\( n = 6 \)). (I) Summarized table of kinetic parameters \( K_M \), \( V_{\text{max}} \) and \( K_{\text{cat}}/K_M \).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DEA/NO</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 ppm Cysteine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average ( K_M )</td>
<td>7.28 ± 0.18</td>
<td>17.38 ± 4.67</td>
</tr>
<tr>
<td>Average ( V_{\text{max}} )</td>
<td>0.41 ± 0.03</td>
<td>0.53 ± 0.08</td>
</tr>
<tr>
<td>Average ( K_{\text{cat}}/K_M )</td>
<td>0.47 ± 0.02</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td><strong>16 ppm Cysteine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average ( K_M )</td>
<td>4.42 ± 0.47</td>
<td>8.74 ± 2.61</td>
</tr>
<tr>
<td>Average ( V_{\text{max}} )</td>
<td>0.10 ± 0.01</td>
<td>0.042 ± 0.004</td>
</tr>
<tr>
<td>Average ( K_{\text{cat}}/K_M )</td>
<td>0.20 ± 0.01</td>
<td>0.05 ± 0.01</td>
</tr>
</tbody>
</table>
3.5 Effects of GSNO on CSE

After purging with 2 ppm or 16 ppm oxygen, CSE was exposed to various concentrations of GSNO (0.5, 1, 10, 20, and 50 µM) for 10 min. The CSE solution was passed through a buffer exchange column (Thermo Scientific) to remove any NO\textsubscript{x} ions that might have formed during the incubation period. A control CSE fraction was identically treated with 2 ppm or 16 ppm oxygen concentrations, but without GSNO. A constant amount of control and NO-exposed CSE (~1.4 µM) were exposed to varying amounts of Cys. The amount of H\textsubscript{2}S produced continuously was measured by PDMS method (Faccenda, Wang et al. 2012). Kinetic parameters are shown in Figure 3.6. In the presence of 2 ppm oxygen, the $K_M$ value increased with increasing GSNO concentrations, with the maximum increase of ~1.8-fold at 20 µM GSNO (Figure 3.6 (B)). This illustrates that the binding affinity has decreased. $V_{\text{max}}$ did not have significant change upon incubation of CSE with GSNO (Figure 3.6 (C)). $K_{\text{cat}}/K_M$ values decreased with increasing GSNO concentrations and the largest decrease in $K_{\text{cat}}/K_M$ was ~1.7-fold at 50 µM (Figure 3.6 (D)). At 16 ppm oxygen, a similar pattern with $K_M$, $V_{\text{max}}$ and $K_{\text{cat}}/K_M$ was observed. $K_M$ values increased with the increasing GSNO concentration with the maximum increase at 50 µM GSNO (~2.1 fold) (Figure 3.6 (F)), also indicates a lowering in the binding affinity of the substrate. $V_{\text{max}}$ values did not change significantly (Figure 3.6 (G)). As a result, $K_{\text{cat}}/K_M$ has a decreasing trend with maximum of ~1.7-fold decrease at 50 µM GSNO (Figure 3.6 (H)). When comparing the 2 ppm and 16 ppm conditions, the increases in the $K_M$ values were similar (~1.8- and ~2.1-fold, respectively). These results signify that oxygen in this situation does not affect the interaction of NO with CSE. The lowered Cys binding affinity is thought to result from the direct S-nitrosylation
of enzyme thiols, thus resulting in the observed alterations in the catalytic efficiency of the enzyme.

\[\text{Control} \quad \bullet \quad 0.5\mu\text{M} \quad \square \quad 1\mu\text{M} \quad \triangle \quad 10\mu\text{M} \quad \times \quad 20\mu\text{M} \quad \blacklozenge \quad 50\mu\text{M}\]
Figure 3.6 H₂S production of GSNO-treated CSE under 2ppm or 16 ppm of oxygen. The enzyme was measured for H₂S kinetics using the PDMS cuvette-based method. Reactions were maintained at 37 °C and the CSE concentration in each reaction is ~1.4 μM. (A) Representative plot of the initial rate (v₀) of CSE (which was purged at 2 ppm oxygen and incubated with 50 μM GSNO) catalyzed H₂S formation as a function of [Cys]. (B) The binding affinity (Kₘ) of control and GSNO-treated CSE at 2 ppm oxygen with Cys. (C) The substrate concentration of maximum velocity (Vₘₐₓ) of control and NO donor treated CSE with 2 ppm oxygen with Cys. (D) The substrate concentration of catalytic efficiency (K₉⁹/Kₘ) of control and NO donor treated CSE with 2 ppm oxygen with Cys. (E) Representative plot of the initial rate (v₀) of CSE (which was purged at 16 ppm oxygen and incubated with 50 μM GSNO) catalyzed H₂S formation as a function of [Cys]. (F) The binding affinity (Kₘ) of control and GSNO-treated CSE at 2 ppm oxygen with Cys. (G) The substrate concentration of maximum velocity (Vₘₐₓ) of control and NO donor treated CSE with 16 ppm oxygen with Cys. (H) The substrate concentration of catalytic efficiency (K₉⁹/Kₘ) of control and NO donor treated CSE with 16 ppm oxygen with Cys. Errors bars represent standard deviation (n = 3). (I) Summarized table of kinetic parameters.

<table>
<thead>
<tr>
<th>GSNO</th>
<th>Control</th>
<th>0.5 μM</th>
<th>1 μM</th>
<th>10 μM</th>
<th>20 μM</th>
<th>50 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 ppm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg Kₘ</td>
<td>8.99 ± 1.74</td>
<td>11.44 ± 0.55</td>
<td>10.99 ± 1.78</td>
<td>11.97 ± 1.61</td>
<td>16.75 ± 1.14</td>
<td>14.79 ± 3.31</td>
</tr>
<tr>
<td>Avg Vₘₐₓ</td>
<td>0.28 ± 0.018</td>
<td>0.37 ±</td>
<td>0.0003</td>
<td>0.37 ± 0.002</td>
<td>0.26 ± 0.033</td>
<td>0.35 ± 0.015</td>
</tr>
<tr>
<td>Avg K₉⁹/Kₘ</td>
<td>0.28 ± 0.038</td>
<td>0.28 ± 0.013</td>
<td>0.29 ± 0.048</td>
<td>0.19 ± 0.049</td>
<td>0.18 ± 0.020</td>
<td>0.17 ± 0.0024</td>
</tr>
<tr>
<td>16 ppm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avg Kₘ</td>
<td>8.34 ± 0.55</td>
<td>10.67 ± 1.15</td>
<td>11.85 ± 0.62</td>
<td>11.57 ± 0.60</td>
<td>12.59 ± 0.51</td>
<td>17.47 ± 1.29</td>
</tr>
<tr>
<td>Avg Vₘₐₓ</td>
<td>0.35 ± 0.072</td>
<td>0.40 ± 0.010</td>
<td>0.38 ± 0.064</td>
<td>0.385 ± 0.005</td>
<td>0.43 ± 0.14</td>
<td>0.44 ± 0.031</td>
</tr>
<tr>
<td>Avg K₉⁹/Kₘ</td>
<td>0.36 ± 0.018</td>
<td>0.33 ± 0.044</td>
<td>0.28 ± 0.015</td>
<td>0.28 ± 0.011</td>
<td>0.30 ± 0.003</td>
<td>0.22 ± 0.001</td>
</tr>
</tbody>
</table>
3.6 NO effects on H$_2$S production of RAW 264.7 cells

The PDMS-permeability method was used for the detection of H$_2$S generated by cultured cells. In this study, NO inducer, NOS inhibitor or both were added to suspensions of RAW 264.7 macrophages, which were then transferred into individual PDMS wells, placed into a cuvette and stirred. The H$_2$S produced by the cells was diffused through the PDMS bottom layer and subsequently detected by reacting with DTNB at 37˚C. There was no detectable H$_2$S in resting RAW cells. The rate of H$_2$S release from LPS-stimulated RAW cells was ~0.09 nmol/min/10$^6$ cells (Figure 3.7). Using the standard curve obtained by this method (Figure 3.3), this corresponds to ~0.58 nmol sulfide in 100 μL (5.8 μM sulfide or 1.0 μM free H$_2$S). The subsequent H$_2$S production of RAW cells with the NOS inhibitor, L-NAME after LPS stimulation, increased ~3.8-fold to ~0.35nmol/min/10$^6$ cells or 2.13 nmol (21.3 μM sulfide or 3.83 μM free H$_2$S). This increased rate of H$_2$S production in the presence of NOS inhibitor was decreased ~3.8 fold by the CSE inhibitor PAG. This indicates that the H$_2$S detected was produced enzymatically. These results also suggest that the increased NO (or NO$_x$) concentration in response to LPS in RAW cells depressed the H$_2$S levels. The reason that explains this result is that NO directly reacting with H$_2$S or regulating the H$_2$S synthesis pathways.
Figure 3.7 Rates of H$_2$S release from RAW 264.7 macrophages. Treated cells (~10$^6$) were lifted and added to PDMS wells containing PLP (50 µM) and Cysteine (1 mM) and the rate of H$_2$S release was determined using the PDMS cuvette-based method. Errors bars represent standard deviation (n = 3).
CHAPTER 4

DISCUSSION

4.1 PDMS-based H₂S Assay Method and Assessment

The reported biological concentrations of H₂S vary from nanomolar to the millimolar range (10,83,84). Many different methods were used to obtain these results. This has raised questions about the accuracy of measurements, especially for methods using H₂S–reactive chemical indicators. For example, the high H₂S concentrations could be due to false detection from other sulfide–containing species, oxidized metabolites of H₂S, or sulfide released as a result of indirect measurement or the probes may not be specific for H₂S. The most commonly used chemical probe is zinc acetate for trapping H₂S, followed by reaction with phenylenediamine, forming ethylene blue as a chromogenic indicator for H₂S (Scheme 2). These chemicals require reaction with H₂S, and several other steps of sample manipulation including of the pH environment of the reaction (85).

Based on the drawbacks of the existing methods, our lab developed the PDMS–permeability method for H₂S assay. The method must have the H₂S selectivity to become a valid H₂S assay. The maximum H₂S-permeability rate was found to be 244 ± 13 ppm (m)/min (or 7.2 ± 0.4 mM/min) free H₂S (detected as aqueous sulfide) from the H₂S donor Na₂S at pH 6.0. At pH 7.4 and pH 8.0 the rates were ~63% and 5.4% of the rate at pH of 6.0. Other thiols, such as dithiothreitol and glutathione had ~560-fold and ~750-fold lower permeability at pH 6.0 and pH 7.0 respectively. This indicates the practicality of the PDMS-based method for the specific measurement of H₂S gas in biological samples.
The detection limit of the PDMS-permeability method was estimated and expressed as aqueous sulfide, which is $9.2 \pm 1.9$ ppb(m) (or $\sim 0.51 \mu$M in 1.6 mL of buffer) of free H$_2$S. In comparison with other GC-based methods, it can detect H$_2$S as low as 1 ppt to 2 ppb (86,87), which indicates that our method is not the most sensitive one. However, our method can detect H$_2$S specifically and continuously, without complex sample preparation. Therefore, our method serves as a better choice for the inexpensive kinetic measurement of free H$_2$S and does not need specialized equipment.

A major advantage of the PDMS-based assay is that H$_2$S can be detected continuously by using virtually any available colorimetric or fluorimetric thiol-specific reagent once it crosses the PDMS-membrane. In addition, in–vivo H$_2$S concentrations in the synthesis location and the target cells are different. The location where CSE or CBS is producing H$_2$S would obviously have a higher content of H$_2$S compared to the target cells which are far away from the H$_2$S synthesizing location and actually consume H$_2$S (84,88). The advantage of using the PDMS-based method is detecting H$_2$S at the original producing location, before it crosses a membrane, which would provide us knowledge of H$_2$S being produced and H$_2$S that is being metabolized. It is known that H$_2$S is produced in the cytoplasm and oxidized in the mitochondria (89). Applying this assay by simply substituting cellular extract in the place of cellular suspensions may also be of use to assess this intracellular H$_2$S production.

Beside the advantages discussed above, the PDMS-based method does have drawbacks. One noticeable disadvantage is the relatively thick ($\sim 100 \mu$M) PDMS membrane bottom layer and $\sim 20$ s lag between H$_2$S addition/release and detection (Figure 3.3 (A)). Additionally, the thick membrane may prevent some H$_2$S diffusion, thus
preventing thiols from being detected. Both of these factors could account for the detection limit of H$_2$S using this method, however, this issue can be solved by manufacturing thinner membranes.

4.2 Determination of $K_M$ and $V_{\text{max}}$ of CSE and CBS for cysteine and homocysteine

The PDMS permeability method was used to determine the kinetic parameters of recombinant CSE and CBS (Table 3.3 and Table 3.4) and compared with literature values where possible. For the kinetic parameters of CSE, the $K_M$ determined for cysteine was 3.98 ± 2.44 mM, which is comparable to the literature value of 2.75 mM (13). The reason that may explain the slight difference between the PDMS-measured $K_M$ value and the literature value is that PDMS method was performed at physiological pH. The literature value was assayed at pH 8.2, which is optimal for H$_2$S generation by CSE and CBS. In addition, more of the total sulfide will traverse the PDMS membrane as free H$_2$S at pH ~6 compared to higher pH values. CSE $K_M$ value for homocysteine was determined to be 7.87 ± 4.75 mM. The $K_M$ that we detected is almost three times higher than the literature value (2.7 ± 0.85 mM) which was tested using a lead acetate-based method with spectrophotometry measuring a lead sulfide precipitate. The issue regarding this method is that the heavy metal ion (Pb$^{2+}$)–protein interactions may induce the artificial overproduction of H$_2$S (90). Zebrafish CBS enzyme kinetic parameters are shown in Table 3.4. The binding affinity $K_M$ values of CBS for HCys and Cys are 7.21 ± 1.18 mM and 9.39 ± 1.35 mM respectively. Zebrafish recombinant CBS has a relatively low binding affinity to its substrates compared with human recombinant CSE. The human recombinant CBS in literature has a binding affinity of 1.1 ± 0.7 mM for homocysteine. Similar to the CSE literature $K_M$ value, this kinetic assay also utilized heavy metal to
detect \( \text{H}_2\text{S} \). In human CBS, the allosteric effector AdoMet could bind to the CBS domain of the protein, hence the reaction rate \( V_{\text{max}} \) increases by \( \sim 2 \) to 3–fold without changing the \( K_M \) (23,32,35). Therefore, the CBS activity could be controlled by modulating this allosteric domain.

### 4.3 Effects of DEA/NO on recombinant CBS and Effects of DEA/NO and GSNO on recombinant CSE

As discussed, both NO and \( \text{H}_2\text{S} \) could be generated in a biological environment and could cross-talk. It has been found that NO could bind to the heme of human CBS and inhibit enzyme activity (48). Furthermore, our lab indicates that NO or \( \text{NO}_x \) may influence \( \text{H}_2\text{S} \) production by regulating \( \text{H}_2\text{S} \)-generating enzyme (CBS or CSE) activities (91). Hence for the purpose of studying NO’s function of regulating the \( \text{H}_2\text{S} \) biosynthesis pathway, the effects of DEA/NO and GSNO on CSE in a biological environment were examined. DEA/NO is a type of diazeniumdiolate, which has NO covalently connected to a diethylamine. GSNO is a nitrosothiol (71) and NO can be spontaneously released (72). GSNO is different from DEA/NO. This is because the NO releasing mechanism of GSNO (Scheme 3 (A)) is different than for DEA/NO (Scheme 3 (B)). GSNO is reported to be a more efficient S-nitrosylating agent compared to DEA/NO (92). Additionally, Sinha et al. demonstrated that different chemistries of NO release lead to different biological responses (72). Thus, different mechanisms acting on CSE by NO released from these two NO donors would be expected. This was examined by mimicking two types of physiological environments: 2 ppm and 16 ppm oxygen. 2 ppm oxygen represents a hypoxic condition in ischemia/reperfusion injury, and 16 ppm oxygen mimics the normal oxygen level in the lung, heart, brain and all tissues. Here the
evaluation examined the three parameters: $K_M$, $V_{\text{max}}$, and $K_{\text{cat}}/K_M$. The comparisons between the control and DEA/NO-treated CSE kinetic parameters were made. At 2 ppm or 16 ppm condition with DEA/NO treatment, $K_M$ increased ~2.4 or ~2 fold compared with control (Figure 3.5), respectively, which indicates the possible structural changes induced by NO binding that could lower the Cys binding affinity to CSE. The lowered binding affinity subsequently attenuated enzyme activity. Since the $K_M$ values changed differently at 2 ppm and 16 ppm for DEA/NO treated CSE, a possible reaction mechanism may apply to the anaerobic environment, and another possible reaction mechanism may apply to aerobic environment. GSNO was also used to check the effects of NO on H$_2$S-producing enzyme. H$_2$S production was measured at both 2 ppm and 16 ppm oxygen with different concentrations of GSNO, ranging from 0.5 to 50 μM. $K_M$ values have a maximum increase of ~2 fold with increasing concentration of GSNO for both 2 ppm and 16 ppm oxygen environment. The similar increase of $K_M$ values indicates that oxygen may not play a role in the GSNO-treated CSE catalytic reaction. Therefore, the modification of GSNO on CSE may follow the NO modification under low oxygen level.

For all of the NO-treated CSE, the most noticeable changes are the lowered binding affinity and subsequent depressed catalytic activity. This may be due to the reaction of NO with cysteine residues on CSE. There are 10 cysteines on each monomer of human recombinant CSE. A few of them are close to the PLP-binding catalytic site. A proposed mechanism of direct reaction between nitric oxide and thiols without the presence of oxygen may explain this structure change mechanism at 2 ppm oxygen. NO can induce disulfide formation (93) (Scheme 5). In this mechanism, NO directly oxidizes
thiols to a disulfide: RSSR. This proposed mechanism has been found as a major product in RAW cells under low oxygen condition (94).

In the presence of oxygen (16 ppm), NO may react with oxygen to form 'NO₂, and 'NO₂ can react with another NO molecule to produce N₂O₃ (75). N₂O₃ could then react with the RSH group of cysteine of CSE resulting in the formation of RSNO (72,74,75) (Scheme 6). Another potential pathway would be 'NO₂ formed from the reaction of 'NO with oxygen reacts with thiol (RSH) to form a thyl radical (RS'), and then another 'NO comes to react with it to form S-nitrosothiols (Zhang, Andrekopoulos et al. 2009) (Scheme 6). As a result, the S-nitrosylated cysteine may change the structure or folding of the protein, and hence affect the catalytic properties of CSE. S–nitrosylation is predicted to occur primarily in the hydrophobic domains of proteins, which is kinetically favourable (93). According to Hess et al. and Liu et al., both NO and oxygen are hydrophobic, and hence the microenvironment that would support S-nitrosylation should be hydrophobic (95,96). Based on findings, we propose that the possible S-nitrosylation in CSE should be in its hydrophobic microdomains. Figure 4.1 highlighted the hydrophobic domain of CSE, where the possible modification would take place. S-nitrosothiols formed during from S-nitrosylation have physiological functions which could be used in other signaling pathways. In a recently published paper, Filipovic reported that a new compound: thionitrous acid (HSNO) could be formed from H₂S with low molecular weight thiols or S-nitrothiols (Scheme 7), which can freely cross the cellular membrane and act as a source of NO and as a transnitrosating agent (77).

The other way in which NO could affect enzyme activity is the modification of tryptophan and tyrosine. NO was found to directly scavenge tyrosine and tryptophan.
radicals in amino acids, peptides and proteins (97). In the case of the Tyr residue, Tyr’ could react rapidly with nitric oxide derivative, nitrogen dioxide (\('\text{NO}_2\) to form 3-nitrotyrosine (Scheme 8 A). Alternatively, NO may react with Trp’ to form N-nitro species (Scheme 8 B). One of these two residues may be in the catalytic site or important for protein folding, therefore their modification could result in depressed enzyme activity.

![Figure 4.1 Highlighted hydrophobic domain of CSE shown in yellow, where S-nitrosylation may occur.](image)

\[
2\cdot \text{NO} + 2 \text{RSH} \rightarrow \text{RSSR} + 2\text{HNO}
\]

**Scheme 5: The reaction of NO with thiols in the absence of oxygen**

\[
\begin{align*}
\cdot \text{NO} + 1/2 \text{O}_2 & \rightarrow \cdot \text{NO}_2 \\
\cdot \text{NO} & \rightarrow \text{N}_2\text{O}_3 \\
\text{RSH} & \rightarrow \text{RSNO}
\end{align*}
\]

**Scheme 6: Reactions of NO with thiols in the presence of oxygen**
(1) $\text{HS}^\bullet + \bullet \text{NO} \rightarrow \text{HSNO}$
(2) $\text{H}_2\text{S} + [\text{H}_2\text{NO}_2]^+ \rightarrow \text{HSNO} + \text{H}_3\text{O}^+$
(3) $\text{RSNO} + \text{H}_2\text{S} \rightarrow \text{HSNO} + \text{RSH}$

Scheme 7: Reactions for the formation of \text{HSNO}

(A)

(B)

Scheme 8: Reactions of NO with Tyr (A) and Trp (B).
4.4 NO inducer and NO suppresser effects on H$_2$S production in RAW 264.7 cells

The PDMS-based H$_2$S assay was used to monitor the H$_2$S production from stimulated and unstimulated RAW 264.7 cells. LPS is a known NOS inducer which could increase the expression of CSE (41,68) and L-NAME is a NOS inhibitor. In this study, both the NOS inducer and inhibitor were incubated with RAW cells for 12 h, with the L-NAME incubation occurring after LPS stimulation. The production of H$_2$S by CSE was then measured. Previous studies show that LPS induces NO production by inducing iNOS expression. According to the recent work of Zhu et al., LPS stimulation could increase H$_2$S production by CSE (27,80), and this activation may depress the NO production by interacting with NO equivalents, such as NO$^+$ and N$_2$O$_3$ (68). This experiment is aimed to test this hypothesis: NO$_x$ reacts with H$_2$S. By inhibiting NO production using L-NAME, H$_2$S production increased by ~3.8 fold (Figure 3.7). These results provide evidence that stimulated NO or NO$_x$ production in RAW cells inhibits H$_2$S production by either direct reaction with H$_2$S or by interfering with its biogenesis pathway. From the study of PDMS with NO in the normal and low levels of oxygen environment, it is possible that NO and oxygen are concentrated in the hydrophobic PDMS environment, reacting to form N$_2$O$_3$ (91). The latter can readily react with sulfide. This phenomenon is expected to happen in the hydrophobic microdomains of cells leading to a direct scavenging of H$_2$S. It is also possible that NO or NO$_x$ could modulate the H$_2$S-producing pathway by directly acting on the activity and expression of CSE and CBS as proposed in the NO effects on both enzymes.
4.5 The cross-talk between \( \text{H}_2\text{S} \) and NO

The study of cross-talk between \( \text{H}_2\text{S} \) and NO became popular recently, due to the evidence of NO and \( \text{H}_2\text{S} \) signaling pathways interacting with each other, where one could intensify the other’s physiological effect (98) and this study has been focused on mechanisms that are not yet fully understood. \( \text{H}_2\text{S} \) and NO are known to directly react with one another. My study attempted to determine the direct effects of NO donors on CSE activity. Results suggest that DEA/NO and GSNO may have a potential role in regulating CSE-dependent \( \text{H}_2\text{S} \) production by changing the stability and activity of a protein (78). The resulting modification: RSNO could be used for further physiological purpose, such as regulating enzyme activity by modifying the thiol-dependent process (75), control the vascular tone (99), or could be useful for S-nitrosothiol-mediated pathways (100). These results suggest that direct NO donor could be a potential therapeutic compound for partially inhibiting CSE-dependent \( \text{H}_2\text{S} \) production in patients who have hyperhomocysteinemia (28). Controlling the release of \( \text{H}_2\text{S} \) could also be a potential method to attenuate cardiovascular-related disease. Slow NO donors may be also useful in controlling \( \text{H}_2\text{S} \)-induced inflammation. For example, it has been shown that \( \text{H}_2\text{S} \) can induce NO production (61), and NO donor inhibits LPS-induced \( \text{H}_2\text{S} \) biogenesis (101). Therefore, NO and \( \text{H}_2\text{S} \) may function to regulate the inflammatory response (45). Therefore, managing \( \text{H}_2\text{S} \) biosynthesis could be a helpful method to reduce \( \text{H}_2\text{S} \)-related diseases, but further tests are necessary to determine the amount of NO that is necessary to lower \( \text{H}_2\text{S} \) production in biological systems. Similarly, \( \text{H}_2\text{S} \) donor could control NO production. During endotoxic shock, \( \text{H}_2\text{S} \) inhibits iNOS expression (102). Thus, these
studies provide evidence of the interaction between NO and H$_2$S and investigate the potential methods for controlling the concentration of one in the production of the other.
CHAPTER 5
CONCLUSION AND FUTURE WORK

H₂S and NO are two gaseous molecules which have important roles in regulating physiological functions. The evidence shows that the two signaling molecules regulate each other in controlling these physiological functions. This project is aimed to study the cross talk between NO and H₂S. Due to the disadvantages of the existing H₂S assays, we developed a continuous accurate H₂S assay, which uses H₂S-selective PDMS-permeable membranes. PDMS was molded into wells that can fit into a 96-well plate or a cuvette for continuous measurements. The method was validated using H₂S-producing enzymes CSE and CBS. The resulting kinetic parameters of CSE were compared with the literature values and were within the literature range when using cysteine as the substrate. Then, this method was used to examine the NO donor effects on H₂S-producing enzyme: CSE. The lowered binding affinities led to depressed catalytic activities of enzymes. The results hint that NO may modify enzyme residues and these residue modifications may affect their catalytic efficiency. Further, H₂S production by RAW 264.7 macrophages was also examined using the PDMS method. The results suggest that stimulated NOₓ inhibits H₂S production by either direct reaction with H₂S or by interfering with its biogenesis pathway. As a result, these studies raise the possibly that NO has the potential to regulate H₂S production.

Future work involves the use of mass spectrometry to identify the specific residues that are modified by NO. For instance, if the S-nitrosylation is achieved under NO treatment, it is important to find which cysteine is being modified. If the modification
is not on cysteine, NO would either react with tyrosine or tryptophan residues. Locating the modification would allow as to manipulate the H$_2$S production by the enzymes.
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