Efficient Inhibition of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) by Sulfuration with Solubilized Elemental Sulfur

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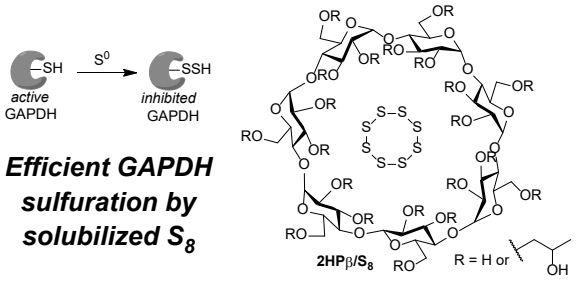
Abstract

Hydrogen sulfide (H_2S), carbon monoxide (CO), and nitric oxide (NO) have garnered increasing scientific interest in recent decades due to their classifications as members of the gasotransmitter family of signaling molecules. Due to the versatility of sulfur redox chemistry in biological systems, H_2S specifically is being studied for its ability to modulate cellular redox environments, particularly through the downstream production of oxidized sulfur species. A major mechanism of this regulation is through a posttranslational modification known as persulfidation, where oxidized sulfur atoms are appended to free cysteine in proteins. Currently, it is difficult to discern the activity of H_2S itself versus these oxidized sulfur species, particularly sulfane sulfur (S^0). We have previously developed a method of solvating S_8, a source of pure S^0, to more accurately study persulfidation and sulfuration in general. Here, we apply this pure S^0 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which has previously been shown to be inhibited by S^0-containing polysulfides via persulfidation. Using solvated S^0, we demonstrate that native, reduced GAPDH can be completely shut down by sulfuration with S^0. Further, oxidized GAPDH activity cannot be rescued using S^0, demonstrating that it is the oxidation of reduced GAPDH by S^0 that curtails its activity. We also compare inhibition of GAPDH by pure S^0 to different polysulfides and demonstrate the modulating effects that pendant alkyl groups have on GAPDH inhibition. These results highlight the promise of this novel, simplified system for the study of S^0.

Graphical Abstract:



Introduction

The evolution of life on Earth has been shaped by hydrogen sulfide (H_2S). Approximately 2.4 billion years ago, Earth's atmosphere shifted from a reducing to an oxidizing environment that was rich in O_2.[1] This crucial event likely contributed to the evolution of multicellular life on Earth, because O_2 reduction delivers among the largest free energy release per electron transfer of all elements.[2] This feature, along with the stability of O_2, enabled the development of specialized and compartmentalized features associated with more complex lifeforms. Prior to this shift to an oxidizing environment, Earth's atmosphere contained much higher concentrations of H_2S,[3, 4] and it was under these conditions that early single-celled life began. Sulfur-reducing microbes used abundant sources of oxidized sulfur as metabolic electron acceptors to generate H_2S and CO_2,[5] and sulfur-oxidizing species used H_2S as an electron donor for anoxygenic photosynthesis.[6] Even today, the influence of H_2S on Earth's earliest lifeforms is reflected in the very fabric of eukaryotic multicellular life billions of years after H_2S was displaced by O_2 as a primary energy source. For example, sulfide-oxidizing α-protobacteria that developed a symbiotic relationship with an archaeon host many have given rise to present-day mitochondria.[7] Such unions may have contributed to the survival of anaerobic species during the transition to an aerobic atmosphere, which may have also been aided by the ability of mitochondrian symbiotes (and even mitochondria today[8] through sulfide:quinone oxidoreductase) to produce ATP from H_2S.[9] These cellular remnants of our ascension from the primordial H_2S world are reflected in our mitochondria, cellular responses to hypoxia,[10] and complex cellular sulfur signaling systems.[8]

Today H_2S joins carbon monoxide (CO) and nitric oxide (NO) as a trio of endogenously produced signaling molecules known as the gasotransmitters.[11-14] These small, membrane-permeable gases act in numerous pathways throughout cells, sometimes acting in concert to achieve complex processes including angiogenesis and vasodilation.[15] Of the three molecules,

H_2S is thought to have had the greatest influence on the naissance of eukaryotic cells.[7, 9] Much of this contribution to evolutionary history may be due to accessibility of -2 to +6 sulfur oxidation states.[16] Beginning from fully-reduced sulfur, H_2S can be oxidized in cells to form polysulfides, which contain sulfane sulfur (S^0).[17]

Prior work on the effects of H_2S treatment in cells has revealed benefits such as reduction of oxidative stress,[18-20] prevention of gastric ulcers,[21] and enhancement of wound healing rates.[22-24] Recent evidence suggests, however, that S^0 species may be responsible for much of the action originally associated with H_2S treatment.[25] Much of this activity may be due to the oxidative post-translational modification known as persulfidation, or sulfhydration, in which free cysteine residues react with S^0 sources to form persulfides (-SSH) or higher-order sulfurated species ($-SS_{n\geq1}H$).[26-30] Since the sulfur atom in cysteine and H_2S share a -2 redox state, H_2S cannot directly persulfidate proteins unless they have been previously oxidized, which typically occurs through the formation of disulfide bonds, nitrosothiols, or sulfenic acids. By modifying cysteine residues, persulfidation changes both the structure and function of proteins and can serve as a regulatory, redox-sensitive enzyme "switch". A prominent example of a cellular process regulated by persulfidation is vasodilation, which is mediated by the persulfidation of Cys_{43} in the Kir 6.1 subunit of K_{ATP} channels[31] and results in hyperpolarization and subsequent vasorelaxation of endothelial cells.

Building from the recognition that persulfidation is a key regulator of sulfur biology, methods to modulate or shunt this reactivity are important areas of investigation. A number of S^0-containing donors, ranging from persulfide donors[32-39] to discrete polysulfides[40-42] have been developed and used to investigate how S^0 and persulfidation impacts various systems. However, cellular treatments with different S^0 donor species often leads to diverging results.[43]

Simplifying the approach to S^0 delivery, we recently developed a system for the solvation of elemental sulfur (S_8), a stable species composed entirely of S^0 atoms, using 2-hydroxypropyl β-cyclodextrin (2HPβ).[44] This solubilized 2HPβ/S_8 can be efficiently reduced by thiols to generate H_2S, is cell permeable, and can confer protection against oxidative stress in macrophages. Building from this system, we demonstrate here the high efficacy of the 2HPβ/S_8 system in inhibiting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by persulfidation/sulfuration and compare these results to different common S^0 delivery sources (Figure 1).

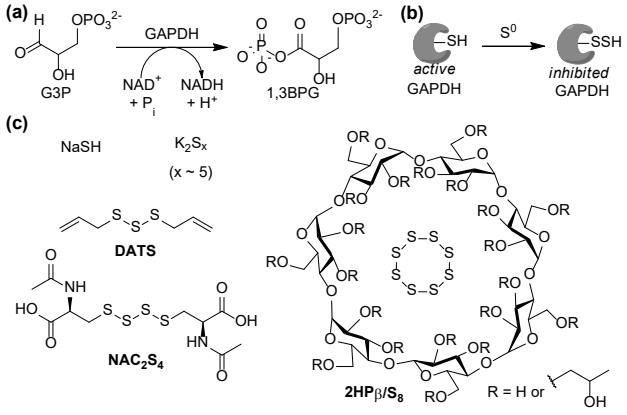


Figure 1. (a) GAPDH conversion of glyceraldehyde 3-phosphate (G3P) to 1,3-bisphosphoglycerate (1,3BPG). (b) Persulfidation of GAPDH results in enzyme inhibition. (c) Sulfide and sulfane-sulfur sources used to investigate GAPDH persulfidation and inhibition in this study.

Results and Discussion

Based on the ability of synthetic sulfane-sulfur containing species to persulfidate cysteine residues, we wanted to compare the reactivity of common S^0 donors and determine whether

2HPβ/S_8 can effectively participate in similar reactions. To evaluate the effectiveness of 2HPβ/S_8 in these reactions, we studied the persulfidation and subsequent inhibition of GAPDH. Earlier work by Mutus and co-workers showed that treatment of GAPDH with inorganic polysulfides resulted in persulfidation of Cys_{156} and Cys_{247} and subsequent inhibition of GAPDH activity whereas treatment with H_2S had no effect on GAPDH activity.[45] Based on this prior work and the ease of monitoring GAPDH activity, we reasoned that the GAPDH platform would be a simple system to compare the persulfidation efficiency of different sulfane sulfur sources.

Measuring K_M and V_{max} of GAPDH and the Saturating G3P Concentration

We first measured the K_M and V_{max} of purified, lyophilized GAPDH to determine the ideal concentration of the glyceraldehyde-3-phosphate (G3P) substrate for subsequent inhibition assays. Briefly, initial rates of NAD^+ reduction by GAPDH at constant NAD^+ concentration were plotted against G3P concentrations (0-2 mM) using the method described previously.[45] Using a calibration curve for NADH concentration and fitting the resultant data provided, K_M = 0.59 mM and V_{max} = 0.16 µM NADH/sec (Figure 2).

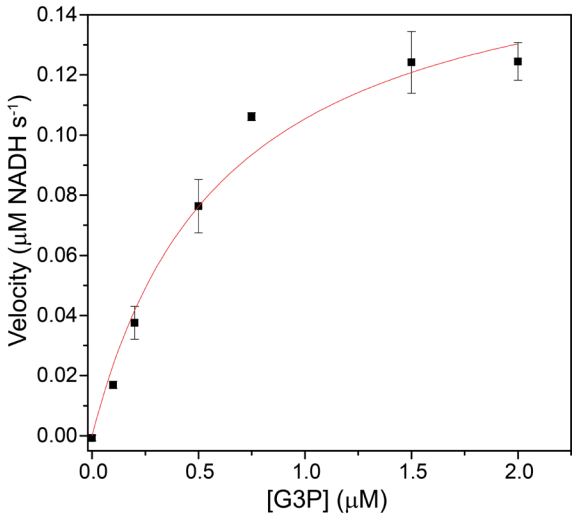


Figure 2. Michaelis-Menten curve for the kinetic relationship between GAPDH and G3P concentration. The K_M was calculated as 0.59 mM G3P, and the V_{max} as 0.16 μM NADH/sec.

Polysulfides, but not H_2S, Decrease GAPDH Activity

In prior work, both H_2S (in the form of NaSH or Na_2S) and potassium polysulfide ("K_2S_x") were evaluated for GAPDH inhibition activity under different redox conditions.[45] Because the sulfur atom in H_2S is in the fully reduced -2 state, H_2S should not react with or modify reduced GAPDH. Conversely, polysulfides contain S^0 and should be able to persulfidate or sulfurate free cysteine residues. Consistent with this chemistry, Mutus and co-workers showed that treatment of reduced GAPDH with NaSH did not impact GAPDH activity, whereas K_2S_x treatment reduced enzyme activity by ~60%. These results contradicted earlier reports of GAPDH activity enhancement after sulfide treatment.[28] A key point is that NaSH cannot persulfidate reduced proteins, although it can persulfidate oxidized cysteine residues such as Cys-SOH or Cys-SNO.[26] Similarly, NaSH solutions, especially if handled in air, likely contain small amounts of oxidized polysulfides, which may also contribute to the observed activity. Such adventitious oxidation may contribute to these reports of H_2S modification of GAPDH activity because proteins *in vitro* and *in cellulo* could have oxidative post-translational modifications unless measures are taken to ensure free and reduced cysteine residues and a uniform redox state. We repeated the GAPDH activity experiments with NaSH and K_2S_x on reduced GAPDH (Figure 3), which provided results that closely matched the previously reported data from Mutus and co-workers. NaSH treatment did not modify GAPDH activity (Figure 2a) whereas K_2S_x treatment reduced GAPDH activity by ~70% (Figure 2b).

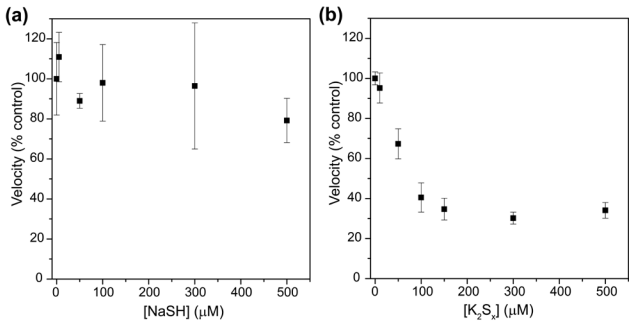


Figure 3. GAPDH inhibition by 30-minute treatments of (a) NaSH and (b) K_2S_x. NaSH treatment does not result in persulfidation or modification of GAPDH activity, whereas treatment with K_2S_x results in persulfidation and inhibition of GAPDH activity by ~70%. All data were acquired in triplicate at 37 °C by measuring production of NADH.

One limitation of using K_2S_x as a source of inorganic polysulfides, however, is that K_2S_x is a mixture of polysulfides that readily disproportionates into other polysulfides and reactive sulfur species.[46] Similarly, because K_2S_x is not one specific polysulfide species, it is difficult to know accurate concentrations of the resultant polysulfides, or S^0, in solution. For the purposes of this study, we assumed an average formula of K_2S_5 based on the elemental makeup of this inorganic salt. Due to this uncertainty, however, the S^0 concentrations from K_2S_x solutions should be viewed as approximate rather than exact. In addition, K_2S_x disproportionation also likely generates small amounts of H_2S in solution. Such adventitious H_2S generation from K_2S_x may help to explain the observation by Mutus and co-workers that the activity of GAPDH that had been oxidized to mixed disulfides with glutathione disulfide (GSSG) could be partially rescued by treatment with K_2S_x, which was also observed after treatment with NaSH and dithiothreitol (DTT). Taken together, this ambiguity highlights the need for more well-defined sources of S^0 and presents

a valuable opportunity to evaluate whether 2HPβ/S_8, which only contains sulfur atoms in the S^0 oxidation state, can more effectively inhibit GAPDH activity.

2HPβ/S_8 Strongly Inhibits GAPDH Activity

We next investigated the impact of 2HPβ/S_8 on GAPDH inhibition with concentrations of S_8 ranging from 0-100 μM (0-800 μM S^0). Under these conditions, we observed strong inhibition, with complete inhibition occurring at 100 μM S_8 (Figure 4). The concentration of S_8 required to achieve the 60% reduction in GAPDH activity observed from 200 μM K_2S_x treatment was less than 20 μM (160 μM S^0). One striking difference in GAPDH activity between treatment with K_2S_x and 2HPβ/S_8 is that GAPDH inhibition plateaus at high concentrations of K_2S_x treatment, whereas 2HPβ/S_8 resulted in complete inhibition near 100 μM S_8. Although the exact cause of this plateau behavior is unclear, a plausible explanation is that higher K_2S_x concentrations lead to higher H_2S generation, which would lead to an equilibrium state in which the GAPDH enzyme is never fully oxidized or inhibited. These limitations again highlight the potential of using solubilized S_8 as a means of studying S^0 and persulfidation and sulfuration under conditions with a simpler sulfur redox landscape.

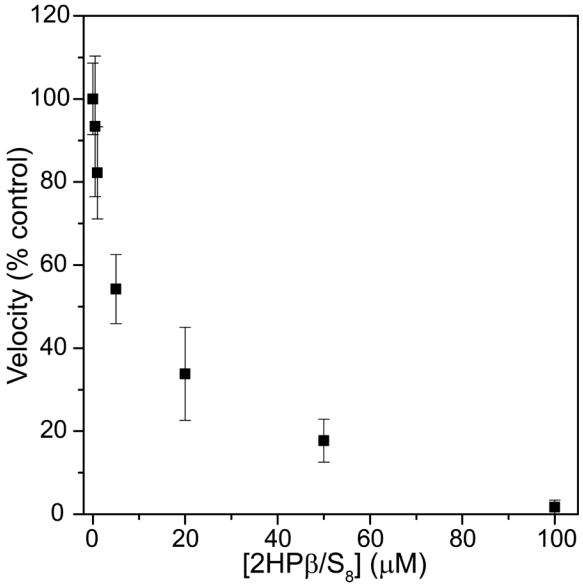


Figure 4. Inhibition of GAPDH with 30-minute treatments of 2HPβ/S_8. Complete GAPDH inhibition is observed at high [2HPβ/S_8] is observed. All data were acquired as initial rates in triplicate at 37 °C by measuring production of NADH.

Sulfane Sulfur does not Rescue Oxidized GAPDH Function

Having demonstrated that 2HPβ/S_8 results in efficient inhibition of reduced GAPDH activity, we next evaluate how 2HPβ/S_8 affects oxidized GAPDH activity. We also reasoned that the 2HPβ/S_8 could provide further insights into the unexpected prior observation that both K_2S_x and NaSH partially rescue the function of GAPDH oxidized with GSSG.[45] Our expectation was that the disproportionation of K_2S_x likely generates reducing byproducts,[46] which were responsible for the observed GAPDH activity rescue, although the mixture of polysulfides and H_2S may also have enhanced activity. Based on this logic, treatment of oxidized GAPDH with 2HPβ/S_8 should not increase GAPDH activity when oxidized. To test this hypothesis directly, we incubated GAPDH with excess GSSG, while also incubating reduced GAPDH with tris buffer only. Both parallel samples were incubated at room temperature for two hours, passed through

PD-10 desalting columns packed with Sephadex G-25 resin for buffer exchange, then subjected to the activity assay with 2HPβ/S_8 (Figure 5). As expected, no rescue of function was observed for the oxidized GAPDH sample treated with 2HPβ/S_8, whereas the reduced GAPDH sample showed sharp inhibition with increasing 2HPβ/S_8. These results indicate that S^0 alone does not rescue oxidized GAPDH activity, because rescue requires reducing the enzyme back to its native form. These data also highlight the benefits of using S_8 delivery because the observed effects can more reliably be attributed to S^0 atoms that do not have the disproportionation challenges of polysulfides.

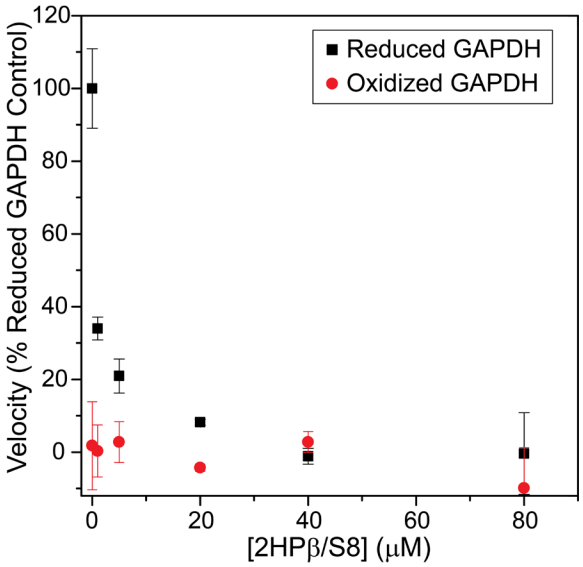


Figure 5. Initial rates of either native (black) or oxidized (red) GAPDH after 2HPβ/S_8 treatment. Reduced GAPDH was incubated with either GSSG or untreated tris buffer for two hours before 30-minute incubation with 2HPβ/S_8. 2HPβ/S_8 treatment strongly inhibits native GAPDH by oxidation and cannot rescue the function of oxidized GAPDH. All data points were acquired in triplicate at 37 °C by measuring production of NADH.

Organic Polysulfides also Inhibit GAPDH

To further the comparison of 2HPβ/S_8 with other sources of sulfane sulfur, we also investigated organic polysulfides.[47-51] For these investigations, we used both the naturally-occurring polysulfide diallyl trisulfide (DATS) and the synthetic *N*-acetylcysteine tetrasulfide (NAC_2S_4).[40] When we applied DATS to GAPDH, we observed a very similar result to that observed for K_2S_x treatment with 500 μM DATS (500 μM S^0) resulting in a ~50% reduction in GAPDH activity (Figure 6a). Taking into account the number of S^0 atoms per molecule, DATS is a less efficient at inhibiting GAPDH activity when compared with 2HPβ/S_8, which achieved a 50% reduction in GAPDH activity between 5 – 10 μM (40 – 80 μM S^0). It is also possible that the tri- versus tetra-sulfides have inherently different sulfane sulfur donating abilities, and prior reports have shown differing cytotoxicities of a series of organic polysulfides with different chain lengths.[43] To further investigate this activity with other polysulfides, we treated GAPDH with NAC_2S_4 (Figure 6b), which sharply curtailed almost all enzyme activity at only 5 μM (10 μM S^0) and completely inhibited GAPDH activity between 25 – 50 μM (50 – 100 μM S^0). These results highlight the efficacy of NAC_2S_4 at oxidizing GAPDH.

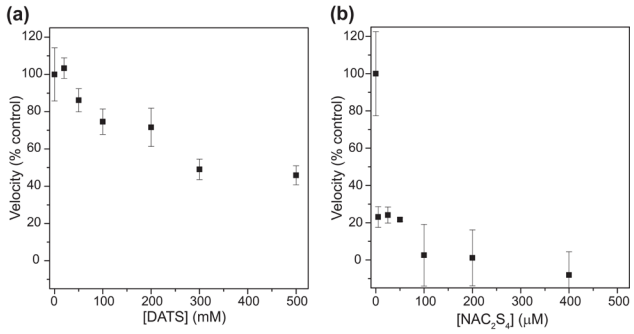


Figure 6. Inhibition of GAPDH after 30-minute treatment with organic polysulfides (a) DATS and (b) NAC_2S_4. All data points were acquired in triplicate at 37 °C by measuring production of NADH.

Conclusions

Persulfidation and sulfuration are key post-translational modification connected to the interplay between H_2S and other reactive sulfur species. To discern which cellular activities are modulated by H_2S versus S^0, it is vital to have systems that allow for the direct delivery of S^0. Organic polysulfides are stable sources of S^0, but the activity is also dependent on the pendant alkyl groups on the polysulfide. We showed that significant GAPDH inhibition variability is observed for organic polysulfides when comparing the activity of DATS and NAC_2S_4. By contrast, the 2HPβ/S_8 system provides a simple and reliable method for S^0 delivery and superior GAPDH inhibition when compared to previously investigated inorganic polysulfides.

Based on the complex redox landscape of reactive sulfur species, it is often difficult to disentangle the activity of H_2S from downstream oxidized sulfur species, many of which can modify the structure and function of proteins and result in cell-protective effects.[17] The direct delivery of pure S^0 in biological studies provides an important approach toward untangling the complex redox and chemical pathways associated with reactive sulfur species. Much as how controllable H_2S donors have become essential tools for investigating the role of H_2S in different systems, we anticipate that expanded development S^0 donors will similarly advance investigations into the role of sulfane sulfur in complex environments.

Materials and Methods

Materials

Reagents were purchased from Sigma-Aldrich, Cayman Chemical Company, Fisher Scientific, Oakwood Chemical, and Strem Chemicals. 2HPβ/S_8[44] and NAC_2S_4[40] were

prepared as previously described. Lyophilized glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from rabbit muscle was purchased from Sigma and used in all experiments. Enzyme kinetic data was recorded on a Cary 60 or Cary 100 UV-vis spectrophotometer at 37 ºC.

Calibration Curve for Enzyme Velocity

Various concentrations of NADH were dissolved in 20 mM pH 7.8 tris buffer and added to 500 µL assay buffer (20 mM tris pH 7.8, 100 mM NaCl, 1 mM oxidized nicotinamide adenine dinucleotide (NAD^+), 10 mM sodium pyrophosphate, 20 mM sodium arsenate, 0.1 mg/mL bovine serum albumin (BSA)) for a total volume of 1 mL in plastic cuvettes. Each cuvette also contained 1 mM G3P. The absorbance values were measured at 340 nm on a Cary 100 Uv-vis at 27 ºC.

Determination of Saturating Glyceraldehyde-3-Phosphate Concentration

GAPDH was dissolved in 20 mM pH 7.8 tris buffer to yield a solution of 1 mg/mL. A 10 µL aliquot of this solution was added to 10 µL tris buffer in microfuge tubes, which were then incubated at 37 °C for 30 minutes. After incubation, 1 µL of solution was removed from each tube and added to 500 µL assay buffer in plastic 1.5 mL cuvettes. The cuvettes were placed into the spectrophotometer and allowed to equilibrate at 37 °C. The reaction was initiated by adding 500 µL of glyceraldehyde-3-phosphate (G3P) (Sigma) in pH 7.8 tris for final reaction concentrations of 0 – 2 mM G3P. Kinetic scans were acquired at 340 nm on a Cary 100 UV-vis for 2 minutes at 37 °C. Each kinetic experiment was performed in triplicate. Data was analyzed by subtracting the initial absorbance and dividing by the total reaction time. K_M values were calculated with the GRG Nonlinear solving method in Microsoft Excel.

GAPDH Inhibition Assays

We followed the reduced GAPDH enzyme assay as previously described.[45] Lyophilized GAPDH was dissolved in 20 mM pH 7.8 tris buffer at a concentration of 1 mg/mL. A 10 µL aliquot

of this solution was added to 10 µL tris buffer in microfuge tubes containing the test compounds. The vehicles test points were pH 7.4 PBS buffer for NaSH and K_2S_x, 5% 2-hydroxypropyl β-cyclodextrin (2HPβ) for S_8, pH 7.8 tris buffer for diallyl trisulfide (DATS), and 2.5% DMSO for *N*-acetylcysteine tetrasulfide (NAC_2S_4). Solutions of S_8 in 2HPβ were prepared as previously described.[44] Each experiment was performed as described above on a Cary 60 UV-vis at 37 °C for two minutes, performed in triplicate, and adjusted to the vehicle.

Comparison of Oxidized and Reduced GAPDH Activity

We followed the oxidized GAPDH enzyme assay as previously described.[45] Briefly, two solutions of lyophilized GAPDH were solvated to 2 mg/mL in 2.5 mL 20mM Tris pH 7.8. One GAPDH solution was treated with equal volume of 20 mM GSSG in Tris for 2 hours (final concentration 10 mM), and the other GAPDH solution was simultaneously incubated in an equal volume of untreated Tris, so that both solutions had a final GAPDH concentration of 1 mg/mL. Both samples were run through PD-10 desalting columns packed with Sephadex G-25 resin (GE). Inhibition assays with S_8 were then performed as described above.

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