

Supramolecular Activation of S₈ by Cucurbiturils in Water and Mechanism of Reduction to H₂S by Thiols: Insights into Biological Sulfane Sulfur Trafficking

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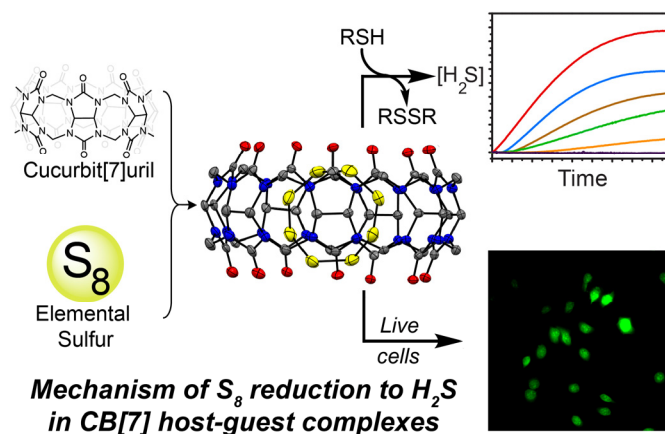
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Abstract:

Reactive sulfur species (RSS) play critical roles in diverse chemical environments. Molecules containing sulfane sulfur (S^0) have emerged as key species involved in cellular redox buffering as well as RSS generation, translocation, and action. Using cucurbit[7]uril (CB[7]) as a model hydrophobic host, we demonstrate here that S_8 can be encapsulated to form a 1:1 host guest complex, which was confirmed by solution state experiments, mass spectrometry, and X-ray crystallography. The solid state structure of CB[7]/ S_8 shows that the encapsulated S_8 is available to nucleophiles through the carbonyl portals of the host. Treatment of CB[7]/ S_8 with thiols results in efficient reduction of S_8 to H_2S in water at physiological pH. We establish that encapsulated S_8 is attacked by a thiol within the CB[7] host and that the resultant soluble hydropolysulfide is ejected into solution, where it reacts further with thiols to generate soluble sulfane sulfur carriers and ultimately H_2S . The formation of these intermediate is supported by observed kinetic saturation behavior, competitive inhibition experiments, and alkylative trapping experiments. We also demonstrate that CB[7]/ S_8 can be used to increase sulfane sulfur levels in live cells using fluorescence microscopy. More broadly, this work suggests a general activation mechanism of S_8 by hydrophobic motifs, which may be applicable to proteins, membranes, or other bimolecular compartments that could transiently bind and solubilize S_8 to promote reaction with thiols to solubilize and shuttle S_8 back into the redox labile sulfane sulfur pool. Such a mechanism would provide an attractive manifold in which to understand the RSS translocation and trafficking.



Introduction:

Reactive sulfur species (RSS) play critical roles in diverse chemical environments ranging from biological chemistry to global sulfur cycles.¹⁻² This importance is even reflected in terrestrial evolution, with early chemoautotrophic sulfur-reducing microbes using oxidized forms of sulfur as electron acceptors to generate H₂S and CO₂ when Earth's atmosphere was much more reducing.³ Remnants of our sulfur-reliant past are found in contemporary biology in our mitochondria, which likely evolved from a symbiotic fusion between sulfide-oxidizing α -proteobacteria and sulfur-reducing archaea.⁴⁻⁶ Building from this evolutionary importance, interest in biological RSS has been reinvigorated in the last two decades, in part due to the discovery that hydrogen sulfide (H₂S) is an endogenously produced signaling molecule.⁷⁻¹¹ H₂S joins nitric oxide (NO) and carbon monoxide (CO) in a group of endogenous gaseous molecules often referred to as gasotransmitters.¹² H₂S is now accepted to play key roles in diverse signaling and regulatory pathways and has been investigated extensively as both a research tool and potential therapeutic agent in different diseases affecting the cardiovascular, neuronal, gastrointestinal, and other systems.¹³⁻¹⁴ The chemical mechanisms by which H₂S exerts biological action is intrinsically tied to a complex and interconnected array of RSS. Although sulfur can access oxidation states from -2 to +6 in biology,¹⁵ much of established RSS redox and signaling occurs within the fully reduced (S²⁻) to the sulfane sulfur (S⁰) states. Common entities within this 2 electron redox manifold include H₂S, thiols, persulfides (RSSH), organic hydropolysulfides (RS(S)_nSH), and organic / inorganic polysulfides (RS(S)_nSR, HS(S)_nSH). These and related sulfane sulfur containing compounds form a redox-labile RSS pool with key roles in diabetes mellitus, high blood pressure, schizophrenia, as well as other diseases.^{14, 16-17} Redox-labile RSS with S¹⁻ or S⁰ oxidation states often generate species with enhanced reactivity, which highlights why this pool of compounds may be particularly important in cellular signaling processes. As an example, persulfidation of protein Cys residues is a key post-translational modification that can modify protein activity and also that that protects proteins from irreversible oxidation during aging.¹⁸ More broadly, this pool

of soluble sulfane sulfur containing species plays key and emerging roles in cellular redox buffering as well as RSS generation, translocation, and action.

In parallel with the rapidly expanding biological roles of RSS has been the development and application of chemical tools for the delivery of both fully reduced and sulfane sulfur species.¹⁹⁻²² This work has led to significant advances in both the development of practical tools for RSS delivery and also in the expansion of fundamental RSS chemistry that informs on biological action. For example, small molecule RSS donors have shown high efficacy in animal models for conditions ranging from cardiovascular disease and ischemia/reperfusion injury to gastrointestinal inflammation and wound healing.²³⁻²⁵ Similarly, contemporary investigations have led to the discovery of new fundamental chemistry of persulfides and polysulfides,²⁶⁻²⁸ new approaches for responsive RSS delivery,²⁹⁻³⁰ and new interconnectivities with other small signaling molecules.³¹⁻³⁵ An attractive approach for sulfane sulfur delivery would be to use systems with only S⁰ atoms. A significant challenge with this approach, however, is that the simplest form of sulfane sulfur, S₈, is essentially insoluble in water ($\sim 1.96 \times 10^{-8} \text{ M}^{-1}$).³⁶ This solubility is significantly lower than the generally-accepted low micromolar levels of sulfane sulfur in cellular environments.³⁷⁻³⁸ More broadly, combining the vibrant sulfane sulfur redox landscape with the low solubility of S₈ raises the yet unanswered question of why S₈ is not deposited or precipitated in mammalian organisms.

An emerging platform to address these questions and limitations is to use macrocyclic receptors as potential host molecules for S₈. As a proof of concept toward this approach, we recently showed that up to 2 mM S₈ could be solubilized in 50 wt% solutions of 2-hydroxypropyl β -cyclodextrin (2HP β) in water and that 2HP β /S₈ decreased NO₂⁻ production in Raw 264.7 macrophage cells stimulated with proinflammatory lipopolysaccharides.³⁹ Similarly, treatment of isolated GAPDH enzyme with 2HP β /S₈ modulates enzyme activity through sulfuration of a key GAPDH Cys residue.⁴⁰ Although both of these examples suggest that solubilized S₈ may be bioavailable, the mechanism of S₈ and the interplay between soluble sulfane sulfur species

remains unclear. Understanding the mechanism by which complementary host molecules can solubilize and activate S_8 would provide important insights into the biological sulfur redox economy. Furthermore, these studies would advance our understanding of why S_8 is not a common deposited thermodynamic product in biological environments, even though small molecule polysulfides can extrude S_8 directly.⁴¹ With the goal of advancing our understanding of these fundamental questions, we report here the use of a cucurbituril supramolecular host as a model system to provide a detailed mechanistic investigation into the binding, activation, and reduction of S_8 by hydrophobic host motifs (Figure 1).

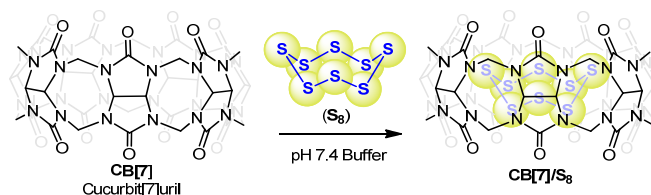


Figure 1. S_8 binding to cucurbit[7]uril in water.

Results and Discussion

CB[7]/ S_8 Binding and Characterization

Macrocyclic receptors, such as calixarenes (CX), cyclodextrins (CD), and cucurbit[n]urils (CB[n]s) have been studied extensively due to their well-established host-guest chemistry and ability to bind different guests. CB[5,6,7,8,10] can be prepared by an acid-catalyzed condensation between glycouril and paraformaldehyde and have been used previously to increase the aqueous solubility and bioavailability of different biologically-relevant guests.⁴²⁻⁴⁵ In particular, CB[n]s are particularly attractive hosts because of their discrete sizes, hydrophobic interior, uniformity of functional groups around portal, water solubility, high binding affinity for hydrophobic guests, and high biocompatibility.⁴⁶ Guest binding in CB[n]s is primarily driven size compatibility of the CB[n] cavity and potential interactions of charged or hydrogen bonding motifs with the carbonyl portals.

Because of these characteristics, we surmised that CB[*n*]s would be ideal hosts for S₈ based on the tunable size and potential for hydrogen bonding of incoming thiol nucleophiles to the CB[7] carbonyl-lined portals.

The structural rigidity of CB[*n*]s provides well-defined interior cavities that can be matched with specific guest to maximize host-guest size complementarity. Prior work has shown that both synthetic and biological host-guest structures maintain an ideal packing coefficient, defined as volume of the guest divided by the volume of the host cavity, of 0.55 ± 0.09 .⁴⁷ Based on the volume of S₈ (~149 Å³), we expected that CB[7] (~279 Å³) would be an ideal host for S₈ based on the calculated packing coefficient of 0.53 for CB[7]/S₈. To test this hypothesis, we prepared the CB[6], CB[7], and CB[8] homologs using the Pellingier method,⁴⁸ and treated aqueous solution of each host in pH 7.4 PBS buffer with excess S₈ for 24 hours. After filtration of the heterogeneous solutions, we measured the UV-Vis absorbance of each sample to determine whether S₈ ($\lambda_{\text{max}} = 263 \text{ nm}$; $\epsilon = 6730 \text{ M}^{-1}\text{cm}^{-1}$) had been solubilized. As expected, we observed a significant increase in the S₈ absorbance for solutions of CB[7], whereas significant increases in S₈ absorbance were not observed for CB[6] or CB[8]. Using this method, we could readily solubilize $1.0 \pm 0.3 \text{ mM}$ S₈ in 20 mM solutions of CB[7], which is significantly more efficient than the 2 mM solubility of S₈ in 50% w/w (~360 mM) solutions of 2HP β from prior work.³⁹

Having established that S₈ can be solubilized by CB[7], we next set to measure the binding affinity (K_a) and binding stoichiometry. Because of the low solubility of S₈ in water and lack of distinct spectroscopic signals for free and bound S₈, we used constant activity method to measure the K_a for S₈ binding to CB[7].⁴⁹ In this method, the total S₈ in solution (S_t) was measured as a function of increasing CB[7] concentration (0.5-2.0 mM) in the presence of excess S₈. The presence of solid S₈ ensures that the activity of S₈ in free solution remains constant at different CB[7] concentrations. Under these conditions, the concentration of S₈ bound in CB[7] is equal to $S_t - s_0$, where s_0 is the solubility of S₈ in water ($1.96 \times 10^{-8} \text{ M}$). Using this method, eq 1 can be

used to measure K_a for binding from the y-intercept, and also the binding stoichiometry (n) from the slope of the plot.

$$\log \frac{S_t - s_0}{s_0} = \log K_a + n \log [\text{CB}[7]] \quad \text{eq (1)}$$

The resultant log-log plot of $(S_t - s_0)/s_0$ versus $[\text{S}_8]$ provided a linear plot with a slope of 0.99, which is consistent with 1:1 host-guest binding (Figure 2). In addition, the y-intercept afforded $\log(K_a) = 7.06 \pm 0.22$, which is significantly higher than the prior S_8 binding in the 2HP β / S_8 system. In further support of 1:1 binding in solution, we also measured the ESI-MS spectrum of an aqueous solution of $\text{CB}[7]/\text{S}_8$, which showed a peak at 710.0673 m/z ($[\text{CB}[7] + \text{S}_8 + 2\text{H}^+]^{2+}$, which matches the expected exact mass of 710.0673 m/z and isotopic distribution expected for the $\text{CB}[7]/\text{S}_8$ complex. Taken together, these measurements support formation of a 1:1 host-guest complex between S_8 and $\text{CB}[7]$.

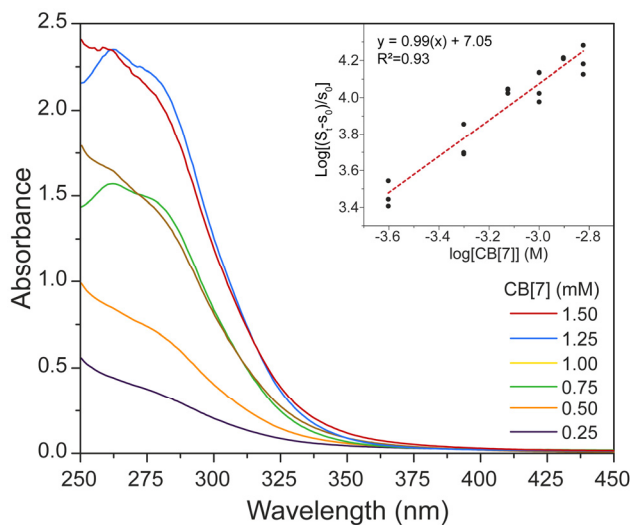


Figure 2. UV-vis spectra of the absorbance of $\text{CB}[7]/\text{S}_8$ with increasing concentrations of $\text{CB}[7]$ dependent (0.25-1.50 mM) in the presence of excess S_8 . Inset: Log-log plot for binding constant and binding stoichiometry of S_8 in $\text{CB}[7]$.

Further supporting formation of a 1:1 host-guest complex, we grew crystals suitable for single-crystal X-ray diffraction by slow evaporation of a saturated aqueous solution of $\text{CB}[7]/\text{S}_8$

using molecular sieves to increase the rate of evaporation. The resultant host guest complex crystallized in $Pca2_1$ with 4 CB[7]/S₈ molecules per asymmetric unit and 16 molecules in the unit cell (Figure 3). Each S₈ molecule is fully encapsulated in a CB[7] host, and bound S₈ is accessible to solvent through the carbonyl portals. Because none of the CB[7] or S₈ molecules sit on a special position in the orthorhombic space group, the incommensurate symmetry of the CB[7] and S₈ does not lead to symmetry-generated disorder within the unit cell. Each of the 4 crystallographically distinct CB[7]/S₈ molecules are well-ordered within the structure and both host and guest have well-defined thermal parameters.

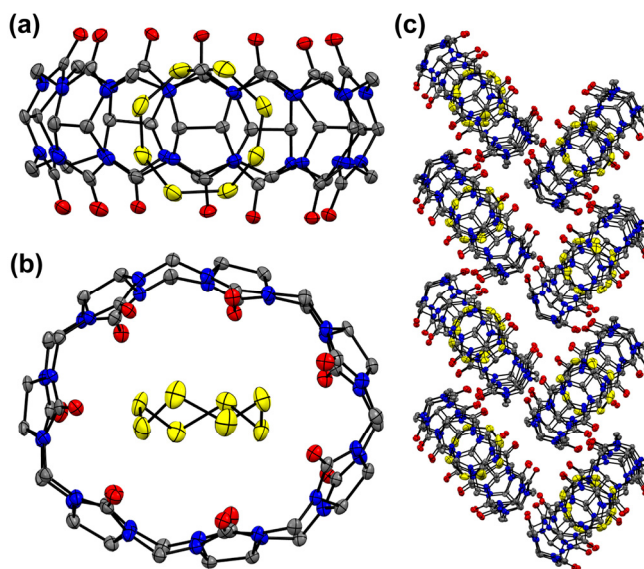


Figure 3. (a) Side on and (b) top view of the molecular structure of CB[7]/S₈. (c) Unit cell packing showing the CB[7]/S₈ herringbone alignment. Ellipsoids are shown at 50% probability levels. Red, grey, blue, and yellow ellipsoids represent O, C, N, and S, respectively. Hydrogen atoms and solvent molecules are omitted for clarity.

When examining the molecular structure more closely, we noticed that each CB[7] in the CB[7]/S₈ structure is significantly distorted from circularity. To better understand the magnitude of this distortion, we first calculated the shortest and longest radii of the CB[7] by calculating the centroid of each CB[7] defined by the 14 *sp*³ glycouril carbons and measuring the radii between the centroid and each of the 14 *sp*³ carbons. Comparing these radii showed shortest and longest

radii of 5.47 and 6.20 Å, respectively ($\Delta_r = 0.73$ Å). We next calculated the mathematical ellipticity, or deviation from circularity, for each CB[7] to better compare each CB[7] in the CB[7]/S₈ structure with other CB[7] host-guest complexes. In our analysis of prior CB[7] host-guest complexes, we limited our search to CB[7] structures in which the guest was fully encapsulated within the host cavity. We excluded structures in which the guest interacted directly with the carbonyl portal or with the exterior of the CB[7] host, since these structures may result in additional intermolecular forces contributing to host deformation. To benchmark the normal ellipticity of “empty” CB[7], we used data from published X-ray structures of CB[7] that did either had no guest or only solvent molecules. These measurements also help to account for normal distortions that may arise from crystal packing forces in the solid state structures. We measured centroids and individual radii as described above for each structure (Table S1). Working under the assumption that the perimeter of each CB[7] was identical based on the uniformity of covalent bond distances, we used the above radius measurements to calculate the mathematical ellipticity of each CB[7] host. On the basis of this analysis, we found that the average ellipticity for ‘empty’ CB[7] was 0.064 ± 0.037 , which was not statistically different from the prior CB[7] host guest complexes (0.066 ± 0.031). By contrast, the average ellipticity of the 4 CB[7] molecules in the CB[7]/S₈ host-guest complexes was 0.14 ± 0.02 , which is significantly more distorted. Closer analysis of the structure shows that this structural deviation of the CB[7] host leaves two sulfur atoms on each face of the CB[7]/S₈ complex accessible to solvent or nucleophiles, which provides a key structural insight into how S₈ can be activated by thiol-based nucleophiles in solution (*vide infra*).

Bioavailability CB[7]/S₈

Because CB[7] has been used previously to delivery encapsulated guests in cellular environments,⁵⁰⁻⁵² we were curious whether CB[7]/S₈ could be used to deliver S₈ and increase sulfane sulfur levels in live cells.⁵³ To test this directly, we used the sulfane-sulfur responsive fluorescent probe SSP4 to determine whether intracellular S⁰ levels were modified by treatment with CB[7]/S₈ (Figure 4).⁵⁴ We incubated HeLa cells with either 10 μM CB[7], 100 μM K₂S_x, or 10

μM CB[7]/ S_8 for 5 hours and then washed the cells and replaced the media to remove any extracellular species. After subsequent treatment with 20 μM SSP4 and further incubation for 15 minutes, the cells were washed again and the media was replaced prior to fluorescence imaging. Treatment of HeLa cells with CB[7] alone did not result in an increase in SSP4 fluorescence, which confirms that CB[7] alone does not increase intracellular S^0 levels. By contrast, treatment with CB[7]/ S_8 resulted in a significant increase in SSP4 fluorescence, which matched the positive sulfane sulfur control experiment using K_2S_x . These data demonstrate that CB[7]/ S_8 is can effectively deliver sulfane sulfur to live cells, which highlighting the generality and biocompatibility of using CB[7] for S_8 delivery.

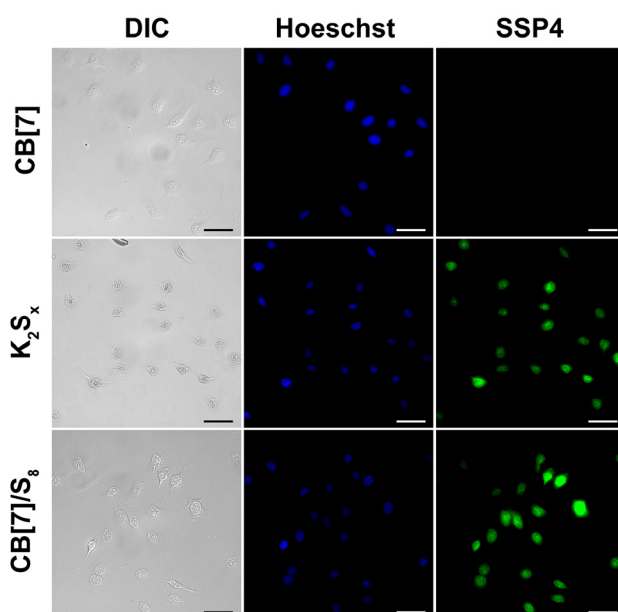


Figure 4. Fluorescent images of sulfane sulfur in live HeLa cells using the SSP4 fluorescent probe. Cells were treated with either 10 μM CB[7], 100 μM K_2S_x , or 10 μM CB[7]/ S_8 . Scale bar = 50 μm .

CB[7]/ S_8 Reactivity and Thiol Activation Mechanism

Having established both the molecular structure and biological compatibility of CB[7]/ S_8 , we next investigated the chemical accessibility of the bound S_8 toward thiol-based reductants to

generate H₂S. A small library of biologically-relevant and water soluble thiols were used to probe the chemical accessibility and activation of bound S₈. We initially aimed to use the methylene blue (MB) assay⁵⁵ measuring released H₂S, but we found that the MB dye produced upon reaction with H₂S is readily encapsulated in CB[7], which prohibited use of this common approach.⁵³ To overcome this challenge, we used a Unisense H₂S responsive electrode to measure H₂S concentrations. In addition to providing a real-time response with no sample workup, the electrode is also significantly more sensitive for H₂S than the MB assay.

Because reduction of each S⁰ atom to H₂S requires two equivalents of thiol, we started our investigations with the dithiol dithiothreitol (DTT) to simplify the reaction. As expected, treatment of CB[7]/S₈ (80 μM S⁰) with DTT resulted in quantitative reduction of the bound S₈ to H₂S over the course of 90 minutes (Figure 5). Similarly, treatment of CB[7]/S₈ with biologically-relevant thiols, such as cysteine (Cys), glutathione (GSH), or homocysteine (Hcy), also resulted in efficacious reduction of S₈ to H₂S, (56-69% over 90 min). In comparison, *N*-acetyl cysteine (NAC) and penicillamine (Pen) are far less reactive toward CB[7]/S₈. Using the above data, we fit the resultant H₂S release curves to measure k_{obs} for each thiol to determine whether thiol or thiolate was the active nucleophile. If the thiolate is the active nucleophile in S₈ activation reaction, then we would expect to observe an inverse relationship between k_{obs} and thiol p*K*_a. As expected, the resulting plot of log(k_{obs}) versus log(p*K*_a) was linear, with more acidic thiols resulting in faster S₈ reduction from CB[7]/S₈, which is consistent with thiolate being the active nucleophile in solution. Closer inspection of the H₂S generation curves for the series of thiols above shows immediate H₂S production in the case of DTT, whereas treatment with other thiols result in a brief induction period. This induction period suggests that an intermediate required for H₂S generation may initially accumulate during the thiol-mediated reduction process.

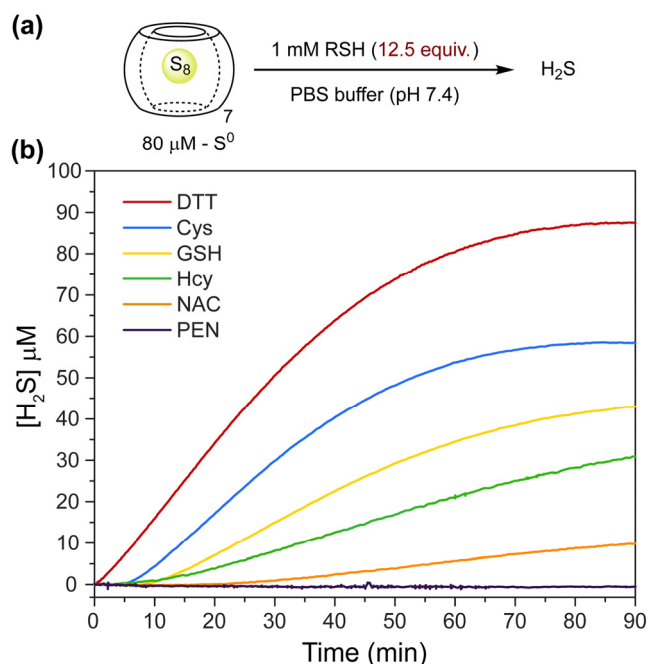


Figure 5. (a) Reaction conditions for thiol-triggered release of H₂S from CB[7]/S₈. (b) H₂S release from CB[7]/S₈ (80 μM S⁰) in the presence of different thiols. The experiments were performed in triplicate.

To better understand the mechanism of S₈ reduction by thiols in CB[7]/S₈ we envisioned two primary mechanisms that could be operative. Both mechanisms start with initial thiolate attack on the encapsulated S₈ to open the S₈ ring to form a thiol-bound hydropolysulfide (RS-S₈H) (Figure 6). In Mechanism I, the resultant RS-S₈H remains encapsulated in the CB[7], and subsequent reduction steps occur within the host. By contrast, in Mechanism II, the RS-S₈H intermediate is ejected from the CB[7], and each subsequent reduction step happens in solution. In both mechanisms, S₈ reduction and subsequent H₂S formation should show a rate dependence on thiol concentration, but would show saturation behavior if the initial thiol attack on the CB[7]/S₈ was no longer rate limiting at high thiol concentration.

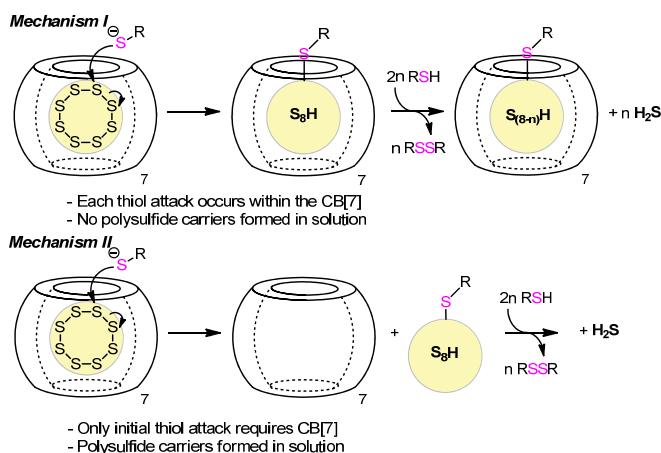


Figure 6. Propose mechanisms for reduction of S_8 in $CB[7]/S_8$ by thiols to generate H_2S . Mechanism I proceeds through polysulfide carries within the $CB[7]$ host, whereas Mechanism II generates polysulfide carriers in solution.

To test these scenarios, we measured H_2S production from $CB[7]/S_8$ ($80 \mu M S^0$) under pseudo first order conditions with increasing concentrations of Cys as the model thiol substrate (Figure 7). As expected, a plot of $\log(k_{obs})$ vs $\log([Cys])$ was initially linear, but showed saturation behavior at concentrations above 10 mM Cys (Figure 7b). These results suggest that at high $[Cys]$, attack of Cys on $CB[7]/S_8$ is rate limiting and once the resultant polysulfides are formed they are quickly reduced to H_2S . At lower $[Cys]$, however, the rates of Cys attack on $CB[7]/S_8$ and the rate of reduction of the resultant polysulfides are comparative, which leads to the observed $[Cys]$ dependence. In addition, increased $[Cys]$ abolished the induction period for H_2S formation (Figure 7a). Taken together, these data suggest that at low $[Cys]$, polysulfide carriers accumulate prior to further reduction by Cys to generate H_2S , whereas at high $[Cys]$, the formed polysulfide carriers are reduced to H_2S immediately upon formation.

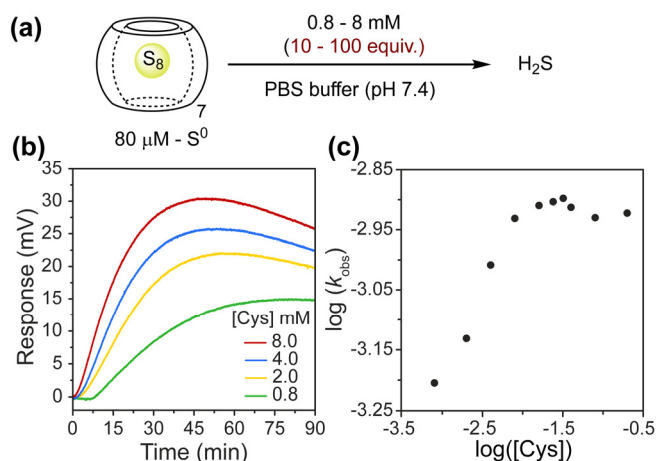


Figure 7. (a) Reaction scheme of thiol-dependent H₂S release from CB[7]/S₈ (b) Representative H₂S response upon treatment of CB[7]/S₈ (80 μM S⁰) with increasing [Cys] (0.8-8 mM). (c) The plot of log(*k*_{obs}) vs log[Cys] show saturation behavior.

To further investigate the role of CB[7] and potential intermediates formed during the thiol-mediated reduction of S₈ from CB[7]/S₈ we performed a series of experiments using proflavine (Pro) as a competitive guest for CB[7] (*K*_a = 1.65 × 10⁷ M⁻¹).⁵⁶ To differentiate between Mechanism I and Mechanism II, we added Cys (1 mM) and increasing concentration of Pro (0-4 mM) to a solution of CB[7]/S₈ (80 μM S⁰) and monitored H₂S production. If the H₂S generating steps of S₈ reduction are occurring solely within the CB[7] (Mechanism I), then we would expect increasing [Pro] to provide a dose-dependent decrease in the rate of H₂S production (Figure 8a). Conversely, if the H₂S generating steps of S₈ reduction are occurring outside of CB[7] (Mechanism II), then the initial rates of H₂S production should be identical because they reflect polysulfide reduction in solution, which is a CB[7] independent process, but the total H₂S generated should decrease with increasing [Pro] because there would be less initial CB[7]/S₈ in solution (Figure 8b). When performing these experiments, we observe identical initial H₂S production rates but overall decreased H₂S production with increasing [Pro], which is consistent with Mechanism II (Figure 8c). In a complementary experiment, we initiated the reaction of CB[7]/S₈ (80 μM S⁰) with Cys (1 mM) and added excess Pro midway through the reaction. If the CB[7] is required for each step in H₂S generation (ie Mechanism I), then we would expect to observe an immediate halt in H₂S

production upon Pro addition. If, however, CB[7] is only required for the initial step for S_8 activation and free polysulfides and related soluble sulfane sulfur carriers are the resting intermediate for the reaction (ie Mechanism II), then we would not expect to see any change in H_2S production upon Pro addition. Under these conditions, addition of Pro 28 minutes after Cys addition did not significantly change H_2S production when compared the identical experiment without Pro addition, which again supports Mechanism II (Figure 8d). When taken together, these experiments confirm that the CB[7] is only required for the initial S_8 activation step and that polysulfides and other soluble sulfane sulfur carriers are the resting intermediates for the observed reduction chemistry.

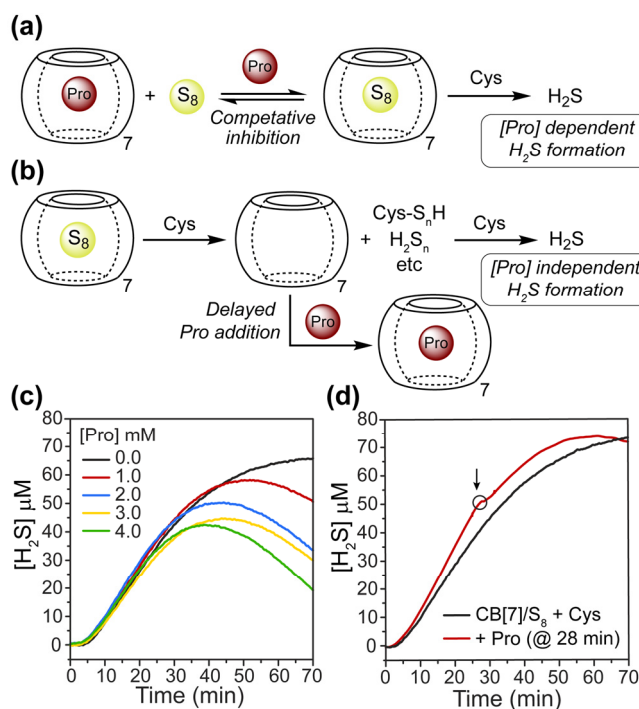


Figure 8. (a) Treatment of CB[7]/ S_8 with proflavine (Pro) would decrease the rate of H_2S production if each H_2S -generating step occurs within CB[7]. (b) Treatment of CB[7]/ S_8 with Pro would not impact H_2S release if free polysulfides are the resting intermediate. (c) Measured H_2S production from CB[7]/ S_8 (80 μM S^0) with Cys (1 mM) with increasing $[Pro]$ (0-4 mM). (d) H_2S production is unchanged when Pro is added during the course of the reaction of CB[7]/ S_8 (80 μM S^0) with Cys (1 mM), confirming that polysulfides are the resting intermediate.

If Mechanism II is the primary pathway for H₂S formation, then we expect that polysulfides and related soluble S⁰-containing RSS are formed during the reaction. We next investigated whether these RSS intermediates could be trapped by using the fluorescent alkylating agent monobromobimane (mBB).⁵⁷⁻⁵⁸ Depending on the RSS species present, quenching with mBB results in different products, which can be differentiated by HPLC. For example, H₂S and inorganic polysulfides react with 2 equiv. of mBB to produce sulfide dibimane or doubly-labeled polysulfides, respectively, whereas other sulfhydryl-containing species such as persulfides, organic polysulfides, react with 1 equiv. of mBB to generate singly-labeled fluorescent derivatives. Based on this expected reactivity, we treated CB[7]/S₈ (80 μM S⁰) with Cys (1 mM), allowed the reaction to proceed for 30 min, quenched the reaction with excess mBB, and analyzed the resultant products by HPLC. When comparing the resulting chromatogram with those from control experiments for mBB trapping of H₂S, persulfides, and polysulfides, we observe clear formation of H₂S and polysulfides, further confirming the generation of free sulfane-sulfur carries in solution.

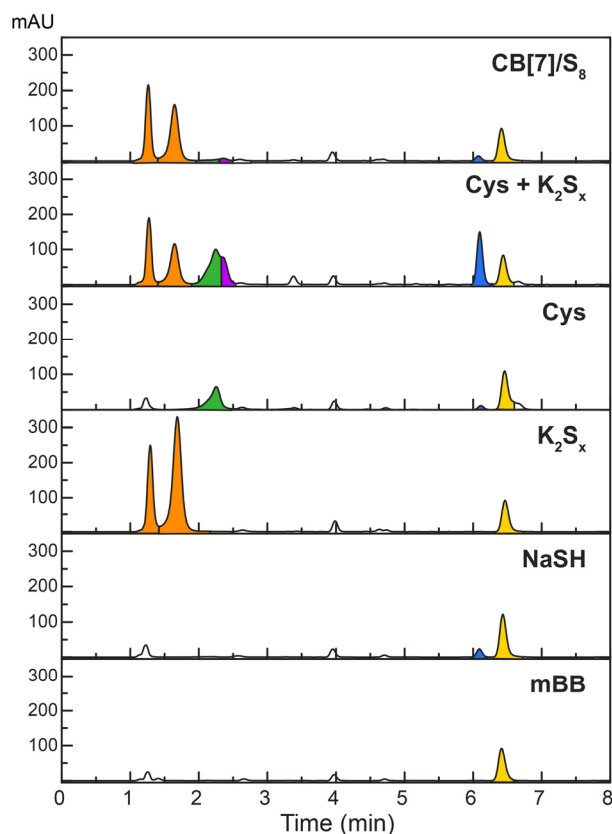


Figure 9. HPLC traces for CB[7]/S₈ the reaction of CB[7]/S₈ treated with Cys and quenched with mBB. Comparisons with background trapping reactions show significant formation of organic and inorganic polysulfides formed during the reaction.

Combining the above investigations allows for a more comprehensive mechanism of S₈ activation by CB[7] (Figure 11). S₈ is encapsulated in CB[7] in water to form the CB[7]/S₈ complex. The structure of the CB[7] allows for thiolate to access and attack the bound S₈ and eject soluble sulfane sulfur carriers in solution. These carriers then react further with thiols to generate other sulfane sulfur containing RSS, including inorganic polysulfides as well as Cys-ligated organic polysulfides and hydropolysulfides. Each of these classes of sulfane sulfur carriers has been implicated previously in the roles of sulfane sulfur action and translocation in cellular environments.^{37, 59-60} In the presence of excess thiol or other reducing agents, the sulfane sulfur atoms in these carriers will be reduced fully to H₂S.

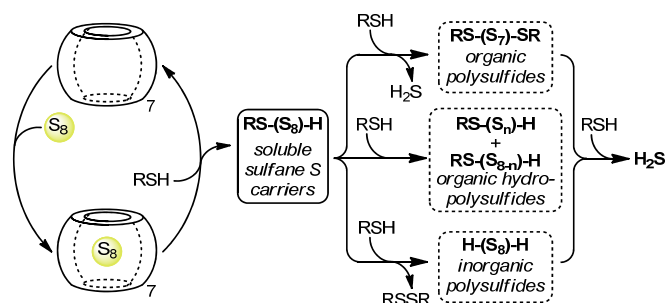


Figure 10. Mechanism of S₈ activation by CB[7] to generate soluble sulfane sulfur carriers en route to H₂S generation.

More broadly, this determined mechanism provides an attractive manifold for the general activation of S₈ by hydrophobic motifs to generate redox-labile soluble sulfane sulfur carriers. Based on the dynamic sulfur redox economy in all living organisms, why is S₈ formation and subsequent precipitation not observed in cellular environments under physiological conditions? An attractive hypothesis is that available hydrophobic motifs, whether they be in proteins, membranes, or other bimolecular compartments, may transiently bind and solubilize S₈ to promote reduction by GSH or Cys to solubilize and shuttle S₈ back into the redox labile sulfane sulfur pool. Such a mechanism would provide an attractive manifold in which to understand the translocation and trafficking of RSS in complex redox environments.

Conclusion

Sulfane sulfur is a key component of the redox labile sulfur pool, yet S₈ is insoluble in water and inaccessible to common thiol-mediated redox chemistry. We demonstrated that S₈ can be bound and solubilized in water using a CB[7] supramolecular host and that this solubilized form of S₈ can increase sulfane sulfur levels in live cells. The structure of the CB[7]/S₈ complex was determined by X-ray crystallography and confirmed that S₈ binds within the CB[7] cavity with a 1:1 stoichiometry. Treatment of the CB[7]/S₈ complex with thiols resulted in S₈ reduction to H₂S with high efficiency and a linear dependence of H₂S production rate on thiol pK_a. Furthermore, saturation behavior for H₂S production was observed for thiols and competitive inhibition studies

using proflavine demonstrated that only the initial thiol attack on the S_8 occurs within the CB[7] cavity and that soluble sulfane sulfur carriers are formed in solution. Alkylative trapping experiments with mBB further supported the formation of polysulfides and hydropolysulfides intermediates. In addition to expanding the supramolecular chemistry of S_8 as a biologically-relevant guest, the mechanistic work provides a useful paradigm for understanding S_8 uptake and activation by hydrophobic motifs and the interplay of S_8 with the redox labile sulfane sulfur pool.

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