Single-Molecule Chemistry

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S-Nitrosothiol Chemistry at the Single-Molecule Level**

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Nitric oxide (NO) and its relatives (notably RSNO and HNO) perform important roles as regulatory molecules in health and disease.[1] NO activates guanylate cyclase by binding to its ferrous heme (see the Supporting Information, Figure S1). The lifetime of NO is short, being milliseconds to seconds,[1b] and therefore it is unlikely that NO diffuses more than approximately 100 μm, which is in the range of a few cells, from the site at which it is generated. Through poorly understood mechanisms,[1b,2] NO is also converted in vivo into small molecule and protein *S*-nitrosothiols (RSNOs). RSNOs modulate cellular activity in ways that differ from the direct action of NO.[3] Furthermore, RSNO derivatives of albumin and hemoglobin are sufficiently long-lived to be transported in the circulation.[4] In addition, NO is passed from one thiol to another by transnitrosation, which can be facilitated by specific protein–protein interactions,[2a,5] and protein function can be modulated by S nitrosation.[6] Protein SNOs are also converted into other derivatives, such as *S*-glutathionylated proteins,[7] thus generating a complex signaling web (Figure S1). RSNO also act as a source of HNO (nitroxyl),[8] which is also a locally acting regulatory molecule with biological effects that differ from and even oppose those of NO.[1c,8a,b]

Although the actual effectors (NO, RSNO, HNO etc.) are not always clearly distinguished, NO has been implicated in numerous medical disorders beyond its well-known role in erectile function. Examples include disorders involving the cardiovascular system,[9] neuronal cell death in trauma and disease,[6,10] the immune system,[11] and cancer.[9] RSNOs and several agents that generate either NO or HNO are under development as therapeutic agents.[8a,9,12]

Here, we have used the protein nanoreactor approach[13] to examine RSNO chemistry at the single-molecule level. Reactant molecules are tethered within the lumen of a protein pore, staphylococcal α-hemolysin (αHL), and a non-perturbing current carried through the pore by aqueous ions registers changes in molecular structure that occur during bond-making and bond-breaking events, thus revealing the kinetics of the steps involved in a series or cycle of reactions (Figure 1 a). The technique is sufficiently sensitive to distinguish enantiomers[14] or determine a deuterium isotope effect,[15] and herein we show that the two atoms, -NO, in RSNO are readily detected. Clearly, it would not be possible to examine the single-molecule chemistry of RSNO in a meaningful way with bulky fluorescent reagents, the main alternative to our approach. We have investigated the lifetime of the proposed nitroxyl disulfide intermediate, $RSN(O)SR^-$, in transnitrosation, examined the competition between transnitrosation and S thiolation by *S*-nitrosoglutathione, and studied the reaction between RSNO and sulfite. The latter is especially relevant to the exacerbating effect in asthma of sulfite[16] acting through the NO pathway.[17]

S-Nitrosoglutathione (GSNO) mediates cell signaling through transnitrosation,[1a,18] which is the transfer of the

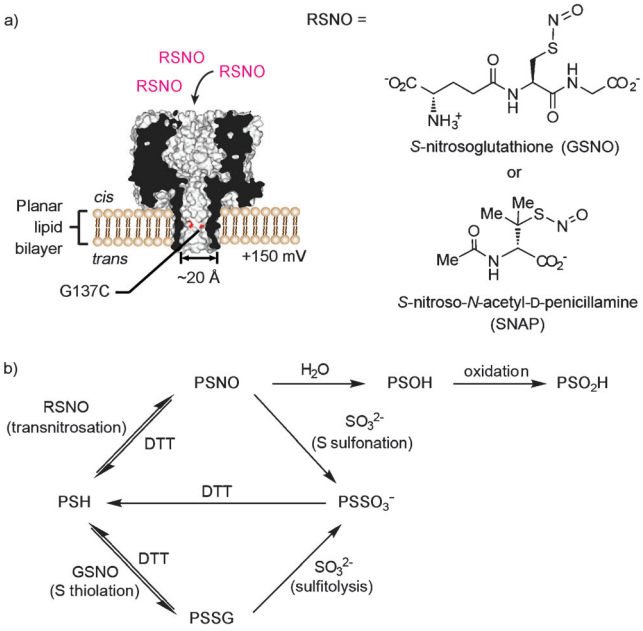

Figure 1. *S*-Nitrosothiol chemistry observed at the single-molecule level. a) The single-channel electrical recording technique. Cross-section of an α-hemolysin (αHL) pore, $(G137C\text{-}D8)_7$ (P7SH), located in a planar lipid bilayer. Cysteine residues at position 137, near the middle of the transmembrane β barrel, are highlighted in red. The homoheptameric P7SH has seven cysteine residues within the lumen of the pore. The internal diameter of the transmembrane barrel is approximately 20 Å. *S*-Nitrosothiols (RSNOs) were added to the *cis* compartment, which is the side where the cap domain of the αHL pore resides and is connected to ground. The *trans* compartment was held at +150 mV. The structures of *S*-nitrosoglutathione (GSNO) and *S*-nitroso-*N*-acetyl-D-penicillamine (SNAP) are shown on the right. b) *S*-Nitrosothiol chemistry observed by using the nanopore approach. PSH: $(WT)_6(G137C\text{-}D8)_1$, an αHL pore with a single free thiol in the lumen; PSNO: the *S*-nitrosothiol derivative of PSH; $PSSO_3^-$: the *S*-sulfonate derivative; PSSG: the glutathione mixed disulfide; PSOH: the sulfenic acid; PSO_2H: the sulfinic acid; DTT: DL-dithiothreitol.

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NO group to protein cysteine residues, and by S glutathionylation, which involves the formation of a mixed disulfide, again with protein cysteine residues (Figure S1).[19] We examined the reaction of GSNO with the cysteine residues inside the transmembrane β barrel of the homoheptameric αHL pore $(G137C\text{-}D8)_7$ (P7SH) (Figure 1a). The addition of GSNO to the *cis* side of P7SH (Figure 1a and Figure 2a) produced a series of small steps in the transmembrane current ($\Delta I = -1.2 \pm 0.3$ pA, 107 steps out of a total of 121 from 34 experiments). A maximum of seven steps was observed, a number, which corresponds to the total number of cysteine residues in each P7SH pore (Figure 2a). The heteroheptamer $(WT)_6(G137C\text{-}D8)_1$ (PSH), which contains only one cysteine residue, showed a single-step change (success rate 83%, $n =$ 60 experiments, see the Supporting Information, Table S1). Because a different RSNO, *S*-nitroso-*N*-acetyl-D-penicillamine (SNAP), produced similar current steps with P7SH ($\Delta I = -1.1 \pm 0.3$ pA, 41 steps from 14 experiments), the steps were attributed to transnitrosation reactions.

The proposed associative *N*,*N*-dialkylsulfanyl aminoxide intermediate ($RSN(O)SR^-$, see the Supporting Information, Figure S3a)[20] was not observed even in experiments ($n = 3$) performed with GSNO at both lower pH and reduced temperature (pH 6.0 and 5°C). It has been suggested that the intermediate is short-lived;[20a] and according to our measurements, if it does exist, the lifetime must be well under 200 μs (Figure S3). It was claimed by Perissinotti et al. that high concentrations of the *N*,*N*-dialkylsulfanyl aminoxide of cysteine ethyl ester can be observed in methanol.[21] However, the ^{1}H NMR spectrum obtained by these authors is also consistent with disulfide formation from the reaction of the *S*-nitrosothiol and the thiol, which occurs slowly, at least in aqueous solution[22] (see also the section entitled "Second-order rate constants for S glutathionylation" in the Supporting Information).

In addition to the transnitrosation steps, much larger current jumps ($\Delta I = -20 \pm 5$ pA, 14 steps out of 121 steps from 34 experiments, that is, 12%) occurred less frequently with P7SH in the presence of GSNO at pH 7.4 (Figure 2b and Table 1). The magnitude of these steps suggested that they might arise by S glutathionylation and this was confirmed by generating the *S*-glutathionyl derivative of PSH by an independent route (see the Supporting Information, Figure S4, $\Delta I = -20 \pm 3$ pA ($n = 3$)). S Glutathionylation by GSNO was reduced to a single observation in 51 steps at pH 8.4. The pH dependence of S glutathionylation by GSNO suggests that the acidity of the microenvironment of the reacting thiolate might dictate whether transnitrosation or S thiolation takes place, with S thiolation favored by lower pH. SNAP did not undergo S thiolation at pH 7.4 or pH 8.4 (Table 1), presumably owing to greater steric hindrance at the tertiary SNO group.[23]

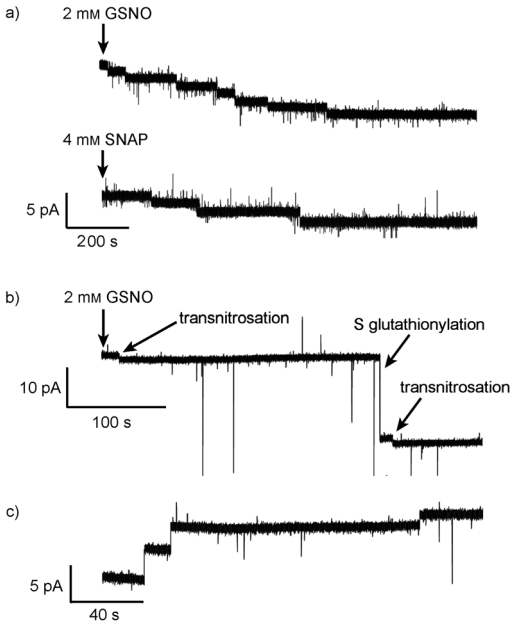


Figure 2. Transnitrosation, S thiolation and S sulfonation. a) Seven successive transnitrosations by GSNO observed with P7SH. Similar changes in transmembrane current were observed when SNAP was used instead of GSNO. GSNO or SNAP was added to the *cis* compartment. b) S Glutathionylation by GSNO under the same conditions as in (a). Two transnitrosation steps (indicated) can also been seen. c) Three successive S sulfonations by sulfite ion on the *S*-nitrosothiols of P7SNO. Sodium sulfite (12 mM) was added to the *cis* side of preformed P7SNO obtained from the reaction of GSNO (2 mM, *cis*) with P7SH. The three S sulfonation steps are of different amplitudes because the environment within the pore is altered after each reaction. Note the increase in pore conductance upon S sulfonation. Conditions: 2 M KCl, 80 mM 3-morpholinopropane-1-sulfonic acid (MOPS), 100 μM ethylenediaminetetraacetate (EDTA), pH 7.4, at +150 mV and 22±1°C.

Table 1: Transnitrosation reaction rate constants, k_t, measured by single-channel current recording with P7SH.

	pH	Number of experiments[a]	Total number of reaction steps[b]	Number of S thiolation steps	k_t [$M^{-1}s^{-1}$][c]
GSNO	7.4	34	121	14	1.0±0.2
	8.4	14	51	1	2.5±0.7
SNAP	7.4	14	41	0	1.4±0.2
	8.4	12	45	0	4.5±1.1

[a] One P7SH pore was used in each experiment. So, the number of experiments represents the number of P7SH pores examined. [b] This is the sum of the transformations (transnitrosation and S thiolation) observed in all the experiments. [c] k_t is the single-molecule bimolecular rate constant for the transnitrosation between RSNO and the protein thiol group. The mean value ± s.d. is given. The k_t values were statistically corrected for the number of remaining thiol groups in P7SH (see the Supporting Information). Conditions: 2 M KCl, 80 mM MOPS, 100 μM EDTA, pH 7.4 or 8.4, 22±1°C and +150 mV.

By using spatially separated reagents,[24] multiple turnovers of transnitrosation of the thiol group in PSH were observed at pH 8.4. GSNO and DL-dithiothreitol (DTT) were added to the *cis* and *trans* compartments, respectively (Figure 3a). Titration experiments (Figure 3d) showed linear dependencies of the reciprocals of the mean dwell times at the unreacted pore level PSH (τ_{PSH}) and at the *S*-nitrosothiol level PSNO (τ_{PSNO}) on the concentrations of GSNO and DTT, respectively (Figure 3b and c); these data allowed the estimation of second-order rate constants for the forward

transnitrosation reaction between PSH and GSNO ($k_t = 10 \pm 1\,M^{-1}s^{-1}$) and the reverse transnitrosation between PSNO and DTT ($k_t = 17 \pm 1\,M^{-1}s^{-1}$).

The reaction between RSNO and sulfite ion (SO_3^{2-}) has been reported previously,[25] with RSH as a proposed product. However, the reaction products were not characterized

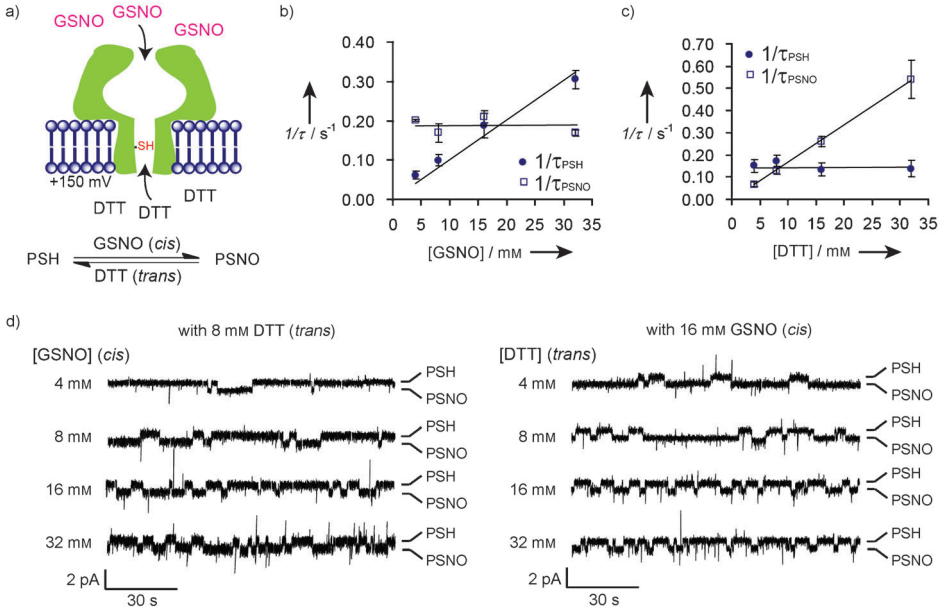


Figure 3. Reversible transnitrosation. a) Reversible transnitrosations in the PSH nanoreactor in the presence of GSNO (*cis*) and DTT (*trans*). Conditions: 2 M KCl, 80 mM MOPS, 100 μM EDTA, pH 8.4, at +150 mV and 22 ± 1 °C. b) Reciprocals of the mean dwell times for both the PSH ($1/\tau_{PSH}$) and PSNO ($1/\tau_{PSNO}$) levels versus the concentration of GSNO. The same concentration of DTT (8 mM) was used throughout this set of experiments. c) As in (b), but the concentration of DTT was varied and a fixed concentration of GSNO (16 mM) used. Each point represents the mean ± s.d. for three experiments. d) Single-channel recordings under the above conditions at various concentrations of GSNO and DTT. The levels corresponding to PSH and PSNO are marked. The two conductance levels differ by 0.7 ± 0.1 pA ($n = 6$).

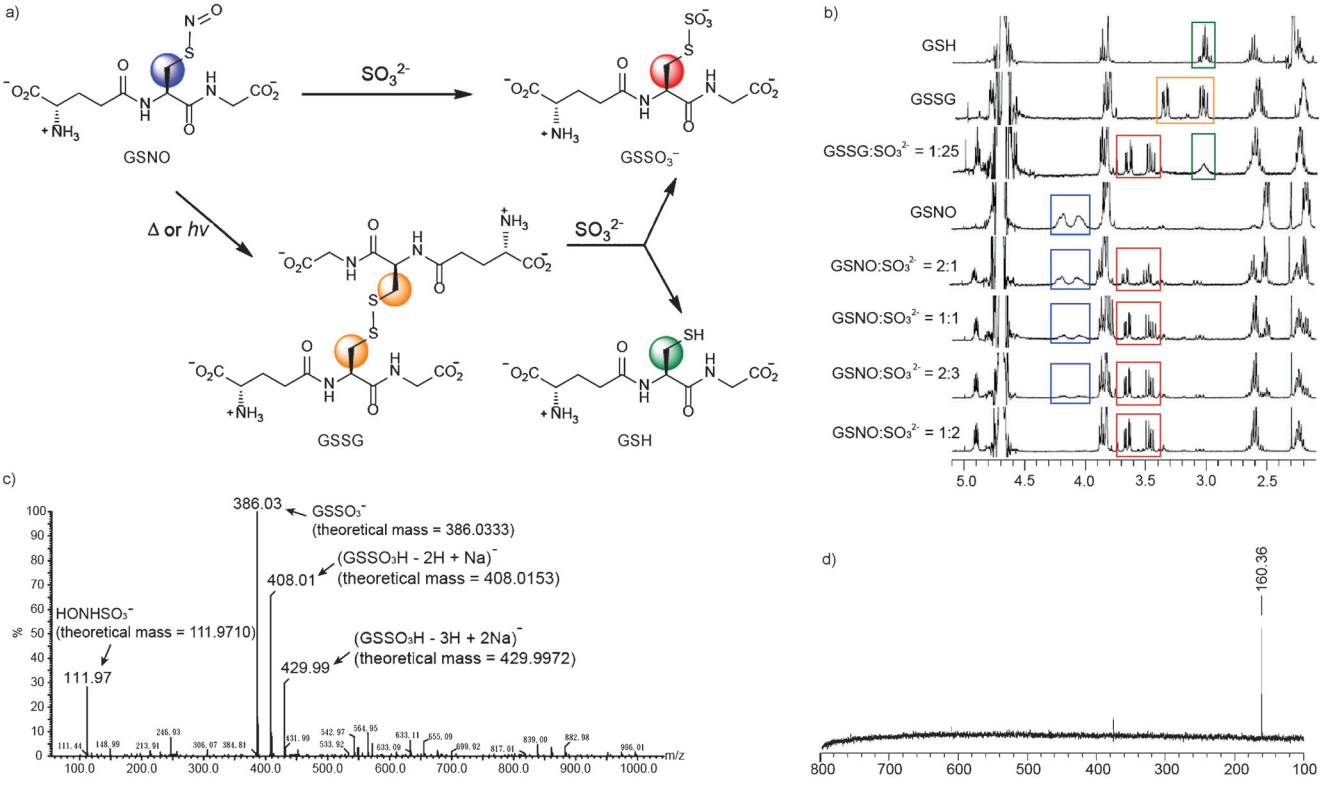


Figure 4. Product characterization for the reaction between GSNO and sulfite ion in bulk solution. a) Reaction scheme. b) Stacked 400 MHz ^{1}H NMR spectra (5.1–2.1 ppm) in D_2O containing 2 M KCl, 80 mM sodium phosphate, 100 μM EDTA, pD 7.4, at 22 ± 1 °C. Glutathione derivatives at 10 mM were used in each experiment. The reactions were complete within 10 min after mixing, and the spectra were collected within 1 h. Peaks from the CH_2S protons are framed with colors corresponding to the various glutathione derivatives highlighted in (a). c) Electrospray ionization mass spectroscopy (ESI-MS, negative ion mode) showing the peak corresponding to $GSSO_3^-$. The sample was prepared by mixing GSNO and sodium sulfite in a 1:2 ratio in water and the products were analyzed without purification. d) ^{15}N NMR spectrum (50 MHz, 800–100 ppm) after the reaction between GS^{15}NO (83 mM) and sodium sulfite mixed in a 1:2 ratio in the same buffer as in (b). The peak at 160.4 ppm corresponds to [^{15}N]hydroxylamine-*N*-sulfonate (see also Figure S8).

spectroscopically. Furthermore, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was used to detect thiol formation, and the disulfide bond of this reagent is also cleaved by sulfite. To clarify the chemistry between sulfite and RSNO, a detailed spectroscopic and kinetic study was performed with GSNO (for details see the Supporting Information). ^{1}H NMR spectroscopy and mass spectrometry (Figure 4b and c) showed that *S*-sulfoglutathione ($GSSO_3^-$) was formed from GSNO and sulfite at pH 7.4 and 22 °C. The reaction stoichiometry determined by ^{1}H NMR titration experiments revealed that each GSNO consumes two sulfite ions (Table 2). ^{15}N NMR and mass spectrometry on the products

Table 2: Determination of the reaction stoichiometry for S sulfonation of GSNO by ^{1}H NMR.

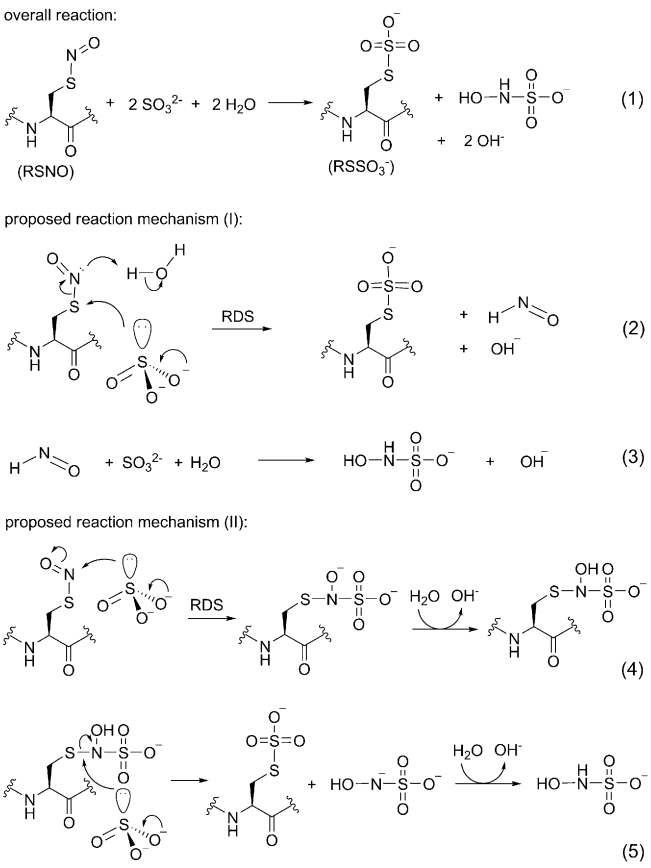
Molar ratio of GSNO to SO_3^{2-}[a]	GSNO/$GSSO_3^-$ ratio[b]	Expected GSNO/$GSSO_3^-$ ratio if GSNO reacted with SO_3^{2-} in 1:2 stoichiometry
2:1	2.5 ± 0.6	3.0
1:1	0.9 ± 0.3	1.0
2:3	0.4 ± 0.3	0.3
1:2	0	0
1:25	0	0

[a] GSNO (10 mM) was mixed with various concentrations of sulfite ion. [b] The product ratio (mean ± s.d.) was determined from the peak areas of the CH_2S protons in ^{1}H NMR spectra (Figure 4b, $n = 4$). Owing to the decomposition of GSNO to GSSG before the collection of NMR spectra, the ratio of GSNO to $GSSO_3^-$ was calculated as the molar ratio of (GSNO + 2GSSG) to $GSSO_3^-$.

of the reaction between $GS^{15}NO$ and sulfite identified hydroxylamine-*N*-sulfonate as the nitrogen-containing product (Figure 4c and d and Figure S8). Nitric oxide (NO) or nitroxyl (HNO), which might be formed as short-lived intermediates, could not be detected by electron paramagnetic resonance (EPR) after trapping with oxymyoglobin and metmyoglobin.[8a] The kinetics of the reaction between GSNO and sulfite were also followed by monitoring the disappearance of GSNO at 334 nm with a stopped-flow UV/Vis spectrometer (see the Supporting Information). The reaction rate showed a first order dependence on each reactant ($d[GSNO]/dt = k_{SO_3^{2-}}[GSNO][sulfite]$); $k_{SO_3^{2-}}$, at pH 7.4, was $124 \pm 3\ M^{-1}s^{-1}$ at 22 °C and $264 \pm 5\ M^{-1}s^{-1}$ at 37 °C. The latter agrees with a previously reported value for the disappearance of GSNO.[25b]

With these findings in hand, the S sulfonation of protein SNO was examined at the single-molecule level. P7SNO was first formed by treating P7SH with GSNO. The subsequent addition of sulfite to the *cis* compartment elicited several steps in the transmembrane current ($\Delta I = +1.4$ to $+3.7$ pA, for each step) (Figure 2c). The second-order rate constant $k_{SO_3^{2-}}$ was $82 \pm 40\ M^{-1}s^{-1}$ (11 steps from 6 experiments) at pH 7.4 and 22 °C. No intermediates were observed.

The observations above can be rationalized by two possible mechanisms. In the first, sulfite attacks the sulfur atom of P7SNO in a rate-determining step, with concerted protonation at the nitrogen atom leading to the formation of the *S*-sulfonate species and nitroxyl (HNO) (reaction 2, Scheme 1). HNO is then consumed by a second sulfite ion.



Scheme 1. Proposed reaction mechanisms for S sulfonation of RSNO by sulfite ion. The overall reaction observed at pH 7.4, 22 °C is shown in reaction 1. The first proposed mechanism involves nucleophilic attack at the sulfur atom of RSNO by sulfite ion with concerted protonation at the RSNO nitrogen atom (reaction 2) to form the *S*-sulfonate ($RSSO_3^-$) and HNO in a rate-determining step. The second proposed mechanism involves the nucleophilic attack at the nitrogen atom of RSNO by sulfite ion in the rate-determining step (reaction 4). RDS = rate-determining step.

Indeed, it has been reported that sulfite reacts rapidly with HNO to form hydroxylamine-*N*-sulfonate,[26] which would also explain our inability to trap and detect HNO by EPR. In vivo, the released HNO would react rapidly with thiols, which are present at high concentrations compared to sulfite, converting them to disulfides or sulfinamides ($RS(=O)NH_2$) and sulfinates (RSO_2^-).[8b] Nucleophilic attack with concerted protonation (reaction 2, Scheme 1) circumvents the difficulty posed by the fact that the nitroxyl anion (NO^-) is a triplet in the ground state, and as such is spin forbidden to act as leaving group.[8c,27] A second mechanism involves the direct attack of sulfite at the nitrogen atom of P7SNO in a rate-determining step to form P7S-N(OH)-SO_3^- (reaction 4, Scheme 1). A substitution reaction at the sulfenamide sulfur atom in the P7S-N(OH)-SO_3^- intermediate with a second sulfite ion yields the *S*-sulfonate product, $P7SSO_3^-$, together with hydroxylamine-*N*-sulfonate as the leaving group (reaction 5). Because only one step was observed in the single-molecule experiment, P7S-N(OH)-SO_3^- and $P7SSO_3^-$ would have to have similar conductance values for our observations to be consistent with this pathway. Furthermore, in the similar

S thiolation reaction, the immediate products are the disulfide and HNO.[28] Therefore, all things considered, we favor the first mechanism for S sulfonation, which results in the generation of free HNO (Scheme 1).

GSNO and other small molecule and protein SNOs are present in the human airways. GSNO (0.2–0.5 μM in normal lungs[17b]) exerts bronchodilatory effects by transnitrosation of a range of ion channels and receptor systems, leading to airway smooth muscle relaxation.[17a] While the mechanisms of sulfite allergy remain unclear,[16] we propose that one origin of its asthmatic effect is the depletion of GSNO ($k_{SO_3^{2-}} = 264\ M^{-1}s^{-1}$ at pH 7.4, 37 °C) and protein SNOs by conversion into their *S*-sulfonate analogues. Furthermore, the proposed generation of HNO, which has different physiological effects to free NO,[8b] might alter the normal nitrergic signaling cascade responsible for human airway dilation, leading to breathing difficulties.

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