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Rapid Reaction of Hydrogen Sulfide with the Neutrophil Oxidant Hypochlorous Acid to Generate Polysulfides

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H_2S has been recognized as a signaling molecule and mediator of inflammation. Here, we report the kinetics and mechanism of its reaction with the neutrophil oxidant hypochlorous acid. Stopped flow studies, carried out at high pH, showed this reaction to be extremely fast, with a second-order rate constant extrapolated to be 2×10^9 M^{-1} s^{-1} at pH 7.4. The reaction produces polysulfides rather than polythionates and may represent a novel pathway for protein Cys-sulfhydration, a recently proposed mechanism for H_2S signaling.

Hydrogen sulfide has been shown to be produced in almost all tissues of the human body via trans-sulfuration pathways during the metabolism of cysteine by two pyridoxal phosphate-dependent enzymes: *cystathione-γ-lyase* (CSE) and *cystathione-β-synthetase* (CBS) (*1*). H_2S is currently under active investigation to elucidate its biological functions and explore its therapeutic potential (*2*). It acts as a vasorelaxant as demonstrated by hypertension in CSE-deleted mice (*3*), it is proposed to increase the lifespan and thermotolerance in *Caenorhabditis elegans* (*4*), and it is capable of inducing hybernation in mice by decreasing oxygen consumption and reducing the metabolic rate (*5*). Along with nitric oxide and carbon monoxide, it is now regarded as a physiological gasotransmitter. Although many investigations suggest that, unlike NO or CO, it acts primarily via opening K_{ATP} channels (*6*), a recent report proposed that in analogy with NO-mediated nitrosothiol formation, H_2S signals through the formation of protein persulfides (ProteinCys-SSH) (*3*).

The chemistry of the reactions of H_2S with biomolecules is poorly understood. For example, its cardioprotective (*7*) and neuroprotective (*8, 9*) effects were associated with its ability to scavenge physiological oxidants, but the kinetics and products of these reactions were not studied, and the physiological concentrations of H_2S are controversial. In this contribution, we have investigated the reaction of H_2S with hypochlorous acid (HOCl). HOCl is a neutrophil-derived oxidant that is thought to be primarily responsible for clearing invading microbes and has a major role in inflammation (*10*). It is produced in the myeloperoxidase-catalyzed oxidation of chloride by H_2O_2 (*11*). Of particular relevance to this study, the mediatory role of H_2S in leukocyte action is increasingly recognized (*12*).

Mixing OCl^- with HS^- resulted in a marked change in the UV-spectrum; see Figure S1 (Supporting Information). The largest absorbance change was an increase at 290 nm, and therefore, the kinetics of the reaction was followed at this wavelength. At physiological pH, the reaction was too fast to follow, but it could be monitored by stopped-flow spectroscopy at high OH^- and relatively low reactant concentrations. Pseudo first-order conditions were employed by keeping the concentration of HS^- at a large excess over [OCl^-]. The observed exponential kinetic traces (see Figure S2a, Supporting Information) indicate that the rate law exhibits first order dependency on the concentration of OCl^-. This was corroborated by the fact that the observed pseudo first-order rate constants remained the same when changing the concentration of OCl^- at constant pH, [HS^-], and ionic strength (not shown). Increasing the concentration of HS^- resulted in a linear increase in the observed rate constants (Figure S2b, Supporting Information) indicating that the reaction is also first order for [HS^-].

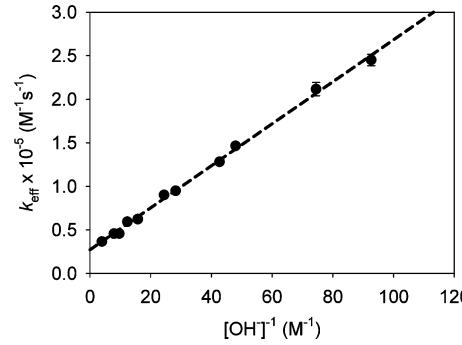

Figure 1. Hydroxide ion concentration dependency of the rate of the reaction of HS^- with OCl^- under pseudo first-order conditions. Data points represent the effective rate constants (that were calculated by dividing the obtained pseudo first-order rate constants by [HS^-]) at different hydroxide ion concentrations. The dashed line represents the linear least-squares fit of the data.

The rate of the reaction was inversely dependent on [OH^-] (Figure 1). This indicates that HOCl is more reactive than OCl^- and is consistent with analogous reactions of the hypohalites and with the protonated form of the oxidant being a better electrophile (*13*−*16*). However, the fact that the line on Figure 1 has a positive intercept indicates that OCl^- reacts with a measurable rate under these conditions. The following mechanism is consistent with these observations:

$$OCl^- + H_2O \rightleftharpoons HOCl + OH^- \quad K_1 = K_w/K_a^{HOCl} \tag{1}$$

$$HS^- + HOCl \xrightarrow{k_2} \text{products} \tag{2}$$

$$HS^- + HOCl \xrightarrow{k_3} \text{products} \tag{3}$$

where K_w is the ionic product of water, and K_a^{HOCl} is the acid dissociation constant of HOCl ($pK_a^{HOCl} = 7.4$) Using the pre-

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equilibrium approximation for reaction 1, the following rate equation can be derived from this model:

$$\frac{-\mathrm{d}[HS^-]}{\mathrm{d}t} = \left(k_3 + \frac{k_2 K_1}{[OH^-]}\right)[OCl^-][HS^-] \quad (4)$$

On the basis of this rate equation, k_{eff} in Figure 1 will be defined as $k_{eff} = (k_2K_1/[OH^-] + k_3)$. Because an excess of $[HS^-]$ was used over $[OCl^-]$, it is not necessary to correct k_2 or k_3 with a proportionality constant (*17*). Therefore, $k_3 = (2.7 \pm 0.2) \times 10^4\ M^{-1}\ s^{-1}$ can be obtained from the intercept and $k_2 = (4.8 \pm 0.1) \times 10^9\ M^{-1}\ s^{-1}$ from the slope of the line of Figure 1 using $pK_w = 13.7$ and $pK_a^{HOCl} = 7.4$. These rate constants are close to the analogous rate constants for OCl^- and HOCl reacting with the thiolate form of cysteine: $1.9 \times 10^5\ M^{-1}\ s^{-1}$ and $1.2 \times 10^9\ M^{-1}\ s^{-1}$, respectively (*14, 18*). The sulfhydryl group of cysteine is mostly protonated at physiological pH (*19*). However, the two pK_a values of H_2S are 6.76 and 19.2, respectively (*20*), which means that at pH 7.4, 81% would be present as HS^- and 19% as H_2S. The negative charge on the sulfur in HS^- makes it a better nuclephile compared to H_2S, and therefore, it is the more reactive form toward electrophiles. The S^{2-} form, which would be an even better nucleophile, has very little abundance at pH < 14.

On the basis of the $[OH^-]$ dependency at high pH, the reaction of HOCl with HS^- is 5 orders of magnitude faster than that of OCl^-. If we assume that H_2S has negligible reactivity with HOCl at pH 7.4 and that the reaction proceeds predominantly via HS^- and HOCl, then the lower limit of the apparent rate constant between the two reactants at pH 7.4 can be extrapolated to the value of $2 \times 10^9\ M^{-1}\ s^{-1}$.

We also investigated the products of the reaction. The peaks at 290 and 380 nm in the UV-spectrum at pH 7 suggest the formation of polysulfides (*21*). We found a linear correlation between $[OCl^-]_0$ and the observed absorbance change at both pH 7.4 and pH 13, with the linear fits passing through the origin (Figure S3, Supporting Information). This would be consistent with a mechanism analogous to that described for cysteine (*14*) in which the initial oxidation step is followed by the formation of a disufide (HS-S$^-$) as in reactions 5−9.

$$OCl^- + H_2O \rightleftharpoons HOCl + OH^- \qquad \textit{pre-equilibrium} \quad (5)$$

$$HS^- + HOCl + HSCl + OH^- \qquad k_2 = 4.8 \times 10^9\ M^{-1}\ s^{-1} \quad (6)$$

$$HS^- + OCl^- + H^+ \rightarrow HSCl + OH^- \qquad k_3 = 2.7 \times 10^4\ M^{-1}\ s^{-1} \quad (7)$$

$$HSCl + H_2O \rightarrow HSOH + Cl^- + H^+ \qquad \textit{fast} \quad (8)$$

$$HSOH + HS^- \rightarrow HS_2^- + H_2O \qquad \textit{fast} \quad (9)$$

Alternatively, reactions 6 and 7 could proceed via OH^+ transfer to directly give HSOH. However, the Cl^+ transfer mechanism would be consistent with a large body of literature on the two electron oxidation of nucleophiles with hypohalous acids; see refs *14*−*19* and the references therein. The disulfide species can undergo further exchange reactions to give polysulfides (reaction 10), where the number of atoms of zerovalent sulfur per polysulfide can vary between 1 and 8 depending on the pH and the relative amount of the oxidized versus reduced forms (*21*).

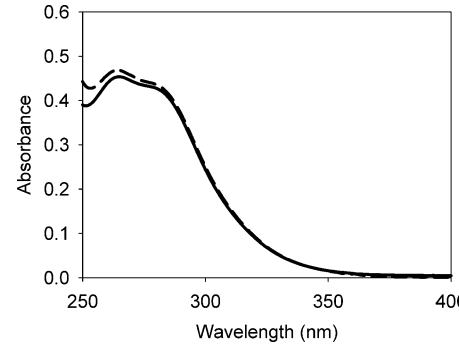


Figure 2. Qualitative and quantitative analysis of the product(s) of the reaction of HS^- with OCl^-. OCl^- (2.5 mM) was reacted with 150 mM HS^- at pH 7.4 (in 50 mM phosphate buffer). The reaction mixture was acidified by the addition of H_3PO_4 to a final pH of ∼2. After 2 h of incubation in the dark at room temperature, the yellow precipitate was extracted into $CHCl_3$, and the dashed line represents the spectrum of the organic phase. The solid line shows the UV-spectrum of 0.5 mM authentic cyclooctasulfur in chloroform. On the basis of the similarities of the two spectra and a previous qualitative analysis of a similar precipitate by tandem mass spectrometry (*22*), we conclude that acidification of the reaction mixture resulted in the precipitation of cyclooctasulfur. Because of a 5-fold dilution during extraction, the solid line represents the expected amount of S_8 formation upon a 100% conversion of OCl^- to S_8 in the above experiment. The similar absorbance of the two samples suggests that OCl^- generates predominantly polisulfides rather than polythionates when reacted with an excess of HS^- (for further details see Supporting Information).

$$nHS_2^- \rightleftharpoons HS_{(n+1)}^- + (n-1)HS^- \qquad (n = 1-8) \quad (10)$$

Under the conditions of our kinetic analysis (high pH and an excess of $[HS^-]$ over the oxidant), the dominant form (>99%) is the disulfide (*21*). However, at pH 7.4 in a 1 to 1 mixture of HS^- and S_0, the two dominant species would be HS_4S^- (∼60%) and HS_3S^- (∼40%) (*21*).

As reactions 8 and 9 are fast (compared to reactions 6 and 7) at pH > 13 (see Stopped-Flow Studies in Supporting Information), we have no information on the nature or the rate of these reactions or the protonation state of the intermediates. However, these rates could be smaller than that of reaction 6 at physiological pH, where reaction 6 becomes very fast.

Polysulfides are relatively stable at higher or neutral pH, but under acidic conditions, they precipitate from aqueous solution as elemental sulfur.

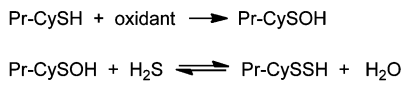
$$HS_{(n+1)}^- + H^+ \rightleftharpoons n/8S_8 + H_2S \qquad (n = 1-8) \quad (11)$$

Upon acidification of our reaction mixtures, we observed the formation of a yellow precipitate. The precipitate dissolved in chloroform, resulting in an UV-spectrum analogous to that of cyclooctasulfur (Figure 2). The amount of precipitated S_8 at different $[OCl^-]$ was quantified using a previously reported procedure (*22*) (see Supporting Information and Figure 2). At an excess of $[HS^-]$ over $[OCl^-]$, the majority of the oxidizing equivalents were converted to elemental sulfur, suggesting a minor contribution (if at all) of pathways that would result in the formation of polythionates (oxysulfur species, such as $S_2O_3^{2-}$, $S_4O_6^{2-}$, SO_3^{2-}, or SO_4^{2-}).

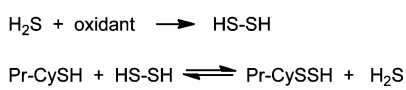
Although the calculated second order rate constant of the reaction of HS^- with HOCl at pH 7.4 means the reaction is highly favorable, whether H_2S acts as an antioxidant toward HOCl (*9, 23*) will depend on its local concentration. Different authors have reported widely varying values for physiological

Scheme 1. Proposed Models for the Formation of Protein Persulfides

Model A



Model B



Protein persulfides were generated by (A) direct oxidation of the protein Cys thiol or (B) direct oxidation of H_2S.

concentrations of H_2S. However, the most recent studies favor a relatively low value (*24*), suggesting that H_2S has little importance as an antioxidant. However, HOCl could deplete H_2S at sites of inflammation, possibly producing bioactive products and influencing its signaling function. For example, it has recently been proposed that H_2S signals via protein sulfhydration (*25*) to give persulfide derivatives on Cys residues. To form a persulfide from a thiol and H_2S, one molar oxidizing equivalent is needed. The oxidant could either react with the Cys to give a sulfenic acid, which could react further with HS^- to give the persulfide (model A, Scheme 1). Alternatively, the oxidant could target HS^- directly to give polysulfides, as shown in this study, followed by a reaction with the Cys thiol to give the persulfide (model B, Scheme 1).

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Supporting Information Available: Experimental details, concentration dependencies of the rate law, spectral titration, and quantitative analyses of the product(s). This material is available free of charge via the Internet at http://pubs.acs.org.

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