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Original Contribution

Insights into the mechanism of the reaction between hydrogen sulfide and peroxynitrite

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ABSTRACT

Hydrogen sulfide and peroxynitrite are endogenously generated molecules that participate in biologically relevant pathways. A revision of the kinetic features of the reaction between peroxynitrite and hydrogen sulfide revealed a complex process. The rate constant of peroxynitrite decay, $(6.65 \pm 0.08) \times 10^3\ M^{-1}\ s^{-1}$ in 0.05 M sodium phosphate buffer (pH 7.4, 37 °C), was affected by the concentration of buffer. Theoretical modeling suggested that, as in the case of thiols, the reaction is initiated by the nucleophilic attack of HS^- on the peroxide group of ONOOH by a typical bimolecular nucleophilic substitution, yielding HSOH and NO_2^-. In contrast to thiols, the reaction then proceeds to the formation of distinct products that absorb near 408 nm. Experiments in the presence of scavengers and carbon dioxide showed that free radicals are unlikely to be involved in the formation of these products. The results are consistent with product formation involving the reactive intermediate HSSH and its fast reaction with a second peroxynitrite molecule. Mass spectrometry and UV–Vis absorption spectra predictions suggest that at least one of the products is $HSNO_2$ or its isomer HSONO.

Hydrogen sulfide[1] is being considered a new signaling molecule in mammals after the discovery of its formation in vivo and its modulatory effects. It is produced in tissues of mammals through the activity of cystathionine β-synthase and cystathionine γ-lyase, two pyridoxal phosphate-dependent enzymes, and probably also of mercaptopyruvate *S*-transferase [1]. Although the concentrations of hydrogen sulfide measured in vivo are not high—just tens of nanomolar [2]—several effects, including neuromodulator and vasodilator properties [3,4], have been described in research model systems, and the administration of hydrogen sulfide and its modulation or production are of possible pharmacological interest.

In the context of efforts to rationalize the potential of hydrogen sulfide to act as a scavenger of biological oxidants, the reaction with peroxynitrite[2], the product of the fast recombination of nitric oxide with superoxide radicals, drew the attention of two research groups [5,6]. Two independent determinations of the rate constant reached slightly different results. Carballal et al. [5] reported a value of $(4.8 \pm 1.4) \times 10^3\ M^{-1}\ s^{-1}$ and Filipovic et al. [6] reported a value of $(8 \pm 2) \times 10^3\ M^{-1}\ s^{-1}$, both at 37 °C and pH 7.4. Carballal et al. proposed a reaction mechanism analogous to the classic one proposed for thiols with peroxynitrite [7–13]. Like in the case of low- and high-molecular-weight thiols, the proposed reaction mechanism involved the formation of a sulfenic acid-like product (HSOH) after the attack of HS^- on ONOOH. This intermediate would react

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[1] The term "hydrogen sulfide" is used in this text for the mixture of H_2S and HS^- present in aqueous solution according to the working pH, unless otherwise specified. The IUPAC-recommended names are sulfane and hydrogen sulfide for H_2S and sulfanide and hydrogen(sulfide)(1−) for hydrosulfide anion, HS^-.

[2] "Peroxynitrite" is used to refer to the species in equilibrium, $ONOO^-$ and ONOOH. The IUPAC-recommended names are oxoperoxonitrate(1−) and hydrogen oxoperoxonitrate, respectively.

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with a new HS^- molecule to produce HSSH. Nevertheless, Filipovic et al. noticed the formation of an unexpected yellow product. The stability of this new product seemed to be low in the presence of oxygen. In addition, mass spectrometry results suggested the formation of $HSNO_2$ or its isomers. Another set of plausible reaction mechanisms was proposed, including concerted reactions and radical processes.

The two groups joined efforts to better understand the puzzling reaction between hydrogen sulfide and peroxynitrite, and this work provides new clues for the elucidation of its mechanism.

1. Materials and methods

1.1. Reagents and solutions

$Na_2S \cdot 9H_2O$ of high purity was purchased from J.T. Baker. Peroxynitrite was synthesized as previously described [5,14], diluted in 2 mM NaOH, and quantified by measuring absorbance at 302 nm ($\varepsilon_{302\ nm} = 1670\ M^{-1}\ cm^{-1}$) [15]. Phosphate and formate buffers were of high quality. The use of sodium hydroxide was avoided in the preparation of phosphate buffers. When sodium hydrogen carbonate was added, the final pH was adjusted to the desired value. DMPO[3], desferrioxamine, mannitol, potassium peroxymonosulfate, and cysteine stocks were prepared in ultrapure water. Sodium hypochlorite stock solution was diluted in 2 mM NaOH and measured at 292 nm. Hydrogen peroxide was diluted in ultrapure water and quantified at 240 nm. Tetranitromethane stock was prepared in phosphate buffer. H_2S_2 was synthesized following a previously reported protocol [16].

1.2. Kinetic measurements and UV–Vis spectral characterization of products

Kinetic measurements were performed in stopped-flow instruments using single-wavelength or photodiode array detectors (Applied Photophysics SX20 and μSFM-20 Bio-Logic stopped-flow module equipped with a J&M TIDAS high-speed diode array spectrometer with combined deuterium and tungsten lamps). Anaerobic conditions were achieved by displacing O_2 from vials and solutions with N_2 and flushing instruments with N_2-saturated water.

1.3. Mass spectrometry characterization

Reaction was performed in 20 mM ammonium formate buffer (pH 8.0) with or without dilution in acetonitrile and injected at −20 or +4 °C, respectively, using cryo-mass electron spray ionization (ESI). Spectra were recorded on an ultra-high-resolution ESI–time-of-flight (TOF) maXis mass spectrometer (Bruker Daltonics) and processed in data analysis software provided by the manufacturer.

1.4. Computational methods

1.4.1. Isolated species

To obtain information about the potential energy surface and the mechanism of the reaction under investigation, and to perform UV–Vis absorption spectra predictions, we performed several electronic structure calculations using the Gaussian 03 program [17]. The structures of the reactant complex (HS^-/ONOOH), product complex ($HSOH/NO_2^-$), and transition state (TS) of the first step of the reaction along with possible prospective intermediates were optimized both in vacuo and in the presence of up to four water molecules at different levels of theory: density functional theory (DFT) using the *PBE* functional and *MP2*, employing a double-zeta plus polarization (*dzvp*) Gaussian basis set [18]. Frequency calculations were performed in all cases. Intrinsic reaction coordinate and time-dependent DFT (TD-DFT) calculations were performed at the *PBE/dzvp* level of theory.

1.4.2. Aqueous solution absorption spectra prediction

This study was carried out using an all-electron Gaussian basis set density functional code developed by Nitsche et al. [19]. This code was interfaced with an Amber classical force field, to carry out hybrid quantum mechanics–molecular mechanics (QM–MM) simulations. Absorption spectra were calculated from an average of an ensemble of instantaneous configurations obtained from a QM–MM Born-Oppenheimer 50-ps molecular dynamics, which combined DFT at the *PBE/dzvp* level and the TIP3P force field to describe the H_2O solvent molecules. A set of configurations extracted from these QM–MM trajectories was then computed at the real-time TD-DFT level, using exactly the same *PBE/TIP3P* hamiltonian as the one adopted to perform the molecular dynamics. This methodology has proven to yield reliable predictions of absorption properties of molecules in solution or in complex environments [20].

1.5. Simulations and data analysis

Kinetic data were analyzed and fitted using the Pro-Data Viewer software (Applied Photophysics), Bio-Kine32 software (Bio-Logic), or Origin 6.1. Kinetic simulations were performed with GEPASI [21].

2. Results and discussion

2.1. Rate constant of peroxynitrite decay at 302 nm

In the previous reports, Carballal et al. had used NaHS, whereas Filipovic et al. had used Na_2S [5,6]. Thus, the difference in rate constants determined by both groups was initially thought to be related to the hydrogen sulfide source (NaHS vs Na_2S), because NaHS stocks can contain significant amounts of impurities [22]. However, new determinations of the rate constant for peroxynitrite consumption using Na_2S led to results quite similar to the previous ones using NaHS, consistent with the fact that the NaHS used before had been titrated [5]. Another difference in the procedures was the buffer concentration (potassium phosphate 0.3 M used by Filipovic et al. vs 0.1 M used by Carballal et al.). As observed in Fig. 1, the second-order rate constant was $(9.1 \pm 0.3) \times 10^3\ M^{-1}\ s^{-1}$ (37 °C, pH 7.4) with 0.3 M potassium phosphate buffer, but the rate constant was $(6.65 \pm 0.08) \times 10^3\ M^{-1}\ s^{-1}$ with 0.05 M buffer. Also, the decay of peroxynitrite alone was faster in the high-concentration buffer. These observations can be explained by the effect of buffer concentration—not ionic strength—on the pK_a of peroxynitrous acid according to previous reports [23,24]. It has been shown that the higher the phosphate concentration, the higher the pK_a of peroxynitrous acid. We can argue that when the pK_a increases, a higher amount of peroxynitrous acid (ONOOH; the reactive species toward HS^-) is present in solution at pH 7.4 and thus its spontaneous decay as well as its consumption by HS^- is faster.

From the value of $(6.65 \pm 0.08) \times 10^3\ M^{-1}\ s^{-1}$ at pH 7.4 (37 °C, 0.05 M sodium phosphate buffer) we can extrapolate a pH-independent rate constant of $(5.6 \pm 0.6) \times 10^4\ M^{-1}\ s^{-1}$ assuming pK_a's of 6.7 and 7.0 for peroxynitrous acid and hydrogen sulfide, respectively [23,25]. Compared to the rate constants of low-molecular-weight thiolates, we can conclude that HS^- has a slightly lower nucleophilic character than aliphatic thiolates [5], probably because of the absence of the substituent methylene group and its inductive effect.

[3] *Abbreviations used:* DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DTPA, diethylenetriaminepentaacetic acid; TS, transition state; QM–MM, quantum mechanics–molecular mechanics; DFT, density functional theory; TD-DFT, time-dependent DFT.

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