



Reactions between nitrosopersulfide and heme proteins

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ABSTRACT

When nitrosothiols react with excess hydrogen sulfide, H₂S, they form several intermediates including nitrosopersulfide (SSNO⁻). The stability and importance of this species has been debated. While some data suggest SSNO⁻ can be a relatively stable source of NO activity, others suggest that the species degrades too quickly. We find the species to be relatively stable in isolation. Due to the abundance and prominence of iron-containing proteins throughout the human body, it is important to establish the interaction of ferrous- and ferric-iron containing proteins with SSNO⁻. Study of the reactions of SSNO⁻ with heme proteins can also provide information about the potential in vivo stability and spontaneous reactivity of this species. We have used time-resolved electron paramagnetic resonance and UV–Vis absorption spectroscopy to study the reactions of SSNO⁻ with heme proteins. Iron-nitrosyl hemoglobin is formed when SSNO⁻ is reacted with deoxyhemoglobin and deoxygenated methemoglobin, suggesting NO formation from SSNO⁻. However, the yields of nitrosyl hemoglobin in reactions of SSNO⁻ with deoxyhemoglobin are much less than when SSNO⁻ is reacted with deoxygenated methemoglobin. Very little to no nitrosyl hemoglobin is formed when SSNO⁻ is reacted with carboxyhemoglobin, HbCO, and when SSNO⁻ is reacted with oxygenated hemoglobin, minimal methemoglobin is formed. Taken together, these data confirm the release of NO, but indicate a vacant heme is necessary to facilitate a direct heme-SSNO⁻ reaction to form substantial NO. These data also suggest that the ferric iron in methemoglobin potentiates SSNO⁻ reactivity. These results could potentially impact NO and sulfide bioavailability and reactivity.

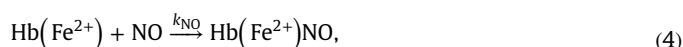
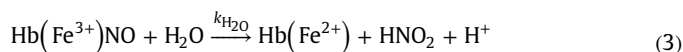
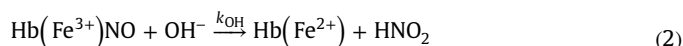
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1. Introduction

Nitric oxide (NO) produced by nitric oxide synthase (NOS) in the endothelium must diffuse into the smooth muscle to activate its heme-containing target, soluble guanylate cyclase (sGC). The reactivity of NO with heme-containing proteins is implicated in the inhibition of mitochondrial respiration and NO cytotoxicity where the targets are cytochrome c oxidase and catalase, respectively [1–3]. NO bioavailability is diminished by its reaction with oxyhemoglobin [4]. In the presence of oxygen, NO can form nitrosothiols which have been thought to be more stable than NO and still carry NO activity [5]. NO reactivity with ferrous and ferric heme proteins influences its signaling in substantial ways [6–8].

When the ferriheme of methemoglobin (metHb) reacts with NO, it binds to and subsequently reduces the heme at both acidic and alkaline pH values, but is fastest at higher pH [9,10]. Hence,

nitrosyl hemoglobin (HbNO) formation from metHb requires two NO, one to reduce and the other to bind, and the rate of reduction is dependent on [OH⁻] (Eqs. (1)–(4)) [9,10],



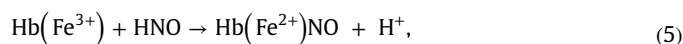
where Hb(Fe³⁺) is methemoglobin, Hb(Fe³⁺)NO is the ferriheme-nitrosyl adduct, Hb(Fe²⁺) is reduced ferrous hemoglobin, and Hb(Fe²⁺)NO is iron-nitrosyl hemoglobin. The equilibrium constant, *K*, at pH 7.4 is $1.3 \pm 0.1 \times 10^4 \text{ M}^{-1}$, the rate constant, *k*_{H₂O}, is

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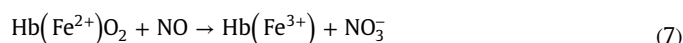
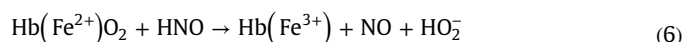
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$1.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and the rate constant, k_{OH} , is $3.2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ [9]. In the case of myoglobin, metmyoglobin binds NO to form the ferric nitrosyl species with an equilibrium constant $K = 1.3 \pm 0.1 \times 10^4 \text{ M}^{-1}$, but subsequent reduction of the ferric nitrosyl does not occur between pH 6.0 and 7.2 [9]. Only the hydroxyl ion facilitates the reduction of metmyoglobin, not water like Hb as in Eq. (3).

Nitroxyl (HNO) also reduces ferriheme containing globins to yield nitrosyl species, as shown in Eq. (5),



where Hb (Fe^{3+}) is metHb, HNO is nitroxyl, and $\text{Hb}(\text{Fe}^{2+})\text{NO}$ is HbNO. The reduction of ferriheme depicted in Eq. (5) is expected to occur with a rate constant similar to that for metmyoglobin which has been reported to be $8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ [11]. The reaction of HNO with oxyhemoglobin (oxyHb) yields metHb and NO, which leads to NO dioxygenation by excess oxyHb (Eqs. (6) and (7)) [12,13]. NO dioxygenation occurs when the O_2 -ligated ferrous heme of oxyHb reacts with NO, yielding metHb and nitrate (Eq. (7)) [13–15],



where oxyhemoglobin, $\text{Hb}(\text{Fe}^{2+})\text{O}_2$ reacts with HNO or NO on the order of $10^7 \text{ M}^{-1} \text{ s}^{-1}$ [13].

H_2S , like NO, is a gasotransmitter that has many biological effects, including the potentiation of NO signaling [16,17]. Endogenously produced H_2S is stored in sulfane sulfur, such as, polysulfides, persulfides, thiosulfate, etc. [18]. At neutral pH, un-stored H_2S is predominantly HS^- due to a pK_a of 6.8 [19]. Although NO and HS^- are likely nonreactive, HS^- and NO^+ , which occurs in S-nitrosothiol (RSNO), or sulfide radical (HS^\bullet) and NO are reactive [19–21].

As a product of the chemical reaction of RSNO with HS^- and a long-term activator of sGC, nitrosopersulfide (SSNO^-) has gained interest for its physiological potential [21]. As shown in Eqs. (8) and (9),



nucleophilic attack of HS^- on the sulfur of RSNO may result in thionitrous acid (HSNO) formation and HNO formation upon a subsequent nucleophilic attack of HS^- on HSNO (Eqs. (8) and (9)) [20,21]. Although HSNO is likely a product of this reaction, its labile nature excludes it as a candidate for the prolonged sGC activation observed by Cortese-Krott and colleagues [20]. The prevalence of persulfide (HSS^-) at high concentrations of sulfide leads to formation SSNO^- ,



where nucleophilic attack of HSS^- on the RSNO results in the formation of SSNO^- (Eq. (10)) [20]. For this reason, SSNO^- formation is prevalent at 1:1 or higher HS^- to RSNO concentration ratios [20]. When concentration ratios of 1:1 and 2:1 (HS^- :RSNO) are used, SSNO^- is formed within 10 min and there is substantial NO released when compared to RSNO alone [20]. Cortese-Krott et al. show sustained sGC activation and cGMP accumulation upon SSNO^- decomposition along with a gradual increase in absorbance at 290–300 nm, indicating polysulfide formation [20]. These data support homolytic cleavage of SSNO^- as a mechanism to

obtain NO from SSNO^- . SSNO^- decomposition by homolytic cleavage as shown by Eq. (11),



results in NO and the disulfide radical (SS^\bullet), which form polysulfides [20]. In this way, the once oxidized redox congener of NO, RSNO, can behave as a reservoir for NO bioactivity.

Precontracted aortic rings that were preincubated with a sGC inhibitor showed diminished relaxation in response to GSNO and SSNO^- [21], confirming a role for sGC in GSNO and SSNO^- -induced relaxation. Although application of an NO scavenger effectively mitigated GSNO-induced relaxation, it had no effect on SSNO^- -induced relaxation [21]. These data suggest a mechanism for SSNO^- -induced relaxation in which NO is not “released” from SSNO^- , but sGC activation is obtained. A possible explanation may be HNO formation or direct SSNO^- and sGC interaction. sGC is a heme containing protein and direct interaction between SSNO^- and sGC cannot be ruled out. Although some believe SSNO^- is physiologically relevant, still others suggest that SSNO^- is too unstable to be physiologically relevant [20,22]. In this study, we sought to determine the stability and spontaneous reactivity of SSNO^- in the presence of heme-containing proteins.

2. Experimental procedures

2.1. Chemicals

MAHMANONOate and Angeli's salt were purchased from Cayman Chemical Company. Equine skeletal muscle myoglobin, lyophilized bovine liver catalase, TRIZMA hydrochloride (Tris HCl), phosphate buffered saline (PBS) pH 7.4, monosodium phosphate, disodium phosphate, diethylenetriaminepentaacetic acid (DTPA) and potassium hexacyanoferrate (III) ($\text{K}_3\text{Fe}(\text{CN})_6$) were purchased from Sigma. Sodium hydroxide (NaOH) solution, 1 N, was purchased from Fisher Scientific. $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ was obtained from Acros Organics. S-nitrosoglutathione (GSNO) was formed from the protocol by Hart [23] with adaptations.

2.2. Reagent preparation

Methemoglobin and metmyoglobin were prepared by mixing oxyhemoglobin or equine skeletal muscle myoglobin, respectively, with $\text{K}_3\text{Fe}(\text{CN})_6$ in 10-fold excess in PBS, pH 7.4. A stock of $\text{K}_3\text{Fe}(\text{CN})_6$ was prepared in PBS pH 7.4 and the globin/ $\text{K}_3\text{Fe}(\text{CN})_6$ mixtures were gently agitated for 20 min to ensure thorough mixing. Then, the mixtures were filtered through a sephadex G-25 M column containing 0.15% Kathon. Concentration was determined by UV-visible spectroscopy. Lyophilized bovine liver ferricatalase stocks were prepared in 100 mM Phosphate Buffer, pH 7.4 and centrifuged at $13,000 \times g$ to remove any debris. Concentration was determined spectrally, $\epsilon_{405} = 1.49 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ [24]. Stocks of 10 mM NaOH with and without 0.1 mM DTPA were made in deionized (DI) water and degassed with N_2 or Ar gas. Stocks of PBS pH 7.4 with and without 0.1 mM DTPA were made and degassed with N_2 or Ar. DI water and 20 mM TRIS HCl pH7.4 were also degassed.

In a 5 mL polypropylene tube capped with a rubber stopper, Na_2S crystals were rinsed with DI water and immediately degassed with N_2 or Ar until dried. The addition of 3–4 mL of degassed 10 mM NaOH with 0.1 mM DTPA to the degassed Na_2S crystals yielded stock concentrations that ranged between 10 and 25 mM. Na_2S stock concentrations were ascertained using UV-visible spectroscopy by diluting the stock into 2 mL of degassed DI water in a degassed 1 cm quartz cuvette, $\epsilon_{232} = 7700 \text{ M}^{-1} \text{ cm}^{-1}$ [21]. Stocks were freshly made and used within 6 h.

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