

Catalytic generation of N_2O_3 by the concerted nitrite reductase and anhydrase activity of hemoglobin

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Nitrite reacts with deoxyhemoglobin to form nitric oxide (NO) and methemoglobin. Though this reaction is experimentally associated with NO generation and vasodilation, kinetic analysis suggests that NO should not be able to escape inactivation in the erythrocyte. We have discovered that products of the nitrite-hemoglobin reaction generate dinitrogen trioxide (N_2O_3) via a novel reaction of NO and nitrite-bound methemoglobin. The oxygen-bound form of nitrite-methemoglobin shows a degree of ferrous nitrogen dioxide (Fe(II)-NO_2^+) character, so it may rapidly react with NO to form N_2O_3 . N_2O_3 partitions in lipid, homolyzes to NO and readily nitrosates thiols, all of which are common pathways for NO escape from the erythrocyte. These results reveal a fundamental heme globin- and nitrite-catalyzed chemical reaction pathway to N_2O_3 , NO and S-nitrosothiol that could form the basis of *in vivo* nitrite-dependent signaling. Because the reaction redox-cycles (that is, regenerates ferrous heme) and the nitrite-methemoglobin intermediate is not observable by electron paramagnetic resonance spectroscopy, this reaction has been 'invisible' to experimentalists over the last 100 years.

Recent studies have revealed that the ubiquitous circulating anion nitrite (NO_2^-) is a vasodilator and an intrinsic signaling molecule^{1–5}. The vasodilator activity of nitrite is associated with an allosterically controlled heme-based reduction of nitrite to NO by deoxygenated hemoglobin (deoxyHb)^{6,7}. Nitrite infusions into the human circulation increase blood flow at near-physiological concentrations^{2,8}. This vasodilation is temporally associated with increases in red cell heme iron-nitrosylated hemoglobin (HbFe(II)-NO , designated as $[\text{FeNO}]^7$ using the Enemark-Feltham notation⁹) and to a lesser extent S-nitrosated hemoglobin (SNO-Hb, hemoglobin nitrosated at the β -93 cysteine)².

Although the *in vitro* incubation of nitrite with deoxygenated red cells and hemoglobin solutions produces vasodilation, tissue NO-dependent cyclic GMP (cGMP, **1**) accumulation, gas-phase NO generation and NO-dependent inhibition of mitochondrial oxygen consumption, the mechanism of NO escape from the red cell after nitrite reduction by hemoglobin remains elusive^{2,10,11}. Indeed, an important challenge to the nitrite reductase hypothesis and other erythrocyte-NO export theories is explaining how the NO can escape heme autocapture¹. Modeling calculations have shown that only 0.1 pM NO would be produced outside a red blood cell at steady

state, even at supraphysiological nitrite levels, unless additional mechanisms exist to limit the scavenging reactions of NO with hemoglobin¹². A similar paradox can be seen in the cardiomyocyte, in which nitrite inhibits cellular respiration during hypoxia via the nitrite reductase activity of deoxymyoglobin, despite the fact that the high concentrations of myoglobin in the cardiomyocyte might be expected to inhibit NO-dependent signaling^{13,14}. We and others have therefore considered the possibility that the nitrite-hemoglobin reactions (i) are compartmentalized at the red cell membrane to limit cytoplasmic scavenging, or (ii) generate NO_x species such as N_2O_3 that can concentrate in the membrane or in hydrophobic membrane channels and thereby diffuse out of the red blood cell^{7,12,15}. N_2O_3 is a logical candidate for such an intermediate because (i) it is a primary nitrosating species capable of generating red cell S-nitrosothiols^{15–18}, which clearly form as a side product of the nitrite-hemoglobin reaction², (ii) it is small and uncharged, which facilitates its diffusion through the red cell membrane and (iii) it can homolyze to NO and NO_2^+ , allowing for export of NO (refs. 12,15).

If N_2O_3 is formed in the reaction of nitrite and deoxyhemoglobin, then nitrosated products should be detectable. We previously reported

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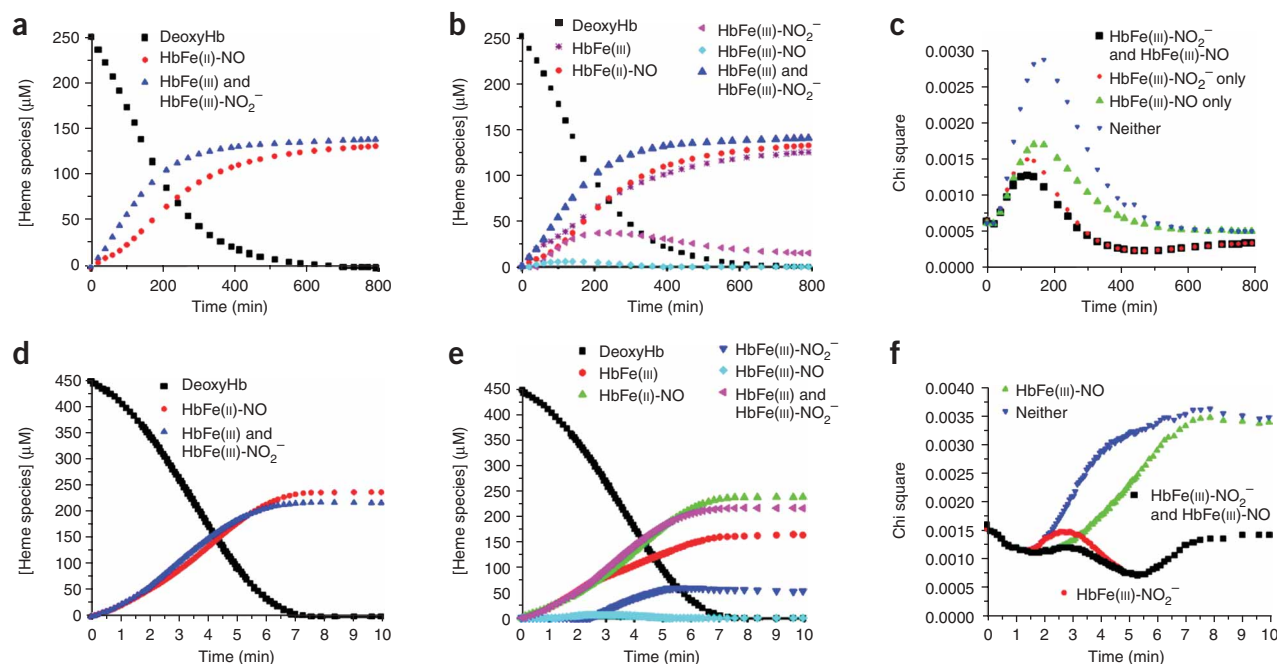


Figure 1 HbFe(III)-NO₂⁻ (nitrite-MetHb) is the intermediate in the deoxyhemoglobin-nitrite reaction. **(a)** Disappearance of deoxyhemoglobin and formation of HbFe(II)-NO and total MetHb (sum of MetHb and HbFe(III)-NO₂⁻) over time in the reaction of 250 μM deoxyhemoglobin (in heme) with 125 μM nitrite at pH 7.4 and 37 °C. Reaction progress was monitored by absorption spectroscopy. **(b)** Concentrations of various heme species over time for the reaction shown in **a**. Heme species include deoxyhemoglobin, MetHb, HbFe(II)-NO, HbFe(III)-NO₂⁻ and HbFe(III)-NO. Total MetHb is labeled as 'HbFe(III) and HbFe(III)-NO₂⁻' and is equal to the sum of these two species. **(c)** Comparison of chi-squared values from the experiment in **a** fitted using four combination sets of standard spectra, which include deoxyhemoglobin, MetHb, HbFe(II)-NO and additional standard spectra of either HbFe(III)-NO₂⁻ alone, HbFe(III)-NO alone, both species or neither species. **(d)** The disappearance of deoxyhemoglobin and formation of HbFe(II)-NO and total MetHb (sum of MetHb and HbFe(III)-NO₂⁻) over time in the reaction of 450 μM deoxyhemoglobin with 5 mM nitrite at pH 7.4 and 37 °C. **(e)** Concentrations of various heme species over time for the reaction shown in **d**. Note that the plot of 'HbFe(III) and HbFe(III)-NO₂⁻' is equal to the sum of these two species, which are also plotted separately. **(f)** Comparison of chi-squared values from the experiment in **d** fitted using four combination sets of standard spectra, which include deoxyhemoglobin, MetHb, HbFe(II)-NO and additional standard spectra of either HbFe(III)-NO₂⁻ alone, HbFe(III)-NO alone, both species or neither species.

that during nitrite infusions in humans, in addition to the expected products of methemoglobin (MetHb) and HbFe(II)-NO, SNO-Hb is also formed². Others have also shown that the reaction of deoxyHb and nitrite forms S-nitrosothiols, including SNO-Hb (refs. 19–22). Some of these investigators have suggested that the formation of SNO-Hb from nitrite involves the formation of HbFe(II)-NO followed by the transfer of the NO⁺ group to Cys93 during the allosteric transition from the T quaternary state (tense, mostly deoxygenated) to the R quaternary state (relaxed, mostly oxygenated), or via the formation of a stable HbFe(III)-NO (refs. 22,23). The latter species, which is designated {FeNO}⁶ according to the Enemark-Feltham notation⁹ and which has also been written as MetHb-NO and HbFe(II)-NO⁺, is actually a single form in which the electron has a higher probability of being closer to the iron than to the ligand.

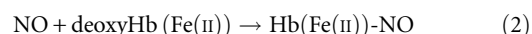
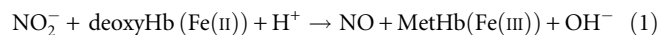
In the current study we observed S-nitrosation but we were not able to detect the putative HbFe(III)-NO intermediate, and the nitrosation that we did observe could not be explained by allosteric intramolecular transfer of heme-bound NO to the cysteine. In contrast, we found that the nitrite anion directly binds to MetHb, and this results in an electronic configuration that is silent when observed by electron paramagnetic resonance (EPR) spectroscopy. Based on density functional theory (DFT) calculations, we propose that the nitrite-bound MetHb has Fe(II)-NO₂⁻ character that reacts rapidly with NO to form N₂O₃. In this reaction, deoxyhemoglobin redox cycles and catalyzes the conversion of two nitrite ions into N₂O₃. To our knowledge, this represents a novel mechanism for both anaerobic and

metal-catalyzed N₂O₃ formation and S-nitrosation, and it likely represents a major pathway for NO-dependent signaling in a hypoxic heme-rich environment.

RESULTS

Fe(III)-NO₂⁻ forms during the nitrite-deoxyHb reaction

We have previously characterized the anaerobic reaction of deoxyhemoglobin with nitrite as an allosteric second-order reaction⁷. As predicted by equation (1) and (2), we measured an equal ratio of MetHb to HbFe(II)-NO at the end of the reaction.



However, others have observed that more MetHb than HbFe(II)-NO is made in the course of this reaction, particularly at low nitrite-to-hemoglobin ratios, which they attribute to the formation of an HbFe(III)-NO intermediate^{11,23}. This intermediate has nitrosonium ion character as HbFe(II)-NO⁺ that could potentially nitrosate thiols to make S-nitrosothiols (SNOs).

As HbFe(III)-NO has a clearly distinguishable visible spectrum, it should be possible to determine whether this species is formed as a significant intermediate using least-squares deconvolution of visible spectra taken during the time course of the reaction between nitrite and deoxyHb. Using reference spectra with and without the HbFe(III)-NO species in the reference standard, we compared the

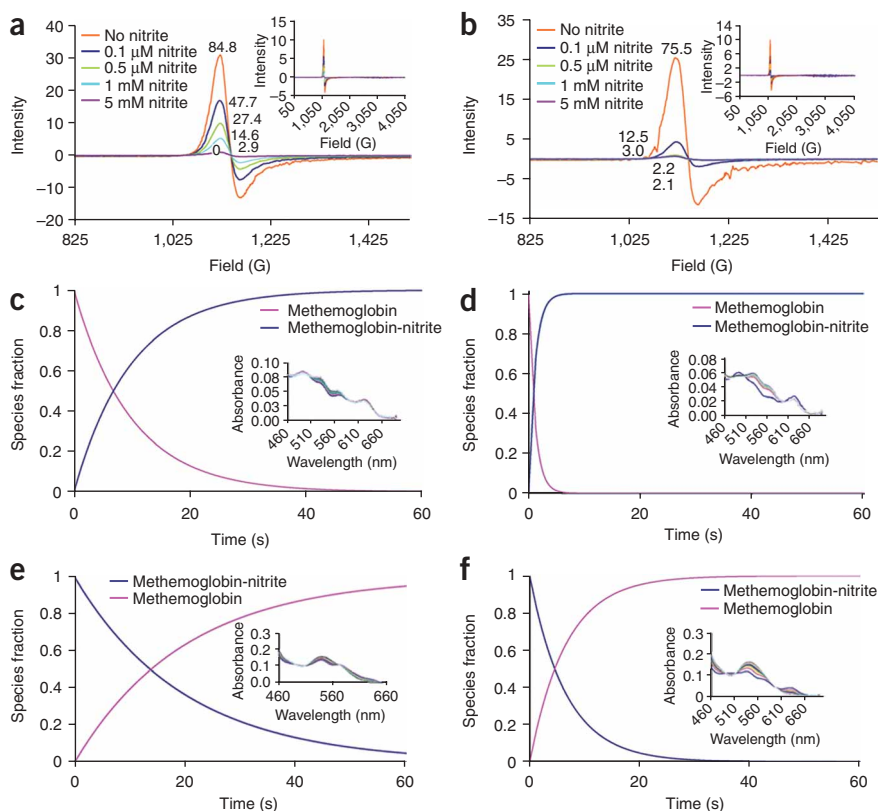


Figure 2 EPR silence of HbFe(III)-NO_2^- . **(a)** Samples were frozen 5 min after nitrite addition to 84.8 μM MetHb. The concentrations of remaining MetHb are indicated adjacent to each tracing. No low-spin MetHb signals were detected (inset). The average dissociation constants of HbFe(III)-NO_2^- were 75, 271, 284 and 285 μM for 0.1, 0.5, 1 and 5 mM added nitrite, respectively. Intensity is given in arbitrary units. **(b)** An analogous experiment performed at pH 6.5. The measured concentrations of MetHb remaining after the addition of nitrite are indicated next to curves. The average dissociation constants of HbFe(III)-NO_2^- were 7, 18, 28 and 145 μM for 0.1, 0.5, 1 and 5 mM added nitrite, respectively. **(c)** The kinetics of nitrite (4 mM) association with MetHb (50 μM) at pH 7.4 using stopped-flow absorption spectroscopy. The observed rate of nitrite binding averaged $0.12 \pm 0.01 \text{ s}^{-1}$ ($n = 6$). Inset depicts raw data. **(d)** The kinetics of nitrite (4 mM) association with MetHb (50 μM) at pH 6. The observed rate of nitrite binding averaged $0.76 \pm 0.11 \text{ s}^{-1}$ ($n = 6$). Inset depicts raw data. **(e)** The kinetics of nitrite dissociation from MetHb for the reaction of 2.5 mM HbFe(III)-NO_2^- with 2.5 mM KCN at pH 7.4. The rate of nitrite dissociation averaged $0.053 \pm 0.002 \text{ s}^{-1}$ ($n = 3$). Inset depicts raw data. **(f)** The kinetics of nitrite dissociation from MetHb for the reaction of 2.5 mM HbFe(III)-NO_2^- with 2.5 mM KCN at pH 6. The rate of nitrite dissociation averaged $0.153 \pm 0.001 \text{ s}^{-1}$ ($n = 3$). Inset depicts raw data.

derived yields and residuals over time, assuming that the smallest residual was due to inclusion of all proper heme species in the deconvolution (Fig. 1a,b). Five basis spectra were used; they included deoxyhemoglobin, MetHb and HbFe(II)-NO species, while HbFe(III)-NO_2^- (formed when nitrite binds to MetHb) and HbFe(III)-NO (putative intermediate) were added in isolation and together to discern their respective contributions to the reaction constituents.

Approximately equal product yields of HbFe(II)-NO and total MetHb were observed (Fig. 1), both at the low (Fig. 1a,b), equimolar and high nitrite/hemoglobin (Fig. 1d,e) ratios, which is consistent with equation (1) and (2) and our previous studies. Notably, we observed a significant amount of HbFe(III)-NO_2^- but no HbFe(III)-NO when both species were included in the regression analysis (Fig. 1b,e). At all nitrite/hemoglobin ratios we observed significantly lower chi-squared residuals when the HbFe(III)-NO_2^- basis spectra was included (Fig. 1c,f). Addition of HbFe(III)-NO did not further lower the chi-squared values. Notably, inclusion of HbFe(III)-NO alone often resulted in residuals that were nearly as high as when neither species was included. We also performed additional experiments at room temperature (20–25 °C), and HbFe(III)-NO was not observed when 1 mM deoxyHb was reacted with 250 μM nitrite for 180 min. In all these cases ($n = 3$), regression analysis reported no HbFe(III)-NO at any of the time points when all the basis spectra were used. These data suggest that HbFe(III)-NO_2^- , and not quasi-stable HbFe(III)-NO , is an intermediate of the deoxyhemoglobin-nitrite reaction. Similar results were observed in the deoxymyoglobin-nitrite reaction¹³. Importantly, we also confirmed the absence of an HbFe(III)-NO species in these reactions using a new chemiluminescence-based approach that we discuss below.

Analysis of HbFe(III)-NO_2^-

To confirm the stoichiometry of the reactions described above, we performed freeze-quench EPR measurements of products during the course of the nitrite-deoxyhemoglobin reaction. MetHb yields by EPR were consistently lower than those measured by UV-Vis absorption spectroscopy. To further probe this observation we added increasing concentrations of nitrite to MetHb and found that, like nitrite bound to bacterial nitrite reductase and other ferric heme proteins^{24,25}, HbFe(III)-NO_2^- is effectively EPR silent (Fig. 2a). The large low-field peak due to high-spin MetHb around $g = 6$ decreased as nitrite was added, and no concomitant signal for the low-spin MetHb was seen at higher magnetic fields (Fig. 2a). Notably, the EPR-silent species was more stable at lower pH, as evidenced by the faster disappearance of the $g = 6$ signal at equivalent nitrite concentrations at pH 6.5 (Fig. 2b). We also confirmed the tighter binding of nitrite to MetHb at lower pH using stopped-flow absorption measuring the association and dissociation rates of nitrite-bound MetHb (Fig. 2c–f). Thus, both absorption and EPR spectroscopy show that nitrite binds to MetHb more tightly at lower pH. The dissociation constant measured by examination of silencing of the $g = 6$ EPR signal (as described in the Methods) is much lower than that calculated using absorption spectroscopy (reported to be around 1–5 mM, refs. 26,27). More extensive EPR silencing was observed with lower nitrite concentrations, resulting in EPR-derived dissociation constants for HbFe(III)-NO_2^- of 75 μM for 100 μM of added nitrite and 285 μM for 5 mM added nitrite at pH 7.4, and 7 μM for 100 μM added nitrite and 145 μM for 5 mM of added nitrite at pH 6.5. These data suggest that under physiological conditions in the erythrocyte, a substantial fraction of nitrite would be bound to MetHb rather than remain free in solution.

The EPR silence of HbFe(III)-NO₂⁻ could occur because of line broadening resulting from *g* strain, as considered for other ferric heme proteins^{24,25}. To explore this possibility and other aspects of the electronic configuration of HbFe(III)-NO₂⁻, we have performed density functional theory (OLYP/STO-TZP) calculations on various six-coordinate nitrite-bound ferric porphyrins. The proximal ligand in most of these calculations is imidazole with an acetic acid side chain that hydrogen bonds to the imidazole NH group, while the N-bound ("nitro") or O-bound ("nitrito") nitrite hydrogen bonds to another imidazole on the distal side (Fig. 3). For all our models, the nitro form was found to be more stable than the *O*-nitrito form by about 7 kcal mol⁻¹. However, the relative orientation of the nitrite and proximal imidazole planes (coplanar or perpendicular) made little difference (< 1 kcal mol⁻¹) in the energy of these species. For every conformation of every species examined (whether nitro or *O*-nitrito), the two

alternative d_π¹ states (²A' and ²A'' for C_s point group symmetry) proved to be within 0.5 kcal mol⁻¹ of each other; which suggests that rapid fluctuation between these states may be responsible for the lack of an EPR signal for HbFe(III)-NO₂⁻. These results may also explain recent reports of an EPR-silent intermediate in the nitrite-deoxyhemoglobin reaction that is detectable by reductive chemiluminescence^{11,22}.

Unlike nitrate, nitrite may be a noninnocent ligand. A recent DFT analysis²⁸ emphasizes the π-accepting character of N-bound nitrite and the high electron affinities of ferric-nitro porphyrins. Here, we find that both our six-coordinate ferric-nitro and *O*-nitrito models have similar electron affinities of about 2.0 eV, which is unusually high for an electroneutral metalloporphyrin but not so high as to preclude the existence of these species. The *O*-nitrito ligand is noninnocent in certain conformations (Fig. 3), which may be viewed as a certain

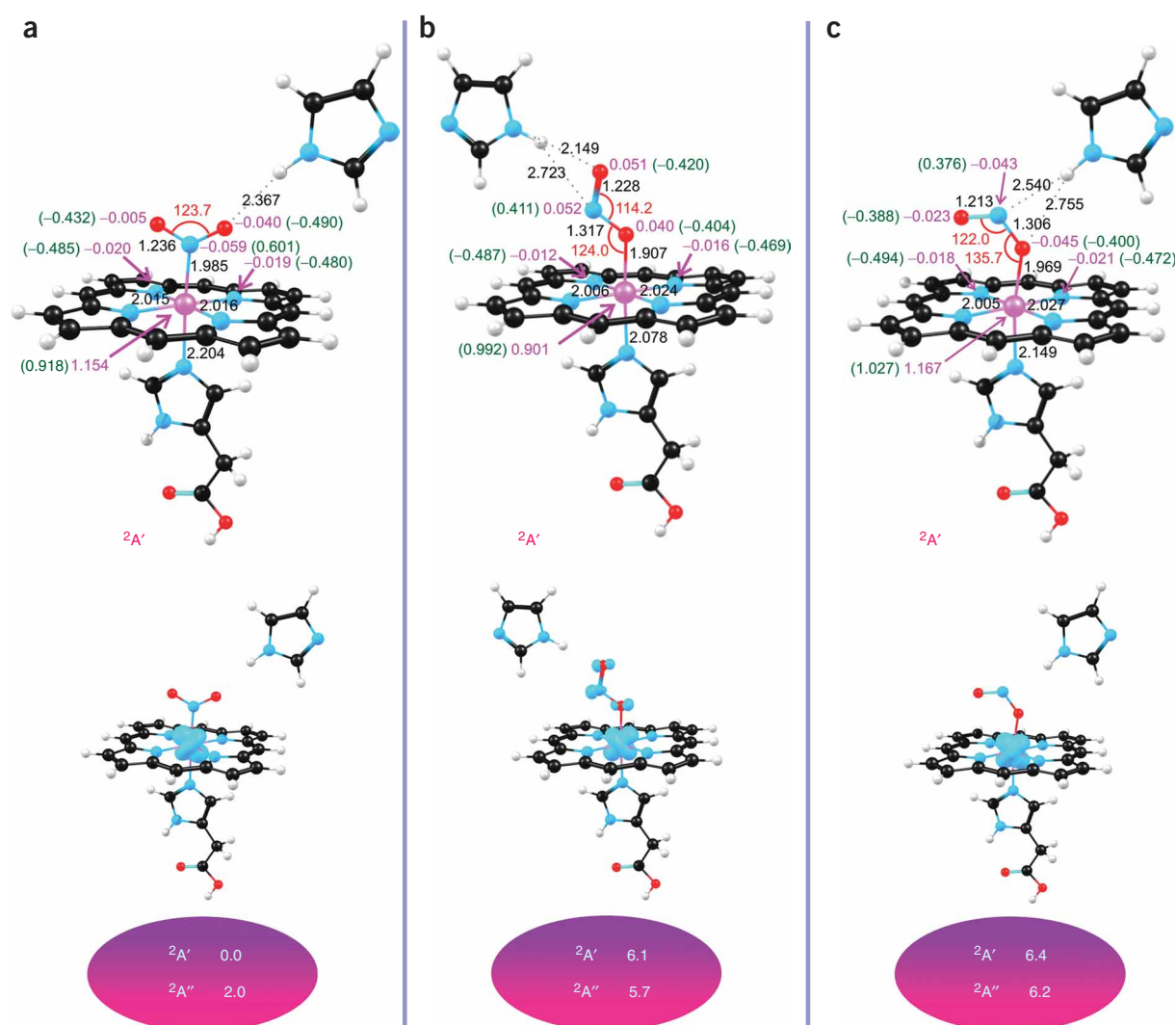


Figure 3 Electronic configuration of HbFe(III)-NO₂⁻. (a–c) Selected OLYP/STO-TZP results for an Fe(III)-nitro model (a) and two Fe(III)-*O*-nitrito models (b and c). Each of these models was restricted to C_s symmetry, which allowed the separate optimization of both the ²A' and ²A'' states, which correspond to the unpaired electron occupying one or the other of the two d_π (d_{xz} or d_{yz}) orbitals. The three diagrams in the top row present selected optimized bond distances (Å, in black), Mulliken spin populations (in magenta) and charges (in green in parentheses) for the ²A' states of the three models. The middle row shows the corresponding spin density plots, in which the excess spin density is shown in cyan. Shown against the oval insets at the bottom of the figure are the energies of the various states studied (in kcal mol⁻¹), relative to the ²A' state of the model in a. Observe that although the *O*-nitrito forms in b and c are higher in energy than the form in a, the ²A' and ²A'' states are very similar in energy for all three forms.

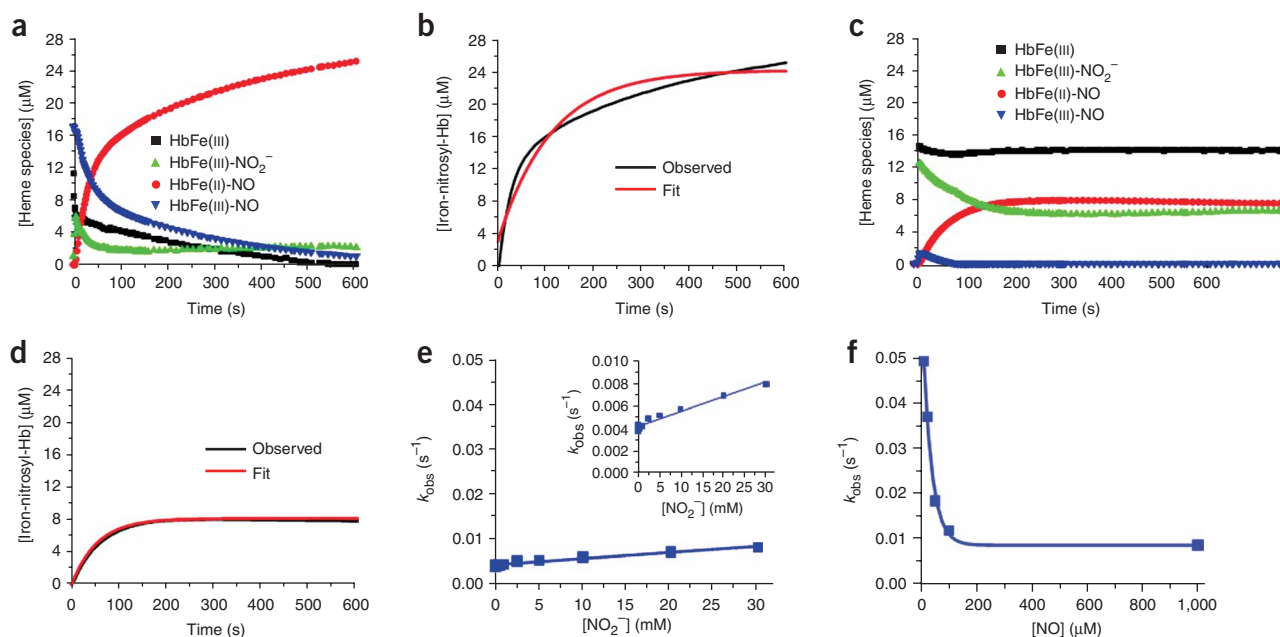
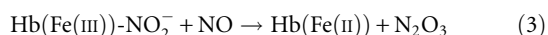


Figure 4 Reductive nitrosylation of HbFe(III)-NO₂⁻ catalyzed by NO. (a) 1 mM NO was added to a pre-equilibrated solution of 30 μM deoxygenated MetHb and 5 mM nitrite. (b) The observed change in HbFe(II)-NO concentration for the reaction shown in a was compared with that predicted by fitting to a single exponential that yielded an observed rate constant of 0.0086 s⁻¹. (c) 50 μM NO was added to a pre-equilibrated solution of 30 μM deoxygenated MetHb and 5 mM nitrite. (d) The observed change in HbFe(II)-NO concentration for the reaction shown in c was compared with that predicted by fitting to a single exponential that yielded an observed rate constant of 0.018 s⁻¹. (e) The observed rates of HbFe(II)-NO were determined for mixtures of 30 μM deoxygenated MetHb, 1 mM NO and a range of nitrite concentrations at pH 7.4. The line shown is a fit to the data. The inset shows the same data replotted on a different scale. The rate of reductive nitrosylation that we observe when no nitrite is added is higher than that reported previously²¹, which is perhaps due to the fact that we performed our experiments at a higher temperature (25 °C versus 37 °C) and we used less NO (1.8 mM versus 1 mM (from 500 μM ProliNO)). (f) The observed rates of HbFe(II)-NO formation were determined for mixtures of 30 μM deoxygenated MetHb, 5 mM nitrite and a range of NO concentrations at pH 7.4. The line shown is a fit to the data.

Fe(II)-NO₂[•] character. The existence of an Fe(II)-NO₂[•] nature of nitrite bound to an Fe(III) porphyrin has been considered previously in other contexts^{29,30}.

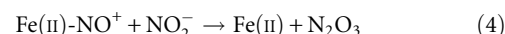
The reaction of NO with HbFe(III)-NO₂⁻

We therefore hypothesized that a highly electron-hungry HbFe(III)-NO₂⁻ intermediate with Fe(II)-NO₂[•] character may undergo a radical-radical reaction of the coordinated nitrite with NO, thereby leading to the formation of N₂O₃. Synthetic ferric-nitro porphyrins also have a similar means of relieving their electron deficiency by reacting with NO, which leads to the formation of highly stable {FeNO}⁶ ferric-nitronitrosyl complexes^{31,32}. NO formed in the nitrite-deoxyhemoglobin reaction could react with the Fe(II)-NO₂[•] intermediate at very rapid reaction rates. This general reaction scheme is shown in equation (3):



This reaction must be distinguished from reductive nitrosylation, in which two molecules of NO react with MetHb. The first NO molecule binds to MetHb (forming {FeNO}⁶ or Fe(II)-NO⁺ (nitrosonium cation)). The nitrosonium ion reacts with water or another nucleophile, and the second NO binds to the newly formed ferrous heme. Others have shown that nitrite, which is a contaminant of NO solutions, could catalyze the reductive nitrosylation reaction, thereby increasing the observed rate by approximately four-fold in the presence of 20 mM nitrite and 1–2 mM NO (ref. 21). They suggest that nitrite accelerates the

reaction through a mechanism in which nitrite reacts with the ferrous-nitrosonium intermediate to yield deoxyhemoglobin and N₂O₃:

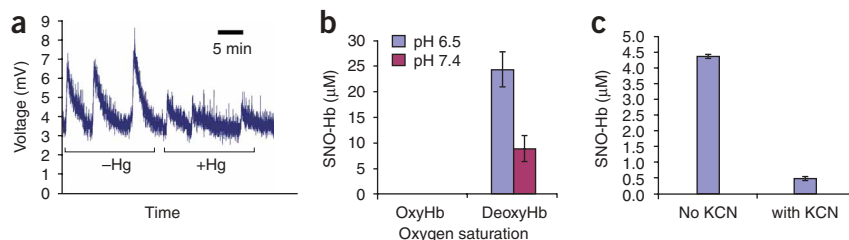


Although this mechanism could accelerate MetHb reduction by NO and lead to N₂O₃ formation, it is still too slow to compete with NO heme reactions and is difficult to reconcile with more rapid rates of reductive nitrosylation observed by others in the field^{22,33}.

We therefore considered an alternative solution. Previous experiments examining nitrite effects on rates of reductive nitrosylation were conducted at high NO concentrations, such that NO may have preferentially bound to the MetHb and thereby prevented or greatly diminished nitrite binding to MetHb (NO binds MetHb with approximately 10 to 100 times the affinity of nitrite)^{26,27,34,35}. Such competitive binding of NO would inhibit generation of the HbFe(III)-NO₂⁻ complex, which may be involved in catalytic reductive nitrosylation.

To test this hypothesis, we examined the effects of varying nitrite and NO concentrations on the rate of MetHb reduction and HbFe(II)-NO formation (Fig. 4). We first examined this process in the presence of excess NO (1 mM), and we observed rates that changed with increasing nitrite concentration and that were similar to those reported earlier²¹. We measured the change in all heme species over time by absorption spectroscopy (Fig. 4a), and we performed a single exponential fit to HbFe(II)-NO formation as a function of time

Figure 5 Nitrite-mediated nitrosothiol formation. (a) Formation of SNO-Hb in the reaction of 5 mM deoxygenated hemoglobin with 1 mM nitrite and 1 mM GSH, at pH 6.5 for 30 min at room temperature (20–25 °C). The figure represents raw data of either direct individual injections (–Hg) or individual injections treated with 5 mM HgCl₂ for 3 min (+Hg). (b) Hemoglobin (5 mM) was reacted with 1 mM nitrite and 1 mM GSH in PBS with 100 μM DTPA, at either pH 6.5 or pH 7.4, for 30 min. These reactions were carried out with 100% oxygenated Hb (oxyHb) and 98 ± 1% deoxyHb, followed by six-fold dilution in SNO-stabilization solution (modified 2C assay, *n* = 3). The average concentration of SNO-Hb was 0 μM (oxyHb) and 24.44 ± 3.52 μM (deoxyHb) at pH 6.5, and 0 μM (oxyHb) and 8.94 ± 2.49 μM (deoxyHb) at pH 7.4. (c) DeoxyHb (300 μM) was reacted with nitrite (1 mM) for 30 min at pH 6.5 in the presence or absence of KCN (5 mM). SNO-Hb was subsequently measured using the modified 2C assay. Error bars represent one s.d. from the mean (*n* = 3). When this experiment was repeated with 2.5 mM KCN, SNO-Hb was measured to be 1.6 ± 0.5 μM, compared with 4.3 ± 1.4 μM when the reaction was repeated in parallel without KCN. All errors are reported as one s.d.



(Fig. 4b). It is clear from this fit that under these conditions the kinetics of HbFe(II)-NO deviate from a single exponential process and involve a fast and slow phase. Such deviation suggests that at least two underlying processes are modifying the concentration of HbFe(II)-NO. A plot of the observed rate constant as a function of nitrite concentration (Fig. 4e) is linear, giving an apparent second-order rate constant of 0.13 M^{–1} s^{–1} (which is similar to that reported previously²¹), and represents nitrite-catalyzed reductive nitrosylation. However, fitting to a single exponential fit strongly weights the slower process and obscures the presence of the faster process. As NO concentration is reduced, the amplitude of the slow process is decreased and the faster process is revealed, as illustrated when we performed the experiment under conditions identical to those in Figure 4a but with 50 μM NO rather than 1 mM NO (Fig. 4c,d,f). The exponential fit is now a much better description of the data (Fig. 4d), and the observed rate constant now actually increases as NO concentration is decreased (Fig. 4f). Although this simple kinetic analysis perhaps does not do justice to the underlying complexity of the reactions, it illustrates that a hitherto unappreciated second reaction with faster kinetics than nitrite-catalyzed reductive nitrosylation is occurring in the initial stages of this reaction, and this faster reaction dominates as NO concentration is lowered. These data are consistent with a reaction such as that shown in equation (3)—one that occurs with a larger observed rate constant than reductive nitrosylation and that provides evidence for a direct reaction between NO and HbFe(III)-NO₂[–].

N₂O₃ formation in the reaction of nitrite and deoxyHb

To examine whether the nitrite-deoxyhemoglobin reaction generates a nitrosating agent, we used a modified copper-cysteine (2C) reductive chemiluminescent assay (see Methods) to examine SNO formation. When 5 mM Hb was incubated with 1 mM nitrite, significant (*P* < 0.004 for pH 6.5 and *P* < 0.02 for pH 7.4) SNO-Hb is formed (Fig. 5a,b). The most SNO is made under deoxygenated conditions and lower pH (Fig. 1a,b and Supplementary Fig. 1 online). The total amount of S-nitrosation measured when nitrite is mixed with glutathione and Hb is greater than that measured in the absence of the Hb (Supplementary Fig. 1). Studies examining the phosphate concentration dependence of SNO-Hb formation support the intermediacy of N₂O₃ (Supplementary Fig. 1). Notably, 23 μM SNO-Hb is produced when 5 mM deoxygenated Hb is mixed with 1 mM nitrite and 1 mM glutathione (GSH, 2) for 30 min (Fig. 5b). When the nitrite concentration is reduced to the levels obtained in studies of nitrite infusions in humans (approximately 250 μM (refs. 2,36)),

2.5 μM SNO-Hb is formed, which is similar to levels measured *in vivo*² (Supplementary Fig. 1). When we incubated 300 μM deoxyHb with 1 mM nitrite for 30 min in the presence of KCN, the amount of SNO-Hb formed was greatly diminished relative to the same incubation when the KCN was absent (Fig. 5c), which demonstrates dependence of SNO-Hb formation on the presence of HbFe(III)-NO₂[–]. The effect of KCN was reproducible when performed in high-phosphate buffer (Supplementary Fig. 2 online), which demonstrates that the inhibition by KCN is not due to an increase in the solution pH.

N₂O₃ formation by the reaction of NO with HbFe(III)-NO₂[–]

As shown above (Fig. 4), NO reacts rapidly with the HbFe(III)-NO₂[–] complex. To determine whether this reaction generates a freely diffusible nitrosating agent, which would be consistent with the formation of N₂O₃ predicted from equation (3), we assembled a novel experimental system (Fig. 6a). Nitrite and MetHb were placed in a purge vessel and continuously bubbled through with helium gas. Helium was then bubbled through a second chamber (trap) containing GSH and diethylenetriamine pentaacetic acid (DTPA, 3) (GSH serving as an N₂O₃ reactant trap), and the accumulation of S-nitrosothiols in this second chamber was measured by reductive chemiluminescence. Raw chemiluminescence data indicated that the addition of NO to a solution of MetHb and nitrite results in the accumulation of higher levels of S-nitrosoglutathione (GSNO, 4) in the trap than with either NO or nitrite alone (Fig. 6b). When 375 nmol of MetHb (75 μM in solution) was reacted with 50 nmol of NO (10 μM in solution) after brief pre-equilibration with 5 μmol of nitrite (1 mM in solution), 3.82 ± 2.92 nmol of GSNO was detected in the trap solution (Fig. 6c). GSNO formation was significantly lower when nitrite was excluded from the reaction, such that only 0.2 ± 0.08 nmol of GSNO formed in the absence of nitrite (*P* < 0.02). Note that this experiment reveals not only gas-phase generation of a nitrosating agent but also the anaerobic formation of a nitrosating agent (as all experiments are conducted with helium).

We also examined the extent of hemoglobin iron nitrosylation and S-nitrosation (Fig. 6d) within the reaction solution itself. There was no significant difference in SNO-Hb formation whether MetHb was reacted with NO alone (1.44 ± 0.16 nmol) or with both nitrite and NO (1.16 ± 0.3 nmol). Notably, the amount of HbFe(II)-NO in the reaction solution was significantly (*P* < 0.03) lower when MetHb was reacted with both nitrite and NO (3.0 ± 0.74 nmol) relative to when MetHb was reacted with NO alone (4.65 ± 1.12 nmol) (Fig. 6e). This suggests that although the NO, in the absence of nitrite, can reduce

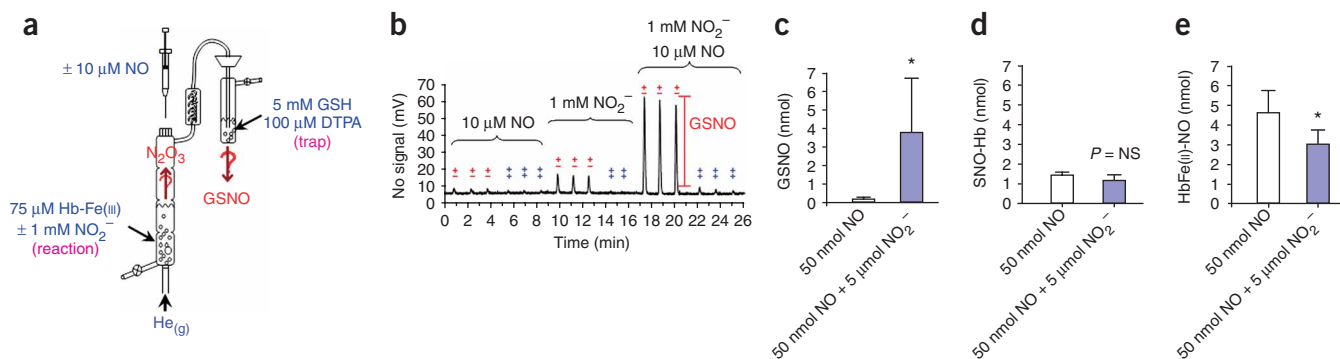


Figure 6 NO-catalyzed reduction of HbFe(III)-NO₂[−] generates gas-phase N₂O₃. **(a)** Experimental setup used to detect the formation and release of N₂O₃ into the gas phase. MetHb was pre-equilibrated with nitrite before the addition of NO. The first (“reaction”) vessel was purged with helium and maintained under positive pressure. **(b)** Detection of GSNO by reductive chemiluminescence. Before injection into tri-iodide (see Methods), aliquots of the trap vessel solution were pretreated with either acidified sulfanilamide alone (±) or with mercuric chloride followed by acidified sulfanilamide (‡). **(c)** Average concentrations of GSNO formed in the trap vessel of the reactions of 375 nmol MetHb and 50 nmol NO with 5 μmol nitrite, which produced 3.82 ± 2.92 nmol GSNO compared with 0.2 ± 0.08 nmol GSNO when nitrite was excluded. **(d)** Average SNO-Hb concentrations formed in the purge vessel of reactions in **c**. 1.16 ± 0.31 nmol SNO-Hb was detected in the presence of nitrite, compared with 1.44 ± 0.16 nmol SNO-Hb when nitrite was excluded from the reaction (no significant difference). **(e)** Average HbFe(II)-NO concentrations formed in the purge vessel of reactions in **d**. 4.65 ± 1.12 nmol HbFe(II)-NO was detected in the presence of nitrite, compared with 3.02 ± 0.74 nmol HbFe(II)-NO when nitrite was excluded. **P* < 0.05 for the paired analysis of mean daily experiments (*n* = 9 sets of experiments) by the Wilcoxon matched pairs test. Error bars represent one s.e.m.

MetHb via the uncatalyzed nitrite-independent reductive nitrosylation pathway, this mechanism generates less N₂O₃ (limited GSNO in the trap vessel, **Fig. 6c**), and HbFe(II)-NO remains in the reaction solution. In contrast, NO-mediated reduction of HbFe(III)-NO₂[−] simultaneously produces N₂O₃, which is then purged out of solution such that the concentration of HbFe(II)-NO in the reaction vessel is significantly lower (*P* < 0.03) (**Fig. 6e**) and the concentration of GSNO in the trap vessel is significantly higher (*P* < 0.02) (**Fig. 6c**).

Examination of other proposed mechanisms for nitrosation

One proposed mechanism for SNO-Hb formation is the intramolecular transfer of NO generated in the deoxyHb-nitrite reaction from the ferrous heme to the β-93 cysteine following the T-to-R conformational change upon hemoglobin oxygenation^{20,23,37}. However, such an allosterically controlled intramolecular transfer of NO from the heme to the cysteine during oxygenation is not observed by EPR and is not balanced electronically³⁸. Moreover, this mechanism could not apply to our observations here, as the most nitrosation is observed under deoxygenated conditions—without subsequent oxygenation.

A second proposed mechanism for SNO-Hb formation in the nitrite-deoxyhemoglobin reaction is the formation of a MetHb bound with NO intermediate (HbFe(III)-NO, or HbFe(II)-NO⁺) that could lead to nitrosation (equation (5))^{22,23}:



where R represents any thiol-containing group (for example, glutathione or cysteine). However, we found that HbFe(III)-NO₂[−] is the major spectral intermediate in the deoxyHb-nitrite reaction, rather than MetHb-NO (**Fig. 1**). Because it can be difficult to definitively identify minor species among many others using absorption spectroscopy, we developed an additional sensitive and specific chemiluminescence-based assay for HbFe(III)-NO. Given that NO dissociates from HbFe(III)-NO at a rate of 1 s^{−1} (ref. 34), we reasoned that HbFe(III)-NO should be detectable when injected into a purge vessel in-line with a gas-phase chemiluminescent NO analyzer, without the need for any additional chemistry. To prevent capture of NO released from HbFe(III)-NO by any ferrous deoxyhemoglobin present in

solution, we purged the nitric oxide analyzer with CO (mixed 1:1 with argon). The molar excess of CO would bind any ferrous hemes in milliseconds and thus would prevent NO autocapture. Using this sensitive chemiluminescence-based assay, we confirmed that HbFe(III)-NO is not a stable species because it undergoes reductive nitrosylation (with rapid kinetics consistent with the data presented in **Figure 4**) and rapidly transfers NO to any ferrous hemes when present (**Supplementary Fig. 3** online). In addition, we demonstrated that there is no detectable HbFe(III)-NO that forms during the reaction of nitrite with deoxyHb (**Supplementary Fig. 3**).

Another possible mechanism for SNO formation and reductive nitrosylation involves an oxygen transfer reaction (equation (6)):



Oxygen transfer from nitrite to a variety of substrates, including NO, has been demonstrated for Fe(III) porphyrins³⁰. In the context of HbFe(III)-NO₂[−], oxygen transfer would directly lead to iron-nitrosyl-Hb and the NO₂ radical. A second NO could react with the NO₂ radical to form N₂O₃ with subsequent nitrosation. Alternatively, the NO₂ radical could oxidize cysteinyl thiol to form a thiol radical, and a second NO could then react to form SNO-Hb.

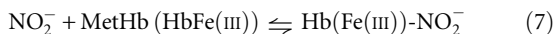
We tested whether the oxygen transfer mechanism has a role in our Hb experiments in two ways. First, we examined whether incubation of HbFe(III)-NO₂[−] leads to formation of iron-nitrosyl-Hb by following the reaction spectroscopically. We did this with several compounds shown to be efficient oxygen-trapping substrates³⁰, including CO and dimethyl sulfide. In these experiments, no iron-nitrosyl-Hb formation was observed. In addition, we found no evidence for formation of dimethyl sulfoxide by gas chromatography after incubation of MetHb-nitrite with dimethyl sulfide. Second, we studied iron-nitrosyl-Hb formation when ¹⁵N-labeled nitrite bound to MetHb was combined with ¹⁴N nitric oxide. Using EPR one can determine the source of the nitrogen after formation of HbFe(II)-NO in this reaction because the hyperfine structure of HbFe(II)-¹⁴NO produces a triplet, whereas HbFe(II)-¹⁵NO produces a doublet in EPR (**Supplementary Fig. 4** online). If the oxygen transfer mechanism (equation (6)) occurs, the iron nitrosyl would be entirely HbFe(II)-¹⁵NO, as it is derived from the

nitrite. If, on the other hand, the $\text{HbFe(III)}\text{-NO}_2^- + \text{NO}$ reaction to form N_2O_3 is the mechanism (equation (3)), the reaction would yield a mixture of $\text{HbFe(II)}\text{-}^{14}\text{NO}$ (formed when the bound NO comes from the ^{14}N nitric oxide that was added to the $\text{HbFe(III)}\text{-NO}_2^-$) and $\text{HbFe(II)}\text{-}^{15}\text{NO}$ (formed when the bound NO comes from homolysis of the N_2O_3). In this experiment the major product was $\text{HbFe(II)}\text{-}^{14}\text{NO}$, which suggests that oxygen transfer does not occur in the reaction of NO with $\text{HbFe(III)}\text{-NO}_2^-$ (Supplementary Fig. 4). Based on the data presented here, none of the alternative reaction mechanisms of SNO formation are as plausible as the $\text{HbFe(III)}\text{-NO}_2^- + \text{NO}$ reaction mechanism (equation (3) above).

DISCUSSION

Accumulating evidence suggests that the ubiquitous circulating anion nitrite (NO_2^-) is a vasodilator and intrinsic signaling molecule^{1–4}. Though the *in vitro* incubation of nitrite with deoxygenated red cells, deoxyhemoglobin and deoxymyoglobin¹³ results in NO-dependent signaling, the rapid inactivating reactions of NO with deoxy- and oxyhemoglobin create a paradox as to how this signaling is physiologically possible^{1,39–42}.

However, findings presented in this work identify a novel heme-nitrite chemistry that provides a possible solution to this paradox. Nitrite reacts with deoxyhemoglobin to generate MetHb and NO (equation (1)). Nitrite also binds to MetHb to form $\text{HbFe(III)}\text{-NO}_2^-$ with a dissociation constant of about 1 mM at neutral pH (ref. 26); we in fact found the K_d to be much lower at limiting nitrite concentrations and/or lower pH ($K_d = 7 \mu\text{M}$ at pH 6.5 based on extent of EPR silencing).



We have previously shown that $\text{HbFe(III)}\text{-NO}_2^-$ is formed in the nitrite-deoxyhemoglobin reaction; now we find that this species is the major intermediate in this reaction⁶. Both experimental precedence^{31,32,43} and the DFT calculations indicate that $\text{HbFe(III)}\text{-NO}_2^-$ may have either an N-bound or O-bound nitrite. Both forms have high electron affinities and should avidly react with NO via a radical-radical-like pathway that generates N_2O_3 .



Note that according to the final stoichiometry of this reaction pathway (equation (9)), hemoglobin is catalytic and functions as an allosterically regulated enzyme that converts two nitrite ions into a molecule of N_2O_3 :



The biggest challenge to our favored $\text{HbFe(III)}\text{-NO}_2^- + \text{NO}$ mechanism is that this reaction must compete with the reaction of NO and ferrous hemes in the red blood cell, which is rate-limited by the diffusion of NO through the protein into the heme pocket⁴². Although our data strongly support the $\text{HbFe(III)}\text{-NO}_2^- + \text{NO}$ mechanism, they do not completely rule out the possibility that, under physiological conditions in a red blood cell, other mechanisms are involved. Kinetic analyses suggest that some compartmentalization within the red cell would be necessary for the $\text{HbFe(III)}\text{-NO}_2^- + \text{NO}$ reaction to effectively compete with NO + ferrous heme reactions in the red cell.

The production of N_2O_3 by nitrite-heme reactions could facilitate export of NO bioactivity from the erythrocyte via multiple pathways. First, N_2O_3 is the primary nitrosating species capable of forming red cell S-nitrosothiols, which form as a side product of the nitrite-hemoglobin reaction and may be exportable. Second, N_2O_3 is a small and uncharged molecule, which promotes its concentration

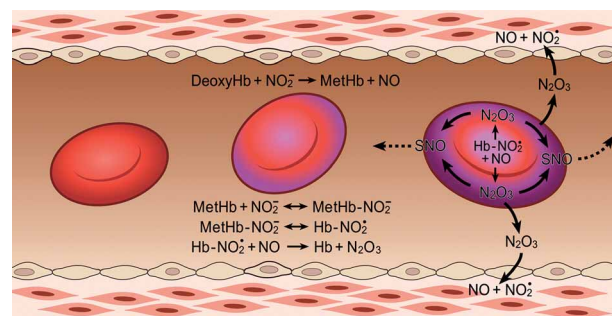


Figure 7 Model of nitrite- and Hb-mediated N_2O_3 export and nitrosation. Hemoglobin deoxygenation (purple) occurs preferentially at the submembrane of the red blood cell as it traverses the arteriole. Nitrite reacts with deoxyHb to make MetHb and NO. Much of this NO binds to hemes of deoxyHb or undergoes dioxygenation to form nitrate and MetHb from oxyHb. MetHb binds nitrite to form an adduct with some $\text{Fe(II)}\text{-NO}_2$ character (Hb-NO_2^-). This species reacts quickly with NO to form N_2O_3 , which can diffuse out of the red cell, later forming NO and effecting vasodilation and/or forming nitrosothiols (SNOs). Low-molecular-weight nitrosothiols may contribute to exportable vasodilatory activity. The figure is not drawn to scale. Not all reactions are shown (for example, hydrolysis of N_2O_3).

and diffusion through the red cell membrane. Finally, N_2O_3 can homolyze to NO and $\text{NO}_2^\bullet$, allowing for NO export¹².

These concerted pathways leading from nitrite-heme chemistry to export of NO or other related, potentially vasodilatory species out of the red cell are illustrated in Figure 7. Given the dimensions of the red blood cell (a biconcave disc), one expects some N_2O_3 with a lifetime of 1 ms and a diffusion coefficient of $1,000 \mu\text{m}^2 \text{s}^{-1}$ to diffuse out of the erythrocyte. In addition, it is quite possible that different isomers of N_2O_3 have different and perhaps longer lifetimes, thereby further facilitating potential export^{44,45}. As hemoglobin deoxygenates from artery to vein, the rate of nitrite reduction by deoxygenating hemoglobin increases, producing more NO, which can then react rapidly with MetHb-bound nitrite to form N_2O_3 . The primary nitrite reductase reaction that generates NO is allosterically regulated, and the rate is maximal as hemoglobin desaturates to 50% (at the hemoglobin P_{50}). This results in maximal rates of nitrite reduction to NO as oxygen and pH decrease⁷. According to the chemistry described here, the formation of N_2O_3 is also promoted as pH decreases. As summarized in our model, once N_2O_3 has formed it may be exported from the red blood cell and homolyze to form NO, which explains nitrite-mediated vasodilation^{2,10}. The likelihood of direct export of N_2O_3 is diminished by the presence of millimolar concentrations of thiol in the red cell. However, some degree of compartmentalization within the red cell would facilitate NO export. For example, deoxygenation near the membrane surface would enhance N_2O_3 formation there (Fig. 7). Further enhancement of NO release may take the form of a previously hypothesized nitrite reductase metabolon located within a lipid raft in the red cell membrane⁴⁶.

This nitrite-hemoglobin reaction also provides a novel and kinetically appealing mechanism for S-nitrosothiol formation. If N_2O_3 is formed in the nitrite-hemoglobin reaction, then in addition to NO and $\text{HbFe(II)}\text{-NO}$, S-nitrosated products should be detected. We previously reported that during nitrite infusions in humans, both iron-nitrosyl hemoglobin ($\text{HbFe(II)}\text{NO}$) and SNO-Hb form in blood². We propose that these reactions involving a nitrite-bound ferric heme form a general route for NO and S-nitrosative signaling under physiological hypoxia, with a number of heme globins subserving this function at different oxygen tensions. Hemoglobin would

function as a nitrite reductase at oxygen tensions of 60–20 mm Hg, near the hemoglobin P_{50} , whereas myoglobin, neuroglobin and cytoglobin would deoxygenate at oxygen partial pressures below 5–10 mm Hg (ref. 7). The formation of N_2O_3 is kinetically appealing as it may compete with NO-heme reactions that otherwise limit nitrosation chemistry. According to this paradigm, nitrite is the main stable NO reservoir in blood and tissues and forms from NO synthase during normoxia^{36,47}. Nitrite can then be reduced to NO and N_2O_3 along the physiological oxygen and pH gradient by the heme globins. In this context, the heme globins are allosterically regulated enzymes that are responsive to tissue metabolism (oxygen and proton levels) and that catalyze the conversion of two nitrite anions into a molecule of N_2O_3 .

These experiments therefore reveal fundamental new metal- and nitrite-catalyzed chemical reaction pathways that generate free NO, N_2O_3 and nitrosothiol. These reactions could constitute the basis of *in vivo* nitrite-dependent hypoxic signal transduction, and more globally, they reveal a possible mechanism for NO signaling in heme-rich environments, such as the erythrocyte and cardiomyocyte.

METHODS

Reagents. Details of routine sample preparation are provided in **Supplementary Methods** online.

Chemiluminescence assay used to measure S-nitroso species. Several assays were considered in performing this study: the tri-iodide (3I) assay, the Cu-cysteine-CO (3C) assay, the ascorbic assay/cupric chloride assay and the modified 2C assay. Detailed information comparing these assays and validating them is provided in **Supplementary Methods**. We found that all of these methods were workable, but given the fact that the 3C assay detects HbFe(II)-NO (**Supplementary Fig. 5** online and **Supplementary Methods**), we found the modified 2C assay to be the most useful for these studies.

To avoid detection of HbFe(II)-NO in the 2C assay, we pretreated our samples with potassium ferricyanide (as part of the SNO stabilization solution), thereby oxidizing the Hb to MetHb, which eliminates HbFe(II)-NO and makes the use of CO unnecessary as MetHb does not capture NO effectively. The modified 2C assay used here to measure SNO is a variation of the 2C assay previously described⁴⁸. The modification involves treatment with SNO stabilization solution (10 mM *N*-ethylmaleimide (NEM), 4 mM ferricyanide, 100 μ M DTPA, final concentrations⁴⁹) for 0.5 to 1 h. The ferricyanide oxidizes both HbFe(II) and HbFe(II)-NO to MetHb to prevent autocapture of NO released from SNO-Hb, and it eliminates the HbFe(II)-NO signal; the NEM blocks free thiols to prevent artifactual SNO-Hb formation. After incubation with SNO stabilization solution, the sample was run through two G-25 sephadex columns (GE Healthcare) to remove excess ferricyanide. Validation of the modified 2C assay included verification that the assay does not give a signal for HbFe(II)-NO or NEM-blocked Hb. Similar tests were performed for the 3C and 3I assays, and in all cases HbFe(II)-NO was made by adding NO-saturated buffer to excess Hb so that the Hb became partially nitrosylated (with no free NO). NEM treatment was performed on oxygenated samples and verified using the Ellman's reagent (5,5'-dithiobis(2-nitrobenzoate)) for free thiols. To ensure that no false-positive SNO-Hb signal was given from HbFe(II)-NO, we performed our modified 2C assay on HbFe(II)-NO prepared by adding NO buffer to excess deoxyHb. HbFe(II)-NO did not produce a significant signal whether the samples were pretreated with NEM before deoxygenation or not. We also observed that EPR signals from HbFe(II)-NO completely disappear after incubation with SNO stabilization solution. Standard samples gave the expected signals (**Supplementary Fig. 6** online).

Nitrosation of small-molecular-weight molecules incubated together with nitrite and Hb or RBCs (such as GSH) was assayed by filtering out the protein using a centricron filter (Millipore). The filtrate was then injected directly into the NOA using the 2C assay.

Absorption spectroscopy. Details for absorption spectroscopy are provided in **Supplementary Methods**. Basis spectra used for kinetic analysis are also

provided (**Supplementary Fig. 7** online). The improvement in a fit by the addition of a new species was considered significant (thereby justifying the inclusion of the additional species) when the residuals (chi square) were reduced by a factor of two or more.

EPR spectroscopy. Details for EPR measurements are given in **Supplementary Methods**. EPR spectroscopy was used to determine the dissociation constant of nitrite-bound MetHb at various concentrations of nitrite. The dissociation constant is given by $K_d = [\text{MetHb}][\text{nitrite}]/[\text{MetHb-nitrite}]$. We mixed known amounts of MetHb ($[\text{MetHb}]_i$, also confirmed by EPR) with known amounts of nitrite ($[\text{nitrite}]_i$). EPR spectra were used to determine $[\text{MetHb}]$ after nitrite addition (this concentration is the unliganded MetHb present in the expression for K_d). Nitrite-bound MetHb was assumed to be completely EPR silent, so $[\text{MetHb-nitrite}]$ was determined from the difference of $[\text{MetHb}]_i$ and $[\text{MetHb}]$.

Generation and detection of gas-phase N_2O_3 by reductive nitrosylation.

Details of this assay are given in **Supplementary Methods**. In the anaerobic purge vessel, 1 mM nitrite was added 4 min after MetHb (75 μ M) and allowed to equilibrate with the MetHb for another 4 min before injection of 5 μ M Proli NONOate. The reaction was stopped 20 min after injection of the last reagent. The trap vessel contained 10 ml of 5 mM reduced L-glutathione and 100 μ M DTPA in 0.01 M phosphate-buffered saline (PBS), pH 7.4. When the reaction was terminated, the trap solution was divided into 1 ml aliquots and immediately frozen at -80°C for subsequent analysis. One aliquot of the trap solution was analyzed by absorbance spectroscopy to ensure absence of trap solution contamination by MetHb from the purge vessel. The reaction solution from the purge vessel was collected and passed through a sephadex G-25 column (Amersham Biosciences) to remove excess nitrite, and the hemoglobin was quantified by absorbance spectroscopy, aliquoted into 1 ml fractions and frozen at -80°C for subsequent analysis. All aliquots were stored in dark (foil-wrapped) Eppendorf tubes until analyzed in triplicate for their nitrosothiol and iron-nitrosyl-heme content using the ozone-based chemiluminescence assay as previously described. Sample analysis was subsequently performed using reductive chemiluminescence⁴⁹. 1 ml aliquots of each sample were reacted either with 100 μ l PBS (2 min) followed by 100 μ l 5% acidified sulfanilamide in 1 N HCl (5 min) or with 100 μ l 50 mM mercuric chloride in PBS (2 min) followed by 100 μ l 5% acidified sulfanilamide in 1 N hydrochloric acid (5 min) before injection into a purge vessel containing 6 ml tri-iodide solution (2 g potassium iodide and 1.3 g iodine dissolved in 40 ml water and 140 ml acetic acid) in line with the NOA.

Statistical analysis. Data from all gas-phase N_2O_3 -generation experiments was analyzed using GraphPad Prism 4.0 (GraphPad Software Inc.) and is reported as mean $\pm$ s.e.m. Wilcoxon matched pairs test was used to compare average values of GSNO, SNO-Hb and iron-nitrosyl-Hb with and without nitrite addition for nine sets of experiments. Results were considered statistically significant at $P < 0.05$.

DFT calculations. DFT calculations were carried out with the OLYP (refs. 50,51) generalized gradient approximation (GGA), triple- ζ plus polarization Slater-type orbital basis sets, and a fine mesh for numerical integration of the matrix elements. The results were checked against a number of other functionals and found not to vary to any significant extent. The ADF 2006 (Scientific Computing and Modeling) program system was used for all calculations.

Note: Supplementary information and chemical compound information is available on the Nature Chemical Biology website.

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AUTHOR CONTRIBUTIONS

The bulk of the experiments were performed by S.B. and R.G., who also helped in the design of these experiments and in writing the paper. A.G. and J.C. performed DFT calculations and wrote the text associated with these. D.B.K.-S. and M.T.G. directed the research, designed the experiments and wrote most of the paper. Significant other intellectual contributions (and some writing) were made by R.P., S.B.K. and N.H. Other experiments were performed by J.H., A. Jeffers, A. Jiang, X.H., I.A., R.S., Z.H. and A.M.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at <http://www.nature.com/naturechemicalbiology/>.

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