Generating S-Nitrosothiols from Hemoglobin

MECHANISMS, CONFORMATIONAL DEPENDENCE, AND PHYSIOLOGICAL RELEVANCE*

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Background: The mechanism for production of N2O3 from MetHb, nitrite, and NO is controversial.

Results: An Hb intermediate attributed to heme-bound N2O3 is characterized.

Conclusion: Partially met-R state Hb can function as a generator of long lived forms of bioactive NO.

Significance: The results provide insight into how Hb reactivity with nitrite can be harnessed physiologically and therapeutically.

In vitro, ferrous deoxy-hemes in hemoglobin (Hb) react with nitrite to generate nitric oxide (NO) through a nitrite reductase reaction. In vivo studies indicate Hb with nitrite can be a source of NO bioactivity. The nitrite reductase reaction does not appear to account fully for this activity because free NO is short lived especially within the red blood cell. Thus, the exporting of NO bioactivity both out of the RBC and over a large distance requires an additional mechanism. A nitrite anhydrase (NA) reaction in which N2O3, a potent S-nitrosating agent, is produced through the reaction of NO with ferric heme-bound nitrite has been proposed (Basu, S., Grubina, R., Huang, J., Conradie, J., Huang, Z., Jeffers, A., Jiang, A., He, X., Azarov, I., Seibert, R., Mehta, A., Patel, R., King, S. B., Hogg, N., Ghosh, A., Gladwin, M. T., and Kim-Shapiro, D. B. (2007) Nat. Chem. Biol. 3, 785–794) as a possible mechanism. Legitimate concerns, including physiological relevance and the nature of the mechanism, have been raised concerning the NA reaction. This study addresses these concerns demonstrating NO and nitrite with ferric hemes under near physiological conditions yield an intermediate having the properties of the purported NA heme-bound N2O3 intermediate. The results indicate that ferric heme sites, traditionally viewed as a source of potential toxicity, can be functionally significant, especially for partially oxygenated/partially met-R state Hb that arises from the NO dioxygenation reaction. In the presence of low levels of nitrite and either NO or a suitable reductant such as L-cysteine, these ferric heme sites can function as a generator for the formation of S-nitrosothiols such as S-nitrosoglutathione and, as such, should be considered as a source of RBC-derived and exportable bioactive NO.

The significant and vital role of nitric oxide (NO)3 in maintaining vasculature integrity is well established. NO functions as a modulator of vascular tone, inflammation, inflammatory responses, wound healing, neurotransmission, pain, and many other key biological functions (1–4). Enzymatic generation of NO through different nitric-oxide synthase (NOS) proteins appears to be the primary source of NO. There are also NO-related molecules that can function either as a potential source of non-NOS-derived NO (e.g. nitrite) or as much longer lived species such as S-nitrosothiols (5, 6) that can transport NO bioactivity over much longer distances/time periods. These NO-related species are often referred to as bioactive forms of NO because they either can be converted to NO or manifest much of the functional activity of NO. Understanding the functionality of these bioactive forms of NO and how they are generated are questions of current interest for the following: (i) exposing important physiological/biochemical pathways; (ii) understanding the role of NO in pathophysiology; and (iii) developing strategies that can harness the impressive therapeutic potential of NO.

Hemoglobin is emerging as the second major participant/source in the mechanism for generating vascular NO and bioactive NO. However, it functions as an NO scavenger primarily through the rapid reaction of NO with heme-bound oxygen to yield ferric heme and nitrate (the NO dioxygenation reaction) (7–9). This NO-depleting reaction is asserted to be a major factor in the mechanism of acellular Hb toxicity and is likely to exacerbate pro-inflammatory conditions where NO levels are already low because of underlying endothelial dysfunction (10–12). However, studies now suggest that both deoxy-Hb (five coordinate Fe2+) and MetHb (Fe3+) can be a generator of both NO and bioactive forms of NO such as S-nitrosothiols (10, 11, 13–27).

The NO-generating capability of Hb, an allosteric protein, is one proposed basis for the phenomenon of hypoxic vasodilation in arterioles (19, 28–34). The conformation- and modification (chemical and mutagenic)-sensitive NO-generating capability of acellular Hbs have also been invoked to explain why some acellular Hb-based oxygen carriers (HBOCs) are not only nonvasoconstrictive (11, 35–37) but also vasodilatory (11,
for RBC physiological function, it may still represent one of the factors involved in the gating mechanism regulating access of endothelial NOS-generated NO to the smooth muscle (38, 39).

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**Hemoglobin as a Source of Bioactive NO**—There are several reactions that can potentially account for how Hb participates, not only in RBC-associated NO bioactivity (10, 11), but also in the mechanism for vasodilatory activity on the part of certain HBOCs (11, 35–37, 40). The potential role of Hb as a source of bioactive NO started with the proposal that Hb could transport and deliver NO in an allosterically controlled manner through the formation of SNO-Hb (20, 41–43). SNO-Hb is an Hb derivative in which the reactive thiols associated with the two Cys-β93 undergo SNO. Both the formation of the Cys-β93 SNOs and the efficacy for the transfer or release of these SNO-associated nitric oxides were proposed to be sensitive to the quaternary structure of the Hb tetramer (6, 21, 33, 42, 44, 45). Although SNO-Hb has been observed in vivo, both its functional significance and the mechanism of its formation still remain uncertain. The ability of PEGylated Hbs without reactive thiols to function as vasodilators (11, 46, 47) indicates that SNO-Hb formation is not a requirement for this activity and implies a significant role for other Hb-derived mechanisms. Similarly, there are studies that show SNO-Hb is not essential for generating NO-associated bioactivity by RBCs (34).

Although formation of SNO-Hb is not an absolute requirement for RBC physiological function, it may still represent one of several S-nitrosothiol species capable of transporting and transferring NO bioactivity. The initial proposal that SNO-Hb could be formed directly through a direct NO transfer from a ferrous NO heme on the β chains of the Hb tetramer is not supported by recent measurements (10). The questions of i) how the NO can act as a source of S-nitrosation for either the Hb thiols or RBC thiols, such as glutathione and l-cysteine, are still very much an open and active investigative pursuit. Because SNO-containing molecules are likely to be the basis for the long lived transportable NO bioactivity, there is considerable interest in the intriguing and still unanswered questions, which are being addressed in this study, as to by what reactions can Hb create reactive species capable of S-nitrosating reactive thiols on either Hb or other thiol-containing molecules.

Nitrite appears to be either an essential or an important reagent in the production of bioactive NO through Hb both in the RBC (14, 16, 31, 33, 48, 49) and as an acellular component in the circulation (11, 50). Significant amounts of acellular Hb in the circulation occur in hemolytic diseases and with the infusion of HBOCs. The role of nitrite is dramatically seen in results (11) that show that reversal of l-Nω-nitroarginine methyl ester-induced vasoconstriction by vasodilatory HBOCs require the addition of low concentrations of nitrite, which have no impact when infused without the added HBOCs. l-NG-nitroarginine methyl ester shuts down endothelial production of NO (i.e. endothelial NOS), thereby dramatically reducing the closely correlated NO and nitrite levels in the circulation.

A role for nitrite in NO production from Hb began to emerge when it was shown (22, 29, 51, 52) that deoxy-Hb can catalyze the conversion of nitrite to NO though a nitrite reductase reaction. In this reaction, five-coordinate ferrous heme sites within Hb react with nitrite to produce NO and ferric heme. Furthermore, the rate of the reaction is allosterically controlled. Studies on partially oxygenated Hb (32, 49), chemically modified Hbs (31, 53, 54), sol-gel-encapsulated Hbs (55), and Hb dimers bound to haptoglobin (50) all indicate the R state deoxy-hemes manifest a faster initial rate for this reaction by a factor of approximately 10 over the corresponding T state deoxy-hemes. R and T states refer here, respectively, to the high and low oxygen affinity states associated with the quaternary structures of the fully oxygenated and fully deoxygenated derivatives of Hb. There is evidence that it is the redox potential (56, 57) that contributes to the R/T- and protein-specific sensitivity of the nitrite reductase reaction. The redox potential is dependent on the quaternary structure of Hb with the T state having the higher value (favoring the reduced state of the iron). This allosterically responsive behavior links this reaction with hypoxic vasodilation. As part of the mechanism, a half-oxygenated R state Hb would have the maximum NO-generating efficacy in the NR reaction (32, 49, 53) because it represents a compromise between accessible reactive deoxy-heme sites and the higher reactivity associated with the R state (i.e. too much deoxygenation results in T state formation and too much oxygenation results in too few sites available for the reaction with nitrite). The importance of this reaction is also apparent from HBOC studies. Those Hbs with the highest rates for NR typically show the lowest levels of induced vasoconstriction. Furthermore, those Hbs with the highest rates for the NR reaction are most able to reverse l-NG-nitroarginine methyl ester induced vasoconstriction in the presence of low levels of added nitrite (levels insufficient to elicit activity in the absence of Hb) (11).

Although the NR activity of Hb is a suggestive candidate for the biochemical/biophysical basis for Hb-mediated production of bioactive NO, there are limitations that indicate that it may be only part of a bigger picture (31, 49). As discussed previously, the production of free NO per se may not be adequate for long distance transfer of NO reactivity because it is readily scavenged by Hb and has a limited capacity to survive long distance diffusion (51, 58, 59). Thus, prolonged NO survival and escape from a RBC is very questionable for free NO.

The missing element in the picture may be the proposed nitrite anhydrase (NA) reaction (10, 59, 60) of met-heme with nitrite and NO to yield N₂O₃, a potent S-nitrosating agent. In the initial publications, the proposed reaction has nitrite binding to the ferric heme and then NO reacts with the bound nitrite to form an intermediate with N₂O₃ transiently bound to the heme. Experiments in which the met-nitrite derivative of Hb is trapped in a sugar-based glassy film show that upon exposure of the glassy film to NO gas, the very slow diffusion of the NO into the protein results in the formation of a species having a distinct visible spectrum that resembles that of the ferrous NO derivative, but it is significantly different with both of the two Q band peaks red-shifted slightly by a few nanometers (61). A similar spectrum has been generated from sol-gel-encapsulated met-nitrite Hb bathed in a buffer with a large excess of nitrite when small aliquots of NO-containing buffer are added (27). The formation of this spectroscopically distinct species is associated with an increase in the fluorescence spectrum of dianiminofluoro-
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rescein (DAF). Under the anaerobic conditions of those specific experiments, the most plausible explanation for the increase in DAF fluorescence is N$_2$O$_3$ production rather than NO or any other NO-derived species. Based on these results, it has been proposed that this spectroscopically distinct species is the NA intermediate in which N$_2$O$_3$ is bound to a ferrous heme (27, 61, 62). In these experiments, nonphysiological conditions, including high concentrations of nitrite, were used to generate this intermediate.

Aside from the nonphysiological conditions employed in the studies used to generate the purported intermediate, there are additionally concerns regarding the viability of the NA mechanism in vivo. The original mechanism has nitrite binding first to the heme followed by NO binding to heme-bound nitrite; however, NO has a much higher affinity for ferric heme than does nitrite making it unlikely that the ferric nitrite derivative would form in the presence of NO. Although the mechanism in which nitrite binds to heme followed by NO binding to the heme-bound nitrite to form N$_2$O$_3$ can occur, modeling studies indicate that the reverse pathway in which NO binds to the heme followed by nitrite binding to the heme-bound NO is favored (60).

Recent work shows that under low NO concentration conditions, reductive nitrosylation ferric heme to form ferrous NO-heme can be fast (13). Given the low affinity of nitrite for ferric heme, the question arises as to how the NA reaction can be competitive with reductive nitrosylation under physiological conditions. However, in that same study it was also shown that the ability of ferric NOHb to S-nitrosate GSH (presumably through the Hb $^{\text{NO}}$ $\leftrightarrow$ HbNO $^{\text{S}}$ character of ferric NOHb) is substantially increased in the presence of even small amounts of nitrite, which is suggestive of the NA or a related operative mechanism.

This study is designed to address the concerns over the viability of the NA reaction in vivo. The following progression is followed in pursuing this objective. The intermediate is further characterized spectroscopically and most significantly is shown to form with much lower concentrations of nitrite and NO (from either NO-saturated solutions or added NONOates). The issue of the nitrite/NO-binding sequence is addressed through several different protocols. Sol-gel encapsulation is used to evaluate how conformation impacts the partitioning between reductive nitrosylation and formation of the intermediate. The functional properties of the intermediate are explored using DAF fluorescence under anaerobic conditions to assess N$_2$O$_3$ formation and both mass spectrometry and HPLC to evaluate the capacity of the intermediate to S-nitrosate glutathione. The results support the assertions that the NA reaction is indeed a plausible mechanism for Hb-mediated production of S-nitrosothiols and that the spectroscopically distinct intermediate is a heme-bound N$_2$O$_3$ derivative of Hb.

**EXPERIMENTAL PROCEDURES**

**Solution Preparation**—Samples were prepared in 0.05 M Bis-Tris, pH 7.0 or 7.4, or in PBS at pH 7.4. Human HbE was purified from red blood cells (RBC) obtained from transgenic mice expressing human HbE as described previously (57, 64, 65). HbE was shown previously to have similar allosteric and oxygen-binding properties as HbA, but relative to HbA, it appears to have a higher redox potential as manifested in a lower rate for nitrite reductase activity in both the T and R state and a faster rate of reduction by L-cysteine (57). HbE is used in this study to assess the potential role of the redox potential in modulating the partitioning between reductive nitrosylation and nitrite anhydrase activity. Aquo-met solution samples were prepared by oxidizing O$_2$Hb using K$_3$Fe(CN)$_6$ added in a 2:1 ratio to heme, which was subsequently removed using a spin column prepared from G10-Sephadex.

**Sol-gel Preparation**—Hemoglobin was encapsulated in a thin film of tetramethylorthosilicate-derived sol-gels according to published procedures (27, 55). Basically, tetramethylorthosilicate is hydrolyzed using 2 mM HCl and then mixed in a 1:1 volume ratio with protein (0.8–0.9 mM heme) solubilized in 0.05 M BisTris OAc buffer, pH 7.0. The resulting solution is spun in a 1-cm wide NMR tube until gelation occurs, creating a thin film of an Hb-containing gel on the bottom fourth of the tube. A final concentration of 0.40–0.45 mM in heme for the encapsulated Hb is required to produce a high optical quality thin film. After the sol-gel is allowed to age at 4°C for 2–6 days in the dark to ensure that the hydrogel polymerization is complete, the bathing buffer of the sol-gel is then changed as needed to suit the specific measurement. Typically, the samples are bathed in 2 ml of buffer. Samples are prepared anaerobically when required.

Aquo-MetHb T state sol-gel samples were prepared by encapsulating the deoxy-Hb derivative, in the presence of the potent allosteric effector L35 (2-[4-(3,5-dichlorophenylureido) phenoxyl]-2-methylpropionic acid) (66) at 1:1.1, heme/L35. This allosteric effector remains potent even at high pH values where organic phosphate-based effectors (e.g. inositol phos- phate and diphasphoglycerate) lose efficacy. L35, a gift from Drs. Iraj and Parvis Lalezari, was added to help stabilize the T state population and thus allow for a clear distinction between the T and R state reactions at pH 7.0. After the above noted aging step, T state gels were oxidized to the ferric (met) redox state using K$_3$Fe(CN)$_6$ in a 2:1 ratio. The ferricyanide was flushed out of the sol-gel using several washings of buffer, once conversion to aquo-MetHb was complete. The bathing buffer was then replaced with a fresh buffer that matched the needs of the specific measurement.

R state aquo-met samples were prepared by first encapsulating the CO or O$_2$-ligated Hb derivative. When starting with the CO derivative, the aged sol-gel sample is photolysed in the presence of oxygen to facilitate the replacement of the CO with the dioxygen ligand. Once the sample has been fully converted to the oxyHb derivative, it is then purged with argon to remove free oxygen and facilitate the loss of dissociated oxygen. The sample is then oxidized using the same technique described for the deoxy samples.

**Reductive Nitrosylation** (67–70)—All buffer and protein solutions were prepared anaerobically by purging with argon gas. Sol-gel samples were prepared in an oxygen-free glove box. The addition of NO to MetHb results in an initial formation of NO MetHb followed by a pH- and NO concentration-dependent reduction to form ferrous NOHb. In this study, NO is added to MetHb under several different pH conditions, and the
progression to ferrous NOHb is then monitored using absorption spectroscopy. In these studies, NO is added either in aliquots of NO-saturated buffer (2 mM saturated solution) or aliquots with a known concentration of diethylamine NONOates (Sigma) (amine compounds known to release NO in a time- and pH-dependent manner). To solutions of MetHb (0.30 mM heme) in 0.05 M BisTris, pH 7.0, varying aliquots of NO-saturated buffer or NONOate relative to heme were added, and the samples were allowed to evolve as a function of time. Samples of aquo-MetHb encapsulated in the sol-gel (0.45 mM heme) were treated likewise, adding the NONOate to the bathing buffer (0.05 M BisTris, pH 7.0) of the sol-gel. Spectra were scanned at regular time intervals and deconvoluted as described below, and the evolution of populations generated was plotted as a function of time.

**NA Reaction (59)**—All buffer and protein solutions were prepared anaerobically by purging with argon gas. Sol-gel samples were prepared in an oxygen-free glove box. The NA reaction was followed by monitoring the buildup and decay of a spectrum attributed to an intermediate described in earlier work (61) that is attributed to the formation of a species in which N2O3 was bound to the heme as a result of a reaction involving ferric heme, NO, and nitrite. Aliquots of NONOate-saturated buffer, or NONOate, were added to Met-Hb in solution (0.30 mM heme) or in the sol-gel (0.45 mM heme) films, in the presence of varying levels of nitrite, followed by a monitoring of the evolution of hemoglobin populations as a function of time. Populations were followed using absorption spectra and spectral deconvolution techniques as described below. To create a mixed population of aquo-MetHb and nitrite-bound to ferric Hb, saturating concentrations of nitrite (30 mM) were added to the sol-gel, and the binding was followed by absorption. Once the nitrite was fully bound, the bathing buffer of the sol-gel was removed, so a mixed population of nitrite-bound and -unbound was achieved. To this mixed population, NO was added as an aliquot of the saturated solution, and the samples were allowed to evolve. Spectra were scanned at regular time intervals and deconvoluted as described below.

**Characterization of Hb Samples**—The UV-visible absorption spectrum of the solution phase and sol-gel phase Hb samples was used to evaluate both the redox state and ligation status for all prepared hemoglobin derivatives. Samples were monitored either on a Lambda 2 (PerkinElmer Life Sciences) or an Evolution 300 (Thermo Scientific, Piscataway, NJ). The latter has a larger dynamic range allowing for monitoring samples with higher optical density, thus also facilitating the clear observation of the intense Soret absorption band in the 400–440-nm regime.

**Quaternary Status**—The quaternary state status of the sol-gel-encapsulated samples was established by converting the resulting MetHb samples back to the CO derivative (addition of a small aliquot of dithionite and flushing with CO) and then generating the full time course from several nanoseconds out to 1.0 min of the CO recombination trace subsequent to photodissociation using a nanosecond pulse at 532 nm to photodissociate the CO and a weak continuous wave blue output at 441.6 nm from a HeCd laser to monitor the recombination process as described in detail in earlier publications (55, 71–75).

The kinetic traces were compared with previous traces generated from well characterized sol-gel-encapsulated COHb samples stabilized in either the T or R quaternary state (55, 71, 72, 74–76).

**Spectral Deconvolution and Population Analysis**—The absorption spectra of evolving samples (gel or solution) was repetitively scanned at regular time intervals (30–90 s, longer intervals for the gel samples) over extended periods of time. Each spectrum was deconvoluted using a basis set consisting of Fe(II)NO, Fe(III)NO2, aquo-, and hydroxyl-met, Fe(III)NO, which were prepared individually, as described previously (55, 57). Included in the basis set was a unique spectrum of an intermediate, derived as described previously (27, 50, 57). The mathematical expression used for the fitting was a program within Mathcad (version 14.0, PTC Needham, MA). The spectral analysis basically consists of a summation of data points that yield a theoretical curve calculated from the input basis set spectra. The theoretical fit is compared with the unknown data curve. The residuals (the difference between the experimental spectral data and the theoretical fit) were calculated and were 10−3 or less for each data point. The fit required the use of the unique intermediate spectrum to reproduce the sample data and to minimize the residuals, as described previously (27, 50, 57).

**HPLC Identification of S-Nitrosothiols**—All buffer and protein solutions were purged with argon gas. Sol-gels were prepared anaerobically (in an oxygen-free glove box). To determine whether the intermediate would facilitate transfer of the NO to a thiol receptor, samples of sol-gel-encapsulated aquo-met hemoglobin in 0.05 M BisTris, pH 7.0 (0.45 mM heme), were allowed to react with nitrite (30 mM) and NO in the form of NONOate. At the appearance of the intermediate, the buffer was removed, and the sample was carefully rinsed. A solution of GSH (5 mM) and l-Cys (0.5 mM) was added, and the sample was allowed to evolve. Aliquots of the bathing buffer were removed anaerobically, at various time points and run on a Zorbax C-18, 5-μm reverse phase HPLC column (DuPont), eluted with 10 mM phosphate, 10 mM tetrabutylammonium bisulfate, 5% acetonitrile. Elution times were compared with those of authentic standards (GSNO, GSSG, and l-Cys, obtained from Sigma) prepared and run using the same elution buffer. As a control, a sol-gel of aquo-met hemoglobin was reduced with dithionite, carefully rinsed to remove all dithionite, and reacted with NONOate in 0.05 M BisTris, pH 7.0, to form Fe(II)NO. After carefully removing all of the NONOate, the thiols were added and allowed to react for the same times. Aliquots of the bathing buffer were removed at intervals similar to the previous sample.

**DAF-FM and DAF-2**—All buffer and protein solutions were prepared anaerobically by purging with argon gas. The fluorescein derivatives DAF-FM and DAF-2 were widely used to detect the presence of nitric oxide (for oxygen containing samples or N2O3 in the absence of oxygen) (27, 61, 77–82). A recent study (77) showed that in the presence of dioxygen, NO2 rather than N2O3 was likely to be the primary agent altering the DAF resulting in the enhanced fluorescence. In this study, the use of anaerobic conditions essentially eliminates that mechanism. As a consequence of this anaerobically designed method, the changes in fluorescence can confidently be attributed to N2O3 formation. Both fluorophores have a low quantum yield, but in
the presence of NO or N$_2$O$_3$, a product with a much higher quantum yield was produced. The 480- or 497-nm excited emission spectrum of either DAF-FM or DAF-2 (diaminofluorescein derivatives, Sigma) also shifts to higher wavelengths (515–530 nm) in the presence of NO or N$_2$O$_3$. Emission spectra were acquired using a Quanta Master Model QM 4/2000SE enhanced performance spectrofluorometer (PTI International, Lawrenceville, NJ). The following describes the two general protocols used with DAF. A stock solution of the fluorophore was prepared in DMSO. To detect the presence of N$_2$O$_3$, 2 eq solutions of aquo-MetHbA were prepared in 0.05 M BisTris, pH 7.0 (0.30 mM in heme). To one sample, 1 mM NO$_2$ was added, followed by 1:1 NONOate. To the second sample, only 1:1 NONOate was added without any nitrite. Both samples were allowed to evolve. When the intermediate spectrum was observed in the sample with nitrite, then 2.5 $\mu$L DAF-FM was added to both samples, and the samples were scanned. About 1 h later, the samples were scanned again. Results were then compared. In a second experiment, using a sol-gel, 0.1 mM NO$_2$ was added to a sample of MetHbA in PBS, pH 7.4, followed by 5 mM L-Cys. To initiate the formation of the intermediate, the L-Cys reduced the MetHb to deoxy-Hb, which reacts with the nitrite to form NO. After the absorption spectrum showed clear evidence for the formation of the intermediate, the nitrite plus thiol-containing buffer was removed; the sample was flushed with fresh buffer (free of nitrite and added thiols), and then DAF-2 was added (10 $\mu$L).

**Identification of Reaction Products Using Mass Spectrometry, Preparation of MS Standards—**Samples of L-Cys, DTPA, and GSH were solubilized in HPLC-purified water to use as standards for MS. GSNO was prepared by bubbling purified NO gas (through a concentrated NaOH solution) into an aerated water solution sample of GSH and verified by its characteristic UV spectrum (absorption maximum at 334 nm) (83, 84). Standard solutions were desalted and extracted as described below for the sol-gel samples of HbA.

**Reaction Products—**Sol-gel samples of aquo-MetHbA and a sol-gel without Hb in phosphate buffer, pH 7.4, were incubated with nitrite, thiols, and DTPA (a copper chelator to prevent hydrolysis of SNO). An aliquot of buffer was removed anaerobically at the onset of the reaction and again at the appearance of the intermediate spectrum (observed spectroscopically). The aliquots were desalted using a SepPak filter (Waters), eluted from the cartridge using 100% acetonitrile, and after adding 0.1% formic acid, the aliquots were immediately analyzed by ESI-MS, LTQ ion-trap mass spectrometer (ThermoFinnigan). Spectra were compared with standard solutions of L-Cys, GSNO, DTPA, and GSH prepared in HPLC water and analyzed in the same manner. Samples were run in the positive-ion mode with capillary voltage and temperature set at 4 kV and 225 °C, respectively, in the 50–800 mass-to-charge (m/z) range. For ESI-MS/MS (MS2) and ESI-MS/MS/MS (MS3) analysis, a normalized collision energy of 20% was applied, and the sheath (N$_2$) and collision helium gas pressures were 1.5 millitorr and 4 p.s.i., respectively. Samples were analyzed by infusion using a syringe pump operating at 5 $\mu$L/min flow rate.

**RESULTS**

**Spectrum of the Intermediate**

Identification and characterization of the proposed N$_2$O$_3$-heme Hb intermediate would greatly facilitate evaluation and testing of the nitrite anhydrase function of Hb and its potential relevance to physiological processes. To this end, the presented results build upon and extend the earlier high nitrite concentration studies in trehalose glass and sol-gel films that provide evidence of a spectroscopically distinct species that is ascribed to this proposed intermediate. This work extends these studies to include solution phase as well as sol-gel-encapsulated samples under conditions that employ both high and much lower nitrite concentrations. The spectral analysis of the intermediate is also extended to include the peak in the Soret region of the absorption spectrum, along with the distinct Q band.

Fig. 1a shows the evolution of the full absorption spectrum, including both the Soret and Q band regions, for a solution of MetHb (0.3 mM in heme) at pH 7.0 to which is added nitrite (1:1 nitrite/heme) and NO (released from NONOates added in slight excess to the heme concentration). It can be seen that over a 73-min time period, the Soret band shifts from 406 to 418 nm. This spectrum has also been generated at lower pH values and remains similar to what is shown in Fig. 1. This Soret peak is distinct from the standard equilibrium T state ferrous NOHb spectrum (generated when NOHb is in low pH buffer with allosteric effectors) by the manifestation of a significantly blue-shifted shoulder reflective of the five coordinate ferrous NO heme in the $\alpha$ subunits of the $T$ state tetramer. Fig. 1b shows an expansion of the Q band region over the same time interval. The Q band evolution shows a progression from a predominantly aquo-met population to a population that has Q band peaks at 539 and 569 nm. These are essentially the same Q band peak positions reported in the earlier work on glass-embedded and sol-gel-encapsulated samples (27, 61). In those experiments the starting Hb population was predominantly the met-nitrite Hb derivative due to the high concentration (ranging between 20 mM and 0.1 M) of initially added nitrite.

Fig. 1, c and d, emphasize the spectroscopic distinction between the ferric NOHb derivative and the intermediate through a comparison of the evolution of a thin film of a sol-gel-encapsulated MetHb sample bathed in an excess of added pH 7.4 buffer containing the following: (i) nitrite (20 mM) and NO (0.25 mM) (Fig. 1c); and (ii) the same NO-containing buffer but without nitrite (Fig. 1d). Fig. 1c shows the formation of the “intermediate” spectrum for the nitrite plus NO sample; and Fig. 1d shows that, in the presence of a comparable amount of NO without nitrite, the evolved spectrum is characteristic of the met-NOHb derivative. The spectrum attributed to the intermediate is stable in the absence of further addition of NO or NONOates. As reported previously for samples of MetHb in the presence of excess nitrite (27), it was again observed that continued addition of NO (aliquots of NO-saturated buffer or progressive release of NO from added NONOates), either to solution phase or to sol-gel phase nitrite MetHb samples, resulted in a progressive buildup of the intermediate. Once there was a substantial buildup of the intermediate population, the additional added or released NO initiated a precipitous con-
version of the intermediate spectrum to that of the end point ferrous NOHb population, which is considered a dead end species due to the very low off rate of NO from ferrous NO heme derivatives. Table 1 contains the Soret and Q band peak positions of both the intermediate and the stable Hb derivatives, including those ferric and ferrous species that might contribute to the evolving spectra described below. It can be seen that the Q bands of the intermediate are clearly distinct from other Hb derivatives, including those that are potential participants in the NO- and nitrite-based reactions addressed in this study.

The peak position of the Soret band and the shape of the Q bands are consistent with the intermediate being a low spin ferric or ferrous heme derivative. This distinct and consistent spectrum attributed to the intermediate is used as part of the basis set to fit the mix of spectra occurring during nitrite- and NO-initiated reactions of MetHb discussed in the subsequent sections. The individual spectra of these species were used as a basis set to generate the time-dependent evolution of the populations of Hb derivatives under systematically varied conditions. This approach produces plots showing the evolution of different populations of Hb species under varying solution conditions. The presented data represent the best fit using linear combinations of basis set spectra from several Hb derivatives. The residuals for these fits are less than 10^{-3}. Not including the intermediate spectrum invariably yields a poorer fit for samples having both NO and nitrite added. It is important to note that the spectrum associated with the intermediate can be generated under conditions where it is both stable and dominant (~80% of the total Hb population in solution and sol-gel phase samples and almost 100% in glassy matrices).

**TABLE 1**

| Ligation state | Soret | Q band | Other |
|----------------|-------|--------|-------|
| HbFeIIINO      | 418   | 545, 575 |       |
| (R state)      |       |        |       |
| HbFeIIINO      | 417, 395 (shoulder) | 544, 575 |       |
| (T state)      |       |        |       |
| HbFeIIICN      | 420   | 540    |       |
| Deoxy-HbA      | 430   | 555    | 757–758 (band III) |
| (T state)      |       |        |       |
| Deoxy-HbA      | 430   | 558, 559 | 759–765 (band III) |
| (R state)      |       |        |       |
| Intermediate   | 417   | 539, 569 |       |
| HbFeIIINO_{2}  | 411, 412 | 537, 568 | 630   |
| HbFeII H_{2}O  | 406   | 500, 538, 576 | 630   |
| HbFeII OH^a    | 409   | 500, 540, 576 | 630^a |

^a The samples were measured in 0.05 M BisTris, pH 7.0; for HbFeII OH, 0.05 M Bis-Tris, pH 9.0, band at 630 nm indicates residual aquomet.
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**Role of Nitrite in the Low Concentration Limit**

Fig. 2 shows the effect of added nitrite (1 mM) on the evolution of solution phase MetHb populations at pH 7.4 in the presence of a slight excess of NO (from NONOates). In the absence of added nitrite (Fig. 2a), the ferric NOHb is initially formed and then undergoes reductive nitrosylation yielding ferrous NOHb. In the presence of the excess nitrite (Fig. 2b), the purported intermediate is rapidly formed and continues to slowly build until ~80% when it declines as ferrous NOHb concomitantly builds up. A very similar decrease in the fractional contribution of the intermediate with a corresponding buildup of the ferrous NOHb population is seen (27) when NO (added as aliquots of NO-saturated buffer) is titrated into a solution of ferric MetHb in the presence of a very large excess of nitrite as noted above. Under those conditions (0.1 mM nitrite levels), where there is no detectable ferric NOHb population, the population of intermediate initially increases with each added aliquot of NO-saturated buffer and remains stable over time. Once the population of intermediate reaches the 70–80% level, added NO results in a decrease in the population of intermediate and a correlated appearance of ferrous NOHb. In Fig. 2b, the time-dependent changes in the buildup and subsequent decrease of the intermediate is attributed to the continued release of NO from the NONOates. The results obtained with the NO-saturated buffer do not show the dramatic time-dependent changes as anticipated due to the rapid availability of the “free” reactive NO (vis à vis slow release of NO from the NONOates). Under conditions of much lower micromolar levels of NO, it was observed that in the absence of added nitrite the ferric NOHb population can revert back to the aquo-MetHb species without substantial reductive nitrosylation. However, even under these low NO conditions, the presence of even small amounts (tens of micromolar) of nitrite will result in the formation of the intermediate.

**Issue of Mechanistic Sequence, Nitrite Followed by NO**

In the initially proposed NA mechanism, NO reacts with the heme-bound nitrite to form N₂O₃. The lower binding affinity of nitrite for ferric heme relative to that of NO (85, 86) raised questions as to the validity of this temporal sequence. Subsequent simulation studies (60) indicated that N₂O₃ could be formed with either NO binding first, followed by nitrite reacting with ferric bound NO, or through the initially proposed sequence in which nitrite binds first (31, 59, 87). The formation of the above described intermediate can be used to compare the efficacy of the two pathways. Figs. 3–5 depict the consequence of adding NO to samples of MetHb + nitrite (denoted by red arrows at the time point when added), either in a single burst (aliquot of NO-saturated buffer), in slow release mode from NONOates, or in repetitive additions of NONOates. Fig. 3a shows the results of a small aliquot of NONOate containing buffer added to a spectroscopically determined mixed population of aquo-MetHb and nitrite MetHb in a buffer-bathed thin sol-gel film. It can be seen that the nitrite MetHb population is eliminated concomitantly with the appearance of the purported intermediate. The impact on the aquo-MetHb population is relatively minor, consistent with the slow releasing NO preferentially interacting with the heme-bound nitrite population.

**Sequence 1, Solution Phase Met-Hb + Low Concentration of Nitrite + NO**—Fig. 3b shows the influence on the Hb populations of several additions of aliquots of NONOates to a solution phase MetHb sample (0.29 mM heme) containing a low concentration of added nitrite (75 μM). At this low concentration of added nitrite, there was essentially no detectable initial nitrite MetHb population. Under these conditions, the addition of NONOate results in the initial formation of ferric NOHb followed by formation of the intermediate. It can be seen that when the population of intermediate is high, additional aliquots of added NONOates (indicated by the red arrows) result in the formation of ferrous NOHb similar to the pattern described above for samples with much higher initial nitrite concentrations.

**Sequence 2, Sol-gel-Encapsulated Met Hb + Low Concentration of Nitrite + NO**—Fig. 3c shows the evolving Hb populations when NO-saturated buffer was added to a sol-gel-encapsulated sample that had nearly equal starting populations of aquo-MetHb and nitrite MetHb. In this case, the rapid addition of an aliquot of NO-saturated buffer results in an initial population of ferric NOHb that decreases as the intermediate builds up. In this sample, the addition of NO impacts both ferric populations but the nitrite MetHb population appears to decrease to a greater degree than the aquo-met population.

**Sequence 3, Solution Phase Met Hb + Low Concentration of Nitrite + l-Cys**—Fig. 4 shows how the intermediate can be generated from MetHb in the presence of nitrite through a mechanism where the generation of NO occurs not through addi...
both nitrite (2.0 mM) and 1-Cys (5 mM) as well as added 10 mM GSH (which when added alone is not a very effective reductant of MetHb compared with 1-Cys), there is again the appearance of the intermediate, but it is subsequently replaced with ferrous NOHb. The inset in Fig. 4b shows the Q band spectral changes indicative of a reduction over time of MetHb by 1-Cys (5 mM). It can be seen that the time scale for the reduction matches the time scale for the buildup of intermediate shown in Fig. 4b.

**Issue of Mechanistic Sequence, NO Followed by Nitrite**

Sequence 4, Sol-gel-encapsulated Met Hb + NO + Low Concentration of Nitrite—Fig. 5 shows two examples with an initial addition of NO to a sol-gel-encapsulated MetHb sample followed by the addition of nitrite subsequent to the buildup of a population of ferric NOHb. In one case, Fig. 5a, the initial addition of NO is in the form of an aliquot of NO-saturated buffer that converts ~60% of the initial met population to ferric NOHb. It can be seen that the addition of nitrite (0.5 mM) results in a complete loss of the ferric NOHb population, partial loss of the MetHb population, a buildup of the intermediate at a level that closely matches the drop in the ferric NOHb population, and a buildup of a nitrite MetHb population that approximately matches the drop in the MetHb population. A second addition of nitrite has minimal impact on the populations as would be anticipated if there were no free NO left in the buffer. The explanation that the absence of any additional spectral change with the further addition of nitrite, due to the depletion of available NO, is supported by the observation seen in the figure that the subsequent addition of an aliquot of NO-saturated buffer does indeed trigger another large increase in the population of the intermediate. The pattern clearly shows that both NO and nitrite are needed to produce the intermediate. Once the intermediate population exceeds ~70%, this buildup of intermediate is then followed by a decrease in the intermediate accompanied by the appearance and buildup of a ferrous NOHb population as observed for other samples. Fig. 5b depicts results from a similar experiment but with NONOates being added as the source of initial NO. The figure shows a progressive buildup of the ferric NOHb population subsequent to the addition of NO-releasing NONOates. The addition of nitrite results in the loss of the ferric NOHb population and a corresponding buildup of the population of the intermediate that remained stable for 2 days (in the cold at ~4 °C).

**Reductive Nitrosylation Versus Intermediate Formation, Conformation Dependence**—The above results are consistent with preferential binding to ferric heme of NO relative to nitrite under conditions where both are at low concentrations. Ferric NOHb can undergo reductive nitrosylation (13, 67, 69, 89) or, if there is nitrite present, formation of the intermediate that we attribute to heme-bound N2O3. Because both can be relatively rapid, the question arises as to whether there is a conformational dependence associated with the partitioning of two reactions. The following several figures illustrate the recurring pattern in which the T state favors reductive nitrosylation, whereas the R state favors the pathway to intermediate formation. Fig. 6, a and b, show, respectively, the reductive nitrosylation and intermediate formation occurring for a sol-gel-encapsulated T state MetHb sample (prepared from deoxy-HbA+ effectors).
Both samples are exposed to the same low level of NO (via NONOates), but the sample used for Fig. 6b also has added 0.25 mM nitrite. A similar comparison is made for sol-gel-encapsulated R state MetHb samples in Fig. 7, a and b, where the samples are exposed to the same NONOate and NONOate/nitrite conditions used in Fig. 6. It can be seen that for the R state samples the reductive nitrosylation is slower, and the stability of the intermediate is more persistent (no evidence of ferrous NOHb formation).

Fig. 8 compares the populations generated from T and R state sol-gel-encapsulated MetHb in the presence of a large excess of nitrite when the NO is slowly produced via the reduction of MetHb by L-cysteine/glutathione. Here, the R state Hb is a well characterized high oxygen affinity Cys-β93 cross-linked Hb. It was observed from the figure that the intermediate per-

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**FIGURE 4.** Effect of L-Cys on the evolution of populations of MetHb in the presence of added nitrite. a, solution phase aquo-MetHb (0.29 mM heme), in 0.05 M BisTris, pH 7.0, 0.28 mM NO2−, followed by addition of 0.15 mM L-Cys; b, sol-gel-encapsulated aquo-MetHb bathed in PBS, pH 7.4, followed by addition of 5 mM L-Cys and 10 mM GSH + 2.0 mM NO2−; inset is the time course for the reduction of 0.59 mM (heme) aquo-met solution subsequent to the addition of 5 mM L-Cys.

**FIGURE 5.** a, time evolution of the populations of an aquo-MetHbA sol-gel in PBS, pH 7.4, + NO solution (0.250 mM) (red arrow); the second and third additions were NO2− (0.5 mM) added at the black arrow, followed by two additions of NO (0.1 mM) each added at the red arrows. b, time evolution of the populations of an aquo-MetHbA sol-gel in 0.05M BisTris, pH 7.4, 2:1 NONOate/heme to form ~50% FeIIINO/FeIIIHb. At this point 5 mM NO2− was added.

**FIGURE 6.** Time evolution of the populations of Hb sol-gels. a, prepared as [deoxy-HbA + L35 + inositol hydrogen phosphate] in 0.05 M BisTris, pH 7.0, oxidized with K3Fe(CN)6, to which 1.5:1 NONOate/heme was added. b, prepared in the same manner, to which 1:1 NO2−/heme was added, followed by 1.5:1 NONOate/heme.
sists to a much greater degree for the R state derivative, whereas the T state produces far more ferrous NOHb product.

**Effect of Redox Potential**

Differences in the redox potential have been invoked to account for the R/T differences in the rates for the nitrite reductase reaction (10, 49, 56, 57, 90). To test the relevance of the redox potential in the current processes, we compared reductive nitrosylation and intermediate formation between HbA and HbE. HbE has been shown to have the higher redox potential (with respect to reduction of MetHb via L-Cys) and a lower rate for the nitrite reductase reaction (for both T and R states) (57). Figs. 9 and 10 compare the two hemoglobins with respect to reductive nitrosylation and intermediate formation, respectively. HbE undergoes a much more rapid reductive nitrosylation (Fig. 9) that is consistent with its higher redox potential. Fig. 10 depicts the more rapid and persistent population of intermediate formed for HbA. As with T state MetHbA samples, the intermediate formed with the MetHbE sample undergoes significant conversion to ferrous NOHb. The corresponding MetHbA samples exhibit a long lived stable population of intermediate.

**Evidence of N₂O₃ Formation from DAF Fluorescence**

The functional relevance of the intermediate hinges on whether it has the capability to nitrosate thiols as would be anticipated based on the assignment of the intermediate as a heme-N₂O₃-containing Hb species. In earlier studies focusing on the reaction under anaerobic conditions of NO with ferric
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nitrite Hb in glassy matrices and in solution/sol-gel, DAF fluorescence (77, 79–81) was used to support the claim that this reaction produces N$_2$O$_3$ (27, 61). This study extends the earlier work both with respect to DAF fluorescence and to direct evidence of GSNO formation being correlated with intermediate formation. In Fig. 11, the increase in the DAF fluorescence with time is shown subsequent to the addition of DAF-2 to a sol-gel-encapsulated MetHb sample under anaerobic conditions. In this case, the addition of L-Cys to nitrite (0.1 M)-containing bathing buffer was used to initiate formation of the intermediate. After the absorption spectrum showed clear evidence for the formation of intermediate based on the wavelength positions of the Q bands, the nitrite plus thiol-containing buffer was removed; the sample was flushed with fresh buffer free of nitrite and added thiols, and then DAF was added. It can be seen that upon addition of DAF-2 to the flushed sol-gel sample, there is a progressive increase in the DAF fluorescence. Similar results are obtained from solution phase samples using much lower concentrations of nitrite.

Fig. 12a shows the change in DAF fluorescence over a 1-h interval starting when DAF is added to a solution phase sample of MetHb to which is added NONOates (1:1 in heme) and 1 mM nitrite. The DAF is added anaerobically after the absorption spectrum has evolved to indicate that the intermediate has formed. It can be seen that the fluorescence increased over the 1-h interval subsequent to DAF addition. In Fig. 12b, the absence of any change in DAF fluorescence over the same interval is shown for an identically treated sample but without any added nitrite. The DAF is added after the same time interval as for the sample used to generate the results in Fig. 12a. Similar anaerobic experiments were conducted on samples with the same concentrations of added materials but with the DAF present from the start. In these cases no change in DAF fluorescence was observed for the following: (i) MetHb samples before and...
after addition of 1 mM nitrite; and (ii) buffer to which is added 1 mM nitrite or NONOates. The addition of 1:1 NONOates to a DAF-containing MetHb sample produced an initial very small increase in fluorescence but no further evolution with time. NONOates added to a comparable DAF-containing solution under aerobic conditions produced a large increase in DAF fluorescence.

**Mass Spectrometry-based Evidence of N₂O₃ Formation, Intermediate-associated GSNO Production**—The production of reactive $S$-nitrosating agents in the reaction of MetHb in the presence of nitrite and either thiols or NO (NONOates) is implied by the observations made in the presence of DAF in solution samples and in the sol-gel samples. The DAF results are consistent with the formation of the proposed $N₂O₃$-heme intermediate. The stability of the intermediate in the absence of excess sources of NO suggests that the likely mode for a reaction between the generated $N₂O₃$ and added thiols would be through a direct interaction between the added thiol-containing reagent and the heme-bound $N₂O₃$. MS was used to determine whether GSH, a small physiologically relevant thiol-containing molecule, could be $S$-nitrosated (GSNO) through the intermediate in an experiment that parallels the above described DAF sol-gel measurement in which $\mathit{L}$-Cys, GSH, and nitrite are added to the bathing buffer of a sol-gel-encapsulated MetHb sample. Aliquots of the reaction buffer bathing the thin film of sol-gel were removed and analyzed by MS at the onset of the experiment, at a time coincident with the appearance of the intermediate (via absorption spectroscopy), and at the end of the experiment. On the same time scale, control samples were also taken for MS evaluation both from a standard buffer solution and a similar buffer that is bathing a Hb-free sol-gel. These two protein-free controls were incubated in parallel with the Hb-containing sol-gel sample. The three samples contained all of the same non-Hb components (nitrite, $\mathit{L}$-Cys, GSH, and DTPA). The resulting MS-generated spectra were compared with standard spectra of GSH, GSNO, $\mathit{L}$-Cys, and DTPA that were prepared and analyzed in the same manner.

The results from the MS measurements are shown in Fig. 13. At the onset of the experiment, the characteristic spectrum of GSH is evident both in the bathing buffer from the sol-gel sample and in the buffer from the Hb-free controls. As the experiment progressed, a second aliquot of buffer from the sol-gel sample, taken when the absorption spectrum of the intermediate noticeably developed, showed the presence of GSNO and a reduced level of free GSH. The GSNO signal from the final aliquot of buffer taken from the sol-gel sample at the end of the experiment showed a reduction in the GSNO level compared with the earlier aliquot. The GSNO signature was not found in any of the buffer controls in the absence of the protein.

**HPLC Based Evidence of N₂O₃ Formation, Intermediate Associated GSNO Production**—A sol-gel-encapsulated MetHb sample was equilibrated with 30 mM nitrite-containing buffer. After a 20-min equilibration time (to allow diffusion of the nitrite through the sol-gel film), NONOates were added anaerobically to the buffer (1:1 heme). Once the absorption spectrum indicated that the intermediate had formed, the buffer was removed, and the sample was rinsed to remove excess nitrite. GSH (5 mM)-containing buffer was then added to the flushed sample. Four aliquots from the added buffer were removed for HPLC analysis after the GSH was added and the Hb spectrum recorded. The first aliquot was removed immediately post-GSH addition and the other three at ~20, 40, and 60 min post-GSH addition. The standards (GSH, GSNO, and GSSG) were run on the HPLC as well. The GSNO peak was not evident in the very first aliquot taken within 10 min of adding the GSH-containing buffer, but the next three aliquots showed an increase and then a decrease in GSNO with time as seen in Fig. 14, consistent with what was observed in the MS experiment. The observed decrease in GSNO following the initial buildup could be due to any number of possible reactions that result in loss of the NO from GSNO. The exact cause is the focus of future studies. A MetHb sol-gel sample, which was reduced to deoxy and then converted to ferrous NOHb (after addition of NONOates), was then flushed with fresh buffer containing GSH as above. Aliquots of this buffer were run on the HPLC as above but showed no evidence of GSNO formation.

**DISCUSSION**

The presented results make plausible the proposal that nitrite/NO/MetHb mechanisms can result in $S$-nitrosation of accessible reactive thiols under conditions that approach physiological. The results include identifying a key intermediate associated with these mechanisms and evaluating its properties, including pathways of formation. This intermediate was first introduced by Kim-Shapiro and co-workers (59) as a step in the NA reaction of Hb. Evidence is presented here that validates the significance of this intermediate as a long lived source of NO bioactivity. The importance of this intermediate is that it represents a mechanism whereby Hb either in the RBC or associated with plasma acellular Hb species obtains, retains, and transports the capacity for $S$-nitrosation over an extended time period. Evidence is accumulating that $S$-nitrosated versions of both small molecules, such as GSH and cysteine as well as larger thiol-containing molecules, such as albumin and Hb, are emerging as long lived sources of bioactive NO accessing distinct and often compartmentalized signaling pathways (1, 5, 6, 18, 91–97).

**Physical Properties of the Intermediate**—The presented results support the claim that NO and nitrite react with ferric heme sites in Hb to produce a species with an absorption spectrum distinct from that of the NO-bound derivatives of either ferric or ferrous Hb (27, 61) as well as other characterized ferric and ferrous Hb species (see Table 1). The consistent appearance of this intermediate-associated spectrum under the various conditions used in this study and earlier studies, including high and low concentrations of added nitrite, solution phase samples at different pH values in various buffers, sol-gel-encapsulated samples, and samples embedded in glassy matrices, together provide strong evidence that this species is a true reaction intermediate and not a sample specific artifact. The key requirements appear to be ferric heme sites, preferably in an R state Hb tetramer (or the stable $\alpha\beta$ dimer as occurs when complexed to haptoglobin), to which is added nitrite and NO. The source of NO used in these experiments derives from one of the following: NO containing buffer, NO released from added NONOates, or NO generated from the Hb nitrite reductase.
activity initiated through the reduction of ferric heme by L-Cys. It is anticipated that therapeutically promising NO-releasing nanoparticles (98–100) can also function as a sustained source of NO to drive these reactions.

Redox Status of the Intermediate—In an earlier study using cyanide binding as an assay for the presence of ferric heme, the results were suggestive of at least partial ferrous character (27). If the hemes in the intermediate existed as fully ferrous heme derivatives, one would anticipate that once formed these hemes would be highly vulnerable to having excess NO displace the bound ligand (allegedly N₂O₃) resulting in a ferrous NO heme derivative. This argument is based on the very high affinity (very high on rates and very low off rates) for NO binding to ferrous heme derivatives of Hb and Mb. If the hemes in the intermediate are indeed ferrous in character, then an absence of a facile displacement/replacement of the proposed N₂O₃ ligand with NO could be due to the following: (i) a very low off rate for the heme-bound N₂O₃ ligand; and/or (ii) reduced NO occupancy in the distal heme pocket when N₂O₃ is bound to the heme. The concept that the distal heme pocket occupancy of a ligand such...
as NO can be modulated by steric constraints has been demonstrated quite dramatically through the use of recombinant Hbs. Mutated residues in the distal heme pocket modulate the rate for NO dioxygenation reactivity by sterically altering NO occupancy when oxygen is bound to the heme (7–9).

An alternative explanation for why NO does not appear to displace the ligand associated with the intermediate is that the heme is a true ferric species. If the heme is a ferric species then the issue becomes one of whether added NO preferentially displaces the ferric ligand associated with the intermediate (N$_2$O$_3$) or binds to a free ferric heme to form either ferric NO heme or more intermediate if nitrite is also present. Given that the binding affinity of NO for ferric heme sites is a fraction of that for ferrous sites and that the presence of nitrite enhances the reactivity of the NO at ferric sites, it is not unreasonable that “vacant” ferric heme sites could be more reactive toward added NO than the sites having the N$_2$O$_3$ ligand. In this scenario, the ferric heme-coordinated N$_2$O$_3$ would have excess negative charge (Fe$^{3+}$–N$_{23}$), which could be accommodated by an imidazole proton from the distal histidine; however, if the heme is ferrous in character, there would be less need for stabilization of the now neutral N$_2$O$_3$ by the distal histidine. The possibility that excess negative charge could be localized on either the iron or on a distal oxygen of the coordinated N$_2$O$_3$ is reminiscent of how the ferric NO heme species is characterized. As a consequence we express this species as Fe$^{3+}$–N$_{23}$ ⇔ Fe$^{2+}$–N$_2$O$_3$ in analogy to the Fe$^{3+}$–NO ⇔ Fe$^{2+}$–NO$^+$ representation for the ferric NO heme derivative (10, 13, 22, 63, 101).

The redox potential of the heme in Hb is responsive to quaternary state and mutation. Additionally, there are indications that the positioning of the distal histidine is also responsive to allosteric control. The dependence of both redox potential and position of the distal histidine raises the possibility that the redox state of the intermediate is under allosteric control. An allosterically controlled balance between the redox state of the iron and the ability of the distal histidine to stabilize a negative charge could provide a mechanism that controls the reactivity/affinity of the intermediate. One possibility is that the R state favors the ferric-N$_{23}$ species, and the T state favors ferrous-N$_2$O$_3$. Studies are underway to further examine this possibility as well as to evaluate whether the R and T forms of the intermediate have lesser and greater propensity to react or undergo ligand replacement by ferrous ligands such as NO, CO, and O$_2$.

**Functional Properties of the Intermediate**—That the ligand purported to be the intermediate is capable of nitrosation is indicated by the DAF fluorescence results along with the MS and HPLC results; GSNO formation, concomitant with the formation of the intermediate, is consistent with but not definitive proof that the ligand associated with the intermediate is capable of nitrosation. The two obvious candidates are the nitrosonium ion (NO$^+$) and N$_2$O$_3$, which ultimately nitrosates through the formation of the nitrosonium ion upon dissociation. Evidence has been put forth that ferric NO heme exists as Fe$^{3+}$–NO ⇔ Fe$^{2+}$–NO$^+$, which would account for the response of the DAF fluorescence to the anaerobic addition of NO to MetHb. That the species formed when NO and nitrite are both present and is distinct from the ferric NO species is supported by the following: (i) the clearly different spectrum that emerges when nitrite and NO are added together to MetHb; (ii) the enhanced production of GSNO when both ligands are present (13); and (iii) the continuing buildup of DAF fluorescence when nitrite and NO are both added. Given these related findings, the proposed heme-bound N$_2$O$_3$ is the most reasonable ligand associated with the intermediate.

As discussed above, the intermediate may also have the redox ambiguity associated with the ferric NO heme species, and as a consequence, in analogy with the ferric NO species, it is represented as Fe$^{3+}$–N$_{23}$ ⇔ Fe$^{2+}$–N$_2$O$_3$. If the R state intermediate favors a ferric species (because of the reduced redox potential), then the bound N$_2$O$_3$ would have an excess negative charge that would likely be stabilized by the distal histidine. The higher redox potential of the T state would favor an intermediate that is more ferrous in nature, with stabilization through the distal histidine less of an issue.

**Mechanism of Formation**—The following is a summary of possible mechanistic sequences for the formation of the N$_2$O$_3$ intermediate based on the current results and earlier studies.

When NO is slowly titrated into a sample of MetHb in the presence of a large excess of nitrite (resulting in 100% of the starting population detectable as the ferric nitrite derivative of Hb, Reaction 1), the intermediate is the only species generated (Reaction 2).

\[
\text{Fe}^{3+} + \text{NO}^- (\text{excess}) \rightarrow \text{Fe}^{3+} - \text{NO}^- \quad \text{REACTION 1}
\]

\[
\text{Fe}^{3+} - \text{NO}^- + \text{NO} + \text{NO}_2^- (\text{excess}) \rightarrow \{\text{Fe}^{3+} - \text{N}_2\text{O}_3\} \leftrightarrow \text{Fe}^{2+} - \text{N}_2\text{O}_3 \quad \text{REACTION 2}
\]

In these and all subsequent numbered reactions, only the ferrous/ferric designation is written out instead of the usual Hb
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designation to emphasize that we are discussing heme sites within the Hb tetramer and not necessarily a fully ferrous or fully ferric tetramer. As discussed above, the intermediate is represented as the N₂O₃-containing species with both ferric and ferrous character. Once the population of the intermediate builds and reaches at least 70%, continued addition of NO results in the appearance of the ferrous NO derivative of Hb with the likely release of N₂O₃ (Reaction 3), consistent with the possibility that NO has a higher affinity for the heme-bound nitrite compared with the N₂O₃-bound heme (see above).

Below this ~70% switch over point, the intermediate appears stable for many hours. The same overall pattern of intermediate formation followed by ferrous NO formation is seen when much lower concentrations of nitrite are used. However, when a bolus of NO is added to Hb in the presence of a low concentration of nitrite, the ferric NO derivative appears and then decays as the population of the intermediate builds up (Reactions 4 and 5).

\[
\text{Fe}^{3+} + \text{NO}^{-} + \text{NO} \text{ (excess)} \rightarrow \text{Fe}^{3+} - \text{NO} + \text{NO}^{-} \]

\text{REACTION 4}

\[
\text{Fe}^{3+} + \text{NO} + \text{NO}^{-} \rightarrow \{\text{Fe}^{3+} - \text{N}_2\text{O}_3\} \leftrightarrow \text{Fe}^{2+} - \text{N}_2\text{O}_3 \]

\text{REACTION 5}

Slower sustained release of NO from added NONOates in the presence of low levels of nitrite appears to minimize the initial buildup of the ferric NO Hb population and maximize a more direct formation of intermediate (Reaction 6).

\[
\text{Fe}^{3+} + \text{NO}^{-} + \text{NO} \text{ (slow release from NONOates)} \rightarrow \{\text{Fe}^{3+} - \text{N}_2\text{O}_3\} \leftrightarrow \text{Fe}^{2+} - \text{N}_2\text{O}_3 \]

\text{REACTION 6}

Together, these two patterns suggest that local concentration of NO in and around the distal heme pocket (dhp) dictates whether NO reacts with nitrite within the dhp or whether excess NO displaces/replaces nitrite from the dhp resulting in the formation of the ferric NO derivative (Reactions 7 and 8). The brace marked with the dhp subscript refers to the distal heme pocket simultaneously containing the two designated potential ligands (NO and N₂⁻).

\[
\text{Fe}^{3+} \{\text{NO} + \text{NO}^{-}\}_{\text{dhp}} \rightarrow \{\text{Fe}^{3+} - \text{N}_2\text{O}_3\} \leftrightarrow \text{Fe}^{2+} - \text{N}_2\text{O}_3 \]

\text{REACTION 7}

\[
\text{Fe}^{3+} \{\text{NO} + \text{NO}^{-}\}_{\text{dhp}} + \text{NO} \rightarrow \text{Fe}^{3+} - \text{NO} + \text{NO} + \text{NO}^{-} \]

\text{REACTION 8}

With respect to the significance of the binding sequence, the present results show that the intermediate can be generated with either binding sequence. It is clear that nitrite has enhanced affinity for the ferric NO heme species relative to aquo-ferric heme (Reaction 9) and that added NO preferentially reacts with heme-bound nitrite relative to aquo-ferric heme (Reaction 10).

\[
\text{Fe}^{3+} - \text{NO} + \text{Fe}^{3+} + \text{NO}^{-} \rightarrow \{\text{Fe}^{3+} - \text{N}_2\text{O}_3\} \leftrightarrow \text{Fe}^{2+} - \text{N}_2\text{O}_3 \]

\text{REACTION 9}

\[
\text{Fe}^{3+} - \text{NO}^{-} + \text{Fe}^{3+} + \text{NO} \rightarrow \{\text{Fe}^{3+} - \text{N}_2\text{O}_3\} \leftrightarrow \text{Fe}^{2+} - \text{N}_2\text{O}_3 \]

\text{REACTION 10}

From these solution phase and sol-gel phase studies, it is not clear whether the added NO binds directly to the heme-bound nitrite or first displaces the nitrite and then binds to the heme followed by the nitrite reacting with the heme-bound NO.

The previously described trehalose results (61, 62) support the plausibility of a pathway for the formation of the intermediate as follows: (i) the nitrite can bind first to the heme followed by NO, either reacting directly with the heme-bound nitrite to form the intermediate, or (ii) NO displaces the nitrite, which can remain within the dhp, whereas the NO binds to the heme and then has the dhp-localized nitrite react with heme-bound NO to form the intermediate. The present results do not rule out either sequence; however, they do favor, as the dominant pathway, a mechanism where NO binds first to the ferric heme followed by nitrite binding. It is not clear as to whether the results supporting the nitrite binding first mechanism actually reflect a displacement of the nitrite by NO followed by a potentially rapid reaction of the dissociated/displaced nitrite back to the heme-bound NO. Support for a totally intra-dhp process is based on the observations that ligand escape from the distal heme pocket for glass-embedded Hb or Mb is extremely slow and that the glass experiments show slow formation of the intermediate without noticeable ferric NO heme formation from the met-nitrite Hb derivative as external NO within the glass slowly gains access to the distal heme pocket. If NO has to bind first to the heme prior to formation of the intermediate, then the trehalose glass results imply a mechanism with the nitrite/NO ligand exchange step occurring totally within the distal heme pocket. Thus, the glass experiment supports mechanisms where either the NO and heme-bound nitrite can rapidly exchange, resulting in ferric NO with nitrite in the immediate vicinity or the NO being able to bind to the heme-bound nitrite. Although the affinity of the ferric heme is much higher for the NO relative to the nitrite, the reported slow off-rate for the heme-bound nitrite makes it plausible that the NO could interact with heme-bound nitrite as well as replace nitrite, once the nitrite dissociates.

Intermediate formation can be initiated through an NR pathway as initially proposed (31, 59, 60, 87). The NR pathway generates both the needed ferric heme and NO (Reaction 11).

\[
\text{Fe}^{2+} + \text{NO}^{-} \rightarrow \text{Fe}^{3+} + \text{NO} \text{(NR)} \]

\text{REACTION 11}

If there is an abundance of five coordinate ferrous heme sites, as is the case in most of the in vitro experiments to date, the NO will generate ferrous NO heme derivatives. If, however, the ferrous heme is slowly generated in the presence of nitrite as in the case of a slow reduction of ferric heme via Cys (Reaction 12),
then there is an enhanced probability of forming the intermediate instead of the “dead end” ferrous NO derivative. The question remains as to whether there is an intermediate formed during the NR reaction that can result in both NO and nitrite occupying the dhp, thereby favoring the formation of the intermediate under conditions of low external NO (Reaction 13).

\[ \text{Fe}^{3+} + \text{L-Cys} \rightarrow \text{Fe}^{2+} + \text{L-Cys} \]

**REACTION 12**

\[ \text{Fe}^{2+} + \text{NO}_{2}^{-} \text{(excess)} \rightarrow \text{Fe}^{3+} + [\text{NO} + \text{NO}_{2}]_{\text{dhp}} \rightarrow \{\text{Fe}^{3+} + \text{NO} + \text{NO}_{2}^{-}\} - \text{N}_{2}\text{O}_{3}^{-} \leftrightarrow \text{Fe}^{2+} - \text{N}_{2}\text{O}_{3}^{-} \]

**REACTION 13**

**Physiological Relevance and Potential Biomedical Significance**—The current results create a framework where several if not all of the proposed Hb-based pathways can be integrated into a uniform scheme and where physiological and pathophysiological conditions dictate which elements of the scheme are dominant. All of the potential participating elements are subject to physiological and external control as follows: nitrite, NO, and R and T state ferric hemes, reactive thiols, GSH, and L-Cys. Although ferric NO can contribute to S-nitrosothiol formation via nitrosonium ion derived from Fe(III), it is concluded from earlier work (13) and this study that the presence of nitrite enhances Hb-mediated S-nitrosothiol formation with the formation of the heme-N$_2$O$_3$ intermediate being the basis for this enhanced reactivity.

The results of this study further indicate that there are multiple pathways to generate this intermediate. The desired R state ferric heme can be generated either from five coordinate ferrous hemes in partially oxygenated Hb undergoing the NR reaction, which also produces the needed NO, or from an NO dioxygenation reaction converting an oxy heme site into a ferric heme site, which can then react with NO and nitrite to yield the intermediate. NO can be generated locally through the nitrite reductase reaction initiated by a partially deoxygenized oxy Hb reacting with nitrite or from a partially ferric oxyHb species that is reduced via L-Cys (or other suitable reductants) to the mixed five coordinate ferrous/oxyHb species that can then undergo the NR reaction.

A major significance of this intermediate lies in its longevity, implying a potential for a prolonged capacity to transport NO bioactivity out of the RBC via the allosterically controlled generation of S-nitrosothiols. With the likelihood that the formation and reactivity of the intermediate is under allosteric control, it becomes possible that the mechanism generating the intermediate is linked to the mechanism for RBC-mediated hypoxic vasodilation in the arterioles. Important questions remain that pose real challenges to the physiological relevance. Most notable is the relative probability that the NO/nitrite combination can access a ferric heme site and form the intermediate in the presence of oxy heme sites that react rapidly with NO through the NO dioxygenase reaction.

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