Red blood cells (RBCs) act as O₂-responsive transducers of vasodilator and vasoconstrictor activity in lungs and tissues by regulating the availability of nitric oxide (NO). Vasodilation by RBCs is impaired in diseases characterized by hypoxemia. We have proposed that the extent to which RBCs constrict vs. dilate vessels is, at least partly, controlled by a partitioning between NO bound to heme iron and to Cysβ93 thiol of hemoglobin (Hb). Hemes sequester NO, whereas thiols deploy NO bioactivity. In recent work, we have suggested that specific micropopulations of NO-ligated Hb could support the chemistry of S-nitrosohemoglobin (SNO-Hb) formation. Here, by using nitrite as the source of NO, we demonstrate that a (T state) micropopulation of a heme-NO species, with spectral and chemical properties of Fe(III)NO, acts as a precursor to SNO-Hb formation, accompanying the allosteric transition of Hb to the R state. We also show that at physiological concentrations of nitrite and deoxyHb, a S-nitrosothiol precursor is formed within seconds and produces SNO-Hb in high yield upon its prompt exposure to O₂ or CO. Deoxygenation/reoxygenation cycling of oxyHb in the presence of physiological amounts of nitrite also efficiently produces SNO-Hb. In contrast, high amounts of nitrites or delays in reoxygenation inhibit the production of SNO-Hb. Collectively, our data provide evidence for a physiological S-nitrosothiol synthase activity of tetrameric Hb that depends on NO-Hb micropopulations and suggest that dysfunction of this activity may contribute to the pathophysiology of cardiopulmonary and blood disorders.
with SNO-Hb produced upon prompt oxygenation (12, 15–17). However, our kinetic analysis (12), like other work on nitrite/Hb interactions (25–27), was performed at supraphysiological nitrite/Hb ratios (typically nitrite:Hb 1:10–10:1 vs. ~1:1,000 in vivo), with a global spectral deconvolution approach that was not adapted to recognize “minority species.” We now examine the product distribution of reactions of nitrite and deoxyhemoglobin by using a modified approach in which spectral deconvolution is used to test for the presence of minority species formed at physiologically relevant concentrations, whereas studies of the chemical reactivity of these species are used to verify their identity. We show that product distributions vary substantially as a function of time after reagent mixing (seconds to hours), and nitrite to heme ratio (1:1,000–1:1). At physiological concentrations and time scales (approximate venous residence times in vivo), the reaction leads to high-yield production of a species with properties of Fe(III)NO that converts efficiently to SNO-Hb upon oxygenation (or CO exposure). In contrast, SNO-Hb production is suppressed at higher nitrite concentrations, apparently reflecting the formation of alternative NO-Hb micropopulations not competent for this chemistry. Moreover, delays in sample oxygenation drastically reduce SNO yields. This behavior presumably reflects the reactive nature of the SNO precursor that, absent prompt oxygenation, yields dead-end products. Overall, our data strengthen the case that Hb operates under physiological conditions as a SNO synthase, converting NO into SNOs through an allosterically modulated heme-NO redox reaction.

Experiments and Results

Reaction of NaNO₂ with DeoxyHb Assayed by UV-Vis Spectroscopy. Modified spectral analysis method. We analyze product development in the reactions of nitrite (10–400 μM) with deoxyHb (250 μM) by spectral deconvolution of UV-Vis spectra in which experimental spectra are modeled as a linear combination of component spectra (12, 14, 16). In previous studies, we included in the basis of spectral components all Hb spectra that could be reasonably expected under the given experimental conditions. However, the calculated best-fit spectra still show small systematic deviations from experimental ones, both in our studies and related studies by others (28, 29). In estimating the precision of the fitted parameter values, we included effects of parameter correlation and systematic error of the model and, thus, reported conservative error estimates of ~10 μM (a concentration exceeding the NO-Hb species of physiological relevance; refs. 3, 7, 15, and 17).

Here we adopt a use of the deconvolution method that is better adapted to recognize the minority species that form in physiological situations. First, we identify the minimal basis set that provides a reasonable simulation of the experimental spectrum. We then add additional suspected components to the spectral basis, repeat the analysis, and test for significance of such additions (30). In the present study, the minimal model includes deoxyHb, methHb, oxyHb, and Fe(II)NO Hb as basis spectra. With this procedure, we find that the addition of an Fe(III)NO component significantly improves the fit and provides a better improvement than addition of other possible components. Quantitatively, the improvement is significant with greater than 99.9% confidence in every experimental trial at the point of maximal Fe(III)NO concentration (F test). We report analyses made with the minimal basis augmented by the Fe(III)NO component. Additional experimental details are documented in Fig. 6, which is published as supporting information on the PNAS web site.

Results. Under supraphysiological conditions of nitrite excess (400 μM), the ratio of products [Fe(III)]/[Fe(II)NO] was close to unity (1:1), in basic agreement with previous reports by several laboratories (refs. 12 and 26; Fig. 1A); a slight excess of Fe(III) over Fe(II)NO, however, was observed during the reaction. In addition, we detected, as described above, a trace concentration of a species that was analyzed as Fe(III)NO. The concentration of this species peaked after ~40 min of reaction and subsequently decayed to zero (Fig. 1 A and B). With limiting nitrite, the ratio of [Fe(III)]/[Fe(II)NO] products showed substantial departures from unity, as exemplified in Fig. 1A (75 μM nitrite). Most interestingly, Fe(III)NO was observed to rise to a sustained level, without decay, under these reaction conditions (Fig. 1 A and B).

In Fig. 2 we illustrate the trends followed by the final levels of Fe(III)NO (Fig. 2A), Fe(III) and Fe(II)NO levels (Fig. 2B), and the [Fe(III)]/[Fe(II)NO] ratio (Fig. 2C) as a function of [NaNO₂] over the range 10–400 μM (here and in subsequent descriptions of reaction conditions, [NaNO₂] refers to the starting concentration of NaNO₂). Although the ratio of [Fe(III)]/[Fe(II)NO] varies over this range, it appears to approach unity if [NaNO₂] is in excess, or as [NaNO₂]/[heme] approaches zero. The trend in Fe(III)NO levels also exhibits a biphasic response, increasing roughly linearly with increasing [NaNO₂] up to 250 μM, but decreasing after this point. This result implies a connection to the occupancy of Hb heme by ligands {Fe(II)NO} or “holes” {Fe(III)} and, thus, to Hb allostery. The
turning point in the trend occurs at a nitrite level at which the hemes are half-occupied, that is, at which further reactions would lead to a transformation of Hb from its T to R quaternary structure (15, 31). The same principle applies to time-dependent behavior of Fe(III)NO in reactions with excess nitrite, exemplified in Fig. 1A and B (400 μM nitrite): After half-saturation of the protein, there is a decline in concentration of Fe(III)NO.

**SNO-Hb Formation. Correlation with Fe(III)NO.** Inasmuch as heme-[Fe(III)NO] has been demonstrated previously to be an effective intermediary en route to SNO-Hb production (16), and the linkage of SNO-Hb formation to Hb allosteroy has been established (1, 7), we conjectured that the allosterically coupled loss of Fe(III)NO described above might reflect an allosterically linked conversion of the Fe(III)NO species to SNO-Hb. To investigate this possibility, we assessed SNO-Hb formation in reactions of deoxyHb with nitrite over the range 50–350 μM and compared the trends in SNO-Hb levels to trends in Fe(III)NO. The results are illustrated in Fig. 3A. Remarkably, the trend in SNO-Hb formation precisely complements the trend in Fe(III)NO loss: The amount of SNO formed is directly proportional to the deviation of Fe(III)NO levels from their linear dependence on [NaNO₂] that is evident when [NaNO₂] < [heme] (proportionality constant = 0.67 ± 0.05; R = 0.98). In Fig. 3C, we show the trends as a function of both [NaNO₂] and heme occupancy to underscore the connection with Hb allostery.

To explore this point further, we subjected mixtures of deoxyHb and limiting amounts of nitrite to oxygenation, after the nitrite reaction was completed (Fig. 3B). Again, remarkably, we found that SNO-Hb was produced after oxygenation in direct proportion to the concentration of Hb[Fe(III)NO] before oxygenation (proportionality constant = 0.89 ± 0.06; R = 0.97). In other words, declines in [Fe(III)NO] induced by ligation of vacant hemes are matched by an equivalent increase in [SNO-Hb]. Evidently, the heme-[Fe(III)NO] species is a precursor for SNO-Hb, with chemical interconversion coupled to the allosteric transition of Hb from T to R state.

**Temporal characteristics and physiological conditions.** In the SNO-Hb paradigm (7, 13–17), SNO-Hb production would be optimized under physiological conditions, where nitrite or NO is ∼1 μM, Hb is in great excess, and deoxygenated Hb is available for no more than tens of seconds (the venous residence time is ∼30 seconds). To investigate the significance of this temporal constraint, a significance already suggested by the detrimental effect of aging of HbNO preparations on SNO formation (4), 250 μM deoxyHb and 1 μM NaNO₂ were mixed and analyzed promptly by photolysis/chemiluminescence. Notably, at 10 seconds after mixing, a positive signal is obtained, amounting to roughly half of the nitrite concentration (565 nM ± 10%; n = 5). Gel filtration (G25) eliminates the signal (Fig. 4C). Control experiments conducted with nitrate alone, or nitrite plus glutathione, gave essentially no photolysis/chemiluminescence signal (=2% of nitrite concentration), establishing that the signal is derived from the protein. Similarly, no signals were obtained upon incubation of nitrite with metHb, carboxyHb, or from deoxyHb pretreated with N-ethylmaleimide. In further control experiments, we confirmed that signals produced by Hb[Fe(II)NO] and SNO-Hb are not altered by gel filtration. Collectively, the data indicate the rapid formation (within seconds) of a nitrite-derived species weakly associated with Hb, the generation of which is promoted by vacant reduced hemes and thiols and/or T state quaternary structure of Hb, and from which NO is liberated by photolysis.

If this species were the SNO precursor characterized by UV-Vis spectroscopy, then occupation of the hemes should...
effect its conversion to SNO-Hb. To test this idea, mixtures of deoxyHb and nitrite, prepared as described above, were exposed (at 10 sec after mixing) to a bolus of O2 or CO, then purified via gel chromatography (G25) to isolate protein-bound NO products. Remarkably, a 10-sec exposure to CO or O2 produces SNO-Hb in concentrations equal to the SNO precursor concentrations measured before the gas bolus (Fig. 4B). Delaying oxygenation lowers SNO-Hb yields, presumably due to interfering reaction(s) of the SNO precursor (Fig. 4B). In addition, raising the nitrite concentration led to diminished yields of SNO-Hb (approaching zero SNO at 50–100 μM nitrite), as previously described in experiments with NO (15, 17). For example, at 100 μM nitrite/250 μM deoxyHb (×10 sec), yields of SNO-Hb after oxygenation were 250 ± 212 nM (n = 3).

**Oxygenation/deoxygenation cycling.** Because of chain heterogeneity in O2 dissociation rates, β-chains will preferentially deoxygenate under hypoxic conditions, effectively increasing α-chain interactions with nitrite relative to α-chains (16, 32). Thus, inasmuch as the efficiency of SNO-Hb production appears linked to NO localization to the β-chain (16), rapid deoxygenation of oxyHb in the presence of nitrite should increase the percent yield of SNO-Hb on reoxygenation. To test this prediction, 250 μM oxyHb and 1 μM NaNO2 were mixed, rapidly deoxygenated with argon (~1 min), and reoxygenated and purified via G25 chromatography. OxyHb (deoxygenation/reoxygenation) in the presence of nitrite produces SNO-Hb (206 nM ± 25; n = 4) that comprises 92% of all protein-bound NO (224 nM ± 10), in contrast to the lower SNO yields (65–70%) from deoxyHb samples incubated with nitrite for 10 sec and subsequently bolused with CO or O2 (Fig. 4).

**Discussion**

The data reported here provide considerable insight into the reaction of Hb with nitrite. The distribution of products, including nitrosylHb (FeNO) and methHb [Fe(III)], depends on the nitrite concentration. Further, not only is an intermediate with an Fe(III)NO spectrum detected, but its evolution has been monitored to show different fates depending on the allosteric state of Hb. In particular, when nitrosylHb and methHb accumulate in sufficient amounts to trigger a transition to the R state, the intermediate is lost with a corresponding gain in SNO product. The most significant feature of our results is the quantitative relationship between the SNO-Hb precursor formed in T state under physiological conditions and the SNO-Hb obtained from it after allosteric transition to the R state. Notably, Pezacki et al. (18, 24) have described a similar reaction in reverse: Upon deoxygenation, SNO-Hb furnishes a βFe(III)NO. The physiological significance of βFe(III)NO is suggested by the report of Nagababu et al. (25) that the in vivo HbFeNO pool is predominantly an EPR silent Fe(III)/NO hybrid.

Doyle (26) pioneered the quantitative exploration of interactions between deoxyhemoglobin and (excess) nitrite and described a heuristic reaction scheme in which one equivalent of nitrite yields one equivalent of Fe(III) and Fe(II)NO (Eq. 1). Subsequent work examining the kinetics and product distribution of this reaction under a range of conditions (12, 25, 33) has continued to employ nitrite concentrations and [NaNO2]/[deoxy-heme] ratios well above physiological values. A clue to the importance of reactant concentrations on this chemistry emerged from EPR studies, which revealed, in the aftermath of mixing very high concentrations of nitrite and deoxyHb, essentially equal subunit populations of nitrosyl heme [Fe(II)NO] but also preferential processing of NO within the framework of mixing very high concentrations of nitrite and deoxyHb, essentially equal subunit populations of nitrosyl heme [Fe(II)NO] whereas spectra obtained under conditions that simulate key aspects of the in vivo situation exhibit substantial βFe(II)NO preference (16). In addition, the importance of duration of reaction on product distribution was recognized: Aging of NO-deoxyHb samples that is incurred over the lengthy course of the nitrite reaction enables competing chemistry, including redistribution of NO from α- to α-chains, reductive loss of NO to HNO and quenching of radicals in Hb (4, 7, 13–15, 34). Because previous work has suggested collectively that the efficacy of transfer of NO groups from heme to βCyS-93 (to form a bioactive SNO) might require not only physiological NO/Hb ratios and HbNO concentrations (< 1 μM) but also preferential processing of NO within the β-chain (14–17), the conditions used in prior work were not optimized for the study of SNO formation.

In the present study, the kinetics and product stoichiometry of nitrite reactions were reexamined, with a focus on physiological conditions. This focus includes the following: (i) variation of [NaNO2] from the supraphysiological range down to concentrations detected in vivo; (ii) examination of reactions over time intervals comparable with the physiological situation (venous residence time ≤30 sec); and (iii) oxygenation of reaction mixtures over seconds to simulate the lung (and use of Hb at concentrations compatible with full O2 saturation over such intervals) (4, 15, 17). Our results demonstrate that, under
Fe(II)NO a photolyzable NO signal within seconds (Fig. 4), an observation to preserve endogenous levels of SNO-Hb in RBCs (4). Chemiluminescence and observed, allosterically linked conversion to SNO-Hb. The observed chemistry of this species, namely facile, quantitative, identifies as Fe(III)NO. This assignment is buttressed by the presence. Here we took an alternative approach to analysis of low protein-bound NO are lower when nitrite is added directly to deoxyHb followed by bolus (as in Fig. 4) with O2 (hatched bar) or CO (filled bar). Results are presented as mean ± SE of four to five experiments.

Conditions that characterize the physiological realm, Hb operates on nitrite as a SNO synathese:

$$\text{HS-Hb}[\text{Fe(II)}] + \text{NO}_2^- + 4\text{O}_2 + \text{H}^+ \rightarrow \text{SNO-Hb}[\text{Fe(II)NO}]_2 + \text{H}_2\text{O}. \quad [2]$$

We have previously reported that Hb can synthesize SNO through a variety of processes involving $\beta$Fe(III)NO and that nitrite-derived FeNO is converted to SNO after oxygenation (3, 16). These processes formally involve an Fe(III)NO intermediary. We and others (16, 25, 29), however, had not been able to identify the production of Fe(III)NO in reactions between Hb and nitrite, although Rifkind et al. (23, 25) have inferred its presence. Here we took an alternative approach to analysis of UV-Vis spectra that has enabled the detection of a minority species, identified as Fe(III)NO. This assignment is buttressed by the observed chemistry of this species, namely facile, quantitative, allosterically linked conversion to SNO-Hb.

Under physiological nitrite levels, we used photolysis-chemiluminescence and observed ~50% conversion of nitrite to a photolyzable NO signal within seconds (Fig. 4), an observation in line with earlier ideas of Rifkind et al. (25), who proposed that nitrite is consumed rapidly by deoxyHb to form an EPR-silent Fe(II)NO$^-$ equivalent in T state. We further determined that exposure of the weakly associated deoxyHb/nitrite-derived complex to O2 or CO produces an equivalent yield of SNO-Hb. Thus, the initial complex formed in deoxyHb may be viewed as a SNO precursor that is under allosteric control and whose reactivity is similar to Fe(III)NO or Fe(II)NO$^+$. Notably, oxyHb deoxynated in the presence of physiological amounts of nitrite, conditions that we have shown should favor NO binding to $\alpha$-vs. $\beta$-chains (16), and then reoxygenated produces SNO-Hb concentrations nearing 100% of protein-bound NO (Fig. 5). Thus, in the realm of interactions of nitrite with hemoglobin of physiological relevance (physiological concentrations, ratios, and timescales), SNO-Hb is the major product. Raising the nitrite concentration to nonphysiological levels, or increasing incubation times with Hb, allows for alternative reactions yielding bio-inactive products [e.g., Fe(II)NO and N2O], thereby drastically reducing SNO-Hb yields upon oxygenation (Fig. 4B).

Our results are reminiscent of previous studies that found a requirement for immediate oxygenation of submicromolar FeNO to efficiently produce SNO-Hb in vitro (7, 14, 15, 17) and to preserve endogenous levels of SNO-Hb in RBCs (4).

The distribution of met and nitrosyl species in the nitrite reaction can be rationalized in a model that includes the effects of quaternary structure and chain heterogeneity. In T state, $k_{\alpha_\text{ox}} = k_{\beta_\text{ox}} > k_{\alpha_\text{red}}$ and $k_{\alpha} > k_{\beta}$. (32, 35) ($k_{\alpha}$ and $k_{\beta}$ represent specific rate constants for ligand binding and for heme-iron oxidation, respectively, and the superscript refers to the Hb subunit). Under limiting conditions, nitrite will preferentially oxidize $\alpha$-chains. If the NO lost from the $\alpha$Fe(III)NO intermediate in the oxidation tends to transfer within an $\alpha/\beta$ dimer (36), then this chemistry would furnish dimers of composition [Fe(III)NO][Fe(II)NO] (16), to the extent that the buildup of such dimers is faster than NO redistribution to $\alpha$Fe(II). As [NaNO2] increases into the intermediate range, however, oxidation on the $\beta$-chains will be increasingly competitive, which will result in a significant pool of dimers of composition [Fe(III)NO][Fe(II)NO], Because heme iron within the $\alpha$-chains are, in the T state, oxidized more rapidly that those of $\beta$-chains in T state ($k_{\alpha} > k_{\beta}$) (32), the $\alpha$Fe(II)NO that forms will be more likely to yield Fe(III) and HNO and, thus, add to the rate of metHb production while decreasing the total yield of Hb[Fe(II)NO]. Indeed, in previous analyses, both we and others (15, 26) have documented the coincident production of HNO (via detection of its byproducts, hydroxylamine and dinitrogen oxide) and metHb. Moreover, previous work by Gow and Stamler (15) emphasized not only the importance of chain heterogeneity in NO-mediated oxidation of hemes, but also the NO/Hb ratio (and the NO concentration), which determines the allosteric state of Hb. In the R state (high NO/Hb ratio), the ligand off rates are decreased, thus mitigating NO-mediated oxidation (15). Indeed, increasing [NaNO2] predictably leads to a predominately R state population with altered reactivity, as first noted by Doyle et al. (26) and recently detailed by others (27, 29).

In this situation, a decrease in the rate of dissociation of NO/HNO decreases the NO-mediated oxidation; consequently, the [Fe(III)]/[Fe(II)NO] ratio shifts back toward unity. This model predicts a mass balance relationship between [NaNO2], the products Fe(III), Fe(II)NO, Fe(III)NO, and SNO reaction products that is in complete accord with all of our experimental results (see Fig. 7, which is published as supporting information on the PNAS web site).

In summary, the chemistry of nitrite/Hb interactions under physiological conditions (like that of NO/Hb interactions) is very different from that observed at the high nitrite to Hb ratios that have been used in most previous work (12, 26, 27, 29, 33). We have shown that conditions that closely replicate the physiological situation, entailing brief exposures of deoxyHb or rapidly saturated oxyHb to limiting (physiological) nitrite, and which include cycles of oxygenation or deoxygenation/reoxygenation, respectively, yield SNO-Hb as a major product. The amount of SNO-Hb produced is far greater than the amount of NO that would be predicted to form according to published rates of nitrite reduction (26), reflecting instead the production and reaction of an Fe(II)NO/SNO precursor. It is interesting to note that current methodologies used to assay NO species, with the exception of photolysis, cannot detect either Fe(III)NO or N2O$,^-$ thereby potentially assay SNOs that are in equilibrium with Fe(III)NO or Fe(II)NO$^+$. This inability may be of importance in understanding certain discrepancies between reported levels of NO species detected in biological systems (7). Finally, it should be noted that, although the results presented here coordinate the observation of Fe(II)NO with oxygenation-induced SNO-Hb formation, the possibility that a SNO precursor can involve a direct reaction of nitrates with thiols of Hb finds precedent in previous work (37, 38) and is not excluded by our experiments.
Materials and Methods

Preparation of Deoxymethemoglobin. Highly purified adult human hemoglobin (HbA0; Apex Biosciences, Durham, NC) was reduced with sodium dithionite in an anaerobic glove box. Excess dithionite was removed with gel filtration chromatography (Sephadex G25 resin; Amersham Pharmacia Biosciences). Purified samples were prepared daily and stored anaerobically between experiments. In some experiments, deoxyHb samples were incubated with N-ethylmaleimide before starting the reaction. Excess N-ethylmaleimide was removed via gel chromatography.

Preparation of Oxygenmethemoglobin. Deoxymethemoglobin was prepared as described above and subsequently oxygenated with room air.

Preparation of Carboxyhemoglobin. Deoxymethemoglobin was prepared as described above and placed in a 3-ml septum-top glass cuvette. Samples were treated with a gentle stream of 1,000 ppm anaerobic CO gas for 30 min before measurement.

Preparation of Methemoglobin. HbA0 was incubated with an excess of potassium ferricyanide for 5 min. Excess potassium ferricyanide was removed with gel chromatography.

Reaction of Deoxymethemoglobin and Nitrite. Five hundred micromolar deoxymethemoglobin and an equal volume of NaNO2 buffer solution (NaNO2 plus 100 mM phosphate, pH 7.4) were mixed by vortexing inside an anaerobic glove box. Samples were placed in a 0.5 mm, 0.1 mm, or 0.2 mm screw-top cuvette (Spectrocell, Orelund, PA). Initial nitrite concentrations used in spectroscopic measurements varied from 1–500 μM with deoxyHb concentration constant at 250 μM, giving [NaNO2]/[DeoxyHb] concentrations from 1:250–2:1.