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# Responses of normal and sickle cell hemoglobin to *S*-nitroscysteine: implications for therapeutic applications of NO in treatment of sickle cell disease☆

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**Abstract**

Factors which govern transnitrosation reactions between hemoglobin (Hb) and low molecular weight thiols may define the extent to which *S*-nitrosated Hb (SNO-Hb) plays a role in NO in the control of blood pressure and other NO-dependent reactions. We show that exposure to *S*-nitrosylated cysteine (CysNO) produces equivalent levels of SNO-Hb for Hb $A_0$ and sickle cell Hb (Hb S), although these proteins differ significantly in the electron affinity of their heme groups as measured by their anaerobic redox potentials. Dolphin Hb, a cooperative Hb with a redox potential like that of Hb S, produces less SNO-Hb, indicating that steric considerations outweigh effects of altered electron affinity at the active-site heme groups in control of SNO-Hb formation. Examination of oxygen binding at 5–20 mM heme concentrations revealed increases due to *S*-nitrosation in the apparent oxygen affinity of both Hb $A_0$ and Hb S, similar to increases seen at lower heme concentrations. As observed at lower heme levels, deoxygenation is not sufficient to trigger release of NO from SNO-Hb. A sharp increase in apparent oxygen affinity occurs for unmodified Hb S at concentrations above 12.5 mM, its minimum gelling concentration. This affinity increase still occurs in 30 and 60% *S*-nitrosated samples, but at higher heme concentration. This oxygen binding behavior is accompanied by decreased gel formation of the deoxygenated protein. *S*-nitrosation is thus shown to have an effect similar to that reported for other SH-group modifications of Hb S, in which R-state stabilization opposes Hb S aggregation. 

*Keywords:* Nitrosation; Sickle cell; Metal chelator; SNO-Hb; Oxidation potential

*Abbreviations:* Hb, hemoglobin; Hb $A_0$, purified hemoglobin of adult human; SNO-Hb, *S*-nitrosylated Hb; CysNO, *S*-nitrosylated cysteine; EDTA, ethylenediamine tetraacetic acid; DPTA, diethylenetriamine pentaacetic acid; GSH, reduced glutathione; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid

☆ This work is dedicated to Maurizio Brunori, who introduced me (C.B.) to hemoglobin research and whose elegant studies of oxygenation and oxidation processes in heme proteins inspired us to take a closer look at these interrelated phenomena in relation to the interactions of NO with normal and sickle cell hemoglobin.

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## 1. Introduction

Hemoglobin (Hb) plays an important biological role as a respiratory protein. Another important biological role of Hb is emerging from intensive studies focused on interactions of Hb with NO and the role this may play in the control of blood pressure and other NO-dependent reactions. Nitrosation of sulfhydryl groups at the highly conserved cysteine residues at position 93 on the two β chains of the Hb tetramer creates *S*-nitrosated Hb (SNO-Hb). SNO-Hb forms in vivo as well as in vitro, and its potential role in blood pressure regulation was brought out by the collaborative work of Stamler and ourselves [1]. This work stimulated further research on interactions of Hb with NO in many laboratories because of its far-reaching implications [2–6]. Release of NO from SNO-Hb has the potential to bring about reactions normally associated with free NO. Unlike SNO-Hb, free NO is a very reactive molecule, whose lifetime in the complex cellular milieu would be expected to be very short. It is this characteristic of NO that delayed the discovery of NO-dependent reactions in smooth muscle relaxation, platelet inhibition, neurotransmission, and immune regulation [7–11]. What is learned about Hb-based NO interactions and transport will have far-ranging applications in these disparate fields. Notably, studies have shown that Hb nitrosated on its sulfhydryl groups can act as a vasodilator [1,12], with the potential of transmitting the relaxation possibilities and other aspects of NO biochemistry far away from the site of NO generation.

Relevant reactions of NO-containing compounds with the sulfhydryl groups of Hb are summarized below. In addition to transnitrosation reactions (Eq. (1)) and copper catalyzed *S*-nitrosothiol decomposition (Eq. (2)), *S*-nitrosothiols can transfer the RS-group to Hb (Eq. (3)), resulting in formation of a mixed disulfide of Hb and the NO donor (e.g. *S*-nitrosylated cysteine (CysNO) and Hb can form cysteinyl-Hb). SNO-Hb itself can serve as the *S*-nitrosothiol (Eq. (3)) and react with other thiols, creating Hb-S-SR with release of RS-NO or $NO^-$ (9, 10). The nitrosonium ion, $NO^-$, can react with $HbFe^{III}$ to form $HbFe^{II}NO$. Additionally, Spencer et al. [13] reported a two-step process whereby *S*-nitrosoglutathione (GSNO) reacts first with deoxy Hb to produce met Hb, reduced glutathione (GSH), and NO free radical (Eq. (4)), with the NO• subsequently binding to deoxy Hb or reacting with oxy Hb. Finally, as shown in Eq. (5), apart from these NO-transfer reactions, NO• can interact with oxygen to form effective nitrosating compounds, generally denoted as $NO_x$ [8], that can nitrosate thiols and lead to formation of SNO-Hb.

$$\text{RS-NO} + \text{Hb-SH} \rightarrow \text{Hb-S-NO} + \text{RSH} \quad (1)$$

$$2\text{RS-NO}/\text{Cu}^{\text{II}} \rightarrow \frac{1}{2}\text{RSSR} + \text{NO}\bullet + \text{HbO}_2 \rightarrow \text{HbFe}^{\text{III}} + \text{NO}_3^- \quad (2)$$

$$\text{RS-NO} + \text{Hb-SH} \rightarrow \text{Hb-S-SR} + \text{NO}^- \rightarrow \text{NO}^- + \text{HbFe}^{\text{III}} \rightarrow \text{HbFe}^{\text{II}}\text{NO} \quad (3)$$

$$\text{RS-NO} + \text{HbFe}^{\text{II}} \rightarrow \text{HbFe}^{\text{III}} + \text{RSH} + \text{NO}\bullet \rightarrow \text{NO}\bullet + \text{HbFe}^{\text{II}} \rightarrow \text{HbFe}^{\text{II}}\text{NO} \rightarrow \text{NO}\bullet + \text{HbFe}^{\text{II}}\text{O}_2 \rightarrow \text{HbFe}^{\text{III}} + \text{NO}_3^- \quad (4)$$

$$\text{NO}_x + \text{Hb-SH} \rightarrow \text{Hb-S-NO} \quad (5)$$

The effects of metals and experimental conditions on these reaction pathways and reaction products are further described in this report of in vitro studies with isolated Hbs and low molecular weight RSNO compounds. These reactions are highly relevant for understanding NO-dependent reactions with Hb and developing therapeutic uses of NO. The relative significance of oxygen-mediated reactions (via $NO_x$ compounds as in Eq. (5)) and transnitrosative mechanisms (via low molecular weight RSNO donors as in Eq. (1)) for generation of SNO-Hb in vivo are still unclear. A recent report that metal chelators inhibit *S*-nitrosation of Hb [14] is indicative of this still changing view of SNO-Hb biochemistry, since transnitrosative mechanisms that lead to SNO-Hb formation (Eq. (1)) are known to be favored by removal of metal chelators, and without the cystinylation reactions enhanced by metal contaminants (vide infra).

The low levels of SNO-Hb in blood make it difficult to resolve questions associated with SNO-Hb and its role in vivo. Alterations in SNO-Hb levels can result from as yet unidentified constituents of blood that control SNO-Hb formation.

More information on the biochemistry of SNO-Hb is clearly needed. Experiments reported here clarify further the relative importance of electronic and steric controls of SNO-Hb formation and degradation. Other experiments are reported that *S*-nitrosation acts to stabilize the R-state condition at physiologically relevant temperatures and concentrations, that under these conditions deoxygenation is not sufficient to cause NO release from purified SNO-Hb. These experiments uncovered an indirect spectrophotometric approach to the investigation of SNO-Hb reactions in solution.

NO is being studied for its potential use as a therapeutic agent for treatment of sickle cell disease. At high concentration, sickle cell Hb (Hb S: β6 Glu→Val) aggregates when deoxygenated, giving rise to the adverse effects associated with sickle cell disease (see reviews by Eaton and Hofrichter [15,16]). Studies done in vivo may be clarified by the in vitro studies reported here, which show that *S*-nitrosation of Hb S opposes aggregation of the deoxygenated protein at physiologically relevant concentrations and temperatures.

For therapeutic applications NO may be administered at low levels in breathing gas ([5,6,17,44]), where it can act directly as a vasodilator. Alternatively, NO administration in varied forms can lead to reactions of NO with oxygen that can yield nitrosating agents, which can lead to formation of nitrosated thiols such as SNO-Hb as in Eq. (5). NO-based therapies for sickle cell disease under investigation have the potential to improve tissue oxygenation, decrease platelet adhesion and aggregation, and decrease Hb S polymerization. Head et al. [17] reported that low concentrations of NO (80 parts per million by volume of NO in air for 45 min) increase the oxygen affinity of sickle erythrocytes in vitro and in vivo. They drew the conclusion that Hb S polymerization must have been reduced directly (by heme nitrosylation) or indirectly (by SNO-Hb formation), since previous studies showed a strong correlation between the extent of polymer formation and lowered oxygen affinity exhibited by Hb S in concentrated solutions and in red blood cells [18–20,45]. Winslow [21] documented that the reduced blood oxygen affinity exhibited by erythrocytes containing Hb S is due to intracellular polymerization, with the correlate that treatments that reduce polymerization will also increase blood oxygen affinity. The results of Head and coworkers are in conflict with those reported by Gladwin et al. [6] in which NO in breathing gas was shown to produce a small increase of SNO-Hb in sickle cell patients, but not to the extent that oxygen affinity was altered. As noted in Section 4, variations in experimental methods and differences between equilibrium conditions and 'apparent' $P_{50}$ values may underlie these apparent discrepancies.

We previously reported that *S*-nitrosated forms of Hb A and Hb S at low (<1 mM heme) concentrations have increased oxygen affinity relative to unmodified Hb, with increased R-state character that is most evident at low levels of oxygen saturation [22]. This finding prompted us to suggest that *S*-nitrosation of Hb S might be viewed as a possible therapeutic approach to alleviating sickle cell disease. In support of this hypothesis, we show in this report that *S*-nitrosation of Hb S brings about alterations of its concentration-dependent oxygen binding behavior that are correlated with decreased polymer formation by the deoxygenated protein.

## 2. Experimental procedures

### 2.1. Sample preparation

*S*-nitrosation reactions of Hb $A_0$, Hb S (β6 Glu→Val) and Bottlenose Dolphin (*Tursiops truncatus*) Hb were studied after cell lysis, anion removal and chromatographic purification of the Hb as described elsewhere [23,22]. *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES), $KNO_3$ (Sigma) and KCl (Fisher > 99%), were dissolved and adjusted to pH values indicated. Metal chelators, when used, were 0.1 mM diethylenetriamine pentaacetic acid (DPTA) or 0.05 mM ethylenediamine tetraacetic acid (EDTA), which gave equivalent results in the studies reported here. Metal chelators were essential in the preparation of low-met samples of Hb S and SNO-Hb A0 and SNO-Hb S at high concentration. High concentrations of purified Hbs were

achieved by centrifugation of samples in Centricon filters.

### 2.2. Oxygen binding

Oxygen binding measurements at low protein concentrations (~0.1 mM) for normal and *S*-nitrosated samples were done using a tonometric method based on that of Riggs and Wolbach [24] with modifications as previously described [23,22,25]. Oxygen binding measurements at high (10–20 mM) concentration required special precautions (low temperature procedures, low light, added metal chelators) to minimize met Hb formation as the samples were concentrated. Oxygen binding measurements at high concentration were made in a thin membrane sandwich using an oxygen electrode to monitor oxygen levels. For this purpose we used a modified Hemoscan instrument (Aminco, Inc.) operated in a step-wise mode. Dynamic error in continuous measurements using this technique was previously described [26] and leads to *apparent* oxygen affinity determinations even in the step-wise mode of operation when the Hb contained in the thin film has polymerized (see Section 4). The binding curves referenced or shown in this paper are composites of data points from multiple experiments using samples with met Hb levels <12%.

### 2.3. Hb S gelation assays

The effects of *S*-nitrosation of Hb S on its aggregation propensity were tested by direct visual observation of gel formation, using a method previously validated by Benesch et al. [18]. Hb samples were observed in a water-jacketed chamber in which deoxygenated samples were stirred continuously and kept anaerobic by a gentle flow of humidified nitrogen. The use of a stirring bar facilitated temperature changes and detection of complete Hb gelation. Samples were extensively deoxygenated in tonometers in an ice bath prior to introduction to the sample chamber, with care taken to add the sample below the flow of nitrogen. Oxygen scavengers such as sodium dithionite could not be used to ensure complete deoxygenation, since their reductive action would cause the release of NO from SNO-Hb. The extent of gelation was evaluated 30 min after bringing 0.25 ml of a completely deoxygenated solution of Hb S at pH 6.8 at 7 °C up to 37 °C, a temperature switch that enables formation of Hb S polymers in samples whose concentrations are above the minimum gelling concentration. This time period was sufficient for comparative qualitative tests of gelation of normal and SNO-Hb S i.e. the unmodified protein consistently forms complete gels in less than 30 min at 37 °C. The samples were maintained for at least 30 min under deoxy conditions prior to the temperature jump to 37 °C to ensure the removal of any oxygen picked up during the transfer from tonometers to jacketed chamber. An oxygen electrode in the side-arm of the chamber was used to check for any leakage of oxygen into the system.

### 2.4. Electrospray-ionization mass spectrometry

Previously described mass spectrometry methods [27] were modified for characterization of changes in mass associated with NO transfers to and from thiols of both high and low molecular weight categories. The method used here, described in more detail elsewhere [22] allows for determinations of the types and levels of *S*-nitrosated species present in changing mixtures. When used in combination with spectral assays, this technique enabled us to determine the extent of SH-group nitrosation of Hb under varied conditions. ES-MS measurements were made on a Micromass-VG Quattro BQ triple quadrupole mass spectrometer equipped with a pneumatically assisted electrostatic ion source operating at atmospheric pressure. Samples are introduced by loop injection into a stream of appropriate solvent flowing at 6 μl/min. Mass spectra are typically acquired in the multichannel analyzer mode from $m/e$ 980 to 1190 with a scan time of 2 s. The mass scale is calibrated with appropriate standards such as horse heart myoglobin ($M_r$ 16951.48) with a resolution corresponding to a peak width at half height of 0.9 Da for $m/z$ 1000. The mass spectra are transformed to a molecular mass scale using software (MaxEnt) supplied by the manufacturer.

### 2.5. Analysis of extent of S-nitrosation of Hb samples

Hb nitrosation following exposure to CysNO was determined by electrospray ionization mass spectrometry (ESI-MS) or by spectral deconvolution analysis as described elsewhere [22]. Prior to spectral evaluation, the samples were subjected to Sephadex G25 chromatography with 2% borate, 0.1 mM DPTA buffer, pH 9.2 (carried out at 4 °C) to standardize the sample pH and buffer conditions and to remove any low molecular weight material. The samples were degassed in tonometers and subjected to spectral analysis before and after treatment with dithionite (sodium hydrosulfite, Tech, Acres Chemical Co.). Dithionite addition rapidly removes any residual oxygen, reduces any met Hb present and releases NO from the RS-NO linkage. The NO is effectively captured by the reduced heme, and the spectrum that results is that of partially NO-Hb. NO gas (National Welders, CP grade), further purified by passage through 5 M and then 1 M NaOH, is added to the degassed, dithionite-treated sample, and the spectrum of fully NO-Hb is obtained after a 10-min equilibration period. Comparisons of the partially and fully NO-Hb spectra allow the fraction of *S*-nitrosation prior to dithionite treatment to be determined. Multi-component analysis of spectra containing several species was done with a software program supplied by Hewlett-Packard and used on a Hewlett-Packard Diode Array Spectrophotometer.

### 2.6. Preparation and detection of S-nitrosothiols

Our assays followed methods for the preparation and handling of low molecular weight RSNO compounds as described by Stamler and Feelisch [8]. Spectrophotometric assays were as previously described [1,28,29]. *S*-nitroso derivatives of Hbs generated by transnitrosation reactions were made immediately after preparation of the low molecular weight RSNO donor, since experimental conditions can alter the stability of SNO-compounds. Most synthesis of RSNO compounds in organic solvents involves $N_2O_4$. These are hampered by preparation and handling of this reagent because it is a powerful oxidant not compatible with many systems. We prepared the low molecular weight RSNO NO donor (CysNO) used here by reaction of cysteine with acidified $NO_2^-$ [30]. We used our previously described methods [22] to generate highly SNO-Hb (90–100% β93 groups modified), which was then concentrated to the desired level. Mixtures of concentrated stock SNO-Hb and unmodified Hb were used to obtain solutions with varied levels of *S*-nitrosation. This procedure results in mixtures where *S*-nitrosation of β93 groups is essentially 100% on some Hb tetramers, and absent on others. Spectral deconvolution methods and electrospray mass spectrometry as described in our earlier work [22] and used in this study showed that the *S*-nitrosated proteins used in oxygen binding and gelation studies had no other modifications than at the β93 groups.

## 3. Results

### 3.1. Mass spectra of normal and sickle cell hemoglobin reacted with CysNO

Knowledge of the ways SNO-Hb can be formed in vitro and in vivo is required to exploit the potential of NO-based therapies for blood pressure regulation or treatment of sickle cell disease. State-of-the-art ESI-MS provide an unparalleled opportunity to simultaneously monitor *S*-nitrosation and NO transfer reactions in an extremely wide range of molecular weight compounds. As shown in Figs. 1–3, this technique makes it possible to directly quantify the time-dependent formation of NO adducts on α or β chains of *mixtures* of Hb and changes in NO adduction of low molecular weight thiols. The mass spectrum shown in Fig. 1 is that of Hb $A_0$ and a small amount of glycated Hb $A_0$ reacted with CysNO in the presence of metal chelators. It contains major peaks in the $m/z$ region of 1200–1240. Three of the peaks are assigned to forms of α-globin+heme that differ only in an associated cation (H+, Na+, or K+). The other peaks are assigned to β-globin. β-globin-SNO is clearly detected (mass difference of 40 from the parent β-globin) and amounts to as much as 100% of the total β-globin under some reaction conditions tested.

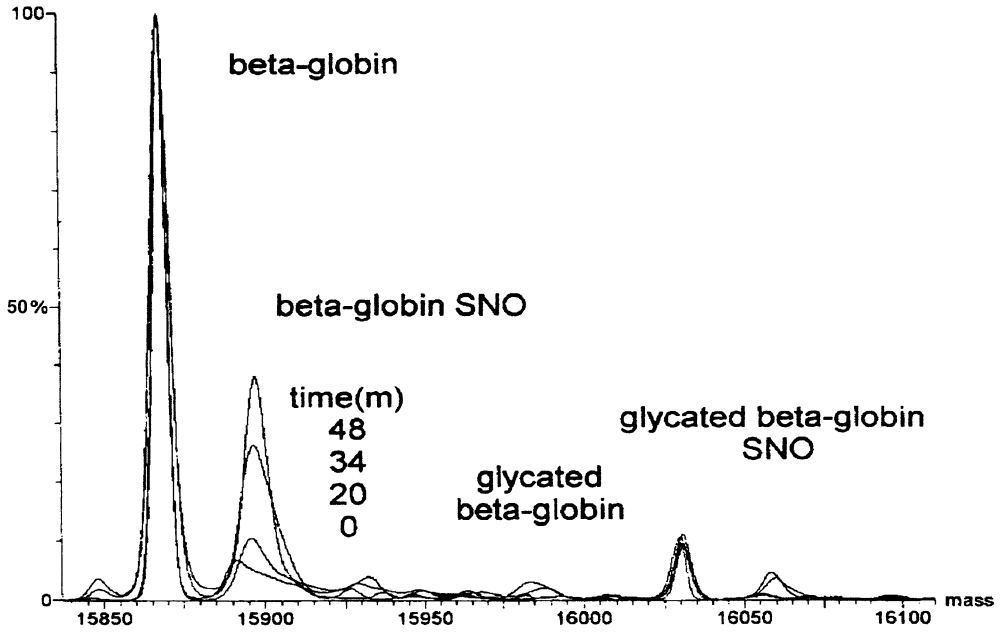


Fig. 1. ESI-MS data on the time course of *S*-nitrosation of 1 mM Hb $A_0$ and a low level of glycosylated Hb $A_0$ exposed to a fourfold excess of CysNO in borate buffer containing 0.1 M DPTA, 25 °C, pH 9.2. Mass spectra show the normal β-globin mass at 15866, as well as progressive β-globin NO-adduction (30 mass units from the parent β-globin and the glycosylated β-globin) as the sole modifications that occur.

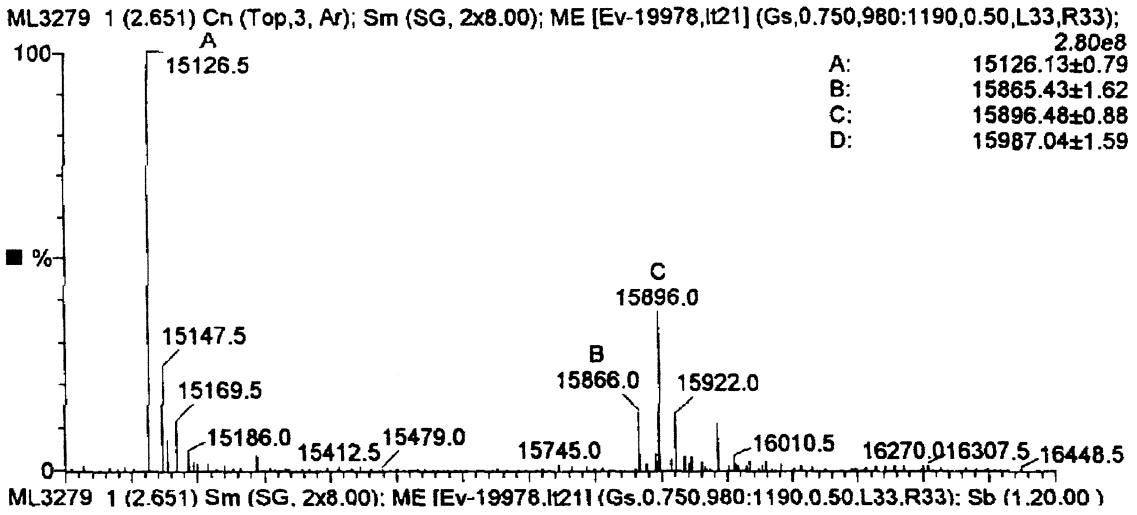


Fig. 2. ESI-MS data on reactions of Hb $A_0$ with high (tenfold) excess of CysNO to Hb in borate buffer containing 0.1 M DPTA, 25 °C, pH 9.2. Note the appearance of 30-mass unit nitrosated adducts (at 15896) of the β globin (normally at 15866), doubly nitrosated β-globin (at 15926) and some NO α-globin NO-adducts (at 15156) at this ratio of CysNO to Hb. As noted in the text, the 'buried' SH groups of the α and β chains are less modified than the external SH group at β93.

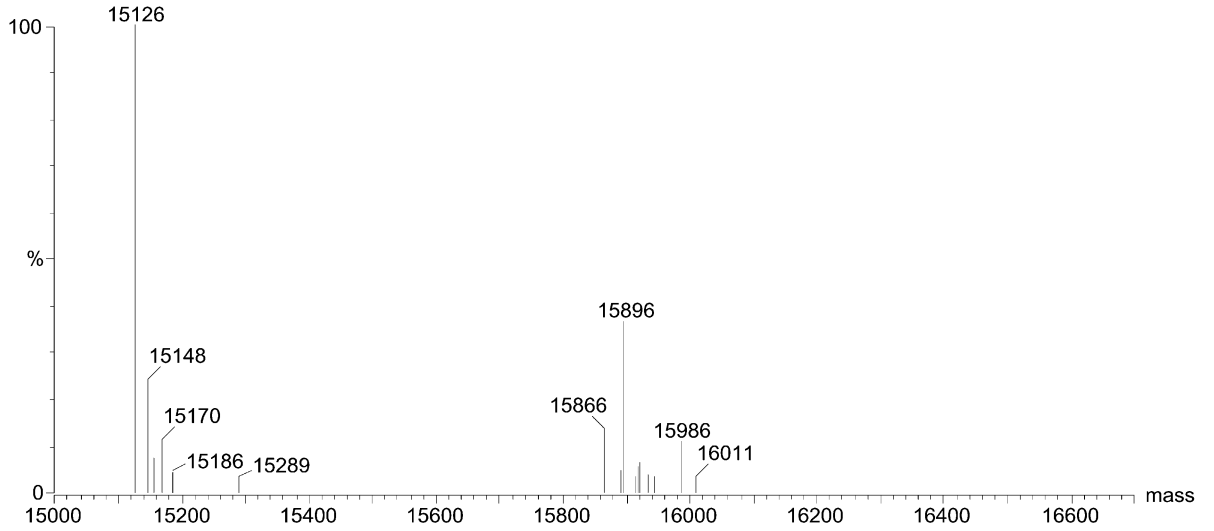


Fig. 3. ESI-MS data on reactions of Hb $A_0$ with CysNO as in Figs. 1 and 2 but in the *absence* of a metal chelator. Note the appearance of cystinylated β-globin (at 15986) at somewhat less than half the level of the β-globin NO-adduct (at 15896) under these reaction conditions.

The only product formed as a result of exposure of Hb to a $2x-4x$ excess of CysNO *in the presence of metal chelators* (DPTA or EDTA) is nitrosated β-globin. Both Hb $A_0$ and glycated Hb $A_0$ show time-dependent increases in SNO-Hb formation in the representative data of Fig. 1, illustrating the power of ESI-MS to follow transnitrosation reactions with mixtures of proteins. In studies of Hb and CysNO reactions under similar conditions, followed by separation of the globins by reverse phase HPLC, Ferranti et al. [27], reported that βCys93 is the preferred site of in vitro NO-adduct formation. As shown below, the 'buried' SH groups of βCys12 and αCys104 form NO-adducts *only* in the presence of prolonged exposure to elevated levels of CysNO or at low protein concentration.

### *3.2. Reactions resulting from elevated CysNO ratios and contaminating metals*

Modification of internal SH groups as noted by Ferranti et al. [27] was confirmed in our studies when Hb was exposed to a $10x-20x$ excess of CysNO over heme. As shown in Fig. 2, these modifications of α and β internal SH groups are evident as 40 mass unit changes of the α-globin (which have no external SH groups) and some doubly nitrosated β-globin. These modifications may not be of physiological significance, since they are improbable at the intracellular concentrations reported for Hb and red blood cell thiols. However, in vitro studies are frequently done at lower Hb concentration and with much higher ratios of CysNO or other NO donors. In such studies the nitrosation of internal thiols could complicate studies of SNO-Hb biochemistry due to the functional repercussions of interfering with the interfacial regions of the Hb tetramer.

Fig. 3 shows effects of metal contaminants on the reaction of Hb and CysNO. NO-Hb and met Hb are spectrally obvious products that result from the metal-catalyzed disruption of the RSNO linkage of CysNO that liberates NO. NO-Hb forms when the liberated NO is captured at deoxy heme sites, while met Hb and nitrate form when the liberated NO reacts with oxyHb (Eqs. (2) and (4)). Less spectrally obvious effects are evident in mass spectrometric studies. As shown in Fig. 3, the presence of contaminating metals can result in appreciable disulfide formation between SH groups of Cys and Hb (as in Eq. (3)). Mass peaks of NO

Table 1
CysNO effects on Hb $A_0$, Hb S and Bottlenose Dolphin Hb

| Protein | μM Hb | μM CysNO | % *S*-nitrosation | % *S*-cystinylation |
|---|---|---|---|---|
| Hb $A_0$ + DPTA | 25 | 250 | 36 | none |
| Hb $A_0$ + DPTA | 33 | 333 | 50 | none |
| Hb $A_0$ + DPTA | 50 | 500 | 58 | none |
| Hb $A_0$ | 50 | 500 | 73 | 20 |
| Hb S | 50 | 500 | 71 | 26 |
| Bottlenose Dolphin Hb | 50 | 500 | 58 | 8 |

The table shows responses of these structurally distinct Hbs to 10 min of exposure to varied levels of CysNO. ESI-MS data revealed the influence of protein type and of the presence or absence of metal chelator (0.1 M DPTA) on the percentages of SNO-Hb and *S*-cystinylated Hb formed as a result of this treatment.

adducts on β chains appear, along with mass peaks associated with cystinylation of the β-chains. These products can be largely avoided by use of metal chelators.

### *3.3. CysNO reactions with Hb $A_0$, Hb S, and Bottlenose Dolphin Hb*

The differences between met and oxy Hb in *S*-nitrosation reactions [1] could be due to conformational differences or differences in β93 SH-group reactivity due to differences in the electron affinity of the nearby β-chain heme groups (see Section 4). To clarify this issue, the impact of varied heme-group redox potential was investigated, using Hb $A_0$, Hb S and Bottlenose Dolphin Hb as transnitrosation targets. Both Hb S (Sickle Cell Hb: β6 Glu → Val) and Hb of the Bottlenose Dolphin (*Tursiops truncatus*) have appreciably shifted redox potentials as shown by Nernst plots of the oxidation process, with increased ease of anaerobic oxidation relative to Hb $A_0$ [31]. Improved methods of ESI-MS allowed us to follow both *S*-nitrosation and cystinylation reactions in Hb $A_0$ and in the two redox-shifted Hbs. Table 1 documents the dose and Hb-dependent changes in levels of β-chain NO adducts and the chelator-dependent levels of cystinylation that resulted from exposure of varied types of Hb to CysNO.

In reactions without added metal chelators the cystinylation reaction induced by exposure to CysNO is approximately half as effective as *S*-nitrosation (i.e. the fraction of nitrosated β-globin is twice that of cystinylated β-globin throughout the time course of the reaction). The presence of multiple reactions disallowed fitting the reaction time-course to the simple expression shown as Eq. (1). Qualitatively, when low levels of Hb (25–50 μM in heme) are mixed with $2x$–$20x$ excess of CysNO over heme, the reactions have half-times in the order of 2–8 min, with the expected increase in reaction rate at the higher CysNO levels. These are fairly slow reactions and may not be fast enough to form SNO-Hb in the course of Hb transit through the lungs. The release of NO from SNO-Hb by simple transnitrosative transfer of NO to GSH, the dominant low molecular weight thiol of red blood cells, has also been reported to be a fairly slow reaction (also see [2]).

As indicated in Table 1, there are no significant differences between the nature or time course of appearance of CysNO-induced reaction products formed by Hb $A_0$ and Hb S, indicating that their significantly different heme redox potentials do not have a major effect on SH reactivity and subsequent SNO-Hb formation. Data obtained using spectral deconvolution assays to monitor SNO-Hb formation in Hb $A_0$ and Hb S supported this conclusion i.e. equivalent levels of spectrally detectible SNO-Hb were generated for these proteins under a given protocol in triplicate assays. However, as shown in Table 1, Bottlenose Dolphin Hb, with a redox potential like that of Hb S, forms appreciably less ($\sim$0.8) the amount of SNO-Hb after a 10-min exposure to a tenfold excess of CysNO than either of the human Hbs, indicating that differences in structurally similar Hbs can affect the extent of SNO-Hb formed via transnitrosation reactions between Hb and low molecular weight thiols.

### 3.4. A new spectroscopic approach to following SNO-Hb formation or degradation

We investigated the possibility that the spectral changes associated with increased or decreased oxygen binding as the *S*-nitrosation of Hb varies could be used as an indirect method to monitor fast reactions of formation or degradation of SNO-Hb. The basic concept is that, at fixed and relatively low levels of oxygen, the reactions that form SNO-Hb will be followed by increased $O_2$ binding and a readily-measured change in absorbency.

To evaluate the effectiveness of this approach, we added a twofold excess of CysNO to a solution of partially oxygenated Hb in a large volume tonometer. A relatively fast (<1 min) absorbance change occurs after CysNO addition, indicative of a transition of Hb to a more oxygenated condition as CysNO transfers NO to the SH groups of Hb and creates SNO-Hb. Following this, there are slower (10–30 min) absorbance changes as the light beam of the measuring instrument causes the release of NO from *S*-nitrosated compounds. As expected (Eq. (4)), the NO released results in the formation of NO-Hb, with some met Hb formation resulting from reaction of NO with oxy Hb. A multi-component curve-fitting program (supplied by the manufacturer of the Hewlett-Packard Spectrophotometer) enabled us to resolve these contributions to the absorbance changes. We were pleased to find that even in the relatively bright light beam of the diode-array spectrophotometer, the photodecomposition process was relatively slow compared to the transnitrosation reaction. To make use of this approach, as in other spectral assays of RSNO compounds, care must be taken to use low-intensity detection beams to avoid complications associated with the photosensitivity of the RSNO bond.

### 3.5. Formation of SNO-Hb at high (intraerythrocytic) concentration with low met Hb content

Modification of previously published procedures was required to generate SNO-Hbs at high (5–20 mM) concentration without excessive met Hb formation. The transnitrosation reactions used to create variably *S*-nitrosated preparations of Hb at either high or low concentration have the net result shown simplistically in the equilibrium representation given by Eq. (1) of Section 1. CysNO was the low molecular weight NO donor used for studies reported here. Concentrated samples of SNO-Hb $A_0$ and Hb S with low met Hb content (<5% oxidized heme) were obtained by carrying out all preparative procedures at 4 °C, minimizing light exposure, and using metal chelators to avoid metal-catalyzed decomposition of the S-NO linkage. The nitrosation of β93 SH-groups was accomplished by exposure of 1–3 mM Hb to a fourfold excess of CysNO for 2–3 min. The CysNO was removed by passage through a Sephadex G-25 column, and the eluted SNO-Hb was then concentrated for use in studies that approximate those found in vivo. In spite of these precautions, many samples that took weeks to prepare were discarded prior to or after oxygen binding determinations due to excessive (>12%) met Hb formation.

Our attempts to *S*-nitrosate samples at intraerythrocytic concentrations led to protein precipitation and excessive met Hb formation. As noted in Section 2, SNO-Hb samples with low (<5%) levels of met Hb were generated (at 1–3 mM heme), concentrated, and then mixed with unmodified concentrated Hb samples to obtain solutions with the desired levels of *S*-nitrosation. The initial mixtures of concentrated SNO-Hb thus contained tetramers with both β93 groups nitrosated, along with tetramers with unmodified SH groups. Subsequent transnitrosation reactions may have randomized the occurrence of *S*-nitrosated groups on Hb tetramers in solution, but this was not ascertained.

### 3.6. Stability of concentrated SNO-Hb upon deoxygenation

The extent of *S*-nitrosation of Hb samples after removal of the low molecular weight NO donor was determined by spectral deconvolution analysis under standard conditions (with samples at approx. 60 μM heme in 2% borate, 0.1 mM DPTA buffer, pH 9.2), or by electrospray mass spectrometry analysis (see Section 2).

Assays to determine the extent of *S*-nitrosation were done on sample aliquots taken from partially

*S*-nitrosated solutions at heme concentrations of 10–20 mM. Aliquots were also removed from the thin membrane sandwich in the Hemoscan *after* determinations of oxygen-binding curves, to assay the consequences of a full oxy–deoxy–oxy cycle. The assay results showed no significant loss of NO from SNO-Hb samples. Estimates of SNO-Hb by mass spectrometry values were within 10%, and spectral deconvolution assays gave values of $\pm 5\%$ for the level of *S*-nitrosation before and after oxygen binding determinations. The variance observed before and after deoxygenation with these methods is within the experimental error typical for these assays. Thus high Hb concentration is not sufficient to cause a significant loss of NO from the SNO-Hb derivative during deoxygenation.

### *3.7. Effects of S-nitrosation on oxygen binding by Hb $A_0$ at high (10–20 mM heme) concentrations*

Studies of the effects of *S*-nitrosation at Hb concentrations like those in red blood cells have not previously been reported. Representative oxygen binding curves for Hb $A_0$ (closed symbols) and its *S*-nitrosyated derivative (open circles) carried out with the Hemoscan at high (10 mM heme) protein concentration are shown in Fig. 4. The effect of *S*-nitrosation at these concentrations is to increase the affinity of oxygen binding, an effect similar to that reported in earlier studies. The shift in oxygen binding parameters associated with *S*-nitrosation for 5–20 mM samples of Hb $A_0$, with $\Delta \log P_{50}$ ranging from 0.19 to 0.3 in ten separate experiments, is similar to that previously reported at lower ($\sim$0.1 mM heme) concentrations where $\Delta \log P_{50} \cong 0.25$ [22,2]. The shift observed in samples at high concentration shows appreciable scatter, possibly due to greater variations in met Hb formation.

### *3.8. S-nitrosation effects on oxygen binding by Hb S at high (5–20 mM) protein concentration*

Oxygen binding parameters for normal and partially *S*-nitrosated solutions of purified Hb S were determined at 19 mM heme, well above the protein's minimum gelling concentration. As shown in representative Hill plots in Fig. 5, 30 and 60% *S*-nitrosated samples have significantly increased oxygen affinity compared to unmodified Hb S. The level of *S*-nitrosation of a 20 mM Hb S sample was 77.6% before oxygen binding, and 75.6% afterwards. Thus, as found for SNO-Hb $A_0$, deoxygenation does not trigger NO release from SNO-Hb S, even under conditions that allow for polymerization of the deoxy-state of Hb S. The Hill plots of oxygen binding show greater changes due to *S*-nitrosation at low oxygen saturation ($\sim$10%) than at high saturation ($>$50%), as expected and observed for R-state stabilization

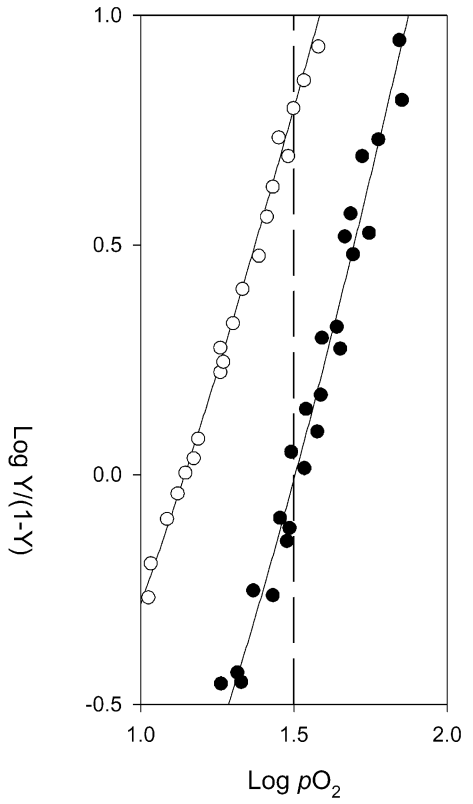


Fig. 4. Oxygen binding by 10 mM Hb $A_0$ (filled circles) and its *S*-nitrosated derivative (open circles). Both conditions were examined with the protein in a thin double-membrane-sandwich in a Hemoscan as described in Section 2. The proteins were in 0.05 M HEPES buffer at pH 7.5, 20 °C. At least three separate determinations of the normal and *S*-nitrosated condition are represented. Spectral deconvolution assays indicated that $\sim 70 \pm 5\%$ of the β93Cys groups were nitrosated in the modified samples before and after oxygen binding.

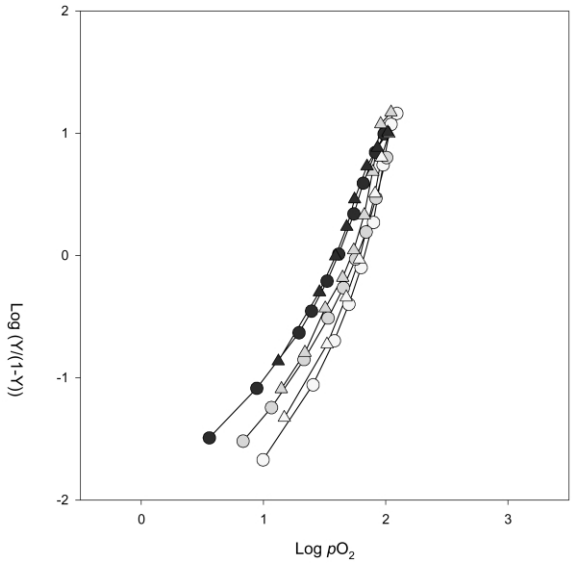


Fig. 5. Hill plots of duplicate determinations (circles and triangles) of oxygen binding by 19 mM solutions of Hb S (far right) and 60% SNO-Hb S (far left) and 1:1 mixtures of these (middle curves) where 30% of the β93 SH-groups are modified. Oxygen binding was examined with the protein enclosed in a thin double-membrane-sandwich in a Hemoscan as described in Section 2. The samples were in $KPO_4$ with 0.5 mM KEDTA, pH 6.8 at 37 °C and were maintained in the deoxygenated condition for 20–25 min prior to step-wise introductions of air.

brought about by thiol modification by other SH reagents [22,32].

There is a concentration-dependent effect in oxygenation of Hb S that is affected by *S*-nitrosation that we attribute to decreased polymer formation in the *S*-nitrosated samples. This is shown in Fig. 6, which presents data for 30 and 60% SNO-Hb S along with data on unmodified Hb S over a range of protein concentrations. The data was obtained using the Hemoscan in a step-wise mode, using the same solution conditions (samples at pH 6.8 in potassium phosphate buffer, 37 °C) used by Benesch et al. [18], in which the heme concentration dependence of oxygen-binding data was shown to provide an indirect measure of the protein's minimum gelling concentration. Our data for unmodified Hb S (open circles) is in general agreement with the data reported by Benesch et al. (filled circles). Our data has more scatter than shown in the earlier papers, but shows the same dramatic increase in $P_{50}$ values above the minimum gelling concentration of Hb S (approx. 12.5 mM under these conditions) as previously reported by Benesch et al. [18].

We were disappointed to find that highly SNO-Hb S shows a concentration-dependent increase in apparent oxygen affinity, indicating that polymerization still occurs at high heme concentration. However, the data of Fig. 6 suggests that an increase in the minimum gelling concentration is brought about by *S*-nitrosation. The shift appears to be from ~12.5 for the unmodified protein to ~15.5 mM for the 30% *S*-nitrosated samples under these measuring conditions, with a less well-

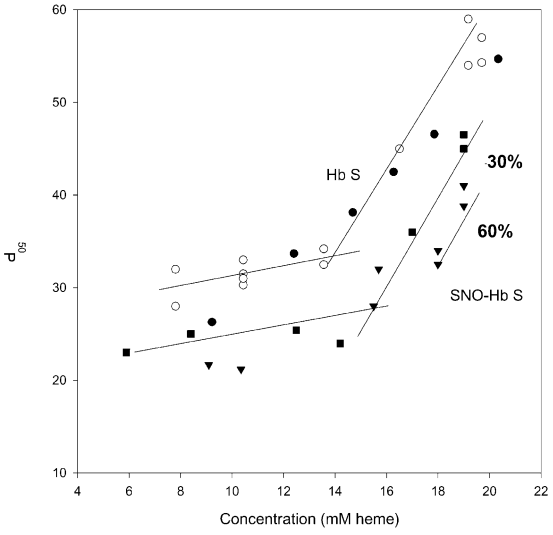


Fig. 6. Effects of *S*-nitrosation and protein concentration on oxygen binding by Hb S and its partially *S*-nitrosated derivatives. Purified samples of Hb S at the indicated concentrations were in 0.1 M $KPO_4$ buffer containing 0.05 mM EDTA or 0.1 mM DPTA, pH 6.8, 37 °C. Oxygen binding data were obtained with a modified Hemoscan apparatus as described in Section 2. The filled circles of the plot are from Benesch et al. [18], while open circles show our more recent data obtained under the same experimental conditions but with a step-wise mode of Hemoscan operation. The slope-change at ~12.5 mM for unmodified Hb S provides an estimate of the minimum gelling concentration of the unmodified protein under these conditions (see text). *S*-nitrosation effects are shown for samples with ~30% β93 SH-groups modified (filled squares) and for ~60% β93 SH-groups modified (filled triangles).

defined shift in minimum gelling concentration for 60% *S*-nitrosated samples.

The concentration-dependent increase in apparent oxygen affinity shown in Fig. 6 for Hb S was not seen with samples of Hb $A_0$. Our use of a step-wise mode (instead of constantly increasing oxygen levels) reduces dynamic error and probably accounts for the more constant level of apparent affinity of our data compared to those of Benesch et al. [18] at concentrations below 12.5 mM, prior to onset of polymer formation by deoxygenated Hb S. The Hemoscan measures oxygen binding by Hb held in a thin double-membrane sandwich by allowing a measured quantity of air into its sample chamber, which results in an increase in oxygen tension that is experienced by the sample and the measuring oxygen electrode. The spectral response as the deoxygenated protein becomes progressively more oxygenated can be somewhat delayed by the permeation and binding of oxygen to Hb in the membrane sandwich. Delay of the sample response results in a dynamic error, which can usually be compensated by use of the step-wise (rather than continuous) mode of operation of the Hemoscan, allowing the spectral changes in the sample to reach equilibrium [26]. Polymerization of deoxygenated Hb S, which occurs above the minimum gelling concentration, introduces a large dynamic factor and considerable hysteresis into measurements of oxygen binding by the Hemoscan and related methods. The absence of a true equililbrium in Hemoscan (or related types of assays) can be readily observed for Hb S at high concentrations by keeping the oxygen tension constant at a point where the oxygen affinity *appears* to be greatly reduced. Slow spectral changes in the direction of greater oxygen binding occur (as Hb S polymers slowly melt), showing that the solution is not truly at equilibrium.

The '$P_{50}$' determinations under these conditions give apparent values of the oxygen binding process, and are not true equilibrium measurements. Accordingly, the values of '$P_{50}$' of Fig. 6 can be influenced by the time the samples are maintained in a deoxygenated condition, as well as the time allowed for 'equilibrium' to be established after an increase in oxygen levels. As shown by the good agreement between replicate curves in Fig. 5, we were able to achieve reasonably consistent data even at high (19 mM heme) concentrations by holding samples for 20–45 min periods in the deoxygenated condition, and using 10 min equilibration steps after each increase of oxygen level. We sought to establish the lowest level effective in altering the inflection point (indicative of minimum gelling concentration), but the data obtained with low ($<30\%$) levels of *S*-nitrosation had too much scatter for this purpose.

### *3.9. Inhibitory effects of S-nitrosation on Hb S gelation*

The effect of *S*-nitrosation on Hb S aggregation/gelation was further tested by direct, visible, observation of gelation after bringing deoxygenated samples of variably nitrosated Hb S at 7 °C up to 37 °C, a temperature switch that is known to enable formation of Hb S polymers. Assays of sample aliquots before and after these studies showed increased met formation, but no appreciable change in level of *S*-nitrosation (values typically within $\pm 10\%$).

Table 2 summarizes the gelation behavior observed with deoxygenated normal and SNO-Hb S samples at varied protein concentrations. As indicated in Table 2, the presence of 30% SNO-Hb S significantly decreases Hb S polymerization relative to controls for samples at 12.5 mM heme, and results in incomplete gelation at 15 mM heme. At higher protein concentration (samples at 19 mM heme) even 60% *S*-nitrosated samples form gels that disallow movement of a stirring bar in the chamber. These results were consistently obtained for matched sets of normal and SNO-Hb S samples (equal concentrations and levels of met Hb) under the specified conditions. Considerable met Hb can form as samples are stirred for an hour or more at 37 °C, but use of matched *S*-nitrosated samples and controls in these studies allowed us to determine that the inhibition of gelation was attributable to *S*-nitrosation rather than met Hb formation. These results are in accord with the oxygen binding data, and support our hypothesis that *S*-nitrosation opposes gelation of deoxygenated Hb S.

Table 2
Effects of *S*-nitrosation on Hb S gelation

| Degree of *S*-nitrosation of Hb S (%) | 10 mM Hb | 12.5 mM Hb | 15 mM Hb | 19 mM Hb |
|---|---|---|---|---|
| 0 | − | ± | + | + |
| ~30 | − | − | ± | + |
| ~60 | − | − | − | + |

Table entries summarize results of visual determinations of the presence or absence of gelation of deoxygenated Hb S at varied Hb concentrations with and without *S*-nitrosation (see Section 2). In the data below, + indicates complete gelation (i.e. stirring bar immobile), ± indicates incomplete gelation, and − indicates no appearance of Hb S polymer. Hb S for these studies was in 0.1 M $KPO_4$, pH 6.8. Samples with ~60% SH groups *S*-nitrosated were generated by exposure of 1–3 mM samples to 2:1 ratios of CysNO to heme at 4 °C for 5 min and concentration to the heme levels indicated. Samples with ~30% SH groups *S*-nitrosated were generated by 1:1 mixing of the 60% samples with purified Hb S. Samples were matched for degree of met Hb, which was less than 12%, although samples with met Hb levels as high as 35% gave similar results. Aliquots from the samples showed no significant loss of SNO-Hb during these studies.

## 4. Discussion

Knowledge of SNO-Hb formation pathways is important for development of possible therapeutic applications of NO. Both oxygen-mediated and transnitrosative pathways for SNO-Hb formation are sensitive to the availability of Hb's SH groups, which are known to be sensitive to the quaternary T↔R equilibrium of Hb [33]. Functional and crystallographic studies have shown that the β93Cys residues at which NO is bound in Hb are more accessible in the high affinity conformation of oxy (R-state) Hb than in deoxy (T-state) Hb [33–35]. The quaternary conformation of the protein in the R-state thus allows for more effective formation of SNO-Hb in oxygenated relative to deoxygenated Hb.

Electronic modes of communication between the SH and heme groups of Hb also appear significant in making *S*-nitrosation of liganded Hb more facile than for the deoxy protein. An electron-exchange aspect of the heme-SH interaction of significance to Hb's interactions with NO was indicated by our finding that oxidized (met) Hb is more reactive with CysNO than oxy Hb and that its β chains are more readily nitrosated [1]. These results on SH-group reactivity in Hb complement studies of the redox chemistry of low molecular weight *S*-nitrosothiols [30]. Electron-exchange possibilities of Hb may affect reactivity of its SH groups in ways not possible with the low molecular weight thiols. In particular, an electron exchange pathway between β-heme and β-SH groups exists [25] and electronic communication of events at the heme could influence the SH groups where *S*-nitrosation occurs. Moreover, our recent work has shown that the oxidized (met Hb) form of SNO-Hb is thermodynamically favored over deoxy SNO-Hb, and thus may be the form from which NO bioactivity is recovered [31]. The possibility of an effective electronic control of the reactivity of the SH group by events at the heme site was tested by comparing SNO-Hb formation in Hbs with varied redox potentials. Since levels or rates of SNO-Hb formation did not correlate with redox potential, we are led to conclude that steric effects outweigh electronic contributions associated with the state of the heme.

Our ESI-MS data confirms and extends earlier reports [1,27,4,29,30] that low molecular weight nitrosated thiols (RSNO species) will react with Hb and can transfer NO to its thiol groups. As shown in Fig. 2 and Fig. 3, the nitrosation of β93Cys is favored over reaction with internal (buried) thiols, but cystinylation occurs along with nitrosation if metal contaminants are not excluded from the reaction. The metal-enhanced formation of SNO-Hb and cystinylated derivatives appears to follow oxygen-mediated pathways as shown in Eq. (2) and Eq. (5).

Cystinylation along with nitrosation of Hb was previously noted in studies of SNO-Hb [36] and was considered a necessary part of *S*-nitrosation events associated with exposure of Hb to nitrosated glutathione (GSNO). The 'SNO-Hb' characterized by these authors had 43% of the β chains SH groups mono-nitrosated and 28% cystinylated. It is important to note that the size and nature of the SH modifier alters Hb function [37], so that this 'SNO-Hb' is not equivalent to the SNO-Hb we characterized [22] in which the β93 SH-group was the only SH group nitrosated and the cystinylation reaction was avoided.

In vivo mechanisms for recovery of NO from SNO-Hb are a matter of on-going debate. As we first hypothesized and later demonstrated, the NO of SNO-Hb cannot be accommodated in the normal deoxy (T) structure of Hb [1,22]. This constraint on the normal R↔T equilibrium could facilitate NO release to tissues at low oxygen pressures as originally suggested [1]. We showed in subsequent studies, however, that purified solutions of SNO-Hb can be cycled between oxy and deoxy states *without* loss of NO [22]. In this report we show that this is also true for SNO-Hb at intraerythrocytic concentrations. Thus high Hb concentration is not sufficient to cause a significant loss of NO from the SNO-Hb derivative during deoxygenation of purified Hb $A_0$ or Hb S.

In vivo triggers of NO release from SNO-Hb are still under investigation. We documented that absorbance changes associated with the shift in oxygen affinity associated with *S*-nitrosation of Hb can be observed when the oxygen tension is held constant. This constitutes a new method of following the reaction that can be used to monitor the formation or degradation of SNO-Hb under varied conditions. This new approach may prove useful in clarifying the triggering mechanisms operative in vivo for NO release from SNO-Hb.

Researchers in several laboratories have shown that Hb $A_0$ and Hb S have identical oxygen affinities and responses to 2,3-diphosphoglycerate at low ($<1$ mM) heme concentration. We confirmed this finding, and demonstrated that *S*-nitrosation due to CysNO exposure occurs at equal rates for purified Hb $A_0$ and Hb S and that the resulting R-state shifts associated with *S*-nitrosation for Hb $A_0$ and Hb S are also the same within experimental error.

Hb $A_0$ and Hb S show pronounced differences at high concentration, where deoxygenation results in aggregation of Hb S but not Hb $A_0$ (see reviews by Eaton and Hofrichter [15,16]). For unmodified Hb S, the formation of Hb S polymers in phosphate buffers at concentrations like those in red cells is accompanied by decreases in the protein's apparent oxygen affinity. Using the continuous mode of Hemoscan operation, Benesch et al. [18] showed that polymer formation by Hb S at high (intracellular) Hb concentrations shifts the apparent $P_{50}$ for oxygen binding to higher oxygen levels than required for half saturation at lower concentrations [18]. Benesch et al. were careful to point out that the binding curves for concentrated solutions of Hb S obtained under continuously changing oxygen tensions have considerable hysteresis, and thus do not reflect true ligand-binding equilibria. The advantage to the non-equilibrium measurements done with the Hemoscan or related approaches, as pointed out by Benesch et al, is that a titration of concentration effects on oxygen binding by Hb S can be used as an effective micro-method for estimation of its minimum gelling concentration.

The indirect Benesch technique was used to study the influence of *S*-nitrosation on Hb S polymer formation. The oxygen-binding parameters we obtained for Hb $A_0$ and Hb S at high concentration by use of this method had more scatter than anticipated from earlier published results. However, informative qualitative results were obtained in spite of this difficulty. The representative data shown in Fig. 5 and Fig. 6 clearly show that concentrated samples exhibit an R-state stabilization of Hb S brought about by *S*-nitrosation, and suggest that this effect increases the minimum gelling concentration. This indirect evidence was supported by direct observation of the effects of *S*-nitrosation on deoxy Hb S gelation. The combined data indicate an increase of the minimum gelling concentration from $\sim$12.5 to $\sim$15.5 mM associated with 30% *S*-nitrosation under our measuring conditions. This roughly brackets the effectiveness of this type of modification, which was less than hoped for at these high levels of *S*-nitrosation. Further study will be required to determine if *S*-nitrosation can be used for effective inhibition of the sickling process in red blood cells.

It has long been recognized that it is the quaternary conformation assumed by Hb when deoxygenated, the T-state, that favors aggregation of Hb S (see discussion by Nagel and Bookchin [38]). Heme ligation opposes Hb S polymer formation by favoring the protein's R-state conformation. NO ligation of heme groups appeared to be an exception, since polymerization of fully nitrosylated Hb S was reported by Briehl and Salhany [39]. This was subsequently shown to be due to the fact

that the studies of Briehl and Salhany were done in the presence of inositol hexaphosphate, which strongly stabilizes the T-state of NO-Hb [40,33].

R-state stabilization resulting from SH-group modifications of Hb was documented in earlier studies [32]. Several types of Hb S modifications (carbamolylation or acetylation, Schiff-base adducts, and SH-group modifications) favor the R-state conformation and have been shown to inhibit Hb S aggregation (see Abraham et al. [41] and reviews by Eaton and Hofrichter [15,16]). It has also been shown that formation of mixed disulfides by covalent attachment of glutathione or cysteine to the cysteine groups at the β93 position significantly favors the R-state of Hb S and inhibits polymer formation [42,43]. Garel et al. [20] showed that eight different thiol reagents could cross the red cell membrane, react with β93 SH-groups, and inhibit sickling of erythrocytes containing Hb S. The degree of inhibition induced by these compounds showed a high correlation with the extent of increase of apparent oxygen affinity of the red blood cells.

The opposition of Hb S gelation by *S*-nitrosation of Hb S reported here is thus in accord with the behavior of Hb S modified by other sulfhydryl reagents, where increases in oxygen affinity and inhibition of aggregation are associated with stabilization of Hb's R-state conformation. Further studies are required to quantify this effect and determine the level of *S*-nitrosation necessary for effective inhibition of red blood cell sickling. It also remains to be determined if low levels of *S*-nitrosation would sufficiently increase the delay time for polymerization to provide a therapeutic effect. Both kinetic and equilibrium aspects of the gelation process have to be considered in development of effective anti-sickling agents. How modifiers of Hb S affect the kinetics of polymer formation may be of paramount importance, as pointed out by Eaton and Hofrichter [15,16]. There is a general linkage between equilibrium and kinetic aspects of polymer formation, but quantitative assessments of altered delay times have not been addressed in this exploratory study of *S*-nitrosation effects on Hb S polymer formation. It is encouraging, however, to consider that NO-Hb, SNO-Hb and NO-induced met Hb could each contribute in its own way to R-state stabilization that opposes Hb S aggregation. A combination of NO-based therapies thus merits further study as a promising new approach to relieving the symptoms of sickle cell disease.

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## References

[1] L. Jia, C. Bonaventura, J. Bonaventura, J.S. Stamler, *S*-nitrosohaemoglobin: a dynamic activity of blood involved in vascular control, Nature 380 (1996) 221–227.

[2] R.P. Patel, N. Hogg, N.Y. Spencer, B. Kalyanaraman, S. Matalon, V.M. Darley-Usmar, Biochemical characterization of human *S*-nitrosohemoglobin: effects on oxygen binding and transnitrosation, J. Biol. Chem. 274 (1999) 15487–15492.

[3] A.J. Gow, J.S. Stamler, Reactions between nitric oxide and haemoglobin under physiological conditions, Nature 391 (1998) 169–173.

[4] A.J. Gow, B.P. Luchsinger, J.R. Pawloski, D.J. Singel, J.S. Stamler, The oxyhemoglobin reaction of nitric oxide, Proc. Natl. Acad. Sci. 96 (1999) 9027–9032.

[5] M.T. Gladwin, A.N. Schechter, J.H. Shelhamer, F.P. Ognibene, The acute chest syndrome in sickle cell disease: possible role of nitric oxide in its pathophysiology and treatment, Am. J. Respir. Crit. Care Med. 159 (1999) 1368–1376.

[6] M.T. Gladwin, F.P. Ognibene, L.K. Pannell, F.S. Nichols, M.E. Pease-Fye, J.H. Shelhamer, A.N. Schechter, Relative role of heme nitrosylation and β-cysteine 93 nitrosation in the transport and metabolism of nitric oxide by hemoglobin in the human circulation, Proc. Natl. Acad. Sci. 97 (2000) 9943–9948.

[7] N. Hogg, V.M. Darley-Usmar, M.T. Wilson, S. Moncada, Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide, Biochem. J. 281 (1992) 419–424.

[8] M. Feelisch, J.S. Stamler, Donors of nitrogen oxides, in: M. Feelisch, J.S. Stamler (Eds.), Methods in Nitric Oxide Research, Wiley, New York, NY, 1996, pp. 71–115.

[9] H. Rubbo, M. Tarpey, B.A. Freeman, Nitric oxide and reactive oxygen species in vascular injury, in: C. Rice-

Evans, B. Halliwell, G.G. Lunt (Eds.), Free Radicals and Oxidative Stress: Environment, Drugs, and Food Additives, Portland Press, London, 1995, pp. 33–45.
[10] J.R. Lancaster, Simulation of the diffusion and reaction of endogenously produced nitric oxide, Proc. Natl. Acad. Sci. 91 (1994) 8137–8141.
[11] W. Martin, J.A. Smith, D.G. White, The mechanism by which hemoglobin inhibits the relaxation of rabbit aorta induced by nitrosodilators, nitric oxide, or bovine refractor penis inhibitory factor, Br. J. Pharmacol. 89 (1986) 563–571.
[12] J.S. Stamler, L. Jia, J.P. Eu, T.J. McMahon, I.T. Demchenko, J. Bonaventura, K. Gernert, C.A. Piantadosi, blood flow regulation by *S*-nitrosohemoglobin in the physiological oxygen gradient, Science 276 (1997) 2034–2037.
[13] N.Y. Spencer, H. Zeng, R.P. Patel, N. Hogg, Reaction of *S*-nitrosoglutathione with the heme group of deoxyhemoglobin, J. Biol. Chem. 275 (2000) 36562–36567.
[14] A. Romeo, A. Filosa, J.A. Capobianco, A. English, Metal chelators inhibit *S*-nitrosation of Cysβ93 in oxyhemoglobin, J. Am. Chem. Soc. 123 (2001) 1782–1783.
[15] W.A. Eaton, J. Hofrichter, Hemoglobin S gelation and sickle cell disease, Blood 70 (1987) 1145–1266.
[16] W.A. Eaton, J. Hofrichter, Sickle cell hemoglobin polymerization, Adv. Protein Chem. 40 (1990) 63–279.
[17] C.A. Head, C. Brugnara, R. Martinez-Ruiz, R.M. Kacmarek, K.R. Bridges, D. Kuter, K.D. Bloch, W.M. Zapol, Low concentrations of nitric oxide increase oxygen affinity of sickle erythrocytes in vitro and in vivo, J. Clin. Invest. 100 (1997) 1193–1198.
[18] R.E. Benesch, R. Edalji, S. Kwong, R. Benesch, Oxygen affinity as an index of hemoglobin S polymerization: a new micromethod, Anal. Biochem. 89 (1978) 162–173.
[19] R.M. Bookchin, Y. Uneda, R.L. Nagel, L.C. Landau, Functional abnormalities of whole blood in sickle cell anemia, in: W.S. Caughey (Ed.), Biochemical and Clinical Aspects of Hemoglobin Abnormalities, Academic Press, New York, NY, 1978, pp. 57–66.
[20] M.C. Garel, C. Domenget, F. Galacteros, J. Martin-Carubi, Y. Beuzard, Inhibition of erythrocyte sickling by thiol reagents, Mol. Pharmacol. 26 (1984) 559–565.
[21] R.M. Winslow, Blood oxygen equilibrium studies in sickle cell anemia. in: J.I. Hercules, G.L. Cottam, M.R. Waterman, A.N. Schechter (Eds.), Proceedings of the Symposium on Molecular and Cellular Aspects of Sickle Cell Disease, DHEW. Pub. No. 76-1007, 1976, pp. 235–275.
[22] C. Bonaventura, G. Ferruzzi, S. Tesh, R.D. Stevens, Effects of *S*-nitrosation on oxygen binding by normal and sickle cell hemoglobin, J. Biol. Chem. 274 (1999a) 24742–24748.
[23] C. Bonaventura, S. Tesh, K.M. Faulkner, D. Kraiter, A.L. Crumbliss, Conformational fluctuations in deoxy hemoglobin revealed as a major contributor to anionic modulation of function through studies of the oxygenation and oxidation of hemoglobins $A_0$ and deer lodge β2(NA2)His→Arg, Biochemistry 1998 (37) (1997) 496–506.
[24] A.F. Riggs, R.A. Wolbach, Sulfhydryl groups and the structure of hemoglobin, J. Gen. Physiol. 39 (1956) 585–605.
[25] C. Bonaventura, G. Godette, S. Tesh, D.E. Holm, J. Bonaventura, A.L. Crumbliss, L.L. Pearce, J. Peterson, Internal electron transfer between hemes and Cu(II) bound at cysteine β93 promotes methemoglobin reduction by carbon monoxide, J. Biol. Chem. 274 (1999b) 5499–5507.
[26] G.N. Lapennas, J.M. Colacino, J. Bonaventura, Thin-layer methods for determination of oxygen binding curves of hemoglobin solutions and blood, in: E. Antonini, L. Rossi-Bernardi, E. Chiancone (Eds.), Methods of Enzymology, Academic Press, New York, NY, 1981, pp. 449–470.
[27] P. Ferranti, A. Malorni, G. Mamone, N. Sannolo, G. Marino, Characterization of *S*-nitrosohaemoglobin by mass spectrometry, FEBS 400 (1997) 19–24.
[28] D.J. Barnett, J. McAninly, D.L.H. Williams, Transnitrosaton between nitrosothiols and thiols, J. Chem. Soc. Perkin. Trans. 2 (1994) 1131–1133.
[29] R. Rossi, L. Lusini, F. Giannerini, D. Giustarini, G. Lungarella, P. De Simplicio, A method to study kinetics of transnitrosation with nitrosoglutathione: reactions with hemoglobin and other thiols, Anal. Biochem. 254 (1997) 215–220.
[30] N. Arulsamy, D.S. Bohle, J.A. Butt, G.J. Irvine, P.A. Jordan, E. Sagan, Interrelationships between conformational dynamics and the redox chemistry of *S*-nitrosothiols, J. Am. Chem. Soc. 121 (1999) 7115–7123.
[31] C.H. Taboy, K.M. Faulkner, D. Kraiter, C. Bonaventura, A.L. Crumbliss, Concentration-dependent effects of anions on the anaerobic oxidation of hemoglobin and myoglobin, J. Biol. Chem. 275 (2000) 39048–39054.
[32] K. Imai, H. Morimoto, M. Kotani, H. Watari, W. Hitata, M. Kuroda, Studies of the function of abnormal hemoglobins. i. an improved method for automatic measurement of the oxygen equilibrium curve of hemoglobin, Biochim. Biophys. Acta 200 (1970) 189–196.
[33] M.F. Perutz, J.V. Kilmartin, K. Nagai, A. Szabo, S.R. Simon, Influence of globin structures on the state of the heme. Ferrous low spin derivatives, Biochemistry 15 (1976) 378–387.
[34] E. Antonini, M. Brunori, Hemoglobin and myoglobin in their reactions with ligands, North-Holland, Amsterdam, 1971.
[35] L.L. Chan, P.H. Rogers, A. Arnone, Crystal structure of the *S*-nitroso form of liganded hemoglobin, Biochemistry 37 (1998) 16459–16464.
[36] M. Wolzt, R.J. MacAllister, D. Davis, M. Feelisch, S. Moncada, P. Vallance, A.J. Hobbs, J. Biol. Chem. 274 (1999) 28983–28990.
[37] I. Khan, F. Dantsker, U. Samuni, A.J. Friedman, C. Bonaventura, B. Manjula, S.A. Acharya, J.M. Friedman,

Modified hemoglobin: kinetic and conformational consequences, Biochemistry 40 (2001) 7581–7592.
[38] R.L. Nagel, R.M. Bookchin, Mechanisms of Hb S gelation, in: R.D. Levere (Ed.), Sickle Cell Anemia and Other Hemoglobinopathies, Academic Press, Inc, San Francisco, 1975.
[39] R.W. Briehl, J.M. Salhany, Gelation of sickle hemoglobin. III. Nitrosyl hemoglobin, J. Mol. Biol. 96 (1975) 733–743.
[40] A. Szabo, M.F. Perutz, Equilibrium between six- and five-coordinated hemes in nitrosylhemoglobin: interpretation of electron spin resonance spectra, Biochemistry 15 (1976) 4427–4428.
[41] D.J. Abraham, M.F. Perutz, S.E.V. Phillips, Physiological and X-ray studies of potential antisickling agents, Proc. Natl. Acad. Sci. 80 (1983) 324–328.
[42] W. Hassan, Y. Beuzard, J. Rosa, Inhibition of erythrocyte sickling by cystamine, a thiol reagent, Proc. Natl. Acad. Sci. 73 (1976) 3288–3292.
[43] M.C. Garel, Y. Beuzard, J. Thillet, C. Domenget, J. Martin, F. Galacteros, J. Rosa, Binding of 21 thiol reagents to human hemoglobin in solution and in intact cells, Eur. J. Biochem. 123 (1982) 513–519.
[44] A.M. Atz, D.L. Wessel, Inhaled nitric oxide in sickle cell disease with acute chest syndrome, Anesthesiology 87 (1997) 988–990.
[45] W. Poillon, B.C. Kim, Q. Castro, Intracellular hemoglobins polymerization and the clinical severity of sickle cell anemia, Blood 9 (1998) 1777–1783.