Effects of S-Nitrosation on Oxygen Binding by Normal and Sickle Cell Hemoglobin*

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S-Nitrosated hemoglobin (SNO-Hb) is of interest because of the allosteric control of NO delivery from SNO-Hb made possible by the conformational differences between the R- and T-states of Hb. To better understand SNO-Hb, the oxygen binding properties of S-nitrosated forms of normal and sickle cell Hb were investigated. Spectral assays and electrospray ionization mass spectrometry were used to quantify the degree of S-nitrosation. Hb A0 and unpolymerized Hb S exhibit similar shifts toward their R-state conformations in response to S-nitrosation, with increased oxygen affinity and decreased cooperativity. Responses to 2,3-diphosphoglycerate were unaltered, indicating regional changes in the deoxy structure of SNO-Hb that accommodate NO adduction. A cycle of deoxygenation/reoxygenation does not cause loss of NO or appreciable heme oxidation. There is, however, appreciable loss of NO and heme oxidation when oxygen-binding experiments are carried out in the presence of glutathione. These results indicate that the in vivo stability of SNO-Hb and its associated vasoactivity depend on the abundance of thiols and other factors that influence transnitrosation reactions. The increased oxygen affinity and R-state character that result from S-nitrosation of Hb S would be expected to decrease its polymerization and thereby lessen the associated symptoms of sickle cell disease.

Hemoglobin (Hb) is of central importance to human health in its role as a respiratory protein. Another chapter in the study of the human health significance of Hb is beginning, focused on NO uptake and delivery by Hb and the role this plays in the control of blood pressure and other NO-dependent reactions. Nitrosation of sulphydryl groups on the Hb tetramer creates S-nitrosated Hb (SNO-Hb), which has been shown to play an important role in NO uptake and delivery (1). S-Nitrosated forms of proteins such as Hb can be formed via interaction with nitrosating agents formed upon interaction of NO and oxygen (NO2) and by NO-exchange reactions (transnitrosations) with nitrosated forms of low molecular weight thiols such as cysteine and glutathione. Conversely, the low molecular weight thiols can act as NO acceptors in transnitrosation reactions where NO is donated by S-nitrosated proteins (2, 3).

Hb-based NO transport via SNO-Hb is significant because it can greatly extend the range of NO-dependent reactions. 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 (4–8). What is learned about Hb-based NO transport will have far-ranging applications in these disparate fields.

The studies reported here concern the oxygen binding properties of variably S-nitrosated adult human hemoglobin (Hb A0) and sickle cell hemoglobin (Hb S) that has a Glu substitution at β6. Although physiological levels of S-nitrosation of Hb are too low for oxygen transport to be significantly affected, the linkage between S-nitrosation and oxygen binding by Hb can affect the stability and subsequent vasoactivity of SNO-Hb. Understanding this linkage is critical for understanding the NO-dependent reactions of Hb in normal and sickle cell erythrocytes, in cell-free Hb-based blood substitutes, or in pharmaceuticals. As will be shown, S-nitrosation of Hb A and unpolymerized Hb S results in increased oxygen affinity via partial stabilization of their high affinity, R-state, conformations. Since R-state-stabilized Hb S does not readily polymerize (9), this finding prompts us to suggest that S-nitrosation of Hb S may be viewed as a possible therapeutic approach to alleviating sickle cell disease.

Functional and crystallographic studies have shown that the Cys-β93 residues at which NO is bound as NO+ in SNO-Hb are more accessible in the high affinity conformation of oxy (R-state) Hb than in deoxy (T-state) Hb (10–12). This conformational sensitivity results in a rate dependence for SNO-Hb formation that mirrors the greater relative exposure of Cys-β93 in conditions that favor the R-state and was invoked to explain the decreased stability of the deoxy form of SNO-Hb (1). Although deoxy SNO-Hb is less stable than the oxy form, we found that purified deoxy SNO-Hb is sufficiently stable to allow oxygen-binding studies to be carried out over a period of several hours. However, as will be documented, loss of NO from SNO-Hb during a cycle of deoxygenation/re-oxygenation can occur under simulated in vivo conditions where NO acceptors such as glutathione are present.

Because the sulphydryl groups at Cys-β93 are in a conformationally sensitive position on the Hb tetramer, the S-nitrosation of Hb would be expected to have heterotropic allosteric effects on ligand binding by the heme groups at the four active sites of the tetramer. The following results document the ex-
istence and nature of these thioesteric effects. We show that S-nitrosation promotes increased oxygen affinity and thus acts in opposition to anionic allosteric effectors that decrease oxygen affinity. Hb A0 and Hb S show similar responses to S-nitrosation, as expected based on their similar structures in the region of Cys-β93 (13). The shift of SNO-Hb forms toward higher oxygen affinity probably involves a regional conformational alteration of the deoxyHb tetramer that prevents His-β146 from making its normal contribution to T-state stability. This was previously shown to be the case in Hb in which the SH groups at Cys-β93 were modified by N-ethylmaleimide (11, 14).

We previously showed that increasing anion levels can modulate Hb function by decreasing the frequency and extent of conformational fluctuations that control the accessibility of the heme groups where oxygen is bound (15). In this report we show that these same considerations underlie the anion-dependence of oxygen binding to SNO-Hb and its decreased stability in the presence of low molecular weight thiols.

**EXPERIMENTAL PROCEDURES**

**Sample Preparation**—Samples of Hb A0 and Hb S were prepared by using the ammonium sulfate method, stripped of organic phosphate cofactors and purified by chromatography as described previously (16). The amounts of oxidized Hb (metHb), oxygenated Hb (oxyHb), and hemichrome were determined by spectral analysis by methods published by Winterbourn and co-workers (17). Samples that contained any detectable hemichrome or greater than 5% metHb were discarded. For experiments with SNO-Hb, the stock Hb solutions, typically 1–3 mM in heme units (Fe porphyrin units), were made just before use and were never frozen. Other experiments where metHb formation was less critical used stock Hb solutions that were stored in liquid nitrogen prior to use. To generate samples with progressively higher levels of metHb, samples were repetitively deoxygenated and re-oxygenated with interuse. To generate samples with progressively higher levels of metHb, the metal chelators used were 0.1 mM DTPA or 0.05 mM EDTA, which usually found for CysNO is due to artifactual contamination of metal-catalyzed formation of disulfides (5). The short half-life of CysNO to Hb for short time intervals in the dark in 2% borate-EDTA or DTPA buffer, pH 9.2, largely avoided metHb formation and nitrosation of internal SH groups. These conditions also minimized metal or light-induced release of NO from CysNO and subsequent NO-induced Hb oxidation. Inclusion of a metal chelator in all buffers was essential to prevent the metal-catalyzed formation of disulfides (5). The short half-life usually found for CysNO is due to artificial contamination of buffers with copper or other redox-active trace metals (21). Free copper and iron in off-the-shelf buffers may frequently be in the nanomolar range, levels sufficient to alter reactions from the pathways they would take in the absence of redox metals.

**RESULTS**

**Formation of Variably S-Nitrosated Hb**—Modification of previously published procedures was required for generation of S-nitrosated Hb suitable for measurements of oxygen binding and anaerobic redox reactions. We found that exposing high (>500 μM) concentrations of oxyHb at 4 °C with low ratios of CysNO to Hb for short time intervals in the dark in 2% borate-EDTA or DTPA buffer, pH 9.2, largely avoided metHb formation and nitrosation of internal SH groups. These conditions also minimized metal or light-induced release of NO from CysNO and subsequent NO-induced Hb oxidation. Inclusion of a metal chelator in all buffers was essential to prevent the metal-catalyzed formation of disulfides (5). The short half-life usually found for CysNO is due to artificial contamination of buffers with copper or other redox-active trace metals (21). Free copper and iron in off-the-shelf buffers may frequently be in the nanomolar range, levels sufficient to alter reactions from the pathways they would take in the absence of redox metals.

**Transnitrosation reactions to create SNO-Hb have the net result shown simplistically in the equilibrium representation below, where R represents a low molecular weight thiol.**

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\text{NO} + \text{Hb} \leftrightarrow \text{RSH} + \text{SNO-Hb}
\]

**Reaction 1**

The extent of S-nitrosation of Hb samples after removal of the low molecular weight NO donor was typically determined by spectral deconvolution analysis under standard conditions as described under “Experimental Procedures.” Fig. 1 shows representative spectra for deoxygenated SNO-Hb. The mixture of deoxyg Hb is NO-Hb that results from dithionite addition, and the final NO-Hb spectrum obtained after NO addition to fully occupy the heme binding sites. The spectrum of deoxygenated SNO-Hb indicates the presence of a low level of metHb that exceeds that of the deoxyg Hb control sample, but shows no evidence of NO ligation of the heme. The amount of NO ligan-
cared to the heme that appears after the NO-linkage to Cys-β3 is disrupted by dithionite is used to estimate the extent of S-nitrosation. For some samples the extent of S-nitrosation was also quantified by the Saville reaction (22, 23), with a standard curve generated with precisely determined quantities of nitrite. The results obtained were within 10% of the values obtained with our deconvolution assays.

The experimental conditions used in our experiments were established by detailed studies of reactions between CysNO and Hb, in which we used an innovative approach to electrospray ionization mass spectrometry that avoided the use of organic solvents. Using this technique, we obtained spectra of deoxygenated SNO-Hb (before dithionite addition) that was greater than that observed with deoxygenated control, which accounts for their peak differences at 556 nm.

We determined the effects of S-nitrosation on oxygen binding by Hb A0 in the presence of 2,3-diphosphoglycerate (DPG) at 50-fold excess over tetramer concentration. The asymmetric shifts in oxygen affinity associated with S-nitrosation are similar in character to those associated with Met-Hb formation. The Hill plots of Fig. 3 show that the R-state shifts induced by metHb formation are similar in character to those associated with S-nitrosation. The shifts associated with S-nitrosation are, however, much larger than can be attributed to Met-Hb formation brought about by S-nitrosation are very similar to alterations reported for Hb in which the SH groups are modified with N-ethylmaleimide.

S-Nitrosylation and Met-Hb Formation—Some metHb is formed during S-nitrosation reactions as a result of NO (liberated from CysNO) interacting with oxygenated Hb. By limiting the levels of CysNO used and time of exposure, it was possible to generate SNO-Hb samples with low levels of metHb for use in oxygen binding experiments. To estimate the effect of increased levels of metHb, we carried out oxygen binding studies with unmodified Hb A0 with progressively higher levels of metHb. The Hill plots of Fig. 3 show that the R-state shifts induced by metHb formation are similar in character to those associated with S-nitrosation. The shifts associated with S-nitrosation are, however, much larger than can be attributed to metHb formation in the SNO-Hb samples. Under our assay conditions, where levels of metHb were minimized, the shifts in log $P_{50}$ attributable to the presence of metHb in the S-nitrosated samples are less than 10% of the shifts observed.

Stability of SNO-Hb—Prior studies have shown deoxy SNO-Hb to be less stable than oxy SNO-Hb (1). Deoxygenation is a necessary step in the oxygenation studies reported here, a condition that was expected to cause some loss of NO due to instability of deoxy SNO-Hb. Our preparations, however, showed no appreciable loss of NO during oxygen binding experiments. As shown in Fig. 4, the preparations of oxy SNO-Hb used in these studies were stable for over a week without loss of NO when stored at 4 °C at high protein concentration. Samples stored in the deoxygenated condition or at lower protein concentration were less stable and had greater loss of NO and more metHb formation. The data shown in Fig. 4 are for aliquots withdrawn from oxy and deoxy samples of about 80% S-nitrosated Hb, 1 mM in heme, held at 4 °C. The aliquots were brought to standard conditions for spectral deconvolution as-

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Fig. 1. Spectra representative of those used in deconvolution assays of S-nitrosation. Spectra shown are for a deoxygenated SNO-Hb sample (dotted line) prepared as described under “Experimental Procedures,” the same after treatment with dithionite (dashed line), and then after exposure to saturating levels of NO (dashed line). The solid line is for a deoxygenated Hb A0 control. The spectrum of deoxygenated SNO-Hb (before dithionite addition) has a greater amount of metHb than the deoxygenated control, which accounts for their peak differences at 556 nm.

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FIG. 2. A portion of the mass spectrum observed for S-nitrosated Hb A₀. The multiply charged state of α-globin + heme exhibits only cationic adduction, whereas β-globin shows 50% conversion to the S-nitrosated form. The m/z assignments are: 1211.7 = [α-globin + heme + 13H⁺]/13; 1213.3 = [α-globin + heme + 12H⁺ + Na⁺]/13; 1214.9 = [α-globin + heme + 12H⁺ + K⁺]/13; 1221.3 = [β-globin + 13H⁺]/13; 1223.5 = [β-globin-SNO + 15H⁺]/13; and 1225.1 = [β-globin + 12H⁺ + K⁺]/13. The Hb was electrosprayed in 0.02 M ammonium bicarbonate, pH 9.04, without use of organic solvents.

FIG. 3. Progressive effects of S-nitrosation and metHb formation on Hill plots of oxygen binding by Hb A₀. Hill plots shown are composites of several experiments. Purified 60 μM Hb samples were in 0.05 M HEPES buffer containing 0.5 mM EDTA or 0.1 mM DTPA, pH 7.5, 20 °C. In A, data are shown for unmodified Hb A₀ (closed circles), and for Hb A₀ with about 30% (open circles), 40% (closed triangles), and 80% (open triangles) of the Cys-93 groups nitrosated. MetHb levels, measured before and after oxygen binding, were 1.5–3%, 2.9–4.2%, 5.1–9%, and 4.8–9.4%, respectively. In B, data obtained with a 50-fold excess of DPG over Hb tetramer are shown for unmodified Hb A₀ (closed circles) and for about 80% S-nitrosated Hb A₀ (open triangles). MetHb levels, measured before and after oxygen binding, were 4.7–6.7% and 7.2–9.7%, respectively. Fig. 3C shows effects of increasing levels of metHb in Hb A₀. MetHb levels, measured before and after oxygen binding, were 1.5–3% (closed circles), 5.1–6.9% (open circles), 5.4–7.7% (closed triangles), 8.6–9.8% (open triangles), and 12.8–15.6% (closed squares).

says as described under “Experimental Procedures.” The stability of SNO-Hb as demonstrated in this figure is important in allowing for its storage or shipment prior to use in functional studies.

Low Molecular Weight Thiols Destabilize S-Nitrosated Hb—In contrast to the stability noted above, appreciable diminution of S-nitrosation of Hb A₀ occurs when experiments are carried out in the presence of a 5-fold excess of reduced glutathione (GSH). The bar graph of Fig. 5 illustrates the destabilizing influence of GSH at several levels of S-nitrosation (achieved by exposure to different levels of CysNO for varied times). Previous work demonstrated that GSH can accept NO from SNO-Hb in transnitrosation reactions (1). Fig. 5 shows that SNO-Hb is partly but not completely destabilized when deoxygenation occurs in the presence of this high level of GSH. In vivo conditions may result in more loss of NO during the deoxygination process. Fig. 5 also shows that the loss of NO from SNO-Hb is roughly the same in the presence and absence of 2,3-diphosphoglycerate. Appreciable increases in metHb levels occur when oxygenation of SNO-Hb is carried out in the presence of glutathione, with metHb levels typically 25–27% after oxygen equilibria, in contrast to <10% metHb in its absence. This is a consequence of GSH interactions with SNO-Hb, since control experiments showed no effect of GSH on oxygen affinity or metHb levels for unmodified Hb A₀ (data not shown).

The increase in metHb probably results from interactions between oxyHb and free NO that is released from either SNO-Hb or S-nitrosated glutathione during oxygen equilibria.

Oxygen Equilibria of Hb S with Varied Levels of Nitrosation of Cys-93—We determined the effects of S-nitrosation on oxygen binding by purified Hb S in HEPES buffer containing EDTA or DTPA and in the presence of a 50-fold excess of 2,3-diphosphoglycerate over tetramer concentration. No aggregation of Hb S was expected or observed upon deoxygenation at the concentrations (60 μM in heme) used in these experiments. Aggregation-dependent effects that are evident at much higher protein concentration were not studied. The representative results presented in Fig. 6A are for approximately 80% S-nitrosated Hb S and 0% S-nitrosated controls in chloride-free HEPES/EDTA buffer at pH 7.5. Fig. 6B shows a similar pair in the presence of DPG. In these studies, both control and S-nitrosated samples of Hb S were more prone to autoxidation than comparable samples of Hb A₀. The sample handling required for our studies made it difficult to obtain data with low levels of metHb S, and many data sets were not useful due to high metHb levels. The results shown are for control and S-nitrosated forms of Hb S samples with relatively low levels of metHb measured before (<9%) and after (<15%) oxygen binding.

The initial stages of oxygenation of unpolymerized Hb S are
partially shift of log as asymmetric shifts away from the control data. The leftward oxygen binding studies in 0.05 M HEPES/DTPA buffer, pH 7.5, 20 °C S SNO-Hb.

Spectral deconvolution assays to determine the extent of SNO-Hb. The aliquots were subjected to a second chromatography through Sephadex G-25 prior to spectral analysis.

Differences in thiosteric and anionic mechanisms of control of Hb function are indicated by the results presented here and by previous studies on other SH-modified Hbs. Notably, S-nitrosation results in equivalent shifts of log in the presence

most affected by S-nitrosation (ΔlogP_{10} > ΔlogP_{50}), resulting in asymmetric shifts away from the control data. The leftward shift of logP_{10} is approximately the same in the presence and absence of 2,3-diphosphoglycerate, resulting in equivalent decreases in cooperativity to about two thirds of the normal values. These results are very similar to those described above for S-nitrosation of Hb A_{2}.

DISCUSSION

Understanding the properties of SNO-Hb is critical for better understanding of the NO-dependent reactions moderated by Hb. Results presented here show that S-nitrosation stabilizes the high affinity R-state conformation of Hb, as previously reported for Hbs reacted with N-ethylmaleimide, iodoacetamide, and other SH group modifiers (11, 14, 24–27). Thiosteric effects brought about by S-nitrosation of Cys-93 residues in the oxy (R-state) conformation compared with the deoxy (T-state) conformation of Hb was invoked to account for the more facile S-nitrosation of liganded (R-state) Hb and the greater stability of SNO-Hb in the liganded form (1). The results presented here expand upon these earlier findings and illustrate that deoxygenation alone is not sufficient to cause release of NO from SNO-Hb. NO is accommodated in the altered deoxy structure of SNO-Hb, making it possible to deoxygenate the protein without significant loss of NO. However, we show that the presence of glutathione destabilizes SNO-Hb and allows for NO transfer away from SNO-Hb during a cycle of deoxygenation and re-oxygenation. In red cells, where thiols such as GSH can serve as NO acceptors, the conformational sensitivity of SNO-Hb would facilitate NO release upon deoxygenation. These findings are relevant to the increased vasodilatory action reported for oxy SNO-Hb in the presence of glutathione (1).
or absence of 2,3-diphosphoglycerate, the responses of normal and SNO-Hb to 2,3-diphosphoglycerate are equivalent, and 2,3-diphosphoglycerate does not significantly affect the release of NO from SNO-Hb in a cycle of deoxygenation/re-oxygenation in the presence of glutathione. These observations indicate the existence of regional rather than global effects associated with thioesteric and anionic effectors, a conclusion reached earlier by Perutz and co-workers in regard to the anion sensitivity of Hb in which the SH groups at Cys-393 were modified by N-ethylmaleimide (11). This regional control of oxygen affinity makes it possible for SNO-Hb forms to exhibit varied oxygen affinities, dependent on the nature of the parent Hb and its environment.

The regional effects associated with SH group modification have been shown to result, in large part, from disruption of the normal salt bridge between His-β146 and Asp-β94, which in turn decreases the proximal-side pull on the heme-iron that normally confers T-state character on deoxygenated Hb (11, 12, 29). It does not appear that SH group modification by S-nitrosation appreciably alters the β chain anion-binding site in Hb A0 or in Hb S, since in both proteins the magnitude of the 2,3-diphosphoglycerate effect is equivalent for the normal and S-nitrosated forms. The similarity of responses of Hb A0 and Hb S to S-nitrosation mirrors their structural similarities as shown by x-ray crystallography (13).

Anion-induced shifts in the Hill plot asymptotes are not interpretable as solely due to their preferential binding to the T-state, a feature that led Minton and Imai (30) to suggest that a minimum of three states was required to describe Hb function. We recently advanced a new paradigm of Hb function in which anion-induced shifts in the apparent T-state are explained by anion-dependent alterations in the conformational fluctuations that expose “buried” sites (15). The functional properties of SNO-Hb as described above are supportive of this paradigm. This paradigm also rationalizes the absence of a 2,3-diphosphoglycerate effect on the loss of NO from SNO-Hb in the presence of glutathione, since Cys-393 in the altered deoxy state of S-nitrosated Hb is relatively free of steric hindrance and is thereby insensitive to anion-induced decreases in conformational fluctuations.

Differences in thioesteric and anionic mechanisms of control of Hb function may be of considerable significance for understanding the linkage between the oxygen affinity and polymerization of Hb S and for designing better treatments to alleviate sickle cell disease. Treatments that increase the oxygen affinity of Hb S generally decrease polymerization and the associated red cell morphological changes associated with sickle cell disease (9). Significantly, Hb S derivatives with SH groups modified with glutathione (26) or other thiol reagents (25, 27) have decreased tendencies to polymerize. Accordingly, we have initiated studies to determine if Hb S gelation is significantly inhibited by NO ligation or S-nitrosation at levels that might be achievable in vivo.

The data thus far available on the vasodilatory effects of SNO-Hb show that oxidation of the metal center affects NO release and thereby the NO-dependent physiological reactions associated with SNO-Hb (1). In the studies reported here, Hb S oxidation was much more pronounced than that of Hb A0, suggestive of altered NO uptake and delivery for this variant form of Hb even in its unpolymerized state. The greater tendency of Hb S to autoxidize has been noted previously, and the resistance to malaria associated with Hb S has been postulated to arise in part as a consequence of greater oxidative events in red cells containing Hb S than for red cells containing only Hb A0 (34, 35). Consequently, the R-state shifts in Hb S induced by S-nitrosation and by oxidation may be joint determinants of the role played by Hb S in malarial resistance, Hb-linked vasodilation, and sickle cell disease.

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Addendum—A paper by Patel et al. (28), published subsequent to acceptance of this manuscript, supports our finding that S-nitrosation of Hb A increases its oxygen affinity. A major difference in results reported is that these researchers found unaltered cooperativity for SNO-Hb A. This is in marked contrast to our observation that cooperativity is reduced in SNO-Hb relative to unmodified Hb and also in contrast to the pattern of behavioral alterations commonly associated with βG modification by other SH-specific reagents. A difference in methodology is that we overcame problems of metHb formation during exposure of Hb to CysNO, while they compensated for this problem by use of a metHb reductase system after SNO-Hb formation. There is, however, agreement that the SH group modification with NO does not abolish the quaternary conformational changes associated with oxygenation and deoxygenation that underlie allosteric control of NO delivery from SNO-Hb.

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