HEME REDOX PROPERTIES OF S-NITROSATED HEMOGLOBIN A₀
AND HEMOGLOBIN S: IMPLICATIONS FOR INTERACTIONS OF
NITRIC OXIDE WITH NORMAL AND SICKLE RED BLOOD CELLS

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**List of Abbreviations**

Hb A₀, purified adult human hemoglobin; Mb, myoglobin; Hb S, sickle cell hemoglobin; SNO-Hb (%), S-nitrosated hemoglobin, indicating %β93Cys groups modified; Hb-NO, NO bound to heme of Hb; NEM-Hb, β93Cys modified by reaction with N-ethylmaleimide; PDS-Hb, β93Cys modified by reaction with 4,4'-dithiodipyridine; CPA-Hb A₀, carboxypeptidase A-digested Hb A₀; SNO-CPA-Hb A₀, carboxypeptidase A-digested β93Cys nitrosated hemoglobin A₀; GSH, glutathione; GSNO, S-nitrosated glutathione; MOPS, 3-[N-morpholino]propanesulfonic acid; NHE, normal hydrogen electrode; IHP, inositol hexaphosphate; HEPES, N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]; DPG, 2,3-diphosphoglycerate.
Summary

S-nitrosated hemoglobin is remarkably stable and can be cycled between deoxy, oxygenated or oxidized forms without significant loss of NO. Here we show that S-nitrosation of adult human hemoglobin (Hb A₀) or sickle cell Hb (Hb S) results in an increased ease of anaerobic heme oxidation, while anions cause redox shifts in the opposite direction. The negatively charged groups of the cytoplasmic domain of Band 3 protein also produce an allosteric effect on S-nitrosated Hb. Formation and deoxygenation of a SNO-Hb/Band 3 protein assembly does not in itself cause NO release, even in the presence of glutathione; however, this assembly may play a role in the migration of NO from the red blood cells to other targets and may be linked to Heinz body formation. Studies of the anaerobic oxidation of Hb S revealed an altered redox potential relative to Hb A₀ that favors met-Hb formation and may therefore underlie the increased rate of autoxidation of Hb S under aerobic conditions, the increased formation of Heinz bodies in sickle cells, and the decreased lifetime of red cells containing Hb S. A model for the interrelationships between the deoxy, oxy, and met forms of Hb A₀ and Hb S, and their S-nitrosated counterparts, is presented.
Introduction

The reactivity of heme proteins with nitro and nitroso compounds has been under intense scrutiny since Ignarro et al reported the ability of some of these compounds to activate an αβ heterodimer enzyme containing a b-type heme, sGC, involved in the relaxation of the endothelium (1-4). Nitric oxide (NO) has been observed to react with Fe-porphyrin complexes in various oxidation states (Fe$^{2+}$, Fe$^{3+}$ and Fe$^{4+}$), and to rapidly bind to the heme of deoxy hemoglobin (deoxyHb) with an association rate constant on the order of $10^7$-$10^8$ M$^{-1}$s$^{-1}$ (5). NO preferentially binds to the α chain heme groups of deoxy Hb (Hb-NO) at equilibrium in solution and in red blood cells (5-9). Reaction of NO with oxygenated heme groups results in heme oxidation and nitrate formation. This process, like NO binding to deoxy Hb, occurs rapidly and with a similar rate constant (10-12). As shown in this report, reactions of NO at the sulphydryl groups of Hb also promote met-Hb formation.

Several lines of study have shown that S-nitrosated Hb (SNO-Hb) can be formed in vivo and in vitro, and although present at low concentration, may be of importance in blood pressure regulation. Hb contains a highly conserved cysteine residue at position β93 whose reactions with NO may account for its persistence in hemoglobin’s evolutionary history. A dynamic cycle of SNO-Hb formation in the lungs and NO-release in the tissues was implicated by finding the presence of greater levels of SNO-Hb in aortic relative to venous blood (13). This cycle has, however, been brought into question. Notably, Gladwin and co-workers did not see higher levels of SNO-Hb in aortic blood, even in patients given low levels of NO in breathing
It is now well established that the formation of SNO-Hb is under allosteric control (13). Functional and crystallographic studies demonstrate that the β93Cys residues at which NO is bound in SNO-Hb A₀ are more accessible in the high affinity conformation of oxy (R-state) Hb than in deoxy (T-state) Hb (6,15,16). This conformational sensitivity results in a rate-dependence for SNO-Hb formation that mirrors the greater relative exposure of β93Cys in conditions that favor the R-state. Allosteric considerations were also invoked to explain the decreased stability of the deoxy form of SNO-Hb (13), but allosteric control of NO release from SNO-Hb in vivo is still under debate (14,17,18). In a purified condition, free of red blood cell constituents, SNO-Hb is sufficiently stable to allow oxygen-binding studies (7) and oxidation-reduction studies (this report) to be carried out over a period of several hours without significant loss of NO from the SNO-Hb derivative.

Stamler and co-workers recently presented evidence showing that interactions of SNO-Hb with the Band 3 protein on the erythrocyte membrane can facilitate NO release from SNO-Hb (19). Band 3 protein, also known as anion-exchanger AE1, mediates anion exchange through the erythrocyte membrane and interacts strongly with Hb A₀ (20-22). We show in this report that the cytoplasmic domain of Band 3 protein stabilizes the low affinity conformation of SNO-Hb A₀, as previously reported for unmodified Hb A₀ (22), but that these interactions do not in themselves cause release of NO from the SNO-Hb/Band 3 protein assembly. The release of NO may require a transport pathway and a series of transnitrosation reactions, but this has
We previously reported that S-nitrosated forms of Hb A₀ and Hb S have increased oxygen affinity, with increased R-state character that is most evident at low levels of oxygen saturation (7). This finding, in light of the higher solubility of R-state Hb S relative to its T-state, prompted us to suggest that S-nitrosation of Hb S might be viewed as a possible therapeutic approach to inhibition of Hb S polymer formation and alleviation of sickle cell disease (7). As part of a study directed toward exploring this possibility, we report here the redox properties of unmodified and S-nitrosated forms of Hb A₀ and Hb S. As will be shown, the redox properties of Hb S were found to differ from those of Hb A₀. Moreover, S-nitrosation shifts the redox potential of Hb A₀ toward greater ease of oxidation, with smaller effects on Hb S. The shift of SNO-Hb forms of Hb A₀ and Hb S toward the R-state, with higher oxygen affinity and greater ease of oxidation, probably involves a regional conformational alteration of the deoxy Hb tetramer that prevents β146His 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 β93Cys were modified by N-ethylmaleimide (16,23). The NO of SNO-Hb thus acts in a similar fashion as other sulfhydryl reagents and increases oxygen affinity and the ease of anaerobic oxidation.

The *in vitro* studies reported here suggest that oxidative and nitrosative reactions in red blood cells containing Hb S could be appreciably altered. These alterations may help explain the well-documented tendency of Hb S to oxidize more quickly than Hb A₀ under aerobic conditions.
conditions, the shorter lifetime of red blood cells containing Hb S, and the contribution of Hb S to malarial resistance (24,25).

The biological significance of this work includes the description of possible SNO-Hb reactivity patterns and their relevance to blood pressure regulation. Additional information regarding band 3/SNO-Hb complex formation complements that of Stamler and co-workers (19) with respect to the mechanism for NO release from SNO-Hb in vivo.

**Materials and Methods**

*Materials:* Ru(NH₃)₆Cl₃ (Strem Chemical Co. >99%), HEPES (Sigma >99%), KCl (Fisher Scientific >99%), EDTA (Sigma), sodium bicarbonate (Sigma), potassium borate (Sigma), potassium nitrite (Sigma), glutathione (Sigma) and L-cysteine (powder, Aldrich) were used as received.

*Sample Preparations:* Hemolysates of cells containing native human hemoglobin (Hb A₀) and sickle cell Hb (Hb S) were used to prepare purified Hb by the ammonium sulfate method (26). Samples were stripped of organic phosphates by passage through a mixed bed resin as previously described (6), using TMD-8 resin (Sigma). All samples were subjected to chromatographic purification with a FPLC system (Pharmacea, Inc.) in which computer assisted ion-exchange liquid chromatography was carried out using Q Sepharose as an anion
exchange column material that allows for fast flow (5 ml/min) of elution buffers. Well-separated Hb types (Hbs A, S, F, etc.) were eluted using a linear gradient of 0 to 0.15M NaCl in 0.05 M Tris buffer at pH 8.3. Samples were stored at 4°C for a maximum of 4 days. The cytoplasmic domain of band 3 was expressed and purified to >95% as evidenced by a single band on an SDS-PAGE gel using techniques and procedures described elsewhere (27).

Hb samples reacted with N-ethylmaleimide (NEM-Hb) and with 4,4’-dithiodipyridine (PDS-Hb) were prepared as previously described (7). The reaction with NEM was carried out using a 1:3 ratio heme:NEM in 0.05 M bis-Tris, pH 7.2, while PDS was used at a ratio of 3:7 heme:PDS. The PDS was dissolved in ethanol before being added to 0.05 M HEPES buffer at pH 7.5. Both reactions were done at 37°C, followed by Sephadex G25 chromatography to separate the Hb from the low-molecular-weight reagent. Carboxypeptidase A-digested Hb A0 (CPA-Hb A0) was prepared by treating the CO derivative of Hb A0 with carboxypeptidase A (Sigma Chem. Co., Type 1-DFP) at an enzyme-to-protein ratio of 1:50. The mixture was incubated at 37°C for two hrs and then dialyzed at 4°C against 0.05M Tris buffer, pH 8.3. This enzymatic digestion under the conditions employed removes the C-terminal His and Tyr of the β-chains, as verified by electrospray ionization mass spectrometry. The CPA-digested Hb A0 was subsequently run through a compact G25 column in order to put it in the buffer of choice. Photolysis of the CO-Fe bond with repeated evacuation and flushing with nitrogen removed CO prior to use in experiments with the unliganded derivative. S-nitrosated forms of Hb A0 and Hb S were prepared and the level of SNO-Hb quantified as previously described, using S-nitrosated cysteine as the NO donor (7). Similar procedures were used to generate S-nitrosated
CPA-Hb. In all cases, aliquots of the unmodified protein solutions were used as reference samples. S-nitrosated forms of Hb A₀ and Hb S were handled carefully at low temperatures and in the absence of ambient light.

Sample concentrations and oxidation states were determined spectrophotometrically using published extinction coefficients (28). Samples containing spectrally detectable levels of hemichrome (29) were discarded. The relative levels of oxidized Hb (met-Hb) and oxygenated Hb (oxy-Hb) were determined by spectral analysis using either a Cary Model 2300 UV/Vis/NIR spectrophotometer or a Hewlett Packard Diode Array UV/Vis spectrophotometer.

**Mass spectrometry:** Mass measurements were made on a Micromass Quattro LC (Altrincham, UK) triple quadrupole mass spectrometer equipped with a pneumatically assisted electrostatic ion source operating at atmospheric pressure and in a positive ion mode. Hb samples in 50% aqueous acetonitrile containing 1% formic acid were analyzed by loop injection into a stream of 50% aqueous acetonitrile flowing at 10 µL/ min. Spectra were acquired in the multi-channel analyzer (MCA) mode from m/z 600-1400 (scan time 5 sec). The mass scale was calibrated using the multiply charged envelope of the α chain of Hb A₀ (MW 15126.38). The raw mass spectra were transformed to a molecular mass scale using a maximum entropy based method (MaxEnt) which uses the MemSys5 program (MaxEnt Solutions Ltd., Cambridge UK) and is part of the Micromass MassLynx software suite. Transformations were performed from 860-1400 m/z using a resolution of 1 amu.
**Spectroelectrochemical experiments:** Our spectroelectrochemical technique was slightly modified from our previous reports to facilitate study of nitrosated Hb A₀ and Hb S (30-33). Specifically, to minimize loss of NO from SNO-Hb A₀, our experimental protocol was modified so that the experimental time was kept to a minimum by using larger applied potential increments. Exposure to light was minimized by a shutter and the applied potential was not allowed to fall below ca. -120 mV (NHE). Addition of 0.5 mM EDTA to the HEPES buffer when studying SNO-Hb A₀ and SNO-CPA-Hb A₀ was found to improve the quality of the data and also minimized loss of NO from SNO-Hb A₀. With these precautions, loss of bound NO was shown to be < 5% as determined by ESI-MS, where the cone voltage was set at 33 volts. HEPES was selected as the supporting buffer for its non-complexing nature and stability, as well as the absence of spectral and electrochemical interferences. A stock solution of 2.0 M KCl in 0.05 M HEPES/ 0.5 mM EDTA at pH 7.5 was used to prepare the working solutions for the spectroelectrochemistry. Nanopure water was used at all times and all solutions were stored at 4°C.

For each spectroelectrochemical experiment, a solution containing ca 5 mM Ru(NH₃)₆Cl₃ and 0.05 M HEPES (with or without 0.5 mM EDTA) at pH 7.5 with specific concentrations of KCl in a 5 mL pear-shaped flask was connected to a vacuum line for deoxygenation of the solution using a slight vacuum for 15 min followed by purging with N₂ for 15 min (pump-purging). This procedure was repeated twice followed by addition of Hb A₀ and additional pump-purging with gentle swirling to minimize bubbling and insure the
complete removal of oxygen from the working solution. Final concentrations were typically 0.06 - 0.08 mM in heme.

Spectroelectrochemical experiments were carried out in an anaerobic optically transparent thin layer electrode (OTTLE) cell made in-house as described previously (32,33). A salt bridge was constructed using a Pasteur pipette plugged at the bottom with an agar gel so as to connect the Ag/AgCl reference (Bioanalytical Systems Inc.) electrode to the working electrode. The salt bridge solution was composed of 0.2 M KCl in 0.05 M HEPES (± 0.5 mM EDTA) at pH 7.5 and was degassed and then flushed with N₂ for one hr. The OTTLE cell was purged with N₂ for 15 min. prior to injecting the protein solution.

A typical increment of 40 to 50 mV was applied to the system starting at approximately +400 mV down to –120 mV (vs. NHE). At each applied potential the absorbance was monitored until no change was detected. The back-to-back reduction-oxidation-reduction sweeps were performed to determine the reproducibility of our data, and showed minimal loss of the protein (< 10%) and gave reproducible results. Nernst plots were then derived from the observed changes in absorbance as previously described (30-34). All Nernst plots represent applied potentials E (mV) relative to NHE.

Oxygen equilibria: Oxygen binding measurements were performed tonometrically, using a modified spectrophotometric method based on that of Riggs and Wolbach (26). The S-nitrosated samples were kept in the dark except during spectral analysis (< 30 sec for data
collection with a Hewlett-Packard M diode array spectrophotometer) and very few data points were collected in each set to minimize the time under low oxygen conditions where Hb autoxidation was most pronounced.

Results

*Spectroelectrochemistry of Hb A0 and Hb S:* We previously determined that oxygen binding curves (Hill Plots), and oxidation curves (Nernst Plots), have informative differences in regard to how anionic effectors modulate Hb function (31-34). To follow up on this parallel data analysis between oxygen binding and anaerobic oxidation, we used spectroelectrochemical methods to compare the redox behavior of Hb A0 and Hb S under varied conditions that are comparable to our oxygen binding studies. As described in Methods, use of an optically transparent electrode cell (OTTLE cell) and a cationic mediator allows us to probe both anion effects and effects of SH modifications on the anaerobic oxidation process.

Figure 1 represents the Nernst plot for sickle cell hemoglobin (Hb S) in the presence and absence of allosteric effectors, chloride and 2,3-diphosphoglycerate. Hb S is a variant of Hb A0 with an “external” substitution of β6 glu → val. Except at high protein concentrations where the deoxy form polymerizes (giving rise to the adverse effects of sickle cell disease), the oxygen binding curves of Hb S are very similar to those of Hb A0. Surprisingly, we found that stripped Hb S (freed of exogenous anions) has a more negative $E_{1/2}$ than does Hb A0 ($E_{1/2} = 70$
mV for Hb S in contrast to $E_{1/2} = 85$ mV for Hb A$_0$; Table I). Anions such as 2,3-diphosphoglycerate shift the redox potential positive for both proteins, as illustrated in Figure 1 and Table I. Under all conditions examined, the Hb S samples showed negative shifts in redox potentials relative to Hb A$_0$. The effects of anion binding on the redox potential are mirrored by oxygen affinity shifts that reflect the anionic stabilization of the T-state. As documented below, opposite shifts of the redox potential occur when the SH-groups of Hb A$_0$ are modified by NO or other SH reagents.

*Stability of SNO-Hb during spectroelectrochemical experiments:* Hb specifically derivatized by nitrosation at the $\beta$93Cys position was generated as described in Methods. Although the S-NO linkage in S-nitrosated Hb (SNO-Hb) is known to be susceptible to reductive cleavage, we found that SNO-Hb is not degraded by our electrochemical mediator, and that SNO-Hb can undergo a complete redox cycle during the spectroelectrochemistry experiment without significant loss of NO. Experimental verification of this statement follows below.

Electrospray ionization mass spectrometry (ESI-MS) confirmed that the potentials applied during a spectroelectrochemical experiment did not affect the degree of S-nitrosation of the sample studied. A SNO-Hb A$_0$ standard sample (0.08 mM in heme) in 0.05 M HEPES/0.5 mM EDTA at pH 7.5 was prepared. Three separate aliquots of the standard sample were removed and one aliquot was exposed to the Ru(NH$_3$)$_6$Cl$_3$ mediator, another was carried through a complete spectroelectrochemical reduction experiment over the course of *ca* 1 hr, and the third was carried through a complete redox cycle (reduction-oxidation-reduction) with *ca* 12
hrs of experimental manipulation. ESI-MS methods described in more detail elsewhere (7) showed the β-chain nitrosation as a 30 mass unit shift from the parent β-globin chain, such as is illustrated in Figure 2 of Reference 7. Our ESI-MS data obtained here show the same level of nitrosation, within 5%, for the standard sample and each of the aliquots. These results confirm that no significant loss of NO from SNO-Hb A₀ occurred during the course of our experiments.

Spectral assays provide an independent confirmation of our ESI-MS results. A solution of the redox mediator, Ru(NH₃)₆Cl₃, was prepared and added to one of two aliquots of a solution containing 0.08 mM SNO-Hb in 0.05 M HEPES / 0.5 mM EDTA buffer at pH 7.5. The two samples (blank and that containing 5 mM Ru(NH₃)₆Cl₃) were incubated for 5 hours at 20°C (expected time necessary to perform a spectroelectrochemical experiment). The degree of S-nitrosation was then determined for both samples by spectral deconvolution as described in detail elsewhere (see Figure 1 in Ref. 7) (7). Although the treated sample was completely oxidized by the mediator, it showed a negligible loss (~2%) in the degree of S-nitrosation (63% vs 65% of β93Cys derivatized in the control). The control did not show any loss of cysteine-bound NO over the course of the experiment. These results are consistent with a minimal loss of NO due to extended exposure of the sample to the electrochemical mediator.

*Spectroelectrochemistry of S-nitrosated Hb A₀ and Hb S:* Preparations of partially S-nitrosated forms of Hb A₀ and Hb S were made using either met- or oxy-Hb A₀ as a starting material. In all experiments of this report the modification of the parent protein was solely that associated with nitrosation at the β93Cys position. In our SNO-Hb preparations there were no internal
sulphydryls modified and no disulfides formed (see Methods). Figure 2 shows Nernst plots for Hb A₀ and 90% S-nitrosated Hb A₀. As illustrated, the presence of NO as a modifier of β93 cysteine prompts a change in the electronic environment of the heme. For Hb A₀, we determined that the percentage of S-nitrosation (with up to 95% of the β93Cys derivatized) correlated with a negative shift in $E_{1/2}$ (ca. 10–40 mV), with a larger negative shift for higher percent derivatization.

Studies of the redox behavior of S-nitrosated Hb S revealed that this species is only slightly shifted toward more negative potentials in comparison with the non-modified protein. Table I documents the results obtained, along with comparative data on the effects of SH-modification by other reagents as described below. The apparent difference of 6 mV between Hb S and SNO-Hb S is much smaller than the shift that occurs as a result of comparable S-nitrosation of Hb A₀.

**Spectroelectrochemistry of irreversibly SH-modified Hbs:** Stable and irreversible derivatization of the β93 SH-groups of Hb results from reaction with either NEM or PDS. The reaction goes to completion for both NEM and PDS modifiers, with 100% of β93Cys being modified for each reagent. At our reaction conditions only the external β93Cys residues were derivatized, avoiding modification of internal sulphydryls (see Methods). These modified Hbs exhibit $E_{1/2}$ values that are shifted 30 and 40 mV, respectively, more negative than native Hb A₀. As expected based on previous studies, (6) the irreversibly SH-modified forms also show increased
O$_2$ affinity. These results are compared to those associated with NO-induced SH-group modifications in Table I.

Spectroelectrochemistry of R-state-stabilized SNO-CPA-Hb A$_0$: Digestion with carboxypeptidase A (CPA) removes the C-terminal histidine ($\beta$146) and tyrosine ($\beta$145) residues from Hb A$_0$. These amino acids are involved in the formation of a salt bridge that stabilizes the T state of Hb A$_0$. The deletion of these amino acids results in a Hb A$_0$ form that cannot undergo an R to T transition and is locked in the R state (6). Figure 2 shows the Nernst plots for normal and S-nitrosated forms of the R-state Hb, CPA-Hb A$_0$, along with comparable plots for Hb A$_0$. The results illustrate that the R-state protein is more easily oxidized (Nernst plot shifted to more negative potentials) relative to Hb A$_0$. Moreover, no redox differences were found between CPA-Hb A$_0$ and its S-nitrosated counterpart, while as shown in Figure 2, S-nitrosation of Hb A$_0$ leads to a shift of the E$_{1/2}$ of the protein that favors its oxidation.

Deoxygenated S-nitrosated Hb A$_0$ exhibits spectral characteristics like those of CPA-Hb A$_0$ that reflect its R-state character. As presented in Figure 3 for the nitrosated and non-nitrosated Hb A$_0$ we observe a slight broadening and shift of the Soret $\lambda_{\text{max}}$ for deoxy Hb A$_0$ to shorter wavelength upon S-nitrosation. In addition, the molar absorptivity of deoxy SNO-Hb A$_0$ was estimated to be ca. 98000 M$^{-1}$cm$^{-1}$, corresponding to a decrease of 15% from the molar absorptivity for the deoxy state of unmodified Hb A$_0$. These spectral differences, previously noted for R-state chains and dimers of Hb,(6) reinforce the idea that modification of $\beta$93Cys by nitrosation decreases the ability of the protein to fully attain the normal T state conformation.
Interactions of Hb A₀ and SNO-Hb A₀ with the cytoplasmic domain of Band 3 protein: As reported in Table I, S-nitrosation increases the oxygen affinity of Hb, while binding of the cytoplasmic domain of Band 3 protein to SNO-Hb decreases its oxygen affinity. These shifts are in accord with previous studies of the effects of S-nitrosation (7) and of Hb/Band 3 protein interactions (22). During these experiments, there was significant heme oxidation and the observed oxygen affinity shifts have to be considered in the context of our earlier report (1) that an increase in level of met-Hb will increase the oxygen affinity of SNO-Hb A₀ and Hb A₀. The observation of enhanced heme oxidation of SNO-Hb A₀ after addition of the cytoplasmic domain of Band 3 protein is consistent with prior reports of increased oxidation of Hb upon interaction with Band 3 protein (35). The decrease in oxygen affinity (in spite of increased oxidation) that accompanies formation of the SNO-Hb/Band 3 protein assembly confirms earlier reports that the cytoplasmic domain of Band 3 protein can bind to the β-chain anion binding site of Hb A₀ and mimic the effects of 2,3-diphosphoglycerate and other anionic allosteric effectors with respect to oxygen affinity (22).

Having determined the nature of the oxygen affinity shifts brought about by interactions between SNO-Hb A₀ and the cytoplasmic domain of Band 3 protein under our experimental conditions, we then sought to determine if the interactions of Band 3 protein with SNO-Hb A₀ would cause the release of NO. Spectral deconvolution assays were performed to address this question. As in the previous experiments, we noted that mixing the cytoplasmic domain of Band 3 protein with S-nitrosated Hb A₀ (SNO-Hb A₀) increased heme oxidation. However,
there was no significant loss of NO from the SNO-Hb A₀/Band 3 protein assembly during an oxy-deoxy-oxy cycle (complete oxygen removal, a one hour period of incubation at 25°C after oxygen removal, and re-oxygenation).

The level of derivatization of β93Cys in our preparation of SNO-Hb A₀ was about 60% (± 5%) prior to addition of the cytoplasmic domain of Band 3 protein. After the addition experiment the amount of NO released by dithionite addition indicated that 50% (± 5%) of the sample was S-nitrosated. When the same study was carried out in the presence of equimolar amounts of glutathione along with the Band 3 protein domain, S-nitrosation was estimated at 30 (±5)% after the sample was passed through a G25 column to remove any glutathione or nitrite or nitrate that had formed. This greater loss (30% loss with GSH vs 10% loss without GSH) of NO from SNO-Hb was not attributable to Hb’s interactions with the Band 3 protein domain, since we previously observed a similar glutathione-dependent decrease in the stability of SNO-Hb A₀ (7). Our results confirm those of Patel and co-workers (17) that GSH is both ineffective and slow in its transnitrosation reactions with SNO-Hb. We find this to be true even in the presence of the Band 3 protein domain. The significant finding from these studies is that formation and deoxygenation of the SNO-Hb A₀/Band 3 protein assembly does not, in itself, cause NO release, and that GSH does not function effectively as a NO receiver for the Hb/Band 3 complex.

Our experimental protocol for spectral deconvolution assays calls for use of a G25 column to remove low molecular weight species prior to the dithionite-induced release of NO
from S-nitroso linkages (7). This procedure would have removed any free NO or low molecular weight NO derivatives from the mixture and eliminates the possibility of regenerating NO from any low molecular weight forms (such as nitrite). Moreover, there were no detectable NO-heme adducts present prior to dithionite treatment, indicating that little or no NO was trapped at the heme prior to dithionite treatment. As reported by Spencer et al (36), GSNO, if formed to a significant extent, could have generated some NO-heme as a result of its interactions with deoxy Hb.

While our study of SNO-Hb interactions with the cytoplasmic domain of Band 3 protein does not rule out transfer of NO from SNO-Hb A₀ to SH-groups on Band 3 protein, it does provide convincing evidence that NO is not effectively released from the protein mixture even in the presence of equimolar glutathione. In the absence of other factors that might influence the reaction in red blood cells, the conformational shifts in purified SNO-Hb induced by deoxygenation in the presence of Band 3 protein, or induced by deoxygenation of the Hb/Band 3 protein complex in the presence of glutathione, do not force the release of NO. Accordingly, the \textit{in vivo} mechanism for release of NO from SNO-Hb A₀ remains undefined.

\textbf{Discussion}

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, in which the focus is
on NO uptake and delivery by Hb A₀ and the role this plays in the control of blood pressure and in oxidative and nitrosative reactions. The significance of this report stems from our exploration of what can and cannot happen once nitrosation of the β93 sulfhydryl group occurs.

Our results address the physiologically significant changes brought about by anions and SH modifiers that alter Hb conformation (allosteric controls) and Hb redox potential (electronic controls). These changes regulate Hb-based NO uptake and delivery as well as its oxygen transport functions. Intriguing differences in the redox potentials of Hb A₀ and Hb S were found that may alter the oxidative and nitrosative reactions in red blood cells containing Hb S. This finding also clarifies a long-standing puzzle regarding the greater ease of aerobic oxidation (autoxidation) of Hb S. It is now clear that although the substitution in Hb S is “external”, its structural consequences reach to the heme site where a shift in the redox potential of Hb S makes the protein more susceptible to oxidation.

Quantitative studies of the redox (oxidation-reduction) equilibria of respiratory heme proteins were begun by Taylor and Hastings (37) who studied the equilibrium between ferrous and ferric myoglobin. As reviewed elsewhere, (6) the oxidation-reduction equilibria of Hb under varied experimental conditions has been the subject of many investigations. Inconsistencies in the earlier data can be attributed to Hb-interactions with the oxidizing or reducing agents used in the redox titrations (6,38-41). Our spectroelectrochemical approach allows us to explore the redox behavior of Hb with higher resolution and reproducibility than
previously possible. Significantly, the cationic mediator used in the studies reported here allows us to probe SH-modified Hbs and the anionic control mechanisms operating in these systems.

Earlier studies performed by Brunori and Antonini (6) and Banerjee and Cassoly (42) demonstrated the impact on anaerobic oxidation of chemically modifying the β93 cysteine residue of Hb. These authors, using different SH-modifiers, observed a ca 30 mV negative shift in redox potential relative to native Hb. Our results confirm and extend these earlier studies, and show that modification of the β93 SH group by NO and a number of other thiol reagents results in species more easily oxidized than unmodified Hb A0. By analogy to the well-documented anionic allosteric effectors that stabilize the T-state (31), we propose that these thiosteric modifiers influence the E1/2 of the protein by stabilizing the protein’s R-state. NO, however, holds the distinctive position of being the only thiosteric modifier known to operate in vivo. While the concentration of SNO-Hb in vivo is too low to have a significant effect on oxygen binding, this Hb form could have a considerable influence on NO transport and metabolism.

Figures 2 and 3 and results shown in Table I demonstrate that S-nitrosation and other chemical modifications of the β93 cysteine of Hb produce shifts toward more negative potential and higher oxygen affinity. The mechanism by which E1/2 and P1/2 are shifted seems to be associated with a shift toward the R-state conformation. As a test of this model, we investigated CPA-Hb A0 and its S-nitrosated derivative. CPA-Hb A0, from which the C-terminal His and Tyr have been removed by digestion with carboxypeptidase A, has a more
negative $E_{1/2}$ value relative to Hb A$_0$, consistent with it being locked in the high O$_2$ affinity (R-state) conformation (6,43). The finding that S-nitrosation of CPA-Hb A$_0$ does not affect its redox potential is in accord with the conclusion that SH-modifiers exert their influence by stabilizing the R-state of Hb.

Hypothetically, the formation of the S-nitrosated derivative of oxidized (met) Hb can be associated with either a transnitrosation reaction between met-Hb A$_0$ and SNO-deoxy-Hb (Eq. 1), or a redox process by which one electron is transferred from SNO-deoxy-Hb to an existing met-Hb A$_0$ molecule (Eq. 2). (In the Equations below the heme site of met-Hb reactant is arbitrarily labeled using *.)

$$\text{met-Hb}^* + \text{SNO-deoxy-Hb} \rightarrow \text{SNO-met-Hb}^* + \text{deoxy-Hb} \quad (1)$$

$$\text{met-Hb}^* + \text{SNO-deoxy-Hb} \rightarrow \text{deoxy-Hb}^* + \text{SNO-met-Hb} \quad (2)$$

Our anaerobic oxidation results show that oxidation of SNO-deoxy-Hb by met-Hb to give SNO-met-Hb (Eq.2) is thermodynamically feasible. The half-potentials are a minimum of 10-15 mV apart, which is sufficient for a thermodynamic electron exchange between the modified and non-modified Hb, and is reasonable in light of the concentrations reported for met-Hb and SNO-deoxy-Hb in vivo ([met-Hb]>>[SNO-Hb]) (44). Moreover, previous work has demonstrated the existence of an internal electron exchange pathway between the $\beta$-chain heme groups and the SH-groups at position $\beta$93Cys (45,46). This pathway would facilitate the electron exchange mode represented by Equation 2. The rates for electron exchange in
Equation 2 have not been determined, and may be much faster than those observed in previous studies of Cu-treated Hb (45,46).

*Paradigm for NO metabolism and storage in vivo:* Our studies of the redox behavior of normal and S-nitrosated forms of Hb A₀ and Hb S show that NO-interactions at the SH groups of Hb have significant effects on the protein’s heme groups. Met-Hb is known to be formed as a result of the interactions of NO with oxy-Hb, and we find that NO interactions with the SH groups also favor met-Hb formation. Accordingly, we suggest a possible paradigm for Hb-based transport, storage and metabolism of NO *in vivo* that is shown schematically in Figure 4. This paradigm, in which met-Hb plays a significant role, is based on results reported herein for SH-modified forms of Hb A₀ (NEM-Hb A₀, PDS-Hb A₀, SNO-Hb A₀), and CPA-Hb (SNO-CPA-Hb), as well as reports from the literature (3,6,17,36,42,47,48). Possible responses to low oxygen conditions are shown, although the physiological condition is rarely “free” of oxygen, and metHb levels are usually low. There are many conditions where erythrocytes are greatly retarded in their flow through the capillaries, creating low oxygen conditions. In diabetes, sickle cell disease, hereditary stomatocytosis, polycythemias, normal thrombotic disorders and infarcts, etc., movement of the red blood cells past obstructions may be so slow that complete deoxygenation can in fact occur. There is no question that evolution adapts to such stress conditions as well as normal physiological function.

Healthy human adults typically have *ca.* 2 – 3 % of their circulating hemoglobin oxidized daily, (28) creating a small pool of met-Hb in red blood cells. Spencer *et al* (36)
reported that the interaction of deoxy Hb A₀ with S-nitrosated glutathione (GSNO) leads to the formation of NO, met-Hb A₀ and glutathione (GSH) (Eq. 3), followed by the formation of a heme-NO adduct (Hb-NO) (Eq. 4).

\[
\begin{align*}
\text{Hb A}_0 + \text{GSNO} & \rightarrow \text{met-Hb A}_0 + \text{NO} + \text{GSH} \\
\text{Hb A}_0 + \text{NO} & \rightarrow \text{Hb-NO}
\end{align*}
\]

This process could play an important role in increasing the pool of available met-Hb on one hand, and in eliminating excess NO in red blood cells on the other hand. Although met-Hb occurs as a small (generally < 1%) fraction of the total Hb (∼ 20 mM in heme in erythrocytes) met-Hb levels are sufficient to be involved in the storage and metabolism of biologically relevant levels of NO. This concept is supported by the stability of met-SNO-Hb in vitro (this work and unpublished observations), its negatively shifted redox potential (this work) and its recognized vasodilatory activity (13).

Figure 4 represents our model for possible inter-relationships between the deoxy, oxy-, and met- forms of Hb A₀ and their S-nitrosated counterparts. This scheme illustrates the six pathways that have been studied in vitro and may be involved in the NO biochemistry of hemoglobin in vivo. Cycle A represents the well-documented oxygenation cycle that depends on environmental P_{O₂}. Cycle B represents the equilibrium between met and deoxy-Hb, showing that deoxy-Hb can be oxidized to form met-Hb via a one electron reduction of S-nitrosated glutathione (top of the cycle), leading to the formation of NO and glutathione. (Other processes, like the interaction of NO with oxy-Hb, can also produce met-Hb.) The second half of cycle B (going from met-Hb to deoxy-Hb) requires the intervention of a reducing agent. We
have shown here that S-nitrosated Hb A₀ has a low enough E₁/₂ to reduce met-Hb A₀ to deoxy-Hb A₀, leading to the formation of a stable SNO-met-Hb species (interface between cycles B and D). This electron exchange process at the B/D cycle interface is thermodynamically feasible in vitro and can theoretically lead to reactivation of met-Hb A₀ to deoxy-Hb A₀ and storage of NO as SNO-met-Hb, which has been demonstrated to have vasodilatory properties (13). The reaction depicted in C shows a possible route to generate SNO-met-Hb that bypasses any low-level concentration of SNO-deoxy-Hb. The driving force to go "directly" to SNO-met-Hb from SNO-oxy-Hb via path C would be to maintain the low-spin state of the heme, by having Fe undergo both an electron exchange and a ligand exchange. We know that the R to T transition of the protein is directly correlated to the spin-state change of the Fe, and can therefore influence the unloading of NO from the β93 cysteine in erythrocytes. Reaction F represents a probable oxygen-driven equilibrium between the oxy and deoxy form of the S-nitrosated protein. Significantly, our in vitro experiments show that both SNO-deoxy-Hb A₀ and SNO-met-Hb A₀ are remarkably stable (i.e. do not release NO) if no low molecular weight NO-carriers such as cysteine or glutathione are present in the medium. This observation is important; as it strongly suggests that for NO unloading from SNO-Hb (or the SNO-Hb/Band 3 protein assembly) to occur, an acceptor for a transnitrosation reaction must be present (Figure 4, reaction G), or possibly an intracellular reductant that releases NO from the SNO complex. We are presently investigating the possibility that electrons travel from the β chain heme site to the SNO site by the internal electron transfer pathway that we have previously documented to exist (46).
High levels of met-Hb have been associated with formation of Heinz bodies that associate with Band 3 protein in red blood cells (35,49,50). The reactions of met-Hb may also play a role in NO transport, metabolism and storage. The results described above reveal that at least three types of NO-interactions with Hb favor met-Hb formation. They also show that met-Hb is well suited for storing NO as SNO-Hb. SNO-met-Hb, sequestered at the membrane as a Hb/Band 3 complex may also aid in the delivery of NO to tissues. Notably, the conformation of met-Hb can be adjusted from R toward T by modifying the composition of the protein environment, i.e. by increasing the concentrations of various allosteric effectors such as Cl\(^-\) and 2,3-diphosphoglycerate in the protein environment (15,31). Because NO cannot be accommodated at the \(\beta93\text{Cys}\) position in the normal T-state, anion binding to Hb in either its met or deoxy state might be expected to facilitate the release of NO from SNO-met-Hb. However, anion binding is clearly insufficient to cause NO release. Band 3 protein, like small anions, can act as an allosteric effector that shifts Hb toward its T state, but the association of SNO-Hb with Band 3 protein does not in itself cause the release of NO. The intriguing possibility remains that association of SNO-Hb with Band 3 protein may position NO for release from the red blood cell and facilitate NO-dependent vasoactivity \textit{in vivo}. However, as noted above, as yet unidentified interactions of SNO-Hb with red blood cell constituents appears to be required for facilitated NO release.

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Table I  Influence of anions and β93 cys-modifications on the redox and oxygen affinity of  Hb A₀, CPA-Hb A₀ and Hb S.

| Protein                        | \(P_{50}\) a \(\text{(mm Hg)}\) | \(E_{1/2}\) b \(\text{(mV vs NHE)}\) |
|--------------------------------|---------------------------------|-----------------------------------|
| Hb A₀                          | 1.07                            | 85(3)                             |
| Hb A₀ + 0.2 M KCl              | 1.8                             | 113(2)                            |
| Hb A₀ + 12.5 x DPG c           | 6.8                             | 114(2)                            |
| Hb A₀ + 12.5 x IHP c           | 33.9                            | 135(5)                            |
| SNO-Hb A₀ (90%)                | 0.59                            | 45(4)                             |
| SNO-Hb A₀ (60%)                |                                 | 74(3)                             |
| SNO-Hb A₀ (55%)                |                                 | 76(3)                             |
| PDS-Hb A₀ + 0.2 M KCl          | 0.56\(^d\)                      | 55(8)                             |
| NEM-HbA₀ + 0.2 M KCl           | 0.51                            | 45(12)                            |
| CPA-Hb A₀                      |                                 | 13(2)                             |
| SNO-CPA-Hb A₀ (60%)            |                                 | 13(2)                             |
| SNO-Hb A₀ (65%)/ Band 3 protein| 15.49\(^e\)                     |                                   |
| Hb S                           | 0.97                            | 70(5)                             |
| Hb S + 12.5 x DPG c            | 6.7                             | 110(2)                            |
| Hb S + 0.2 M KCl               |                                 | 103(2)                            |
| Hb S + IHP                      | 31.6                            |                                   |
| SNO-Hb S (65%)                 | 0.57                            | 64(3)                             |
a 0.05 M HEPES, pH 7.4, 20°C unless noted otherwise, with 0.5 mM EDTA for SNO-Hbs.

b 0.06-0.08 mM heme, 0.05 M HEPES (0.5 mM EDTA), 1 mM Ru(NH$_3$)$_6$Cl$_3$, pH 7.5, 20°C.

Values based on 2 – 10 measurements; estimated uncertainties in parentheses.

c Concentration of DPG or IHP 12.5 fold over that of heme (0.06 – 0.08 mM).

d 0.06-0.08 mM heme, 0.05 M HEPES, 0.15 M KCl, pH 7.1, 20°C.

e 0.06 mM heme, 05 M HEPES, 0.5 mM EDTA, 25 °C, pH 6.5.
Figure Captions

Figure 1

Influence of allosteric effectors on the redox properties of Hb S. Nernst plot symbols: (●) Hb S, (■) Hb S + 0.2 M KCl, (▲) Hb S + 1 mM DPG. Conditions: [heme] = 0.06 mM to 0.08 mM in 0.05 M HEPES, 1 mM Ru(NH₃)₆Cl₃, 20°C at pH 7.5.

Figure 2

Influence of S-nitrosation on the redox properties of Hb A₀ and CPA-Hb. Nernst plot symbols: (●) Hb A₀, (▲) SNO-Hb A₀ (90% β93 nitrosylated), (○) CPA-Hb and (∇) SNO-CPA-Hb (65% β93 nitrosylated). Conditions: [heme] = 0.06 mM to 0.08 mM in 0.05 M HEPES, 0.5 mM EDTA, 1 mM Ru(NH₃)₆Cl₃, 20°C at pH 7.5.

Figure 3

Spectra of (A) Hb A₀ and (B) SNO-Hb A₀ (65% β93 nitrosylated) as a function of applied potential. Arrows represent direction of spectral changes while sweeping an externally applied potential from +400 mV to -120 mV. Conditions: [heme] = 0.06 mM to 0.08 mM in 0.05 M HEPES, 0.5 mM EDTA, 1 mM Ru(NH₃)₆Cl₃, 20°C at pH 7.5.
Figure 4

Paradigm for NO delivery and storage in vivo as it relates to the Hb A₀ cycle. R and T states are noted in the gray boxes. Symbolic representations which emphasize the Fe oxidation states are as follows: HbFeII-O₂, oxy-hemoglobin; SNO-HbII-O₂, β93cys-NO modified oxy-hemoglobin; HbFeII, deoxy-hemoglobin; SNO-HbFeII, β93cys-NO modified deoxy-hemoglobin; HbFeIII, met-hemoglobin; SNO-HbFeIII, β93cys-NO modified met-hemoglobin; HbFeII-NO, hemoglobin heme-NO adduct. See text for detailed explanation.
Figure 1
Figure 2
Figure 3
Heme redox properties of S-nitrosated hemoglobin Ao and hemoglobin S: Implications for interactions of nitric oxide with normal and sickle red blood cells
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