Heme Nitrosation of Deoxyhemoglobin by S-Nitrosoglutathione Requires Copper*

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NO reactions with hemoglobin (Hb) likely play a role in blood pressure regulation. For example, NO exchange between Hb and S-nitrosoglutathione (GSNO) has been reported in vitro. Here we examine the reaction between GSNO and deoxyHb (HbFeII) in the presence of both Cu(I) (2,9-dimethyl-1,10-phenanthroline (neocuproine)) and Cu(II) (diethylenetriamine-N,N,N',N",N",N"-pentaacetic acid) chelators using a copper-depleted Hb solution. Spectroscopic analysis of deoxyHb (HbFeI1/GSNO incubates shows prompt formation (<5 min) of ~100% heme-nitrosylated Hb (HbFeIINO) in the absence of chelators, 46% in the presence of diethylenetriamine-N,N,N',N",N",N"-pentaacetic acid, and 25% in the presence of neocuproine. Negligible (<2%) HbFeI1/GSNO was detected when neutroproine was added to copper-depleted HbFeI1/GSNO incubates. Thus, HbFeI1NO formation via a mechanism involving free NO generated by Cu(I) catalysis of GSNO breakdown is proposed. GSNO is a source of reducing equivalents because extensive GSSG was detected in HbFeI1/GSNO incubates in the absence of metal chelators. No S-nitrosation of HbFeI1 was detected under any conditions. In contrast, the NO released from GSNO is directed to Cys93 of oxyHb in the absence of chelators, but only methHb formation is observed in the presence of chelators. Our findings reveal that the reactions of GSNO and Hb are controlled by copper and that metal chelators do not fully inhibit NO release from GSNO in Hb-containing solutions.

Possible exchange of NO between thiols and hemoglobin (Hb) in red blood cells (RBCs) has been the focus of intense interest recently (1, 2). It has been suggested that GSNO or S-nitroso-L-cysteinyl could act as an NO donor to Cys93 of bovine serum albumin (6). For Hb to function as a blood pressure regulator in an O2-sensitive manner, release of NO from Cys93 of HbFeI1 is necessary (7). One possibility is that NO is delivered to tissues via S-nitrosation from Cys93 to GSH or another thiol (8) that promotes NO transport across the RBC.

HbSNO + RSH ⇄ HbSH + RSNO

REACTION 1

Recently it has been suggested that delivery to tissues of all the NO bound to Cys93 of Hb would result in extensive vasodilation, which would be fatal (9). Hence, it was proposed that most of the NO released from Cys93 is actually captured by HbFeII. Capture of NO released from GSNO is also possible and would compete with the exit of NO from the RBC. In fact, Spencer et al. (10) reported direct reductive cleavage of GSNO by HbFeII and capture of the released NO by another FeII center (HbFeI1).

HbFeII + GSNO + H+ ⇄ HbFeIII + GSH + NO
HbFeIII + NO ⇄ HbFeINo

REACTIONS 2 AND 3

A trace amount of Cu(I) serves as a highly efficient catalyst of S-nitrosothiol breakdown (11). We have suggested that neocuproine, a Cu(I)-specific chelator, inhibits NO release from GSNO in solutions of HbFeI02 (4). Therefore, we considered it likely that neocuproine would also inhibit NO release from GSNO in solutions containing HbFeI1. To distinguish between direct reductive cleavage of GSNO by HbFeI1 (Reaction 2) and Cu(I)-catalyzed release (Reaction 4), it is necessary to remove all trace copper or prevent its turnover via redox cycling using GSH (Reaction 5) or another donor in the Hb-containing solutions.

CuI + GSNO + H+ ⇄ CuII + GSH + NO

REACTIONS 4 AND 5

Here we report the results of a detailed examination of HbFeI1/GSNO incubates after 5 min in the presence of preferential chelators of Cu(I) (neocuproine) and Cu(II) (DTPA). Solutions of HbFeI1 that were not diazylated and solutions that underwent exhaustive dialysis versus EDTA were used. Our direct spectroscopic and ESI-MS analyses reveal that NO re-

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1 The abbreviations used are: Hb, hemoglobin; DTPA, diethylenetriamine-N,N',N",N",N",N"-pentaaetic acid; ESI, electrospray ionization; MS, mass spectrometry; FTIR, Fourier transform infrared; GSNO, glycine N2,N2-L-γ-glutamylS-nitroso-L-cysteiny1); HbFeIII, metHb; HbFeI, deoxyHb; HbFeIINO, heme-nitrosylated Hb; HbFeIINO2, oxyHb; HbSNO, Hb S-nitrosated at Cys93; ICP, inductively coupled plasma; NaPi, sodium phosphate buffer; neocuproine, 2,9-dimethyl-1,10-phenanthroline; RBC, red blood cell.

2 A. A. Romeo, J. A. Capobianco, and A. M. English, unpublished observations.
lease from GSNO is <2% in the presence of neocuproine in HbFeIII/GSNO solutions containing dialyzed Hb. HbFeIII/NO formation, and hence GSNO breakdown, is ~100% within 5 min in the absence of chelators. HbFeIII/NO formation is decreased by ~50–75% in the presence of DTPA and neocuproine and in solutions containing dialyzed Hb without neocuproine. Because trace copper was found in all reagents by ICP-MS, these observations are consistent with Cu(I)-catalyzed release of NO (Reaction 4).

The source of the reducing equivalents for the prompt Cu(I)-catalyzed reductive cleavage of GSNO is also of interest. Extensive GSSG was formed in HbFeIII/GSNO incubates in the absence of metal chelators, indicating that GSH is the main source of reducing equivalents under these conditions (Reaction 5). We detected less GSSG than expected by ESI-MS in HbFeIII/GSNO incubates containing DTPA, although we observed ~50% GSNO breakdown. This suggested HbFeII as a possible additional source of reducing equivalents to [Cu(II)-(DTPA)]2− because related EDTA complexes were shown to be reductively active with Hb (12). Careful examination of the absorption spectrum of HbFeII prepared all H2O solutions. The reactions were carried out in 200 mM NaPi, pH 7.2, using 10 mM diethylenetriaminepentaacetic acid (DTPA; ICN) and 650 μg of dialyzed Hb without neocuproine. The key results of this comparison are that in the absence of metal chelators, indicating that GSH is the main source of reducing equivalents under these conditions (Reaction 5). We detected less GSSG than expected by ESI-MS in HbFeIII/GSNO incubates containing DTPA, although we observed ~50% GSNO breakdown. This suggested HbFeII as a possible additional source of reducing equivalents to [Cu(II)-(DTPA)]2− because related EDTA complexes were shown to be reductively active with Hb (12). Careful examination of the absorption spectrum of HbFeII.

The realization that commonly used Cu(I) (neocuproine) and Cu(II) chelators (EDTA and DTPA) may not always prevent copper turnover is an important consideration in deciphering the mechanisms of S-nitrosothiol signaling and NO biochemistry in general. Although neocuproine, a tight binding Cu(I) chelator (Kd ≈ 1.2 × 10−19 M) (13, 14), is a better inhibitor than DTPA of GSNO breakdown (Reaction 4) in Hb-containing solutions, dialysis of the Hb samples and neocuproine addition were necessary to obtain negligible GSNO breakdown.

Finally, the prompt changes in HbFeIV/O2 on incubation with GSNO are compared with those observed for the HbFeII incubates. The key results of this comparison are that in the absence of chelators, S-nitroso-CysSSg of the oxy protein is extensive, and this competes with NO capture by the FeIIO2 heme to form HbFeIII and NO2−. In contrast, no S-nitrosation of the deoxy protein is detected, suggesting that all of the NO released from GSNO is captured by the FeII heme.

**EXPERIMENTAL PROCEDURES**

**Materials**

Human hemoglobin A was obtained from Sigma and used without further purification. Nanopure water (specific resistance, 18.2 MΩ-cm) obtained from a Millipore Simplicity water purification system and treated with Chelex-100 (Sigma) to remove trace metal ions was used to prepare all H2O solutions. The reactions were carried out in 200 mM sodium phosphate buffer, pH 7.2 (NaPi), prepared from sodium phosphate salts (Fisher) in nanopure H2O. Stock solutions of 15 mM diethylenetriamine-N,N,N’,N”-tetraacetic acid (DTPA; ICN) and 650 μM 2,9-dimethyl-1,10-phenanthroline (neocuproine; Sigma) were prepared in NaPi. Stock solutions of 250 mM GSNO (Cayman) in NaPi were prepared just before use in a glove bag (Aldrich) under nitrogen, and the GSNO concentrations were determined spectrophotometrically (ε333.5 nm = 774 m M−1 cm−1) (15). GSH and GSSG were obtained from Sigma, and N2 and NO gases were from Praxair. NO was purged into a 10% KOH solution before use (16).

**Methods**

**Preparation and Optical Spectroscopy of Hb Samples**—Typically 1 g of lyophilized metHb (HbFeII) from the bottle was dissolved in 2 ml of NaPi. After 2 min of centrifugation at 12,000 rpm, the precipitate was discarded, and the red solution of HbFeIII was stored at 4°C prior to use. An aliquot (10 μl) of the Hb solution was pipetted onto a 13-mm CaF2 window of a dismountable FTIR type cell (Harrick). The cell was immediately assembled using a 6-μm Teflon spacer (Harrick) and placed in a custom-made bracket in a Beckman DU 650 UV-visible spectrophotometer. HbFeII concentrations were found to be 32 mM in heme assuming ε500 nm = 10 μM−1 cm−1/heme and ε630 nm = 4.4 μM−1 cm−1/heme (17, 18). This was confirmed by diluting the samples 10−fold, adding potassium ferricyanide and excess BCN (BDH chemicals) and allowing the absorbance of the CN adduct at 11.0 μM−1 cm−1/heme in a 1-cm cuvette on a Beckman spectrophotometer. HbFeII was prepared in the glove bag under N2 by treating HbFeIII with equimolar sodium dithionite (Fisher) (19) followed by desalting on a 1.6 × 2.5-cm HiTrap Sephadex G-25 column (Amersham Biosciences), and the HbFeII concentration was calculated using ε500 nm = 12.5 μM−1 cm−1/heme (20). HbFeIII was prepared from HbFeII by introducing a small volume of air into the sample using a syringe; for example, 720 μl of air was added to 200 μl of a 32 mM heme sample. A single addition of O2 in slight excess (8.5 μmol of O2/μmol of heme) yielded fully oxygenated Hb as indicated by the Soret spectrum recorded following a 5-min equilibration. HbFeIII and HbFeII were prepared from HbFeIII and HbFeII on exposure to NO gas. The optical spectra were recorded using a scan time of 1200 nm/min. Use of the FTIR type cell to record the optical spectra of the products formed in the HbFeIII/GSNO incubates allowed measurements to be made at close to physiological concentrations of Hb (2–4 mM) (21).

**Preparation of Dialyzed Hb Samples**—Approximately 500 mg of HbFeIII was dissolved in 5 ml of 100 mM Na2EDTA (Sigma), pH 7.0, and dialyzed against 100 mM NaPi, pH 7.2, for 30 min. The solution was then dialyzed versus 10 mM NaPi, pH 7.0, which was replaced with fresh solution six times in 24 h. The dialysis was continued versus EDTA-free H2O, which was replaced with fresh solution 12 times in 48 h. After dialysis, HbFeIII was lyophilized and dissolved in NaPi to a concentration of 30 mM heme.

**ICP-MS Analysis**—A PE Sciex Elan 6000 ICP-MS with a cross-flow nebulizer and a Scott type spray chamber was used to determine the amount of copper in the Hb and GSNO samples and in the buffers (see Table I). The RF power was 10,000 W, and the argon flow was 0.85 liter/min, which gave the best sensitivity as determined by the recommended optimization procedure. The optimum lens voltage was determined by maximizing rhodium sensitivity, and the data were acquired in the pulse count mode (22). Stock Hb solutions in NaPi were added to 50 μl of 30% H2O2 (ACP Chemicals, Inc.) and 500 μl of concentrated HNO3 (OmniTrace Ultra high purity, EM Science) to give a final heme (iron) concentration of 4.3 mM, and the samples were ashed using a Bunsen burner. The residue was dissolved in 10 ml of 5% (v/v) HNO3, and ICP-MS analyses for copper and iron were performed. An internal standard of 9 mM (0.500 ppm) manganese prepared from a 1000 ppm manganese standard solution (ACP Chemicals, Inc.) was added to all of the solutions. The standard curves were prepared by diluting 1000 ppm copper and iron standard solutions (ACP Chemicals, Inc.) in 5% (v/v) HNO3, and nanopure water to give 0–8 μM (0–0.500 ppm) copper and 0–9 μM (0–0.500 ppm) iron. All of the reported ICP-MS data are the results of at least triplicate experiments, and in all cases the standard deviations were <5%.}

**FTIR Analysis**—Approximately 20 μl of Hb (28 mM heme) in NaPi was added by syringe onto a 13-mm CaF2 window in the glove bag under N2 where necessary. The FTIR cell was immediately assembled with a 250-μm Teflon spacer (Harrick), and the spectra were recorded at 25°C on a Nicolet Magna-IR 550 spectrometer with a MCT detector cooled to 77 K and purged with dry air from a Whatman FTIR Purge (model 75-52). All of the reported spectra are averages of 500 scans recorded in 5.52 min at a resolution of 2 cm−1 using a Happ-Genzel apodization with a velocity and aperture of 4.4303 cm/s and 2, respectively. Omnic (Nicolet) software was used for subtraction, base-line correction, smoothing, and Fourier transform self-deconvolution employing a half-width-at-half-height of 0.6 cm−1 and an enhancement (K factor) of 1. Subtraction of water vapor absorption from the spectra was performed by the method of Dong et al. (23, 24).

**ESI-MS Analysis**—Stock Hb solutions (28 mM heme) in NaPi were diluted 10−fold with H2O to give ~0.5 μg/μl protein. The aliquots were infused into the ESI source of the mass spectrometer (ThermoFinnigan SSQ 7000) by flow injection from the high performance liquid chromatography (Aglient 1090) using a 100-μl loop (but no column) at 50 μl/min with 5% CH3CN (0.05% trifluoroacetic acid) as the mobile phase. Stock (250 mM) GSNO, GSH, GSNO, and GSSG solutions in 200 mM NaPi, pH 7.2, were diluted 500-fold with H2O and 10-fold to 50 μM with 75% CH3CN (0.05% trifluoroacetic acid), and their mass spectra were evaluated as for the Hb solutions.

**Multi-component Analysis of the Optical Spectra of Incubates**—The absorbance at a given wavelength is the sum of the absorbances (A) of all species at that wavelength: A = A1 + A2 + A3 + . . . = ε1[X] + ε2[O2] + ε3[NO] + . . .
RESULTS

ICP-MS Analysis—Because the stability of S-nitrosothiols is highly dependent on the copper content, all of the solutions were examined by ICP-MS for trace copper. The results are summarized in Table I and reveal that 19 μM copper was found in 5 mM Hb (20 mM heme) solutions. This decreased to 2 μM copper following dialysis versus EDTA. NaNi buffer (200 mM) and the 250 mM stock solutions of GSH and its derivatives were found to contain −1 μM copper (Table I). These values differ from those reported previously using atomic absorption spectroscopy, where −50 μM copper was found in solutions containing 5 mM Hb, but <1 μM copper was found in 5 mM dialyzed Hb as well as in NaNi (4). Because ICP-MS is more sensitive, more accurate, and did not show matrix effects (as verified using a manganese internal standard) compared with atomic absorption spectroscopy (25), the present copper analyses are considered more reliable.

Optical Absorption Spectra—Fig. 1a compares the spectra of HbFeII and HbFeIII and their NO adducts in the Soret and visible regions. As expected, high spin FeII and FeIII hemes exhibit Soret maxima at 430 and 405 nm, respectively, and visible bands at 556 nm (FeII) and 500, 540, and 580 nm (FeIII). Soret maxima are observed at 418 nm (ε = 150 mM−1 cm−1; FeII(NO) and 416 nm (ε = 137 mM−1 cm−1; FeIII(NO)) for the heme-NO adducts of Hb. The corresponding visible bands are at 545 and 575 nm (FeII(NO)) and 540 and 565 nm (FeIII(NO)). These values agree with those reported previously (19, 26).

The products formed on mixing HbFeII and GSNO under anaerobic conditions were directly probed by comparing their spectra with those in Fig. 1a. Evidence for heme-iron nitrosylation is clearly seen in the Soret and visible bands of the HbFeII/GSNO incubates without chelators (Fig. 1b). The Soret maximum blue-shifted from 430 to −418 nm within 5 min of mixing HbFeII and GSNO, and the visible region resembles that of HbFeIINO in Fig. 1a. Multi-component analysis of the 5-min spectrum reveals almost complete conversion of HbFeII to HbFeIINO (Table II). Thus, rapid release of NO from GSNO occurred in the HbFeII/GSNO sample.

Heme nitrosylation is also evident 5 min after mixing HbFeII and GSNO in the presence of 200 μM DTPA (Fig. 1c). The extent of nitrosylation is less because the blue shifting of the Soret maximum is less, and the visible maximum of HbFeII at 556 nm is still evident, indicating that DTPA decreases the amount of NO released from GSNO. Multi-component analysis of the spectrum of the 5-min HbFeII/GSNO/DTPA incubate reveals the presence of 46% HbFeII(NO), 44% HbFeII(NO,dotted line), HbFeII(NO,dotted and dashed line), and HbFeII (dashed line). The corresponding values for the expected iron states in the HbFeII/GSNO incubates are: 169 (405) and 116 (415) for HbFeIINO, 102 (405) and 125 (415) for HbFeII, and 92 (405) and 137 (415) for HbFeIII.

To compare the heme species formed in HbFeII/GSNO incubates with those formed in HbFeII(O2)/GSNO incubates, the spectra of the latter were recorded. The Soret spectra of HbFeII(O2)/GSNO (Fig. 3, a and c) reveal that HbFeIII is formed within 5 min, and multi-component analysis uncovered the...
DeoxyHb and GSNO

**TABLE II**

Hb species present in Hb/GSNO incubates based on analysis of their visible spectra

| Incubate                              | HbFeII | HbFeIII | HbFeII(NO) | HbFeIII(NO) | HbFeII(O2) |
|---------------------------------------|--------|---------|------------|-------------|------------|
|                                       | mm     | mm      | mm         | mm          | mm         |
| HbFeIII/GSNO                          | 0.15   |         |            | 14.85       |            |
| HbFeIII/GSNO/DTPA                    | 6.59   | 1.28    | 6.97       | 0.16        |            |
| HbFeIII/GSNO/neocuproine             | 11.2   |         | 3.8        |             |            |
| HbFeII(II)/dial/GSNO                 | 10.05  |         | 4.95       |             |            |
| HbFeII(II)/dial/GSNO/neocuproine     | 14.72  |         | 0.28       |             |            |
| HbFeII(II)/GSNO                      |        |         |            |             | 6.85       |
| HbFeII(II)/GSNO/DTPA                 |        |         |            |             | 8.15       |
| HbFeII(II)/GSNO/neocuproine          |        |         |            |             | 11.5       |
| HbFeII(II)/neocuproine               |        |         |            |             | 13.2       |

*HbFeII(dial) was dialyzed versus EDTA (see “Methods”).

15 mM (heme) Hb plus 15 mM GSNO were incubated at room temperature in 200 mM sodium phosphate buffer, pH 7.2. Spectra were recorded 5 min after the start of the incubation. The wavelengths used in the multi-component analysis were the Soret maxima of the expected components, and the extinction coefficients are given under “Methods.”

Results corroborate those from the analysis of the visible spectra, which revealed that the major Hbs are HbFeII(NO) and HbFeIII in the 5-min HbFeII(GSNO) incubates in the presence of DTPA and neocuproine, respectively. Furthermore, no evidence for HbSNO formation is seen in Fig. 4a, because the ν(SH) absorption of Cysβ93 does not appear to lose intensity on exposure to GSNO (Fig. 4a, spectrum 1 versus spectra 2 and 3 and spectrum 6 versus spectra 4 and 5).

**Fig. 3.** Effect of 15 mM GSNO and DTPA on the visible absorption spectra of 15 mM (heme) HbFeIII(O2), a, HbFeII(NO) (dashed line) and HbFeII(NO) + GSNO after 5 min (solid line), b, HbFeII(NO) (dashed line) and HbFeIII(NO) + GSNO + 200 μM DTPA after 5 min (solid line), c, observed spectrum from a for HbFeII(NO) + GSNO after 5 min (solid line); sum (line with short dashes) of the spectra of 6.85 mM HbFeIII(NO) (with long dashes) and 8.15 mM HbFeII(NO) (dotted line), d, observed spectrum from b for HbFeII(NO) + GSNO + 200 μM DTPA after 5 min (solid line); sum (line with short dashes) of the spectra of 3.1 mM HbFeIII(NO) (with long dashes) and 11.9 mM HbFeII(NO) (dotted line). See legend for Fig. 1 for experimental details.

1) exhibits the three ν(SH) peaks at 2576, 2563, and 2558 cm⁻¹ assigned previously to Cysβ93, Cysβ112, and Cysα104, respectively (29, 30).

The spectrum of HbFeII plus GSNO (Fig. 4a, spectrum 4) exhibits a peak for Cysβ93 ν(SH) at 2584 cm⁻¹, which is close to that of HbFeII(NO) (2585 cm⁻¹). The ν(SH) band for Cysβ93 is also at 2584 cm⁻¹ in the HbFeII(GSNO/DTPA spectrum (Fig. 4a, spectrum 5), but in the presence of both DTPA and neocuproine (Fig. 4a, spectrum 2) Cysβ93 possesses the same ν(SH) (2576 cm⁻¹) as HbFeIII alone (Fig. 4a, spectrum 1). These results corroborate those from the analysis of the visible spectra, which revealed that the major Hb species are HbFeII(NO) and HbFeIII in the 5-min HbFeII(GSNO) incubates in the presence of DTPA and neocuproine, respectively. Furthermore, no evidence for HbSNO formation is seen in Fig. 4a, because the ν(SH) absorption of Cysβ93 does not appear to lose intensity on exposure to GSNO (Fig. 4a, spectrum 1 versus spectra 2 and 3 and spectrum 6 versus spectra 4 and 5).

Loss of Cysβ93 ν(SH) absorption is clearly evident in Fig. 4b (spectrum 1). Thus, HbSNO formation does occur in the HbFeII(NO)/GSNO incubates in the absence of metal chelators as we reported previously (4). The addition of DTPA prevents loss of Cysβ93 ν(SH) intensity, but the spectrum is not identical to

**Fig. 2. Multi-component analysis of the visible absorption spectra obtained 5 min after mixing 15 mM (heme) HbFeII and 15 mM GSNO. a, observed spectrum from Fig. 1c for HbFeII + GSNO + DTPA (solid line); sum (line with short dashes) of the spectra of 1.28 mM HbFeII (dashed line), 0.16 mM HbFeII(NO) (line with dashes and two dots), 6.97 mM HbFeIII(NO) (line with long dashes), and 6.59 mM HbFeII (line with dashes and dots). b, observed spectrum from Fig. 1f for dialyzed HbFeII + GSNO + 150 μM neocuproine (solid line); sum (line with short dashes) of the spectra of 0.28 mM HbFeII(NO) (line with long dashes) and 14.72 mM HbFeIII (dotted and dashed line).

Absorbance vs. Wavelength (nm)

- FTIR Spectra—FTIR spectroscopy is a valuable probe of protein thiols because the SH stretching vibration ν(SH) falls in a spectral window (~2500 cm⁻¹) with minimum H₂O and protein absorption (29–31). Although ~5 mM Hb is necessary to observe the weak IR ν(SH) absorption (29–31), comparable Hb concentrations are found in RBCs (~3 mM) (21). The FTIR spectrum of HbFeII in the ν(SH) region recorded in the absence and presence of GSNO and metal chelators (Fig. 4a, spectrum 1) exhibits the three ν(SH) peaks at 2576, 2563, and 2558 cm⁻¹ assigned previously to Cysβ93, Cysβ112, and Cysα104, respectively (29, 30).

The spectrum of HbFeII plus GSNO (Fig. 4a, spectrum 4) exhibits a peak for Cysβ93 ν(SH) at 2584 cm⁻¹, which is close to that of HbFeII(NO) (2585 cm⁻¹). The ν(SH) band for Cysβ93 is also at 2584 cm⁻¹ in the HbFeII(GSNO/DTPA spectrum (Fig. 4a, spectrum 5), but in the presence of both DTPA and neocuproine (Fig. 4a, spectrum 2) Cysβ93 possesses the same ν(SH) (2576 cm⁻¹) as HbFeIII alone (Fig. 4a, spectrum 1). These results corroborate those from the analysis of the visible spectra, which revealed that the major Hb species are HbFeII(NO) and HbFeIII in the 5-min HbFeII(GSNO) incubates in the presence of DTPA and neocuproine, respectively. Furthermore, no evidence for HbSNO formation is seen in Fig. 4a, because the ν(SH) absorption of Cysβ93 does not appear to lose intensity on exposure to GSNO (Fig. 4a, spectrum 1 versus spectra 2 and 3 and spectrum 6 versus spectra 4 and 5).

Loss of Cysβ93 ν(SH) absorption is clearly evident in Fig. 4b (spectrum 1). Thus, HbSNO formation does occur in the HbFeII(NO)/GSNO incubates in the absence of metal chelators as we reported previously (4). The addition of DTPA prevents loss of Cysβ93 ν(SH) intensity, but the spectrum is not identical to
tion, smoothing, and Fourier transform self-deconvolution were performed only in the HbFeIIO₂ incubates in the absence of metal chelators (Fig. 4 spectrum 1) but not in their presence (data not shown). These MS results support those from FTIR spectroscopy in that HbSNO is formed only in the HbFeIIO₂ incubates (4). No peak corresponding to S-nitrosation of the β-subunit of HbFeI⁺ was observed under any conditions with or without metal chelators (Fig. 5a) or in the dialyzed HbFeII samples. On the other hand, S-nitrosation of the β-subunit was detected following incubation of HbFeIIO₂ with GSNO in the absence of metal chelators (Fig. 5b) but not in their presence (data not shown). These MS results support those from FTIR spectroscopy in that HbSNO is formed only in the HbFeIIO₂/GSNO incubates in the absence of metal chelators (Fig. 4b, spectrum 2 versus spectrum 4).

Mass Spectral Analysis—ESI-MS was used to probe HbSNO formation in the HbFeIIO₂/GSNO incubates (4). No peak corresponding to S-nitrosation of the β-subunit of HbFeI⁺ was observed under any conditions with or without metal chelators (Fig. 5a) or in the dialyzed HbFeII samples. On the other hand, S-nitrosation of the β-subunit was detected following incubation of HbFeIIO₂ with GSNO in the absence of metal chelators (Fig. 5b) but not in their presence (data not shown). These MS results support those from FTIR spectroscopy in that HbSNO is formed only in the HbFeIIO₂/GSNO incubates in the absence of metal chelators (Fig. 4b, spectrum 2 versus spectrum 4).

In the low m/z region of the ESI mass spectra, the low molecular weight products formed in the HbFeIIO₂/GSNO incubates can be monitored. In the absence of metal chelators, a weak GSNO (m/z 337) and an intense GSSG peak (m/z 613) are observed (Fig. 6b) that are not present in the spectrum of Hb alone (Fig. 6c). In mass spectrum of the HbFeI⁺/GSNO/nectroproline incubate (Fig. 6d), GSNO is the dominant glutathione species, and little GSH is present, consistent with decreased GSNO breakdown in the presence of the Cu(I)-specific chelator. In Fig. 6c, the GSNO intensity is ~50% of that in the presence of neocuproine, indicating that DTPA is less effective in inhibiting the release of NO from GSNO in Hb solutions. However, a well defined GSSG peak (m/z 613) is not observed in Fig. 6c as expected if GSH is the major source of electrons for the reduction of HbFeIIO₂ alone (Fig. 4b, spectrum 2 versus spectrum 4). Direct monitoring of the heme shows 23% conversion of HbFeIIO₂ to HbFeI⁺ within 5 min in the HbFeIIO₂/GSNO/DTPA incubate (Fig. 3 and Table II). Thus, the FTIR data confirm that DTPA does not prevent release of NO from GSNO (Reaction 4), but it does inhibit S-nitrosation of Cysβ₁⁰⁰ (Fig. 4b, spectrum 2 versus spectrum 1). The FTIR spectrum of the HbFeIIO₂/GSNO/neocuproine incubate is essentially identical to that of HbFeIIO₂ alone (Fig. 4b, spectrum 3 versus spectrum 4), which is consistent with the optical results where 88% HbFeIIO₂ was found in the neocuproine incubate (Table II).

Mass Spectral Analysis—ESI-MS was used to probe HbSNO formation in the HbFeIIO₂/GSNO incubates (4). No peak corresponding to S-nitrosation of the β-subunit of HbFeI⁺ was observed under any conditions with or without metal chelators (Fig. 5a) or in the dialyzed HbFeII samples. On the other hand, S-nitrosation of the β-subunit was detected following incubation of HbFeIIO₂ with GSNO in the absence of metal chelators (Fig. 5b) but not in their presence (data not shown). These MS results support those from FTIR spectroscopy in that HbSNO is formed only in the HbFeIIO₂/GSNO incubates in the absence of metal chelators (Fig. 4b, spectrum 2 versus spectrum 4).

In the low m/z region of the ESI mass spectra, the low molecular weight products formed in the HbFeIIO₂/GSNO incubates can be monitored. In the absence of metal chelators, a weak GSNO (m/z 337) and an intense GSSG peak (m/z 613) are observed (Fig. 6b) that are not present in the spectrum of Hb alone (Fig. 6c). In mass spectrum of the HbFeI⁺/GSNO/neocuproine incubate (Fig. 6d), GSNO is the dominant glutathione species, and little GSH is present, consistent with decreased GSNO breakdown in the presence of the Cu(I)-specific chelator. In Fig. 6c, the GSNO intensity is ~50% of that in the presence of neocuproine, indicating that DTPA is less effective in inhibiting the release of NO from GSNO in Hb solutions. However, a well defined GSSG peak (m/z 613) is not observed in Fig. 6c as expected if GSH is the major source of electrons for the reduction of HbFeIIO₂ (Reactions 4 and 5). A relatively intense GSH peak persists in Fig. 6c, and also the optical spectra reveal that HbFeI⁺ is formed in the HbFeIIO₂/GSNO/DTPA incubate (Table II). Because HbFeI⁺ is not observed in the HbFeIIO₂ incubates in the absence of DTPA, [Cu(II)(DTPA)]²⁻ was added to a solution of HbFeII to determine whether this complexed form of copper could oxidize the heme. Multi-component analysis of the absorption spectrum of 4 μM HbFeIIO₂ and 16 μM [Cu(II)-(DTPA)]²⁻ after 5 min of incubation revealed the presence of HbFeIIO₂;
FIG. 7. Visible absorption spectra of 4 μM (heme) HbFeII with 16 μM [Cu(II)(DTPA)]2−. Dashed line, HbFeII; solid line, HbFeII + [Cu(II)(DTPA)]2− after 5 min. [Cu(II)(DTPA)]2− was prepared by mixing equimolar solutions of CuSO4 and DTPA. The experimental details are as given in the legend to Fig. 1, except that a 1-cm quartz cuvette was used.

21% HbFeIII (Fig. 7). This can be compared with 19% HbFeIII formation in the HbFeIII/GSNO/DTPA incubate (Table II).

2. DISCUSSION

Spencer et al. (10) have reported that HbFeIII can directly reduce GSNO and that the released NO is captured by additional FeII heme. Because the release of NO from GSNO is known to be Cu(I)-catalyzed (32), we monitored HbFeIII/GSNO incubates by optical and FTIR spectroscopies to probe changes occurring at the heme and Cysβ35 centers, respectively. In addition, the incubates were analyzed by ESI-MS to examine changes in the mass of the protein and to determine the low molecular weight species produced.

The optical spectra shown in Fig. 1 clearly reveal that trace Cu(I) is required for the release of NO from GSNO (Reaction 4). Essentially no HbFeIII NO is formed in the HbFeIII/GSNO incubate containing both copper-depleted Hb (i.e. dialyzed Hb in Table I) and neocuproine. The combined data in Tables I and II indicate that total inhibition of Cu(I) catalysis of GSNO breakdown requires removal of most of the copper as well as neocuproine addition to Hb-containing solutions. Thus, the results summarized in Table II are not consistent with direct reduction of GSNO by HbFeIII (10) (Reaction 2) when equimolar heme and GSNO are present. Given the rapid (<5 min) Cu(I)-catalyzed release of NO from GSNO (Fig. 1b), it is likely that copper-catalyzed reductive cleavage of GSNO also occurs in vivo.

The next question that arises is, of course, what is the source of reducing equivalents to regenerate Cu(I) following electron transfer to GSNO to release NO (Reaction 4)? Possible electron donors to Cu(I) are GSH (Reaction 5) and HbFeII. With GSH as a donor, two molecules of NO would be released or two heme FeIII NO adducts would be formed per molecule of GSSG produced. The mass spectrum reveals extensive formation of GSSG in the HbFeIII/GSNO incubates in the absence of chelators (Fig. 6b). Although a peak corresponding to its Na+ adduct occurs at m/z 635, the expected GSSG peak at m/z 613 is not clearly evident in HbFeIII/GSNO incubates containing DTPA. Nonetheless, GSSG does release NO, as evidenced by the loss of GSSG peak intensity relative to the base peak at m/z 638 (Fig. 6, c versus d) and the formation of HbFeII NO (Fig. 1c and Table II). Thus, reduction of [Cu(II)(DTPA)]2− by HbFeIII was considered, which would account for the HbFeIII absorbance seen in Fig. 2a. From Table II, 7.13 mM NO is released (6.97 mM HbFeIII NO and 0.16 mM HbFeII NO) from the 15 mM GSNO present in the HbFeIII/GSNO/DTPA incubate. If all of the reducing equivalents were to come from GSH oxidation (Reaction 5), then 3.56 mM GSSG should be produced. However, HbFeIII oxidation provides 1.44 milliequivalents of reduc tant (1.28 mM HbFeIII and 0.16 mM HbFeIII NO), which would decrease the concentration of GSSG produced to 2.84 mM. This is approxi mately one-third of the GSSG produced in HbFeIII/GSNO incubates where a strong GSSG peak is observed at m/z 613. The lower than expected intensity of the GSSG (m/z 613) peak in Fig. 6c is attributed to ion suppression. The production of HbFeIII in the HbFeIII incubates in the presence but not the absence of DTPA (Table II) suggested that [Cu(II)(DTPA)]2− may accept an electron from HbFeII, which was confirmed. The addition of authentic [Cu(II)(DTPA)]2− to HbFeIII led to oxidation of 21% of the heme (Fig. 7). Electron transfer between [FeII(EDTA)]2− and HbFeIII has been reported previously (12).

No loss of ν(SH) intensity was detected by FTIR when HbFeII was treated with GSNO with or without metal chelators (Fig. 4a). This is consistent with the proposal that the allosteric transition of Hb controls Cysβ35 S-nitrosation (33). The FTIR results also show that Cu(I) is required for HbFeIII NO formation because neocuproine inhibited blue shifting of the Cysβ35 ν(SH) peak (Fig. 4a, spectra 2 and 3) that accompanies heme nitrosylation (29). The reactions in the HbFeIII/GSNO incubates reported here are shown in Scheme 1. Clearly the oxidation of HbFeII by [Cu(II)(DTPA)]2− is unlikely to be of importance in vivo, but the requirement of copper for NO release from GSNO shown in Scheme 1 could well have physiological relevance.

The key result that copper is also required for S-nitrosation of Cysβ35 of HbFeII NO (4) can be deduced from Figs. 4b and 5b. S-Nitrosation of Cysβ35 is observed only in the absence of metal chelators (Fig. 4b, spectrum 1, and Fig. 5b). Thus, we propose Scheme 2 for the reactions of NO with HbFeII NO. NO is targeted to Cysβ35 in the absence of chelators, but in the presence of DTPA, all of the NO released from GSNO is targeted to the FeIII O2 heme (Figs. 3 and 4b) and converted to NO3− (Reaction 6). Scheme 2 predicts that efficient Cu(II)-catalyzed S-nitrosation of Cysβ35 will preserve the biological activity of NO by preventing its conversion to nonvasoactive NO3−. The capture of NO by HbFeIII (Scheme 1) should not lead to loss of its vasoactive power as long as FeIII NO adduct formation is reversible on a physiologically relevant time scale. It has been proposed that the FeIII heme centers of partially oxygenated Hb, as found in the RBC (1), have a much lower affinity for NO than fully deoxyg enated HbFeII NO (8). Thus, copper control of NO reactivity with Hb, plus weaker binding of NO to HbFeIII in vivo than in vitro, could explain why RBC Hb does not act as a sink for most of the NO produced in the vascular system. A possible copper catalyst in vivo is copper-zinc superoxide dismutase, which is abundant in the RBC (34).

It is of interest to compare the extent of the prompt changes in HbFeIII and HbFeIII NO on incubation with GSNO. When 15 mM GSNO is incubated with 15 mM (heme) HbFeIII in the absence of metal chelators, 99% is converted to HbFeIII NO within 5 min, whereas only 46% of HbFeIII NO2 is converted to...
HbFeIII under the same conditions. This is easy to understand when we consider that ~7.5 nM NO released from GSNO was targeted to Cysβ35 in HbFeIII but not in HbFeII (Figs. 4 and 5). In the DTPA incubates 44% HbFeIII versus 77% HbFeIII remain, and in necuprein incubates 75% HbFeII versus 88% HbFeIII remain after the same 5-min period. Clearly, the is blue-shifted (409 nm) from that at 5 min (411 nm) (Fig. 1 of GSNO by HbFeII but are consistent with catalysis of GSNO breakdown by trace copper and NO generation in the reac-
tions. Because HbFeII is stable in the presence of metal chelators is due to inhibition of GSNO breakdown, but the lower consumption of HbFeIII relative to HbFeII cannot be attributed to NO trapping by Cysβ35 in the former because this does not occur in the presence of chelators (Fig. 4b). More efficient NO trapping by HbFeII (35) than by HbFeIII (k = 3–5 × 107 M−1 s−1 for Reaction 6) (36) would give rise to the higher HbFeII consumption in the incubates. Also, the reaction of NO with any free O2 in the HbFeIII incubate would decrease the amount of HbFeIII consumed.

In this work we also examined the spectra of the HbFeIII/GSNO incubates after 1 h. The results obtained indicate that the prompt products undergo further reaction over longer times. For example, the Soret maximum after 1 h of HbFeIII/GSNO incubate still shows a maximum at 418 nm but with decreased intensity (data not shown). Because the spectrum of authentic HbFeIII(NO) in the absence of any glutathione-derived species is stable for >1 h, the HbFeIII(NO) initially formed must react with some reagent in the incubate.2 The Soret maximum of the HbFeIII(GSNO)/DTPA incubate after 1 h (data not shown) is blue-shifted (409 nm) from that at 5 min (411 nm) (Fig. 1c). We initially attributed this blue shift to increased HbFeIII(NO) formation caused by NO-driven reduction of HbFeII using reducing equivalents from GSH. However, the addition of GSH to authentic HbFeIII(NO) also gave rise to a Soret maximum at 411 nm and not the expected HbFeIII(NO) peak at 418 nm. The biological relevance of these slow reactions is questionable because, for example, any HbFeIII formed in the RBC would be rapidly converted to HbFeII by methemoglobin reductase (37).

CONCLUSIONS

The results reported here provide insight into the mechanism of heme nitrosoylation following mixing of HbFeII with GSNO. The data presented are inconsistent with direct reduction of GSNO by HbFeII but are consistent with catalysis of GSNO breakdown by trace copper and free NO generation in the reactions. Because HbFeII is stable in the presence of GSNO when free copper is rigorously excluded from the system, direct reduction of GSNO by HbFeII is unlikely to be of biological significance. Trace copper also controls targeting of NO released from GSNO to the Cysβ35 and FeIII centers of HbFeII. Thus, we conclude that copper catalysis of S-nitrosation and S-denitrosation plays a key role in preserving the vasoactivity of NO in the RBC.

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