Metal Chelators Inhibit S-Nitrosation of Cysβ93 in Oxyhemoglobin

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The role in blood flow of S-nitrosohemoglobin (HbSNO), where Hb is S-nitrosated at Cysβ93, is currently the subject of intense debate.[1] HbSNO presumably controls delivery of the vasorelaxant nitric oxide to hypoxic tissues in an oxygen-sensitive manner.[1a,b] In support of a role for Hb in NO transport, a significant fraction of NO in the blood is in the form of HbSNO, with concentrations ~10 times higher in arterial (~300 nM) vs venous (~30 nM) blood,[1c] although more recent studies did not detect arterial−venous HbSNO gradients.[1f] HbSNO formation may also provide red blood cells (RBCs) with a mechanism to counteract platelet activation.[2] Despite its potential physiological importance, the mechanism of HbSNO formation in vivo remains unknown, but a trans-S-nitrosation equilibrium with low-molecular-weight nitrosothiols, such as nitrosoglutathione (GSNO), is a central postulate:[1]

$$\mathrm{GSNO} + \mathrm{HbSH} \rightleftharpoons \mathrm{GSH} + \mathrm{HbSNO} \qquad (1)$$

We have *directly* probed spectrometrically the protein products formed on mixing Hb and GSNO. Cysβ93 S-nitrosation was detected only in oxyHb and, unlike trans-S-nitrosation,[3a,b] *requires* the presence of metal ions.

Fourier transform infrared spectroscopy (FTIR) is a valuable probe of protein thiols.[4] The SH stretching vibration ν(SH) falls in a spectral window (~2500 cm^{-1}) with minimum H_2O and protein absorption.[4] The FTIR spectra of human oxy- and deoxyHb were recorded alone and in the presence of GSNO, the nitroso form of the dominant thiol in RBCs.[5] Although ~6 mM Hb is necessary to observe the weak IR ν(SH) absorption,[4] comparable Hb concentrations are found in RBCs (~5 mM). The FTIR spectrum of oxyHb in Figure 1a exhibits the three ν(SH) peaks at 2586, 2566, and 2556 cm^{-1} assigned previously to Cysβ93, Cysβ112, and Cysα104, respectively.[4b,c] The oxyHb/GSNO spectrum does not exhibit a 2586-cm^{-1} peak (Figure 1b),

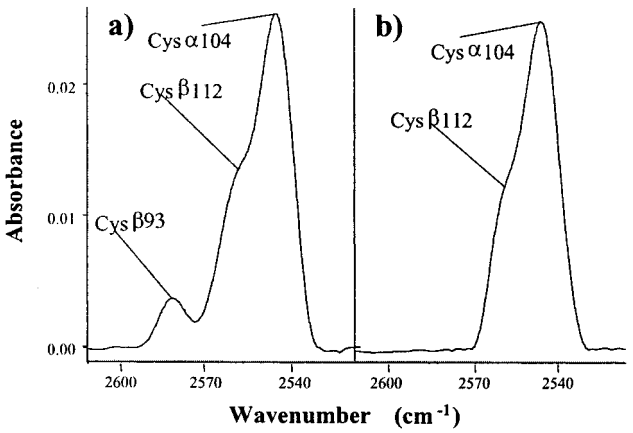

Figure 1. FTIR spectra in the ν(SH) region of (a) 30 mM (heme) oxyHb and (b) 28 mM (heme) oxyHb with 28 mM GSNO. Experimental procedures: OxyHb was prepared from human metHb (Sigma) as described previously.[7] The Hb samples (20 μL) in 200 mM sodium phosphate buffer (pH 7.2) were added by syringe onto a 13-mm CaF_2 window with 250-μm Teflon spacer. The cell was immediately assembled, 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). Each spectrum is the average of 500 scans recorded in 6 min [starting at $t = 2$ min following mixing of oxyHb and GSNO (Cayman) for sample b] at 2-cm^{-1} resolution with an aperture of 32. Omnic (Nicolet) software was used for subtraction, baseline correction, smoothing, and Fourier self-deconvolution (HWHH 8.2 cm^{-1}, K factor 1) of the spectra.

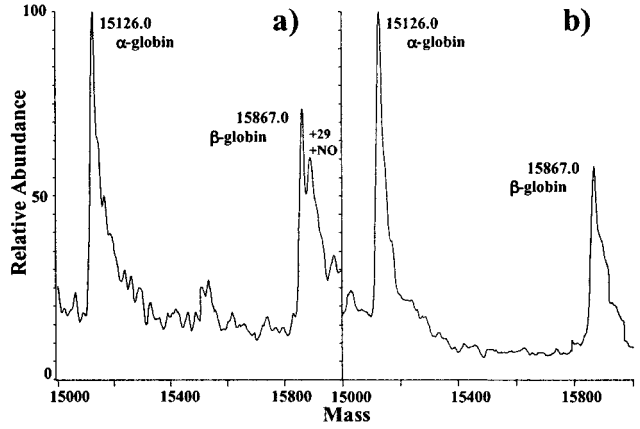

Figure 2. Deconvolved electrospray mass spectra of the heme-free Hb subunits from the reactions of (a) oxyHb with GSNO and (b) oxyHb with GSNO and DTPA. Experimental procedures: Samples prepared as in Figure 1 [500 μM DTPA was added to (b)] were diluted 10^3-fold with H_2O to give ~0.5 μg/μL Hb. Aliquots (100 μL) were infused into the electrospray source of the mass spectrometer (Finnigan SSQ 7000) by flow injection from the HPLC (HP1090) at 50 μL/min using a 75% CH_3-CN (0.05% TFA) isocratic elution. Under these conditions, Hb dissociates into free heme and α- and β-subunits. The unresolved shoulders on the subunit peaks at high mass are due to sodium adducts.

consistent with S-nitrosation of the Cysβ93 residues, which are the two reactive thiols in Hb.[1a] This was confirmed by mass spectrometry; the deconvolved mass spectra of the heme-free subunits from oxyHb reveal a new peak corresponding to β-globin plus NO (+29 Da for CysH → CysNO) on GSNO exposure whereas the mass of α-globin remains unaltered (Figure 2a). DeoxyHb also exhibits three ν(SH) peaks but Cysβ93 ν(SH) absorption is observed in the deoxyHb/GSNO spectrum and no β-globin nitrosation was detected in the mass spectra of either

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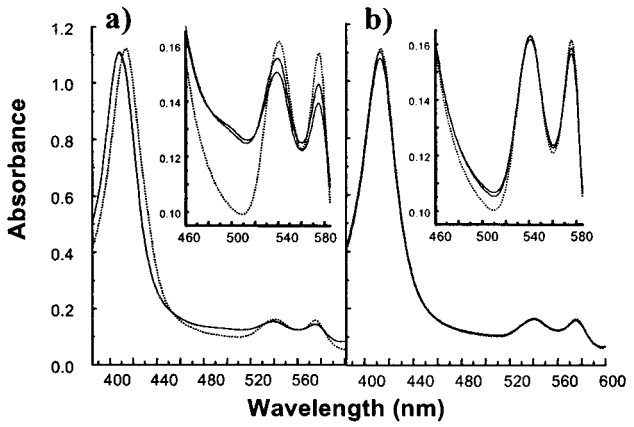

Figure 3. UV−vis absorption spectra of 15 mM (heme) oxyHb and 15 mM (heme) oxyHb with 15 mM GSNO. (a) No metal chelators present; (b) 200 μM DTPA (diethylenetriamine-*N,N,N′,N″,N″*-pentaacetic acid; ICN) and 120 μM neocuproine (2,9-dimethyl-1,10-phenanthroline; Sigma) present. Experimental procedures: Samples (10 μL) in 200 mM sodium phosphate buffer (pH 7.2) were added to the FTIR cell (6-μm Teflon spacer) as described in Figure 1, and the cell was placed in a custom-made bracket in the spectrophotometer (Beckman DU 650). Spectra were recorded (1200 nm/min) at 25 °C for oxyHb (- - -), and for oxyHb with GSNO (—) at $t = 2$ and 15 min.

deoxyHb sample (data not shown). Thus, our FTIR and mass spectral data support the findings, obtained by indirect measurements by Stamler and co-workers, that Cysβ93 readily undergoes S-nitrosation in oxyHb (R-state) but not in deoxyHb (T-state).[1c] This presumably allows Hb to act as an allosterically controlled NO buffer.[1a−c]

The UV−vis spectra provide further insight into the mechanism of HbSNO formation. Evidence for partial heme-iron nitrosylation is seen in the Soret and visible bands of the deoxyHb/GSNO sample and in its blue-shifted (2584 cm^{-1}) Cysβ93 ν(SH) (data not shown), which falls between that of $HbFe^{II}$−NO (2585 cm^{-1}) and deoxyHb (2578 cm^{-1}).[4b,c] GSNO reduction by deoxyHb, which would release the free NO required for heme nitrosylation, has been reported:[6]

$$HbFe^{II} + GSNO \rightleftharpoons HbFe^{III} + GS^- + NO \qquad (2)$$

$HbFe^{II}$−NO formation under the present conditions (heme:GSNO = 1:1) would require GSH oxidation by $HbFe^{III}$−NO, and GSSG was detected by mass spectrometry (data not shown). A blue-shift in the Soret of the oxyHb/GSNO sample (Figure 3a) is evidence of metHb formation, which has a Soret maximum at 405 nm.[7] Reaction 3 has not been reported, but since GSNO is

$$HbFe^{II}{-}O_2 + GSNO \rightleftharpoons HbFe^{III} + GS^- + O_2 + NO \qquad (3)$$

known to oxidize free O_2^-,[8] it may also oxidize the $Fe^{III}O_2^-$ center of oxyHb.

Additional metHb would be produced by reaction 4, which is well documented:[9]

$$HbFe^{II}{-}O_2 + NO \rightleftharpoons HbFe^{III} + NO_3^- \qquad (4)$$

NO release from GSNO could also be catalyzed by ubiquitous copper impurities:[3]

$$RSNO + Cu^I \rightleftharpoons RS^- + NO + Cu^{II} \qquad (5)$$

To distinguish between NO generation by reactions 3 and 5, the experiments were repeated in the presence of chelators. The Cu^{II} chelator DTPA did not inhibit formation of metHb, but in the presence of the Cu^I chelator neocuproine, the growth of metHb absorption in the oxyHb/GSNO sample was negligible (Figure 3b), indicating that NO is generated mainly via reaction 5.

Surprisingly, in the presence of 200 μM DTPA, no significant loss of Cysβ93 ν(SH) intensity was observed in the oxyHb/GSNO FTIR spectrum, which resembled that in Figure 1a. Mass spectral analysis confirmed that β-globin S-nitrosation was inhibited by DTPA (Figure 2b). Consistent with these results, amperometric measurements of NO consumption indicated that Cu^{II} induces rapid HbSNO formation in the presence of *free* NO.[10] Thus, we propose that, in the *absence* of chelators, GSNO decomposes to give free NO (reaction 5), which rapidly S-nitrosates Cysβ93 of oxyHb by reaction −5. Cysβ93 is S-nitrosated rather than GSH since Cu^{II}-catalyzed RSNO formation is not kinetically competitive with RSSR formation ($RS^- + Cu^{II} \rightleftharpoons {}^1/_2RSSR + Cu^I$) for low-molecular-weight thiols.[3,10] If all the Cu^{II} present is chelated by DTPA, NO generated by reaction 5 is consumed by reaction 4, thus converting oxyHb to metHb with no HbSNO formation. A copper-catalyzed mechanism for HbSNO formation in vivo is plausible since RBCs contain ~20 μM copper.[11] Intriguingly, two copper-binding sites[12] have been identified in Hb in vitro, but their Cu loading in RBCs has not been reported. We detected ~50 μM Cu in 5 mM human Hb (Sigma) by atomic absorption analysis (data not shown). Thus, the products observed here reflect competition between Hb,[12] GSH, and GSSG[3c] for copper in the absence of added chelators.

In summary, the data presented are *inconsistent* with HbSNO formation via simple trans-S-nitrosation (NO^+ transfer; reaction 1) and with the putative trans-S-nitrosation equilibrium and rate constants derived from experimental data where HbSNO formation was only inferred, not observed.[1e,13] Our results strongly point to a mechanism involving Cu^{II} catalysis of Cysβ93 S-nitrosation (reaction −5) by *free* NO generated by Cu^I catalysis of RSNO breakdown (reaction 5). The crux of this hypothesis is the identification of the Cu catalysts in vivo.

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