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Rates of release of nitric oxide from HbSNO and internal electron transfer ☆

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Abstract

The discovery that hemoglobin (Hb) in erythrocytes contains a fraction of β-Cys-93 thiols as the nitrosylated derivative (HbSNO) led to the suggestion that this species is involved in transporting and releasing nitric oxide, which is the signal for local vasodilation. The release of NO from HbSNO requires an electron transfer to facilitate release and to regenerate the cysteine thiol via one-electron reduction in the absence of added thiols. An alternative mechanism, which has received much attention, transfers the nitrosyl group to an external thiol, which in turn would have to be reduced. The observed first order rate constant for the spontaneous oxidation of the ferrous heme of deoxy HbSNO is $1.0 \times 10^{-4}\,s^{-1}$ in the absence of thiols. Under the same conditions, native Hb is stable. The oxidation of HbSNO occurs with the same rate constant that can be derived for the rate reported for the formation of HbNO from HbSNO. These similarities suggest that both processes involve the same reaction: internal electron transfer and direct release of nitric oxide.

1. Introduction

Hemoglobin is the major constituent of red blood cells and is responsible for transporting oxygen [1]. It has a tetrameric quaternary structure, with each subunit

☆ This paper was intended for inclusion in the issues dedicated to Frank H. Westheimer on the occasion of his 90th birthday.

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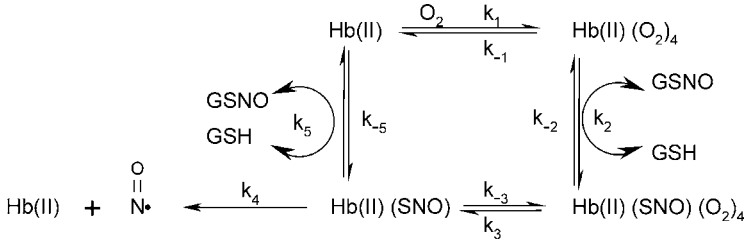
having a ferrous heme group that reversibly binds oxygen. The four globin chains of hemoglobin occur as a pair of αβ dimers.

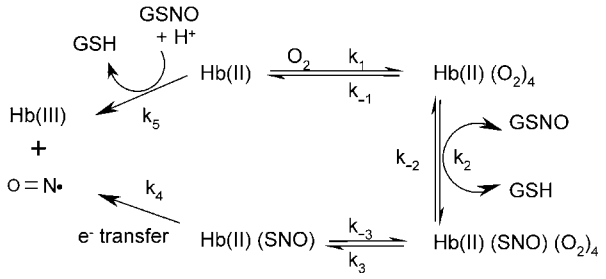
Nitric oxide has been shown to provide a signal to the blood vessel endothelia that causes the surrounding smooth muscle to relax [2]. Stamler and co-workers have reported that a small amount of hemoglobin in red cells is nitrosylated (HbSNO) specifically, the thiol of one β-Cys-93 is nitrosylated [3,4]. They suggest that HbSNO is a circulating source of nitric oxide that promotes vasodilation in response to reduced oxygen concentration. HbSNO is formed when oxyhemoglobin reacts with endogenous nitrosylated thiols, such as glutathione S–NO [4]. In the laboratory, HbSNO is synthesized by reacting Hb with *S*-nitroso *N*-acetylpenicillamine (SNAP) [5,6].

NO is readily transferred to Hb from R–SNO compounds, such as *S*-nitroso-glutathione (GSNO), in the oxygenated state [7]. The rate of transfer of NO from HbSNO to GSH increases in the deoxy state [4], and this has been assumed to be associated with changes in steric effects [8]. The release of NO from HbSNO has been observed in the absence of added thiols at low oxygen pressures. The process was assumed not to be related to any changes in the state of heme irons [4]. Transfer of NO to thiols has been assumed to be the major pathway for NO release but has only been shown to be faster than the release of free NO when there is a 100-fold excess of GSH over HbSNO [4] (Scheme 1).

In this report we provide evidence for release of nitric oxide occurring as a result of an internal electron transfer from the heme to the electrochemically labile S–N



Scheme 1.



Scheme 2.

bond [9]. This process results in release of NO and an oxidation of the iron in the adjacent β heme [10], Scheme 2. When *S*-nitroso thiols are added to deoxygenated hemoglobin, the hemoglobin is partially oxidized and NO gas is released, Eq. (1) [8].

$$\text{Hb Fe}^{2+} + \text{RSNO} + \text{H}^{+} \rightarrow \text{Hb Fe}^{3+} + \text{GSH} + {}^{\cdot}\text{NO} \quad (1)$$

The rate of release of nitric oxide from HbSNO by an internal electron transfer mechanism should be first order

$$\text{Rate} = k_{\text{obs}}[\text{HbSNO Fe(II)}]. \quad (2)$$

Our observation that release of NO from HbSNO leads to more rapid oxidation at the β heme than at the α supports the second mechanism [10].

Yamamoto [11] reported that the signals from the protons on the heme methyl groups of hemoglobin undergo a paramagnetic shift in the ^{1}H-NMR when the ferrous heme is oxidized and coordinated to a high field ligand, such as azide ion. This phenomenon results in resolution of the signals from the α and the β ferric methyls in a region distinct from the rest of the protein (δ15–δ30). Furthermore, it is known that in a mixture of native ferrous–heme hemoglobin and hemoglobin with oxidized dimers, there is a slow equilibration of oxidation states among all the hemes of all subunits in the solution [12].

HbSNO (with both β subunits nitrosylated), upon spontaneous loss of its NO groups, undergoes a spectral change that indicates apparent formation of "half-methemoglobin" with half the hemes containing iron in the ferric state [10]. The observed enhanced oxidation of the β hemes [10], should result from the proximity of the β hemes to the SNO groups at β-Cys-93. The eventual distribution of oxidation of the α and β iron centers will result from the subsequent equilibration of electrons among the subunits [12]. A kinetic analysis of heme oxidation during denitrosylation (in the absence of thiol acceptors and metal contaminants) is necessary for testing the proposed mechanism of the NO-release reaction. Thus, we measured the rate of metHb formation from deoxygenated HbSNO. We compare it to the rate of conversion of HbSNO to Hb derived from data reported by Stamler and coworkers [3,4]. The similar rates for selective heme oxidation and release of nitric oxide are consistent with a mechanism in which NO release is triggered by an internal electron transfer.

2. Materials and methods

Commercial reagents were used without further purification. Water was doubly distilled and de-ionized. Purified carbonmonoxy human hemoglobin A was obtained from Hemosol (9.6 g/dL). ^{1}H-NMR spectra were taken at 500 MHz for proteins and 300 MHz for all other samples. UV/Vis spectroscopy was performed in quartz cells in a temperature-controlled sample compartment with gas control ports and an oxygen sensor. Protein samples were concentrated using centrifugation tubes or stirred-cell ultra-filtration, using a YM-10 membrane.

2.1. S-nitroso N-acetylpenicillamine (SNAP)

Sodium nitrite (0.10 g, 14×10^{-4} mol) was added to *N*-acetylpenicillamine (0.10 g, 5×10^{-4} mol) in 5 mL water, acidified with one drop of concentrated hydrochloric acid in a 10 mL round bottom flask covered with foil. The mixture was stored at 4 °C for 10 min and then added to a foil covered G-25 Sephadex (2.5 × 15 cm) column equilibrated with water to separate the SNAP from excess inorganic nitrite. The green (SNAP) fraction was collected and lyophilized (0.092 g, 90%).

2.2. Nitrosylated Hb (HbSNO)

This synthesis was scaled-up based on previous work [6]. A solution of carbonmonoxy hemoglobin A (10.0 mL, 1.5×10^{-5} mol) was passed through a column containing Sephadex G-25 (5 × 15 cm) equilibrated with 0.05 M sodium borate, pH 9.0, and 0.005 M EDTA to chelate any metal contaminants. The eluted protein was converted into oxyhemoglobin by irradiation under flowing oxygen for 2 h at 0 °C. The hemoglobin was reacted with SNAP (0.09 g, 4×10^{-4} mol) at 4 °C for 15 min in an opaque (foil-wrapped) round bottom flask. The entire sample was passed through a foil-wrapped column of G-25 Sephadex (phosphate buffer, pH 7.4, ionic strength 0.1 M, 5 × 15 cm) and the hemoglobin fraction was collected.

2.3. Methemoglobin (metHb)

A solution of carbonmonoxy hemoglobin A (12.0 mL, 1.8×10^{-5} mol) was reacted with potassium ferricyanide (0.09 g, 2.70×10^{-4} mol) for 3 h at room temperature. The sample was passed through a column of Sephadex G-25 equilibrated with 0.1 M phosphate buffer, pH 7.4. The eluent was concentrated by ultra-filtration, exchanged with water and lyophilised.

2.4. UV/Vis

A sample of HbSNO was deoxygenated for 16 h at 25 °C under N_2 pressure and then re-oxygenated for 30 min with air. A parallel control experiment was performed using native hemoglobin. Absorption spectra of hemoglobin (40 μM) were taken for solutions in phosphate buffer at 25 °C.

2.5. ^{1}H-NMR

The degree of oxidation of the α and β hemes was monitored by ^{1}H-NMR. The nitrosylated sample (HbSNO) was concentrated to 10 mL by ultra-filtration and exchanged twice with 30 mL of deuterium oxide. After the sample was concentrated to 10 mL, a process which lasted until 3 h from nitrosylation, sodium azide was added (0.005 g, 10-fold excess) and the samples were deoxygenated under argon at 25 °C in a foil-wrapped flask. ^{1}H-NMR measurements were taken after 2 and 48 h of deoxygenation for the nitrosylated sample. A control was performed whereby the sodium

azide was added to the HbSNO moments prior to ^{1}H-NMR measurements, to assess the influence of the azide ligand on the rate of oxidation. Measurements on the aliquot, sealed in an NMR tube filled with argon, removed after 2 h of deoxygenation, were taken (500 MHz, water suppression, 45 °C) every 30 min for 15 h. A single ^{1}H-NMR spectrum was taken of the HbSNO after 48 h as well as of each of the controls. Lyophilized methemoglobin was dissolved in deuterium oxide. The metHb ^{1}H-NMR spectrum was recorded (500 MHz, water suppression, 45 °C) for comparison.

3. Results and discussion

3.1. Half-metHb after denitrosylation, detected by its absorption spectrum

Two SNO bonds are formed per tetramer, and in theory two equivalents of Fe(II) are oxidized in an internal electron transfer mechanism. The degree of oxidation for an extensively deoxygenated sample of HbSNO was shown to be $48 \pm 2\%$ methemoglobin [13], Fig. 1. A mixture of ferrous and ferric hemoglobin can be quantitatively analyzed where the hemes are oxygentated [13]. Thus, the sample that had been nitrosylated was oxygenated prior to analysis. Native hemoglobin and HbSNO are relatively stable when oxygenated. Thus, reoxygenation of a deoxygenating sample provides a measured of the extent of heme oxidation at the time of oxygenation. The partial oxidation that we observe confirms that denitrosylation results in heme oxidation and also that the initial HbSNO sample was fully nitrosylated. The spectrum is a composite of those of oxygenated native hemoglobin and methemoglobin at the same concentration.

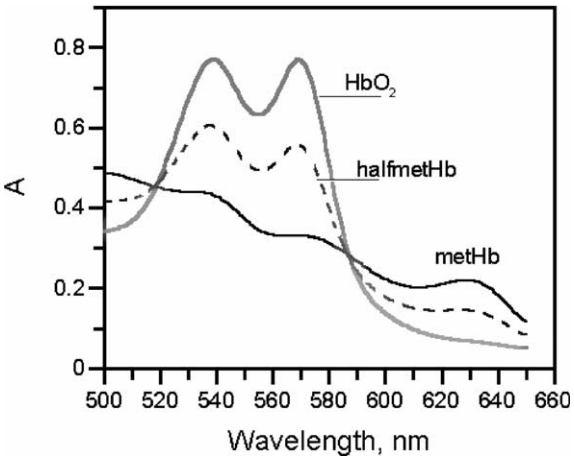


Fig. 1. Spectra reveal the extent of heme oxidation upon denitrosylation. Samples of native human hemoglobin and methemoglobin were briefly oxygenated prior to measurement. *S*-nitrosylated hemoglobin was deoxygenated for 16 h at 25 °C, and then reoxygenated for 30 min with air. The extent of heme oxidation in the previously *S*-nitrosylated sample is $48 \pm 2\%$.

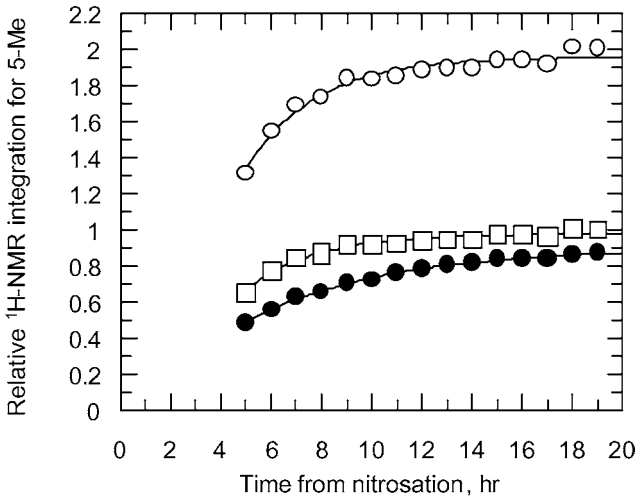


Fig. 2. The rate of NO release from deoxygenated HbSNO. The amount of overall metHb formation (-○-) was calculated as the sum of the integrations of the signals from the 5-Me protons, normalized to 2.00 at $t = 19$ h. The integration of the α subunit (-●-) is consistently below the average oxidation per subunit (-□-) as the overall oxidation increases, an epiphenomenon of the electron exchange between the subunits. The rate of appearance of the oxidized β heme is kinetically complex and is not shown.

3.2. Rate of heme oxidation

The rate of release of NO can be correlated to the extent of heme oxidation in a deoxygenated sample of HbSNO (Fig. 2, -○-). The electron exchange among the hemes can also be observed as the extent of α heme oxidation (Fig. 2, -●-). The latter increases steadily until the distribution of Fe(III) is at equilibrium among all species (Fig. 2, -□-).

The formation of methemoglobin arises from oxidation of the β hemes while the final distribution of oxidized hemes results from equilibration of electrons among the subunits [12]. Fitting the rate of overall metHb formation, as observed from integration of the signal from the protons of the heme 5-Me groups, to the integrated first order rate expression, gives the observed first order rate constant for β heme oxidation.

$$k_{\text{obs}} = 1.00 \pm 0.12 \times 10^{-4}\,\text{s}^{-1}/\text{tetramer}.$$

The rate constant for α oxidation, an epiphenomenon of the β oxidation by NO release, is

$$k_{\text{obs}} = 0.51 \pm 0.03 \times 10^{-4}\,\text{s}^{-1}/\text{tetramer}.$$

The rate of oxidation of the hemes in deoxygenated HbSNO is much greater for the β hemes than the α hemes. As noted earlier, the apparently slower rate of α heme oxidation results from the relatively slow exchange of electrons among the hemes where oxidation occurs in the β hemes [12]. Although oxidation can occur by transfer of an electron to NO (reacting with oxygen) to give NO_3^- [7]. Due to the absence of oxygen in the experiments and the heme-specificity of the oxidation reaction, we ex-

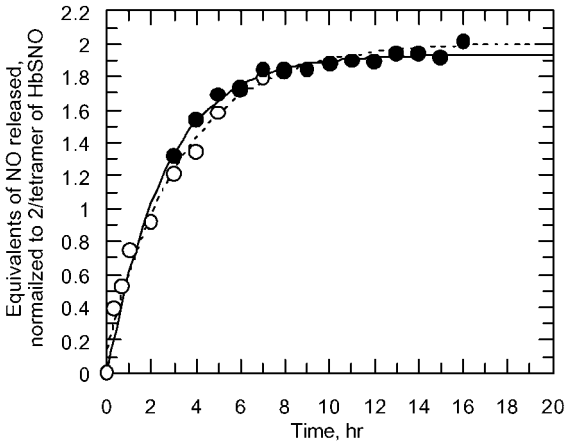


Fig. 3. The rate of NO release from deoxygenated HbSNO, by visible spectroscopy measurements of HbNO recapture (-○-), from Stamler and co-workers [4], compared to the rate of heme oxidation, by ^{1}H-NMR (-●-).

clude a mechanism in which an electron is transferred to NO and oxygen is incorporated to produce nitrate.

To correlate the rate of heme oxidation to NO release, the rate of HbNO formation by recapture under the same conditions was calculated from the data of Stamler and coworkers [4]

$$k_{\text{obs}} = 0.81 \pm 0.15 \times 10^{-4}\,\text{s}^{-1}.$$

The rate constants for the two processes are the same within experimental error (Fig. 3). Since there were no external thiol acceptors present, an internal electron transfer should be required as in the a transfer from ferrous heme to S–NO [10].

The formation of metHb as NO is released may lead to formation of HbNO (iron-coordinated NO). Exposure of deoxygenated Hb to GSNO produces metHb (detected by EPR) and HbNO (detected by visible spectroscopy), Scheme 2 [5]. Although deoxygenated ferric hemoglobin can bind NO, such binding is slower and weaker than in the deoxygenated ferrous species [14]. In our case, we do not detect HbNO from spectroscopic measurements. However, the species may be removed as oxygen and nitrogen sweep the system.

3.3. Proposed mechanism

The generation of NO from the nitrosothiol requires addition of an electron to regenerate the thiol of the cysteine. Our results show that the electron is transferred internally as a response to deoxygenation of the adjacent heme iron in a hemoglobin subunit. When this happens in a red blood cell, the resulting oxidized heme can be reduced to the active ferrous form by methemoglobin reductase [1], allowing for an efficient recovery system. Thus, we can consider that the combination of the internal electron transfer and the reductase in the red cell allows hemoglobin to be a specifically

adapted transporter of nitric oxide in circulation. Other components in the cell, such as those at the surface, do not provide a coupled electron source. Bimolecular processes such as transfer of NO to glutathione from HbSNO would imply that there can be a specific interaction between the protein and glutathione and that a source of electrons other than the heme is available for the regeneration of the cysteine from the resulting radical. Our discovery of the competent electron transfer internally coupled to NO release may explain how the problems of a bimolecular process are overcome.

NO that would be released by internal reduction would be able to bind to free ferrous heme groups. However, binding and release from the ferrous heme is dynamic and the overall release from the erythrocytes will be controlled by an equilibrium among the heme-associating species. When NO is free, it is a dissolved gas and will diffuse out of the cell. Given that the heme it is closest to upon release is oxidized and incapable of tight binding, diffusion away from the immediate hemoglobin will be entropically driven.

The in vivo system is obviously complex and we cannot surmise to what extent NO released from hemoglobin would bind to the endothelium to produce local vasodilation. However, the mechanism provides a basis for further testing and predictions of its physiological consequences.

Acknowledgments

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