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The interplay between heme iron and protein sulfhydryls in the reaction of dimeric *Scapharca inaequivalvis* hemoglobin with nitric oxide☆

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Abstract

The homodimeric hemoglobin from the mollusk *Scapharca inaequivalvis* possesses a single reactive cysteine residue per monomer, Cys92, which is located in the subunit interface in the vicinity of the heme group. The interplay between the heme iron and Cys92 towards the reaction with NO has been investigated by the combined use of electrospray mass spectrometry, FTIR and UV-Visible spectroscopy. When the ferrous liganded or unliganded protein reacts with free NO in solution Cys92 is not modified, but undergoes nitrosation when the hemoglobin is exposed to the nitric oxide releaser *S*-nitrosocysteine. When the ferric protein reacts with free NO under anaerobic conditions the heme iron is reduced and Cys92 is nitrosated. At variance with other hemeproteins investigated to date, in *Scapharca* HbI the heme-iron NO driven reduction is not accompanied by the formation of a ferric iron nitrosyl intermediate in detectable amounts. The results are consistent with the hypothesis that the nitrosating agent is the NO^+ species, which is generated during the NO driven reduction of the ferric heme iron. The possible reaction mechanism is discussed in comparison with recent findings on human hemoglobin and myoglobin. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Nitric oxide (NO) has been shown to act as a physiological messenger which regulates local responses of the cardiovascular, immune and neural systems. Free NO, however, is potentially harmful as it can inhibit cytochrome *c* oxidase directly and/or initiate a cascade of higher nitrogen oxides and radicals that induce the so-called nitrosative stress [1–5]. In vertebrates, free NO is actively scavenged by oxygenated hemoglobin in the blood stream or by myoglobin (Mb) in myocytes and is thereby converted into the non-toxic nitrate [6]. The half-life of NO in oxygenated media is relatively short. NO may be stabilized by formation of *S*-nitrosothiols which can decompose

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☆ Dedication: This work is written in recognition of the 65th birthday of our colleague and friend Maurizio Brunori. We, the authors, salute Maurizio Brunori and his outstanding contributions to science.

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to regenerate NO or can act as ‘NO donors’ in transnitrosation reactions. In this respect, low molecular weight thiols like cysteine and glutathione, and protein sulfhydryls have been suggested to be physiologically relevant [5]. However, free NO does not react directly with sulfhydryls, but needs to be activated, e.g. by metals [7] or by the heme iron.

In human hemoglobin (HbA) and myoglobin, cysteine nitrosation has been determined under a variety of experimental conditions [2–4,8,9]. In HbA, it has been proposed that under physiological oxygen tensions free NO is captured preferentially by the minor population of deoxygenated hemes to produce HbA tetramers carrying three O_2 and one heme–iron bound NO. Thereafter, some of the heme-associated NO molecules would be transferred intramolecularly to the conserved thiol provided by Cysβ93, thus producing *S*-nitroso HbA. Furthermore, transfer of NO to intracellular low molecular mass thiols, such as glutathione and free cysteine may follow [5]. A different reaction pathway has been proposed recently in the reaction of human myoglobin with free NO [8]. Human Mb possesses a solvent exposed cysteine residue (Cys110) that undergoes nitrosation at very low pH in the presence of oxygen when Mb is exposed to free NO generated by $NaNO_2$. The intermediacy of a reactive, higher NO oxide, namely N_2O_3, that acts as thiol nitrosating agent, has been invoked [8,9].

As a whole, these observations suggest that there is no unique pathway for cysteine nitrosation, but rather a complex and multifaceted interplay between NO, the heme iron and sulfhydryl groups. The unusual topological relationship of the only cysteine residue (Cys92) and the heme group in the homodimeric hemoglobin from the mollusk *Scapharca inaequivalvis* (HbI) suggested that this protein may represent a useful model system to shed more light on heme-mediated cysteine nitrosation. In HbI the Cys92 residues are positioned symmetrically in the subunit interface at approximately two helical turns from the proximal histidine, His101, and at approximately one helical turn from Phe97, a major component in the transfer of information between the heme groups [10]. Thus, Phe97 is packed tightly against the proximal histidine in the deoxygenated derivative and is extruded into the interface upon ligand binding. This movement entails a change in the Cys92 environment which becomes less polar due to the release of structural water molecules located in the subunit interface and that of the deoxygenated derivative [10]. It should be pointed out that the Cys92 groups are not involved in hydrogen bonding interactions and are fully accessible to SH reagents of different bulkiness and/or charge in both the liganded and unliganded derivatives [11–13]. In ferric HbI due to the existence of unique pH-linked co-ordination and spin-state equilibria the position of Phe97, and by inference the Cys92 environment, resembles that of the ligand-bound derivative at acid pH values and that of deoxygenated derivative at alkaline ones [14,15].

The data described in the present paper indicate that heme-mediated Cys92 nitrosation does occur in anaerobiosis, most likely by intramolecular transfer of NO^+.

2. Materials and methods

S. inaequivalvis HbI was extracted and purified as described previously [16]. Ferric HbI was obtained by oxidizing the ferrous oxygenated protein with a five-fold molar excess of sodium nitrite in 100 mM Tris–HCl buffer at pH 7.5 and, subsequently, by passing the protein solution through a G-25 column equilibrated with the desired buffer. Under these conditions, selective oxidation of the heme iron occurs without side reactions in particular involving cysteine residues [11]. All buffers (Bis–Tris–HCl pH 6.5 or Tris–HCl pH 7.5 or 8.5) were passed through a Chelex column to remove contaminant metal ions. Thereafter, 1 mM EDTA was added. Control experiments were carried out also in the absence of EDTA.

S-nitroso cysteine (SNOC) was prepared according to Byler et al. [17] by using cysteine hydrochloride (Sigma–Aldrich Co.) and $NaNO_2$ (Sigma–Aldrich Co.). In brief, cysteine hydroclorıde in water (50 mM) was reacted with a ten-fold molar excess of $NaNO_2$ at 2 °C and then precipitated with cold acetone. After repeating the cold acetone wash three times, the SNOC preparation was virtually free of nitrite and nitrate contaminants and contained no unreacted free cysteine nor sulfoxides, as checked by infrared spectroscopy of the dried powder.

The *S*-nitrosation and heme iron nitrosylation reactions were carried out on HbI ferrous and ferric derivatives using NO gas or SNOC. The reaction with SNOC was carried out at 4 °C by adding SNOC in slight molar excess (2–10) over the protein SH groups. After a 25-min incubation time, the mixture was passed through a G-25 column, equilibrated with 50 mM Tris–HCl buffer containing 1 mM EDTA, to remove any unreacted SNOC and was immediately analyzed by mass spectrometry.

Reactions with NO gas were carried out by mixing the protein with water saturated NO solutions under aerobic or anaerobic conditions. Strictly anaerobic conditions were obtained by washing the protein solution with N_2 gas in a tonometer for at least 30 min. Controlled low oxygen pressures were obtained by adding small, known volumes of air into the tonometer containing the deoxygenated protein.

UV-Vis absorption spectra were measured with a HP8453 diode array spectrophotometer equipped with a Peltier thermostatted cell. NO reduction kinetics were measured by mixing a N_2 equilibrated ferric HbI solution with the desired NO saturated buffer in the absence of oxygen in a manually driven HiTech rapid mixing apparatus. Spectra were collected at 3 s time intervals for 1–10 min at 20°.

Single wavelength, fast kinetic measurements were carried out on an Applied Photophysics rapid mixing apparatus (Leatherhead, UK) by mixing HbI solutions (either aerobic or anaerobic) with NO containing buffers over the temperature range 5–35 °C.

Infrared spectra were measured on a Nicolet Magna 760 instrument equipped with an MCT detector. For each sample 512 spectra were averaged at 2 cm^{-1} resolution. The HbI derivatives obtained from the reaction of the protein with NO gas or SNOC were converted first to cyano-met derivatives by addition of a 10 mM solution of NaCN and were concentrated to 8–12 mM heme with Vivaspin II (cut-off 10 kDa) concentrators (Sartorius, Germany). Thereafter, the protein was transferred to a 0.5 mm CaF_2 cell for FTIR measurements.

For mass spectrometry measurements, an aliquot of protein was desalted and analyzed by reverse phase chromatography on a Vydac C4 column (2.1×220 mm, 5 μm particle size, 300 Å pore size) using a linear gradient of 5–80% acetonitrile, containing 0.2% trifluoroacetic acid, in 60 min at a constant flow rate of 150 μl/min, with a Lab-Service HPLC apparatus (Labservice Analytica, Bologna, Italy). Under these conditions, the protein was recovered in the apo form. The column effluent was monitored by an electrospray ion trap mass spectrometer (ES-IT, mod. LCQ, Thermo-Finnigan, San Jose, CA, USA). ESI source conditions were as follows: sheath gas (nitrogen) flow rate, 90 (arbitrary unit); electrospray needle voltage, 50 kV; capillary voltage and temperature, 12 V and 260 °C, respectively; electron multiplier and conversion dynode, 800 V and 15 kV, respectively. The mass spectra were collected over the mass range 50–2000 amu, every 3 s, in the positive ion mode. Protein mass spectra were deconvoluted by software provided with the instrument. All masses are reported as average mass.

3. Results

3.1. Reaction of ferrous HbI with NO

The behavior of ferrous HbI in its reaction with NO gas resembles that of other hemoglobins and myoglobins [18]. Thus, oxygenated HbI mixed with NO containing buffers undergoes a fast oxidation reaction that is accompanied by nitrate production. The reaction is second order and proceeds at a rate of 2×10^7 M^{-1} s^{-1} in 10 mM phosphate buffer pH 7.0 containing 1 mM EDTA at 20 °C. HbI–CO mixed with free NO in the presence of dithionite gives rise to the nitrosyl HbI adduct with the concomitant release of CO from the heme [19]. The reaction of deoxygenated HbI with NO gas yields the typical ferrous heme iron nitrosyl adduct, in agreement with a report by Coletta et al. [19].

Mass spectrometric analysis, conducted on ferrous HbI after reaction with 1 mM NO in the

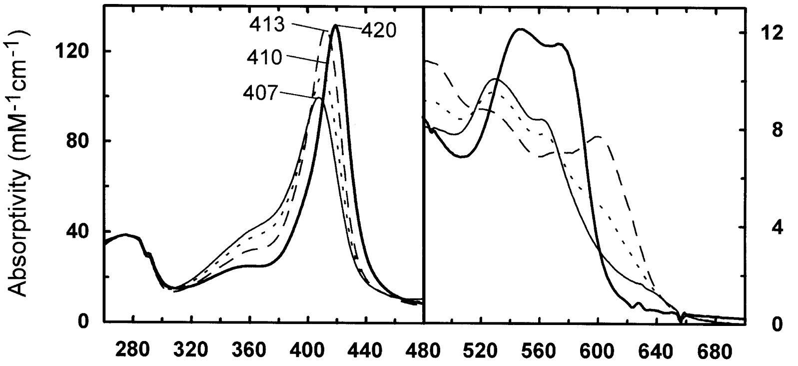

Fig. 1. UV-visible absorption spectra of *S. inaequivalvis* HbI derivatives. Ferric derivatives at pH 6.5 (continuous thin line), 7.5 (dotted line) and 8.5 (dashed line) and ferrous NO derivative at pH 7.5 (continuous thick line). Buffers: 50 mM Bis–Tris–HCl at pH 6.5 and 50 mM Tris–HCl at pH 8.5. All measurements were carried out at 20 °C.

oxygenated, carbonylated and deoxygenated form, reveals the presence of a single peak at 15941 ± 1.6 amu, corresponding to the theoretical mass of the native protein (15943 amu).

3.2. Reaction of ferric HbI with NO

The ferric heme nitrosyl adduct of hemoglobins and myoglobins is known to be unstable in the presence of NO in excess; it is reduced to the ferrous heme nitrosyl adduct in a complex reaction whose mechanism has been elucidated by Addison and Stephanos with reference to HbA, *Glycera* hemoglobin and horse myoglobin [20].

Before presenting the reaction of ferric HbI with NO, it is useful to recall the complex rapidly reversible pH-dependent equilibria of this HbI derivative. Two distinct dimeric high spin species predominate at neutral and alkaline pH values. An aquomet derivative is present in sizeable amounts around neutrality, whereas at pH values above 7.0 an unusual pentacoordinate derivative is formed where the hydroxyl group and not the proximal histidine provides the fifth co-ordinating ligand [21]. These dimeric species dissociate into a monomeric hexacoordinate low-spin hemichrome at acidic pH values. Reference spectra of ferric HbI at acidic, neutral and alkaline pH values are shown in Fig. 1.

The behavior of ferric HbI mixed with NO in excess at pH 7.5 and 20 °C under strictly anaerobic conditions is depicted in Fig. 2a. The spectrum of the typical ferric high spin derivative, peaked at around 410 nm in the Soret region (see Fig. 1), changes within approximately 60 s into the spectrum characteristic of the ferrous heme iron nitrosyl complex with a peak at 420 nm in the Soret region and two shallow bands at 568 and 542 nm (Q_0 and Q_1 bands) in the visible region. The inset shows that the time course can be described by a single exponential. The same behavior is observed when the reaction is carried out at pH values between 6.5 and 8.5 and at temperatures between 5 and 35 °C in the presence or in the absence of 1 mM EDTA. The fact that addition of EDTA has no effect on the reaction is of special importance as it shows that free metal catalysed nitrosation does not occur [7]. The formation of the ferrous heme nitrosyl complex is second order with respect to NO concentration. The apparent rate constant is approximately 32 M^{-1} s^{-1} at pH 7.5; it displays a slight pH dependence as it slows down 3- to 4-fold going from pH 8.5 to pH 6.5 (Fig. 2b). It should be stressed that the ferric-nitrosyl intermediate has never been detected. This behavior is at variance with that of HbA and myoglobins where the ferric-nitrosyl intermediate is formed within the same time regime. The ferric-nitrosyl intermediate can be easily distinguished from the ferrous-nitrosyl complex particularly in the visible region due to the highly structured and blue shifted Q_0 and Q_1 bands [20].

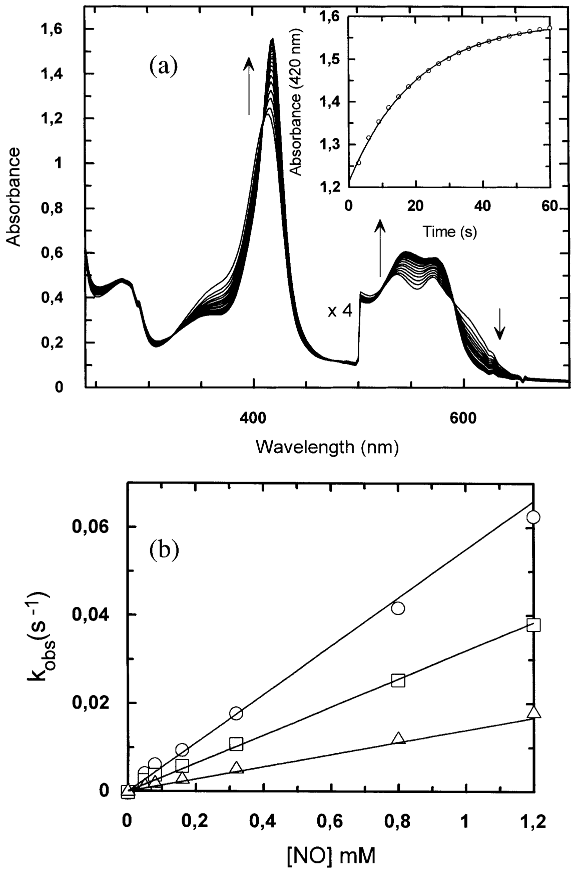

Fig. 2. Reaction kinetics of ferric *S. inaequivalvis* HbI with NO under anaerobic conditions. Panel A: UV-visible spectra of ferric HbI (16 μM) after mixing with a 2 mM NO solution at pH 7.5 in 50 mM Tris–HCl buffer containing 1 mM EDTA. The spectra were collected at 20 °C at 3 s intervals. The inset shows the time course of the reaction at 420 nm; the continuous line represents the fit of the experimental data to a single exponential. Panel B: The observed pseudofirst order rate constants (k) of the reaction between ferric HbI and NO measured at pH 8.5 (○), pH 7.5 (□) and pH 6.5 (△) and 20 °C are reported as a function of NO concentration. Continuous lines represent linear fits of the data; they yield apparent second order rate constants of 52±4 M^{-1} s^{-1} (pH 8.5), 32±5 M^{-1} s^{-1} (pH 7.5) and 14±3 M^{-1} s^{-1} (pH 6.5). Buffers: 20 mM Tris HCl at pH 8.5 and 7.5, 20 mM Bis–Tris HCl at pH 6.5. All buffers contained 1 mM EDTA.

Mass spectrometry, carried out at the end of the reaction of ferric HbI with NO under anaerobic conditions, indicates that iron reduction is accompanied by an increase of 30±1 amu with respect to the native protein, a value that corresponds to a single NO adduct per HbI monomer (Fig. 3). The concomitant disappearance of the free S–H stretching frequency at 2568 cm^{-1} in FTIR measurements indicates that essentially complete nitrosation of Cys92 is taking place (Fig. 4).

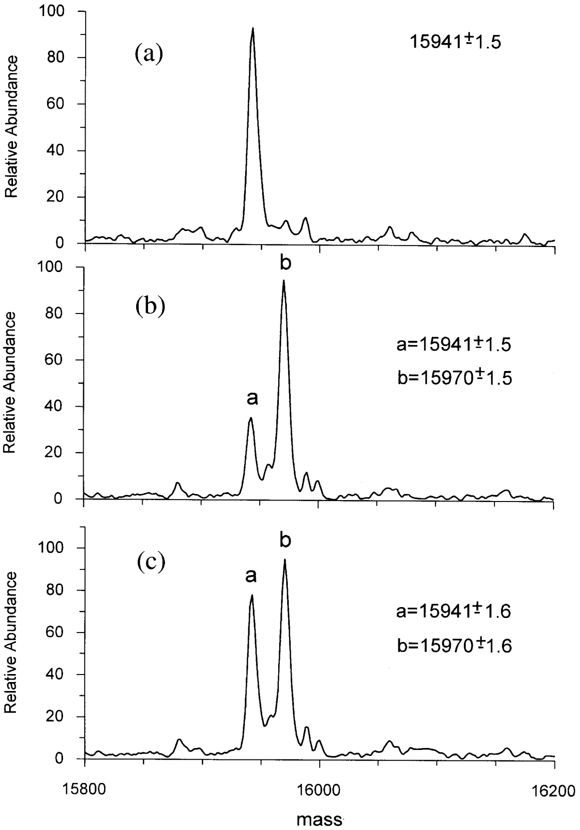

Fig. 3. Transformed electrospray mass spectra of *S. inaequivalvis* HbI derivatives reacted with free NO or *S*-nitroso cysteine. Panel A shows the molecular mass of ferrous HbI reacted aerobically with free NO (10 molar excess over heme); it is identical to that of native HbI (15943 amu, [16]). Panels B and C, respectively, show the mass spectrum of ferric HbI reacted under anerobic conditions with free NO (10 molar excess over heme) and that of oxygenated ferrous HbI reacted with SNOC (2 molar excess over heme). For further details see Materials and methods. The correspondence between the mass of the protein fraction in the ferric state (peak a in panel B) and that of the native protein shows that no side reactions have occurred during the preparation of the ferric derivative with sodium nitrite.

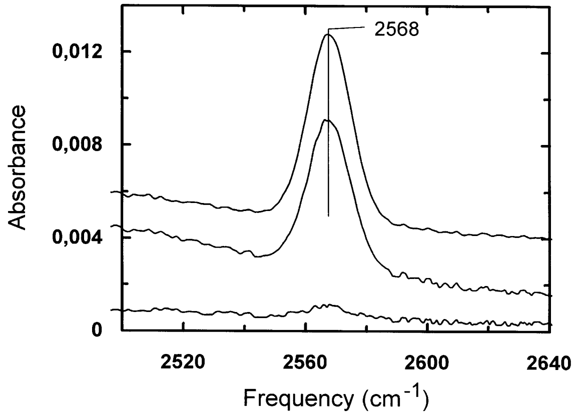

Fig. 4. FTIR spectra of *S. inaequivalvis* HbI in the region of the sulfhydryl group stretching band. The spectra were taken at pH 7.5 in 20 mM Tris–HCl buffer containing 1 mM EDTA and at 20 °C. From top to bottom: native HbI, HbI reacted with NO under aerobic conditions, HbI reacted with NO under anaerobic conditions. The protein was converted to the cyanomet derivatives after the reaction with NO, see Materials and methods for further details. The spectra were normalized to a protein concentration of 20 mM heme.

Preliminary experiments showed that the NO induced reduction of the HbI heme iron is observed also when oxygen is present, provided its concentration is below 40–50 μM. Under these conditions, the amount of NO-reduced protein never attains more than 20%; moreover, the protein is rapidly reoxidized (data not shown). At oxygen concentrations higher than 50 μM no spectral changes are observed. Due to technical difficulties inherent to mass spectrometry and FTIR measurements at low oxygen tensions, the occurrence of Cys92 nitrosation was not assessed. Furthermore, extensive investigations are needed in order to establish the efficiency of the nitrosation process under these conditions of potential physiological relevance.

3.3. Reaction of ferrous HbI derivatives with SNOC

In order to establish whether ferrous HbI can undergo *S*-transnitrosation, the reaction with SNOC was studied. When oxy- or CO-HbI is treated with SNOC in 2–10-fold molar excess over Cys92, *S*-nitrosation occurs as determined by electrospray mass spectrometric analysis. The extent of *S*-nitrosation is approximately 40% at a SNOC/Cys92 ratio as low as 2 (see Fig. 3) and does not increase significantly at higher SNOC/Cys92 ratios. No significant differences were observed between oxy- and CO-HbI, indicating that the intrinsic stability of *S*-nitroso Cys92 is not linked to the nature of the ligand. Similar results were obtained in the absence of EDTA.

4. Discussion

The present investigation clearly shows that in *Scapharca* homodimeric HbI there is an interplay between the heme iron and Cys92 in the processing of free NO. Cys92 nitrosation occurs spontaneously when free NO reacts anaerobically with the ferric protein. We ascribe the occurrence of this reaction to the heme-catalysed production of NO^{+}, which can diffuse to the Cys92 residues that are located in the subunit interface like the heme groups themselves.

When ferric HbI reacts with free NO in anaerobiosis, the NO-driven reduction of the heme iron described for other hemoglobins and myoglobins takes place, but is accompanied by conversion of Cys92 to a nitrosothiol. The NO-driven reduction of the heme iron has distinct features with respect to the same reaction in other hemoglobins and myoglobins [20]. These are manifest in the spectral profiles and in the kinetic records observed after mixing the HbI and NO solutions. First, the ferric heme iron nitrosyl adduct is not detected under any of the experimental conditions analyzed, i.e. pH values from 6.5 to 8.5 and temperatures between 5 and 35 °C, presence or absence of 1 mM EDTA. In contrast, within the time frame used in the present work (seconds to minutes), the ferric heme iron nitrosyl adduct is clearly apparent in the hemoglobin and myoglobin spectra. Second, the overall reaction that leads to the formation of the ferrous heme iron nitrosyl complex (32 M^{-1} s^{-1} at pH 7.5 and 20 °C) occurs faster than in the case of human hemoglobin (5–10 M^{-1} s^{-1}) and horse myoglobin (approx. 0.5 M^{-1} s^{-1}) [20]. At all pH values the overall reaction is second order with respect to NO concentration. In spite of these differences, the anaerobic NO-driven reduction of ferric HbI may be rationalized according to the three basic reaction steps proposed by Addison and Stephanos for hemoglobins and myoglobins [20] if one assumes that the nature of the rate limiting step differs. In the latter proteins, NO binds rapidly to the ferric heme iron (10^4–10^5 M^{-1} s^{-1}) yielding the ferric heme iron NO complex. The bimolecular binding of a second NO molecule governs the subsequent reduction of this adduct and limits the overall rate. It leads to the formation of ferrous nitrosyl hemoglobin and a nitrosonium ion. The absence of a detectable ferric heme iron NO complex in HbI indicates that, in contrast, the bimolecular binding of NO to the ferric heme iron is slow and rate limiting and that NO binding is followed by the immediate donation of the NO unpaired π^* electron to the iron atom itself to give rise to ferrous heme and a nitrosonium ion. It may be hypothesized that the NO^+ species, that is released upon uptake of a second free NO molecule, diffuses locally through the heme pocket along the subunit interface until it reaches Cys92. Indeed, the combined use of mass spectrometry and FTIR measurements shows that Cys92 is converted quantitatively to a nitrosothiol derivative (Figs. 3 and 4). Given the quantitative relationship between the NO-driven heme reduction and Cys92 nitrosation, the latter reaction may be expected to occur also under oxygen tensions close to the physiological ones (<50 μM).

The slow combination rate of ferric HbI with NO is not unique to this ligand as it has been observed previously in the case of fluoride and cyanide [22]. It reflects at least in part the existence of the complex pH-dependent coordination-linked association-dissociation equilibria of ferric HbI recalled above, which entail also a change in the polarity of the Cys92 environment [13]. Thus, the decrease in rate at low pH most likely reflects the reactivity of the hexacoordinated monomeric hemichrome-like species, which requires, displacement of a strong coordination ligand, i.e. the distal histidine, and association of monomers to dimers. Further experiments, however, are needed to establish quantitative relationships concerning this point.

Cys92 nitrosation does not occur in the reaction of free NO with liganded and unliganded ferrous HbI. The oxygenated protein gives rise to the ferric derivative and nitrate at a rate of 2×10^7 M^{-1} s^{-1} which is similar to those reported for vertebrate hemoglobins and myoglobins (2×10^7 and 4×10^7 M^{-1} s^{-1}, respectively). This finding therefore supports our interpretation that Cys92 nitrosation of the ferric protein by free NO indeed involves the intermediacy of the heme iron. As reported for human hemoglobin and myoglobin [5,8], *S*-nitrosation of ferrous HbI was achieved easily via a transnitrosation reaction, e.g. using SNOC as 'NO donor'.

In conclusion, the present study shows that cysteine nitrosation may occur efficiently in the reaction of ferric hemoproteins with free NO under anaerobic conditions through the heme-catalysed production of the reactive NO^+ species. Taken together with the observation that in anaerobiosis ferric horse myoglobin catalyses glutathione nitrosation in the presence of free NO [23], the present data suggest that NO^+ may represent a ubiquitous *S*-nitrosating agent which can be released by 'NO donors' or can be produced in situ by ferric hemoproteins. Although the relevance of this process under low oxygen tensions still needs to be established, the present findings afford the possibility that hemoproteins may regulate NO chemistry according to different pathways depending on oxygen availability. Thus, N_2O_3 can be generated under aerobic conditions or NO^+ under anaerobic ones (or under low oxygen tensions) with the aim of preserving the bioavailability of NO through cysteine nitrosation. In general terms such a scheme is in line with the view of Lipton et al. [24] that oxygen works in concert with, and under the control of, low molecular mass nitrosothiols not only for matching the oxygen requirement of different tissues, but also for controlling the response to oxygen deprivation.

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