Abstract

Truncated hemoglobins (trHbs) are widely distributed in bacteria and plants and have been found in some unicellular eukaryotes. Phylogenetic analysis based on protein sequences shows that trHbs branch into three groups, designated N (or I), O (or II), and P (or III). Most trHbs are involved in the O$_2$/NO chemistry and/or oxidation/reduction function, permitting the survival of the microorganism in the host. Here, a detailed comparative analysis of kinetics and/or thermodynamics of (i) ferrous Mycobacterium tuberculosis trHb N and O (Mt-trHbN and Mt-trHbO, respectively), and Campylobacter jejuni trHb Cj-trHbP nitrosylation, (ii) nitrite-mediated nitrosylation of ferrous Mt-trHbN, Mt-trHbO, and Cj-trHbP, and (iii) NO-based reductive nitrosylation of ferric Mt-trHbN, Mt-trHbO, and Cj-trHbP is reported. Ferrous and ferric Mt-trHbN and Cj-trHbP display a very high reactivity towards NO; however, the conversion of nitrite to NO is facilitated primarily by ferrous Mt-trHbN. Values of kinetic and/or thermodynamic parameters reflect specific trHb structural features, such as the ligand diffusion pathways to/from the heme, the heme distal pocket structure and polarity, and the ligand stabilization mechanisms. In particular, the high reactivity of Mt-trHbN and Cj-trHbP reflects the great ligand accessibility to the heme center by two protein matrix tunnels and the E7-path, respectively, and the penta-coordination of the heme-Fe atom. In contrast, the heme-Fe atom of Mt-trHbO the ligand accessibility to the heme center of Mt-trHbO needs large conformational readjustments, thus limiting the heme-based reactivity. These results agree with different roles of Mt-trHbN, Mt-trHbO, and Cj-trHbP in vivo.

Introduction

Based on phylogeny, the globin superfamily contains three lineages: flavohemoglobins and single domain globins (lineage 1), protoglobins (Pgb) and globin coupled sensors (lineage 2), and truncated hemoglobins (trHbs; lineage 3). Members of the globin superfamily belong to two structural classes: one showing the classical 3-on-3 $\alpha$-helical sandwich (lineages 1 and 2) and one having the 2-on-2 $\alpha$-helical sandwich (lineage 3) (Fig. 1). Although no definitive conclusion can be drawn about the ancestral state of the globin fold, the occurrence of the 2-on-2 fold, but not of an isolated 3-on-3 fold, in all three kingdoms of life suggests that the 2-on-2 is the ancestral fold. In an evolutionary perspective, the predominant function of globins is (pseudo-)enzymatic, with O$_2$ transport and storage being specialized functions associated with the evolution of metazoans [1–6].

TrHbs are widely distributed in bacteria and plants and have been found in some unicellular eukaryotes. They are distantly related to the 3-on-3 globins, showing less than 20% overall identity with the latter. Phylogenetic analysis of protein sequences shows that trHbs branch into three groups, designated N (or I), O (or II), and P (or III). TrHbs belonging to group N display a level of conservation higher than those of groups O and P; however, the conversion of nitrite to NO is facilitated primarily by ferrous Mt-trHbN. Values of kinetic and/or thermodynamic parameters reflect specific trHb structural features, such as the ligand diffusion pathways to/from the heme, the heme distorted pocket structure and polarity, and the ligand stabilization mechanisms. In particular, the high reactivity of Mt-trHbN and Cj-trHbP reflects the great ligand accessibility to the heme center by two protein matrix tunnels and the E7-path, respectively, and the penta-coordination of the heme-Fe atom. In contrast, the heme-Fe atom of Mt-trHbO the ligand accessibility to the heme center of Mt-trHbO needs large conformational readjustments, thus limiting the heme-based reactivity. These results agree with different roles of Mt-trHbN, Mt-trHbO, and Cj-trHbP in vivo.
Figure 1. Consensus phylogenetic tree of major globin groups from the three kingdoms of life. This phylogenetic tree was based on the alignment of 150 sequences representing the following groups of globins: 10 plant non symbiotic 3/3 Hbs, 5 plant symbiotic 3/3 Hbs, 15 bacterial 3/3 globin-coupled sensors (GCSs), 4 3/3 protoglobins (Pgbs), 9 bacterial 2/2 Hbs, 19 bacterial 2/2 Hb2s, 10 bacterial 2/2 Hb3s, 2 Chlamydomonas reinhardtii 2/2 Hbs, 2 ciliate 2/2 Hbs, 3 plant 2/2 Hbs, Thalassiosira pseudonana 2/2 Hb, 20 bacterial 3/3 flavohemoglobins (FHbs), 19 bacterial 3/3 single-domain Hbs, 9 eukaryote 3/3 FHbs, 1 diplomonad Giardia lamblia 3/3 Hb, and 1 mycetozoan Dictyostelium discoideum 3/3 Hb. Cyanidioschyzon merolae and Thalassiosira pseudonana 3/3 single-domain globins, and 3 vertebrate (i.e., human, bird, and fish) 3/3 neuroglobins (Ngbs), cytoglobins (Cygbs), α- and β-globins and myoglobin (Mbs), and 2 urochordate 3/3 Hbs. Modified from [1] (Copyright (2005) National Academy of Sciences, U S A).

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sequence allow the achievement of the simplified fold, keeping at the same time a high affinity for the heme, a suitable ligand access to the heme-Fe atom, and the proper heme-Fe oxidation state. However, specific features, such as ligand entry/exit mechanisms holding to promote diffusion of ligands to/from the heme, the heme distal pocket structure and polarity, as well as ligand stabilization mechanisms distinguish members of the three trHb groups (Fig. 2) [5].

*Mycobacterium tuberculosis* (M. tuberculosis) possesses two trHbs, namely Mt-trHbN and Mt-trHbO, and a 3-on-3 flavohemoglobin, while *Campylobacter jejuni* (C. jejuni) possesses only *Campylobacter jejuni* trHb P (Cj-trHbP) and a single-domain 3-on-3 globin [4,5,7–11].

Most trHbs are involved in the O₂/NO chemistry and/or oxidation/reduction function, permitting the survival of the microorganism in the host [5,11–13]. Noteworthy, Mt-trHbN binds reversibly ioniaizid, a first-line anti-tuberculosis medication in prevention and treatment of tuberculosis, highlighting a direct role of the pro-drug to impair fundamental functions of mycobacteria, e.g. scavenging of reactive nitrogen and oxygen species, and metabolism [14].

Studies performed with *Mycobacterium bovis* Calmette-Guerin (BCG) demonstrated that the inactivation of the glbN gene impairs the ability of stationary phase cells to protect aerobic respiration from NO inhibition, suggesting that Mt-trHbN may play a vital role in protecting *M. tuberculosis* from NO toxicity in *vivo* [12,15]. This functional assessment is supported by the observation that Mt-trHbN catalyzes the rapid oxidation of NO into nitrate [13,16]. Heterologous expression of Mt-trHbN has also been shown to protect *Escherichia coli* against nitrosative stress [17]. On the other hand, Mt-trHbO does not detoxify NO efficiently [18], but displays peroxidase activity, suggesting an oxidation/reduction function [19]. Lastly, Cj-trHbP has been proposed to play a prominent role in *C. jejuni* respiration rather than in protection against reactive nitrogen and oxygen species. A strain of *C. jejuni* lacking trHbP turned out to be disadvantaged with respect to wild-type cells when grown under high aeration, achieving lower growth yields and consuming O₂ at approximately half the rate displayed by wild-type cells. Although Cj-trHbP mutated cells are equally sensitive as the wild-type to NO and oxidative stress, the functional role of Cj-trHbP remains elusive [7,8,11].

Here, a detailed comparative analysis of kinetics and/or thermodynamics of (i) ferrous Mt-trHbN, Mt-trHbO, and Cj-trHbP (Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II), respectively) nitrosylation, (ii) nitrite-mediated nitrosylation of Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II), respectively) nitrosylation, (ii) NO-based reductive nitrosylation of ferric Mt-trHbN, Mt-trHbO, and Cj-trHbP (Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III), respectively) is reported. Ferrous and ferric Mt-trHbN and Cj-trHbP display a very high reactivity towards NO, the conversion of nitrite to NO being facilitated primarily by ferrous Mt-trHbN. This reflects the great ligand accessibility to the heme center of Mt-trHbN, and Cj-trHbP by two protein matrix tunnels and the E7-path, respectively, and the penta-coordination of the heme-Fe atom. In contrast, the accessibility to the heme center of Mt-trHbO needs large conformational readjustments, thus limiting the heme-based reactivity. These results agree with different roles of Mt-trHbN, Mt-trHbO, and Cj-trHbP in *vivo*.

**Materials**

Mt-TrHbN(III), Mt-TrHbO(III), and Cj-trHbP(III) were cloned, expressed and purified as previously reported [20–22]. Mt-TrHbN(II), Mt-TrHbO(II), and Cj-trHbP(II) were obtained, under anaerobic conditions, by adding sodium dithionite (final concen-
The concentration of Mt-trHbN, Mt-trHbO, and Cj-trHbP stock solutions was 3.0 \times 10^{-6} \text{ M}, 4.6 \times 10^{-6} \text{ M}, and 3.2 \times 10^{-6} \text{ M}, respectively.

Gaseous NO (from Aldrich Chemical Co. (Milwaukee, WI, USA) was purified by flowing through a NaOH column in order to remove acidic nitrogen oxides. The stock NO solution was prepared anaerobically by keeping in a closed vessel the degassed 5.0 \times 10^{-3} \text{ M} phosphate buffer solution (pH = 7.0) under NO at $P = 760.0 \text{ mm Hg}$ ($T = 20.0^\circ\text{C}$). The solubility of NO in the aqueous buffered solution is $2.05 \times 10^{-3} \text{ M}$, at $P = 760.0 \text{ mm Hg}$ and $T = 20.0^\circ\text{C}$ [23]. Sodium dithionite (final concentration, 5.0 \times 10^{-4} \text{ M}) was added to NO solutions (final concentration, 1.0 \times 10^{-4} \text{ M to 5.0 \times 10^{-4} M}).

All the other chemicals were obtained from Merck AG (Darmstadt, Germany). All products were of analytical grade and used without purification unless stated.
Methods

Nitrosylation of Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II)

Kinetics of Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) nitrosylation were analyzed in the framework of the minimum reaction mechanism represented by Scheme A [23]:

\[
\text{trHb(II)} + NO \xrightleftharpoons{k_{on}}{k_{off}} \text{trHb(II)} - NO \quad (A)
\]

Values of the pseudo-first-order rate constant \(k\) (i.e., \(k_{on}\)) for Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) nitrosylation were obtained by mixing the trHb[II] solutions (final concentration, 1.5×10^{-6} M, 2.3×10^{-6} M, and 1.6×10^{-6} M, respectively) with the NO solution (final concentration, 5.0×10^{-6} M to 1.2×10^{-4} M) under anaerobic conditions. 

No gaseous phase was present. The nitrosylation of Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) was monitored between 360 and 460 nm.

In view of the linear relationship between the absorbance change and the protein concentration change, values of the pseudo-first-order rate constant \(k\) were obtained according to Eqn. (1) [23]:

\[
[\text{trHB(II)}] = [\text{trHB(II)}]_0 \times e^{-k \times t} \quad (1)
\]

Values of the second-order rate constant for the nitrite-mediated nitrosylation of Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) (i.e., \(k_{on}\)) were obtained from the dependence of \(k\) on the NO concentration (i.e., [NO]), according to Eqns. (2) [23]:

\[
k = k_{on} \times [\text{NO}] + k_{off} \quad (2)
\]

where \(k_{off}\) is the first-order rate constant for trHb[II]-NO denitrosylation.

Values of \(k_{on}\) and \(k_{off}\) for Mt-trHbN[II](-NO), Mt-trHbO[II](-NO), and Cj-trHbP[II](-NO) (de)nitrosylation were obtained at pH 7.0 and 9.0 (1.0×10^{-1} M Bis-Tris propane buffer), and 20.0°C.

Nitrite-mediated nitrosylation of Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II)

Kinetics of the nitrite-mediated nitrosylation of Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP[II] were analyzed in the framework of the minimum reaction mechanism depicted in Scheme B [24–35]:

\[
\text{trHb(II)} + NO_2^- + H^+ \xrightarrow{h_{on}} \text{trHb(III)} + NO + OH^- \quad (B_a)
\]

\[
\text{trHb(III)} + Na_2S_2O_4 \xrightarrow{\text{fast}} \text{trHb(II)} \quad (B_b)
\]

\[
\text{trHb + NO} \xrightleftharpoons{k_{on}}{k_{off}} \text{trHb(II)} - \text{heme–Fe(II)} - NO \quad (B_c)
\]

Values of the pseudo-first-order rate constant \(k\) (i.e., \(h\); reactions (a) and (b); reactions (a) and (c) in Scheme C, respectively) and of the
dissociation equilibrium constant (i.e., \( L = l_{\text{off}}/l_{\text{on}} \)); reaction (a) in Scheme C) for Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) reductive nitrosylation were obtained by mixing the trHb(III) solutions (final concentration, \( 1.5 \times 10^{-4} \) M, \( 2.3 \times 10^{-4} \) M, and \( 1.6 \times 10^{-4} \) M, respectively) with the NO solution (final concentration, \( 2.5 \times 10^{-5} \) M to \( 5.0 \times 10^{-5} \) M) under anaerobic conditions.

No gaseous phase was present. The reductive nitrosylation of Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) was monitored between 360 and 460 nm.

In view of the linear relationship between the absorbance change and the protein concentration change, values of the pseudo-first-order rate constants \( l \) and \( b \) were obtained according to Eqsns (5a)–(5c) [36–44]:

\[
[\text{trHb(III)}]_{0} = [\text{trHb(III)}]_{i} \times e^{-l_{\text{on}}t} \quad (5a)
\]

\[
[\text{trHb(III)}]_{0} = [\text{trHb(III)}]_{i} \times \left( e^{-l_{\text{on}}t} - e^{-b_{\text{on}}t} \right) \quad (5b)
\]

\[
[\text{trHb(III)}]_{0} = [\text{trHb(III)}]_{i} + [\text{trHb(III)}]_{\text{NO}} \quad (5c)
\]

Values of \( l_{\text{on}} \) and \( l_{\text{off}} \) (reaction (a) in Scheme C) were determined from the dependence of \( l \) on [NO], according to Eqn. (6) [23]:

\[
l = l_{\text{on}} \times [\text{NO}] + l_{\text{off}} \quad (6)
\]

Values of \( L \) (reaction (a) in Scheme C) were obtained according to Eqn. (7) [23,41,42]:

\[
\lambda = \frac{[\text{NO}]}{L + [\text{NO}]} \quad (7)
\]

where \( \lambda \) is the molar fraction of the NO-bound Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III). Moreover, values of \( L \) were estimated from the \( l_{\text{off}}/l_{\text{on}} \) ratio [23].

Values of the second-order rate constant \( b_{\text{OH \,-}} \) and of the first-order rate constant \( b_{\text{H}_2\text{O}} \) for the OH– and \( \text{H}_2\text{O}-\text{catalyzed conversion of trHb(II)-NO}^+ \) to trHb(II), respectively (reaction (c) in Scheme C), were determined from the dependence of \( b \) on [OH–] according to Eqns. (8) [36–43]:

\[
b = b_{\text{OH \,-}} \times [\text{OH}^-] + b_{\text{H}_2\text{O}} \quad (8)
\]

Values of \( L \), \( l_{\text{on}} \), \( l_{\text{off}} \), \( b_{\text{OH \,-}} \), and \( b_{\text{H}_2\text{O}} \) for the reductive nitrosylation of Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) (reactions (3a) and (3c) in Scheme C) were obtained between pH 8.4 and pH 9.4 (\( 1.0 \times 10^{-5} \) M Bis-Tris propane buffer), at 20.0°C. Values of \( k_{\text{on}} \) and \( k_{\text{off}} \) for Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) nitrosylation (Scheme A, and reaction (Cd) in Scheme C) were obtained at pH 9.0 and 20.0°C (see above).

The reductive nitrosylation of Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) was also obtained anaerobically by keeping the trHb(III) solutions under purified gaseous NO (760 mmHg), between pH 8.4 and 9.4 (\( 1.0 \times 10^{-5} \) M Bis-Tris propane buffer) and 20.0°C.

**Stopped-flow apparatus**

Kinetic experiments have been carried out spectrophotometrically with the BioLogic SFM 2000 (Cliax, France) rapid-mixing stopped-flow apparatus at single wavelength between 360 nm and 460 nm; the dead-time of the stopped-flow apparatus was \( \sim 1 \) ms and the observation chamber was 1 cm.

**Data analysis**

Kinetic data obtained at different wavelengths have been normalized each other on the basis of the total absorbance change at the specific wavelength. The results are given as mean values of at least four experiments plus or minus the corresponding standard deviation. All data were analyzed using the MATLAB program (The Math Works Inc., Natick, MA, USA).

**Results and Discussion**

**Nitrosylation of Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II)**

Mixing Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) with NO solutions (pH 7.0 and 9.0, 20.0°C) induces a pH-independent shift of the optical absorption maximum of the Soret band from 430–433 nm (i.e., trHb(II)) to 416–418 nm (i.e., trHb(II)-NO) (Fig. 3, and Figs S1 and S2 in File S1, panel A, and Table 1). pH-independent absorbance spectra of Mt-trHbN(II)-NO, Mt-trHbO(II)-NO, and Cj-trHbP(II)-NO obtained mixing Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) with NO solutions correspond to those obtained by adding gaseous NO to the Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) solutions. Moreover, values of \( k_{\text{on}} \) and \( \lambda \) of the absorption spectra in the Soret region of Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) agree with those reported in the literature [8,20,21,43,46].

Under all experimental conditions, the time course of NO binding to Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) corresponds to a mono-molecular process for more than 80% of its course (Fig. 3, and Figs S1 and S2 in File S1, panels B and C). Values of the pseudo-first-order rate constant for Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) nitrosylation (i.e., \( k \); Eqn. (1)) are wavelength-independent at fixed NO concentration (data not shown).

Values of \( k \) increase linearly with the NO concentration (Fig. 3, and Figs S1 and S2 in File S1, panels D and E). The analysis of data reported in Figure 3, and Figures S1 and S2 in File S1, panels D and E, according to Eqn. (2), allowed the determination of values of the second-order rate constant for Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) nitrosylation (i.e., \( k_{\text{on}} \); corresponding to the slope of the linear plots), which are essentially pH-independent (Fig. 3, and Figs S1 and S2 in File S1, panels D and E). The y intercept of the linear plots appears very close to zero (Fig. 3, and Figs S1 and S2 in File S1, panels D and E), indicating that values of \( k_{\text{off}} \) for Mt-trHbN(II)-NO, Mt-trHbO(II)-NO, and Cj-trHbP(II)-NO denitrosylation are lower by at least two-orders of magnitude than values of \( k \) obtained at the lowest NO concentration (i.e., \( k_{\text{off}} < 1 \times 10^{-1} \) s\(^{-1} \)).

Values of \( k_{\text{on}} \) for NO binding to Mt-trHbN(II) and Cj-trHbP(II) vary between \( 1.0 \times 10^{-1} \) M\(^{-1}\) s\(^{-1} \) and \( 2.1 \times 10^{0} \) M\(^{-1}\) s\(^{-1} \) at pH 7.0 and pH 9.0 (Fig. 3, and Figs S1 and S2 in File S1, panels D and E, and Table 2). Values of \( k_{\text{on}} \) for NO binding to Mt-trHbO(II) here determined at pH 7.0 and 9.0 (\( = 1.9 \times 10^{0} \) M\(^{-1}\) s\(^{-1} \) and \( 2.3 \times 10^{1} \) M\(^{-1}\) s\(^{-1} \), respectively; Fig. S1 in File S1, panels D and E, and Table 2) are in agreement with data referring of the slow nitrosylation course, reflecting 80% of the whole process, previously reported at pH 7.5 (\( = 1.8 \times 10^{1} \) M\(^{-1}\) s\(^{-1} \)) [18]. Values of \( k_{\text{on}} \) for NO binding to Mt-trHbO(II) and Cj-trHbP(II) increase
changing the pH from 7.0 to 9.0, whereas values of $k_{on}$ for Mt-trHbN(II) nitrosylation decrease. However, the small magnitude of the pH-dependent change, essentially within the error limit, might suggest that these variations are not statistically relevant.

Values of $k_{on}$ for nitrosylation of ferrous heme-proteins span over eight orders of magnitude (Table 2) ([36,41–43,47–53] and present study), mainly reflecting the ligand accessibility to the heme distal pocket and the coordination of the heme-Fe(II) atom.

Figure 3. Mt-trHbN(II) nitrosylation at 20.0°C. (A) Difference absorbance spectrum of Mt-trHbN(II) minus Mt-trHbN(II)-NO, at pH 7.0. (B) Difference absorbance spectrum of Mt-trHbN(II) minus Mt-trHbN(II)-NO, at pH 9.0. (C) Normalized averaged time courses of Mt-trHbN(II) nitrosylation, at pH 7.0. The NO concentration was $5.0 \times 10^{-6}$ M (trace a) and $1.2 \times 10^{-5}$ M (trace b). The time course analysis according to Eqn. (1) allowed the determination of the following values of $k = 9.2 \times 10^2$ s$^{-1}$ (trace a) and $2.8 \times 10^3$ s$^{-1}$ (trace b). (D) Normalized averaged time courses of Mt-trHbN(II) nitrosylation, at pH 9.0. The NO concentration was $5.0 \times 10^{-6}$ M (trace a) and $1.2 \times 10^{-5}$ M (trace b). The time course analysis according to Eqn. (1) allowed the determination of the following values of $k = 7.4 \times 10^1$ s$^{-1}$ (trace a) and $1.9 \times 10^2$ s$^{-1}$ (trace b). (E) Dependence of the pseudo-first-order rate-constant $k$ for Mt-trHbN(II) nitrosylation on the NO concentration, at pH 7.0. The analysis of data according to Eqn. (2) allowed the determination of $k_{on} = (2.1 \pm 0.3) \times 10^7$ M$^{-1}$ s$^{-1}$. (F) Dependence of the pseudo-first-order rate-constant $k$ for Mt-trHbN(II) nitrosylation on the NO concentration, at pH 9.0. The analysis of data according to Eqn. (2) allowed the determination of $k_{on} = (1.6 \pm 0.3) \times 10^7$ M$^{-1}$ s$^{-1}$. The Mt-trHbN(II) concentration was $1.5 \times 10^{-6}$ M. The NO concentration was $1.0 \times 10^{-6}$ M (panels A and B). Where not shown, the standard deviation is smaller than the symbol. For details, see text.

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Of note, H-bond interactions locking the heme distal residues TyrCD1 and TrpG8 of Mt-trHbO limits ligand access to the heme distal pocket (Fig. 2) [18]. In contrast, the low values of $k_{on}$ for ferrous horse heart cytochrome $c$ and rabbit hemoglobin-heme-Fe (HPX-heme-Fe) nitrosylation reflect the slow rate of hexa- to penta-coordination transition of the heme-Fe(II) atom, which precedes ligand binding ([36,52] and present study). On the other hand, the fast hexa- to penta-coordination conversion of the heme-Fe(II) atom of Arabidopsis thaliana hemoglobin (Hb) class 1 and Glycine max Lb does not affect NO binding [31,34]. Accordingly, the decrease of the $k_{on}$ value for the nitrosylation of ferrous human serum heme-Fe-albumin (HSA-heme-Fe) upon binding of warfarin and ibuprofen to the fatty acid binding site 2 (FA2) has been attributed to the allosteric drug-dependent hexa-coordination of the heme-Fe(II) atom [54–56].

**Nitrosylation of Pathogen Truncated Hemoglobins**

| Derivative          | $\lambda_{\text{max}}$ (nm) | $\varepsilon$ (M$^{-1}$ cm$^{-1}$) | pH   |
|---------------------|-------------------------------|-------------------------------------|------|
| Mt-trHbN(III)       | 406                           | $1.41 \times 10^4$                 | 6.4  |
| Mt-trHbN(III)-OH$^-$| 410                           | $1.25 \times 10^5$                 | 9.4  |
| Mt-trHbN(III)-NO   | 421                           | $1.37 \times 10^5$                 | 6.4 to 9.4 |
| Mt-trHbN(II)        | 432                           | $1.03 \times 10^5$                 | 6.4 to 9.4 |
| Mt-trHbO(II)-NO    | 417                           | $1.22 \times 10^5$                 | 6.4 to 9.4 |
| Mt-trHbO(III)-OH$^-$| 409                           | $1.04 \times 10^5$                 | 6.4  |
| Mt-trHbO(III)-NO   | 412                           | $1.36 \times 10^5$                 | 9.4  |
| Mt-trHbO(II)-NO    | 422                           | $1.46 \times 10^5$                 | 6.4 to 9.4 |
| Mt-trHbO(II)        | 430                           | $9.20 \times 10^4$                 | 6.4 to 9.4 |
| Mt-trHbO(III)-NO   | 416                           | $1.28 \times 10^5$                 | 6.4 to 9.4 |
| Cj-trHbP(III)       | 410                           | $1.41 \times 10^5$                 | 6.4  |
| Cj-trHbP(III)-OH$^-$| 414                           | $1.09 \times 10^5$                 | 9.4  |
| Cj-trHbP(III)-NO   | 420                           | $1.28 \times 10^5$                 | 6.4 to 9.4 |
| Cj-trHbP(II)        | 433                           | $1.19 \times 10^5$                 | 6.4 to 9.4 |
| Cj-trHbP(II)-NO    | 418                           | $1.41 \times 10^5$                 | 6.4 to 9.4 |

| Concentration (M) | pH     | $k_{\text{on}}$ (M$^{-1}$ s$^{-1}$) |
|-------------------|--------|------------------------------------|
| 1.0 $\times 10^{-3}$ | 6.4   | 1.0 $\times 10^5$                  |
| 5.0 $\times 10^{-3}$ | 6.4   | 5.0 $\times 10^4$                  |

Values of $\lambda_{\text{max}}$ and $\varepsilon$ of the absorption spectra in the Soret region of ferric and ferrous derivatives of Mt-trHbN, Mt-trHbO, and Cj-trHbP, 20.0°C.
conversion of trHbN(II) to trHbP(II)-NO (i.e., Log $h_{on}$) increases by one-order of magnitude (Fig. 4, and Figs S3 and S4 in File S1, panel D). However, the increase of $h_{on}$ for the nitrite-mediated conversion of human cytoglobin on pH decrease has been interpreted accounting for the reversible pH-dependent penta-to-hexa-coordination transition of the heme-Fe(II) atom [33].

Values of $h_{on}$ for nitrite-mediated conversion of ferrous heme-proteins range between $7.0 \times 10^{-2}$ M$^{-1}$ s$^{-1}$ and $6.8 \times 10^{5}$ M$^{-1}$ s$^{-1}$ (Table 3) ([25,26,29,31–35] and present study), reflecting the different structural and chemical features of the heme site [5,57–67]. Values of $h_{on}$ for NO$_2^-$ binding to penta-coordinated ferrous Mt-trHbN, Cj-trHbP, carp myoglobin (Mb)-1, carp Mb-2, horse heart Mb, and sperm whale Mb, ranging between $1.8 \times 10^{-2}$ M$^{-1}$ s$^{-1}$ and $1.6 \times 10^{3}$ M$^{-1}$ s$^{-1}$, are among the fastest observed (Table 3). Of note, the low reactivity of Mt-trHbO reflects the unfavorable ligand accessibility to the heme pocket due to the locked conformation(s) of the heme distal residues TyrCD1 and TrpG8 (Fig. 2) [18]. The very different values of the interconversion rate for the hexa- to penta-coordination of the heme-Fe(II) atom modulates kinetics of nitrite binding to *Synechocystis* Hb, *Arabidopsis thaliana* nonsymbiotic Hbs classes 1 and 2, and rice nonsymbiotic Hb class 1, human Cygb, and horse heart cytochrome c [18,31,33,34]. Accordingly, changes of the $h_{on}$ values for the nitrite-mediated conversion of ferrous human neuroglobin (Ng) to its ferrous nitrosylated derivative (from $1.2 \times 10^{-2}$ M$^{-1}$ s$^{-1}$ to $1.2 \times 10^{-1}$ M$^{-1}$ s$^{-1}$) reflect the reversible redox-linked hexa-to-penta-coordination transition of the heme-Fe(II) atom. In fact, under oxidative conditions, the formation of the Cys46-Cys55 bridge stabilizes the high-reactive penta-coordinated heme-Fe(II) atom, thus facilitating the reaction. In contrast, under reductive conditions, the cleavage of the Cys46-Cys55 bridge leads to the formation of the low-reactive hexa-coordinated heme-Fe(II) atom [32]. Similarly, the inhibition of nitrite-dependent conversion of ferrous HSA-heme-Fe to the nitrosylated derivative reflects warfarin binding to the FA2 site with the concomitant penta-to-hexa coordination interconversion of the heme-Fe(II) atom [35]. Lastly, the nitrite-mediated conversion of ferrous human Hb to the nitrosylated derivative is modulated allosterically, inositol hexakisphosphate impairing the reactivity of ferrous human Hb stabilizing the low-reactivity T-state [25,26].

### Table 2. Values of the second-order rate constant for nitrosylation of ferrous globins.

| Heme-protein | $k_{on}$ (M$^{-1}$ s$^{-1}$) |
|--------------|---------------------------|
| Mt-trHbN     | $2.1 \times 10^{5}$a      |
| Mt-trHbO     | $1.9 \times 10^{5}$a      |
| Cj-trHbP     | $1.1 \times 10^{5}$a      |
| Ma-Pgb       | $2.7 \times 10^{7}$       |
| Arabidopsis thaliana Hb class 1° | $2.5 \times 10^{8}$ |
| Glycerine max Lb° | $1.2 \times 10^{8}$      |
| Scapharca inaequivalvis Hbl° | $1.6 \times 10^{7}$       |
| Horse heart Mb° | $1.6 \times 10^{7}$       |
| Sperm whale Mb° | $1.7 \times 10^{7}$       |
| Mouse Ngb° | $1.5 \times 10^{9}$       |
| Tetrameric human Hb° | $2.6 \times 10^{7}$       |
| Horse heart cytochrome c° | 8.3 |
| HSA-heme-Fe° | $6.3 \times 10^{6}$       |
| Ibuprofen-HSA-heme-Fe(II)l | $4.1 \times 10^{5}$ |
| Warfarin-HSA-heme-Fe(II)l | $4.8 \times 10^{5}$ |
| Rabbit HPX-heme-Fe°m | $6.3 \times 10^{3}$ |

*pH 7.0 and 20.0 °C. Present study.
°pH 7.0 and 20.0 °C. From [43].
°pH 7.0 and 20.0 °C. From [53].
°pH 7.0 and 20.0 °C. From [49].
°pH 7.0 and 20.0 °C. From [50].
°pH 9.2 and 20.0 °C. From [41].
°pH 7.0 and 20.0 °C. From [48].
°pH 7.5 and room temperature. From [51].
°pH 7.0 and 20.0 °C. From [47].
°pH 6.5 and room temperature. From [36].
°pH 7.0 and 20.0 °C. From [55].
°pH 7.0 and 10.0 °C. From [52].
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Reactive nitrosylation of Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III)

The reactive nitrosylation of Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) occurs only at pH=8.4. Of note, Mt-trHbN(III)
binds reversibly NO without converting to Mt-trHbN(II)-NO at pH 7.5 [68].

Mixing Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) with NO solutions (between pH 8.4 and 9.4, 20.0°C) induces a shift of the optical absorption maximum of the Soret band from 406–414 nm (i.e., trHb(III)) to 420–422 nm (i.e., trHb(III)-NO) (Fig. 5, and Figs S5 and S6 in File S1, panel A, and Table 1). Then, the optical absorption maximum of the Soret band shifts from 420–422 nm (i.e., trHb(III)-NO) to 416–418 nm (i.e., trHb(II)-NO) (Fig. 5, and Figs S5 and S6 in File S1, panel A, and Table 1).

Absorbance spectra of Mt-trHbN(II)-NO, Mt-trHbO(II)-NO, and Cj-trHbP(II)-NO obtained mixing Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) with NO solutions (Fig. 5, and Figs S5 and S6 in File S1, panel A, and Table 1) correspond to those obtained by: (i) adding gaseous NO to the Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) solutions (Fig. 3, and Figs S1 and S2 in File S1, panel A, and Table 1), and (ii) mixing Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) with nitrite solutions in the presence of dithionite (Fig. 4, and Figs S3 and S4 in File S1, panel A, and Table 1). Moreover, values of \( \lambda_{\text{max}} \) and \( \epsilon \) of the absorption spectra in the Soret region of Mt-trHbN, Mt-trHbO, and Cj-trHbP derivatives agree with those reported in the literature [8,20,21,45,46,68–70].

Over the whole NO concentration range explored, the time course for Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) reductive nitrosylation corresponds to a biphasic process (Fig. 5, and Figs S5 and S6 in File S1, panel A, and Table 1). Values of \( l \) and \( b \) are wavelength-independent at fixed NO concentration (data not shown).

The first step of kinetics for Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) reductive nitrosylation (indicated by \( l \) in Scheme C) is a bimolecular process as observed under pseudo-first order conditions (Fig. 5, and Figs S5 and S6 in File S1, panel C) at all the pH values investigated. Plots of \( l \) versus [NO] are linear (Eqn. (6)), the slope corresponding to \( l_{\text{on}} \). Values of the second-order rate constant \( l_{\text{on}} \) over the pH range explored (i.e., from pH 8.4 to 9.4; Tables S1–S3 in File S1) vary between 1.1 \( \times \) 10^5 M^-1 s^-1 (for Mt-trHbN(III)), between 8.5 \( \times \) 10^3 M^-1 s^-1 and 2.1 \( \times \) 10^4 M^-1 s^-1 (for Mt-trHbO(III)), and between 8.9 \( \times \) 10^4 M^-1 s^-1 and 1.7 \( \times \) 10^5 M^-1 s^-1 (for Cj-trHbP(III)).

The y intercept of plots of \( l \) versus [NO] corresponds to the dissociation rate constant \( l_{\text{off}} \) (Eqn. (6)), whose values over the pH range explored (i.e., from pH 8.4 to 9.4; Tables S1–S3 in File S1) vary between 1.1 \( \times \) 10^5 M^-1 s^-1 and 2.3 \( \times \) 10^5 M^-1 s^-1 (for Mt-trHbN(III)), between 8.5 \( \times \) 10^3 M^-1 s^-1 and 2.1 \( \times \) 10^4 M^-1 s^-1 (for Mt-trHbO(III)), and between 8.9 \( \times \) 10^4 M^-1 s^-1 and 1.7 \( \times \) 10^5 M^-1 s^-1 (for Cj-trHbP(III)).

Between pH 8.4 and pH 9.4, the molar fraction of trHb(III)-NO (i.e., \( x \), Eqn. (7)) increases as a function of free [NO], tending to level off at [NO] > 10 M; the analysis of data according to Eqn.
Table 3. Values of the second-order rate constant for the nitrite-mediated nitrosylation of ferrous globins.

| Heme-protein                              | $k_{on}$ (M$^{-1}$ s$^{-1}$) |
|-------------------------------------------|-----------------------------|
| Mt-trHbN                                  | 1.6×10$^3$                  |
| Mt-trHbO                                  | 3.8×10$^{-1}$               |
| Cj-trHbP                                  | 4.3                         |
| Synechocystis Hb                          | 6.8×10$^1$                  |
| Arabidopsis thaliana Hb class 1$^a$       | 2.0×10$^1$                  |
| Arabidopsis thaliana Hb class 2$^b$       | 4.9                         |
| Rice non symbiotic Hb class 1$^b$         | 3.3×10$^1$                  |
| Carp Mb-1$^d$                              | 5.3                         |
| Carp Mb-2$^d$                              | 1.8                         |
| Horse heart Mb                             | 2.9                         |
| Sperm whale Mb                             | 6.0                         |
| HistE7Ala$^a$                              | 1.8                         |
| HistE7Leu$^a$                              | <0.2                        |
| Mouse Ngb                                 | 5.1                         |
| Human Cygb                                 | 1.4×10$^{-3}$               |
| Human Ngb                                 | 1.2×10$^{-1}$               |
| Tetrameric human Hb                       | 1.2×10$^{-1}$               |
| Horse heart cytochrome                    | 6.0                         |
| HSA-heme-Fe$^i$                           | 1.3                         |
| Warfarin-HSA-heme-Fe$^i$                  | 9.3×10$^{-2}$               |

$^a$ pH 7.4 and 20.0°C. Present study.
$^b$ pH 7.0; unknown temperature [31].
$^c$ pH 7.4 and 25.0°C. From [34].
$^d$ pH 7.4 and 25.0°C. From [32].
$^e$ pH 7.4 and 25.0°C. From [25].
$^f$ pH 7.4 and 25.0°C. From [29].
$^g$ pH 7.0 and 25.0°C. From [33].
$^h$ pH 7.4 and 25.0°C. From [36].
$^i$ pH 7.0 and 25.0°C. From [35].

The second step of kinetics for Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) reductive nitrosylation (indicated by Cb-Cd in Scheme C) follows a [NO]$^-$independent monomolecular behavior (Fig. 5, and Figs S5 and S6 in File S1, panel D). Values of $L$ over the pH range explored (i.e., from pH 8.4 to 9.4; Tables S1–S3 in File S1) vary between 9.3×10$^{-8}$ M and 1.9×10$^{-7}$ M (for Mt-trHbN(III)), between 9.8×10$^{-8}$ M and 2.1×10$^{-7}$ M (for Mt-trHbO(III)), and between 3.1×10$^{-5}$ M and 7.9×10$^{-5}$ M (for Cj-trHbP(III)). According to the trHb(III):NO 1:1 stoichiometry of reaction Ca in Scheme C, the Hill coefficient $n$ is 1.00±0.02. As expected for a simple system [23], values of $L$ correspond to those of $l_{cat}/l_{cat}$, under all experimental conditions (Table 4 and Tables S1–S3 in File S1).

The second step of kinetics for Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) reductive nitrosylation (indicated by Cb-Cd in Scheme C) follows a [NO]$^-$independent monomolecular behavior (Fig. 5, and Figs S5 and S6 in File S1, panel D) at all the pH values investigated. According to Scheme C, the value of $b$ increases linearly on increasing [OH$^-$] (i.e., from pH 8.4 to 9.4; Fig. 5, and Figs S5 and S6 in File S1, panel F, and Tables S1–S3 in File S1). The slope and the y intercept of the plot of $b$ versus [OH$^-$] correspond to $b_{OH^-$} and $b_{H_2O}$ values. Values of $b_{OH^-$} and $b_{H_2O}$ for for Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) reductive nitrosylation are 1.7×10$^2$ M$^{-1}$ s$^{-1}$ and 6.4×10$^{-1}$ s$^{-1}$, 2.4×10$^2$ M$^{-1}$ s$^{-1}$ and 2.9×10$^{-4}$ s$^{-1}$, and 9.1×10$^2$ M$^{-1}$ s$^{-1}$ and 4.8×10$^{-8}$ s$^{-1}$, respectively (Table 4).

Over the whole pH range explored (i.e., from pH 8.4 to 9.4), the reductive nitrosylation of Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) is an irreversible process, as the spectra of Mt-trHbN(II)-NO, Mt-trHbO(II)-NO, and Cj-trHbP(II)-NO revert to Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) instead of Mt-trHbN(III), Mt-trHbO(III), and Cj-trHbP(III) by merely pumping off gaseous NO or bubbling helium through the Mt-trHbN(II)-NO, Mt-trHbO(II)-NO, and Cj-trHbP(II)-NO solutions; however, the denitrosylation process (i.e., the Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) formation) needs about 12 hours to be completed.

Under all experimental conditions, free Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) was never detected spectrophotometrically because of the very rapid reaction of Mt-trHbN(II), Mt-trHbO(II), and Cj-trHbP(II) with NO ($k_{on}$=8.6×10$^4$ M$^{-1}$ s$^{-1}$; Table 1).

Ferric Mt-trHbN, Cj-trHbP, Methanosarcina acetivorans protoglobin (Ma-Pgb), horse heart Mb, sperm whale Mb, and HSA-heme-Fe react rapidly with NO ([41,43,48,55] and present study),
even though the binding of a weak exogenous ligand (i.e., a water molecule) occurs likely at the sixth coordination position of the heme-Fe(III) atom \([5,57,58,66,71]\). As reported above, the low reactivity of Mt-trHbO reflects the unfavorable ligand accessibility to the heme distal pocket \([18]\). Unexpectedly, the very low value of \(k_\text{on}\) for the reductive nitrosylation of penta-coordinated Scapharca inaequalvis HbI may reflect either the non-occurrence of the heme-Fe(III)-NO intermediate or the concomitant nitrosation of the Cys92 residue affecting the heme-Fe reactivity \([38]\). On the other hand, the reactivity of ferric hexa-coordinated Glycine max

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**Figure 5. Reductive nitrosylation of Mt-trHbN(III), at 20.0°C.** (A) Difference absorbance spectra of Mt-trHbN(III) minus Mt-trHbN(III)-NO and of Mt-trHbN(III)-NO minus Mt-trHbN(III) (open and filled circles, respectively), at pH 9.0. (B) Normalized averaged time courses of Mt-trHbN(III) reductive nitrosylation, at pH 9.0. The NO concentration was \(2.5 \times 10^{-5} \text{ M}\) (trace a) and \(2.0 \times 10^{-4} \text{ M}\) (trace b). The time course analysis according to Eqs. (5a)–(5c) allowed the determination of the following values of parameters \(a\), \(l\), and \(b\): trace a - \(a = 0.61, l = 6.0 \text{ s}^{-1}\), and \(b = 2.5 \times 10^{-3} \text{ s}^{-1}\); and trace b - \(a = 0.89, l = 2.6 \times 10^{1} \text{ s}^{-1}\), and \(b = 2.4 \times 10^{-3} \text{ s}^{-1}\). (C) Dependence of \(l\) on [NO] for Mt-trHbN(III) reductive nitrosylation, at pH 9.0. The continuous line was generated from Eqn. (6) with \(l_{\text{on}} = (1.4 \pm 0.2) \times 10^{5} \text{ M}^{-1} \text{ s}^{-1}\) and \(l_{\text{off}} = 1.6 \pm 0.2 \text{ s}^{-1}\). (D) Dependence of \(a\) on [NO] for Mt-trHbN(III) reductive nitrosylation, at pH 9.0. The continuous line was generated from Eqn. (7) with \(L = (1.5 \pm 0.2) \times 10^{-5} \text{ M}\). (E) Dependence of \(b\) on [NO] for Mt-trHbN(III) reductive nitrosylation, at pH 9.0. The average \(b\) value is \(2.5 \times 10^{-3} \text{ s}^{-1}\) (dashed line). (F) Dependence of \(b\) on [OH\(^{-}\)] for Mt-trHbN(III) reductive nitrosylation. The continuous line was generated from Eqn. (8) with \(b_{\text{OH}^{-}} = (1.7 \pm 0.2) \times 10^{2} \text{ M}^{-1} \text{ s}^{-1}\) and \(b_{\text{H}_{2}\text{O}} = (6.4 \pm 0.7) \times 10^{-4} \text{ s}^{-1}\). The Mt-trHbN(III) concentration was \(1.5 \times 10^{-6} \text{ M}\). Where not shown, standard deviation is smaller than the symbol. For details, see text.

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Table 4. Values of kinetic and thermodynamic parameters for reductive nitrosylation of ferric heme-proteins.

| Heme-protein                  | \( k_\text{on} \) (M\(^{-1}\) s\(^{-1}\)) | \( k_\text{off} \) (s\(^{-1}\)) | \( K \) (M) | \( \Delta \text{G}^\circ \text{t} \) (M\(^{-1}\) s\(^{-1}\)) | \( \Delta \text{G}^\circ \text{e} \) (s\(^{-1}\)) |
|-------------------------------|------------------------------------------|---------------------------------|------------|------------------------------------------|------------------------------------------|
| MttrHbN                       | \( 1.4 \times 10^{30} \)                | \( 16 \)                        | \( 1.6 \times 10^{30} \) | \( 1.1 \times 10^{-2} \) | \( 1.7 \times 10^{20} \) | \( 6.4 \times 10^{-6} \) |
| MttrHbO                       | \( 9.2 \times 10^{30} \)                | \( 21 \)                        | \( 1.9 \times 10^{20} \) | \( 2.3 \times 10^{-4} \) | \( 2.4 \times 10^{20} \) | \( 2.9 \times 10^{-6} \) |
| Cj-trHbP                      | \( 1.1 \times 10^{30} \)                | \( 8.1 \)                       | \( 6.5 \times 10^{30} \) | \( 7.4 \times 10^{-2} \) | \( 9.1 \times 10^{20} \) | \( 4.8 \times 10^{-6} \) |
| Ma-Pgb                        | \( 4.8 \times 10^{6} \)                 | \( 26 \)                        | \( 6.1 \times 10^{3} \)  | \( 5.4 \times 10^{-2} \) | \( 2.9 \times 10^{20} \) | \( 4.1 \times 10^{-6} \) |
| Glycine max Lb\(^a\)          | \( 1.4 \times 10^{25} \)                | \( 30 \)                        | \( 2.1 \times 10^{25} \) | \( 2.1 \times 10^{-3} \) | \( 3.3 \times 10^{20} \) | \( 3.0 \times 10^{-4} \) |
| Scapharca inaequivalvis Hbl   | \( 3.2 \times 10^{10} \)                | \( <1 \times 10^{-2} \)         | n.d.       | \( 3.1 \times 10^{-2} \) | \( >2 \times 10^{20} \) | n.d. |
| Horse heart Mb                | \( 6.8 \times 10^{5} \)                 | \( 52 \)                        | \( 1.2 \times 10^{-4} \) | \( 7.6 \times 10^{25} \) | \( 3.9 \times 10^{20} \) | n.d. |
| Speem whale Mb                | \( 1.9 \times 10^{30} \)                | \( 14 \times 10^{3} \)          | \( 7.7 \times 10^{30} \) | n.d.                        | \( 3.2 \times 10^{20} \)                | n.d. |
| Human Ngb Cys46–Cys55\(^a\)  | \( 2.1 \times 10^{6} \)                 | \( 2.5 \times 10^{-30} \)       | n.d.       | \( 1.2 \times 10^{-6} \) | \( >2 \times 10^{20} \)                | n.d. |
| Cys46/Cys55\(^b\)            | \( 2.9 \)                                | \( 20 \times 10^{-30} \)        | n.d.       | \( 1.9 \times 10^{-40} \) | \( >5 \times 10^{20} \)                | n.d. |
| Tetrameric human Hb           | n.d.                                    | n.d.                            | \( 8.3 \times 10^{-30} \) | n.d.                        | \( 3.2 \times 10^{20} \)                | \( 1.1 \times 10^{-30} \) |
| Horse cytochrome c            | n.d.                                    | n.d.                            | \( 7.1 \times 10^{-30} \) | n.d.                        | \( 1.5 \times 10^{30} \)                | n.d. |
| HSA-heme-Fe                   | \( 2.1 \times 10^{40} \)                | \( 2.0 \times 10^{-30} \)       | n.d.       | \( 1.8 \times 10^{-8} \) | \( 4.4 \times 10^{20} \)                | \( 3.5 \times 10^{-60} \) |
| Rabbit HPX-heme-Fe            | \( 1.3 \times 10^{40} \)                | \( \leq 10^{-4} \)              | n.d.       | \( \leq 8 \times 10^{-6} \) | \( \geq 7 \times 10^{-9} \)              | n.d. |

\(^{a}\) pH 9.0 and 20.0°C. Present study.  
\(^{b}\) pH 20.0°C. Present study.  
\(^{c}\) pH 7.2 and 220°C. From [43].  
\(^{d}\) pH 22.0°C. From [43].  
\(^{e}\) pH 7.3 and 20.0°C. From [39].  
\(^{f}\) pH 20.0°C. From [39].  
\(^{g}\) pH 7.5 and 20.0°C. From [38].  
\(^{h}\) Derived from values of \( b \) and [OH\(^-\)] [38], at 20.0°C.  
\(^{i}\) pH 9.2 and 20.0°C. From [41].  
\(^{j}\) Derived from values of \( b \) and [OH\(^-\)] [41], at 20.0°C.  
\(^{k}\) pH 8.79 and room temperature. From [37].  
\(^{l}\) Derived from values of \( l_\text{on} \) and \( L \) [37], at pH 8.79 and room temperature.  
\(^{m}\) Room temperature. From [37].  
\(^{n}\) In “Human Ngb Cys46–Cys55”, the Cys46 and Cys55 residues form an intramolecular disulphide bond.  
\(^{o}\) pH 7.0 and room temperature. From [74].  
\(^{p}\) Derived from values of \( l_\text{on} \) and \( l_\text{off} \) [74], at room temperature.  
\(^{q}\) Derived from values of \( b \) and [OH\(^-\)] [74], at pH 7.0 and room temperature.  
\(^{r}\) In “Human Ngb Cys46/Cys55”, the Cys46 and Cys55 residues do not form the intramolecular disulphide bond.  
\(^{s}\) pH 7.1 and room temperature. From [37].  
\(^{t}\) Room temperature. From [37].  
\(^{u}\) pH 8.35 and room temperature. From [37].  
\(^{v}\) pH 7.0 and 20.0°C. From [42].  
\(^{w}\) 20.0°C. From [42].  
\(^{x}\) pH 7.0 and 10.0°C. From [40].  
\(^{y}\) 10.0°C. From [40].  
\(^{z}\) n.d., not determined.
Lb, horse cytochrome c, and rabbit HPX-heme-Fe ([37,39,40,72] and present study) reflects the hexa- to penta-coordination conversion of the heme-Fe atom ([59,63,73] and present study) (Table 4). As reported for the nitrite-mediated nitrosylation of ferrous human Ngb [32] (Table 3), NO binding to ferric human Ngb is modulated by the formation and cleavage of the Cys46-Cys55 disulphide bridge [74].

Values of $l_N$ for NO dissociation from nitrosylated heme-Fe(III) proteins span over five orders of magnitude (Table 4) ([37–43,74] and present study), reflecting the different stabilization mode of the heme-Fe bound ligand by heme distal residues [5,54,58,59,66,71,73,75–79].

Although values of $l_{ON}$ and $l_{NO}$ binding to heme-Fe(III) proteins are very different (Table 4) ([37–43,74] and present study), values of $l (= l_{ON}/l_{NO})$ are similar (Table 4) ([37–43,74] and present study), indicating the occurrence of kinetic compensation phenomena.

Values of $h_{ON}$ for reductive nitrosylation of heme-Fe(III) proteins, spanning over four orders of magnitude (Table 4) ([37–43,74] and present study), possibly reflect either the different OH$^-$ accessibility to the heme pocket ([5,23,58–60,63,64,67,71,75,80–84]) or the heme-Fe(III) protein reduction potentials [37,39]. Although the values of $h_{ON}$ and $h_{H2O}$ cannot be compared directly, OH$^-$ ions catalyze reductive nitrosylation of heme-Fe(II)-NO$^+$ much more efficiently than H$_2$O. Moreover, the linear dependence of $b$ on [OH$^-$] indicates that no additional elements appear to be involved in irreversible reductive nitrosylation of Mt-trHbN, Mt-trHbO, and Cj-trHbP (present study), as previously reported for related heme-Fe(III) proteins (reaction (c) in Scheme C; Fig. 5, and Figs S5 and S6 in File S1, panel E) [37–43,74].

Reductive nitrosylation of most ferric heme-proteins is limited by the OH$^-$-mediated reduction of the heme-Fe(II)-NO$^+$ complex to heme-Fe(II) (reaction (c) in Scheme C) [37–39,41–43,74]. By contrast, NO binding to hexa-coordinated horse cytochrome c and ferric rabbit HPX-heme (reaction (a) in Scheme C) represents the rate-limiting step [37,40] (Tables 2 and 4 for comparison).

**Conclusion and Perspectives**

The occurrence of different types of trHbs (i.e., trHbN, trHbO and trHbP) in bacteria, plants and some unicellular eukaryotes (Fig. 1) opens the question of their role, also in view of their frequently simultaneous presence in the same organism; this envisages the possibility that different types of trHbs reflect different physiological roles in these organisms [1–6,11].

The high reactivity of Mt-trHbN and Cj-trHbP reflects both the penta-coordination of the heme-Fe atom and the ligand accessibility to the heme pocket (Fig. 2). Indeed, the ligand access pathway through protein matrix tunnels in penta-coordinated Mt-trHbN (Fig. 2) [5,83] and the E7-path in penta-coordinated Cj-trHbP (Fig. 2) [5,92,66] is characterized by the lowest energy barrier, with values close to those displayed by penta-coordinated sperm whale Mb. Of note, ligand entry to and exit from the heme distal pocket of sperm whale Mb is modulated by gating movement of the HisE7 residue [75]. In contrast, the low reactivity of penta-coordinated Mt-trHbO [21] reflects H-bond interactions that lock the heme distal residues TyrCD1 and ThrP60 into a conformation(s) that limits ligand access to the heme distal pocket (Fig. 2) [18,70]. Of note, the E7-path appears to sustain ligand diffusion to the heme distal cavity of Mt-trHbO; indeed, Mt-trHbO hosts two protein matrix cavities instead of the protein matrix tunnel occurring in Mt-trHbN (Fig. 2) [84].

* M. tuberculosis* is massively exposed to NO during its intramacrophagic life, and Mt-trHbN has been shown to play a prominent role in protection from nitrosative stress [16]. Therefore, Mt-trHbN is most likely to function as NO dioxygenase that converts toxic NO into harmless nitrate in the presence of oxygen, and relieves toxicity due to NO and nitrosative stress. This is also consistent with the NO-inducible response of the gene encoding for trHbN in *M. tuberculosis*, both *in vitro* and inside infected macrophages [85]. Of note, also the promoter of the trHbO gene is induced during macrophage infection, though it poorly responds to NO induction [85]. Based on biochemical data, the evidence of trHbO in protection from NO is weak, while it is more likely that this protein is involved in oxygen sensing and aerobic respiration [86]. Similar considerations could hold true for the enteric pathogen *C. jejuni*, which colonizes the intestinal tract of birds and can cause enteritis in humans. Like other pathogens, *C. jejuni* is exposed to NO and other nitrosating species during host infection [87], the expression of both bacterial Cj-trHbP and 3-on-3 globin being upregulated by NO [11]. The 3-on-3 globin plays the major role in resistance to nitrosative stress and aerobically converts NO to nitrate [88], whereas the contribution of trHbP is less prominent. In contrast, both globins are devoid of NO-protective activity under oxygen-limited conditions that normally exist *in vivo* [89]. Therefore, the role of Cj-trHbP is clearly distinct from that of the 3-on-3 globin, being related to O$_2$ metabolism [7,8], likely performing a peroxidase or P450-type of oxygen chemistry [9].

As a whole, the comparison of the biochemical properties of microbial globins, including trHbs, will allow to shed light on their functional diversification and on the molecular bases of microbe ecology.

**Supporting Information**

*File S1  Supporting tables and figures.*

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**Author Contributions**

Conceived and designed the experiments: PA PV MC. Performed the experiments: AdM GRT AP. Analyzed the data: PA PV MC. Contributed reagents/materials/analysis tools: PA AP. Contributed to the writing of the manuscript: PA PV MC.

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