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Nitric oxide scavenging by *Mycobacterium leprae* GlbO involves the formation of the ferric heme-bound peroxynitrite intermediate ☆

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Abstract

Ferrous oxygenated ($Fe(II)O_2$) hemoglobins (Hb's) and myoglobins (Mb's) have been shown to react very rapidly with NO, yielding NO_3^- and the ferric heme–protein derivative (Fe(III)), by means of the ferric heme-bound peroxynitrite intermediate (Fe(III)OONO), according to the minimum reaction scheme:

$$Fe(II)O_2 + NO \xrightarrow{k_{on}} Fe(III)OONO \xrightarrow{h} Fe(III) + NO_3^-$$

For most Hb's and Mb's, the first step (indicated by k_{on}) is rate limiting, the overall reaction following a bimolecular behavior. By contrast, the rate of isomerization and dissociation of Fe(III)OONO (indicated by h) is rate limiting in NO scavenging by $Fe(II)O_2$ murine neuroglobin, thus the overall reaction follows a monomolecular behavior. Here, we report the characterization of the NO scavenging reaction by $Fe(II)O_2$ truncated Hb GlbO from *Mycobacterium leprae*. Values of k_{on} ($=2.1 \times 10^6$ M^{-1} s^{-1}) and h ($=3.4$ s^{-1}) for NO scavenging by $Fe(II)O_2$ *M. leprae* GlbO have been determined at pH 7.3 and 20.0 °C, the rate of Fe(III)OONO decay (h) is rate limiting. The Fe(III)OONO intermediate has been characterized by optical absorption spectroscopy in the Soret region. These results have been analyzed in parallel with those of monomeric and tetrameric globins as well as of flavoHb and discussed with regard to the three-dimensional structure of mycobacterial truncated Hbs and their proposed role in protection from nitrosative stress.

Keywords: *Mycobacterium leprae* GlbO; Truncated hemoglobin; NO scavenging; NO-mediated heme–$Fe(II)O_2$ oxidation; Transient heme–Fe(III)OONO complex formation and decay; Kinetics of heme–$Fe(II)O_2$ oxidation by NO; Optical absorption spectroscopy

Leprosy is an old, still dreaded infectious disease caused by the obligate intracellular bacterium *Mycobacterium leprae* [1]. During infection, *M. leprae* is challenged by the toxic activity of reactive nitrogen species [2], primarily nitric oxide (NO), produced by activated macrophages expressing inducible nitric oxide synthase (iNOS) [3]. Among others, NO reacts with the superoxide radical ($O_2^{\cdot -}$) present at the site of inflammation, yielding peroxynitrite (an hydroperoxide, $ONOO^-$) that rapidly nitrosates tyrosine residues [4]. Both iNOS and nitrotyrosine are detected in lepromatous lesions [5,6], and increased levels of NO metabolites are excreted in urines of leprosy patients [7]. Upregulation of iNOS and local release of reactive nitrogen species have been proposed

☆ *Abbreviations:* $Fe(II)O_2$, ferrous oxygenated heme–protein derivative; Fe(III), ferric heme–protein derivative; Fe(III)OONO, ferric heme-bound peroxynitrite derivative; flavoHb, flavohemoglobin; Hb, hemoglobin; iNOS, inducible nitric oxide synthase; Mb, myoglobin; NO, nitric oxide; trHb, truncated hemoglobin.

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to cause nerve damage [8]. A role of the nitrosative defense in macrophage-mediated host's resistance to *M. leprae* is also inferred by the reduced bacterial suppression observed in iNOS-defective macrophages and iNOS knock-out mice [9].

The ability of *M. leprae* to persist in vivo in the presence of reactive nitrogen species [5–8] implies the presence in this enigmatic bacterium of still undefined NO scavenging/detoxification mechanisms. Comparative mycobacterial genomics has unraveled extensive genome decay in *M. leprae*, which has conserved a minimal set of genes involved in protection from oxidative and nitrosative stress [10,11]. Nearly, 50 genes are induced by NO in the facultative intracellular pathogen *Mycobacterium tuberculosis*, a large subset of which have a suggested or documented role in promoting survival under nitrosative stress [11–13]. Remarkably, most of these genes are dysfunctional or absent in the obligate intracellular pathogen *M. leprae;* only superoxide dismutase reductase (*sodC*, ML1925), alkylhydroperoxide reductase (*ahpC*, ML2042), bifunctional thioredoxin reductase/thioredoxin (*trxB* and *trxC*, ML1818, and ML2703, respectively), NADPH-ferredoxin reductase (*fprA* and *fprB*, ML0666, and 2134, respectively), and truncated hemoglobin (trHb) GlbO (*glbO*, ML 1253) orthologues are present in the *M. leprae* genome [11,14–16].

TrHbs are a family of widely distributed small oxygen-binding hemoproteins displaying a typical 2-on-2 α-helical fold and an apolar protein matrix tunnel linking the protein surface to the heme distal site [16]. TrHbs can be phylogenetically classified into three distinct lineages (designated groups I–III); individual members from each trHb group may be present in the same organism, hinting at a complex functional diversification of these proteins (reviewed in [16–18]). In *M. tuberculosis*, GlbN (group I trHb) has primarily been linked to NO detoxification, while GlbO (group II trHb) has been proposed to function in O_2 uptake/transport and/or redox sensing [16,19–23]. Having retained only one trHb, *M. leprae* GlbO has been proposed to represent merging of both O_2 uptake/transport and NO detoxification properties [15,24].

In the present study, we address kinetics and stoichiometry of NO oxidation by ferrous oxygenated (Fe(II)O_2) *M. leprae* GlbO. We demonstrate that Fe(II)O_2 *M. leprae* GlbO reacts in vitro with NO to produce Fe(III) and NO_3^- in 1:1 molar ratio. This process involves oxidation of the heme–Fe atom, with formation and decay of the transient Fe(III)OONO species.

Materials and methods

Heme–proteins and chemicals. Mycobacterium leprae GlbO was expressed in *Escherichia coli* M15 and purified by Ni(II)–nitrilotriacetic acid–agarose affinity chromatography [24]. Horse heart Mb (purified by crystallization) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and used without further purification. Fe(II)O_2 human Hb was prepared as previously reported [25]. Fe(II)O_2 *M. leprae* GlbO and horse heart Mb were prepared by reducing the heme–Fe-atom with sodium dithionite. The excess of dithionite and by-products was removed by passing the Fe(II) solution through a Sephadex G-25 gel filtration column (Amersham Biosciences Europe GmbH, Freiburg, D) equilibrated in air with 1.0×10^{-1} M phosphate buffer, pH 7.3, at 20.0 °C, in the presence of 5.0×10^{-2} M EDTA [24]. Fe(III) *M. leprae* GlbO and human Hb were prepared by oxidizing the heme–Fe-atom with sodium ferricyanide. The excess of ferricyanide and by-products was removed by passing the Fe(III) solution through a Sephadex G-25 gel filtration column (Amersham Biosciences Europe GmbH, Freiburg, D) equilibrated in air with 1.0×10^{-1} M phosphate buffer, pH 7.3, at 20.0 °C, in the presence of 5.0×10^{-2} M EDTA [24]. Values of ε ($mM^{-1}\,cm^{-1}$) of Fe(II)O_2 *M. leprae* GlbO and horse heart Mb, and of Fe(III) *M. leprae* GlbO and human Hb in the Soret region were determined at pH 7.3 (1.0×10^{-1} M phosphate buffer) and 20.0 °C, in the presence of 5.0×10^{-2} M EDTA, by the pyrimidine–hemochromogen method [25].

Gaseous NO was purchased from Aldrich Chemical (Milwaukee, WI, USA). NO was purified by flowing through an NaOH column in order to remove acidic nitrogen oxides. The NO solution was prepared by keeping 2.0×10^{-2} M phosphate buffer, pH 7.0, in the presence of 5.0×10^{-2} M EDTA, in a closed vessel under NO at 760 Torr, at 20.0 °C. The solubility of NO in the aqueous buffered solution is 2.03×10^{-3} M at 20.0 °C [25,26]. All the other chemicals (from Merck AG, Darmstadt, Germany) were of analytical grade and used without further purification.

Kinetics of Fe(II)O_2 M. leprae GlbO oxidation by NO. Values of the second order rate constant for the NO-induced conversion of Fe(II)O_2 *M. leprae* GlbO to Fe(III)OONO (k_{on}) and of the first order rate constant for the conversion of Fe(III)OONO *M. leprae* GlbO to Fe(III) (h) were determined by rapid-mixing of the Fe(II)O_2 *M. leprae* GlbO (final concentration 1.6×10^{-6} M) solution (1.0×10^{-1} M phosphate buffer, pH 7.3, in the presence of 5.0×10^{-2} M EDTA) with the NO (final concentration, 8.0×10^{-6}–4.0×10^{-5} M range) solution (2.0×10^{-2} M phosphate buffer, pH 7.0, in the presence of 5.0×10^{-2} M EDTA). Kinetics was monitored between 360 and 450 nm [21,27–31]. The dead time of the rapid-mixing stopped-flow apparatus was 1.6 ms.

The time courses were fitted to two consecutive exponential processes according to the minimum reaction mechanism (Scheme 1) [21,27–31]:

$$\mathrm{Fe(II)O_2 + NO \overset{k_{on}}{\rightarrow} Fe(III)OONO \overset{h}{\rightarrow} Fe(III) + NO_3^-} \qquad \text{(Scheme1)}$$

Values of the NO-dependent apparent pseudo-first order rate constant for the formation and decay of the transient Fe(III)OONO species (k and h, respectively) have been determined from data analysis, according to Eqs. (1)–(3) [32]:

$$[\mathrm{Fe(II)O_2}]_t = [\mathrm{Fe(II)O_2}]_i \times e^{-kt} \qquad (1)$$

$$[\mathrm{Fe(III)OONO}]_t = [\mathrm{Fe(II)O_2}]_i \times (k \times ((e^{-kt}/(h-k)) + (e^{-ht}/(k-h)))) \qquad (2)$$

$$[\mathrm{Fe(III)}]_t = [\mathrm{Fe(II)O_2}]_i - ([\mathrm{Fe(II)O_2}]_t + [\mathrm{Fe(III)OONO}]_t) \qquad (3)$$

The value of k_{on} was obtained from the linear dependence of k on the NO concentration (i.e., [NO]) according to Eq. (4) [21,27–31]

$$k = k_{on} \times [\mathrm{NO}] \qquad (4)$$

The static difference absorption spectrum in the Soret region of Fe(II)O_2 *minus* Fe(III) was obtained by subtracting the optical density change of Fe(III) from that of Fe(II)O_2 at each wavelength between 360 and 450 nm.

The kinetic difference absorption spectra in the Soret region of Fe(II)O_2 *minus* Fe(III) and of Fe(III)OONO *minus* Fe(III) were reconstructed from the static difference absorption spectrum of Fe(III) *minus* Fe(III) ($\Delta\varepsilon = 0.0\ mM^{-1}\,cm^{-1}$) plus the optical density changes of the overall process Fe(II)$O_2 \rightarrow$ Fe(III) and of the partial reaction Fe(III)OONO $\rightarrow$ Fe(III) (see Scheme 1) at each wavelength, between 360 and 450 nm, measured in the rapid-mixing experiments (wavelength interval = 5 nm).

The absolute absorption spectrum of Fe(III)OONO in the Soret region was reconstructed from the static absorption spectrum of Fe(III) plus the optical density changes of the partial reaction Fe(III)OONO $\rightarrow$ Fe(III)

(see Scheme 1) at each wavelength, between 360 and 450 nm, measured in the rapid-mixing experiments (wavelength interval = 5 nm).

Data were analyzed using the MatLab program (The Math Works, Natick, MA, USA).

Determination of Fe(III), NO_2^-, and NO_3^- in the reaction of NO with Fe(II)O_2 M. leprae GlbO. Aliquots of the NO (2.03×10^{-3} M) solution (2.0×10^{-2} M phosphate buffer, pH 7.0) were added sequentially to the Fe(II)O_2 GlbO (1.46×10^{-5} M) solution (1.0×10^{-1} M phosphate buffer, pH 7.3, in the presence of 5.0×10^{-2} M EDTA) with a gas-tight Hamilton micro-syringe into a quartz cuvette (2010 μl volume) sealed with a rubber septum. No gaseous phase was present in the quartz cuvette. The equilibrium was achieved within the time for sample preparation (~1 min). Then, optical absorption spectra were recorded between 360 and 450 nm to determine the concentration of the Fe(II)O_2 and Fe(III) species with values of ε (mM^{-1} cm^{-1}) given in Table 1. Furthermore, samples were denatured by heat at 100 °C for 5 min and centrifuged at 13,000*g* for 8 min. The supernatants were assayed for the NO_2^- and NO_3^- content spectrophotometrically at 543 nm by using the Griess reagent and VCl_3 to catalyze the conversion of NO_3^- to NO_2^- [30,33]. Undetectable NO_3^- and $<1.5 \times 10^{-7}$ M NO_2^- were present in the NO and Fe(II)O_2 starting solutions.

Results and discussion

Under aerobic conditions, Hbs and Mbs have been shown to protect cells against nitrosative stress by converting NO to NO_3^-, concomitant with the formation of the Fe(III) derivative. Fe(III) is reduced back to Fe(II)O_2 by metHb- and metMb-reductase or related systems allowing the achievement of one-electron reduction. The NO-detoxification reaction catalyzed by Fe(II)O_2 heme–proteins involves an intermediate state in which $OONO^-$ is bound to Fe(III) before isomerization to NO_3^-. For most Hbs and Mbs, the formation of Fe(III)OONO (indicated by k_{on}; see Scheme 1) is rate limiting, and the overall reaction follows bimolecular behavior. Under conditions where the rate of isomerization and dissociation of Fe(III)OONO (indicated by *h*; see Scheme 1) is rate limiting, the overall reaction follows a monomolecular behavior (see [17,18,21,27–31,34–47]).

Over the whole NO concentration range explored (final concentration, 8.0×10^{-6}–4.0×10^{-5} M range), the time course for the oxidation of Fe(II)O_2 *M. leprae* GlbO corresponds to a biphasic process between 360 and 450 nm (Fig. 1A). Monophasic time courses were observed at $\lambda = 370$, 390, 420, and 430 nm, where Fe(II)O_2, Fe(III)OONO or Fe(III) shows the same optical absorbance (Fig. 1, panel B).

The transient species occurring during the O_2-dependent NO oxidation process facilitated by Fe(II)O_2 *M. leprae* GlbO has the Soret maximum at $\lambda = 411$ nm and the extinction coefficient $\varepsilon = 148$ mM^{-1} cm^{-1} (Table 1). These parameters are similar to those of the Fe(III)OONO derivative of horse heart Mb [29], murine neuroglobin [31], and human Hb [29] (Table 1). This is in agreement with the expectation (see Scheme 1) that the intermediate of the NO oxidation process catalyzed by Fe(II)O_2 *M. leprae* GlbO is indeed the Fe(III)OONO species.

As shown in Fig. 1 (panel C), the first step of kinetics for NO detoxification by Fe(II)O_2 *M. leprae* GlbO (indicated by k_{on}; see Scheme 1) is a bimolecular process. The plot of *k* versus [NO] is linear with a *y* intercept at 0, the slope corresponding to k_{on} (see Scheme 1 and Eq. (4)) is 2.1×10^6 M^{-1} s^{-1}, at pH 7.3 and 20.0 °C (Table 2). By contrast, the second step (indicated by *h*; see Scheme 1) follows a [NO]-independent monomolecular behavior (Fig. 1, panel C), the average value of *h* being 3.4 s^{-1}, at pH 7.3 and 20.0 °C (Table 2).

Values of k_{on} for NO oxidation by Fe(II)O_2 were found to be similar for *M. leprae* GlbO (2.1×10^6 M^{-1} s^{-1}) and *M. tuberculosis* GlbO (6.0×10^5 M^{-1} s^{-1}) [21]; they are ~50–1000-fold lower than those reported for horse heart Mb [29], murine neuroglobin [31], human Hb [29], *M. tuberculosis* GlbN [30], and *E. coli* flavoHb [28] (Table 2). The low reactivity of *M. leprae* and *M. tuberculosis* Fe(II)O_2 GlbO vs NO may reflect the limited access and diffusion of diatomic ligands to the heme distal pocket. In fact, the almost continuous apolar tunnel (spanning about 28 Å through the protein matrix) connecting the heme distal pocket to the surface of *M. tuberculosis* GlbN [48,49] is replaced by two small cavities in *M. leprae* and *M. tuberculosis* GlbO (of ~35 and ~26 $Å^3$ volume to be compared with a volume of ~330 $Å^3$ measured for the apolar tunnel of *M. tuberculosis* GlbN) [16,23,24,50].

The value of *h* for the isomerization and dissociation of Fe(III)OONO *M. leprae* GlbO is ~10–100-fold lower than

Table 1
Spectroscopic data for NO scavenging by Fe(II)O_2 heme–proteins

Heme–protein	Fe(II)O_2	Fe(III)OONO	Fe(III)
M. leprae GlbO[a]	$\lambda_{max} = 412$ nm $\varepsilon = 122$ mM^{-1} cm^{-1}	$\lambda_{max} = 411$ nm $\varepsilon = 148$ mM^{-1} cm^{-1}	$\lambda_{max} = 409$ nm $\varepsilon = 115$ mM^{-1} cm^{-1}
Horse heart Mb	$\lambda_{max} = 418$ nm[a] $\varepsilon = 126$ mM^{-1} cm^{-1} [a]	$\lambda_{max} = 410$ nm[b] $\varepsilon = 138$ mM^{-1} cm^{-1} [b]	$\lambda_{max} = 408$ nm[c] $\varepsilon = 188$ mM^{-1} cm^{-1} [c]
Murine neuroglobin[d]	$\lambda_{max} = 410$ nm $\varepsilon \approx 138$ $mM^{-1}cm^{-1}$	$\lambda_{max} = 408$ nm $\varepsilon \approx 143$ mM^{-1} cm^{-1}	$\lambda_{max} = 412$ nm $\varepsilon \approx 140$ mM^{-1} cm^{-1}
Human Hb	$\lambda_{max} = 415$ nm[e] $\varepsilon = 125$ mM^{-1} cm^{-1} [e]	$\lambda_{max} = 407$ nm[b] $\varepsilon = 165$ mM^{-1} cm^{-1} [b]	$\lambda_{max} = 406$ nm [a] $\varepsilon = 171$ mM^{-1} cm^{-1} [a]

[a] pH 7.3 and 20.0 °C. Present study.
[b] pH 9.5 and 5.0 °C. From [29].
[c] pH 6.4 and 25.0 °C. From [25].
[d] pH 7.0 and 5.0 °C and 20.0 °C. From [31].
[e] pH 7.0 and 20.0 °C. From [25].

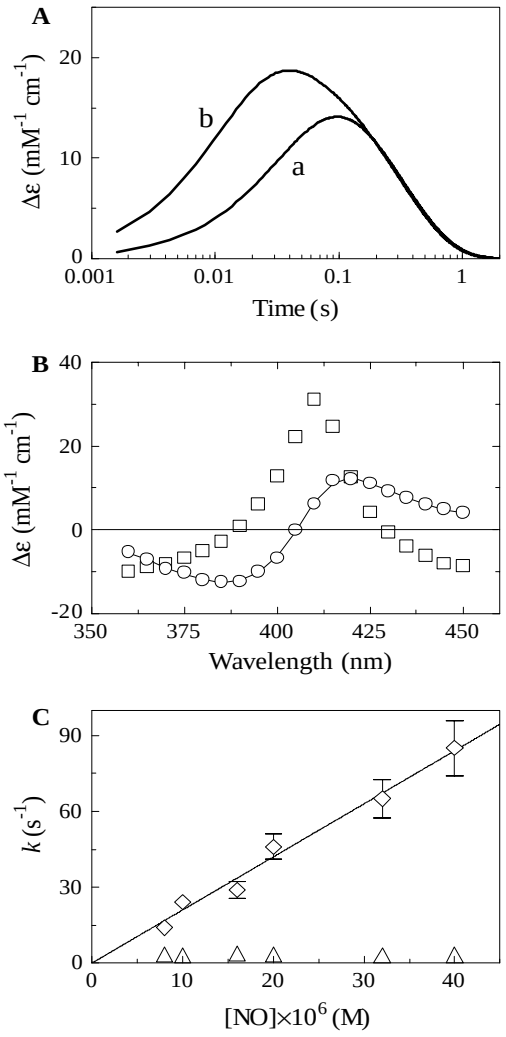

Fig. 1. NO scavenging by Fe(II)O_2 *M. leprae* GlbO. (A) Time course of the NO-induced conversion of Fe(II)O_2 to Fe(III) by means of transient Fe(III)OONO formation, $\lambda = 405$ nm. The NO concentration was 1.0×10^{-5} M (trace a) and 4.0×10^{-5} M (trace b). The Fe(II)O_2 *M. leprae* GlbO concentration was 1.6×10^{-6} M. The analysis of the time course according to Eqs. (1)–(3) [32] allowed us to determine the following parameters: trace a, $k = 24\ s^{-1}$ and $h = 3.2\ s^{-1}$; trace b, $k = 85\ s^{-1}$ and $h = 3.3\ s^{-1}$. Traces a and b are the average of six experiments. (B) Static and kinetic difference absorption spectra (line and symbols, respectively) in the Soret region of Fe(II)O_2 *minus* Fe(III) (line and circles) and Fe(III)OONO *minus* Fe(III) (squares). The kinetic difference absorption spectra in the Soret region of Fe(II)O_2 *minus* Fe(III) (circles) and Fe(III)OONO *minus* Fe(III) (squares) were reconstructed from the static difference absorption spectrum of Fe(III) *minus* Fe(III) ($\Delta\varepsilon = 0.0\ mM^{-1}\ cm^{-1}$) plus the optical density changes of the overall process Fe(II)O_2 → Fe(III) and of the partial reaction Fe(III)OONO → Fe(III) (see Scheme 1) at each wavelength measured in the rapid-mixing experiments (wavelength interval = 5 nm). Values of λ_{max} (nm) and ε ($mM^{-1}\ cm^{-1}$) of the absorption spectra in the Soret region of the Fe(II)O_2, Fe(III)OONO, and Fe(III) derivatives of *M. leprae* GlbO are shown in Table 1. (C) Dependence of the pseudo first order rate constant for the NO-induced conversion of Fe(II)O_2 to Fe(III)OONO (k; diamonds) and of the first order rate constant for Fe(III)OONO isomerization and dissociation (h; triangles) on the NO concentration. The continuous line was calculated according to Eq. (4) [30] with $k_{on} = 2.1 \times 10^6\ M^{-1}\ s^{-1}$, the average value of h being $3.4\ s^{-1}$ (Table 2). Values of k and h represent the average of at least four independent experiments. All data were obtained at pH 7.3 and 20.0 °C. For further details, see the text.

Table 2
Kinetic data for NO scavenging by Fe(II)O_2 heme–proteins

Heme–protein	$k_{on} \times 10^{-6}$ ($M^{-1}\ s^{-1}$)	h (s^{-1})
M. leprae GlbO[a]	2.1	3.4
M. tuberculosis GlbO[b]	0.6	n.d.
M. tuberculosis GlbN[c]	745	n.d.
E. coli flavoHb[d]	⩾600	∼200
Horse heart Mb[e]	44	>340
Murine neuroglobin[f]	>70	≈300
Human Hb	89[g]	>58 [h] > 33[h]

n.d., not determined.
[a] pH 7.3 and 20.0 °C. Present study.
[b] pH 7.5 and 23.0 °C. From [21].
[c] pH 7.5 and 23.0 °C. From [30].
[d] pH 7.0 and 20.0 °C. From [28].
[e] pH 7.0 and 20.0 °C. From [29].
[f] pH 7.0 and 5.0 °C and 20.0 °C. From [31].
[g] pH 7.0 and 20.0 °C. From [29].
[h] The two values represent the decay rates for the α- and β-Hb Fe(III)OONO subunits. From [37].

those reported for NO detoxification by horse heart Mb [29], murine neuroglobin [31], human Hb [29], and *E. coli* flavoHb [28] (Table 2). Remarkably, values of the first order rate constant for O_2 dissociation from mycobacterial GlbOs [21,23] are orders of magnitude lower than those reported for horse heart Mb [25,51], human and murine neuroglobin [52], human Hb [25,51], *M. tuberculosis* GlbN [19,21], and *E. coli* flavoHb [53]. The high stability of heme–Fe-bound ligand in mycobacterial GlbOs may reflect the tight hydrogen bonding to TyrB10 and TrpG8 [16,23,24,50]. By contrast the heme-bound ligands are loosely stabilized by TyrB10 and GlnE11 in *M. tuberculosis* GlbN [48,49] and by HisE7 in horse heart Mb, human and murine neuroglobin, human Hb, and *E. coli* flavoHb [52,54–58].

Titration of Fe(II)O_2 *M. leprae* GlbO with NO resulted in the stoichiometric oxidation of the hemoprotein. As reported in Table 3, the oxidation of Fe(II)O_2 *M. leprae* GlbO mirrors the formation of NO_3^- in the exact molar equivalence, indicating that NO consumption is dependent on Fe(II)O_2 activity rather than on nonspecific reaction. No appreciable NO_2^- production was detected (Table 3). Note that NO_2^- is the main reaction product of the non enzymatic NO oxidation by O_2 [59].

The protection from the nitrosative stress by Fe(II)O_2 needs Fe(III) to be reduced back to Fe(II)O_2 [17,18,30,34,35,38–41,43–47]. Because *M. leprae* GlbO does not contain either a covalently linked flavoreductase domain, otherwise present in flavoHbs [40], or a metHb- and metMb-reductase, oxidation of NO to NO_3^- by Fe(II)O_2 would suggest the existence in *M. leprae* of a reductase partner(s) required for catalytic cycling of Fe(III) to Fe(II)O_2. Searches for functional oxidoreductase genes in *M. leprae* genome identified the products of *trx* (thioredoxin, ML1818), *trxB* (bifunctional thioredoxin reductase/thioredoxin; ML2703), *fdxA* (ferredoxin; ML1489), *fprA* (putative NADPH-ferredoxin reductase; ML0666), and

Table 3
Stoichiometric formation of Fe(III), NO_3^-, and NO_2^- in the reaction of NO with Fe(II)O_2 *M. leprae* GlbO[a]

Reactants		Products		
[NO] × 10^6 (M)	[Fe(II)O_2] × 10^6 (M)	[Fe(III)] × 10^6 (M)	[NO_3^-] × 10^6 (M)	[NO_2^-] × 10^6 (M)
0	14.6	<0.5	<0.5	<0.5
4.0	10.7	3.9	4.0	<0.5
8.0	6.6	8.1	7.9	<0.5
12.1	2.5	12.0	11.9	<0.5
16.1	<0.5	14.5	14.7	<0.5

[a] pH 7.3 and 20.0 °C.

fprB (ferredoxin/ferredoxin NADP-reductase; ML2134) as putative candidates for such function [15].

Because $OONO^-$ is one of the strongest oxidants in biological systems [11,59,60], the NO oxidation process catalyzed by Fe(II)O_2 GlbO might be harmful to leprosy bacilli if the toxic $OONO^-$ intermediate were released during reaction. The release of $ONOO^-$ from Fe(III)$ONOO^-$ *M. leprae* GlbO is unlikely based on the observed reaction stoichiometry (Table 3) and on the hydrogen bonding network defining the heme cavity of mycobacterial GlbO's that prevents the escape of heme-bound ligands [21,23]. Lastly, it should be pointed out that *M. leprae* has retained functional copies of both *sodC*, encoding superoxide dismutase reductase, and of the alkylhydroperoxide reductase *ahpC* gene with the linked *oxyR* gene, which regulates the peroxide stress response in bacteria [11,61]. SodC prevents $OONO^-$ generation by macrophages through dismutation of the superoxide radical ($O_2^{\bullet -}$) [62], while AhpC has been shown to reduce hydroxyperoxide radicals, while displaying also a peroxynitritase activity [63]. The conservation of both these enzymes in *M. leprae* would provide a defense frontline against $OONO^-$ generated by activated macrophages or released accidentally from Fe(III)OONO GlbO.

In conclusion, *M. leprae* GlbO appears to be tailored to provide functional versatility, enabling both NO detoxification and aerobic respiration [20–22,24,50]. This observation would be in keeping with the conservation of only one trHb in *M. leprae*, consistent with the "just enough" evolutionary strategy characteristic of this mysterious intracellular bacterium.

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