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H_2O_2 and $^{\cdot}NO$ scavenging by *Mycobacterium leprae* truncated hemoglobin O

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ABSTRACT

Kinetics of ferric *Mycobacterium leprae* truncated hemoglobin O (trHbO—Fe(III)) oxidation by H_2O_2 and of trHbO—Fe(IV)=O reduction by $^{\cdot}NO$ and NO_2^- are reported. The value of the second-order rate constant for H_2O_2-mediated oxidation of trHbO—Fe(III) is 2.4×10^3 M^{-1} s^{-1}. The value of the second-order rate constant for $^{\cdot}NO$-mediated reduction of trHbO—Fe(IV)=O is 7.8×10^6 M^{-1} s^{-1}. The value of the first-order rate constant for trHbO—Fe(III)—ONO decay to the resting form trHbO—Fe(III) is 2.1×10^1 s^{-1}. The value of the second-order rate constant for NO_2^--mediated reduction of trHbO—Fe(IV)=O is 3.1×10^3 M^{-1} s^{-1}. As a whole, trHbO—Fe(IV)=O, generated upon reaction with H_2O_2, catalyzes $^{\cdot}NO$ reduction to NO_2^-. In turn, $^{\cdot}NO$ and NO_2^- act as antioxidants of trHbO—Fe(IV)=O, which could be responsible for the oxidative damage of the mycobacterium. Therefore, *Mycobacterium leprae* trHbO could be involved in both H_2O_2 and $^{\cdot}NO$ scavenging, protecting from nitrosative and oxidative stress, and sustaining mycobacterial respiration.

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During infection, *Mycobacterium leprae* is faced with the host macrophagic environment, where low pO_2, high CO_2 levels, combined with the toxic activity of reactive nitrogen and oxygen species, including nitrogen monoxide ($^{\cdot}NO$), superoxide ($O_2^{\cdot-}$), and hydrogen peroxide (H_2O_2), contribute to limit the growth of the bacilli. Remarkably, reactive nitrogen and oxygen species produced in vivo during the respiratory burst by monocytic/macrophagic cells are an important cause of host tissue toxicity (i.e., nerve damage) [1–12].

The ability of *M. leprae* to persist in vivo in the presence of reactive nitrogen and oxygen species implies the presence in this elusive mycobacterium of (pseudo-)enzymatic detoxification systems, including truncated hemoglobin O (trHbO) [8–10,13–18]. *M. leprae* trHbO has been reported to facilitate $^{\cdot}NO$ and peroxynitrite scavenging using O_2 and $^{\cdot}NO$ as cofactors [10,16–19]. As reported for some heme-proteins (e.g., hemoglobin (Hb) and myoglobin (Mb)) [20–28], *M. leprae* trHbO may undergo oxidation by H_2O_2, leading to the formation of the highly oxidizing ferryl derivative (trHbO—Fe(IV)=O), which could be responsible for the oxidative damage of the mycobacterium. Remarkably, recent studies [29–36] suggest that $^{\cdot}NO$ and nitrite (NO_2^-) can serve as antioxidants of the highly oxidizing heme-Fe(IV)=O derivative of heme-proteins.

Here, we report kinetics of *M. leprae* trHbO—Fe(III) oxidation by H_2O_2 and of $^{\cdot}NO$- and NO_2^--mediated reduction of *M. leprae* trHbO—Fe(IV)=O. As a whole, *M. leprae* trHbO—Fe(IV)=O, obtained by treatment with H_2O_2, catalyzes $^{\cdot}NO$ detoxification. In turn, $^{\cdot}NO$ and NO_2^- act as antioxidants of *M. leprae* trHbO—Fe(IV)=O. Therefore, *M. leprae* trHbO can undertake within the same cycle not only $^{\cdot}NO$ and peroxynitrite scavenging [9,10,16–19] but also H_2O_2 detoxification (present study).

Materials and methods

Mycobacterium leprae trHbO—Fe(III) was prepared as previously reported [37]. The *M. leprae* trHbO—Fe(III) concentration was determined by measuring the optical absorbance at 409 nm ($\varepsilon_{409nm} = 1.15 \times 10^5$ M^{-1} cm^{-1}) [16]. *M. leprae* trHbO—Fe(IV)=O was prepared by adding 10–25 equivalents of H_2O_2 to a buffered *M. leprae* trHbO—Fe(III) solution. After a reaction time of 10–20 min, the trHbO—Fe(IV)=O solution was stored on ice and used within 1 h [33–35]. The H_2O_2, $^{\cdot}NO$, and NO_2^- solutions were prepared as previously reported [10,16,34].

Kinetics of H_2O_2-mediated oxidation of trHbO—Fe(III) was determined by mixing the trHbO—Fe(III) (final concentration, 2.3×10^{-6} M) solution with the H_2O_2 (final concentration, 1.0×10^{-5} to 5.0×10^{-5} M) solution [23–25].

The time course of H_2O_2-mediated oxidation of trHbO—Fe(III) was fitted to a single-exponential process (Scheme 1) [23–25].

Abbreviations: Fe(III), ferric heme-protein; Fe(IV)=O, ferryl heme-protein; Fe(III)—ONO, *O*-nitrito ferric heme-protein; Hb, hemoglobin; Lb, leghemoglobin; Mb, myoglobin; TrHbO, truncated Hb O.

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Values of k were determined according to Eq. (1) [23–25]:

$$[\text{trHbO—Fe(III)}]_t = [\text{trHbO—Fe(III)}]_i \times e^{-k \times t} \quad (1)$$

The value of k_{on} was determined according to Eq. (2) [23–25]:

$$k = k_{on} \times [H_2O_2] \quad (2)$$

Kinetics for ·NO-mediated reduction of trHbO—Fe(IV)=O was determined by mixing the trHbO—Fe(IV)=O (final concentration, 1.2×10^{-6} M) solution with the ·NO (final concentration, 5.0×10^{-6} to 2.0×10^{-5} M) solution [29–35].

The time course of ·NO-mediated reduction of trHbO—Fe(IV)=O was fitted to a two-exponential process (Scheme 2) [29–35].

Values of h and l were determined according to Eqs. (3)–(5) [29–35,38]:

$$[\text{trHbO—Fe(IV)=O}]_t = [\text{trHbO—Fe(IV)=O}]_i \times e^{-h \times t} \quad (3)$$

$$[\text{trHbO—Fe(III)—ONO}]_t = [\text{trHbO—Fe(IV)=O}]_i \times (h \times ((e^{-h \times t}/(l-h)) + (e^{-l \times t}/(h-l)))) \quad (4)$$

$$[\text{trHbO—Fe(III)}]_t = [\text{trHbO—Fe(IV)=O}]_i - ([\text{trHbO—Fe(IV)=O}]_t + [\text{trHbO—Fe(III)—ONO}]_t) \quad (5)$$

The value of h_{on} was determined according to Eq. (6) [29–35]:

$$h = h_{on} \times [^{\cdot}NO] \quad (6)$$

Kinetics for NO_2^--mediated reduction of trHbO—Fe(IV)=O was determined by mixing the trHbO—Fe(IV)=O (final concentration, 2.9×10^{-6} M) solution with the NO_2^- (final concentration, 2.5×10^{-5} to 2.0×10^{-4} M) solution [33–35].

The time course of NO_2^--mediated reduction of trHbO—Fe(IV)=O was fitted to a single exponential process (Scheme 3) [33–35].

Values of b were determined according to Eq. (7) [33–35]:

$$[\text{trHbO—Fe(IV)=O}]_t = [\text{trHbO—Fe(IV)=O}]_i \times e^{-b \times t} \quad (7)$$

The value of b_{on} was determined according to Eq. (8) [33–35]:

$$b = b_{on} \times [NO_2^-] \quad (8)$$

All the experiments were obtained at pH 7.2 (5.0×10^{-2} M phosphate buffer) and 20.0 °C.

Results

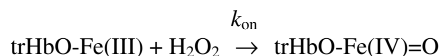
Mixing of the *M. leprae* trHbO—Fe(III) and H_2O_2 solutions is accompanied by a shift of the optical absorption maximum of the Soret band from 409 nm (i.e., trHbO—Fe(III)) [19] to 419 nm (i.e., trHbO—Fe(IV)=O) and a change of the extinction coefficient from $\varepsilon_{409nm} = 1.15 \times 10^5$ M^{-1} cm^{-1} (i.e., trHbO—Fe(III)) [19] to $\varepsilon_{419nm} = 1.06 \times 10^5$ M^{-1} cm^{-1} (i.e., trHbO—Fe(IV)=O).

Over the whole H_2O_2 concentration range explored, the time course for H_2O_2-mediated oxidation of *M. leprae* trHbO—Fe(III) corresponds to a monophasic process between 360 and 460 nm (Scheme 1). Values of k are wavelength-independent at fixed [H_2O_2]. The plot of k versus [H_2O_2] is linear; the slope corresponds to $k_{on} = 2.4 \times 10^3$ M^{-1} s^{-1} (Table 1).

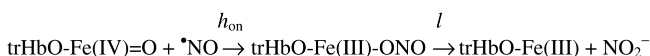
Mixing of the *M. leprae* trHbO—Fe(IV)=O and ·NO solutions brings about a shift of the optical absorption maximum of the Soret band from 419 nm (i.e., trHbO—Fe(IV)=O) to 411 nm (i.e., trHbO—Fe(III)—ONO) and a change of the extinction coefficient from $\varepsilon_{419nm} = 1.06 \times 10^5$ M^{-1} cm^{-1} (i.e., trHbO—Fe(IV)=O) to $\varepsilon_{411nm} = 1.41 \times 10^5$ M^{-1} cm^{-1} (i.e., trHbO—Fe(III)—ONO). Then, the *M. leprae* trHbO—Fe(III)—ONO solution undergoes a shift of the optical absorption maximum of the Soret band from 411 nm (i.e., trHlbO—Fe(III)—ONO) to 409 nm (i.e., trHbO—Fe(III)) [19] and a change of the extinction coefficient from $\varepsilon_{411nm} = 1.41 \times 10^5$ M^{-1} cm^{-1} (i.e., trHbO(III)—ONO) to $\varepsilon_{409nm} = 1.15 \times 10^5$ M^{-1} cm^{-1} (i.e., trHbO—Fe(III)) [19].

Over the whole ·NO concentration range explored, the time course for ·NO-mediated reduction of *M. leprae* trHbO—Fe(IV)=O corresponds to a biphasic process between 360 and 460 nm. The first step (indicated by h_{on} in Scheme 2) is a bimolecular process, while the second step (indicated by l in Scheme 2) is a monomolecular process.

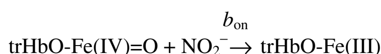
Values of h are wavelength-independent at fixed [·NO]. The plot of h versus [·NO] is linear; the slope corresponds to $h_{on} = 7.8 \times 10^6$ M^{-1} s^{-1} (Table 2). In contrast, values of l are wavelength- and [·NO]-independent; the average value of l is $(2.1 \pm 0.2) \times 10^1$ s^{-1} (Table 2).



Scheme 1.



Scheme 2.



Scheme 3.

Table 1
Values of kinetic parameters for H_2O_2-mediated oxidation of heme-Fe(III)

Heme-protein	k_{on} (M^{-1} s^{-1})
Mycobacterium leprae trHbO[a]	2.4×10^3
Horse heart Mb[b]	5.4×10^2
Sperm-whale Mb[c]	6.6×10^2
Human Mb[d]	3.4×10^4
Horseradish peroxidase[e]	1.7×10^7
Human myeloperoxidase[f]	1.9×10^7
Human eosinophil peroxidase[g]	4.3×10^7
Bovine lactoperoxidase[h]	1.1×10^7
Catalase[i]	1.7×10^7

[a] pH 7.2 and 20.0 °C. Present study.
[b] pH 6.0 and 25.0 °C. From [24].
[c] pH 7.0 and 37.0 °C. From [25].
[d] pH 7.3 and 37.0 °C. From [23].
[e] pH 7.0 and 25.0 °C. From [21].
[f] pH 7.0 and 15.0 °C. From [27].
[g] pH 7.0 and 15.0 °C. From [26].
[h] pH 7.0 and 15.0 °C. From [28].
[i] pH 7.0 and 20.0 °C. From [20].

Table 2
Values of kinetic parameters for ·NO-mediated reduction of heme-Fe(IV)=O

Heme-protein	h_{on} (M^{-1} s^{-1})	l (s^{-1})
Mycobacterium leprae trHbO[a]	7.8×10^6	2.1×10^1
Glycine max Lb[b]	1.8×10^6	$> 5.0 \times 10^1$
Horse heart Mb[c]	1.7×10^7	6.0
Human Hb[d]	2.4×10^7	4.8×10^{-1} 1.2×10^{-1}
Horseradish peroxidase[e]	1.0×10^6	Fast
Human myeloperoxidase[f]	8.0×10^3	Fast
Porcine eosinophyl peroxidase[g]	1.7×10^4	Fast
Bovine lactoperoxidase[g]	8.7×10^4	Fast

[a] pH 7.2 and 20.0 °C. Present study.
[b] pH 7.0 and 20.0 °C. From [35].
[c] pH 7.0 and 20.0 °C. From [33].
[d] pH 7.0 and 20.0 °C. Biphasic kinetics of heme-Fe(III)—ONO decay has been attributed to α- and β-chains. From [34].
[e] pH 7.4 and 20.0 °C. From [29].
[f] pH 7.0 and 25.0 °C. From [30].
[g] pH 7.0 and 25.0 °C. From [32].

Table 3
Values of kinetic parameters for NO_2^--mediated reduction of heme-Fe(IV)=O

Heme-protein	b_{on} ($M^{-1}\ s^{-1}$)
Mycobacterium leprae trHbO[a]	3.1×10^3
Glycine max Lb[b]	2.1×10^2
Horse heart Mb[c]	1.6×10^1
Human Hb[d]	7.5×10^2
Human myeloperoxidase[e]	5.5×10^2

[a] pH 7.2 and 20.0 °C. Present study.
[b] pH 7.0 and 20.0 °C. From [35].
[c] pH 7.5 and 20.0 °C. From [33].
[d] pH 7.0 and 20.0 °C. From [34].
[e] pH 7.0 and 15.0 °C. From [31].

Mixing of the *M. leprae* trHbO—Fe(IV)=O and NO_2^- solutions shows a shift of the optical absorption maximum of the Soret band from 419 nm (i.e., trHbO—Fe(IV)=O) to 409 nm (i.e., trHbO—Fe(III)) [19] and a change of the extinction coefficient from $\varepsilon_{419nm} = 1.06 \times 10^5\ M^{-1}\ cm^{-1}$ (i.e., trHbO—Fe(IV)=O) to $\varepsilon_{409nm} = 1.15 \times 10^5\ M^{-1}\ cm^{-1}$ (i.e., trHbO—Fe(III)) [19].

Over the whole NO_2^- concentration range explored, the time course for NO_2^--mediated reduction of *M. leprae* trHbO—Fe(IV)=O corresponds to a monophasic process between 360 and 460 nm (Scheme 3). Values of b are wavelength-independent at fixed $[NO_2^-]$. The plot of b versus $[NO_2^-]$ is linear; the slope corresponds to $b_{on} = 3.1 \times 10^3\ M^{-1}\ s^{-1}$ (Table 3).

Discussion

Heme-proteins share the ability of detoxifying reactive nitrogen species. Under aerobic conditions, the reaction of the ferrous oxygenated derivative of heme-proteins (heme-Fe(II)—O_2) with ·NO occurs, reflecting the superoxide character of the heme-Fe(II)-bound O_2. The products of this reaction are heme-Fe(III) and NO_3^-. Under anaerobic conditions, ·NO has been reported to be converted to N_2O. ·NO scavenging is considered as a 'pseudo-enzymatic process' since it needs a reductase partner(s) to restore heme-Fe(II) and starting a new catalytic cycle [10,39–47].

Since *M. leprae* survives inside macrophages, it is supposed to avoid the deleterious effects of reactive oxygen species (e.g., H_2O_2), even though how this protection can be accomplished by *M. leprae* is a still open question. Since *M. leprae* lacks a functional catalase (*katG*) gene [48], three alternative mechanisms have been proposed to contribute to H_2O_2 resistance in *M. leprae*: (i) the reduced production of H_2O_2 by *M. leprae*-infected macrophages [49], (ii) the production of alternative (*katG*-independent) catalase

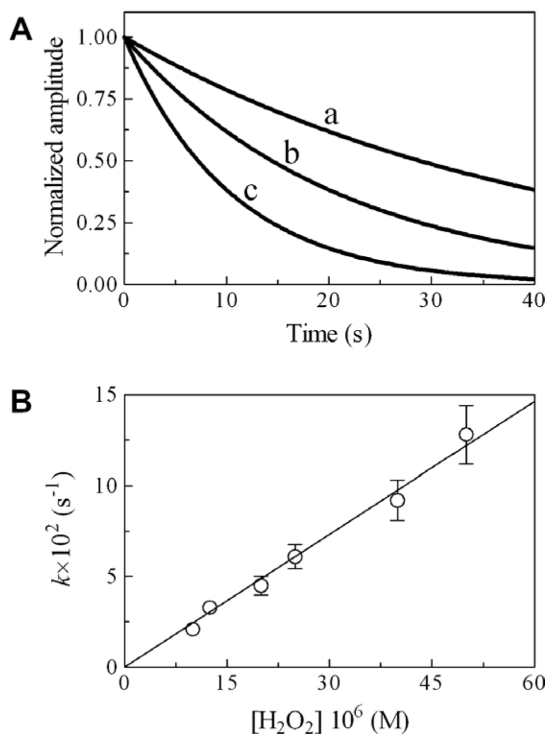


Fig. 1. Kinetics of H_2O_2-mediated oxidation of trHbO-heme-Fe(III), at pH 7.0 and 20.0 °C. (A) Normalized time courses for H_2O_2-mediated oxidation of trHbO-heme-Fe(III). The time course analysis according to Eq. (1) allowed to determine the following values of $k = 2.3 \times 10^{-2}\ s^{-1}$ (trace a), $4.9 \times 10^{-2}\ s^{-1}$ (trace b), and $9.8 \times 10^{-2}\ s^{-1}$ (trace c). Values of k were obtained at $[H_2O_2] = 1.0 \times 10^{-5}$ M (trace a), 2.0×10^{-5} M (trace b), and 4.0×10^{-5} M (trace c). (B) Dependence of k on the H_2O_2 concentration. The analysis of data according to Eq. (2) allowed to determine $k_{on} = 2.4 \times 10^3\ M^{-1}\ s^{-1}$.

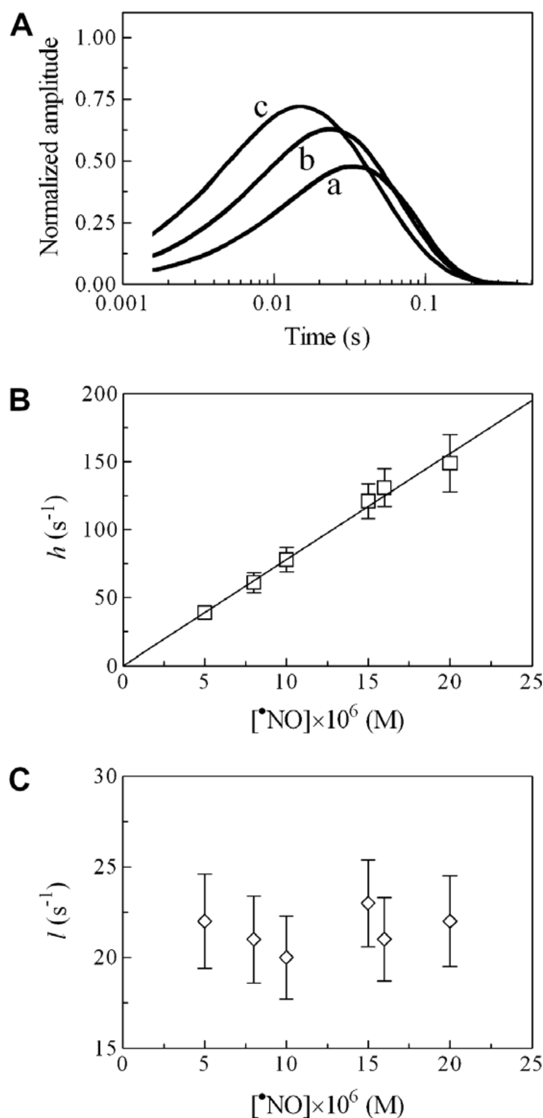


Fig. 2. Kinetics of ·NO-mediated reduction of trHbO-heme-Fe(IV)=O, at pH 7.0 and 20.0 °C. (A) Normalized time courses for ·NO-mediated reduction of trHbO-heme-Fe(IV)=O. The time course analysis according to Eq. (3)–(5) allowed to determine the following values of $h = 3.9 \times 10^1\ s^{-1}$ and $l = 2.2 \times 10^1\ s^{-1}$ (trace a), $h = 7.8 \times 10^1\ s^{-1}$ and $l = 2.0 \times 10^1\ s^{-1}$ (trace b), and $h = 1.5 \times 10^2\ s^{-1}$ and $l = 2.2 \times 10^1\ s^{-1}$ (trace c). Values of h and l were obtained at [·NO] = 5.0×10^{-6} M (trace a), 1.0×10^{-5} M (trace b), and 2.0×10^{-5} M (trace c). (B) Dependence of h on the ·NO concentration. The analysis of data according to Eq. (6) allowed to determine $h_{on} = 7.8 \times 10^6\ M^{-1}\ s^1$. (C) Dependence of l on the ·NO concentration. Values of l are independent of [·NO], the average l value is $(2.1 \pm 0.2) \times 10^1\ s^{-1}$.

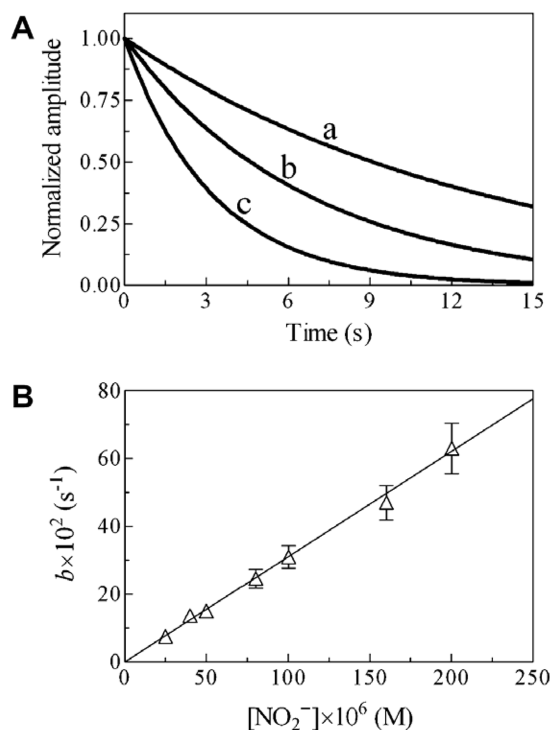


Fig. 3. Kinetics of NO_2^--mediated reduction of trHbO-heme-Fe(IV)=O, at pH 7.0 and 20.0 °C. (A) Normalized time courses for NO_2^--mediated reduction of trHbO-heme-Fe(IV)=O. The time course analysis according to Eq. (7) allowed to determine the following values of $b = 7.6 \times 10^{-2}$ s^{-1} (trace a), 1.5×10^{-1} s^{-1} (trace b), and 3.1×10^{-1} s^{-1} (trace c). Values of b were obtained at $[NO_2^-] = 2.5 \times 10^{-5}$ M (trace a), 5.0×10^{-5} M (trace b), and 1.0×10^{-4} M (trace c). (B) Dependence of b on the NO_2^- concentration. The analysis of data according to Eq. (8) allowed to determine $b_{on} = 3.1 \times 10^3$ M^{-1} s^{-1}.

activity [50], and (iii) the scavenging activity of cell-wall-associated glycolipids [51]. Here, we propose that under anaerobic and highly oxidative conditions, as in the macrophagic environment where *M. leprae* is faced with H_2O_2 [1–12], the rapid formation of *M. leprae* trHbO—Fe(IV)=O occurs, which in turn facilitates ·NO scavenging, leading to the formation of heme-Fe(III) and NO_2^-. Moreover, we suggest that ·NO acts as an antioxidant of the heme-Fe(IV)=O group generated upon reaction of trHbO with H_2O_2 ([33–35] and present study). This reaction does not require partner oxido-reductive enzymes, since the heme-protein oscillates between the heme-Fe(III) and heme-Fe(IV)=O form, being helped by ·NO in keeping efficient the rate of H_2O_2 reduction. In this framework, it becomes comprehensible why *M. leprae* trHbO—Fe(III) does not require a reductase system(s), which indeed has not yet been identified in this elusive mycobacterium [9,10]. In other words, *M. leprae* heme-Fe(III) oxidation to heme-Fe(IV)=O is mediated by H_2O_2 (Fig. 1 and Table 1), while heme-Fe(IV)=O reduction to heme-Fe(III) is facilitated by ·NO (Fig. 2 and Table 2) ([33–35] and present study). Interestingly, catalytic parameters for ·NO scavenging by heme-Fe(II)—O_2 [10] and heme-Fe(IV)=O (Table 2) are similar and high enough to indicate that both reactions could take place in vivo.

Moreover, the reaction of heme-Fe(IV)=O with NO_2^- (Fig. 3), although being significantly slower than that with ·NO (Tables 2 and 3), may play a role when ·NO has been consumed completely, and large concentrations of NO_2^- are present. In contrast to the antioxidant role of ·NO, the reaction with NO_2^- generates $·NO_2$ which could contribute to tyrosine nitration [33]. Intriguingly, high levels of NO_2^-Tyr are detectable in mycobacterial lesions [8,12].

Heme-Fe(IV)=O peroxidases and catalase also facilitate ·NO and NO_2^- detoxification [29,30,32,36]. However, the rate constants are 1–2 orders of magnitude lower than those reported for the heme-Fe(IV)=O derivative of O_2-carriers (e.g., Hb and Mb) (Tables 2 and 3). The structural basis for this difference is not clear and it has been proposed that it might be related to the strong hydrogen bond present in peroxidases between the proximal histidyl residue and a conserved aspartate residue [34]. However, it must be pointed out that in the case of catalase this structural feature is not observed, even though a similar H-bond network has been proposed in the proximal side of the heme [52].

Moreover, the analysis of data reported on Table 2 indicates that the dissociation of the heme-Fe(III)—ONO species and *O*-nitrito isomerization is significantly faster in peroxidases than in heme-Fe(III) O_2-carriers, the rate limiting step being represented by heme-Fe(III)—ONO formation [29,30,32–35].

As a whole, *M. leprae* trHbO—Fe(IV)=O facilitates ·NO detoxification. In turn, ·NO and NO_2^- can serve as antioxidants of the highly oxidizing heme-Fe(IV)=O species. Therefore, *M. leprae* trHbO could be involved in both ·NO and H_2O_2 scavenging without needing a reductase partner(s).

Acknowledgments

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