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H_2O_2 and ·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 ·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 $2.4 \times 10^3\ M^{-1}\ s^{-1}$. The value of the second-order rate constant for ·NO-mediated reduction of trHbO—Fe(IV)=O is $7.8 \times 10^6\ M^{-1}\ s^{-1}$. The value of the first-order rate constant for trHbO—Fe(III)—ONO decay to the resting form trHbO—Fe(III) is $2.1 \times 10^1\ s^{-1}$. The value of the second-order rate constant for NO_2^--mediated reduction of trHbO—Fe(IV)=O is $3.1 \times 10^3\ M^{-1}\ s^{-1}$. As a whole, trHbO—Fe(IV)=O, generated upon reaction with H_2O_2, catalyzes ·NO reduction to NO_2^-. In turn, ·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 ·NO scavenging, protecting from nitrosative and oxidative stress, and sustaining mycobacterial respiration.

During infection, *Mycobacterium leprae* is faced with the host macrophagic environment, where low pH, low pO_2, high CO_2 levels, combined with the toxic activity of reactive nitrogen and oxygen species, including nitrogen monoxide (·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 ·NO and peroxynitrite scavenging using O_2 and ·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 ·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 ·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 ·NO detoxification. In turn, ·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 ·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, ·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).

$$\text{trHbO-Fe(III)} + H_2O_2 \xrightarrow{k_{on}} \text{trHbO-Fe(IV)=O}$$

Scheme 1.

$$\text{trHbO-Fe(IV)=O} + {}^{\bullet}NO \xrightarrow{h_{on}} \text{trHbO-Fe(III)-ONO} \xrightarrow{l} \text{trHbO-Fe(III)} + NO_2^-$$

Scheme 2.

$$\text{trHbO-Fe(IV)=O} + NO_2^- \xrightarrow{b_{on}} \text{trHbO-Fe(III)}$$

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].

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).

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].