



H₂O₂ and ·NO scavenging by *Mycobacterium leprae* truncated hemoglobin O

Paolo Ascenzi^{a,b,*}, Elisabetta De Marinis^a, Massimo Coletta^c, Paolo Visca^{a,b}

^a Department of Biology and Interdepartmental Laboratory for Electron Microscopy, University Roma Tre, Viale Guglielmo Marconi 446, I-00146 Roma, Italy

^b National Institute for Infectious Diseases I.R.C.C.S. 'Lazzaro Spallanzani', Via Portuense 292, I-00149 Roma, Italy

^c Department of Experimental Medicine and Biochemical Sciences, University of Roma 'Tor Vergata', Via Montpellier 1, I-00133 Roma, Italy

ARTICLE INFO

Article history:

Received 28 May 2008

Available online 9 June 2008

Keywords:

Mycobacterium leprae truncated hemoglobin O
H₂O₂ and ·NO scavenging
Nitrosative and oxidative stress
Kinetics

ABSTRACT

Kinetics of ferric *Mycobacterium leprae* truncated hemoglobin O (trHbO—Fe(III)) oxidation by H₂O₂ and of trHbO—Fe(IV)=O reduction by ·NO and NO₂⁻ are reported. The value of the second-order rate constant for H₂O₂-mediated oxidation of trHbO—Fe(III) is $2.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. The value of the second-order rate constant for ·NO-mediated reduction of trHbO—Fe(IV)=O is $7.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$. The value of the first-order rate constant for trHbO—Fe(III)—ONO decay to the resting form trHbO—Fe(III) is $2.1 \times 10^1 \text{ s}^{-1}$. The value of the second-order rate constant for NO₂⁻-mediated reduction of trHbO—Fe(IV)=O is $3.1 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$. As a whole, trHbO—Fe(IV)=O, generated upon reaction with H₂O₂, catalyzes ·NO reduction to NO₂⁻. In turn, ·NO and NO₂⁻ 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 H₂O₂ and ·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 pH, low pO₂, high CO₂ levels, combined with the toxic activity of reactive nitrogen and oxygen species, including nitrogen monoxide (·NO), superoxide (O₂⁻), and hydrogen peroxide (H₂O₂), 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 O₂ 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 H₂O₂, 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 (NO₂⁻) 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 H₂O₂ and of ·NO- and NO₂⁻-mediated reduction of *M. leprae* trHbO—Fe(IV)=O. As a whole, *M. leprae* trHbO—Fe(IV)=O, obtained by treatment with H₂O₂, catalyzes ·NO detoxification. In turn, ·NO and NO₂⁻ 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 H₂O₂ 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 ($\epsilon_{409\text{nm}} = 1.15 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$) [16]. *M. leprae* trHbO—Fe(IV)=O was prepared by adding 10–25 equivalents of H₂O₂ 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 H₂O₂, ·NO, and NO₂⁻ solutions were prepared as previously reported [10,16,34].

Kinetics of H₂O₂-mediated oxidation of trHbO—Fe(III) was determined by mixing the trHbO—Fe(III) (final concentration, $2.3 \times 10^{-6} \text{ M}$) solution with the H₂O₂ (final concentration, 1.0×10^{-5} to $5.0 \times 10^{-5} \text{ M}$) solution [23–25].

The time course of H₂O₂-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-nitroso ferric heme-protein; Hb, hemoglobin; Lb, leghemoglobin; Mb, myoglobin; TrHbO, truncated Hb O.

* Corresponding author. Fax: +39 06 5733 6321.

E-mail address: ascenzi@uniroma3.it (P. Ascenzi).

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]:

$$k = k_{\text{on}} \times [\text{H}_2\text{O}_2] \quad (2)$$

Kinetics for $\cdot\text{NO}$ -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 $\cdot\text{NO}$ (final concentration, 5.0×10^{-6} to 2.0×10^{-5} M) solution [29–35].

The time course of $\cdot\text{NO}$ -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]:

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

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]:

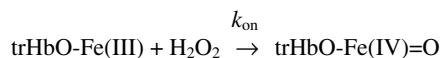
$$b = b_{\text{on}} \times [\text{NO}_2^-] \quad (8)$$

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

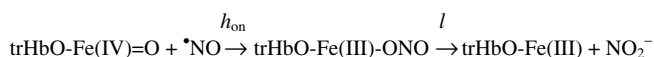
Results

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 $\epsilon_{409\text{nm}} = 1.15 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (i.e., trHbO-Fe(III)) [19] to $\epsilon_{419\text{nm}} = 1.06 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (i.e., trHbO-Fe(IV)=O).

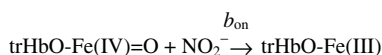
Over the whole H_2O_2 concentration range explored, the time course for H_2O_2 -mediated oxidation of *M. leprae* trHbO-Fe(III) cor-



Scheme 1.



Scheme 2.



Scheme 3.

Table 1

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

Heme-protein	$k_{\text{on}} (\text{M}^{-1} \text{s}^{-1})$
<i>Mycobacterium leprae</i> 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].

responds to a monophasic process between 360 and 460 nm (Scheme 1). Values of k are wavelength-independent at fixed $[\text{H}_2\text{O}_2]$. The plot of k versus $[\text{H}_2\text{O}_2]$ is linear; the slope corresponds to $k_{\text{on}} = 2.4 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (Table 1).

Mixing of the *M. leprae* trHbO-Fe(IV)=O and $\cdot\text{NO}$ 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 $\epsilon_{419\text{nm}} = 1.06 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (i.e., trHbO-Fe(IV)=O) to $\epsilon_{411\text{nm}} = 1.41 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (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., trHbO-Fe(III)-ONO) to 409 nm (i.e., trHbO-Fe(III)) [19] and a change of the extinction coefficient from $\epsilon_{411\text{nm}} = 1.41 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (i.e., trHbO(III)-ONO) to $\epsilon_{409\text{nm}} = 1.15 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (i.e., trHbO-Fe(III)) [19].

Over the whole $\cdot\text{NO}$ concentration range explored, the time course for $\cdot\text{NO}$ -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 $[\cdot\text{NO}]$. The plot of h versus $[\cdot\text{NO}]$ is linear; the slope corresponds to $h_{\text{on}} = 7.8 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Table 2). In contrast, values of l are wavelength- and $[\cdot\text{NO}]$ -independent; the average value of l is $(2.1 \pm 0.2) \times 10^1 \text{ s}^{-1}$ (Table 2).

Table 2

Values of kinetic parameters for $\cdot\text{NO}$ -mediated reduction of heme-Fe(IV)=O

Heme-protein	$h_{\text{on}} (\text{M}^{-1} \text{s}^{-1})$	$l (\text{s}^{-1})$
<i>Mycobacterium leprae</i> trHbO ^a	7.8×10^6	2.1×10^1
<i>Glycine max</i> 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].

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