

Peroxynitrite-mediated oxidation of ferrous carbonylated myoglobin is limited by carbon monoxide dissociation

Paolo Ascenzi^{a,b,*}, Chiara Ciaccio^{c,d}, Massimo Coletta^{c,d}

^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

^d Interuniversity Consortium for the Research on the Chemistry of Metals in Biological Systems (CIRCMSB), Piazza Umberto I 1, I-87100 Bari, Italy

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Abstract

Peroxynitrite-mediated oxidation of ferrous nitrosylated myoglobin (Mb(II)–NO) involves the transient ferric nitrosylated species (Mb(III)–NO), followed by ·NO dissociation and formation of ferric myoglobin (Mb(III)). In contrast, peroxynitrite-mediated oxidation of ferrous oxygenated myoglobin (Mb(II)–O₂) involves the transient ferrous deoxygenated and ferryl derivatives (Mb(II) and Mb(IV)=O, respectively), followed by Mb(III) formation. Here, kinetics of peroxynitrite-mediated oxidation of ferrous carbonylated horse heart myoglobin (Mb(II)–CO) is reported. Values of the first-order rate constant for peroxynitrite-mediated oxidation of Mb(II)–CO (*i.e.*, for Mb(III) formation) and of the first-order rate constant for CO dissociation from Mb(II)–CO (*i.e.*, for Mb(II) formation) are $h = (1.2 \pm 0.2) \times 10^{-2} \text{ s}^{-1}$ and $k_{\text{off}(\text{CO})} = (1.4 \pm 0.2) \times 10^{-2} \text{ s}^{-1}$, respectively, at pH 7.2 and 20.0 °C. The coincidence of values of h and $k_{\text{off}(\text{CO})}$ indicates that CO dissociation represents the rate limiting step of peroxynitrite-mediated oxidation of Mb(II)–CO.

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Carbon monoxide (CO), dioxygen (O₂), and nitrogen monoxide (·NO) are pivotal for life, being (co)substrates, products, and/or modulators in many enzymatic processes such as oxidative respiration, denitrification, carbon and nitrogen fixation, and methanogenesis (see [1–4]).

CO, O₂, and ·NO bind to metal centers, *e.g.* to the sixth coordination position of ferrous heme-proteins (heme-Fe(II)-proteins). ·NO also reacts with ferric Fe centers (*e.g.*, the ferric heme-Fe-atom (Fe(III)) (see [5–12]). Note that NO and CO induce heme-Fe(III)-atom reduction (see [13–18]); in contrast, O₂ facilitates heme-Fe(II)-atom

oxidation (see [5]). O₂ and ·NO derivatives react also with amino acid residues (*e.g.*, Cys, Met, Trp, and Tyr) modulating protein metabolism and functions (see [19–21]).

The reaction of CO with heme-Fe(II)-proteins has received great attention due to its high toxicity (see [22]); recently, CO has been reported to act also as a second messenger (see [23,24]). Furthermore, CO represents an invaluable model system for investigating diatomic ligand binding to metal centers (*i.e.*, the heme) (see [5,6,8,9,25]). Values of the equilibrium constant for CO binding to heme-Fe(II)-proteins range between 10⁷ and 10¹⁰ M⁻¹. Values of the second-order rate constant for heme-Fe(II)-protein carbonylation range between 10⁴ and 10⁷ M⁻¹ s⁻¹. Values of the first-order dissociation rate constant for heme-Fe(II)-protein decarbonylation range between 10⁻² and 10⁻⁴ s⁻¹ (see [5,6,8,9,25–28]). CO binding to heme-Fe(II)-proteins is mainly modulated by heme proximal effects. In particular, the very high carbonylation rate observed

Abbreviation: Mb, myoglobin.

* Corresponding author. Address: Department of Biology and Interdepartmental Laboratory for Electron Microscopy, University 'Roma Tre', Viale Guglielmo Marconi 446, I-00146 Roma, Italy. Fax: +39 06 5517 6321.

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

in some penta-coordinate heme-proteins has been attributed to the close location of the Fe(II)-atom with respect to the heme plane. This can be also achieved by cleavage of the HisF8-Fe heme proximal bond in slow carbonylating penta-coordinate heme-proteins since it leads to fast reacting tetra-coordinated heme-protein systems (see [29–34]).

Values of the second-order rate constant for CO binding to heme-Fe(II)-proteins (*i.e.*, $k_{\text{on(CO)}}$) are easy to determine by the dependence of the observed binding rate constant on the CO concentration. On the other hand, values of the first-order rate constant for CO dissociation from heme-Fe(II)-CO complexes (*i.e.*, $k_{\text{off(CO)}} = k_{\text{obs(CO)}} - k_{\text{on(CO)}} \times [\text{CO}]$) are very difficult to be determined by extrapolating to $[\text{CO}] = 0$ the dependence of the observed binding rate constant (*i.e.*, $k_{\text{obs(CO)}}$) on the CO concentration because of the very low value of $k_{\text{off(CO)}}$ and the large difference with respect to $k_{\text{obs(CO)}}$. Therefore, appropriate methods to determine the first-order rate constant for CO dissociation from heme-Fe(II)-CO species can be through experiments in which either (i) the heme-Fe(II)-CO complex is oxidized by ferricyanide or (ii) CO is replaced by NO, in both cases under the assumption that experimental conditions satisfy the requirement that the rate limiting step is represented by CO dissociation from the heme-Fe(II)-CO adduct. Furthermore, the high affinity of CO renders hard to determine the equilibrium constant for heme-Fe(II)-protein carbonylation ($K_{\text{(CO)}} = k_{\text{on(CO)}}/k_{\text{off(CO)}}$) (see [5,25,35,36]).

The redox potential of peroxynitrite (see [37]) suggests that this chemical is able to oxidize carbonylated heme-Fe(II)-proteins, as already reported for heme-Fe(II)-O₂ and heme-Fe(II)-NO complexes (see [18,38,39]). However, while peroxynitrite-mediated oxidation of heme-Fe(II)-O₂ complexes requires O₂ dissociation, followed by a rapid oxidation of the transient heme-Fe(II) derivative (see [18,38]), peroxynitrite-mediated oxidation of heme-Fe(II)-NO complexes involves the transient heme-Fe(III)-NO species, followed by ·NO dissociation and formation of the heme-Fe(III) derivative (see [18,39]).

Here, kinetics of peroxynitrite-mediated oxidation of horse heart Mb(II)-CO is reported for the first time. The coincidence of values of the first-order rate constant for peroxynitrite-induced oxidation of Mb(II)-CO (*i.e.*, h) and of the first-order rate constant for Mb(II)-CO decarbonylation (*i.e.*, $k_{\text{off(CO)}}$) indicates that CO dissociation represents the rate limiting step of peroxynitrite-mediated oxidation of Mb(II)-CO.

Materials and methods

Ferric horse heart Mb (purified by crystallization; Mb(III)) was obtained from Sigma–Aldrich (St. Louis, MO, USA). The Mb(II)-CO (final concentration 3.2×10^{-6} M) solution was prepared as follows. Mb(II) was obtained by reducing Mb(III) with sodium dithionite. The excess of dithionite and by-products were removed by passing the protein solution through a Sephadex G-10 gel filtration column (Amersham Biosciences Europe GmbH, Freiburg, Germany) equilibrated in air with 5.0×10^{-2} M phosphate buffer, pH 7.2, at 20.0 °C [5]. Then, the Mb(II)-

O₂ solution was gently degassed and the CO (final concentration, 5.0×10^{-4} M) solution was added, under anaerobic conditions [5,25].

CO was purchased from Linde AG (Höllriegelskreuth, Germany). ·NO (from Aldrich Chemical Co., Milwaukee, WI, USA) was purified by flowing through an NaOH column in order to remove acidic nitrogen oxides. The CO and ·NO solutions were prepared by keeping in a closed vessel the 5.0×10^{-2} M phosphate buffer solution (pH = 7.2) under CO or ·NO at $P = 760.0$ mm Hg anaerobically ($T = 20.0$ °C). The solubility of CO and ·NO in the aqueous buffered solution is 1.03×10^{-3} and 2.05×10^{-3} M, respectively, at $P = 760.0$ mm Hg and $T = 20.0$ °C [5].

Peroxyntirite was prepared from either KO₂ and ·NO or from HNO₂ and H₂O₂ [40,41]. Decomposed peroxyntirite was obtained by acidification of the peroxyntirite solution [37]. The solutions of the experiments in the presence of CO₂ were prepared by adding the required amount of a 5.0×10^{-1} M NaHCO₃ solution [42].

All the other products (from Merck AG, Darmstadt, Germany, or Sigma–Aldrich, St. Louis, MO, USA) were of analytical grade and used without purification unless stated.

The value of the first-order rate constant for peroxyntirite-induced conversion of Mb(II)-CO to Mb(III) (*i.e.*, h) was determined by mixing the Mb(II)-CO (final concentration, 3.2×10^{-6} M) solution with the peroxyntirite (final concentration, 1.0×10^{-4} to 5.0×10^{-4} M) solution under anaerobic conditions, at pH = 7.2 (5.0×10^{-2} M phosphate buffer) and $T = 20.0$ °C; no gaseous phase was present (see [18]). Kinetics was monitored between 360 and 460 nm.

The time course of peroxyntirite-induced conversion of Mb(II)-CO to Mb(III) was fitted to a single exponential process according to the minimum reaction mechanism represented by Scheme 1 (Table 1).

The value of h has been determined from data analysis, according to Eq. (1) [5]:

$$[\text{Fe(II)-CO}]_t = [\text{Fe(II)-CO}]_i \times e^{-h \times t} \quad (1)$$

The value of the first-order rate constant for CO dissociation from Mb(II)-CO (*i.e.*, for CO replacement by ·NO; $k_{\text{off(CO)}}$) was determined by mixing the Mb(II)-CO (final concentration, 3.2×10^{-6} M) solution with the ·NO-dithionite (final concentration, 1.0×10^{-4} to 5.0×10^{-4} , and 2.0×10^{-5} M, respectively) solution under anaerobic conditions, at pH = 7.2 (5.0×10^{-2} M phosphate buffer), and $T = 20.0$ °C; no gaseous phase was present (see [25]). Kinetics was monitored between 360 and 460 nm.

The time course of CO dissociation from Mb(II)-CO (*i.e.*, of CO replacement by ·NO) was fitted to a single exponential process according to the minimum reaction mechanism represented by Scheme 2 (Table 1) [25].

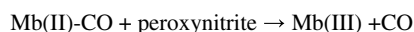
The value of $k_{\text{off(CO)}}$ has been determined from data analysis, according to Eq. (2) [5]:

$$[\text{Fe(II)-CO}]_t = [\text{Fe(II)-CO}]_i \times e^{-k_{\text{off(CO)}} \times t} \quad (2)$$

The difference optical absorption spectra in the Soret region of Mb(II)-CO minus Mb(III) and of Mb(II)-CO minus Mb(II)-NO were obtained under steady-state conditions by subtracting the absorbance of Mb(III) from that of Mb(II)-CO and of Mb(II)-NO from that of Mb(II)-CO, respectively.

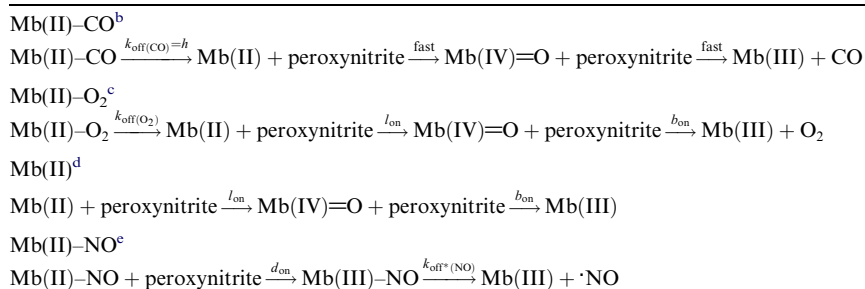
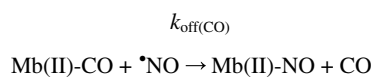
The kinetic difference optical absorption spectra in the Soret region of Mb(II)-CO minus Mb(III) and of Mb(II)-CO minus Mb(II)-NO were reconstructed from the difference optical absorption spectrum of Mb(III) minus Mb(III) and of Mb(II)-NO minus Mb(II)-NO ($\Delta\epsilon = 0.0 \text{ M}^{-1} \text{ cm}^{-1}$) obtained under steady-state conditions plus the absorbance changes of the processes Mb(II)-CO + peroxyntirite → Mb(III) + CO (see Scheme 1) and Mb(II)-CO + ·NO → Mb(II)-NO + CO (see Scheme 2), respectively.

h



Scheme 1.

Table 1

Minimum reaction mechanisms for peroxynitrite-mediated oxidation of horse heart Mb(II) derivatives^a^a For details, see text.^b Present study.^c From [38].^d From [38].^e From [39].

Scheme 2.

The results are given as mean values of at least three 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

Mixing of the Mb(II)–CO and peroxynitrite solutions causes a shift of the optical absorption maximum of the Soret band from 424 nm (*i.e.*, Mb(II)–CO) to 408 nm (*i.e.*, Mb(III)) and a change of the extinction coefficient from $\epsilon_{424 \text{ nm}} = 2.07 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (*i.e.*, Mb(II)–CO) to $\epsilon_{408 \text{ nm}} = 1.88 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (*i.e.*, Mb(III)). The optical absorption spectra of Mb(II)–CO and of Mb(III) obtained here correspond to those reported in the literature (see [5]).

Over the whole peroxynitrite concentration range explored (final concentration, 1.0×10^{-4} to 5.0×10^{-4} M), the time course for the peroxynitrite-mediated oxidation of Mb(II)–CO corresponds to a mono-exponential process for more than 95% of its course (Fig. 1, panel A). Values of the pseudo-first-order rate constant for Mb(III) formation (*i.e.*, h) are wavelength- and [peroxynitrite]-independent (Fig. 1, panel B). The average value of h is $(1.2 \pm 0.2) \times 10^{-2} \text{ s}^{-1}$. These data indicate that the reaction of Mb(II)–CO with an excess of peroxynitrite leads to the oxidation of the heme-Fe(II) atom and to the generation of Mb(III) without involving transient species. As expected, decomposed peroxynitrite does not oxidize Mb(II)–CO.

Although CO₂ reacts with peroxynitrite leading to high reactive and selective oxidant species CO₃^{•-} and [•]NO₂ (see [20,37]), CO₂ (whose concentration corresponds to that of the buffered 5.0×10^{-1} M NaHCO₃ solution) does not affect kinetics of peroxynitrite-mediated oxidation of Mb(II)–CO, under all the experimental conditions (data not shown).

Mixing of the Mb(II)–CO and [•]NO-dithionite solutions causes a shift of the optical absorption maximum of the Soret band from 424 nm (*i.e.*, Mb(II)–CO) to 416 nm (*i.e.*, Mb(II)–NO) and a change of the extinction coefficient from $\epsilon_{424 \text{ nm}} = 2.07 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (*i.e.*, Mb(II)–CO) to $\epsilon_{416 \text{ nm}} = 1.32 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ (*i.e.*, Mb(II)–NO). The optical absorption spectrum of Mb(II)–CO obtained here corresponds to that reported in the literature (see [5]).

Over the whole [•]NO concentration range explored (final concentration, 1.0×10^{-4} to 5.0×10^{-4} M), the time course for the replacement of CO by [•]NO (*i.e.*, for the formation of Mb(II)–NO) corresponds to a mono-exponential process for more than 95% of its course (Fig. 1, panel C). Values of the first-order rate constant for Mb(II)–CO decarbonylation (*i.e.*, for Mb(II)–NO formation; $k_{\text{off(CO)}}$) are wavelength- and [[•]NO]-independent (Fig. 1, panel D). The average value of $k_{\text{off(CO)}}$ is $(1.4 \pm 0.2) \times 10^{-2} \text{ s}^{-1}$. The $k_{\text{off(CO)}}$ value here reported is in excellent agreement with that reported in the literature [5]. These data indicate that the reaction of Mb(II)–CO with an excess of [•]NO-dithionite leads to the formation of Mb(II)–NO without involving transient species, as previously reported (see [25]).

The coincidence of values of h and $k_{\text{off(CO)}}$ (Fig. 1 and Table 2) suggests that peroxynitrite-mediated oxidation of Mb(II)–CO occurs with a reaction mechanism in which CO dissociation represents the rate limiting step; therefore, oxidation by peroxynitrite takes place with unliganded Mb(II). Accordingly, CO₂, facilitating peroxynitrite action (see [20,37]), does not affect Mb(II)–CO oxidation. This mechanism is reminiscent of that reported for ferricyanide-induced oxidation of Mb(II)–CO (see [36]).

The mechanism postulated for peroxynitrite-induced oxidation of Mb(II)–CO holds also in the case of Mb(II)–O₂ and Mb(II), in all cases peroxynitrite appears to react with the Mb(II) species (see Table 1). Therefore, the peroxynitrite-mediated oxidation of Mb(II)–CO and Mb(II)–O₂ occurs with a reaction mechanism in which CO or O₂, that is initially bound to the heme-Fe(II)-atom, dissociates from the carbonylated or oxygenated heme-

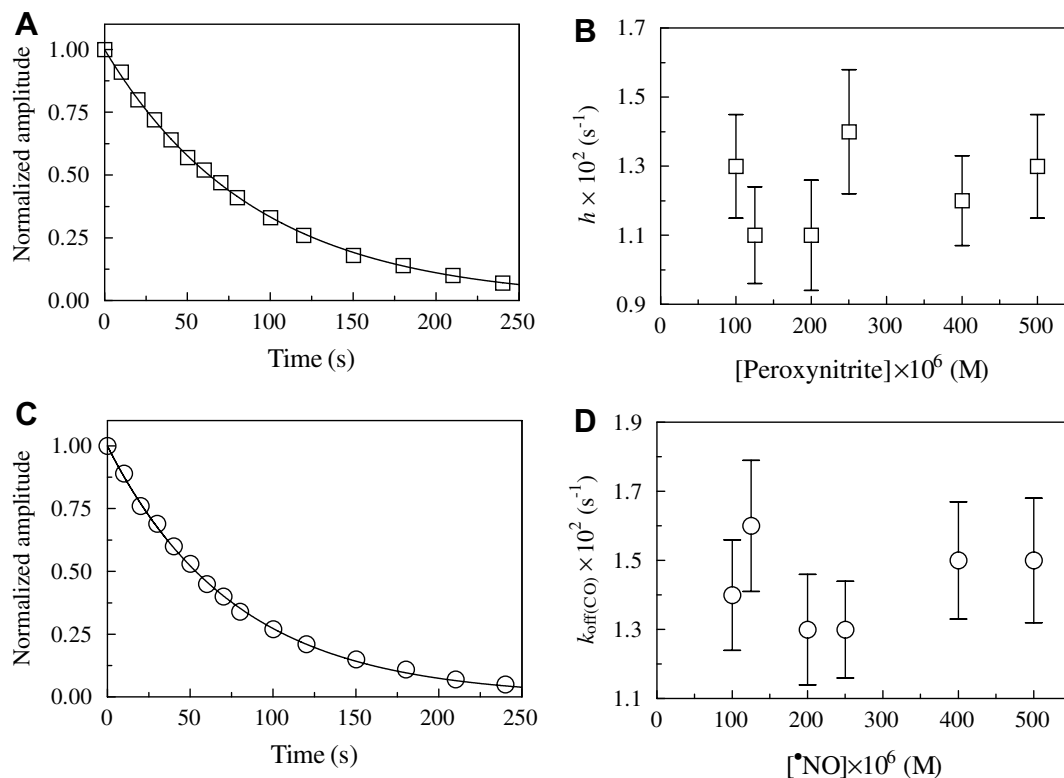


Fig. 1. Kinetics of peroxynitrite-mediated oxidation of Mb(II)–CO and of Mb(II)–CO decarbonylation, at pH 7.2 and 20.0 °C. (A) Normalized averaged time course of the peroxynitrite-mediated conversion of Mb(II)–CO to Mb(III). The time course analysis according to Eq. (1) allowed to determine the following value of $h = 1.1 \times 10^{-2} \text{ s}^{-1}$. The peroxynitrite concentration was $2.0 \times 10^{-4} \text{ M}$. (B) Dependence of h for peroxynitrite-induced conversion of Mb(II)–CO to Mb(III) on peroxynitrite concentration. The average value of h is $(1.2 \pm 0.2) \times 10^{-2} \text{ s}^{-1}$. (C) Normalized averaged time course of Mb(II)–CO decarbonylation. The time course analysis according to Eq. (2) allowed to determine the following value of $k_{\text{off}(\text{CO})} = 1.3 \times 10^{-2} \text{ s}^{-1}$. The $\cdot\text{NO}$ and dithionite concentrations were 2.0×10^{-4} and $2.0 \times 10^{-5} \text{ M}$, respectively. (D) Dependence of $k_{\text{off}(\text{CO})}$ for Mb(II)–CO decarbonylation on $\cdot\text{NO}$ concentration. The average value of $k_{\text{off}(\text{CO})}$ is $(1.4 \pm 0.2) \times 10^{-2} \text{ s}^{-1}$. The Mb(II)–CO concentration was $3.2 \times 10^{-6} \text{ M}$.

Table 2
Kinetic parameters for peroxynitrite-mediated oxidation of horse heart Mb(II) derivatives^a

Heme-protein	Kinetic parameters		
Mb(II)–CO ^b	$k_{\text{off}(\text{CO})} = 1.4 \times 10^{-2} \text{ s}^{-1}$	$h = 1.2 \times 10^{-2} \text{ s}^{-1}$	
Mb(II)–O ₂	$k_{\text{off}(\text{O}_2)} = 1.1 \times 10^1 \text{ s}^{-1}$ ^c	$l_{\text{on}} = 5.4 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ^d	$b_{\text{on}} = 2.2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ^d
Mb(II) ^e	$l_{\text{on}} \sim 10^6 \text{ M}^{-1} \text{ s}^{-1}$	$b_{\text{on}} = 2.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	
Mb(II)–NO ^f	$d_{\text{on}} = 3.1 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$	$k_{\text{off}^*(\text{NO})} \sim 1.2 \times 10^1 \text{ s}^{-1}$	

^a Reaction mechanisms are reported in Table 1. For details, see text.

^b pH = 7.2 and 20.0 °C. Present study.

^c pH = 7.0 and 20.0 °C. From [5].

^d pH = 7.3 and 20.0 °C. From [38].

^e pH = 7.5 and 20.0 °C. From [38].

^f pH = 7.5 and 20.0 °C. From [39].

Fe(II)-protein. In the second step, deoxygenated Mb(II) reacts with peroxynitrite by way of the transient Mb(IV)=O species that precedes the Mb(III) formation (see Tables 1 and 2) (see [18,38]).

Despite the common oxidation mechanism (see Table 1), peroxynitrite-induced oxidation of Mb(II)–CO and Mb(II)–O₂ displays different features, which are related to the relative velocity of the various steps leading to the Mb(III) species [38]. In fact, the first-order rate constant for CO dissociation from Mb(II)–CO (*i.e.*, $k_{\text{off}(\text{CO})}$)

is lower by about one-order of magnitude than kinetics reported for peroxynitrite-induced oxidation of the transient Mb(II) species [38] (Tables 1 and 2), impairing the accumulation of the intermediate Mb(II) species. On the other hand, the first-order rate constant for O₂ dissociation from Mb(II)–O₂ (*i.e.*, $k_{\text{off}(\text{O}_2)}$) [5] is higher by about one-order of magnitude than kinetics reported for peroxynitrite-induced oxidation of the transient Mb(II) species [38] (Tables 1 and 2); therefore, Mb(II) accumulates rapidly before being oxidized by peroxyni-

trite and it can be detected [38]. These data indicate that the different values of $k_{\text{off}(\text{CO})}$ and $k_{\text{off}(\text{O}_2)}$ are the crucial point from which the different rate limiting step of peroxynitrite-induced oxidation of Mb(II)–CO and Mb(II)–O₂ originates.

Peroxynitrite-mediated oxidation of Mb(II)–NO follows a completely different mechanism (Tables 1 and 2), involving the transient Mb(III)–NO species. This reflects the capability of NO to bind both heme-Fe(II)- and heme-Fe(III)-proteins albeit with different thermodynamic and kinetic parameters (see [5,8–12]). At low 'NO concentration (*i.e.*, under conditions where [NO] is sufficient to nitrosylate Mb(II) but not Mb(III)), the dissociation of the transient Mb(III)–NO species (*i.e.*, Mb(III) formation) takes place, Mb(III)–NO denitrosylation kinetics (indicated by $k_{\text{off}^*(\text{NO})}$ in Tables 1 and 2) representing the rate limiting step. At high 'NO concentration (*i.e.*, under conditions where [NO] nitrosylates both Mb(II) and Mb(III)), the dissociation of the transient Mb(III)–NO species (*i.e.*, Mb(III) formation) does not occur, Mb(III)–NO representing the final product (see [18,39]).

As a whole, data here reported represent the first quantitative analysis of peroxynitrite-mediated oxidation of Mb(II)–CO which appears to be limited by decarbonylation kinetics. Peroxynitrite-induced oxidation of Mb(II)–CO and Mb(II)–O₂ is preceded by dissociation of CO and O₂, respectively, values of the second-order rate constants for peroxynitrite-mediated oxidation of Mb(II)–O₂ and Mb(II) (*i.e.*, l_{on} and b_{on}) are similar (see Table 2). In contrast, peroxynitrite-mediated oxidation of Mb(III)–NO does not require the preliminary dissociation of 'NO. Then, Mb(III)–NO undergoes 'NO dissociation (*i.e.*, Mb(III) formation), at low [NO] only (see [18,39]).

Lastly, the different mechanisms describing peroxynitrite-induced oxidation of Mb(II)–CO, Mb(II)–O₂, and Mb(II)–NO (Table 1) (see present study and [38,39]) are important since peroxynitrite-mediated oxidation of ferrous carbonylated, oxygenated, and nitrosylated oxygen carriers may be used to determine the first-order rate constant of CO, O₂, and NO dissociation, respectively. However, some caution is demanded since: (i) ligand dissociation may be faster than heme-Fe(II)-atom oxidation (as in the case of Mb(II)–O₂) [38], and (ii) peroxynitrite may be able to directly oxidize the heme-Fe(II)-atom without requiring the dissociation of the sixth ligand (as in the case of Mb(II)–NO) [39]. As a consequence, for the measurement of the first-order dissociation rate constant of Mb(II)–CO, Mb(II)–O₂, and Mb(II)–NO it is better to monitor ligand binding competition for the heme-Fe(II)-atom (*e.g.*, CO versus NO) (see [25]).

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