The Balancing of Peroxynitrite Detoxification between Ferric Heme-Proteins and CO₂: The Case of Zebrafish Nitrobindin

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Abstract: Nitrobindins (Nbs) are all-β-barrel heme proteins and are present in prokaryotes and eukaryotes. Although their function(s) is still obscure, Nbs trap NO and inactivate peroxynitrite. Here, the kinetics of peroxynitrite scavenging by ferric Danio rerio Nb (Dr-Nb(III)) in the absence and presence of CO₂ is reported. The Dr-Nb(III)-catalyzed scavenging of peroxynitrite is facilitated by a low pH, indicating that the heme protein interacts preferentially with peroxynitrous acid, leading to the formation of nitrite (~91%) and nitrate (~9%). The physiological levels of CO₂ dramatically facilitate the spontaneous decay of peroxynitrite, overwhelming the scavenging activity of Dr-Nb(III). The effect of Dr-Nb(III) on the peroxynitrite-induced nitration of L-tyrosine was also investigated. Dr-Nb(III) inhibits the peroxynitrite-mediated nitration of free L-tyrosine, while, in the presence of CO₂, Dr-Nb(III) does not impair nitro-L-tyrosine formation. The comparative analysis of the present results with data reported in the literature indicates that, to act as efficient peroxynitrite scavengers in vivo, i.e., in the presence of physiological levels of CO₂, the ferric heme protein concentration must be higher than 10⁻⁴ M. Thus, only the circulating ferric hemoglobin levels appear to be high enough to efficiently compete with CO₂/HCO₃⁻ in peroxynitrite inactivation. The present results are of the utmost importance for tissues, like the eye retina in fish, where blood circulation is critical for adaptation to diving conditions.

Keywords: Danio rerio heme-protein; effect of CO₂; kinetics; peroxynitrite detoxification; tyrosine protection; zebrafish nitrobindin

1. Introduction

Nitrosative stress plays a key role in the etiology of human diseases, such as atherosclerosis, inflammation, cancer, and neurological diseases, being particularly relevant in the onset of retinopathies and glaucoma [1–3]. In fact, reactive nitrogen species (RNS) can cause protein, DNA, and lipid nitration, impairing their functions [4–11]. One of the most potent biological nitrosative agents is peroxynitrite (ONOO⁻), which is produced when nitric oxide (NO) and superoxide (O₂⁻) are combined at extremely rapid rates [4,5,9–13]. ONOO⁻ diffuses through membrane anion channels as well as the conjugated peroxynitrous acid ONOOH (pKₐ = 6.9) [13]. ONOO⁻ is relatively stable, while ONOOH decays rapidly, with an apparent half-life of 1-2 s at physiological pH, yielding ~70% nitrate (NO₃⁻) and H⁺, and ~30% nitrogen dioxide (NO₂) and hydroxyl (•OH) radicals via the homolysis of the O–O bond. The secondary reactions of NO₂ and •OH lead to NO₂⁻ and O₂ [4,9–13]. ONOO⁻ reacts with biomolecules mainly by a direct reaction or immediately after ONOOH is homolyzed to NO₂ and •OH [4,9–13].

At the end of the last century, carbon dioxide (CO₂) was reported to react with ONOO⁻ [14,15]. Since the CO₂ concentration is relatively high in vivo (~1.2 × 10⁻³ M at physiological pH in equilibrium with ~2.4 × 10⁻² M HCO₃⁻ with a pKₐ ≈ 6.3 at 25.0 °C), most of the ONOO⁻ rapidly reacts with the CO₂/HCO₃⁻ species (depending on pH),
leading mostly to the formation of 1-carboxylato-2-nitrosodioxidane adduct (ONOOCO$_2^-$), which displays an apparent half-life of 0.5–3 ms. This compound further decays (via homolysis of the O–O bond), yielding the reactive species trioxocarbonate$^*$ (CO$_3^{2-}$) and NO$_2^*$ (3% to 35%), which then proceeds towards CO$_2$ and NO$_3^-$ (or by direct yielding of NO$_2^-$ and CO$_2$ (65% to 90%)[9,11–21]). As a whole, the complexity of the peroxynitrite inactivation mechanism can be sketched, as is shown in Scheme 1 [13,22–41], where Dr-Nb(III) represents the contribution of heme proteins.

![Scheme 1](image-url)

Scheme 1. Dr-Nb(III)-mediated isomerization of peroxynitrite in the absence and presence of CO$_2$ (representing the CO$_2$/HCO$_3^-$/CO$_3^{2-}$ system). The disappearance of the pH-dependent ONOO$^-$/ONOOh species is characterized by the absorption decrease at 302 nm. For the sake of clarity, only the final products of the peroxynitrite inactivation mechanisms (indicated by $k_0$ and $k_0^H$) are reported, while the intermediate species are described in the text.

Moreover, the reaction of ONOO$^-$ with CO$_2$/HCO$_3^-$/CO$_3^{2-}$ redirects the ONOO$^-$/ONOOh-based oxidation of aromatic and aliphatic amino acid residues, facilitating the nitration of tyrosine and tryptophan and limiting the oxidation of methionine and cysteine [5,9–15,20,21,42–45]. Remarkably, NO$_2^*$ and CO$_3^{2-}$ radicals are much stronger oxidant species than their precursors NO, O$_2^*$ and ONOO$^-$/ONOOh [9–13,20,21,46].

Tyrosine nitration through the peroxynitrite pathway (see Scheme 1) is operative in most fish, including zebrafish (Danio rerio), under stressed conditions [47,48]. Further, the effect of the enrichment of CO$_2$ in the atmosphere brings about the raising of CO$_2$ levels also in the oceans [49]. Although the increased levels of CO$_2$ create only limited behavioral effects, they may significantly affect the body response to external insults. In addition, the increased concentration of CO$_2$ in the blood is of particular relevance in fish, for which retinal circulation is of crucial importance due to the high hydrostatic pressure of the environment where they live [50,51].

Here, the kinetics of ONOO$^-$/ONOOh (hereafter peroxynitrite) inactivation by all-$\beta$-barrel ferric Danio rerio nitrobindin (Dr-Nb(III)), in the absence and presence of CO$_2$, are reported and analyzed in parallel with those of all-$\alpha$-helical globins. The present results open a question on the competition between the protecting activity of heme proteins (which inactivate the damaging reactions of peroxynitrite on L-tyrosine nitration) and the non-protecting action of CO$_2$/HCO$_3^-$, which indeed depends on the levels of ferric heme-proteins. Thus, the levels (and the scavenging activity) of most heme proteins might be too low to act as peroxynitrite scavengers in vivo; only circulating ferric hemoglobin (Hb(III)) levels appear to be high enough to efficiently compete with CO$_2$/HCO$_3^-$/CO$_3^{2-}$ in peroxynitrite inactivation.

2. Materials and Methods

2.1. Materials

Dr-Nb(III) was cloned, expressed, and purified as already reported [52]. The Dr-Nb(III) concentration was determined spectrophotometrically by measuring the absorbance
at 407 nm ($\epsilon = 1.57 \times 10^5$ M$^{-1}$ cm$^{-1}$) [32]. Apo-Dr-Nb was prepared, as already reported [33]. Peroxynitrite was obtained from Cayman Chemical (Ann Arbor, MI, USA). The concentration of peroxynitrite was determined spectrophotometrically at 302 nm ($\epsilon = 1.705 \times 10^3$ M$^{-1}$ cm$^{-1}$) [12]. L-tyrosine (obtained from Merck KGaA, Darmstadt, Germany) was dissolved in 5.0 $\times$ 10$^{-2}$ M Bis-Tris propane buffer, at pH 7.0 and 22.0 °C; the final L-tyrosine concentration was 1.0 $\times$ 10$^{-4}$ M [25,30,36,39]. All the other chemicals were purchased from Merck KGaA (Darmstadt, Germany). All chemicals were of analytical grade and were used without further purification.

2.2. Methods

In the absence and presence of CO$_2$, peroxynitrite isomerization by Dr-Nb(III) and apo-Dr-Nb was investigated at pH 5.8, 7.0, and 8.5 (5.0 $\times$ 10$^{-2}$ M Bis-Tris propane buffer) and 22.0 °C, under anaerobic conditions. In the presence of CO$_2$, peroxynitrite isomerization by Dr-Nb(III) and apo-Dr-Nb was investigated by adding NaHCO$_3$ (final concentration, 5.0 $\times$ 10$^{-1}$ M) to the Dr-Nb(III) and apo-Dr-Nb solutions; this NaHCO$_3$ concentration corresponds to different concentrations of CO$_2$, HCO$_3^-$, and CO$_3^{2-}$ depending on pH. After the addition of NaHCO$_3$, the Dr-Nb(III) and apo-Dr-Nb solutions were allowed to equilibrate for at least 5 min; then, the pH was readjusted to the desired pH if needed [25,27,28,30,36,39].

Peroxynitrite isomerization by Dr-Nb(III) and apo-Dr-Nb was investigated via rapid mixing of the Dr-Nb(III) and apo-Dr-Nb solutions (final concentration ranging between 5.0 $\times$ 10$^{-6}$ M and 3.5 $\times$ 10$^{-5}$ M) with the peroxynitrite solution (final concentration ranging between 2.5 $\times$ 10$^{-5}$ M and 2.0 $\times$ 10$^{-4}$ M); no gaseous phase was present. Peroxynitrite isomerization was monitored at 302 nm [9,12,25–27,30,36,39] by the SFM-20/MOS-200 rapid-mixing stopped-flow apparatus (BioLogic Science Instruments, Claix, France); the light path of the observation chamber was 10 mm and the dead-time was 1.3 ms.

Values of the pseudo-first-order rate constant for peroxynitrite isomerization by Dr-Nb(III) and apo-Dr-Nb (i.e., $k_{\text{obs}}$) were determined according to Equation (1) [13,22–41,54,55]:

$$[\text{peroxynitrite}]_t = [\text{peroxynitrite}]_0 \times e^{-k_{\text{obs}} \times t} \tag{1}$$

Values of the second-order rate constant for peroxynitrite isomerization by Dr-Nb(III) (i.e., $k_{\text{on}}$), and of the first-order rate constant for the spontaneous decay of peroxynitrite (i.e., $k_0$), were obtained from the dependence of $k_{\text{obs}}$ on the Dr-Nb(III) concentration, according to Equation (2) [13,22–41,54,55]:

$$k_{\text{obs}} = k_{\text{on}} \times [\text{Dr-Nb(III)}] + k_0 \tag{2}$$

The reaction of peroxynitrite with L-tyrosine was investigated at pH 7.0 and 22.0 °C, as reported elsewhere. The relative nitro-L-tyrosine yield (%) corresponds to (yield with added Dr-Nb(III) or apo-Dr-)/yield with no Dr-Nb(III) or apo-Dr-Nb) $\times 100$ [13,25,30,34–36,39,55,56]. The percentage of NO$_3^-$ and NO$_2^-$ obtained from peroxynitrite isomerization was determined spectrophotometrically at 543 nm by using the Griess reagent and vanadium(III) chloride (VCl$_3$) to catalyze the conversion of NO$_3^-$ to NO$_2^-$, according to literature [25,30,36,39].

Kinetic data were analyzed using the GraphPad Prism program, version 5.03 (GraphPad Software, San Diego, CA, USA). The results are given as the mean values of at least four experiments plus and minus the standard deviation. Data were analyzed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA) and were expressed as mean values ± standard deviation (SD). Statistical analysis was performed using either the Student’s t-test or the one-way ANOVA comparison test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

3. Results and Discussion

In the absence and presence of CO$_2$, the absorbance at 302 nm decreases upon mixing the Dr-Nb(III), apo-Dr-Nb, and peroxynitrite solutions over the whole pH range explored.
According to the literature [13,22–41,54,55], this reflects peroxynitrite isomerization. No absorbance spectroscopic changes were observed in the Soret region.

Under all the experimental conditions, the time course of peroxynitrite isomerization in the absence and presence of Dr-Nb(III), apo-Dr-Nb, and CO₂ was fitted to a single-exponential decay for more than 93% of its course, according to Equation (1) (Figures 1–3, panels A and B). The values of the pseudo-first-order rate constant for peroxynitrite isomerization catalyzed by Dr-Nb(III) (i.e., $k_{\text{obs}}$) increase with the heme protein concentration (Figures 1–3, panels C and D). Previous experiments have shown that, once bound to a heme protein (likely as Fe(III)-ONOOH), peroxynitrite isomerizes to NO₃⁻ at a rate of $\sim 70$ s⁻¹ [13]. Therefore, since the values of $k_{\text{obs}}$ range between 1 × 10⁻¹ s⁻¹ and 8 s⁻¹ (Figures 1–3, panel C), they indicate that, under our conditions, the formation of the Dr-Nb(III)-OONO⁻ species represents the rate-limiting step of the catalytic process. Notably, the Dr-Nb(III)-OONO⁻ conversion to Dr-Nb(III) and NO₃⁻ is faster by at least 10-fold than the formation of the Dr-Nb(III)-OONO⁻ complex.

**Figure 1.** Effect of Dr-Nb(III) on the kinetics for peroxynitrite isomerization, at pH 5.8 and 22.0 °C. (A) Averaged time courses of the Dr-Nb(III)-catalyzed isomerization of peroxynitrite in the absence of CO₂. The time course analysis, according to Equation (1), allowed us to determine the following values of $k_{\text{obs}}$: trace a, 3.1 s⁻¹, $R = 0.998$; trace b, 7.6 s⁻¹, $R = 0.998$. The Dr-Nb(III) concentration was: trace a, 1.0 × 10⁻⁵ M; trace b, 3.0 × 10⁻⁵ M. (B) Averaged time courses of the Dr-Nb(III)-catalyzed isomerization of peroxynitrite in the presence of CO₂. The time course analysis, according to Equation (1), allowed us to determine the following values of $k_{\text{obs}}$: trace a, 7.4 s⁻¹, $R = 0.999$; trace b, 1.3 × 10¹ s⁻¹, $R = 0.998$. The Dr-Nb(III) concentration was: trace a, 1.0 × 10⁻⁵ M; trace b, 3.0 × 10⁻⁵ M. The CO₂ concentration was 1.2 × 10⁻³ M. (C) Dependence of $k_{\text{obs}}$ on the concentration of Dr-Nb(III) in the absence of CO₂. Data were analyzed according to Equation (2) with $k_{\text{n}} = 2.2 \times 10^5$ M⁻¹ s⁻¹ and $k_0 = 8.1 \times 10^{-1}$ s⁻¹; $R = 0.989$, $p = 0.0006$. (D) Dependence of $k_{\text{obs}}$ on the concentration of Dr-Nb(III) in the presence of CO₂. Data obtained were analyzed according to Equation (2) with $k_{\text{n}} = 2.8 \times 10^5$ M⁻¹ s⁻¹ and $k_0 = 4.1$ s⁻¹; $R = 0.991$, $p = 0.0005$. (E) Averaged time courses of peroxynitrite isomerization by apo-Dr-Nb in the absence of CO₂. The time course analysis, according to Equation (1), allowed us to determine the following values of $k_{\text{obs}}$: trace a, 8.3 × 10⁻¹ s⁻¹; trace b, 7.7 × 10⁻¹ s⁻¹. The apo-Dr-Nb concentration was: trace a, 1.0 × 10⁻⁵ M; trace b, 3.0 × 10⁻⁵ M. For clarity, trace b has been up-shifted of 0.3 units. (F) Averaged time courses of peroxynitrite isomerization by apo-Dr-Nb in the presence of CO₂. The time course analysis, according to Equation (1), allowed us to determine the following values of $k_{\text{obs}}$: trace a, 4.3 s⁻¹; trace b, 3.9 s⁻¹. The apo-Dr-Nb concentration was: trace a, 1.0 × 10⁻⁵ M; trace b, 3.0 × 10⁻⁵ M. For clarity, trace b has been up-shifted of 0.3 units. The CO₂ concentration was 1.2 × 10⁻³ M. (G) Dependence of $k_{\text{obs}}$ on the concentration of apo-Dr-Nb in the absence of CO₂. The symbol on the ordinate indicates the value of $k_0$: 8.0 × 10⁻¹ s⁻¹. The average value of $k_{\text{obs}}$ obtained in the presence of apo-Dr-Nb is: 8.1 × 10⁻¹ s⁻¹. (H) Dependence of $k_{\text{obs}}$ on the concentration of apo-Dr-Nb in the presence of CO₂. The symbol on the ordinate indicates the value of $k_0$: 4.1 s⁻¹. The average value of $k_{\text{obs}}$ obtained in the presence of apo-Dr-Nb and CO₂ is: 4.1 s⁻¹. In panels G and H, the differences among the $k_{\text{obs}}$ values are not statistically significant. The peroxynitrite concentration was 2.0 × 10⁻⁴ M. The HCO₃⁻ concentration was 5.0 × 10⁻¹ M. Where not shown, the standard deviation is smaller than the symbol.
Figure 2. Effect of Dr-Nb(III) on the kinetics for peroxynitrite isomerization, at pH 7.0 and 22.0 °C. (A) Averaged time courses of the Dr-Nb(III)-catalyzed isomerization of peroxynitrite in the absence of CO2. The time course analysis according to Equation (1) allowed us to determine the following values of \(k_{\text{obs}}\): trace a, \(7.3 \times 10^{-1}\) s\(^{-1}\), \(R = 0.998\); trace b, 1.8 s\(^{-1}\), \(R = 0.999\). The Dr-Nb(III) concentration was: trace a, \(1.0 \times 10^{-5}\) M; trace b, \(3.0 \times 10^{-5}\) M. (B) Averaged time courses of the Dr-Nb(III)-catalyzed isomerization of peroxynitrite in the presence of CO2. The time course analysis according to Equation (1) allowed us to determine the following values of \(k_{\text{obs}}\): trace a, \(1.5 \times 10^{1}\) s\(^{-1}\), \(R = 0.999\); trace b, \(1.7 \times 10^{1}\) s\(^{-1}\), \(R = 0.998\). The Dr-Nb(III) concentration was: trace a, \(1.0 \times 10^{-5}\) M; trace b, \(3.0 \times 10^{-5}\) M. The CO2 concentration was \(1.2 \times 10^{-3}\) M. For clarity, trace b has been up-shifted of 0.3 units. (C) Dependence of \(k_{\text{obs}}\) on the concentration of Dr-Nb(III) in the absence of CO2. Data were analyzed according to Equation (2) with \(k_{\text{on}} = 4.7 \times 10^{4}\) M\(^{-1}\) s\(^{-1}\) and \(k_{\text{0}} = 3.1 \times 10^{-1}\) s\(^{-1}\); \(R = 0.998\), \(p = 0.0006\). (D) Dependence of \(k_{\text{obs}}\) on the concentration of Dr-Nb(III) in the presence of CO2. (E) Averaged time courses of peroxynitrite isomerization by apo-Dr-Nb in the absence of CO2. The time course analysis according to Equation (1) allowed us to determine the following values of \(k_{\text{obs}}\): trace a, \(3.1 \times 10^{-1}\) s\(^{-1}\); trace b, \(2.7 \times 10^{-1}\) s\(^{-1}\). The apo-Dr-Nb concentration was: trace a, \(1.0 \times 10^{-5}\) M; trace b, \(3.0 \times 10^{-5}\) M. For clarity, trace b has been up-shifted of 0.3 units. (F) Averaged time courses of peroxynitrite isomerization by apo-Dr-Nb in the presence of CO2. The time course analysis according to Equation (1) allowed us to determine the following values of \(k_{\text{obs}}\): trace a, \(1.5 \times 10^{1}\) s\(^{-1}\); trace b, \(1.6 \times 10^{1}\) s\(^{-1}\). The apo-Dr-Nb concentration was: trace a, \(1.0 \times 10^{-5}\) M; trace b, \(3.0 \times 10^{-5}\) M. For clarity, trace b has been up-shifted of 0.3 units. (G) Dependence of \(k_{\text{obs}}\) on the concentration of apo-Dr-Nb in the absence of CO2. The symbol on the ordinate indicates the value of \(k_{\text{0}}\); \(3.1 \times 10^{-1}\) s\(^{-1}\). The average value of \(k_{\text{obs}}\) obtained in the presence of apo-Dr-Nb is: \(3.0 \times 10^{-1}\) s\(^{-1}\). (H) Dependence of \(k_{\text{obs}}\) on the concentration of apo-Dr-Nb in the presence of CO2. The symbol on the ordinate indicates the value of \(k_{\text{0}}\); \(1.5 \times 10^{1}\) s\(^{-1}\). The average value of \(k_{\text{obs}}\) obtained in the presence of apo-Dr-Nb and CO2 is: \(1.5 \times 10^{1}\) s\(^{-1}\). In panels D, G, and H, the differences among the \(k_{\text{obs}}\) values are not statistically significant. The peroxynitrite concentration was \(2.0 \times 10^{-4}\) M. The HCO\(_3^-\) concentration was \(5.0 \times 10^{-3}\) M. Where not shown, the standard deviation is smaller than the symbol.
The analysis of the data shown in Figures 1–3 (panels C and D), according to Equation (2), allowed us to determine the values of $k_{\text{on}}$ and $k_0$, corresponding to the slope and the $y$-intercept of the linear plots, respectively (Table 1). Moreover, the values of $k_0$ were measured in the absence of Dr-Nb(III) via the rapid mixing of the peroxynitrite solution with the appropriate Bis-Tris propane buffer solution (Figures 1–3, panels E and F). Both in the absence and presence of CO$_2$, the values of $k_0$ obtained by the different methods match well with each other (Table 1) and agree well with those previously reported [12,13,25,27,28,30,33,34,36,39,54] (see Table 2).
Table 1. Effect of pH and CO$_2$ on the values of $k_{on}$ and $k_0$ for Dr-Nb(III)-induced isomerization of peroxynitrite at 22.0 °C.

| pH  | $k_{on}$ (M$^{-1}$ s$^{-1}$) | $k_0$ (s$^{-1}$) | $k_{on}$ (M$^{-1}$ s$^{-1}$) | $k_0$ (s$^{-1}$) |
|-----|-----------------------------|------------------|-----------------------------|------------------|
| 5.8 | (2.2 ± 0.3) × 10$^5$         | (8.1 ± 0.8) × 10$^{-1}$ | (2.8 ± 0.3) × 10$^5$         | 4.1 ± 0.4 |
| 7.0 | (4.7 ± 0.5) × 10$^4$         | (3.1 ± 0.3) × 10$^{-1}$ | n.d.                       | (1.5 ± 0.2) × 10$^1$ |
| 8.5 | (1.1 ± 0.1) × 10$^4$         | (4.9 ± 0.5) × 10$^{-2}$ | n.d.                       | (4.8 ± 0.5) × 10$^1$ |

*a The CO$_2$ concentration was 1.2 × 10$^{-3}$ M. 5.0 × 10$^{-2}$ M Bis-Tris propane buffer. n.d., not determined.

Table 2. Effect of pH and CO$_2$ on values of $k_{on}$ and $k_0$ for heme-protein-induced isomerization of peroxynitrite.

| Heme-Protein | $k_{on}$ (M$^{-1}$ s$^{-1}$) | $k_0$ (s$^{-1}$) | $k_{on}$ (M$^{-1}$ s$^{-1}$) | $k_0$ (s$^{-1}$) |
|--------------|-----------------------------|------------------|-----------------------------|------------------|
| Mt-Pgb(III)  | 3.8 × 10$^4$                 | 2.8 × 10$^{-1}$  | n.d.                       | n.d.             |
| Mt-trHbN(III) | 6.2 × 10$^4$                 | 2.7 × 10$^{-1}$  | n.d.                       | n.d.             |
| Ph-trHbO(III) | 2.9 × 10$^4$                 | 2.8 × 10$^{-1}$  | n.d.                       | n.d.             |
| Cj-trHbP(III) | 9.6 × 10$^5$                 | 3.0 × 10$^{-1}$  | 8.8 × 10$^5$                | 2.1 × 10$^1$ |
| Efc-Mb(III)  | 2.9 × 10$^4$                 | 3.5 × 10$^{-1}$  | 7.7 × 10$^4$                | 1.7 × 10$^1$ |
| Pc-Nb(III)  | 1.6 × 10$^4$                 | n.d.            | n.d.                       | n.d.             |
| Hs-Hb(III)  | 1.2 × 10$^4$                 | 3.0 × 10$^{-1}$  | 3.9 × 10$^4$                | 1.7 × 10$^1$ |
| Mt-Nb(III)  | 6.9 × 10$^4$                 | 2.6 × 10$^{-1}$  | n.d.                       | n.d.             |
| At-Nb(III)  | 3.7 × 10$^4$                 | 3.0 × 10$^{-1}$  | n.d.                       | n.d.             |
| Dr-Nb(III)  | 4.7 × 10$^4$                 | 3.1 × 10$^{-1}$  | n.d.                       | 1.5 × 10$^1$ |
| Hs-Nb(III)  | 3.4 × 10$^4$                 | 2.6 × 10$^{-1}$  | n.d.                       | n.d.             |

*pH 7.4 and 20.0 °C. From [55]. b pH 7.0 and 20.0 °C. From [34]. c pH 7.0 and 20.0 °C. From [35]. d pH 7.3 and 25.0 °C. From [36]. e pH 7.0 and 20.0 °C. From [35]. f pH 7.5 and 20.0 °C. From [27]. g pH 7.2 and 25.0 °C. From [40]. h pH 7.1 and 22.0 °C. Present study. i pH 7.1 and 25.0 °C. From [39]. n.d., not determined.

As shown in Table 1, the values of $k_{on}$ for the interaction between Dr-Nb(III) and peroxynitrite were unaffected by CO$_2$/HCO$_3^-$, suggesting that CO$_2$/HCO$_3^-$/CO$_3^{2-}$ does not alter the binding properties of Dr-Nb(III). The values of $k_{on}$ for peroxynitrite isomerization via all-α-helical globins and all-β-barrel nitrobindins ranged between 1.2 × 10$^4$ M$^{-1}$ s$^{-1}$ for Hs-Hb(III) [25] and 6.9 × 10$^4$ M$^{-1}$ s$^{-1}$ for Mt-Nb(III) [40] (Table 2), suggesting that the very different structural organization [40,57–61] is not at the root of the different rate of peroxynitrite isomerization. However, the coordination of the heme-Fe(III) atom, the in-or out-of-plane position of the metal with respect to the pyrrole nitrogen atoms of the porphyrin, the ligand accessibility of the heme-Fe(III) atom, and its Lewis acidity may tune the kinetics of the related peroxynitrite decomposition [36].

To outline the role of the metal center, the values of $k_{obs}$ have been determined as a function of (i) apo-Dr-Nb concentration (at a fixed peroxynitrite concentration) and (ii) peroxynitrite concentration (at fixed Dr-Nb(III) concentration). Apo-Dr-Nb does not induce the isomerization of peroxynitrite; in fact, the values of $k_{obs}$ and $k_0$ match each other in the presence of apo-Dr-Nb (Figures 1–3, panels E–H), as reported for apo-Efc-Mb, apo-Hs-Hb, and apo-Hs-Nb [25,39]. As shown in Figure 4, the values of $k_{obs}$ slightly decrease with an increasing peroxynitrite concentration, reflecting either the slow isomerization process of peroxynitrite-peroxynitrous acid dimers or their slow dissociation preceding the Dr-Nb(III)-catalyzed isomerization of peroxynitrite [30,36].

As shown in Figures 1–3 (panel D) and in Figure 4 (panel B) it turns out that, in the presence of 5.0 × 10$^{-1}$ M CO$_2$/HCO$_3^-$/CO$_3^{2-}$, the role of Dr-Nb(III) in characterizing the rate constant of peroxynitrite isomerization becomes progressively less relevant as the pH is raised since values of $k_0$ for peroxynitrite isomerization obtained in the presence of CO$_2$/HCO$_3^-$/CO$_3^{2-}$ are faster by about two orders of magnitude than those obtained in its absence (Table 1). This indicates that CO$_2$/HCO$_3^-$/CO$_3^{2-}$ dramatically speeds up the
decay of peroxynitrite through direct interaction, without interfering with peroxynitrite binding to Dr-Nb(III) [9,12,13,25,27,28,30,32–34,36].

Figure 4. Effect of peroxynitrite concentration on the values of \( k_{\text{obs}} \) for the isomerization of peroxynitrite by Dr-Nb(III) and apo-Dr-Nb in the absence and presence of CO\(_2\), at pH 7.0 and 22.0 °C. (A) Dependence of \( k_{\text{obs}} \) on the peroxynitrite concentration in the presence of Dr-Nb(III) (open circles) and apo-Dr-Nb (filled circles), in the absence of CO\(_2\) (One-way ANOVA: open circles, ** \( p < 0.01 \), 25 × 10\(^{-6}\) M versus 200 × 10\(^{-6}\) M; filled circles, *** \( p < 0.001 \), 25 × 10\(^{-6}\) M versus 100 × 10\(^{-6}\) M and versus 150 × 10\(^{-6}\) M, **** \( p < 0.0001 \), 25 × 10\(^{-6}\) M versus 200 × 10\(^{-6}\) M). (B) Dependence of \( k_{\text{obs}} \) on the peroxynitrite concentration in the presence of Dr-Nb(III) (open squares) and apo-Dr-Nb (filled squares), in the presence of CO\(_2\) (One-way ANOVA: open squares, *** \( p < 0.001 \), 25 × 10\(^{-6}\) M versus 100 × 10\(^{-6}\) M, **** \( p < 0.0001 \), 25 × 10\(^{-6}\) M versus 150 × 10\(^{-6}\) M and versus 200 × 10\(^{-6}\) M; filled squares, **** \( p < 0.0001 \), 25 × 10\(^{-6}\) M versus 50 × 10\(^{-6}\) M, 100 × 10\(^{-6}\) M, 150 × 10\(^{-6}\) M, and 200 × 10\(^{-6}\) M). The Dr-Nb(III) and apo-Dr-Nb(III) concentration was 5.0 × 10\(^{-6}\) M. The HCO\(_3^−\) concentration was 5.0 × 10\(^{-1}\) M.

To clarify the differential roles of various ferric heme proteins and CO\(_2\)/HCO\(_3^−\)/CO\(_3^{2−}\) levels in peroxynitrite detoxification, the overall effect of pH and CO\(_2\)/HCO\(_3^−\)/CO\(_3^{2−}\) on the observed rate of peroxynitrite isomerization was investigated. Thus, the protonation equilibria of OONO\(^−\)/HOONO and CO\(_2\)/HCO\(_3^−\)/CO\(_3^{2−}\) were considered. The effect of CO\(_2\) on peroxynitrite isomerization in the absence of heme proteins (i.e., \( k_{\text{obs}} \)) can be described by Equation (3):

\[
k_{\text{obs}} = \left( \frac{k_P}{[H^+] + k_P} \right) \times \left\{ k_0 + [L] \times \left( \frac{k_{c1} \times [H^+]^2 + k_{c2} \times [H^+] + k_{c3} \times [H^+] \times k_{c4} \times k_{c5}}{[H^+] + k_{c1} \times [H^+] + k_{c1} \times k_{c2}} \right) \right\} + \left( \frac{[H^+]^2}{[H^+] + k_P} \right) \times \left\{ k_0^H + [L] \times \left( \frac{k_{c1} \times [H^+]^2 + k_{c2} \times [H^+] + k_{c3} \times [H^+] \times k_{c4} \times k_{c5}}{[H^+]^2 + k_{c1} \times [H^+] + k_{c1} \times k_{c2}} \right) \right\}
\]

where \( k_0 \) and \( k_0^H \) are the intrinsic degradation rates of OONO\(^−\) and HOONO, respectively, [H\(^+\)] (= 10\(^{-pH}\)) is the proton concentration, \( k_P \) (= ([OONO\(^−\)] × [H\(^+\)])/[HOONO]) is the protonation constant of peroxynitrite, [L] is the reactant concentration (in our case [HCO\(_3^−\)] = 5.0 × 10\(^{-1}\) M), \( K_{c1} \) ([H\(_2\)O]) (= ([HCO\(_3^−\)] × [H\(^+\)])/[CO\(_2\)] = 10\(^{-6.34}\) is the protonation equilibrium constant between HCO\(_3^−\) and CO\(_2\), \( K_{c2} \) (= ([CO\(_3^{2−}\)] × [H\(^+\)])/[HCO\(_3^−\)]) = 10\(^{-10.25}\) is the protonation equilibrium constant between CO\(_3^{2−}\) and HCO\(_3^−\); \( k_{c1} \) and \( k_{c1}^H \) are the second-order rate constants for the reaction of CO\(_2\) with OONO\(^−\) and HOONO, respectively, \( k_{c2} \) and \( k_{c2}^H \) are the second-order rate constants for the reaction with HCO\(_3^−\) of OONO\(^−\) and HOONO, respectively, and \( k_{c3} \) and \( k_{c3}^H \) are the second-order rate constants for the reaction with CO\(_3^{2−}\) of OONO\(^−\) and HOONO, respectively.

The pH-dependence of peroxynitrite degradation in the absence of CO\(_2\)/HCO\(_3^−\)/CO\(_3^{2−}\) (i.e., [L] = 0, see Equation (3)), allowed us to determine the values of \( k_0 \) (= 3.0 × 10\(^{-2}\) s\(^{-1}\)), \( k_0^H \) (= 8.3 × 10\(^{-1}\) s\(^{-1}\)) and \( k_P \) (= 10\(^{-6.6}\)) (Figure 5, panel A), showing that HOONO decays to NO\(_3^−\) faster than OONO\(^−\), with a p\(K_a\) ≈ 6.8 [13,36]. On the other hand, as already outlined before [9,13,22,27,28,30,36,39], when CO\(_2\)/HCO\(_3^−\)/CO\(_3^{2−}\) is present (i.e.,
[L] = 5.0 × 10^{-1} M), the rate of peroxynitrite degradation becomes much faster and increases with a rising pH (see Table 1 and [36]). Therefore, employing Equation (3) and knowing the values of $K_{c1}$ and $K_{c2}$ (see above), the pH dependence of peroxynitrite degradation (Figure 5, panel B) gives information on the different values of $k_{c1}$ and $k_{c3}^H$ (with i = 1, 2, 3). The inspection of Table 3 allows for the following considerations:

(i) $k_{c1}$, $k_{c2}^H$, and $k_{c3}^H$ are not playing any role, likely because either one or both reactants are too scarcely populated over the pH range investigated for the pseudo-first-order rate constant to have a detectable value, which can then be considered as ≈0 s^{-1};

(ii) At pH ≤ 6.2, the peroxynitrite degradation rate is mostly characterized by the reaction between HOONO and CO$_2$, corresponding to $k_{c1}^H = 8.0$ M$^{-1}$ s$^{-1}$;

(iii) For 6.2 < pH < 8.2, the peroxynitrite degradation rate is mostly characterized by the reaction between OONO$^-$ and HCO$_3^-$, corresponding to $k_{c2} = 6.2$ × 10$^3$ M$^{-1}$ s$^{-1}$;

(iv) For pH ≥ 8.2, the peroxynitrite degradation rate is mostly characterized by the reaction between OONO$^-$ and CO$_3^{2-}$, corresponding to $k_{c3} = 1.8$ × 10$^3$ M$^{-1}$ s$^{-1}$.

Figure 5. pH dependence of the peroxynitrite isomerization rate constant in the absence of CO$_2$ / HCO$_3^-$ / CO$_3^{2-}$ (A), in the presence of 5.0 × 10$^{-1}$ M CO$_2$/ HCO$_3^-$ / CO$_3^{2-}$ (B), and in the presence of 3.0 × 10$^{-2}$ M CO$_2$/ HCO$_3^-$ / CO$_3^{2-}$ (C). In all three panels, the continuous lines correspond to $k_{obs}$, as defined in Equation (3), under different conditions (i.e., [L] = 0 (A), [L] = 5.0 × 10$^{-1}$ M (B), [L] = 3.0 × 10$^{-2}$ M (C), employing the parameters reported in Table 3. In (A, B), the open circles correspond to experimental data obtained from the literature [36] on the peroxynitrite isomerization rate constants as a function of pH under the conditions described above. The continuous lines were obtained according to Equation (3) by the non-linear least-squares fitting of data. In (C), the open circles correspond to values of $k_{obs}$ in the presence of 3.0 × 10$^{-5}$ M Dr-Nb(III) in the absence of CO$_2$/ HCO$_3^-$ / CO$_3^{2-}$ at different pH values (present study). Data shown in (C) indicate that, at pH 5.8, the efficiency of the heme protein (at the indicated concentration) is higher than that of the CO$_2$/ HCO$_3^-$ / CO$_3^{2-}$ system, while at pH 8.5, the efficiency is lower.

Table 3. Values of the parameters fitting data with Equation (3).

| Parameter | Value          |
|-----------|----------------|
| $k_0$ (s$^{-1}$) | (3.0 ± 0.5) × 10$^{-2}$ |
| $k_0^H$ (s$^{-1}$) | (8.3 ± 1.0) × 10$^{-1}$ |
| $K_P$ (M) | (1.6 ± 0.3) × 10$^{-7}$ |
| $K_{c1}$ (M) | (4.6 ± 0.9) × 10$^{-7}$ |
| $K_{c2}$ (M) | (5.6 ± 1.2) × 10$^{-11}$ |
| $k_{c2}$ (M$^{-1}$s$^{-1}$) | (6.2 ± 1.3) × 10$^1$ |
| $k_{c3}$ (M$^{-1}$s$^{-1}$) | (1.7 ± 0.4) × 10$^3$ |
| $k_{c3}^H$ (M$^{-1}$s$^{-1}$) | 8.0 ± 2.1 |

Obviously, the value of $k_{obs}$ and its pH dependence depend on the CO$_2$/ HCO$_3^-$ / CO$_3^{2-}$ levels (i.e., [L]), envisaging that the relative levels of the ferric heme protein and CO$_2$/ HCO$_3^-$ / CO$_3^{2-}$ are crucial in defining the respective role for peroxynitrite detoxification. Of note,
data reported in Figures 1–3 (panel D) refer to [CO₂/HCO₃⁻/CO₃²⁻] = 5.0 × 10⁻¹ M, while in the bloodstream, the physiological levels of CO₂/HCO₃⁻/CO₃²⁻ (~3.0 × 10⁻² M) are about 10-20 fold lower. Therefore, under these conditions, k_obs decreases significantly even in the presence of CO₂/HCO₃⁻/CO₃²⁻, as indicated by Equation (3) (Figure 5, panel C). The pH-dependent mechanism of peroxynitrite degradation can be described by Scheme 2, which reports the different pathways for peroxynitrite degradation.

![Scheme 2](image)

**Scheme 2.** Effect of pH on peroxynitrite degradation. The scheme reproduces the mechanism described by Equation (3), clarifying the meaning of the different parameters; only the significant parameters are reported (see text).

According to Equation (3), the effects of the heme protein and CO₂/HCO₃⁻/CO₃²⁻ levels were simulated (Figure 5, panel C), with the values of the rates at [CO₂/HCO₃⁻/CO₃²⁻] = 3.0 × 10⁻² M and [Dr-Nb(III)] = 3.0 × 10⁻⁵ M (see Figures 1–3, panels C). At acidic pH values, the role of Dr-Nb(III) (= 3.0 × 10⁻⁵ M) is equivalent to that of the CO₂/HCO₃⁻/CO₃²⁻ system (Figure 5C). For alkaline pH values, Dr-Nb(III) levels higher than 3.0 × 10⁻⁵ M would be required to play a relevant role in peroxynitrite detoxification (see Figure 5, panel C).

The relative importance of the heme proteins and CO₂/HCO₃⁻/CO₃²⁻ catalyzing the peroxynitrite isomerization is crucial since, according to the literature [9,12,25,30,36,62,63], ferric heme proteins prevent L-tyrosine nitration, which, instead, occurs either in the presence of apo-Dr-Nb and/or of CO₂/HCO₃⁻/CO₃²⁻ (Figure 6) (see also Scheme 1). In fact, the relative yield of NO₃⁻ and NO₂⁻, obtained from peroxynitrite isomerization catalyzed by Dr-Nb(III), ranged between 89 and 92%, and between 7 and 12%, respectively. However, in the absence of Dr-Nb(III) and/or in the presence of apo-Dr-Nb and/or CO₂/HCO₃⁻/CO₃²⁻, the values of the relative yield of NO₃⁻ and NO₂⁻ ranged between 69 and 74%, and between 7 and 12%, respectively (Table 4). The great relevance of these observations is confirmed by the fact that, under stressed conditions, zebrafish triggers a defense mechanism through nitrosative stress and peroxynitrite production for which its degradation may be, on one side, enhanced by the elevated levels of CO₂ [48,49], but, on the other side, inhibited by the consequent lowering of the pH level. In any event, the correlation between the peroxynitrite degradation by ferric heme proteins and CO₂ levels appears to be relevant within in vivo models of zebrafish, and it may have important consequences for the O₂ supply to poorly oxygenated tissues, such as the retina [50,51].
Table 4. Percentage of NO$_3^-$ and NO$_2^-$ obtained from peroxynitrite isomerization at pH 7.0 and at 22.0 °C.

| Dr-Nb(III) (M) | Apo-Dr-Nb (M) | CO$_2$ (M) | NO$_3^-$ (%) | NO$_2^-$ (%) |
|----------------|----------------|------------|--------------|--------------|
| 0.0            | 0.0            | 0.0        | 73 ± 8       | 28 ± 3       |
| 0.0            | 0.0            | 1.2 × 10$^{-3}$ | 69 ± 7     | 30 ± 3       |
| 0.0            | 3.5 × 10$^{-5}$ | 0.0       | 71 ± 8       | 29 ± 3       |
| 0.0            | 3.5 × 10$^{-5}$ | 1.2 × 10$^{-3}$ | 74 ± 7     | 25 ± 2       |
| 3.5 × 10$^{-5}$ | 0.0            | 0.0        | 89 ± 9       | 12 ± 2       |
| 3.5 × 10$^{-5}$ | 0.0            | 1.2 × 10$^{-3}$ | 93 ± 8     | 7 ± 1        |
| 3.5 × 10$^{-5}$ | 3.5 × 10$^{-5}$ | 0.0       | 91 ± 9       | 10 ± 1       |
| 3.5 × 10$^{-5}$ | 3.5 × 10$^{-5}$ | 1.2 × 10$^{-3}$ | 92 ± 9     | 8 ± 1        |

The peroxynitrite concentration was 2.0 × 10$^{-4}$ M; 5.0 × 10$^{-4}$ M Bis-Tris propane buffer.

4. Conclusions

Ferric heme proteins and the CO$_2$/HCO$_3^-$/CO$_3^{2-}$ system are the major players in peroxynitrite detoxification, for which efficiency depends on both the concentration of these two actors and the pH level. CO$_3^{2-}$ is much more effective at peroxynitrite inactivation, as compared to CO$_2$, with HCO$_3^-$ displaying an intermediate activity. Of note, the CO$_3^{2-}$ levels were much lower than those of the other two components in the system under physiological conditions. Although ferric heme proteins are intrinsically much more effective than the CO$_2$/HCO$_3^-$/CO$_3^{2-}$ system, their levels are usually significantly lower than CO$_2$/HCO$_3^-$/CO$_3^{2-}$. Since over the physiological pH range the rate of peroxynitrite detoxification by CO$_2$/HCO$_3^-$/CO$_3^{2-}$ (i.e., ~3.0 × 10$^{-2}$ M) is ~1.5 s$^{-1}$, values of $k_{\text{obs}}$ ($= k_m \times [\text{heme-Fe(III)}]$), for peroxynitrite scavenging by ferric heme-proteins must be larger than 2 s$^{-1}$. Overall, only the level of the circulating Hs-Hb(III) (~2.0 × 10$^{-4}$ M; corresponding to about 2–3% of the total Hs-Hb in red cells) appears to be sufficient to play a relevant role in the detoxification by peroxynitrite under physiological conditions.

Furthermore, it must be remarked that acidification, which often occurs in the bloodstream of fish when they dive to depth [64–66] and can even be magnified by increased levels of CO$_2$ [49], decreases the effect of the CO$_2$/HCO$_3^-$/CO$_3^{2-}$ system, rendering the role of ferric heme proteins even more crucial. This is especially important in the ocular sys-
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