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Peroxynitrite-dependent modifications of tyrosine residues in hemoglobin. Formation of tyrosyl radical(s) and 3-nitrotyrosine

Review Article

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Summary. Although peroxynitrite is believed to be one of the most efficient tyrosine-nitrating species of biological relevance so far identified, its nitration efficiency is nevertheless limited. In fact, the nitrating species formed through peroxynitrite decay are caged radicals ($^{\bullet}OH/^{\bullet}NO_2$ or, in the presence of carbon dioxide, $CO_3^{\bullet-}/^{\bullet}NO_2$) and the fraction that escapes from the solvent cage does not exceed 30–35%. One exception may be represented by metal-containing compounds that can enhance the formation of nitrotyrosine through a bimolecular reaction with peroxynitrite. Moreover, if the metal is also regenerated in the reaction, the compound is considered a nitration catalysts and the yield of tyrosine nitration enhanced several fold. Examples of peroxynitrite-dependent nitration catalysts are the Mn-superoxide dismutase, some cytochromes and several metalloporphyrins. On the contrary, it has been claimed that some hemoproteins are scavengers of peroxynitrite and play a role in limiting its biodamaging and bioregulatory activity. In this review, we discuss the case of hemoglobin, which is probably the major target of peroxynitrite in blood. This protein has been reported to protect intracellular and extracellular targets from peroxynitrite-mediated tyrosine nitration. This property is shared with myoglobin and cytochrome c. The possible mechanisms conferring to these proteins a peroxynitrite scavenging role are discussed.

Keywords: Peroxynitrite – Hemoglobin – Tyrosine – Ferryl heme – Nitrotyrosine – Oxidative modification

Abbreviations: oxyHb, oxyhemoglobin; metHb, methemoglobin; CO-Hb, carbon monoxide hemoglobin; Mb, myoglobin; Fe^{+4}=O, ferryl heme; 3-nitroTyr, 3-nitrotyrosine

Introduction

The nitration of tyrosine residues appears to represent a prominent *in vivo* pathway of protein oxidative modification occurring in many different pathological conditions (Haddad et al., 1994; Kaur and Halliwell, 1994; Akaike et al., 1996; Torreilles et al., 1999; Thuraisingham et al., 2000; Patel et al., 2000). In general, the detection of 3-nitrotyrosine (3-nitroTyr) has been considered a marker of peroxynitrite production, but should more correctly be considered a marker of $^{\bullet}NO$-related oxidants, since this modification can also occur through different mechanisms (Gunther et al., 1997; Eiserich et al., 1998; Ischiropoulos, 1998; Brennan et al., 2002; Hurst, 2002).

Tyrosine nitration consists in the incorporation of a nitro ($-NO_2$) group at position 3 of the aromatic ring and is believed to depend on the simultaneous availability of tyrosyl ($Tyr^{\bullet}$) and nitrogen dioxide ($^{\bullet}NO_2$) radicals (Scheme 1) (Lymar and Hurst, 1996; Lymar et al., 1996; Lehnig, 1999). The rate-limiting step in tyrosine nitration is its oxidation to $Tyr^{\bullet}$ (Step A in the Scheme 1), which may proceed more slowly than the rate at which $^{\bullet}NO_2$ reacts with the $Tyr^{\bullet}$ ($k = 3 \times 10^9\,M^{-1}\,s^{-1}$, Step B in Scheme 1) (Prutz et al., 1986; Ischiropoulos, 1998). It is interesting to note, however, that *in vivo* tyrosine nitration may be limited due to the effective scavenging of $^{\bullet}NO_2$ and/or $Tyr^{\bullet}$ by antioxidants, such as ascorbate and reactive thiols (Prutz et al., 1986; Gow et al., 1996a).

Peroxynitrite[1] is the product of the near diffusion-limited reaction between nitric oxide ($^{\bullet}NO$) and superoxide

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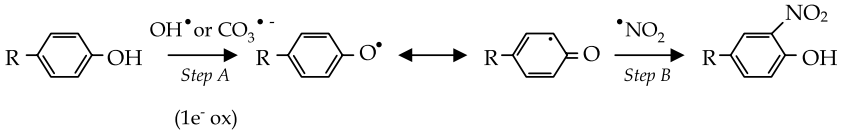
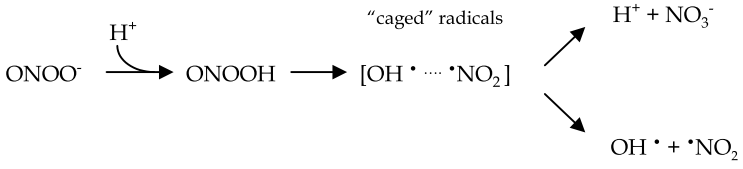
[1] This term refers both to the anion oxoperoxynitrate (1-), $ONOO^-$, and its conjugated acid hydrogen oxoperoxynitrate, ONOOH.

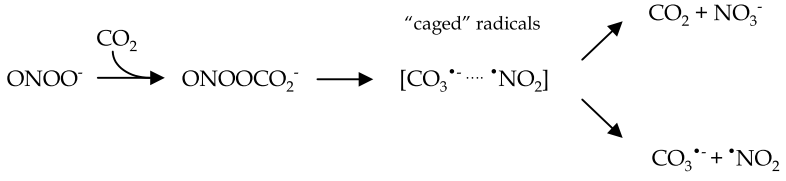
Pathway for 3-nitrotyrosine formation **Scheme 1**



Proton-catalyzed peroxynitrite decay **Scheme 2**



CO_2-catalyzed peroxynitrite decay

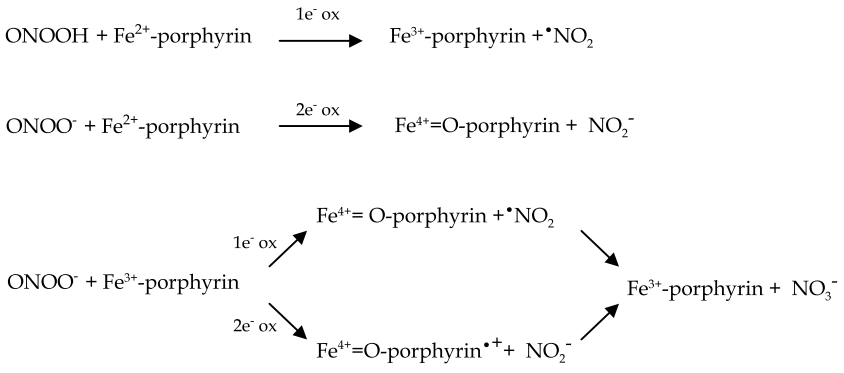


anion ($O_2^{\bullet -}$) (Beckman et al., 1990), and its ability to nitrate tyrosine residues has been widely described since 1992 (Ischiropoulos et al., 1992; Beckman et al., 1992). However, the picture emerging from successive studies is that peroxynitrite is a somewhat selective nitrating agent, since not all proteins or all tyrosine residues of a protein can be nitrated (Gow et al., 1996a; Crow et al., 1997; Estevez et al., 1998; McMillan-Crow et al., 1998; Alvarez et al., 1999; Souza et al., 1999). Although the nature of this selectivity has not yet been completely clarified, it seems likely that several factors may have an important role in targeting nitration to specific tyrosine residues (see below). Since nitration has the effect of decreasing the pKa of the phenoxyl groups (from ~10 to 7.5 in free tyrosine, Sokolovsky et al., 1967), this modification may not only change protein conformation but may also affect the redox and signaling properties of tyrosines, thus contributing to peroxynitrite-mediated cell signaling (Gow et al., 1996b; Kong et al., 1996; Mallozzi et al., 2001).

Peroxynitrite-mediated formation of 3-nitroTyr is not due to a direct reaction of this oxidant with tyrosine residues, but likely involves secondary species formed in the process of peroxynitrite decay to nitrate (Lymar and Hurst, 1996; Lymar et al., 1996; Alvarez et al., 1999). The effective nitrating agent ($^{\bullet}NO_2$) can be formed from peroxynitrite through the proton catalyzed pathway as well as from the reaction with CO_2 (Scheme 2) or with some metal-containing compounds (Scheme 3). One of the prominent effects of CO_2 and metal-containing compounds is to reduce the half-life of peroxynitrite from about 1 s to few milliseconds (Radi, 1998; Romero, 1999), thus largely inhibiting its protonation and hydroxyl-like reactivity (Santos et al., 2000). As suggested (Bonini and Augusto, 2001), the $^{\bullet}OH$ reactivity of peroxynitrite is probably more important in acidic environments, such as in the phagolysosome, at the cell surface and around the negatively charged DNA (Lamm and Pack, 1990), where the negative charges generate an acidic environment.

Peroxynitrite reacts rapidly with CO_2 ($k \sim 10^4\,M^{-1}\,s^{-1}$) and metal-containing compounds ($k = 10^4$–$10^7\,M^{-1}\,s^{-1}$) which, together with thiols ($k \sim 10^3\,M^{-1}\,s^{-1}$), are thought to be the most relevant biological targets of peroxynitrite *in vivo* (Radi et al., 1993; Thomson et al., 1995; Lymar and Hurst, 1996; Lymar et al., 1996; Quijano et al., 1997; Denicola et al., 1998). CO_2 is present in tissues at 1–1.5 mM but thiols and hemoproteins are also highly concentrated (from micromolar to millimolar) and their reaction with peroxynitrite precedes that with other biological targets,

Metal-catalyzed peroxynitrite decay **Scheme 3**



including ascorbate and γ-tocopherol, the major low molecular weight antioxidant defense mechanisms (Lymar and Hurst, 1996). The following paragraphs offer a more in-depth review of the interaction between peroxynitrite and CO_2 or hemoproteins.

Interaction between peroxynitrite and CO_2

The reaction of $ONOO^-$ with CO_2 (Scheme 2) is of particular relevance *in vivo* because it does not detoxify peroxynitrite (Radi et al., 1993). Although the lifetime of peroxynitrite is significantly decreased in the presence of CO_2 (Romero et al., 1999), the diffusion distance is long enough to allow peroxynitrite potentially to cross distances between extra- and/or intracellular compartments (5–20 μm) (Radi et al., 2001). Moreover, its reaction with CO_2 leads to the formation of a short-lived intermediate the nitrosoperoxycarboxylate[2] anion adduct, $ONOOCO_2^-$ (Lymar and Hurst, 1996; Lymar et al., 1996; Denicola et al., 1996) (Scheme 2), which isomerizes to nitrate and CO_2, producing about 30–35% of two reactive radical species, the carbonate anion ($CO_3^{\bullet -}$) and the $^{\bullet}NO_2$ radicals (Scheme 2) (Goldstein and Czapski, 1998; Bonini et al., 1999; Lehnig, 1999). In the presence of CO_2, the $^{\bullet}OH$-dependent reactivity of peroxynitrite and some two-electron oxidations are inhibited, while some one-electron oxidations are favored and nitration of guanine residues in DNA and of tyrosine residues in proteins are significantly increased (Yermilov et al., 1995; Lymar and Hurst, 1996; Lymar et al., 1996; Denicola et al., 1996; Uppu et al., 1996; Pietraforte and Minetti, 1997a, b; Denicola et al., 1998; Scorza and Minetti, 1998; Minetti et al., 1999).

The $^{\bullet}NO_2/CO_3^{\bullet -}$ radicals are potent one-electron oxidants likely involved in the peroxynitrite/CO_2-dependent oxidation of thiols to the corresponding thyil and perthyil radicals (Bonini and Augusto, 2001; Augusto et al., 2002) as well as in the oxidation of a variety of metal complexes and inorganic ions (Huie et al., 1991). The $CO_3^{\bullet -}$ radical is a more selective oxidant of the aromatic residues (rate constants for tyrosine and tryptophan 4.5 and $70 \times 10^7\,M^{-1}\,s^{-1}$, respectively; Chen et al., 1973) than the $^{\bullet}OH$ radical, thus CO_2 promotes the peroxynitrite-dependent one-electron oxidation of tyrosine and tryptophan residues in proteins (Pietraforte and Minetti, 1997a, b).

Is peroxynitrite-dependent tyrosine nitration a selective process?

A selective targeting of peroxynitrite to specific tyrosines (site-specific nitration) has been suggested for some proteins, including Mn-superoxide dismutase (MacMillan-Crow et al., 1998; Quijano et al., 2001), glutamine synthetase (Berlett et al., 1998), SERCA2a isoform of the sarcoplasmic reticulum Ca^+-ATPase (Viner et al., 1999), prostacyclin synthase (Zou et al., 1999), lysozyme, phospholipase A_2, ribonuclease A (Souza et al., 1999) and tyrosine hydroxylase (Blanchard-Fillion et al., 2001). Although the factors driving the nitration reaction have not been completely elucidated, some general hypotheses have been proposed (Berlett et al., 1998; Alvarez et al., 1999; Souza et al., 1999). The number of tyrosine residues and their surface exposure does not account for their relative susceptibility to nitration, as documented by studies performed using proteins with different numbers of tyrosine residues and different exposure of these residues to the solvent (Berlett et al., 1998; Souza et al., 1999). One important factor governing nitration seems to be the localization of tyrosine into hydrophobic domains, since the

[2] Also reported as peroxycarbonate, IUPAC-recommended name is 1-carboxylato-2-nitrosodioxidane.

level of nitration of a hydrophobic tyrosine probe located in a lipid bilayer has been reported to be higher than that measured for tyrosine in an aqueous solution (Zhang et al., 2001). Furthermore, the pH value in the external bulk and inside the protein is crucial for nitration, since the oxidative chemistry of peroxynitrite and the nature of radicals formed from its decay are strictly linked to pH (Crow et al., 1994). Moreover, the presence of neighboring negative charges within a few angstroms of the tyrosine residue can increase the yield of nitration (MacMillan-Crow et al., 1998; Souza et al., 1999; Quijano et al., 2001; Ferrer-Sueta et al., 2002). Overall, the specificity of peroxynitrite-dependent tyrosine nitration seems to depend on the secondary and tertiary structures of proteins and the location and local environment of the tyrosine residue.

Another important contribution to nitration is the closeness of the tyrosine to metal centers (Beckman et al., 1992, MacMillan-Crow et al., 1998; Daiber et al., 2000; Ferrer-Sueta et al., 2002) and the ability of tyrosine residues to function as a sink of other oxidizable residues, such as ferryl species, cysteine, methionine and tryptophan. Finally, it may be important to consider other factors affecting the nitration process such as the increased stability of $Tyr^{\bullet}$ in proteins, the hydrophobic nature of the nitrating agent ($^{\bullet}NO_2$), which is more stable in apolar environments and rapidly hydrolyzed to nitrite and nitrate in aqueous phase.

Interaction between peroxynitrite and heme-containing compounds

Metal-containing compounds, and in particular hemoproteins, react sufficiently fast with peroxynitrite (Table 1) and are sufficiently concentrated in tissues to compete with CO_2. Since the reaction of peroxynitrite with some hemoproteins leads to redox modification of metal centers with formation of high valence oxidation states (Floris et al., 1993; Alayash et al., 1998; Mehl et al., 1999; Minetti et al., 1999; Exner and Herold, 2000; Bourassa et al., 2001), there is debate as to whether these reactions protect from or contribute to peroxynitrite-mediated tissue damage (Floris et al., 1993; Denicola et al., 1998; Alayash et al., 1998; Mehl et al., 1999; Minetti et al., 1999; Cassina et al., 2000; Exner and Herold, 2000; Quijano et al., 2001; Pietraforte et al., 2001). It has been reported that some cytochromes (Mehl et al., 1999; Zou et al., 2000; Daiber et al., 2000) are able to catalyze, rather than to protect, peroxynitrite-mediated tyrosine nitration of target molecules and/or to promote the nitration of its protein tyrosine residues (Cassina et al., 2000; Quijano et al., 2001). Similar effects have been described in the reaction of peroxynitrite with some metalloporphyrins (Mn- and Fe-porphyrins) (Balavoine et al., 1997; Ferrer-Sueta et al., 1997, 1999; Lee et al., 1998a, b).

The mechanism of peroxynitrite reaction with metalloporphyrins has been more deeply investigated and involves the simultaneous formation of high valence metal oxidation states with the possible simultaneous formation of $^{\bullet}NO_2$ (Scheme 3). The high metal oxidation state is formed in a bimolecular reaction with peroxynitrite (i.e. it precedes the isomerization reactions) and oxidizes target tyrosines to $Tyr^{\bullet}$, which in turn reacts with $^{\bullet}NO_2$ to yield 3-nitroTyr (Balavoine et al., 1997; Mehl et al., 1999; Lee et al., 1998a, b; Zou et al., 2000; Daiber et al., 2000). The yield of nitrated products from peroxynitrite can, in these conditions, be enhanced up to 5-fold by the presence of catalytic amounts of these metalloporphyrins (Crow, 2000). The reaction depends on the relative concentrations of the reactants, the pH and the presence of reducing compounds (Lee et al., 1998a, b; Mehl et al., 1999; Ferrer-Sueta et al., 1999).

Table 1. Rate constants for peroxynitrite reactions with hemoproteins

Hemoproteins	k ($M^{-1} s^{-1}$)	T (°C)	pH	Reference
OxyHemoglobin	8.8×10^4	20	7.0	Exner and Herold (2000)
MetHemoglobin	1.7×10^3	37	7.4	Romero et al. (2001)
OxyMyoglobin	5.4×10^4	20	7.3	Exner and Herold (2000)
MetMyoglobin	1.0×10^4	n.s.	7.6	Bourassa et al. (2001)
Cytochrome c^{2+}	1.3×10^4	25	7.4	Thomson et al. (1995)
Myeloperoxidase	6.2×10^6[a]	12	7.2	Floris et al. (1993)
Horseradish peroxidase	3.2×10^6	25	7.2	Floris et al. (1993)
Lactoperoxidase	3.3×10^5	12	7.4	Floris et al. (1993)
Aconitase	1.4×10^5	25	7.6	Castro et al. (1994)
Human Mn-SOD	1.0×10^5	37	7.4	Quijano et al. (2001)

[a] Extrapolated value; *n.s.* = not specified

This picture, which appears to be similar in the case of some cytochromes (Mehl et al., 1999; Zou et al., 2000; Daiber et al., 2000), does not apply, however, to all hemoproteins. One exception is hemoglobin (Hb), one of the most important targets of peroxynitrite in blood (Minetti et al., 2000; Pietraforte et al., 2001). Since the heme oxidation state seems to be important for the interaction of Hb with peroxynitrite, the reactions of oxygenated ferrous (oxyHb) and ferric (metHb) hemoglobin will be analyzed separately.

Mechanism of peroxynitrite interaction with oxyHb

The first study on the reaction of peroxynitrite with oxyHb indicated that peroxynitrite induces heme modifications identical to those elicited by $^{\bullet}NO$ (Schmidt et al., 1994) leading to the rapid oxidation of the heme to metHb (Alayash et al., 1998; Denicola et al., 1998). Subsequently, EPR contributed to the clarification of peroxynitrite/oxyHb interaction (Minetti et al., 1999). This technique, in fact, indicated the formation of a globin-centered $Tyr^{\bullet}$ whose intensity was significantly decreased after heme poisoning with carbon monoxide. The formation of heme-dependent globin radicals centered on tyrosine is characteristic of the interaction of hemoproteins with several peroxides such as H_2O_2 (Davies, 1991; Giulivi and Cadenas, 1998; Gunther et al., 1998; Minetti et al., 1999) and is a consequence of the formation of ferryl ($Fe^{+4}=O$) or perferryl species ($Por^{\bullet+}–Fe^{+4}=O$). It has been suggested, and also demonstrated by visible spectroscopy (Minetti et al., 1999; Exner and Herold, 2000), that $HbFe^{+4}=O$ is formed in the interaction of oxyHb with peroxynitrite. The yields and the kinetics involved in the formation of metHb and $HbFe^{+4}=O$ are strongly affected by pH (Exner and Herold, 2000) and by the presence of CO_2 (Minetti et al., 1999). The reaction of peroxynitrite with CO_2, in fact, competes with that of heme (Denicola et al., 1998; Alayash et al., 1998), and probably allows peroxynitrite to react with heme as a two- rather than a one-electron oxidant, thus inhibiting metHb formation (Minetti et al., 1999). However, the role of CO_2 in peroxynitrite/oxyHb interaction has not been completely clarified. In conclusion, the formation of ferryl heme suggests that peroxynitrite reacts as a two-electron oxidant with oxyHb and that this reaction resembles that with other peroxides (Minetti et al., 1999).

The formation of the transient $HbFe^{+4}=O$ in peroxynitrite-treated oxyHb may be also a route for the oxidation of tyrosine to $Tyr^{\bullet}$. A possible mechanism for the formation of $Tyr^{\bullet}$ may result from $HbFe^{+4}=O$ decay (intramolecular) or through the $HbFe^{+4}=O$-mediated oxidation of a distinct globin molecule (intermolecular). Although the preferred mechanism is presently unknown, the two mechanisms likely have important implications for the tyrosine residues involved.

It is important, however, to bear in mind that $Tyr^{\bullet}$ can be formed also independently of the reaction with heme through the one-electron oxidation pathway of peroxynitrite (Lymar and Hurst, 1996; Lymar et al., 1996; Pietraforte et al., 1997) and this pathway is expected to be more important in the presence of a molar excess of peroxynitrite. In this case, $Tyr^{\bullet}$ is the result of the direct oxidation of tyrosine residues by radicals derived from proton- or CO_2-catalyzed peroxynitrite decay (Scheme 2).

The bimolecular oxyHb/peroxynitrite interaction, leading to the formation of $HbFe^{+4}=O$ and nitrite (Scheme 3), is probably the reason for the ability of this hemoprotein to protect intracellular and extracellular targets from tyrosine nitration (Minetti et al., 1999). Considering that a similar result has also been reported for cytochrome c^{+2} (Pietraforte et al., 2001) and oxymyoglobin (oxyMb) (Pietraforte and Minetti, unpublished results), the protective activity of these hemoproteins represents the major evidence that the ferrous heme is not a catalyst of peroxynitrite-mediated nitration but a peroxynitrite scavenger and that other hemoproteins probably share this property.

Mechanism of peroxynitrite interaction with methemoglobin

In general, peroxides – such as hydrogen peroxide – react more rapidly with ferric than with ferrous hemoproteins and lead to the formation of a species referred to as Compound I or perferryl heme, which is a porphyrin π-cation radical and $Fe^{+4}=O$ heme, ($Por^{\bullet+}–Fe^{+4}=O$-globin). In some hemoproteins, such as Hb and Mb, the porphyrin π-cation radical is quickly reduced by globin to produce the more stable $Fe^{+4}=O$ heme and a globin centered radical, ($Fe^{+4}=O$-$globin^{\bullet}$), but the transient formation of Compound I has been demonstrated by stopped-flow visible spectroscopy (Egawa et al., 2000). In the presence of a reducing substrate, Compound I is reduced, giving only ferryl heme (globin-$Fe^{+4}=O$), also referred to as Compound II.

The study of the reaction between peroxynitrite and metHb is of particular interest since, with the exception of a Zn-porphyrin (Zn(II) tetrakis-(4-benzoic acid porphyrin)) (Quijano et al., 2001), porphyrins and hemoproteins containing a metal (Mn or Fe) at the valence state of +3 are considered catalysts of peroxynitrite-mediated

tyrosine nitration (Balavoine et al., 1997; Ferrer-Sueta et al., 1997, 1999; Lee et al., 1998a, b, Mehl et al., 1999; Zou et al., 2000; Daiber et al., 2000; Bourassa et al., 2001).

Although it is generally accepted that peroxynitrite reacts bimolecularly with the ferrous heme of Mb and Hb (Alayash et al., 1998; Minetti et al., 1999; Exner and Herold, 2000), the mechanisms of the interaction of this oxidant with the Fe^{+3} forms is a matter of debate. This is due mainly to the difficulties encountered in detecting a direct reaction between peroxynitrite and the Fe^{+3} heme, since neither conventional nor stopped-flow visible spectroscopy have been able to identify any heme modification indicative of its oxidation (Alayash et al., 1998; Minetti et al., 1999; Exner and Herold, 2000; Merény et al., 2000; Martinez et al., 2000).

Interestingly, a recent paper (Bourassa et al., 2001) reported that peroxynitrite reacts with the Fe^{+3} heme of Mb and catalyzes peroxynitrite isomerization to nitrate. It has also been demonstrated that some mutants of distal His in Mb not only decompose peroxynitrite at accelerated rates, but are also extensively nitrated at globin tyrosine residues (Herold et al., 2001).

Further indirect evidence of a reaction between peroxynitrite and the Fe^{+3} heme of Hb have been reported (Pietraforte et al., 2001). Firstly, it has been reported that metHb may act as ''peroxynitrite isomerase'' promoting the isomerization of peroxynitrite to nitrate (Scheme 3) and, secondly, that metHb is able to prevent the peroxynitrite-dependent nitration of a tyrosine-containing dipeptide. Since a reduction in both these activities (isomerization and protection from nitration) has been observed when the accessibility of heme to peroxynitrite is hindered by cyanide, it is likely that a bimolecular reaction between peroxynitrite and metHb may occur.

As illustrated in Scheme 3, it has been suggested (Pietraforte et al., 2001) that a ferryl/perferryl species of this hemoprotein is induced by peroxynitrite, coupled with a rapid back-reduction to metHb and nitrate. A fast bleaching of Fe^{+4}=O may be performed by the $^{\bullet}NO_2$ radical, as proposed for Mb (Bourassa et al., 2001). Alternatively, peroxynitrite may oxidize metHb to perferryl heme, which rapidly interacts with nitrite.

Whatever the intermediate formed, in both cases the first step would be the formation of a bimolecular metHb/peroxynitrite complex. Interestingly, spectroscopic evidence for the formation of an intermediate assigned to a peroxynitrito-metHb complex has been hypothesized as a result of the reaction of oxyHb with $^{\bullet}NO$ (Herold, 1999; Herold et al., 2001). This complex is short-lived and quickly decays to metHb and nitrate without evidence of peroxynitrite-mediated oxidative reactions, thus leading the authors to suggest that peroxynitrite does not leave the heme pocket (Herold et al., 2001).

When CO_2 was included in the peroxynitrite/metHb reaction, both the isomerization to nitrate and the protection from nitration were strongly inhibited, indicating that CO_2 competes with heme for peroxynitrite. In fact, the apparent second order rate constant for the peroxynitrite/metHb reaction (Table 1) has been estimated to be lower than that with CO_2.

Similarly to oxyHb, the reaction of peroxynitrite with metHb induces the formation of a long-lived radical(s) assigned to tyrosine (Pietraforte et al., 2001), but it is unclear if the radical is formed through i) a heme-dependent reaction, ii) a reaction with peroxynitrite-derived radicals or iii) both mechanisms.

Characterization of hemoglobin tyrosine residues nitrated by peroxynitrite

Since the formation of $Tyr^{\bullet}$ is the first step in the pathway leading to the formation of 3-nitroTyr (Scheme 1), it is predictable that if both $Tyr^{\bullet}$ and $^{\bullet}NO_2$ radicals are formed in peroxynitrite-hemoprotein interaction, tyrosine residues will be significantly nitrated. Peroxynitrite has been reported to nitrate both the α- and β-chains of oxyHb (Alayash et al., 1998) and also to cause the formation of high molecular weight cross-linkages of Hb (Alayash et al., 1998; Denicola et al., 1998). However, subsequent ascorbate reduction studies (Minetti et al., 2000) showed that, unless a large excess of peroxynitrite is used, the Hb oligomers, which involve cysteine-cysteine and tyrosine-tyrosine cross-linkings, are largely due to modifications occurring under the denaturing conditions employed for Mass Spectrometry and electrophoresis. The nitration of Hb tyrosine residues was, on the contrary, unaffected by treatment with ascorbate. The yields of nitration are strictly correlated with the peroxynitrite/Hb ratio and the presence of CO_2 (Denicola et al., 1996; Alayash et al., 1998; Minetti et al., 2000; Pietraforte et al., 2001).

Hb is a tetrameric protein ($\alpha\alpha\beta\beta$) with three tyrosine residues on each chain: Tyr 24, Tyr 42, and Tyr 140 on the α-chain, and Tyr 35, Tyr 130, and Tyr 145 on the β-chain. Two of these tyrosine residues are nearest to the corresponding heme (αTyr 42 and βTyr 35 shortest distance 4–7 Å), two are more exposed to the solvent (the so called ''penultimate tyrosines'' αTyr 140 and βTyr 145), and the other two are buried and relatively distant from heme (αTyr 24 and βTyr 130, distance from heme $\sim$10 Å) (Perutz et al., 1968).

Table 2. Characterization by liquid chromatography/electrospray mass spectrometry of nitrotyrosine in oxyHb, metHb and CO-Hb after treatment with peroxynitrite

		3-Nitrotyrosine (%)[a]		
		OxyHb	MetHb	CO-Hb
α-chain	Tyr 24	0.03 ± 0.01	0.10 ± 0.01	0.10 ± 0.03
	Tyr 42	4.20 ± 0.72	2.59 ± 0.10	2.50 ± 0.85
	Tyr 140	0.47 ± 0.82	2.22 ± 0.17	1.98 ± 0.48
Total 3-nitroTyr		4.64 ± 1.55	4.82 ± 0.28	4.58 ± 1.36
β-chain	Tyr 35	n.d.	n.d.	n.d.
	Tyr 130	2.82 ± 0.39	2.71 ± 0.27	2.94 ± 0.42
	Tyr 145	n.d.	n.d.	n.d.
Total 3-nitroTyr		2.82 ± 0.39	2.71 ± 0.27	2.94 ± 0.42

[a] Relative yields of nitrated globins were calculated as percentages of the area value associated with extracted ions for modified α and β chains compared with the corresponding unmodified globins. Reproduced from Pietraforte et al. (2001); n.d. = not detectable

The amount of 3-nitroTyr in peroxynitrite-treated oxyHb, metHb and CO-Hb has recently been reported (Pietraforte et al., 2001) and have been summarized in Table 2. The three Hb forms showed comparable nitration yields but a different nitration pattern. These results permit some general considerations. Firstly, the total yield of tyrosine nitration does not depend on the bimolecular heme-peroxynitrite reaction, because similar amounts of nitration were observed with oxyHb, metHb and CO-Hb (Table 2). This finding suggests that nitration is due, at least in part, to the radicals ($CO_3^{\bullet-}/{}^{\bullet}NO_2$) derived in the process of peroxynitrite decay to nitrate. Secondly, the larger nitration of αTyr 42 in oxyHb compared to met- and CO-Hb (Table 2) suggest that the interaction of peroxynitrite with heme promotes the nitration of the Tyr residue nearest to the heme. On the other hand, the isomerization of peroxynitrite to nitrate by the oxyHb heme can explain the lower nitration of αTyr 24 and αTyr 140. Thirdly, the yield of tyrosine nitration in the β-chain was i) lower than that of the α chain, ii) limited to Tyr 130 and iii) apparently unaffected by the heme-peroxynitrite interaction (i.e. comparable in oxy- met- and CO-Hb) (Table 2). The reason for this lower reactivity of the β chain is currently unknown.

The possibility of an autocatalytic mechanism in peroxynitrite-dependent nitration of the tyrosine nearest to heme in cytochrome $P450_{BM\text{-}3}$ (Tyr 334) has recently been reported (Daiber et al., 2000). An analogous mechanism has been proposed for the nitration of Tyr 103 in peroxynitrite-treated metMb (Bourassa et al., 2001). These authors suggested that the oxidation of this tyrosine could be due to the formation of peroxynitrite-dependent Mb ferryl/perferryl species.

As far as Hb is concerned, it has been reported that in the presence of CO_2 the nitration of tyrosine residues is detectable only at 1:1 molar ratio of peroxynitrite/Hb or in excess of peroxynitrite (Minetti et al., 2000). Since under these conditions the yield of tyrosine nitration does not exceed 5% of the respective unmodified subunit (Table 2), we conclude that in the case of Hb the autocatalysis of tyrosine nitration does not occur.

Another example of peroxynitrite-dependent nitration catalysis that fits well with that described above for Cyt $P450_{BM\text{-}3}$ is represented by the non-heme protein Mn-superoxide dismutase. This protein has recently been reported to catalyze the nitration of self (Tyr 34) and target low molecular weight aromatics through the likely formation of Mn-ferryl species (Ferrer-Sueta et al., 2002).

Biological implications

It is reasonable to hypothesize that in blood a large part of peroxynitrite formed in the vasculature would be intercepted by CO_2 or by oxyHb. The high intracellular concentration of oxyHb (20 mM) and its second order rate constant with peroxynitrite (Table 1) indicate that any peroxynitrite crossing the red blood cell will react preferentially with this target (Denicola et al., 1998). It has been estimated that in blood at physiological concentrations of CO_2 and 45% hematocrit about 60% of peroxynitrite generated extracellularly may react with oxyHb (Denicola et al., 1996; Denicola et al., 1998; Minetti et al., 2000).

This 'sink' function of red cells is remarkably facilitated by the huge amount of band 3 (1.2×10^6 copies/cell) in the erythrocyte membrane, which is the major intrinsic membrane protein and the major route of peroxynitrite anion transport (Denicola et al., 1998; Macfadyen et al., 1999).

Importantly, oxyHb is not a catalyst for the tyrosine nitration of either its own globin residues or cellular targets. By protecting biological targets through the scavenging of peroxynitrite to nitrate, oxyHb can be considered in blood a *preventive antioxidant*. Although at an equivalent peroxynitrite/oxyHb molar ratio it has been observed that the reaction with heme increases the yield of Tyr 42 nitration (Table 2), a significant autocatalytic nitration of this tyrosine residue is an unlikely event *in vivo*, because it is unlikely that the amounts of peroxynitrite formed will match the concentration of oxyHb. Even metHb is not a catalyst for tyrosine nitration, but the scavenging of peroxynitrite by this hemoprotein is of minor importance due to its lower concentrations in blood (about 3% of total circulating Hb), its lower rate constant for peroxynitrite and the presence of physiologic concentrations of CO_2.

Finally, a new physiological role as a scavenger of $^{\bullet}NO$ has recently been hypothesized for skeletal and heart muscle Mb (Brunori, 2001; Flögel et al., 2001). Considering that oxyMb reacts fast with peroxynitrite (Table 1), it is likely that Mb could be a powerful peroxynitrite scavenger in those pathological conditions in which the formation of this oxidant has been suggested, such as reperfused ischemic tissues (reviewed by Grisham et al., 1998).

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Note added in proof. Recent studies further supported the hypothesis that hemoglobin and myoglobin can act as efficient peroxynitrite scavengers (Herold S, Shivashankar K, Mehl M (2002) Myoglobin scavenges peroxynitrite without being significantly nitrated. Biochemistry 41: 13460–13472).

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