Mechanisms and Biological Consequences of Peroxynitrite-Dependent Protein Oxidation and Nitration

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SUMMARY

Peroxynitrite modifies proteins through different mechanisms. According to kinetic considerations, direct peroxynitrite reactions with proteins in vivo are most probably restricted to fast reacting thiols as well as metal cofactors. Other amino acidic or prosthetic group modifications, including tyrosine nitration, require the formation of peroxynitrite-derived secondary oxidants. The interaction between peroxynitrite and proteins can lead to peroxynitrite detoxification, such as peroxiredoxin-catalyzed reduction to nitrite or oxyhemoglobin-mediated isomerization to nitrate. Alternatively, peroxynitrite can promote changes in protein function by oxidation and/or nitration of key protein moieties. Considering the low abundance of modified amino acidic residues, their biological relevance is clearer in the case of the gain of a new function. Peroxynitrite reactions with proteins also promote protein aggregation, turnover, signaling and immunological processes. Herein, we review the biochemical mechanisms of peroxynitrite-dependent protein modifications and analyze the impact on protein function in vitro and in vivo.

Keywords: peroxynitrite, 3-nitrotyrosine, oxidative stress, sulfenic acid, kinetics, fast reacting thiols, nitric oxide, nitrogen dioxide, superoxide, free radicals
OVERVIEW OF PEROXYNITRITE BIOCHEMISTRY

Since peroxynitrite was first proposed to be implicated in pathophysiology almost two decades ago (Beckman et al., 1990; Ischiropoulos et al., 1992; Radi et al., 1991a,b), scientists from different fields have made important contributions regarding its routes of formation, mechanisms of reaction, detoxification and cellular actions (Ferrer-Sueta and Radi, 2009; Pacher et al., 2007; Szabo et al., 2007). Peroxynitrite is now known to be implicated in an increasing number of diseases, including neurodegenerative disorders (Torreilles et al., 1999), atherogenesis and related cardiovascular diseases (Uppu et al., 2007; Wattanapitayakul et al., 2000), diabetes (Arora et al., 2008; Zou et al., 2004), and immune and inflammatory disorders (Cross et al., 1997; Oates et al., 1999; Sandhu et al., 2003). Understanding the mechanisms by which peroxynitrite exerts its pathogenic actions can assist in the development of rational treatments for such diseases.

The main route of peroxynitrite formation in biological systems is the fast reaction between superoxide anion (O$_2$•$^-$) and nitric oxide (•NO) radicals. The rate constant of this reaction is in the 10$^9$–10$^{10}$ M$^{-1}$s$^{-1}$ range (Goldstein and Czapski, 1995b; Huie and Padmaja, 1993; Kissner et al., 1997). Thus, the reaction between these two radicals is fast enough to outcompete O$_2$•$^-$-dismutation catalyzed by superoxide dismutase (SOD), at least under conditions of increased •NO production, such as inflammation via inducible NO synthase (iNOS) or sustained activation of the constitutive forms of NOS (Nakamura and Lipton, 2008). When considering sites of peroxynitrite formation, the anionic nature of O$_2$•$^-$ at physiological pH ($pK_a$ HO$_2$ = 4.8), and hence its limited diffusion capability, should be taken into account. This is not the case for •NO, which is a small and neutral lipophilic molecule that can readily diffuse through membranes. Therefore, peroxynitrite formation is favored in those cellular compartments where O$_2$•$^-$ is produced. Other biologically relevant routes for peroxynitrite formation may include the reaction between triplet nitroxyl anion and molecular oxygen, which could occur in selected environments (Shafirovich and Lymar, 2002), as well as metal-bound peroxynitrite formation from the reaction of •NO and ferrous-dioxygen complexes in heme-containing proteins like oxyhemoglobin (Herold, 1998; Romero et al., 2003).

Since peroxynitrous acid has a $pK_a$ of 6.5–6.8 (Goldstein and Czapski, 1995b; Kissner et al., 1997; Pryor and Squadrito, 1995), peroxynitrite predominates as the anion at physiological pH, and hence its diffusion through membranes is limited. In fact, peroxinitrous acid can cross biological membranes by passive diffusion while the anionic species uses anion channels, its permeation being restricted by the number of channels present in the cellular membrane (Denicola et al., 1998; Marla et al., 1997).

Peroxynitrous acid is an unstable species that decays through homolysis of its peroxo bond ($k = 0.9$ s$^{-1}$, pH 7.4, 37°C), resulting in hydroxyl (•OH) and nitrogen dioxide (•NO$_2$) radicals in approximately 30% yields (Gerasimov and Lymar, 1999; Goldstein and Czapski, 1995a). Both radicals participate in secondary reactions that may lead to the oxidation/nitration of different targets. Moreover, both peroxynitrite anion and peroxynitrous acid are strong oxidants that directly react with different biomolecules. Among these, low molecular weight and protein thiols, metal centers and carbon dioxide (CO$_2$) constitute the main targets for peroxynitrite in vivo (for a recent review see Ferrer-Sueta and Radi, 2009). The mechanisms of oxidation vary depending on the target. The direct reaction with thiols involves peroxynitrous acid as the oxidant species (Trujillo and Radi, 2002) and consists of a two-electron oxidation process (Radi et al., 1991b). On the other hand, peroxynitrite anion reacts with CO$_2$ ($k = 4.6 \times 10^4$ M$^{-1}$s$^{-1}$ at pH 7.4 and 37°C) to form a transient intermediate that very rapidly decomposes homolytically to •NO$_2$ and carbonate radical (CO$_3$•$^-$) in ~ 35% yields (Bonini et al., 1999; Denicola et al., 1996; Lymar and Hurst, 1995). These two radicals can in turn lead to secondary or indirect reactions. In the case of metal centers, such as heme proteins or manganese porphyrins, reported mechanisms include one-electron and two-electron oxidations as well as metal-catalyzed isomerization to nitrate (Ferrer-Sueta et al., 1999; Floris et al., 1993; Romero et al., 2003; Thomson et al., 1995; Zou et al., 1999a).
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To rationalize the biological fate of peroxynitrite and its derived radicals, it is necessary to understand the kinetics of the reactions. In this regard, many direct reactions of peroxynitrite have been addressed kinetically, with reported rate constants (k) ranging from $10^2 \text{M}^{-1}\text{s}^{-1}$ to $10^8 \text{M}^{-1}\text{s}^{-1}$, and reactions of $\cdot \text{OH}$, $\cdot \text{NO}_2$, and $\text{CO}_3^{\cdot}$ have been studied as well. It should be taken into account, importantly, that the main targets for peroxynitrite will be dictated not by rate constants but by overall reaction rates, which are determined by the product of rate constant and biological target concentration ($k'$). Care should be taken in determination of the rate constants of peroxynitrite reactions, particularly when stopped-flow methods are not available and competition approaches are used (Trujillo et al., 2008a). Most common misinterpretations arise from the fact that peroxynitrite-derived radicals can also lead to target oxidation, and therefore the use of radical scavengers that react with peroxynitrite-derived radicals but not directly with peroxynitrite itself is highly recommended. Moreover, buffers such as HEPES, MOPS, and Tris, which have been reported to react with peroxynitrite-derived radicals and form products with their own reactivity, should be avoided (Gadelha et al., 1997; Schmidt et al., 1998). Since peroxynitrite anion diffusion through membranes is limited (Denicola et al., 1998; Marla et al., 1997), sites of peroxynitrite formation, diffusion capability and target compartmentalization should also be considered to understand its biological fate. Moreover, the radical nature of peroxynitrite precursors as well as products formed from its decomposition or one-electron oxidations, and the multiple possible interactions among them, add an additional level of complexity.

Peroxynitrite-mediated protein oxidation and nitration have been shown to be biologically relevant processes, leading in many cases to altered protein function, which not only includes protein inactivation but also activation or, most importantly, gain of a new function (for recent reviews see Peluffo and Radi, 2007; Souza et al., 2008b). In some cases, the modification caused by peroxynitrite leads to protein aggregation (Souza et al., 2000b; Zhang et al., 2005b) or degradation (Grune et al., 2001; Souza et al., 2000a). Peroxynitrite has also been reported to be involved in cell signaling processes (Huang et al., 2008). More recently, peroxynitrite-modified proteins have been demonstrated to be immunogenic and have been implicated in the development of inflammatory diseases (Thomson et al., 2007). Finally, the interaction of peroxynitrite with proteins specialized towards its catalytic decomposition results in peroxynitrite detoxification without a net protein modification, either because these proteins catalyze peroxynitrite isomerization (Romero et al., 2003), or as a result of the rapid re-reduction of oxidized protein intermediates by other cellular compounds (Trujillo et al., 2008b).

In this chapter we will present the mechanisms of reaction between peroxynitrite and amino acid residues as well as prosthetic groups in proteins. Selected examples of such protein modifications and their biological consequences will be analyzed.

**PEROXYNITRITE-MEDIATED AMINO ACID AND PROTEIN MODIFICATIONS**

Although virtually all amino acids would be modified if exposed to high enough concentrations of peroxynitrite, its direct reactivity is restricted to cysteine, selenocysteine, methionine, and tryptophan residues, as well as metal prosthetic groups in proteins.

**Cysteine oxidation**

Direct peroxynitrite-mediated thiol oxidation is a two-electron process in which peroxynitrous acid oxidizes thiolates to the corresponding sulfenic acids (RSOH) (Radi et al., 1991b) (Fig. 1). The latter are unstable species that in the presence of accessible thiols form disulfides (RSSR'), within the same or other proteins, or with low molecular weight thiols, leading to mixed disulfide formation. The stoichiometry of this reaction is therefore two thiols oxidized for each peroxynitrite. In selected cases sulfenic acids are stable, and then
yields of thiol oxidation are lower (1:1) (Allison et al., 1973; Carballal et al., 2003; Claiborne et al., 1993). Alternatively, sulfenic acid can react with itself forming a thiosulfinate (RS(O)SR') or react with an amine forming a sulfenamide (RSNHR'). Sulfenic acid can be further oxidized by two electrons forming sulfinic (RSO2H) and sulfonic (RSO3H) acid. To the right (b) are depicted one-electron mechanisms. Thiolates (RS-) react with the radicals derived from peroxynitrite forming the thyl radical (RS*), which can react with itself forming a disulfide or with *NO forming a S-nitrosothiol (RSNO). The thyl radical can also react with dioxygen, eventually leading to sulfinic acid formation, or with another thiolate. The latter reaction gives rise to the disulfide anion radical (RSSR**-), a reductant that can react with dioxygen forming superoxide and a disulfide (RSSR).

In addition to the direct reaction, peroxynitrite-derived radicals, *OH, *NO2, and CO3**- can lead to indirect thiol oxidation, which consists of a one-electron oxidation process to the corresponding thyl radicals (Bonini and Augusto, 2001; Quijano et al., 1997). These radicals may recombine to form disulfide bridges. More frequently, thyl radicals react with oxygen to form thylperoxyl radicals (RSOO*) that can rearrange to sulfonyl radicals (RSO2*) and subsequently sulfinic/sulfonic acid, or further react with another thiol eventually leading to sulfenic acid (Wardman, 1998). Thyl radicals can also react with thiols to form disulfide anion radicals, which in turn can reduce oxygen to form superoxide and disulfide anion radicals (RSSR**-) (Wardman and von Sonntag, 1995). Moreover, thyl radicals react at diffusion-controlled rates with nitric oxide to form S-nitrosothiols (RSNO) (Madej et al., 2008).

The latter can also be formed from the reaction of thiols with dinitrogen trioxide (N2O3) derived from the fast reaction between peroxynitrite-derived *NO2 and *NO (Kharitonov et al., 1995). Therefore, products formed from peroxynitrite-mediated thiol oxidation may differ according to the mechanism of reaction, depending on the concentration of thiol, oxygen and carbon dioxide, and on whether proteins are exposed to peroxynitrite as a bolus (no *NO is present) or generated by fluxes of precursor radicals.
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The kinetics of direct peroxynitrite-mediated thiol oxidation have been determined for several protein and low molecular weight thiols (Fig. 2). The pH dependencies of the rate constants are bell-shaped, according to:

$$k_{2\text{app}} = k_{2\text{pHind}} \left( \frac{[H^+]}{K_a^{\text{ONOOH}} + [H^+]} \right) \left( \frac{K_a^{\text{RSH}}}{K_a^{\text{RSH}} + [H^+]} \right)$$

(1)

where $k_{2\text{app}}$ is the apparent rate constant at a given pH, $k_{2\text{pHind}}$ is the pH independent rate constant, i.e. the intrinsic thiolate reactivity, and $K_a^{\text{ONOOH}}$ and $K_a^{\text{RSH}}$ are the dissociation constants of ONOOH and RSH, respectively (Radi et al., 1991b; Trujillo and Radi, 2002).

For many thiols, including low molecular weight thiols and some protein thiols such as the single thiol group of human serum albumin (HSA), reactivities are related to thiol pKₐ, those thiols with the lower pKₐ reacting faster at pH 7.4 due to increased thiolate fraction (Trujillo and Radi, 2002). On the contrary, intrinsic thiolate reactivity increases with lower thiol pKₐ reflecting changes in thiolate nucleophilicity, on agreement with a reaction mechanism involving the nucleophilic attack of the thiolate on the peroxodic oxygen of peroxynitrite with nitrite as leaving group (Trujillo et al., 2007). However, there are some protein thiols whose reactivities with peroxynitrite are much higher than expected from their pKₐ. These fast reacting thiols include the peroxidatic thiol in peroxiredoxins, and those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Souza and Radi, 1998) and protein tyrosine phosphatases PTP1B (Takakura et al., 1999) (Fig. 2). It is obvious that in these cases, protein factors other than the thiol pKₐ are responsible for this fast reactivity (Trujillo et al., 2007). Further work is required to unravel these factors.
HUMAN SERUM ALBUMIN

HSA is the most abundant protein in the intravascular space (~0.6 mM), constituting 60% of total plasma protein, and is also present extravascularly in extracellular tissue compartments. It contains only one free cysteine, Cys34 (HSA-SH), which is the main plasma thiol, representing > 80% of free thiols in the circulation (Peters, 1996). It has been proposed to participate in the scavenging of oxidant species, mostly due to the presence of the Cys34 thiol. Indeed, HSA-SH is able to react with different oxidants, including peroxynitrite and its derived radicals. The reaction with peroxynitrite occurs with a second order rate constant of $3.8 \times 10^9 \text{M}^{-1}\text{s}^{-1}$ (Alvarez et al., 1999). This reactivity is comparable to that of low molecular weight thiols such as free cysteine ($k = 5.9 \times 10^5 \text{M}^{-1}\text{s}^{-1}$; Radi et al., 1991b) and glutathione ($k = 1.35 \times 10^5 \text{M}^{-1}\text{s}^{-1}$; Koppenol et al., 1992), and is consistent with the apparent pKₐ value of the albumin thiol of 8.3–8.6 (Alvarez et al., 1999), as illustrated in Fig. 2. The reaction with two-electron oxidants leads to a relatively stable sulfenic acid (HSA-SOH) (Carballal et al., 2003), which can react with a thiol such as cysteine leading to a mixed disulfide (HSA-SSR) or be further oxidized to sulfenic (HSA-SO₂H) and sulfonic (HSA-SO₃H) acid. No evidence has been obtained for the formation of sulfenamides or intermolecular disulfide dimers. A recent analysis of the reactivity of albumin sulfenic acid (Turell et al., 2008) supports quantitatively a role for this intermediate in the formation of mixed disulfides and higher oxidation states, which can be observed in ~25% of circulating albumin.

In addition to the direct reaction with peroxynitrite, HSA-SH also reacts with the radicals derived from peroxynitrite homolysis, *OH ($k = 3.8 \times 10^9 \text{M}^{-1}\text{s}^{-1}$; Goldstein and Samuni, 2005), *NO₂ and CO₃⁻ ($k = 5.8 \times 10^8 \text{M}^{-1}\text{s}^{-1}$; Goldstein and Samuni, 2005), leading to thyl radical and other secondary radicals (Gatti et al., 1994). Although the albumin thiol does not react particularly fast with oxidants, it can still be considered an important intravascular scavenger due to its very high concentration, particularly considering that plasma is scarce in antioxidant defenses and low molecular weight thiols. In this regard, oxidized isoforms of albumin have been found to be increased in several pathological conditions (for a review see Turell et al., 2009). These oxidized species are not present when albumin is secreted from liver cells. Thus, they can be considered potential biomarkers of the involvement of oxidative stress processes and constitute a proof of the concept of the scavenger activity of albumin.

PEROXIREDOXINS

Peroxiredoxins (Prxs) are ubiquitous peroxidases that rely on active site Cys residue(s) to reduce peroxides (Hofmann et al., 2002; Wood et al., 2003). The first step in their catalytic cycle, common for all kinds of Prxs, consists of the oxidation of the peroxidatic cysteine to a sulfenic acid derivative. In one-Cys Prxs, reduction back to thiol is achieved directly by the reducing substrate, whose nature is still controversial (Monteiro et al., 2007; Ralat et al., 2006). In two-Cys Prxs, a second (resolving) cysteine residue forms a disulfide bridge with the sulfenic acid in the peroxidatic residue, which in turn is reduced by thioredoxin/thioredoxin reductase at the expense of NADPH (Wood et al., 2003). In 2000, Bryk et al. reported the peroxynitrite-reductase activity of bacterial alkyl hydroperoxide reductase C (AhpC), a typical two-Cys Prx. Afterwards, peroxynitrite reduction to nitrite was demonstrated to be a general property of all kinds of Prxs studied so far (Table 1), which include examples of the different classes of Prx present in microorganisms, plants and animals (Dubuisson et al., 2004; Jaeger et al., 2004; Manta et al., 2008; Nickel et al., 2005; Ogusucu et al., 2007; Peshenko et al., 2001; Sakamoto et al., 2003; Trujillo et al., 2004). The peroxidatic thiol pKₐ in Prxs have been reported to be in the 5–6.3 range for the different Prxs already studied, indicating that it would be mostly (>90%) deprotonated at physiological pH (Bryk et al., 2000; Nelson et al., 2008; Ogusucu et al., 2007; Trujillo et al., 2007). However, the kinetics of peroxynitrite reduction are much faster than expected according to peroxidatic thiol pKₐ (Fig. 2) (Trujillo et al., 2007). The protein factors generating such a fast reactivity are intriguing. As with other peroxide substrates, peroxynitrite-mediated peroxidatic thiol oxidation leads to sulfenic acid...
Table 1  Selected examples of peroxynitrite-mediated amino acid modifications in proteins and their biological consequences

<table>
<thead>
<tr>
<th>Residue</th>
<th>Protein</th>
<th>Rate constant (M$^{-1}$s$^{-1}$)$^a$</th>
<th>Modification</th>
<th>Position</th>
<th>Biological consequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>Bovine peroxiredoxin 6 (1-Cys Prx)</td>
<td>NR</td>
<td>Sulfenic acid</td>
<td>Peroxidatic Cys</td>
<td>Peroxynitrite catalytic reduction$^b$</td>
<td>(Peshenko et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>2-Cys peroxiredoxins (typical)</td>
<td>$10^6$–$10^7$</td>
<td>Intermolecular disulfide</td>
<td>Peroxidatic Cys</td>
<td>Peroxynitrite catalytic reduction$^c$</td>
<td>(Bryk et al., 2000; Manta et al., 2008$^d$; Nickel et al., 2005; Trujillo et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>2-Cys peroxiredoxins (atypical)</td>
<td>$10^7$–$10^8$,d</td>
<td>Intramolecular disulfide</td>
<td>Peroxidatic Cys</td>
<td>Peroxynitrite catalytic reduction$^c$</td>
<td>(Dubuisson et al., 2004; Jaeger et al., 2004; Trujillo et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Protein tyrosine phosphatases</td>
<td>$2$–$22 \times 10^7$</td>
<td>Sulfinic or sulfonic acid</td>
<td>Active site Cys</td>
<td>Protein inactivation</td>
<td>(Takakura et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Human arylamine N-acetyltransferase 1</td>
<td>$5 \times 10^4$</td>
<td>Mostly sulfinic or sulfonic acid</td>
<td>Cys68</td>
<td>Protein inactivation</td>
<td>(Dupret et al., 2005)</td>
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<td></td>
<td>Rabbit glyceraldehyde-3-phosphate dehydrogenase</td>
<td>$2.5 \times 10^5$,d</td>
<td>85% sulfinic or sulfonic acid</td>
<td>Cys149</td>
<td>Protein inactivation</td>
<td>(Souza and Radi, 1998)</td>
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<tr>
<td></td>
<td>DJ-1</td>
<td>$2.7 \times 10^5$,d</td>
<td>Sulfinic acid</td>
<td>Cys106</td>
<td>Protein inactivation Possible gain of chaperone activity</td>
<td>(Andres-Mateos et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Rabbit creatine kinase</td>
<td>$8.8 \times 10^5$,e</td>
<td>Sulfinic or sulfonic acid</td>
<td>Cys283</td>
<td>Protein inactivation</td>
<td>(Konorev et al., 1998)</td>
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<tr>
<td></td>
<td>Human albumin thiol</td>
<td>$3.8 \times 10^4$</td>
<td>Sulfenic acid</td>
<td>Cys34</td>
<td>Not clear change in protein function</td>
<td>(Alvarez et al., 1999)</td>
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<td></td>
<td>Sarco/endoplasmic reticulum calcium ATPase</td>
<td>NR</td>
<td>S-glutathiolation</td>
<td>Cys674</td>
<td>Increased activity</td>
<td>(Adachi et al., 2004)</td>
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</table>

(Continued)
<table>
<thead>
<tr>
<th>Residue</th>
<th>Protein</th>
<th>Rate constant(\text{M}^{-1}\text{s}^{-1})(^a)</th>
<th>Modification</th>
<th>Position</th>
<th>Biological consequence</th>
<th>Reference</th>
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<tr>
<td>Aldose reductase</td>
<td>NR</td>
<td>Sulfinic acid</td>
<td>Cys298</td>
<td></td>
<td>Increased activity</td>
<td>(Kaiserova et al., 2008)</td>
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<tr>
<td>Tau and microtubule-associated protein-2</td>
<td>NR</td>
<td>Disulfide formation</td>
<td>NR</td>
<td></td>
<td>Inhibited microtubule polymerization</td>
<td>(Landino et al., 2004)</td>
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<tr>
<td>Selenocysteine</td>
<td>Glutathione peroxidase</td>
<td>(8 \times 10^6)</td>
<td>Seleninic acid</td>
<td>Sec47</td>
<td>Peroxynitrite catalytic reduction</td>
<td>(Briviba et al., 1998)</td>
</tr>
<tr>
<td>Methionine</td>
<td>(\alpha 1)-Proteinase inhibitor</td>
<td>NR</td>
<td>MetSO</td>
<td>Met358</td>
<td>Protein inactivation</td>
<td>(Moreno and Pryor, 1992)</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>NR</td>
<td>MetSO</td>
<td>Met144–Met145</td>
<td></td>
<td>Protein inactivation</td>
<td>(Galeva et al., 2005)</td>
</tr>
<tr>
<td>Tryptophan and histidine</td>
<td>Succinyl CoA : 3 ketoacid transferase</td>
<td>NR</td>
<td>5-hydroxy 6-nitroTrp</td>
<td>Trp342</td>
<td>Increased activity</td>
<td>(Rebrin et al., 2007)</td>
</tr>
<tr>
<td>Human CuZn superoxide dismutase</td>
<td></td>
<td>(1 \times 10^4)(^f)</td>
<td>His oxidation ((– \text{CO}_2))</td>
<td>His118</td>
<td>Trp32</td>
<td>(Alvarez et al., 2004; Yamakura et al., 2005)</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Human Mn superoxide dismutase</td>
<td>(1 \times 10^5)(^g)</td>
<td>3-nitroTyr</td>
<td>Tyr34</td>
<td>Protein inactivation</td>
<td>(MacMillan-Crow et al., 1998)</td>
</tr>
<tr>
<td>Prostacyclin synthase</td>
<td></td>
<td>(10^6–10^7)(^h)</td>
<td>3-nitroTyr</td>
<td>Tyr430</td>
<td>Protein inactivation</td>
<td>(Schmidt et al., 2003; Zou, 2007)</td>
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<tr>
<td>Microsomal glutathione S-transferase</td>
<td></td>
<td>NR</td>
<td>3-nitroTyr</td>
<td>Tyr92</td>
<td>Increased activity(^i)</td>
<td>(Ji et al., 2006)</td>
</tr>
<tr>
<td>Cytochrome c(^{3+})</td>
<td></td>
<td>ND</td>
<td>3-nitroTyr</td>
<td>Tyr97 or Tyr74(^j)</td>
<td>Increased peroxidase activity. Inhibition of electron transport and apoptosome assembly. Translocation to cytosol.</td>
<td>(Bathyany et al., 2005; Cassina et al., 2000; Gebicka and Didik, 2003; Souza et al., 2008a, and Godoy et al., 2008)</td>
</tr>
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\(^a\)At pH 7.4 and 37°C unless otherwise indicated.
\(^b\)Two physiologically relevant routes for reduction of oxidized enzyme have been described: heterodimerization with glutathione transferase that allows glutathione-mediated reduction (Ralat et al., 2006) and ascorbate-dependent reduction (Monteiro et al., 2007).
\(^c\)Catalytic cycle is complete at thioredoxin (or thioredoxin-related protein)/thioredoxin reductase/NADPH expense.
\(^d\)At 25°C; \(^e\)at pH 6.9.

\(^f\)Reaction with copper cofactor, indirectly leads to histidinyl radical formation; \(^g\)with the manganese cofactor, that indirectly leads to tyrosine nitration. Dityrosine formation is also observed; \(^h\)reaction with heme thiolate cofactor, indirectly leading to tyrosine nitration. \(^i\)Nitration at Tyr92 is responsible for the gain of function. Tyr153 nitration and thiol oxidation are also observed. \(^j\)At higher concentrations of peroxynitrite, dinitrated species (at Tyr97 and Tyr67 or at Tyr74 and Tyr67) and trinitrated species (at Tyr97, 67 and 74) are observed. \(^k\)Identified as site of fibrinogen nitration \textit{in vivo} (Parastatidis et al., 2008). \(^l\)Trp99 is also nitrated by peroxynitrite, but protein inactivation has been ascribed to tyrosine nitration. \(\text{NR}\) = not reported, \(\text{ND}\) = not detected.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Modification Site</th>
<th>Biological Consequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>NR</td>
<td>3-nitroTyr Tyr292, Tyr422 of beta-chain</td>
<td>(Vadseth et al., 2004)</td>
</tr>
<tr>
<td>Human glutathione reductase</td>
<td>NR</td>
<td>3-nitroTyr Tyr106, Tyr114</td>
<td>(Savvides et al., 2002)</td>
</tr>
<tr>
<td>Nerve growth factor</td>
<td>NR</td>
<td>3-nitroTyr Tyr52</td>
<td>(Pehar et al., 2006)</td>
</tr>
<tr>
<td>Cytochrome P450 2B1</td>
<td>NR</td>
<td>3-nitroTyr Tyr190</td>
<td>(Lin et al., 2003)</td>
</tr>
</tbody>
</table>

aAt pH 7.4 and 37°C unless otherwise indicated.
bTwo physiologically relevant routes for reduction of oxidized enzyme have been described: heterodimerization with glutathione transferase that allows glutathione-mediated reduction (Ralat et al., 2006) and ascorbate-dependent reduction (Monteiro et al., 2007).
cCatalytic cycle is complete at thioredoxin (or thioredoxin-related protein)/thioredoxin reductase/NADPH expense.
dAt 25°C;
eAt pH 6.9.
fKinetic data refers to the global reaction between peroxynitrite and the proteins, including the metal cofactors:
gReaction with copper cofactor, indirectly leads to histidinyl radical formation;
hWith the manganese cofactor, that indirectly leads to tyrosine nitration. Dityrosine formation is also observed;
iReaction with heme thiolate cofactor, indirectly leading to tyrosine nitration.
jNitration at Tyr92 is responsible for the gain of function. Tyr153 nitration and thiol oxidation are also observed.
kAt higher concentrations of peroxynitrite, dinitrated species (at Tyr97 and Tyr67 or at Tyr74 and Tyr67) and trinitrated species (at Tyr97, 67 and 74) are observed.
lIdentified as site of fibrinogen nitration in vivo (Parastatidis et al., 2008).
mTrp99 is also nitrated by peroxynitrite, but protein inactivation has been ascribed to tyrosine nitration.
NR = not reported, ND = not detected.
formation, which is in turn reduced by thioredoxin or other reducing agents. However, when peroxynitrite is in excess, over-oxidation of the peroxidatic thiol to sulfenic acid (Peshenko et al., 2001), as well as tyrosine nitration and dimerization, have also been reported (Manta et al., 2008). The functional significance of the latter processes is still to be demonstrated.

The importance of Prxs for peroxynitrite detoxification at the cellular level has been confirmed in several systems. In Saccharomyces cerevisiae there exist five Prxs, including the thiol-specific antioxidant enzymes TSA1 and TSA2. Yeasts deficient in these two proteins were hypersensitive to peroxynitrite, and the phenotype was rescued by the expression of either the tsa1 or tsa2 gene (Wong et al., 2002). In Trypanosoma cruzi, both cytosolic and mitochondrial Prxs efficiently decomposed exogenous and endogenously generated peroxynitrite in intact cells, and protective effects were lost by mutation of the peroxidatic cysteine residues in both enzymes (Piacenza et al., 2008). Peroxiredoxins present in Leishmania chagasi also increased survival during the oxidative stress associated with infection (Barr and Gedamu, 2003). Moreover, mitochondrial Prx 3 protected hippocampal neurons from excitotoxic injury in vivo and decreased associated protein tyrosine-nitration (Hattori et al., 2003). An interplay between •NO and Prxs, by which •NO modulates both redox state and expression of these enzymes, has been proposed (Abbas et al., 2008; Diet et al., 2007).

Selenocysteine oxidation
Selenium-containing compounds have also been reported to react directly with peroxynitrite (Masumoto and Sies, 1996). Most probably, the reacting species include selenolate (RSe\(^{-}\)) and peroxynitrous acid. As for thiols, peroxynitrite-mediated selenol oxidation is a two-electron oxidation process yielding nitrite and seleninic acid (RSeOH) which in turn reacts with thiols completing the catalytic cycle.

GLUTATHIONE PEROXIDASE I
Reduced glutathione peroxidase type I (GPx I) from bovine erythrocytes, which is a tetrameric enzyme, reacts with peroxynitrite with a second order rate constant of \(8 \times 10^{6}\) M\(^{-1}\)s\(^{-1}\) at pH 7.4 and 25°C, and the resulting oxidized form of the enzyme is reduced by the glutathione–glutathione reductase system at the expense of NADPH (Briviba et al., 1998). The concentration of GPx can reach micromolar values in cellular systems (Arteel et al., 1999); therefore, the enzyme should be regarded as a possible target for peroxynitrite, at least in those tissues with higher GPx expression (i.e. 2µM), since the product of rate constant times concentration (\(k' = 16\) s\(^{-1}\), 25°C) is considerable and would be twice as high at 37°C (Trujillo et al., 2008b). Since \(k'\) for the reaction between peroxynitrite and CO\(_2\) is even higher in most cellular compartments (~60 s\(^{-1}\)), research concerning potential reactions between \(^{\cdot}\)NO\(_2\)/CO\(_3\) and selenol-containing compounds would be of interest.

The in vivo role of GPx in protection against peroxynitrite-mediated cytotoxicity is controversial: although experimental evidence towards a protective role exists, it is scarce (Jozsef and Filep, 2003), and animal models have failed to demonstrate such a role. On the contrary, GPx increased apoptosis induced by peroxynitrite in murine hepatocytes (Fu et al., 2001a,b).

Further studies are required to determine whether other GPx types, either selenium or thiol dependent, can directly react with peroxynitrite. Moreover, other selenium-containing proteins, such as thioredoxin reductase and selenoprotein P have also been reported to react with peroxynitrite (Sies and Arteel, 2000), but mechanistic and kinetic studies for those reactions are still to be addressed.

Methionine oxidation
Peroxynitrite oxidizes methionine residues in proteins either by direct or indirect mechanisms. Direct peroxynitrite-mediated methionine oxidation is a two-electron process leading to
methionine sulfoxide formation, for which enzymatic routes of reduction involving methionine sulfoxide reductases have been described (Fomenko et al., 2008; Kim and Gladyshev, 2005; St John et al., 2001). The reaction between peroxynitrite and free methionine is quite slow (3.6 $\times$ 10$^{-2}$ M$^{-1}$s$^{-1}$ at 37°C and pH 7.4; Alvarez et al., 1999; Pryor et al., 1994), and the existence of protein factors that could result in increased peroxynitrite direct reactivity towards methionine residues in proteins has not been addressed. According to Perrin and Koppenol (2000), the reaction is much faster with peroxynitrous acid than with peroxynitrite anion (1700 vs. 8.6 M$^{-1}$s$^{-1}$), and peroxynitrite is mainly reduced to nitrite, although isomerization to nitrate also occurs. Peroxynitrite-derived radicals can oxidize methionine by a one-electron mechanism finally leading to ethylene (Pryor et al., 1994). In the case of methionine residues in peptides or proteins, intramolecular electron transfer reactions from the initial methionine radical cation to other amino acid, namely cysteine, have been proposed (Zhang et al., 2008).

**ALPHA 1-ANTITRYPsin**

Peroxynitrite inactivates alpha 1-antitrypsin, the most abundant extracellular anti-protease in the lung, by oxidizing the methionine residue in the inhibitory active site to methionine sulfoxide (Moreno and Pryor, 1992). Kinetic data for this reaction, that could help to rationalize the importance of this enzyme as a target for peroxynitrite, are lacking. Other enzymes that have been reported to form methionine sulfoxide upon addition of peroxynitrite include bacterial glutamine synthetase in the absence of CO$_2$, whereas in its presence tyrosine nitration is instead the main protein modification observed (Tien et al., 1999).

Peroxynitrite-mediated calmodulin oxidation led to methionine sulfoxide formation at different positions (Galeva et al., 2005; Smallwood et al., 2003), although tyrosine nitration also occurred (Table 1).

**Tryptophan oxidation**

Peroxynitrous acid reacts with tryptophan directly ($k = 37$ M$^{-1}$s$^{-1}$, pH 7.4, 37°C; Alvarez et al., 1996). This direct reaction is not very fast. Therefore, unless accelerated by protein environment, oxidation of tryptophan in most biological compartments would be an indirect process, produced by peroxynitrite-derived radicals. Carbonate radicals react with tryptophan residues faster than with other amino acid residues in proteins ($k = 10^8$ M$^{-1}$s$^{-1}$ vs $10^7$ M$^{-1}$s$^{-1}$ with cysteine or tyrosine residues; Chen and Hoffman, 1973). Hydroxyl radicals and $^1$NO$_2$ are also able to oxidize tryptophan to its radical. In fact, the formation of this radical has been detected by EPR (Pietraforte and Minetti, 1997). The recombination with $^1$NO$_2$ leads to tryptophan nitration, mainly 6-nitrotryptophan, although other nitrated isomers and oxidation products are also formed (Alvarez et al., 1996; Yamakura and Ikeda, 2006). It has been postulated that introduction of a nitro group (-NO$_2$) to the 6- or other positions of Trp residues in proteins could affect the structural role of that Trp in a protein by increasing the bulk at this position and/or affect the electron state of the indole ring through the attractive effect of the nitro group (Yamakura et al., 2005). An antibody against 6-nitrotryptophan allowing its detection in selected proteins, as well as in cellular systems, has been developed (Ikeda et al., 2007).

The formation of a hydroxyl-nitro derivative of tryptophan (5-hydroxy-6-nitrotryptophan) has been shown to occur in vivo in succinyl-CoA:3-oxoacid CoA transferase (SCOT), in rat heart mitochondria. Modified SCOT has been shown to associate with an elevation of its activity and to accumulate progressively with age (Rebrin et al., 2007).

**HUMAN CuZnSOD**

CuZnSOD directly reacts with peroxynitrite with a $k$ of $1 \times 10^4$ M$^{-1}$s$^{-1}$ at pH 7.5 and 37°C, leading to complete protein inactivation (Alvarez et al., 2004). From the pH profile of enzymatic inactivation, peroxynitrite anion was suggested as the oxidizing species. The mechanism of the reaction involves reaction at the copper center of the enzyme active site, forming
•NO₂ and a strong oxidant, possibly a highly oxidizing hydroxyl radical (free or bound to the active site cupric ion), which in turn oxidizes a neighboring histidine residue, as was detected by EPR (Alvarez et al., 2004). The enzyme was also inactivated when exposed to simultaneous fluxes of O₂•⁻ and •NO, even when flux ratios were varied (Demicheli et al., 2007). In the presence of physiological concentrations of CO₂, however, tryptophan nitration is the main protein modification observed. This is in agreement with CO₂ being a preferential target for peroxynitrite at physiologically relevant concentrations of CO₂ and CuZnSOD, leading to CO₃²⁻ and •NO₂ formation, and thereby re-directing the oxidation towards the single tryptophan residue in the enzyme, which results in only a slight decrease in enzymatic activity (Yamakura et al., 2005). In addition, CuZnSOD has been shown to increase yields of peroxynitrite-mediated tyrosine nitration in vitro, most probably by reaction of the oxidizing species formed at the level of cupric ion with tyrosine residues followed by recombination with •NO₂ (Crow et al., 1997; Ischiropoulos et al., 1992). The role of the enzyme in promoting protein nitration in vivo was suggested by the diminished hepatic protein nitration mediated by acetaminophen or lipopolysaccharide in CuZnSOD⁻/⁻ mice (Jian-Hong et al., 2008). Mutations in CuZnSOD have been implicated in familial amyotrophic lateral sclerosis through the gain of a new and toxic function, either the promotion of oxidative processes or the induction of protein aggregation. It has been proposed that mutated forms of CuZnSOD have a decreased affinity for zinc ion, leading to increased superoxide and hence peroxynitrite formation (Beckman et al., 2001; Estevez et al., 1999).

Reactions with metal-containing protein cofactors

Peroxynitrite can directly oxidize transition metals contained in proteins and low molecular weight compounds, particularly those containing heme and non-heme iron, copper and manganese ions, with rates ranging from 10⁴ to 10⁷ M⁻¹s⁻¹ (Table 2).

The oxidation of the metal center can occur through a one-electron process that results in peroxynitrite reduction to •NO₂, or through a two-electron process leading to nitrite (NO₂⁻). Moreover, some metal-containing proteins catalyze peroxynitrite isomerization to nitrate (NO₃⁻). A picture of these different reactions is shown in Fig. 3, which illustrates heme protein oxidations by peroxynitrite.

In the case of the hemoproteins myeloperoxidase and cytochrome P450, reaction with peroxynitrite leads to ferryl-oxo compounds as intermediates plus •NO₂ (Daiber et al., 2000; Floris et al., 1993; Furtmüller et al., 2005). These constitute secondary oxidizing species that can react with exogenous or endogenous targets. Reaction with sacrificial reductants such as ascorbic acid can regenerate the metal center and nitrite, and in such cases the metalloprotein can be considered to catalytically reduce peroxynitrite. In contrast, reaction of the secondary oxidants with critical amino acids nearby may lead to loss of function of the protein. Accordingly, reaction with tyrosine residues, together with the formation of •NO₂, is the basis of metal-catalyzed tyrosine nitration, as will be illustrated by the example of MnSOD, where the initial reaction of peroxynitrite with the metal center leads to the site-specific nitration of nearby tyrosine residues. A similar mechanism is responsible for peroxynitrite-mediated prostacyclin synthase inhibition, involving specific nitration of Tyr430, which is close to the iron-thiolate center of the enzyme (Schmidt et al., 2003; Zou, 2007). Metal-oxo species of transition metals in proteins may alter other amino acids, as indicated above for peroxynitrite-mediated histidine oxidation in CuZnSOD. Similarly, peroxynitrite has been postulated as an alternative oxygen donor for the hydroxylation of proline in hypoxia inducible factor 1α under low oxygen availability, through a series of reactions involving the oxidation of the ferric iron of prolyl hydroxylases (Sumbayev and Yasinska, 2006).

Coordination is critical in modulating heme reactivity towards peroxynitrite. In contrast to penta-coordinated globins, peroxidases and Cyt P450, hexa-coordinated heme proteins such as cytochrome c (Gebicka and Didik, 2003; Thomson et al., 1995) and neuroglobin (Herold et al., 2003).
### Table 2: Mechanisms and kinetics of the reactions of metal-containing proteins with peroxynitrite

<table>
<thead>
<tr>
<th>Protein</th>
<th>k (M$^{-1}$s$^{-1}$)</th>
<th>Conditions</th>
<th>Products</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heme proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxyhemoglobin</td>
<td>$1.7 \times 10^4$</td>
<td>37°C, pH 7.4</td>
<td>Methemoglobin, O$_2^-$ and NO$<em>3^-$; low yields of O = Fe$^V</em>{IV}$Hb</td>
<td>(Romero and Radi, 2005)</td>
</tr>
<tr>
<td>Cytochrome c$^{2+}$</td>
<td>$2.3 \times 10^5$</td>
<td>25°C, pH independent</td>
<td>Fe$^{II}$ cytochrome c and ·NO$_2^-$</td>
<td>(Thomson et al., 1995)</td>
</tr>
<tr>
<td>Cytochrome c$^{3+}$</td>
<td>ND$^c$</td>
<td></td>
<td></td>
<td>(Gebicka and Didik, 2003)</td>
</tr>
<tr>
<td>Myeloperoxidase (Fe$^II$)</td>
<td>$1.3 \times 10^6$</td>
<td>25°C, pH 7</td>
<td>Compound II and NO$_2^-$</td>
<td>(Furtmuller et al., 2005)</td>
</tr>
<tr>
<td>Myeloperoxidase (Fe$^{III}$)</td>
<td>$2 \times 10^7$</td>
<td>12°C, pH independent</td>
<td>Compound II and ·NO$_2^-$</td>
<td>(Floris et al., 1993)</td>
</tr>
<tr>
<td>Lactoperoxidase</td>
<td>$6.8 \times 10^6$</td>
<td>25°C, pH 7</td>
<td>Compound II and ·NO$_2^-$</td>
<td>(Furtmuller et al., 2005)</td>
</tr>
<tr>
<td>Horseradish peroxidase</td>
<td>$3.2 \times 10^6$</td>
<td>25°C, pH 7.4</td>
<td>Compound II and ·NO$_2^-$</td>
<td>(Floris et al., 1993)</td>
</tr>
<tr>
<td>Catalase</td>
<td>ND$^c$</td>
<td></td>
<td></td>
<td>(Floris et al., 1993)</td>
</tr>
<tr>
<td>Cytochrome c oxidase</td>
<td>&gt; $10^6$</td>
<td>RT$^e$, pH 7.4</td>
<td>Two-electron oxidation and NO$_2^-$</td>
<td>(Pearce et al., 1999)</td>
</tr>
<tr>
<td>Metmyoglobin</td>
<td>$7.7 \times 10^4$</td>
<td>20°C, pH 7.0</td>
<td>Metmyoglobin and NO$_3^-$</td>
<td>(Herold and Shivashankar, 2003)</td>
</tr>
<tr>
<td>Methemoglobin</td>
<td>$3.9 \times 10^4$</td>
<td>20°C, pH 7.0</td>
<td>Methemoglobin and NO$_3^-$</td>
<td>(Herold and Shivashankar, 2003)</td>
</tr>
<tr>
<td>Catalase-peroxidase (Mycobacterium tuberculosis)</td>
<td>$1.4 \times 10^5$</td>
<td>37°C, pH 7.4</td>
<td>Compound II and ·NO$_2^-$</td>
<td>(Wengenack et al., 1999)</td>
</tr>
</tbody>
</table>
Continued

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k$ (M$^{-1}$s$^{-1}$)</th>
<th>Conditions</th>
<th>Products</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandin-endoperoxide synthase 1</td>
<td>$1.7 \times 10^7$</td>
<td>8°C, pH 7</td>
<td>Compound I and NO$_2^-$</td>
<td>(Trostchansky et al., 2007)</td>
</tr>
<tr>
<td>Oxygenase domain of inducible NOS$^d$</td>
<td>$2.2 \times 10^5$</td>
<td>pH 7.4</td>
<td>Compound II-like heme and ·NO$_2$</td>
<td>(Marechal et al., 2007)</td>
</tr>
<tr>
<td>Chloroperoxidase$^d$</td>
<td>$3.8 \times 10^6$</td>
<td>23°C, pH 7.1</td>
<td>Compound II and ·NO$_2$</td>
<td>(Gebicka and Didik, 2007)</td>
</tr>
<tr>
<td>Cytochrome P450 102 (<em>Bacillus megaterium</em>)$^d$</td>
<td>$1 \times 10^6$</td>
<td>12°C, pH 6.8</td>
<td>Compound II and ·NO$_2$$^a$</td>
<td>(Daiber et al., 2000)</td>
</tr>
<tr>
<td><strong>FeS-clusters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mitochondrial [4Fe4S] aconitase</td>
<td>$1.1 \times 10^5$</td>
<td>25°C, pH 7.6</td>
<td>[3Fe4S]aconitase and ·NO$_2$</td>
<td>(Tortora et al., 2007)</td>
</tr>
<tr>
<td><strong>Zn-thiolate center</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcohol dehydrogenase (yeast)</td>
<td>2.6-5.2 $\times 10^5$</td>
<td>23°C, pH 7.4</td>
<td>Zn release and thiolate oxidation</td>
<td>(Crow et al., 1995)</td>
</tr>
<tr>
<td><strong>Mn proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Mn superoxide dismutase</td>
<td>$1 \times 10^5$</td>
<td>37°C, pH 7.4</td>
<td>O = Mn$^V$ SOD and ·NO$_2$$^b$</td>
<td>(Quijano et al., 2001)</td>
</tr>
<tr>
<td><strong>Cu$^{2+}$ proteins</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human CuZn superoxide dismutase</td>
<td>$1 \times 10^4$</td>
<td>37°C, pH 7.5</td>
<td>Cu$^{2+}$·OH·SOD + ·NO$_2$</td>
<td>(Alvarez et al., 2004)</td>
</tr>
</tbody>
</table>

$^a$Leading to tyrosine 334 nitration (which is not so near the heme; it is an example of intramolecular electron transfer according to the authors) and thiolate oxidation, which is responsible for enzyme inactivation.

$^b$Leading to Tyr 34 nitration and dimerization.

$^c$RT = room temperature, ND = no appreciable reaction under the experimental conditions employed.

$^d$Heme thiolate proteins.
do not react with peroxynitrite in the ferric state. In the case of the one-electron oxidation of cytochrome c, peroxynitrite reacted with the ferrous but not the ferric form, possibly through an outer sphere electron transfer process (Thomson et al., 1995). In summary, depending on the transition metal center, the outcome of peroxynitrite reaction can be decomposition to nitrate or nitrite or enhancement of one-electron oxidative processes, sometimes leading to protein modification through amino acidic oxidation and/or nitration.

**Oxyhemoglobin**

Peroxynitrite directly reacts with oxyhemoglobin (oxyHb, Hb(Fe$^{2+}$)O$_2$) with a rate constant of $2 \times 10^4$ M$^{-1}$s$^{-1}$ at 37°C and pH 7.4 to yield methemoglobin (metHb, Hb(Fe$^{3+}$)) as final product (Denicola et al., 1998; Romero and Radi, 2005). Analysis of the pH profile revealed that peroxynitrous acid (ONOOH) is the reacting species (Denicola et al., 1998). The mechanism of the reaction has been controversial. However, after a complete analysis of the reaction products and stoichiometry under conditions of excess protein over peroxynitrite (as expected to occur in vivo), we proposed that peroxynitrite reacts with oxyHb displacing superoxide radical from the heme center and yielding a metHb–peroxynitrite compound. This intermediate can rapidly decompose via peroxo bond homolysis to yield ‘caged’ ferrylHb and $\bullet$NO$_2$ that mainly recombine to metHb and nitrate. Only a small fraction of the radicals can diffuse out and are responsible for ‘free’ ferrylHb formation that, by reaction with nearby amino acids, results in low yields of globin-derived radicals and oxidative modifications (Romero et al., 2003).

The reaction of oxyHb with peroxynitrite in the presence of CO$_2$ has also been studied (Bocini and Herold, 2004; Minetti et al., 2000). However, inside red blood cells the rate...
constant value of the reaction between oxyHb and peroxynitrite and the high concentration of this protein (20 mM heme) determine the $k'$ for this reaction as $340 \, s^{-1}$, indicating that the reaction of peroxynitrite with CO$_2$ ($k' = 60 \, s^{-1}$) would not be relevant inside these cells. Within red blood cells, kinetic analysis indicates that peroxiredoxin 2 (240 µM) is the main target for peroxynitrite ($k' = 3360 \, s^{-1}$ at 25°C) (Manta et al., 2008), but oxyhemoglobin may outcompete peroxiredoxin 2 under conditions of bolus peroxynitrite addition or exposure to very high fluxes of peroxynitrite or other peroxides, which could overcome the capability of Prx reduction by the thioredoxin system (Low et al., 2007). In conclusion, oxyHb reaction with peroxynitrite, in contrast to other hemeproteins, does not promote nitration processes and could contribute as a detoxifying pathway for peroxynitrite in red blood cells, converting it to the inert species nitrate and yielding metHb that could be recovered by the enzymatic metHb-reductase system.

**ACONITASES**

Aconitases are iron-sulfur proteins which catalyze the reversible isomerization of citrate and isocitrate via cis-aconitate. While in mitochondria aconitase is part of the citric acid cycle, in the cytosol aconitase is a trans-regulatory factor that controls iron homeostasis at a post-transcriptional level. Both isoforms (mitochondrial, m-; and cytosolic, c-) contain a [4Fe-4S] prosthetic group in which one of the irons, Fe$_\alpha$, is not ligated to a protein residue, and thus can bind to hydroxyl groups of substrates or water (Beinert and Kennedy, 1993). Peroxynitrite reacts with the [4Fe-4S]-cluster of m-aconitase, yielding the inactive [3Fe-4S]-enzyme ($k = 1.1 \times 10^8 \, M^{-1}s^{-1}$) (Castro et al., 1994; Tortora et al., 2007). Carbon dioxide enhances peroxynitrite-dependent inactivation, via reaction of CO$_3$•$^-$ with the [4Fe-4S]-cluster ($k = 3 \times 10^8 \, M^{-1}s^{-1}$) (Tortora et al., 2007). Because of the capacity of peroxynitrite (and secondarily CO$_3$•$^-$) to perform univalent oxidations, its net reactivity towards aconitase will resemble that of O$_2$•$^-$.

The aconitase iron-sulfur cluster has a net oxidation state of +2, with the local positive charge of the exposed Fe$_\alpha$ offering an electrostatic attraction for these anionic oxidants. The transfer of a single electron from the exposed cluster to peroxynitrite (and CO$_3$•$^-$) can destabilize the cluster, causing the loss of Fe$_\alpha$ and the inactivation of the enzyme. Peroxynitrite also nitrates tyrosine residues of m-aconitase (Tyr 151 and Tyr 472 for porcine enzyme) (Han et al., 2005), that are located close to the active site, probably reflecting the site-specific formation of •NO$_2$ following the reaction of peroxynitrite with the active [4Fe-4S] cluster. The reactivity of peroxynitrite toward m-aconitase in vivo is further supported by the fact that proteomic analysis of mitochondria obtained from sepsis, diabetes and animal models of amyotrophic lateral sclerosis, as well as aged rats, reveal that m-aconitase is nitrated (Aulak et al., 2001, 2004; Casoni et al., 2005; Kanski et al., 2005; Turko et al., 2003). Nitrated m-aconitase found in vivo represents a footprint of reactive nitrogen species formed during pathology (and probably a signal for protein degradation (Bota and Davies, 2002; Bota et al., 2002) but does not reflect the degree of enzyme inactivation since tyrosine nitration is not involved in the mechanisms of inactivation (Tortora et al., 2007).

Peroxynitrite also reacts with the Fe-S cluster of c-aconitase in different cell culture types, promoting total cluster disruption with the consequent enzyme inactivation, but turning on its iron-responsive activity (Castro et al., 1998; Soum et al., 2003). Also, and depending on peroxynitrite concentration, Cys oxidation of apo c-aconitase could result in an iron-responsive activity decrease because Cys residues are necessary for protein–RNA interactions (Bouton et al., 1997; Soum et al., 2003).

**Peroxynitrite reactions with coenzymes**

In addition to metal cofactors, other prosthetic groups in proteins have been reported to react with peroxynitrite. For example, peroxynitrite and peroxynitrite-derived radicals react with the dithiol dihydrolipoic acid and its corresponding disulfide, lipoic acid, which...
are cofactors of mitochondrial dehydrogenases and are also found free in different tissues at micromolar concentrations when orally supplemented (Trujillo and Radi, 2002). Peroxynitrite inactivates the pyruvate dehydrogenase complex in vitro, and tyrosine nitration has been postulated as the main cause of protein inactivation, although the role of lipooamide oxidation in the observed inactivation was not evaluated (Richards et al., 2006).

Tetrahydrobiopterin is also oxidized by peroxynitrite (most probably by peroxynitrite-derived radicals). This has been reported to cause NOS uncoupling leading to increased \( \cdot{O_2}^- \) production (Kuzkaya et al., 2003; Sun et al., 2008). This mechanism is considered to be an important contributor in the endothelial dysfunction characteristic of many common clinical disorders.

Folate has also been reported to react with peroxynitrite leading to nitrated derivatives (Nakamura et al., 2002). The fully reduced forms of folate, i.e. tetrahydrofolate and 5-methyltetrahydrofolate, had the most prominent scavenging activity (Rezk et al., 2003). However, kinetic data are not available and it is not known whether the coenzymes react with peroxynitrite itself or with its derived radicals.

**Tyrosine nitration and other oxidative modifications**

**MECHANISMS OF TYROSINE REACTION WITH PEROXYNITRITE**

Peroxynitrite does not directly react with tyrosine (Alvarez et al., 1999). However, tyrosine nitration is widely utilized as a marker of peroxynitrite formation in biological milieu (Kooy et al., 1997). Although other routes for tyrosine nitration do exist (Gunther et al., 1997; van der Vliet et al., 1997), peroxynitrite is without any doubt among the main nitrating agents in vivo (Radi, 2004). Peroxynitrite-mediated tyrosine nitration (as well as dimerization and in some cases, hydroxylation) is an indirect process mediated by free radicals. Acid-catalyzed peroxynitrite homolysis leads to \( \cdot{O_2}^- \) and \( \cdot{NO_2}^- \) formation. In the case of \( \cdot{O_2}^- \), transient formation of a radical adduct with tyrosine can lead to tyrosyl radical but also to 3-hydroxytyrosine (dopa), which has been detected as a peroxynitrite-mediated modification of tyrosine under acidic pH, in proteins exposed to peroxynitrite in vitro and in cellular systems as well as in a model of *Leishmania amazonensis* infection of a mammalian host (Hensley et al., 1997; Linares et al., 2001; Santos et al., 2000). Moreover, both \( \cdot{O_2}^- \) and \( \cdot{NO_2}^- \) react with tyrosine residues to form the critical intermediate, the tyrosyl radicals, which in turn recombine with another tyrosyl radical or \( \cdot{NO_2}^- \) to form 3,3'-dityrosine or 3-nitrotyrosine, respectively (Fig. 4). Protein nitration yields are higher in the presence of \( CO_2 \), not only because of the higher radical yields, but mostly because of the more selective action of \( CO_3^{2-} \) compared with \( \cdot{O_2}^- \) as tyrosine-oxidizing agent. Metal complexes as well as metal-containing proteins can also cause increased tyrosine nitration and dimerization, as described above for CuZnSOD. The fast reaction between several such metal centers with peroxynitrite can lead to equimolar yields of \( \cdot{NO_2}^- \) and highly oxidizing species such as ferryl oxo compounds (Fig. 4). Additional mechanisms of tyrosine nitration have been postulated, namely peroxynitrite-dependent metal-bound nitronium-like species for electrophilic non-radical substitution (Ischiropoulos et al., 1992), although experimental evidence for such a process is lacking.

**TYROSINE NITRATION BY \( \cdot{NO} \) AND \( O_2^{2-} \) FLUXES**

The role of peroxynitrite as a mediator of biological nitration has been lately challenged not only by the discovery of other possible pathways of tyrosine nitration in biological systems but also by the observation that, in homogenous systems, the simultaneous production of \( \cdot{NO} \) and \( O_2^{2-} \) results in nitration yields much lower than those obtained by the bolus addition of previously synthesized peroxynitrite (Pfeiffer and Mayer, 1998; Pfeiffer et al., 2000). Additionally, when peroxynitrite was formed by fluxes of its precursors \( \cdot{NO} \) and \( O_2^{2-} \), the maximum nitration yields were obtained when the rates of precursor formation were nearly identical and decreased in the presence of an excess of either of them, determining bell-shaped nitration
profiles. In fact, the modest yields obtained with physiological fluxes of radicals do not contradict in vivo data, where 3-nitrotyrosine is not an abundant modification even under inflammatory conditions (Radi, 2004). The difference in nitration yield between bolus peroxynitrite versus fluxes of precursors can be easily understood considering that nitration involves recombination between two radical intermediates (Tyr• and •NO2) present at low concentrations; thus, in spite of the fast kinetic constant for the recombination reaction, the low concentrations of the intermediate reactants allow the direct reaction of tyrosine with •NO2 to yield tyrosyl radical (k \(= 3.2 \times 10^5 \text{M}^{-1}\text{s}^{-1}\) at pH 7.4; Prutz et al., 1985) to compete with the reaction of •NO2 with tyrosyl radical (Goldstein et al., 2000). As a consequence of •NO2 consumption, tyrosyl radical dimerization outcompetes 3-nitrotyrosine formation. Moreover, the bell-shaped nitration profile obtained in homogenous systems when one of the peroxynitrite precursors is formed at higher rates than the other is owing to reaction of the excess O2•− with tyrosyl radical or to reaction of excess *NO with *NO2 (Hodges et al., 2000; Sawa et al., 2000), as demonstrated using computer-assisted kinetic simulations. This behavior was hard to reconcile with the solid evidence demonstrating that, in biological systems, peroxynitrite-mediated nitration is associated with an increase in either *NO or O2•− formation. However, this apparent contradiction between in vitro and in vivo results can be solved if we consider two key characteristics of biological systems that prevent the accumulation of the peroxynitrite precursors: the presence of the enzyme SOD and the facile transmembrane diffusion of *NO. SOD is found in different cellular compartments at concentrations in the 1-40\(\mu\)M range (Chang et al., 1988; Halliwell and Gutteridge, 1999; Quijano et al., 2001), and catalyzes O2•− dismutation with a rate constant of 2 \(\times 10^9\) \(\text{M}^{-1}\text{s}^{-1}\) (Fielden et al., 1974; Hsu et al., 1996; Klug-Roth et al., 1973), effectively decreasing O2•− steady state concentrations in the compartment where it is being formed. On the other hand, the lipophilic character of *NO allows it to diffuse outside the cell following its concentration gradient between the places where *NO is formed and consumed, mainly by its reaction with oxyhemoglobin (k = 8 \(\times 10^7\) \(\text{M}^{-1}\text{s}^{-1}\); Herold et al., 2001).
As a consequence, increases in either NO or $O_2^{•−}$ fluxes in vivo will serve to trap more of the partner radical (and increase peroxynitrite formation) instead of accumulating and interfering with nitration pathways as occurs in vitro. Indeed, inclusion of the two ‘drain pathways’ for excess radicals (the presence of SOD and the facile transmembrane diffusion of *NO) in the computer-assisted kinetic model (Quijano et al., 2005) modified the bell-shaped nitration profiles obtained as a function of peroxynitrite precursor flux ratios to a new behavior, where the extent of 3-nitrotyrosine becomes responsive to increases in either $O_2^{•−}$ or *NO, in agreement with in vivo data.

Another important observation was made in studies of SOD inactivation by fluxes of *NO and $O_2^{•−}$ (Demicheli et al., 2007). These studies clearly show that biologically relevant concentrations of SOD compete with *NO, greatly decreasing $O_2^{•−}$ steady state concentration and leading to important increases in *NO steady state that, in turn, better compete with SOD for $O_2^{•−}$; as a consequence, in spite of the dramatic decrease in $O_2^{•−}$ steady state levels, peroxynitrite steady state is only partially affected.

An important dilemma is presented by the fact that glutathione, which is present in cells at mM concentrations, reacts rapidly with *NO$_2$ ($k = 2 \times 10^7 M^{-1}s^{-1}$, (Ford et al., 2002)) and effectively inhibits nitration mediated by peroxynitrite, *NO plus $O_2^{•−}$ fluxes, or peroxidases in the presence of nitrite and H$_2$O$_2$. Therefore, biological tyrosine nitration should predominate in those milieus where glutathione is scarce, such as membranes or extracellular compartments, or under conditions of decreased glutathione concentrations, as occur in pathological conditions associated with oxidative stress (Bharath and Andersen, 2005; Martin et al., 2000). Alternatively, non-*NO$_2$-dependent mechanisms for protein nitration such as oxidation of nitrosotyrosine should be invoked (Demicheli et al., 2007; Gunther et al., 1997). Site-specific nitration, involving the reaction of peroxynitrite within a protein metal center yielding nitrogen dioxide that reacts in situ with a tyrosyl radical formed in a nearby residue, is also a possibility to consider.

EFFECTS OF PROTEIN TYROSINE NITRATION

According to most data, protein tyrosine nitration is a relatively selective process, i.e. only a few proteins get nitrated and, within them, only one or a few tyrosine residues are modified. In the case of metalloproteins, selectivity can be easily explained since nitrated tyrosine residues are usually those close to the metal that catalyzes the nitration process, as will be illustrated by the example of MnSOD. In the case of non-metalloproteins, selectivity is harder to explain. It is clearly not related to protein abundance nor the number of tyrosine residues per protein per se (Souza et al., 1999). When analyzing nitrated proteins from both in vitro experiments and from aged rat tissues, no amino acid consensus sequence or pattern in protein primary structure was apparent (Kanski et al., 2005; Souza et al., 1999). Some common features were recognized that could facilitate specific tyrosine nitration; namely, the presence of acidic residues and of turn-inducing amino acids such as proline and glycine, as well as the absence of cysteine residues in the vicinity of the modified tyrosine (Lin et al., 2003; Lin et al., 2005; Souza et al., 1999; Souza et al., 2008b). In the case of cytochrome P450 2B1, Glu149 has been shown to direct the selective nitration of Tyr190 (Lin et al., 2003; Lin et al., 2005). However, a report of endogenous nitrated brain proteins showed the presence of basic amino acids and cysteine residues close to nitration sites (Sacksteder et al., 2006). Moreover, nitrated tyrosine residues are generally located in loops or irregularly folded regions of secondary structure, although the degree of surface accessibility of tyrosine residues does not always allow prediction of which residue gets nitrated in proteins. Therefore, further investigation is clearly required to understand the molecular basis of the observed selectivity of the tyrosine nitration process. Moreover, the distinct mechanisms of protein tyrosine nitration that could take place simultaneously in vivo, even when considering non-metalloproteins, could add complexity to the picture.
METAL-CATALYZED TYROSINE NITRATION: THE EXAMPLE OF MnSOD

Manganese-superoxide dismutase (MnSOD) is the SOD isoform found in the mitochondrial matrix of eukaryotes and various prokaryotes. This enzyme plays a crucial role maintaining low steady-states concentrations of $O_2^-$. Peroxynitrite reacts with MnSOD leading to a site-specific nitration of the critical tyrosine 34 and inactivation of the enzyme (MacMillan-Crow et al., 1998; Yamakura et al., 1998). Together with prostacyclin synthase, MnSOD is one of the representative examples where the sole nitration of a specific tyrosine residue produces complete enzyme inactivation in vivo (Zou et al., 1999b). The second order rate constants reported for the reaction with human recombinant and *E. coli* MnSOD were $1.0 \times 10^5$ and $1.4 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ at pH 7.4 and 37°C, respectively. The reaction with the apoenzyme (obtained by removing the manganese ion from the active site) had a second order rate constant $<10^4 \text{M}^{-1}\text{s}^{-1}$, and replacing the manganese by zinc did not promote nitration, supporting the central role of the manganese atom in the process (Quijano et al., 2001). The mechanism of reaction proposed is a direct reaction between peroxynitrite and the metal center of the enzyme, forming $\cdot\text{NO}_2$ and the corresponding oxo-manganese complex, that in turn promote site-specific nitration of the critical nearby Tyr34 and inactivation. The enzyme is also capable of promoting nitration of low molecular weight aromatic compounds. Similar inactivation to that observed with authentic peroxynitrite was also obtained when the enzyme was exposed to simultaneous fluxes of $\cdot\text{NO}$ and $O_2^-$, even at flux ratios different from one (Demicheli et al., 2007). The role of Tyr34 nitration in enzyme inactivation was unequivocally demonstrated by generating a recombinant MnSOD in which Tyr34 was translationally replaced by 3-nitrotyrosine. The enzyme retained only 3% enzymatic activity compared to the wild type, indicating that MnSOD nitration at this site, without any other modification, results in enzyme inactivation (Neumann et al., 2008).

Nitration and inactivation of MnSOD have been reported to occur in chronic rejection of human renal allografts (MacMillan-Crow et al., 1996), rat models of angiotensin II-induced hypertension (Guo et al., 2003) and aging (van der Loo et al., 2000). Selective antibodies against MnSOD nitrated at Tyr34 were used to detect it in renal medulla from angiotensin II-infused rats (Xu et al., 2006).

UNCATAYLIZED TYROSINE NITRATION: THE EXAMPLE OF CYTOCHROME $c^{3+}$

The heme protein cytochrome $c$, a mitochondrial peripheral inner membrane protein, participates in electron transfer during cellular respiration. More recently, its role in apoptosis has deserved considerable attention (Jiang and Wang, 2004). In this protein, the iron ion is hexacoordinated, with the fifth and sixth coordination positions interacting with His18 and Met80, respectively. Hence, its reactivity towards peroxides is slow, displaying only a weak peroxidatic activity (Radi et al., 1991). Ferrocytochrome $c$ ($\text{Fe}^{2+}$) reacts with peroxynitrite with a second order rate constant of $1.3 \times 10^4 \text{M}^{-1}\text{s}^{-1}$ at pH 7.4 and 25°C, resulting in the oxidation of the heme group to the ferric state (Thomson et al., 1995), while no significant tyrosine nitration was observed (Cassina et al., 2000). When ferricytochrome $c$ ($\text{Fe}^{3+}$) was treated with peroxynitrite, no reaction was detected at the level of the heme, but tyrosine nitration occurred (Cassina et al., 2000). Using low peroxynitrite fluxes residues Tyr97 and Tyr74 of horse ferricytochrome $c$ were preferentially nitrated (Batthyany et al., 2005). Both residues are far from the heme group (14 Å for Tyr97 and 12 Å for Tyr74) and solvent accessible, suggesting a non-heme-catalyzed nitration reaction. Nitration at Tyr74 promoted rupture of the sixth coordination bond of the heme (with Met80) owing to a steric effect of the nitro group which destabilized the mobile $\Omega$-loop of cytochrome $c$ and transmitted it to the iron center via the nearby Tyr67 (Abriata et al., 2008). These new coordination properties of the heme from the mononitrated species explain the observed increase in peroxidatic activity and also the enrichment of nitration of Tyr67 (which is 7 Å from the heme group) in the dinitrated cytochrome $c$ species, now owing to a heme-catalyzed nitration reaction. Moreover, different mononitrated forms of cytochrome $c$ showed a decreased capability for both mitochondrial electron transfer and apoptosome assembly (Cassina et al.,...
Nitroxydative stress, including rat cerebral cortex after oxygen and glucose deprivation (Alonso et al., 2002), renal ischemia/reperfusion (Cruthirds et al., 2003), and obese mice with leptin synthesis defects (Garcia-Ruiz et al., 2006). Nitrocytochrome c is readily translocated from mitochondria to the cytoplasm and nucleus in non-apoptotic cells and may have antioxidant signaling actions (Godoy et al., 2009).

Other protein modifications: carbonyl formation and protein fragmentation

Protein-bound carbonyls have been extensively used as markers of oxidative damage to proteins (He et al., 1999; Stadtman, 2001). Carbonylation can alter protein function or lead to deleterious intermolecular cross-links and aggregates that preclude their degradation by intracellular proteases. Accumulation of carbonylated proteins has been implicated in the etiology and/or progression of several chronic central nervous system disorders (Butterfield et al., 2006; Picklo et al., 2002). Peroxynitrite is one of the different oxidant species that can lead to protein carbonyl formation (either protein bound or as released products) (Headlam and Davies, 2004; Nowak et al., 2006; Szabo et al., 1997). The mechanism of such modification most probably involves peroxynitrite-derived secondary oxidants. Accordingly, products formed when exposing bovine serum albumin to SIN-1-derived fluxes of peroxynitrite showed a similar profile (though at lower yields) to those formed upon exposing to metal ion/H₂O₂ systems, probably reflecting a common mechanism of reaction, mainly involving aliphatic carbon-centered radicals and subsequent alcoxyl radicals and protein fragmentation (Headlam and Davies, 2004).

Intramolecular electron transfer: when final amino acidic modification differs from initial site of reaction

Peroxynitrite-mediated one-electron oxidations of proteins lead to protein radical formation whose precise location depends both on the mechanisms by which it is formed as well as on the stability of the radical species formed at the particular amino acid residue (Bhattacharjee et al., 2007). The initial site of radical formation may differ from the protein radical finally formed, as has been demonstrated for hydrogen peroxide-mediated myoglobin and peroxynitrite-mediated O₂ oxidations (Bhattacharjee et al., 2007; Romero et al., 2003; Witting et al., 2000). During globin oxidation, radicals have been reported to occur mostly on tyrosine (Davies, 1991; Miki et al., 1989) and/or tryptophan (Gunther et al., 1998) residues, in agreement with radical stabilization by resonance delocalization. In model peptides, electron transfer from tyrosyl radical to cysteine residues to form thiyl radicals was reported to favor disulfide formation and to inhibit tyrosine nitration and dimerization (Zhang et al., 2005a), whereas electron transfer from methionine radicals to tyrosine residues increased tyrosine modifications (Zhang et al., 2008).

Multiple amino acidic modifications

In many cases it is difficult to define which of the protein modification/s is/are the responsible for the observed effect on protein function. Separative techniques that allow the isolation of pure isoforms bearing only one modification (Batthyany et al., 2005), as well as genetic engineering techniques including site-directed mutagenesis (Adachi et al., 2004; Neumann et al., 2008), are invaluable tools for pursuing this aim.

Analytical biochemistry techniques have been used to identify specific proteins modified as well as the modification site(s) both in vitro and in vivo. In this respect, investigation of biological proteomes and protein-translational modifications requires the use of modern and robust mass spectrometry tools usually combined with immunological techniques. However, with few exceptions, available methodologies have had limited success in complex biological samples (reviewed in Ischiropoulos, 2008). Moreover, complementary approaches including pharmacological tools (Szabo et al., 2007) are usually required in order to assign specific protein modifications to peroxynitrite formation in biological systems.
**BIOLOGICAL CONSEQUENCES OF PEROXYNITRITE-MEDIATED PROTEIN OXIDATION AND NITRATION**

The biological implications of protein modifications mediated by peroxynitrite rely on different possible major effects; namely: (i) direct change in protein function; (ii) protein aggregation and subsequent biological responses; (iii) modulation of protein turnover; (iv) modulation of signaling processes; and (v) induction of immunological responses. These effects are described below.

**Loss vs gain of function**

Peroxynitrite-mediated protein modifications, including both oxidation and nitration of specific amino acids, could result in altered protein function. In this regard, tyrosine nitration decreases the pKₐ of the phenolic hydroxyl of tyrosine from ~10 to 7.2–7.5 (Cleighton, 1993), with could have important consequences for protein structure and function (Fig. 5). However, the amount of residues to be nitrated so as to produce a biologically relevant loss of function is a concern. Protein nitration is a relatively widespread *in vivo* modification observed in a large number of proteins, organs and disease conditions. However, its overall yield is typically low. For example, analysis of human bronchoalveolar lavage proteins showed 480 ± 198 and 53 ± 41 µmol of 3-nitrotyrosine per mol of tyrosine residues in asthmatic patients and control

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**Figure 5**

Formation and consequences of protein tyrosine nitration. Protein tyrosine nitration has three major effects: it may affect protein function, modulate phosphorylation cascades and induce an immunological response. Usually nitrated proteins are recognized and degraded by the proteasome system. Tyrosine nitration and dimerization may promote assembly of protein filaments or protein aggregates which will also facilitate hydrophobic interactions; these aggregates become poor proteasome substrates, and can accumulate as intra- or extracellular amyloids. Metabolism of nitrated proteins includes the potential reduction by yet-to-be-established biological reductants or the removal of the nitro group by putative denitrase activities; these processes may or may not involve the intermediate formation of 3-aminotyrosine, which in turn may be readily oxidized back to 3-nitrotyrosine (3-NT).

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subjects, respectively (MacPherson et al., 2001); similarly, the average level of protein-bound 3-nitrotyrosine in urine from healthy subjects was reported in the range of 100 µmol 3-nitrotyrosine/mol tyrosine (Chen and Chiu, 2008). Cysteine oxidation to sulfenic acid/disulfide or other peroxynitrite-mediated amino acidic modifications can potentially lead to pronounced changes in protein structure and function. Since peroxynitrite-mediated cysteine oxidation is usually a reversible process, quantitative determinations are difficult to perform. Taking into account their low abundance, these protein modifications would probably be unable to alter previously existing protein functions. Protein gain of function, on the contrary, could have pronounced biological consequences, even if scarce. The above-described acquisition of an otherwise marginal peroxidatic activity in nitrocytochrome c would be an example. Similarly, oxidation of nerve growth factor by peroxynitrite causes it to acquire an exceptional motor neuron apoptotic activity (Pehar et al., 2006). Other examples where peroxynitrite-mediated protein modification leads to increased activity include the nitration (Tyr92) of rat microsomal glutathione S-transferase (Ji et al., 2006), the S-glutathiolation (Cys674) of sarco/endoplasmic reticulum calcium (Ca\(^{2+}\)) ATPase (SERCA) (Adachi et al., 2004), as well as the oxidation to sulfenic acid of Cys298 in aldose reductase (Kaiserova et al., 2008) (Table 1).

Tyrosine oxidation and nitration involved in protein aggregation
Protein aggregation and filament formation have been implicated as key points in the pathogenesis of certain diseases. \(\alpha\)-Synuclein can be cited as an example in this field; it is a protein particularly susceptible to the effects of nitrating agents, which in addition to inducing tyrosine nitration, also induce oligomer formation via the oxidation of tyrosine residues to form 3,3\(^{-}\)-dityrosine (Souza et al., 2000b). Lewy bodies from Parkinson’s disease and other neurodegenerative diseases are characterized by intracellular \(\alpha\)-synuclein filamentous and aggregation inclusions, which were found to be nitrated, cross linked and ubiquitinated (Giaossen et al., 2000; Shults, 2006; Spillantini et al., 1997). Monomeric nitrated \(\alpha\)-synuclein speeds up the rate of fibril formation and can act as a seed when incubated with the native \(\alpha\)-synuclein, inducing the fibrillation process (Hodara et al., 2004). 3,3\(^{-}\)-Dityrosine formation in assembled \(\alpha\)-synuclein filaments strengthens the filaments to resist denaturing conditions (Souza et al., 2000b), as observed in purified Lewy bodies. In Alzheimer’s disease, Tau protein forms intracellular neurofibrillary tangles. It was shown that peroxynitrite induces tyrosine nitration and 3,3\(^{-}\)-dityrosine formation as in \(\alpha\)-synuclein (Reynolds et al., 2005; Reynolds et al., 2006). In another example, fibrinogen nitration modifies its clotting properties by accelerating thrombin-induced fibrin aggregation and factor XIII cross-linking. A profound distortion of the clot architecture has been observed in the presence of a small percentage of nitrated tyrosine residues, where the clot is transformed into a less elastic structure, more susceptible to embolization (Vadseth et al., 2004). In fact, the level of nitration in circulating fibrinogen is significantly increased under conditions with a high risk of thromboembolic episodes, like coronary artery disease and smoking (Parastatidis et al., 2008; Parastatidis et al., 2007). Endogenous fibrinogen nitration is specific for two tyrosine residues, Tyr292 and Tyr422, located in hole-b in the C-terminal side of the \(\beta\)-chain, and their nitration can accelerate fibrin lateral association by changes in the knob-B: hole-b interaction (Parastatidis et al., 2008).

As a general process, we may imagine a scenario where tiny amounts of nitrated or oxidized tyrosine residues from susceptible proteins (i.e. \(\alpha\)-synuclein, Tau, fibrinogen) act as a starting point to induce a conformation change in the unmodified protein and promote filamentous aggregates.

Protein degradation
Proteasomes are the main intracellular proteolytic systems involved in altered protein removal, and changes in their activity may contribute to the protein aggregation observed in neurodegenerative diseases (Ding and Keller, 2001; Keller et al., 2002). However, isolated
erythrocyte proteasomes exposed to mild oxidative conditions are stimulated, in line with their role in the cellular antioxidant defense (Strack et al., 1996). Peroxynitrite-mediated protein modifications have been reported to alter protein turnover (Fig. 5). Different peroxynitrite-treated proteins exhibited an enhanced proteolytic susceptibility toward degradation by proteasome (Grune et al., 2001). Peroxynitrite treatment caused an overall activation of the hydrolysis of short peptides by the XYZ 20S proteasome, without changing its caseinolytic activity, whereas, in the case of the immunoproteasome, peroxynitrite-mediated oxidation produced a significant conformational change and concomitant decrease in its enzymatic activity (Amici et al., 2003). Moreover, rat brain Lon protease, an ATP-dependent protease that degrades oxidatively modified aconitase in vitro and could play a role in defending against the accumulation of oxidized matrix proteins in mitochondria, is highly susceptible to oxidative inactivation by peroxynitrite. Decline in Lon protease activity preceded electron transport chain dysfunction and was partially reversed by glutathione supplementation of mitochondrial matrix extracts (Stanyer et al., 2008).

**Peroxynitrite as a signaling mediator**

Alterations in cell oxidative metabolism have long been recognized during differentiation and development. The discovery of specific genes and cell signaling reactions that are affected by oxidants led to the hypothesis that reactive oxygen species serve as cellular messengers in gene regulatory and signal transduction pathways. In fact, in the past few years significant experimental efforts have been devoted to explore the relationships between cellular oxidative processes and the modulation of cell signal transduction, collectively called ‘redox signaling’ (Allen and Tresini, 2000; Chung et al., 2006; Kramer and Goodyear, 2007). In this regard, the identification of peroxynitrite’s ability to nitrate tyrosine residues rapidly focused attention on phosphorylation cascades (Brito et al., 1999; Kong et al., 1996) since 3-nitrotirosine cannot be phosphorylated. Though this view was initially strongly considered, further investigation showed that peroxynitrite often promotes instead of inhibits phosphorylation signaling, by mechanisms that involve the inhibition of phosphatases via thiol oxidation and the direct activation of different protein tyrosine kinases via nitro-oxidative modification of essential amino acids. Also, novel signaling molecules can be formed by reaction of peroxynitrite with cellular targets, i.e. the formation of nitrolipids with potent PPAR-γ (peroxisome proliferator activating factor γ) agonist activity. In addition, there is considerable cell specificity in terms of response to peroxynitrite. The complexity of the subject is illustrated here with some recent examples.

(a) Endothelial cells respond to proinflammatory stimuli by producing both *NO and O$_2^•$−. A concomitant barrier dysfunction can be observed, with consequent edema. Protein phosphatase type 2A (PP2A) mediates dephosphorylation and redistribution of tight junction proteins that can cause paracellular leak in epithelial cell monolayers. Exposure of mouse skeletal muscle microvascular endothelial cell monolayers to lipopolysaccharide and interferon-γ increased permeability, which was attenuated by phosphatase inhibitors or silencing the phosphatase. PP2Ac, the catalytic subunit of PP2A, isolated from these cells, showed increased tyrosine nitration, decreased tyrosine phosphorylation and increased phosphatase activity. Therefore for PP2A, peroxynitrite signaling results in an activation (due to tyrosine nitrination) which mediates endothelial barrier dysfunction (Wu and Wilson, 2009). However, other tyrosine phosphatases have been reported to be inactivated by peroxynitrite due to the oxidation of critical cysteine residues (Takakura et al., 1999).

(b) The NF-κB family of transcription factors is an important component of stress-activated cytoprotective signal transduction pathways. Activation mechanisms require phosphorylation, ubiquitination and degradation of the inhibitor protein, IκBα. Ionizing radiation in the therapeutic dose range stimulates NF-κB activity by a mechanism in which IκBα Tyr181 is nitrated as a consequence of constitutive NOS activation,
leading to dissociation of intact IκBα from NF-κB. This mechanism does not appear to require IκBα kinase-dependent phosphorylation or proteolytic degradation of IκBα. Tyrosine 181 is involved in several noncovalent interactions with the p50 subunit of NF-κB, stabilizing the IκBα–NF-κB complex. Evaluation of hydrophobic interactions of the IκBα–p50 complex on the basis of the crystal structure of the complex is consistent with nitration disrupting these interactions and dissociating the IκBα–NF-κB complex (Yakovlev et al., 2007). Therefore, tyrosine nitration is an important post-translational regulatory modification for NF-κB activation.

(c) Protein kinase B (Akt) is a serine-threonine protein kinase that plays key roles in integrating cellular responses to growth factors and other extracellular signals. Akt activation also represents an important protective mechanism to limit apoptotic cell death under conditions of oxidative stress. The activation of Akt critically depends on phosphoinositide-3-kinase signaling. In rat cortical neurons, peroxynitrite rapidly elicited Akt-Ser(473) phosphorylation. Inhibition of the phosphoinositide-3-kinase/Akt pathway abolished the ability of peroxynitrite to prevent apoptotic death. Peroxynitrite-stimulated Akt-Ser(473) phosphorylation led to critical cysteine oxidation in phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase (PTEN). Thus, in this neuronal model, peroxynitrite exerts neuroprotection by inhibiting PTEN and hence activating the anti-apoptotic PI3K/Akt pathway (Delgado-Esteban et al., 2007).

In summary, peroxynitrite exhibits typical properties of a signaling molecule in vitro, either activating or inhibiting several major signal transduction pathways. Efforts in the near future should focus on consolidating data to confirm the relevance of peroxynitrite as a signaling molecule in vivo.

### Protein 3-nitrotyrosine as an antigen

Nitration, as well as other oxidant-induced post-translational protein modifications, has been shown to be responsible for eliciting immune responses against autologous and minimally modified proteins. For example, immunization of rabbits or mice with nitrated proteins or peptides resulted in the generation of specific anti-nitrotyrosine antibodies (Beckmann et al., 1994; Brito et al., 1999; Heijnen et al., 2006). Recently, the molecular mechanisms that may govern the immunologic responses to tyrosine-nitrated proteins and peptides have been explored. Transgenic mice that constitutively express exogenous pigeon/moth cytochrome c are tolerant against immunization with the restricted T-cell epitope that encompasses amino acids 88–103 of this protein, but exhibited a robust immune response when Tyr97 was substituted by 3-nitrotyrosine (Birnboim et al., 2003). The same research group proved that the conversion of Tyr4 to 3-nitrotyrosine in the MHC I-restricted epitope of lymphocytic choriomeningitis virus glycoprotein (gp33) completely abrogated recognition by gp33-specific T cells. Conversely, CD8+ T cells specific against nitrated gp33 were readily elicited by immunization with 3-nitrotyrosine-modified gp33 (Hardy et al., 2008). Additionally, monoclonal antibodies against nitrated peptides from hen egg-white lysozyme were elicited in transgenic mice that express the protein as self (Herzog et al., 2005). Notably, an auto-antibody generated by a hybridome isolated from the spleen of BXD2 mice, which spontaneously develop glomerulonephritis and erosive arthritis, reacted against Tyr286-nitrated enolase but not against the native enzyme or against the enzyme nitrated at the Tyr279 residue (Hsu et al., 2006). An adaptative immune response that exacerbated Parkinson’s disease in 1-methyl-4-phenyl-1.2.3.6-tetrahydropyridine (MPTP)-intoxicated mice was induced by 3-nitrotyrosine-modified α-synuclein, showing a probable connection between autoimmunity and neurodegeneration (Benner et al., 2008). In the case of patients suffering chronic inflammatory conditions such as rheumatoid arthritis, osteoarthritis and systemic lupus erythematosus, high levels of antibodies against 3-nitrotyrosine were found (Khan and Siddiqui, 2006). The levels of immunoglobulins that recognize 3-nitrotyrosine
were also significantly higher in the plasma of subjects with acute lung injury after a major trauma, as compared with both normal control subjects, and subjects with major trauma that did not develop the lung complication (Thomson et al., 2007). Altogether, these data show that protein nitration can generate the emergence of neo-epitopes, able to trigger immune responses against autologous proteins and profoundly influence immunologic responses in autoimmune as well as inflammatory and degenerative diseases.

CONCLUSIONS
In summary, many biological consequences of peroxynitrite formation are related to post-translational protein modifications. These can be performed by peroxynitrite directly, such as oxidation of fast reacting thiols and transition metal cofactors. In other cases, including protein tyrosine nitration, modifications rely on secondary oxidants. Peroxynitrite-dependent protein modifications have been detected in vitro and in vivo and can translate into changes in protein function. Their significance in protein turnover and signaling processes is under intense investigation. Moreover, emerging experimental evidence supports the participation of tyrosine nitration in immune responses. The biochemistry of peroxynitrite–protein interactions is associated with physiological and pathophysiological processes; understanding of this field is opening avenues for the treatment of human disorders.

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Chapter 3

Protein Oxidation and Nitration by Peroxynitrite


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