Amino Acids (2003) 25: 295–311
DOI 10.1007/s00726-003-0018-8

Amino Acids
Printed in Austria

Peroxynitrite reactivity with amino acids and proteins

Review Article

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Received November 15, 2002
Accepted May 8, 2003
Published online September 26, 2003;

Summary. Peroxynitrite, the product of the fast reaction between nitric oxide ($^{\bullet}NO$) and superoxide ($O_2^{\bullet-}$) radicals, is an oxidizing and nitrating agent which is able to traverse biological membranes. The reaction of peroxynitrite with proteins occurs through three possible pathways. First, peroxynitrite reacts directly with cysteine, methionine and tryptophan residues. Second, peroxynitrite reacts fast with transition metal centers and selenium-containing amino acids. Third, secondary free radicals arising from peroxynitrite homolysis such as hydroxyl and nitrogen dioxide, and the carbonate radical formed in the presence of carbon dioxide, react with protein moieties too. Nitration of tyrosine residues is being recognized as a marker of the contribution of nitric oxide to oxidative damage. Peroxynitrite-dependent tyrosine nitration is likely to occur through the initial reaction of peroxynitrite with carbon dioxide or metal centers leading to secondary nitrating species. The preferential protein targets of peroxynitrite and the role of proteins in peroxynitrite detoxifying pathways are discussed.

Keywords: Peroxynitrite – Amino acids – Cysteine – Nitrotyrosine – Nitric oxide – Superoxide

Introduction

Shortly after the discovery of the free radical nitric oxide ($^{\bullet}NO$) as a cellular messenger, its reaction with superoxide ($O_2^{\bullet-}$) to form peroxynitrite[1] was proposed in order to explain the toxicity linked to their excess formation (Beckman et al., 1990; Radi et al., 1991). Indeed, peroxynitrite is a powerful oxidant, more reactive than its precursors nitric oxide and superoxide, and has been implicated in an increasing list of diseases, including atherosclerosis, inflammation and neurodegenerative disorders. Peroxynitrite can react with different biomolecules including proteins, and lead to changes in structure and function. In this paper we address the biochemistry of peroxynitrite in the context of its reactions with amino acids and proteins, which serves to provide a molecular basis for its deleterious effects *in vivo* as well as its possible detoxifying mechanisms.

[1]NOTE: The IUPAC recommended names of nitric oxide, peroxynitrite anion and peroxynitrous acid are nitrogen monoxide, oxoperoxonitrate (1-) and hydrogen oxoperoxonitrate, respectively

Peroxynitrite formation, diffusion and reactivity

The biochemical properties of peroxynitrite are described in previous reviews (Radi et al., 2000; Trujillo et al., 2000; Radi et al., 2001). Its key aspects are summarized in Fig. 1 and outlined briefly in this section.

The main pathway of peroxynitrite formation is the recombination reaction between nitric oxide and superoxide. This reaction is near to the diffusion-controlled limit, with an average rate constant of $10^{10}\,M^{-1}\,s^{-1}$ (Huie and Padmaja, 1993; Goldstein and Czapski, 1995b; Kissner et al., 1997).

$$^{\bullet}NO + O_2^{\bullet-} \rightarrow ONOO^- \quad (1)$$

Since nitric oxide is neutral and hydrophobic, capable of traversing membranes, while superoxide is anionic at neutral pH (pKa = 4.8), peroxynitrite formation will occur predominantly close to the sites of superoxide formation. In turn, peroxynitrite will traverse membranes by passive

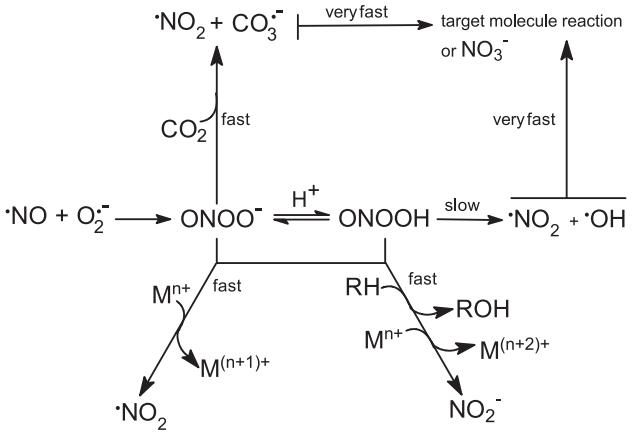

Fig. 1. Overview of peroxynitrite reaction pathways. Peroxynitrite is formed from the diffusion-controlled reaction between nitric oxide and superoxide radicals. Peroxynitrite anion and peroxynitrous acid ($pK_a = 6.8$) promote direct one- or two-electron oxidation reactions in transition metal centers and other biomolecules and yield nitrogen dioxide or nitrite respectively. Peroxynitrite anion can also react fast with carbon dioxide to secondarily yield nitrogen dioxide and carbonate radicals. Alternatively, peroxynitrous acid can undergo homolysis to hydroxyl and nitrogen dioxide radicals. The secondary peroxynitrite-derived radicals can initiate one-electron oxidations in target biomolecules or recombine to yield nitrate

diffusion as its conjugated acid, peroxynitrous acid (ONOOH, pKa = 6.8) or, in the anionic form, through anion channels (Denicola et al., 1998).

Peroxynitrite anion is relatively stable. However, peroxynitrous acid decays rapidly, with an apparent rate constant of 0.9 s^{-1} at 37 °C and pH 7.4. This is due to the fact that peroxynitrous acid homolyzes to form nitrogen dioxide ($^{\bullet}NO_2$) and hydroxyl radicals ($^{\bullet}OH$). Initially formed in a solvent cage, 70% of the radicals recombine inside it forming nitrate, while 30% escape from the cage yielding free hydroxyl and nitrogen dioxide radicals (Beckman et al., 1990; Augusto et al., 1994; Radi et al., 2000). The main product from peroxynitrite decay in the absence of targets is nitrate (Anbar and Taube, 1954; Bohle and Hansert, 1997), while secondary reactions of the radicals can also lead to nitrite and dioxygen, particularly at alkaline pH (Pfeiffer et al., 1997; Coddington et al., 1999).

Peroxynitrite is more reactive than its precursors nitric oxide and superoxide. With one- and two-electron reduction potentials of $E^{\circ\prime}[ONOO^-, 2H^+/{}^{\bullet}NO_2, H_2O] = 1.6-1.7$ V and $E^{\circ\prime}(ONOO^-, 2H^+/NO_2^-, H_2O) = 1.3-1.37$ V, respectively (Merenyi and Lind, 1997; Koppenol and Kissner, 1998), peroxynitrite is a relatively strong oxidant, able to oxidize a wide range of biomolecules. The possible fates of peroxynitrite formed *in vivo* will be determined by kinetic factors; that is, by the rate constant of the reaction of peroxynitrite with the target multiplied by the concentration of the target molecule. Up to date, the kinetics of several tens of peroxynitrite reactions have been determined through stopped-flow spectrophotometry or competition experiments (Radi, 1996; Alvarez et al., 1999; Radi et al., 2000; Ferrer-Sueta et al., 2002). These kinetic studies have enabled us to understand that peroxynitrite reacts with target molecules through two possible pathways. First, peroxynitrite anion or peroxynitrous acid can react directly with a certain target molecule in an overall second-order process. For example, this is the case of thiol oxidation. Second, peroxynitrous acid can first homolyze to form nitrogen dioxide and hydroxyl radicals, which in turn react with the target molecule. The latter processes are first order in peroxynitrite but zero order in target, because the formation of the radicals is rate-limiting ($k = 0.9\ s^{-1}$). To this last type of reaction belong tyrosine nitration and lipid peroxidation. Certainly, molecules that react directly with peroxynitrite (e.g. thiols) will also be oxidized by the nitrogen dioxide and hydroxyl radicals derived from its homolysis.

In principle, the fact that peroxynitrite can form hydroxyl radical provides a novel mechanism that is independent of metal centers for the formation of this extremely potent oxidant. However, as will be shown throughout this chapter, *in vivo* there are present several molecules that react directly with peroxynitrite with relatively high rate constants, so that the contribution of the hydroxyl radical pathway to peroxynitrite toxicity is minimal, and most peroxynitrite (>99%) will react before homolyzing.

One of the most biologically relevant reactions of peroxynitrite is that with carbon dioxide, which is present in biological systems at the relatively high concentration of 1.3–1.5 mM. Carbon dioxide reacts with peroxynitrite with a second-order rate constant of $4.6 \times 10^4\ M^{-1}\ s^{-1}$ at pH 7.4 and 37 °C (Lymar and Hurst, 1995; Denicola et al., 1996), leading to the formation of nitrogen dioxide and carbonate radical ($CO_3^{\bullet-}$) (Bonini et al., 1999). 65% of the radicals formed recombine inside the solvent cage forming nitrate and regenerating carbon dioxide, while the remaining 35% is able to react with target molecules (Goldstein and Czapski, 1997; Lymar and Hurst, 1998). The reaction is thought to proceed through the formation of an adduct, $ONOOCO_2^-$, which has not yet been detected and whose lifetime is estimated to be less than 1 μs (Lymar et al., 1996; Merenyi and Lind, 1997). Carbonate and nitrogen dioxide radicals are strong one-electron oxidants (for a review see (Augusto et al., 2002a)) with reduction potentials of $E^{\circ\prime}[CO_3^{\bullet-}, H^+/HCO_3^-] =$

1.78 V and $E^{o\prime}[^{\bullet}NO_2/\ NO_2^-] = 0.99$ V (Huie et al., 1991; Koppenol et al., 1992; Lymar et al., 2000; Bonini and Augusto, 2001). So carbon dioxide, instead of being a scavenger of peroxynitrite, will rather redirect its reactivity.

Interactions between peroxynitrite and proteins

The principles about peroxynitrite reactivity just pointed out are reflected in the pathways which lead to the modification of proteins. First, peroxynitrite reacts directly with certain amino acidic residues such as cysteine and methionine. Second, prosthetic groups, and particularly transition metal centers, are likely to react fast with peroxynitrite. Third, secondary radicals derived from peroxynitrite (hydroxyl, carbonate and nitrogen dioxide radicals) also react with protein residues. The reactions of peroxynitrite with proteins will be described in detail below.

As for the reactions of peroxynitrite-derived free radicals with amino acids, it should be pointed out that those most susceptible to oxidation are the sulfur-containing (cysteine and methionine) and the aromatic ones (tryptophan, tyrosine, phenylalanine and histidine). These residues have the lowest reduction potentials and react the fastest. However, other residues as well as the peptide bond can be targets for these free radicals too.

Reactions of peroxynitrite with transition metal centers

Indeed, the reactions of peroxynitrite with transition metal centers, particularly those containing heme and non-heme iron, copper and manganese ions, are some of the fastest known for peroxynitrite, and several rate constants that have been determined for protein and non-protein metal centers are shown in Table 1 (see also Fig. 1). The analysis of the kinetics and the products formed from peroxynitrite reaction with different proteins, together with studies performed with low molecular weight model compounds, have allowed us to reach certain generalizations.

Thus, for the reaction of peroxynitrite with a metal center, it can be rationalized that, in the same way as with other Lewis acids (LA) such as the proton or carbon dioxide, the reaction proceeds to form a Lewis adduct which in turn homolyzes to yield $^{\bullet}NO_2$ and the

Table 1. Second-order rate constants of peroxynitrite reactions with protein and non-protein metal centers

Metal center	k ($M^{-1}s^{-1}$)	T (°C)	pH[a]	Reference
Mn(III)-tm-2-pyp[b]	1.85×10^7	37	7.4	(Ferrer-Sueta et al., 1999)
Mn(III)-tm-3-pyp[b]	3.82×10^6	37	7.4	(Ferrer-Sueta et al., 1999)
Mn(III)-tm-4-pyp[b]	4.33×10^6	37	7.4	(Ferrer-Sueta et al., 1999)
Fe(III)-tm-4-pyp[b]	2.2×10^6	37	7.4	(Stern et al., 1996)
Fe(III)-tmps[c]	6.45×10^5	37	7.4	(Stern et al., 1996)
Fe(III)-edta[d]	5.7×10^3	37	7.5	(Beckman et al., 1992)
Ni(II)-cyclam[e]	3.25×10^4	27	NR[h]	(Goldstein and Czapski, 1995a)
Mn(III)-tbap[f]	6.8×10^4	37	7.2	(Quijano et al., 2001)
Zn(II)-tbap[f]	4.9×10^5	37	7.2	(Quijano et al., 2001)
Myeloperoxidase (heme)	6.2×10^6	12	7.2	(Floris et al., 1993)
Lactoperoxidase (heme)	3.3×10^5	12	7.4	(Floris et al., 1993)
Horseradish peroxidase (heme)	3.2×10^6	25	ind[g]	(Floris et al., 1993)
Alcohol dehydrogenase (zinc sulfur cluster)	2.6×10^5	23	7.4	(Crow et al., 1995)
Aconitase (iron sulfur cluster)	1.4×10^5	25	7.6	(Castro et al., 1994)
Cytochrome c^{2+} (heme)	1.3×10^4	25	7.4	(Thomson et al., 1995)
	2.5×10^4	37	7.4	(Thomson et al., 1995)
Metmyoglobin	$1.0–1.4 \times 10^4$	20	7.4	(Bourassa et al., 2001; Herold et al., 2001)
Oxyhemoglobin (monomer)	1.04×10^4	25	7.4	(Denicola et al., 1998)
	$2–3 \times 10^4$	20	NR[h]	(Alayash et al., 1998)
Mn superoxide dismutase (monomer)	2.5×10^4	37	7.4	(Quijano et al., 2001)
Cu,Zn superoxide dismutase (monomer)	9.4×10^3	37	7.5	B. Alvarez et al., manuscript in preparation
Tyrosine hydroxylase	3.8×10^3	25	7.4	(Blanchard-Fillion et al., 2001)

[a] pH: this column shows the pH at which the rate constant was determined, [b] tmpyp: 5,10,15,20,-tetrakis(*N*-metil-4′-pyridyl)porphyrin, [c] tmps: 5,10,15,20,-tetrakis(2,4,6-trimetil-3,5-sulfonatofenil)porphyrin, [d] edta: ethylenediaminetetraacetic acid, [e] cyclam: 1,4,8,11-tetraazacyclotetradecane, [f] tbap: tetrakis-(4-benzoic acid) porphyrin, [g] ind: pH-independent value, [h] NR: not reported

corresponding oxyradical ($^{\bullet}O–LA^{-}$) (Radi et al., 2000; Ferrer-Sueta et al., 2002). The oxyradical may rearrange to yield the corresponding radical of the oxo-compound ($O{=}LA^{\bullet -}$) via oxidation of the Lewis acid:

$$ONOO^{-} + LA \rightarrow ONOO - LA^{-} \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}O{-}LA^{-} \rightarrow {}^{\bullet}NO_2 + O{=}LA^{\bullet -} \quad (2)$$

e.g.:

$$ONOO^{-} + H^{+} \rightarrow ONOO{-}H \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}O{-}H \quad (3)$$

$$ONOO^{-} + CO_2 \rightarrow ONOO{-}CO_2{}^{-} \rightarrow {}^{\bullet}NO_2 + {}^{\bullet}O{-}CO_2{}^{-} \quad (4)$$

$$ONOO^{-} + M^{n+} \rightarrow ONOO^{-}{-}M^{n+} \rightarrow {}^{\bullet}NO_2 + {}^{-\bullet}O{-}M^{n+} \rightarrow {}^{\bullet}NO_2 + O{=}M^{(n+1)+} \quad (5)$$

The rate-limiting step in the overall reaction and the yield of radicals diffusing out of the solvent cage depend on the Lewis acid involved. For instance, for H^{+}, homolysis is rate limiting and the yield of radicals is ~30%, whereas for many Mn complexes the formation of the adduct is the slow step and the radical yield is close to 100% (Ferrer-Sueta et al., 1999). In some cases, the metal oxo-compound has been directly observed, as for example the cytochrome P450 protein chloroperoxidase, where the ferryl intermediate was detected according to its known UV-VIS spectrum (Daiber et al., 2000).

Thus, the reaction of peroxynitrite with a transition metal can lead to the formation of a secondary oxidizing species at the metal center, plus nitrogen dioxide. The oxidizing species may be reduced back by appropriate reductants such as glutathione or ascorbic acid. Now, if the oxidizing species is formed at the metal active site of an enzyme, and reacts with a critical amino acid nearby, the outcome may be loss of function of the enzyme. This site-specific mechanism has been proposed to be operating for manganese superoxide dismutase and prostacyclin synthase, where the initial reaction of peroxynitrite with the metal center leads to the modification of nearby tyrosine residues (Zou et al., 1997; Quijano et al., 2001). Alternatively, the oxyradical or oxo-compound may react with the nitrogen dioxide formed yielding nitrate and thus catalyzing peroxynitrite isomerization (Stern et al., 1996; Herold et al., 2001). In addition, under experimental conditions of excess peroxynitrite, the metal-bound oxidizing species may be reduced back by peroxynitrite itself.

Table 2. Second-order rate constants of the reactions of peroxynitrite with free amino acids, peptides and non-metal containing proteins

Amino acid, peptide or protein	k ($M^{-1}s^{-1}$)	T (°C)	pH[a]	Reference
Glutathione peroxidase (selenocysteine, reduced)[b]	8×10^6	25	7.4	(Briviba et al., 1998)
Glutathione peroxidase (selenocysteine, oxidized)	7.4×10^5	25	7.4	(Briviba et al., 1998)
Peroxiredoxin alkylhydroperoxide reductase (cysteine)	1.51×10^6	NR[c]	7.0	(Bryk et al., 2000)
Protein tyrosine phosphatases (cysteine)	$2–20 \times 10^7$	37	7.4	(Takakura et al., 1999)
Creatine quinase (cysteine)	8.85×10^5	NR	6.9	(Konorev et al., 1998)
Glyceraldehyde 3-phosphate dehydrogenase (cysteine)	2.5×10^5	25	7.4	(Souza and Radi, 1998)
Human serum albumin (whole protein)	9.7×10^3	37	7.4	(Alvarez et al., 1999)
Human serum albumin (cysteine)	3.8×10^3	37	7.4	(Alvarez et al., 1999)
Cysteine	4.5×10^3	37	7.4	(Radi et al., 1991)
Glutathione	1.36×10^3	37	7.4	(Koppenol et al., 1992; Trujillo and Radi, 2002)
Homocysteine	7.0×10^2	37	7.4	(Trujillo and Radi, 2002)
N-Acetylcysteine	4.15×10^2	37	7.4	(Trujillo and Radi, 2002)
Lipoic acid (disulfide)	1.4×10^3	37	7.4	(Trujillo and Radi, 2002)
Selenomethionine	1.48×10^3	25	7.8	(Padmaja et al., 1997)
Methionine	$1.7–1.8 \times 10^2$	25	7.4	(Pryor et al., 1994; Perrin and Koppenol, 2000)
	3.64×10^2	37	7.4	(Alvarez et al., 1999)
N-Acetylmethionine	1.6×10^3	25	6.3	(Perrin and Koppenol, 2000)
Threonylmethionine	2.83×10^2	27	7.4	(Jensen et al., 1997)
Glycylmethionine	2.80×10^2	27	7.4	(Jensen et al., 1997)
Lysozyme	7.0×10^2	37	7.4	B. Alvarez et al., unpublished
Tryptophan	37	37	7.4	(Alvarez et al., 1996)

[a] pH: this column shows the pH at which the rate constant was determined, [b] in the case of proteins, the critical residue is shown in parenthesis, [c] NR: not reported

Peroxynitrite can also oxidize reduced metal centers by two electrons yielding the oxyradical or oxo-compound accompanied by the formation of nitrite instead of nitrogen dioxide. This is particularly relevant in the case of reduced cytochrome *c* oxidase. In the case of the one-electron oxidation of cytochrome *c*, which has all six coordination positions occupied, peroxynitrite reacted with the reduced but not the oxidized form, oxidizing the Fe^{2+} to Fe^{3+}, possibly through an outer sphere electron transfer process (Thomson et al., 1995). In most cases, oxidation of the metal center can be reverted by the appropriate reductant. However, peroxynitrite reaction with aconitase led to the disruption of the iron sulfur cluster (Castro et al., 1994).

In summary, the interactions of peroxynitrite with transition metal centers are complex and, depending on the protein, undergo through a variety of different mechanisms which may ultimately diminish or amplify the oxidative outcome.

Reactions of peroxynitrite with amino acids

The kinetics of the reactions of peroxynitrite with the twenty protein forming amino acids have been determined (Alvarez et al., 1999). The only amino acids that react directly with peroxynitrite are cysteine, methionine and tryptophan. They are the only ones that increase the rate of peroxynitrite decomposition and their second-order rate constants are shown in Table 2, together with other rate constants determined for proteins.

Those amino acids that do not react directly with peroxynitrite (e.g. tyrosine, phenylalanine and histidine) can nevertheless be modified, through the intermediacy of secondary species such as hydroxyl, carbonate and nitrogen dioxide radicals or, in the presence of transition metals, oxidizing species formed from the reaction of peroxynitrite with them.

For some amino acids, plots of the observed rate constant of peroxynitrite decomposition *versus* amino acid

Table 3. Products and intermediates detected in peroxynitrite-damaged amino acids

Amino acid	Product	Reference
Cysteine	Disulfide (RSSR)	(Radi et al., 1991)
	Sulfenic acid (RSOH)	(Radi et al., 1991; Bryk et al., 2000; Carballal et al., 2003)
	Sulfinic acid (RSO_2H)	(Radi et al., 1991)
	Sulfonic acid (RSO_3H)	(Radi et al., 1991)
	Nitrosocysteine (RSNO)	(Balazy et al., 1998; van der Vliet et al., 1998)
	Nitrocysteine ($RSNO_2$)	(Balazy et al., 1998)
	Thiyl radical ($RS^{\bullet}$)	(Augusto et al., 1994)
	Disulphide radical anion ($RSSR^{\bullet-}$)	(Bonini and Augusto, 2001)
	Sulfinyl radical ($RSO^{\bullet}$)	(Bonini and Augusto, 2001)
Methionine	Methionine sulfoxide	(Pryor et al., 1994; Jensen et al., 1997; Perrin and Koppenol, 2000)
Tryptophan	5- and 6-Nitrotryptophan	(Alvarez et al., 1996; Padmaja et al., 1996)
	Hydroxytryptophan	(Alvarez et al., 1996)
	N-Formylkynurenine	(Alvarez et al., 1996; Kato et al., 1997)
	Hydropyrroloindole	(Kato et al., 1997)
	Oxindole	(Kato et al., 1997)
	Tryptophanyl radical	(Pietraforte and Minetti, 1997b)
Tyrosine	3-Nitrotyrosine	(Beckman et al., 1992; Ischiropoulos et al., 1992; van der Vliet et al., 1994; van der Vliet et al., 1995; Ramezanian et al., 1996)
	3-Hydroxytyrosine	(van der Vliet et al., 1994; Ramezanian et al., 1996)
	Dityrosine	(van der Vliet et al., 1994; van der Vliet et al., 1995)
	3,5-Dinitrotyrosine	(Yi et al., 1997)
	Tyrosyl radical	(Pietraforte and Minetti, 1997a)
Phenylalanine	*o*-, *m*- and *p*-Tyrosine	(van der Vliet et al., 1994)
	Nitrophenylalanine	(van der Vliet et al., 1994)
	Nitrotyrosine	(van der Vliet et al., 1994)
	Dityrosine	(van der Vliet et al., 1994)
Histidine	Oxo-histidine	B. Alvarez et al., manuscript in preparation
	Nitrohistidine	B. Alvarez et al., manuscript in preparation
	Histidinyl radical	B. Alvarez et al., manuscript in preparation

concentration show hyperbolic curvatures (Pryor et al., 1994; Alvarez et al., 1996; Alvarez et al., 1999). Although they have not been thoroughly explored, these plots may be explained by the reaction of peroxynitrite with intermediate oxidation products formed from the reactions of amino acids with hydroxyl or nitrogen dioxide radicals.

Reactions of peroxynitrite or its derived radicals with amino acids leads to the formation of oxidized, nitrated and minor amounts of nitrosated products. These are summarized in Table 3.

Cysteine

The reaction of peroxynitrite with the thiols of free cysteine and albumin was the first direct reaction of peroxynitrite that was reported (Radi et al., 1991), and cysteine is the amino acid that reacts the fastest with peroxynitrite. The second-order rate constant of the reactions of peroxynitrite with cysteine, glutathione, homocysteine and the thiol of albumin are $\sim 10^3\,M^{-1}\,s^{-1}$, three orders of magnitude higher than the corresponding reactions of hydrogen peroxide. Furthermore, values up to $10^7\,M^{-1}\,s^{-1}$ have been reported for the reaction of peroxynitrite with very reactive thiols in proteins such as peroxiredoxin, glyceraldehyde 3-phosphate dehydrogenase, creatine kinase and tyrosine phosphatase (Table 2). Plots of the apparent second-order rate constants as a function of pH are bell-shaped, which shows that the protonated form of one species is reacting with the anionic form of the other. Indeed, the rate constants for a series of thiol compounds measured at pH 7.4 increased as the pKa of the thiol decreased, which is consistent with the reaction of the anionic thiolate with peroxynitrous acid (Trujillo and Radi, 2002). In this regard, the unusually high rate constants obtained for the proteins mentioned above can be explained at least partially by the particularly low pKa of the thiols involved.

The reaction of peroxynitrite with thiols yields as products the corresponding disulfides and nitrite. The mechanism likely involves the nucleophilic attack of the thiolate on one of the peroxidic oxygens of peroxynitrous acid, with nitrite as leaving group. An intermediate sulfenic acid (RSOH) is formed, which reacts with another thiol forming the corresponding disulfide. The sulfenic intermediates have been detected in the case of proteins such as peroxiredoxin (Bryk et al., 2000) and albumin (Carballal et al., 2003).

$$RS^- + ONOOH \rightarrow RSOH + NO_2^- \quad (6)$$

$$RS^- + RSOH \rightarrow RSSR + OH^- \quad (7)$$

Besides this second-order reaction, the radicals formed from peroxynitrite homolysis in the absence or presence of carbon dioxide oxidize the thiols to thiyl radicals, which have been detected through EPR with spin traps, and give rise to dioxygen-dependent chain reactions (Augusto et al., 1994; Gatti et al., 1994; Quijano et al., 1997). Subject to the conditions (i.e. concentration of thiol, presence of dioxygen, presence of carbon dioxide), disulfide radical anion ($RSSR^{\bullet -}$) and sulfinyl radical ($RSO^{\bullet}$) have also been detected (Bonini and Augusto, 2001), as well as nitroso and nitrothiols (Balazy et al., 1998).

In addition to low molecular weight molecules, thiol oxidation by peroxynitrite has also been shown in a number of proteins, and in many cases, attributed to loss of function. The oxidation of the thiol may interfere with downstream events, as is the case of thiol-containing tyrosine phosphatases, transcription factors and cysteine proteases.

Disulfides do not react directly with peroxynitrite, since glutathione disulfide did not change the rate of peroxynitrite decay (Trujillo and Radi, 2002). Nevertheless, the particularly reactive disulfide in lipoic acid reacted with peroxynitrite at $1.4 \times 10^3\,M^{-1}\,s^{-1}$ leading to the thiosulfinate or disulfide S-oxide as product (Trujillo and Radi, 2002).

Methionine

The nucleophilic sulfur atom in the side chain of methionine is susceptible to oxidation, and methionine reacts with peroxynitrite with a second-order rate constant in the order of $10^2\,M^{-1}\,s^{-1}$ (Table 2). The main reactive species appears to be peroxynitrous acid. The products formed are methionine sulfoxide and nitrite, with the minor contribution of one-electron pathways to yield secondary products such as ethylene (Pryor et al., 1994; Jensen et al., 1997; Perrin and Koppenol, 2000).

The oxidation of methionine by peroxynitrite has also been observed in proteins such as α1-antitrypsin inhibitor and glutamine synthetase *in vitro* (Moreno and Pryor, 1992; Berlett et al., 1998).

Selenium-containing amino acids

Selenium compounds react with peroxynitrite faster than their sulfur analogs (Table 2) and inhibit the nitration and oxidation of target molecules. The interest in these compounds started with ebselen, which reduces peroxynitrite

to nitrite with a rate constant of $2 \times 10^6\,M^{-1}\,s^{-1}$ forming the selenoxide (Masumoto and Sies, 1996). This is reduced back to ebselen with glutathione as reductant, thus acting as a ''glutathione peroxynitritase'' mimic.

The selenium-containing amino acid selenocysteine is incorporated into a number of selenoproteins through a specific insertion machinery, while selenomethionine is incorporated into proteins at random in place of methionine (Sies and Arteel, 2000). The reactivity of selenomethionine is ten-fold higher than that of methionine, and analogously, selenocystine afforded more protection than cystine in oxidation and nitration assays (Briviba et al., 1996). The selenoxides formed may be reduced by different reductant systems such as glutathione (Assmann et al., 1998). The fact that these selenium compounds reduce peroxynitrite to nitrite in a two-electron process and can in turn be reduced back by physiological reductants makes them interesting both as part of natural defense mechanisms and as pharmacological peroxynitrite scavengers. In this regard, the selenoprotein glutathione peroxidase has been proposed to act as a peroxynitrite reductase (Sies et al., 1997) and selenoprotein P of human plasma can also protect from peroxynitrite (Arteel et al., 1998). The mammalian thioredoxin reductase is another selenoprotein that functions as a peroxynitrite reductase at the expense of NADH, in the presence of selenocystine or ebselen (Arteel et al., 1999a).

Tryptophan

Tryptophan reacts with a second-order rate constant of $37\,M^{-1}\,s^{-1}$ at pH 7.4 and 37 °C (Table 2). The free radicals derived from peroxynitrite can modify it as well, and the formation of tryptophanyl radical has been detected by EPR with spin traps (Pietraforte and Minetti, 1997b). Nitrotryptophan is formed among the products, which is relevant because it could indicate the contribution of pathways derived from nitric oxide to tryptophan damage (Alvarez et al., 1996). Both the formation of tryptophanyl radical and the nitrated products increase in the presence of carbon dioxide (Alvarez et al., 1996; Gatti et al., 1998).

The formation of nitrotryptophan has also been detected *in vitro* in the protein human Cu, Zn superoxide dismutase after exposure to peroxynitrite in the presence of carbon dioxide (Yamakura et al., 2001). Peroxynitrite dependent tryptophan modification can be linked to loss of function in the case of hen egg white lysozyme, which has tryptophan residues important for substrate binding (B. Alvarez et al., unpublished observation).

Tyrosine

Tyrosine cannot react directly with peroxynitrite, as concluded from the fact that it does not increase the rate of peroxynitrite decomposition. Nevertheless, its exposure to peroxynitrite leads to 3-nitrotyrosine, 3-hydroxytyrosine and 3,3′-dityrosine formation, whose structures are shown in Fig. 2.

The reaction occurs through a radical mechanism, as evidenced from the detection of tyrosyl radical and its dimerization product, dityrosine. Thus, the mechanism of peroxynitrite-dependent nitration involves the reaction of tyrosine with hydroxyl or nitrogen dioxide radicals to form tyrosyl radical which recombines with nitrogen dioxide to produce nitrotyrosine. The yield of nitrotyrosine formation is low, less than 10%. The low yield is influenced by the fact that hydroxyl radical is better at adding at the phenolic ring than at abstracting an hydrogen atom from it. The reactions involved in nitration and

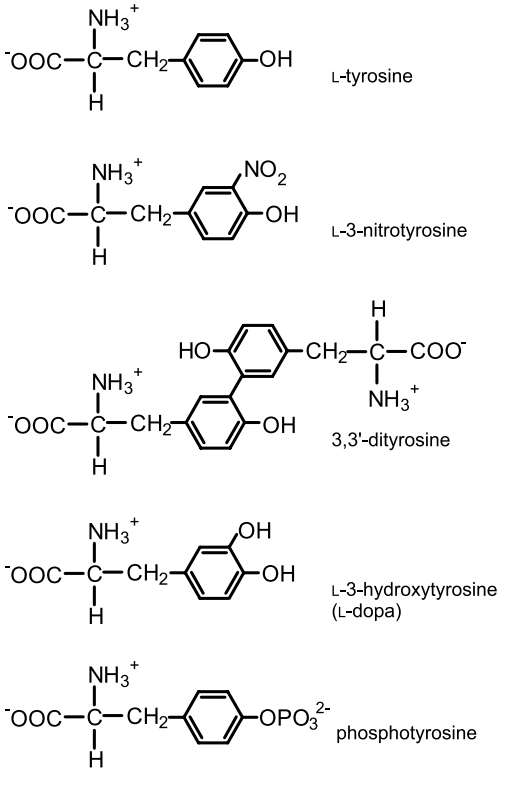

Fig. 2. Tyrosine oxidative modifications by peroxynitrite-dependent reactions. L-3-Nitrotyrosine, dityrosine and L-3-hydroxytyrosine are primary products formed from the reactions of peroxynitrite-derived nitrogen dioxide, carbonate, and hydroxyl radicals with tyrosine. The structure of phosphotyrosine is also shown for comparison. Importantly, tyrosine nitration in the 3-position impedes further phosphorylation in the hydroxyl group (Gow et al., 1996; Kong et al., 1996)

their rate constants are the following (reviewed in Trujillo et al., 2000):

$$ONOOH \rightarrow 0.3\,{}^{\bullet}OH + 0.3\,{}^{\bullet}NO_2 + 0.7\,NO_3^- + 0.7\,H^+$$
$$k = 0.9\,s^{-1} \quad (8)$$

$$TyrH + {}^{\bullet}NO_2 \rightarrow Tyr^{\bullet} + NO_2^-$$
$$k = 3.2\times10^5\,M^{-1}\,s^{-1}\ \text{(Prutz et al., 1985)} \quad (9)$$

$$TyrH + {}^{\bullet}OH \rightarrow 0.95\,TyrOH^{\bullet} + 0.05\,Tyr^{\bullet}$$
$$k = 1.4\times10^{10}\,M^{-1}s^{-1}\ \text{(Solar et al., 1984)} \quad (10)$$

$$Tyr^{\bullet} + {}^{\bullet}NO_2 \rightarrow Tyr\text{-}NO_2$$
$$k = 3\times10^9\,M^{-1}\,s^{-1}\ \text{(Prutz et al., 1985)} \quad (11)$$

$$Tyr^{\bullet} + Tyr^{\bullet} \rightarrow Dityr$$
$$k = 4.5\times10^8\,M^{-1}\,s^{-1}\ \text{(Hunter et al., 1989)} \quad (12)$$

$$2TyrOH^{\bullet} \rightarrow Tyr\text{-}OH + \text{products}$$
$$k = 3\times10^8\,M^{-1}\,s^{-1}\ \text{(Solar et al., 1984)} \quad (13)$$

$$TyrOH^{\bullet} \rightarrow Tyr^{\bullet} + H_2O$$
$$k = 1.8\times10^8\,M^{-1}\,s^{-1} \quad (14)$$

Since the proton-catalyzed homolysis of peroxynitrite is slow relative to second order processes that may occur *in vivo*, it is evident that peroxynitrite-dependent nitrotyrosine formation in biological systems must be mediated by the previous reaction of peroxynitrite with carbon dioxide or metal centers in order to generate secondary oxidizing species that in turn react with tyrosine to form the phenolic radical. Thus, in the presence of carbon dioxide, nitration yields increase due to the efficient hydrogen atom abstraction of carbonate radical to form tyrosyl radical:

$$TyrH + CO_3^{\bullet-} \rightarrow Tyr^{\bullet} + HCO_3^-$$
$$k = 4.5\times10^7\,M^{-1}\,s^{-1}\ \text{(Goldstein et al., 2000)} \quad (15)$$

In addition, low molecular weight copper, iron and manganese compounds increase nitration rates and yields, and so do proteins that contain these transition metals such as hemeproteins, manganese and copper,zinc superoxide dismutase, due to the formation of a metal-bound oxidizing species and nitrogen dioxide, which in turn react with tyrosine (Beckman et al., 1992; Ferrer-Sueta et al., 1997; Zou et al., 1997; Ferrer-Sueta et al., 1999; Quijano et al., 2001).

Protein nitration has been widely detected both *in vitro* and *in vivo* and is examined below.

Histidine

The same as tyrosine, histidine does not react directly with peroxynitrite (Alvarez et al., 1999) but it can be modified by its secondary radicals. Exposure of free histidine to peroxynitrite leads to the formation of a product whose molecular mass is indicative of nitro addition plus loss of water. In histidine-containing peptides, the formation of both oxo- and nitro-derivatives can be detected through mass spectrometry (manuscript in preparation). The fact that histidine is present as metal ligand in a number of proteins makes it a likely candidate for site-specific modification by peroxynitrite, most likely *via* histidinyl radical.

Phenylalanine

Phenylalanine does not react directly with peroxynitrite either (Alvarez et al., 1999). Nevertheless, its exposure to peroxynitrite leads to the formation of *p*-, *m*- and *o*-tyrosine, as well as nitrophenylalanine (van der Vliet et al., 1994).

D-Phenylalanine has been used as a probe for reactive species formation in microdialysis experiments of neurotoxic damage. The formation of *o*- and *m*-tyrosine, as well as nitrophenylalanine and nitrotyrosine, was interpreted as indicative of peroxynitrite formation (Ferger et al., 2001).

Which amino acid reacts most?

The fact that cysteine is the amino acid that reacts the fastest with peroxynitrite indicates that, in the absence of metal centers, selenium amino acids or carbon dioxide, thiol oxidation will be the principal modification introduced by peroxynitrite.

Indeed, as shown in Figure 3, with human serum albumin (HSA) we can see that exposure of relatively high but physiologically significant concentrations of protein (0.27 mM) to peroxynitrite led to complete oxidation of its single thiol, cysteine-34, while only one tyrosine was nitrated per 10 albumin molecules, even though there are 18 tyrosines present in each polypeptide chain. In the presence of carbon dioxide, cysteine oxidation decreased by 50%, since the direct reaction of peroxynitrite with carbon dioxide outcompeted the thiols. Most peroxynitrite then isomerized to nitrate, but the free carbonate and nitrogen dioxide radicals formed were able to oxidize

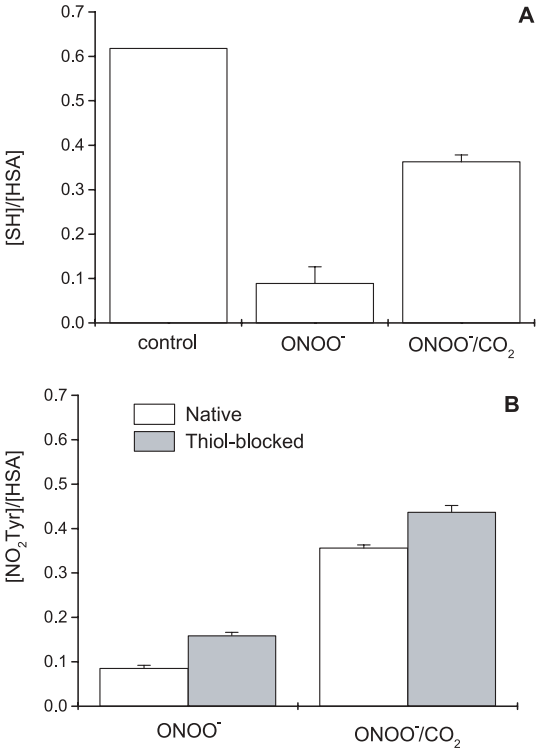

Fig. 3. Thiol oxidation and tyrosine nitration in human serum albumin. Albumin (0.27 mM), either native or thiol-blocked, was exposed to peroxynitrite (1 mM) in phosphate buffer, 0.1 M, pH 7.4, 0.1 mM dtpa, 37 °C, in the presence or absence of 10 mM sodium bicarbonate. Thiols were measured with Ellman's reagent (Panel A) and nitrotyrosine was measured through the increase in absorbance at 430 nm after alkalinization to pH > 10 (Panel B). See also (Alvarez et al., 1999)

the thiol, as expected from their rate constants with cysteine ($\sim 2 \times 10^8\,M^{-1}\,s^{-1}$ for both radicals, (Ross et al., 1998)). Nitration increased four-fold in the presence of carbon dioxide, since carbonate radical is better at forming tyrosyl radical than hydroxyl radical. However, tyrosine nitration still was a minor modification in the presence of carbon dioxide, as only one tyrosine was nitrated per 3 albumin molecules. That part of the carbonate and nitrogen dioxide radicals formed were reacting with the thiol can be evidenced from the fact that blockage of the thiol led to increases in tyrosine nitration, both in the presence and in the absence of carbon dioxide.

Of course, the presence of a transition metal center as well as the accesibility of the different residues may critically affect the outcome.

Protein nitration

Peroxynitrite-mediated tyrosine nitration in biological systems proceeds through a radical mechanism in which peroxynitrite first reacts with carbon dioxide or metal centers forming secondary nitrating species. With regard to the site of nitration within a protein, a certain degree of specificity has been found. Nitration is promoted by exposure of the tyrosine, its location on a loop, its association with a neighboring negative charge and absence of proximal cysteines (Souza et al., 1999). Also, nitration is enhanced in hydrophobic environments, possibly because of the partition and longer half-life of nitrogen dioxide radical (Zhang et al., 2001). Since free radicals mediate the process, the stability of the tyrosyl radical should be critical as well (Guittet et al., 1998). Pathways mediated by carbonate radical are expected to nitrate superficial tyrosines, while the initial reaction of peroxynitrite with an enzymic metal may lead to nitration of tyrosines close to the active site. Proteomic approaches and the availability of techniques to specifically map the site of nitration will surely yield information useful in this sense in the near future.

At first proposed to depend solely on peroxynitrite, other mechanisms have been proposed for nitrotyrosine formation. First, nitration can be mediated by peroxidases such as myeloperoxidase in the presence of hydrogen peroxide and nitrite (Eiserich et al., 1998). Second, nitration can occur from the reaction of tyrosyl radical with nitric oxide followed by further oxidation to yield nitrotyrosine (Gunther et al., 1997). It is useful to recall that nitrogen dioxide by itself is not an efficient nitrating agent. In any cases, in order to attribute nitrotyrosine to peroxynitrite formation, further evidence is needed. In this sense, if nitration is mediated by myeloperoxidase, chlorotyrosine should be observed as well (van der Vliet et al., 1997), while if it is mediated by peroxynitrite, hydroxytyrosine should be expected (Santos et al., 2000; Linares et al., 2001). Notwithstanding the mechanism, there is no doubt that nitration is a hallmark of the contribution of nitric oxide to oxidative damage.

The availability of increasingly sensitive techniques for measuring nitrotyrosine, including the development of specific antibodies, have provided a wealth of information. The list of proteins where nitrotyrosine has been identified is increasing and so is the number of disease states where nitrotyrosine has been detected, at least 50 human diseases and more than 80 conditions modeled in animals or cell culture systems (for a review see Greenacre and Ischiropoulos, 2001). As an example, a proteomic approach using a monoclonal antibody against nitrotyrosine has led to the identification of over 40 different proteins that appear to undergo nitration during inflammatory challenge *in vivo* (Aulak et al., 2001). In some cases, nitrotyrosine formation

is related to loss of function of the proteins. This is the case of manganese superoxide dismutase, where the bulky nitro group may impede accessibility of the substrate to the active site. In addition, nitrotyrosine has a pKa of about 7.5, in contrast to tyrosine which has a pKa of 10. Thus, nitration may introduce a negative charge in a protein. Most importantly, nitration may interfere with signal transduction cascades, since nitrated tyrosyl residues cannot be phosphorylated (Gow et al., 1996; Kong et al., 1996). Furthermore, tyrosine phosphatases have a critical cysteine residue which can be oxidized by peroxynitrite, providing an additional mechanism for signaling alteration (Takakura et al., 1999). These two phenomena are well illustrated in erythrocytes. Low concentrations of peroxynitrite increased phosphorylation of the band 3 of erythrocytes through inhibition of the tyrosine phosphatases, while high concentrations decreased phosphorylation and were accompanied by protein nitration (Mallozzi et al., 1997).

Enzyme inactivation

As shown in Table 4, several enzymes have been reported to be inactivated by peroxynitrite. For irreversible inactivation to occur, the reaction of peroxynitrite with the enzyme must lead to the modification of a critical residue or prosthetic group.

Table 4. Enzymes reported to become inactivated upon exposure to peroxynitrite

Enzyme	Modified residue	Reference
ATPase[b]	ND[a]	(Radi et al., 1994)
Succinate dehydrogenase[b]	ND	(Radi et al., 1994; Rubbo et al., 1994)
Fumarate reductase (*Trypanosoma cruzi*)[b]	ND	(Rubbo et al., 1994)
NADH:ubiquinone oxidoreductase[b]	ND	(Radi et al., 1994) (Riobo et al., 2001)
Cytochrome P450 BM-3[c]	Cys, Tyr	(Daiber et al., 2000)
Cytochrome P450 2B1[c]	Tyr	(Roberts et al., 1998)
Prostacyclin synthase[b,c]	Tyr	(Zou and Ullrich, 1996; Zou et al., 1997)
Inducible nitric oxide synthase[c]	Heme	(Huhmer et al., 1997)
Glutathione peroxidase[b,c]	Selenocysteine	(Asahi et al., 1997; Briviba et al., 1998; Padmaja et al., 1998; Fu et al., 2001)
Alcohol dehydrogenase[c]	Zinc sulfur cluster	(Crow et al., 1995)
Aconitase[b,c]	Iron sulfur cluster	(Castro et al., 1994; Hausladen and Fridovich, 1994; Keyer and Imlay, 1997; Castro et al., 1998)
6-Phosphogluconate dehydratase[b]	Iron sulfur cluster	(Keyer and Imlay, 1997)
Fumarase A[b]	Iron sulfur cluster	(Keyer and Imlay, 1997)
Creatine kinase[b,c]	Cys	(Konorev et al., 1998; Stachowiak et al., 1998)
Glyceraldehyde 3-phosphate dehydrogenase[b,c]	Cys	(Souza and Radi, 1998) (Keyer and Imlay, 1997)
Glutamine synthetase[c]	Tyr, Met	(Berlett et al., 1998)
Succinyl-CoA:3-oxoacid CoA transferase[b]	Tyr	(Marcondes et al., 2001)
Mn superoxide dismutase[b,c]	Tyr	(MacMillan-Crow et al., 1996)
Cu,Zn superoxide dismutase[c]	His	B. Alvarez et al., manuscript in preparation
Tyrosine hydroxylase[b,c]	Tyr, Cys	(Ara et al., 1998; Kuhn et al., 1999; Blanchard-Fillion et al., 2001)
Tryptophan hydroxylase[c]	Cys	(Kuhn and Geddes, 1999)
Ca^{2+}-ATPase[b]	Cys	(Viner et al., 1996; Klebl et al., 1998)
Caspase 3[b,c]	Cys	(Haendeler et al., 1997)
Protein tyrosine phosphatase[c]	Cys	(Takakura et al., 1999)
Nicotinamide nucleotide transhydrogenase[b]	Tyr	(Forsmark-Andree et al., 1996)
Ribonucleotide reductase[c]	Tyr	(Guittet et al., 1998)
Zn^{2+}-glycerophosphocoline cholinephosphodiesterase[c]	Tyr	(Sok, 1998)
NADPH-cytochrome P450 reductase[c]	ND	(Sergeeva et al., 2001)
Glutathione reductase[c]	Tyr	(Francescutti et al., 1996; Savvides et al., 2002)
Glutathione S-transferase[b,c]	ND	(Wong et al., 2001)
Glutaredoxin[b]	ND	(Aykac-Toker et al., 2001)
Protein kinase C[b,c]	Tyr	(Knapp et al., 2001)
Ornithine decarboxylase[b,c]	Tyr	(Seidel et al., 2001)
Xanthine oxidase[c]	Molybdenum center	(Houston et al., 1998) (Lee et al., 2000)
Lysozyme[c]	Trp	B. Alvarez et al., unpublished

[a] ND: not determined, [b] enzyme in cell extracts, *ex vivo* or *in vivo* systems, [c] purified enzyme

In vitro, the inactivation of a certain enzyme is usually determined through exposure of the purified protein to peroxynitrite followed by activity determination. The concentration of peroxynitrite needed to inactivate 50% of the enzyme (IC_{50}) was introduced as a measure of sensitivity to peroxynitrite (Castro et al., 1996). However, many factors affect the IC_{50}, such as the concentration of the enzyme, the presence of contaminants, the rate constant of the reaction of peroxynitrite with critical and non-critical residues, the stoichiometry of the reaction and the amount of peroxynitrite that forms secondary hydroxyl and nitrogen dioxide radicals which may, or may not, inactivate the enzyme (for a review see Radi et al., 2000). Thus, IC_{50} values should be interpreted with caution. For instance, the IC_{50} for aconitase inactivation is 17 μM at 7.25 μM enzyme, but increases to 46 μM peroxynitrite at 24.2 μM enzyme (Castro et al., 1994).

In principle, almost any enzyme can be inactivated at a sufficiently high peroxynitrite concentration. So, in order to extrapolate results obtained with purified enzymes to physiological situations, the kinetic rate constant as well as experiments performed with extracts, cells or other biological systems should be taken into account. In some cases, the losses of activity and amino acid modifications seen *in vitro* have also been observed *in vivo*. For example, nitration and inactivation of manganese superoxide dismutase was observed in chronically rejected human renal allografts (MacMillan-Crow et al., 1996).

Of course, enzymes whose activity is up-regulated by oxidation processes can be activated by peroxynitrite. For instance, the *src* kinase *hck* is activated by peroxynitrite-dependent cysteine oxidation (Mallozzi et al., 2001). Also, matrix metalloproteinases can be activated by peroxynitrite through formation of a mixed disulfide S-oxide or thiosulfinate with glutathione (Okamoto et al., 2001). In addition, peroxynitrite can activate prostaglandin endoperoxide synthase by serving as a substrate for the peroxidase activity of the enzyme (Landino et al., 1996).

Repair

So far, the only two stable amino acid oxidative modifications that can be repaired enzymatically are cysteine disulfide and methionine sulfoxide. It is likely that the enzymatic batteries that deal with these modifications also have a role in the defense of the cell against peroxynitrite, since peroxynitrite can lead to the formation of these oxidation products. Indeed, methionine sulfoxide reductase had a role in protecting bacteria against the toxic effects of reactive nitrogen intermediates (St John et al., 2001).

Proteins affected by oxidative damage have an increased turnover. In this sense, albumin treated with peroxynitrite was degraded faster than native albumin by proteolytic enzymes present in red cell lysates, and nitrated proteins showed increased rates of degradation by the proteasome (Gow et al., 1996; Souza et al., 2000).

Great efforts are being devoted to the search for a denitrase or nitratase enzyme. In this regard, human plasma and homogenates from rat tissues and cultured cells were able to remove from proteins the epitope to nitrotyrosine antibodies (Gow et al., 1996; Kamisaki et al., 1998; Kuo et al., 1999; Irie et al., 2003). However, the existence of a denitrase or nitratase activity separate from proteolysis is still a matter of debate. Neither free nitrotyrosine nor protein nitrotyrosine are reduced by bacterial and other mammalian nitroreductases (Lightfoot et al., 2000). Nevertheless, in a similar way as other nitroaromatic compounds, nitrotyrosine may be enzymatically reduced to the corresponding nitro anion radical ($ArNO_2^{\bullet -}$), which is then oxidized by molecular oxygen to yield $O_2^{\bullet -}$ and regenerate nitrotyrosine (Krainev et al., 1998). An important practical consideration when performing analysis for nitrotyrosine in the lab is that thiol groups in the presence of heme and heat reduce nitrotyrosine to aminotyrosine *in vitro* (Balabanli et al., 1999).

Fates of peroxynitrite in the cytosol

The considerations exposed above clear the way for estimating the fate of peroxynitrite in a certain biological compartment. As an example, we can look at the situation in the cytosol of a cell and calculate the relative rates of the reactions with different cell components by multiplying factors of rate and concentration. The proportion of peroxynitrite that reacts with each component is shown in Fig. 4.

Several conclusions can be drawn from this estimation. First, the amount of peroxynitrite that is able to homolyze to hydroxyl and nitrogen dioxide radicals is minimal. Second, the low molecular weight antioxidants, of which glutathione is the most significant, account for only a small proportion of peroxynitrite and are not able to outcompete other targets. Third, metal- and selenium-containing proteins, but also proteins that do not contain prosthetic groups, are able to react with a high percentage of peroxynitrite. Fourth, carbon dioxide is a significant

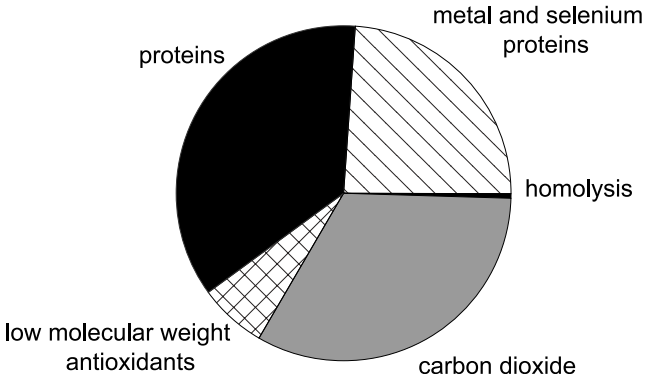

Fig. 4. Fate of peroxynitrite in the cytosol estimated from kinetic rate constants and concentration factors. Concentrations and rate constants were assumed, respectively: carbon dioxide 1.5 mM, $k = 4.6 \times 10^4\,M^{-1}\,s^{-1}$; ascorbate 0.5 mM, $k = 1 \times 10^2\,M^{-1}\,s^{-1}$; glutathione 10 mM, $k = 1.35 \times 10^3\,M^{-1}\,s^{-1}$; uric acic 0.1 mM, $k = 4.7 \times 10^2\,M^{-1}\,s^{-1}$; proteins 15 mM, $k = 5 \times 10^3\,M^{-1}\,s^{-1}$; metal- and selenium-containing proteins 0.5 mM, $k = 1 \times 10^5\,M^{-1}\,s^{-1}$.

target, but it does not account for all the peroxynitrite formed.

Proteins that detoxify peroxynitrite

For a scavenger to be efficient against peroxynitrite, it should be present in the critical cell compartment at a sufficiently large concentration in relation to its rate constant so as to outcompete carbon dioxide, thiols and metal centers. Effective pathways for the regeneration of the scavenger should exist and secondary radicals should not be formed.

Concerning the low molecular antioxidants present in cell systems, ascorbate and uric acid react relatively slowly with peroxynitrite ($k \sim 10^2\,M^{-1}\,s^{-1}$ (Bartlett et al., 1995; Santos et al., 1999). Vitamin E and ubiquinol do not react directly with peroxynitrite (Schopfer et al., 2000, and Botti, Radi et al., unpublished observations). Glutathione, present at millimolar concentrations inside cells, is a better competitor, although, as shown in Fig. 4, it cannot outcompete other targets. Thus, none of the low molecular weight antioxidants by themselves appear to be very effective peroxynitrite scavenger, although a role is increasingly being attributed to them in the scavenging of secondary free radicals derived from peroxynitrite, in the repair of the damage inflicted or in the recycling of appropriate scavengers.

In the last few years, the idea that certain proteins have a natural role in peroxynitrite detoxification is gaining ground. In the same line as the discovery of superoxide dismutase confirmed the formation of superoxide *in vivo*, the existence of proteins with "peroxynitritase" activity is contributing to establish peroxynitrite formation in some cell types and disease states. Candidates for such activity are likely to contain selenium, thiols or metal centers.

In this regard, the selenoprotein glutathione peroxidase has been proposed to have peroxynitrite reductase activity, reducing peroxynitrite to nitrite catalytically at the expense of glutathione, without the intermediate formation of free radicals (Sies et al., 1997). At a concentration of 2 μM, glutathione peroxidase would compete with other targets such as carbon dioxide for peroxynitrite, and the rate of regeneration of the enzyme by glutathione would not be limiting *in vivo* (Arteel et al., 1999b; Sies and Arteel, 2000). It was reported that selenite supplementation of rat liver epithelial cells protected them from peroxynitrite effects (Schieke et al., 1999). In addition, mouse hepatocytes obtained from glutathione peroxidase knockout mice exhibited increased damage than controls when exposed to nitric oxide and superoxide generators (Fu et al., 2001). However, the possible inactivation of the enzyme by peroxynitrite needs to be considered (Asahi et al., 1997; Sies et al., 1997; Briviba et al., 1998; Padmaja et al., 1998; Fu et al., 2001).

Other candidate protective proteins are the peroxiredoxins. These are a family of antioxidant enzymes conserved from bacteria to humans which contain thiols in their active sites. Lately, some bacterial peroxiredoxins (AhpC or alkylhydroperoxide reductase subunit C) have been found to react with peroxynitrite with rate constants about $10^6\,M^{-1}\,s^{-1}$, three orders of magnitude higher than glutathione (Bryk et al., 2000). The products formed are nitrite and sulfenic acid, which in turn forms disulfides. In *Mycobacterium tuberculosis*, the disulfides were found to be reduced back by a thioredoxin-like protein linked to NADH oxidation *via* dihydrolipoamide dehydrogenase and dihydrolipoamide succinyltransferase (Bryk et al., 2002) and AhpC knockouts had increased susceptibility to peroxynitrite (Master et al., 2002). In yeast, mutants in the peroxiredoxins Tsa1p and Tsa2p were hypersensitive to peroxynitrite and nitric oxide donors (Wong et al., 2002).

Also to be considered are the heme peroxidases, which react with peroxynitrite with rates of 10^6–$10^7\,M^{-1}\,s^{-1}$ and can be reduced back by endogenous reductants. However, the reaction of peroxynitrite with the heme peroxidases involves the formation of reactive species such as ferryl heme and nitrogen dioxide (Sampson et al., 1996) and conflicting results exist in the literature (Fukuyama et al., 1996; Brennan et al., 2002; Takizawa et al., 2002).

Thus, the potential role of heme peroxidases in peroxynitrite detoxification requires future investigation.

Last, hemoglobin has been proposed as a significant intravascular scavenger of peroxynitrite. Despite the presence of extracellular targets such as carbon dioxide, at high red blood cell densities such as those present *in vivo*, peroxynitrite is able to reach the cells (Romero et al., 1999). Once inside, the main target for peroxynitrite will be oxyhemoglobin, which is present at a concentration of $\sim$20 mM heme and reacts with peroxynitrite at a rate of $10^4\,M^{-1}\,s^{-1}$. This reaction catalyzes the isomerization to nitrate (Romero, Radi et al., manuscript under revision) as well as leads to the formation of ferryl hemoglobin which can be reduced by glutathione (Augusto et al., 2002b). By this concerted mechanism, oxyhemoglobin may then act as an intravascular peroxynitrite detoxifying system.

Acknowledgements

We thank Gerardo Ferrer for his valuable scientific contributions. This work was supported by Comisión Sectorial de Investigación Científica (Uruguay), Third World Academy of Sciences to BA, the Howard Hughes Medical Institute, Fogarty-National Institutes of Health to RR. RR is an International Research Scholar of the Howard Hughes Medical Institute.

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