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Thiolation and nitrosation of cysteines in biological fluids and cells

Review Article

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Summary. Thiols (RSH) are potent nucleophilic agents, the rates of which depend on the pKa of the sulfhydryl. Unlike compounds having other nucleophile moieties (–OH or $–NH_2$), RSH are involved in reactions, such as conjugations, redox and exchange reactions. Although protein SH groups (PSH) react like non-protein thiols (NPSH), the biochemistry of proteins is much more complex for reasons such as steric hindrance, charge distribution and accessibility of PSH to the solvent (protein conformation). The reaction rates and types of end-products of PSH vary a lot from protein to protein. The biological problem is even more complex because in all compartments and tissues, there may be specific competition between thiols (namely between GSH and PSH), regulated by the properties of antioxidant enzymes. Moreover, PSH are divided biologically into essential and non-essential and their respective influence in the various biological systems is unknown. It follows that during phenomena eliciting a prompt thiol response (oxidative stress), the antioxidant PSH response and reaction mechanisms vary considerably from case to case. For example, in spite of a relatively low pKa that should guarantee good antioxidant capacity, PSH of albumin has much less propensity to form adducts with conjugating agents than NPSH; moreover, the structural characteristics of the protein prevent albumin from forming protein disulfides when exposed to oxidants (whereas protein-thiol mixed disulfides are formed in relative abundance). On the other hand, proteins with a relatively high reactivity, such rat hemoglobin, have much greater antioxidant capacity than GSH, but although human hemoglobin has a pKa similar to GSH, for structural reasons it has less antioxidant capacity than GSH.

When essential PSH are involved in *S*-thiolation and *S*-nitrosation reactions, a similar change in biological activity is observed. *S*-thiolated proteins are a recurrent phenomenon in oxidative stress elicited by reactive oxygen species (ROS). This event may be mediated by disulfides, that exchange with PSH, or by the protein intermediate sulfenic acid that reacts with thiols to form protein-mixed disulfides. During nitrosative stress elicited by reactive nitrogen species (RNS), depending on the oxygen concentration of the system, nitrosation reactions of thiols may also be accompanied by protein *S*-thiolation. In this review we discuss a number of cell processes and biochemical modifications of enzymes that indicate that *S*-thiolation and *S*-nitrosation may occur simultaneously in the same protein in the presence of appropriate interactions between ROS and RNS.

Keywords: Thiols – Nitrosothiols – Protein SH groups – NO – *S*-Thiolation – *S*-Nitrosylation

Abbreviations: CAT, catalase; CSH, cysteine; CGSH, cysteinylglycine; CSSC, cystine; CGSSGC, cysteinylglycine disulfide; DTT, dithiothreitol; GADPH, glyceraldehyde-3-phosphate dehydrogenase; GRX, glutaredoxin; GSH, glutathione; GS-NO, nitroso glutathione; GSSG, glutathione disulfide; Hcy, homocysteine; NOS, nitric oxide synthases; NPSH, non-protein thiols; $ONOO^-$, peroxynitrite; PSH, protein SH groups; PS-NO, *S*-nitroso proteins; PSOH, sulfenic proteins; PSO_2H, sulfinic proteins; PSO_3H, sulfonic proteins; PSSP, protein disulfides; RNS, reactive nitrogen species; RS-NO, nitrosothiols; ROS, reactive oxygen species; RSOH, sulfenic acids; RSH, thiols; RS-SR', mixed disulfides of low molecular weight; RS-SP, protein-thiol mixed disulfides; GS-SP, glutathione-protein mixed disulfides; SOD, superoxide dismutase; GPX, glutathione peroxidase; RBC, red blood cells; RSSR, disulfides

Introduction

Oxygen and NO are physiological molecules that at relatively high concentrations may be toxic. Their metabolism is controlled by a specific antioxidant/oxidant poise. At low to moderate concentrations these reactive species play important roles in regulation of cellular functions. However, under some circumstances not yet clarified at the molecular level their concentrations and activities become disregulated, leading to overproduction of highly reactive oxygen species (ROS) and nitrogen species (RNS) that react readily with various cellular constituents to form biologically less active or inactive derivates. Together with other antioxidants, sulfhydryl groups of thiol compounds (RSH), of both low (NPSH) and high molecular weight (i.e., protein SH groups,

PSH) compounds are involved in the cellular regulation of NO, ROS and RNS concentrations and activities.

PSH are divided in essential and non essential categories. Extensive genetic, enzymatic and chemical studies have clarified the role of essential PSH, as cysteine redox centers implicated in catalysis, quaternary structure, signal transduction and DNA binding. The less studied non-essential PSH are considered simple nucleophilic sites. It is still unknown whether or how they might have a functional linkage with the essential ones. At present, it is unknown how the total thiol population (NPSH + PSH) operates in conjunction with other antioxidant systems in regulating or contrasting the oxygen/NO actions.

Depending on redox states and changes in concentrations of oxygen, NO and thiols, variable mixtures of end-products such as RSSR, PSSP, RS-NO, PS-NO, and mixed disulfides of small (RSSR′) and high molecular weight (RS-SP) are formed in every biological compartment. In fact the metabolic interactions of NO and oxygen with thiols may affect the same compounds that are reversibly controlled by the antioxidant/oxidant poise. All of these compounds are the result of an antioxidant response and some of them, those linked to essential PSH, also fulfill biological functions. However in circumstances of oxidant overproduction or reductant deficiency, the oxidant insult may yield other products of thiol modification (higher oxidative species of NPSH or PSH), such as sulfenic, sulfinic and sulfonic acids. Whereas PSOH production is considered reversible, that of PSO_2H and PSO_3H may contribute to irreversible damages in tissues.

In spite of a very great scientific interest, mechanisms of the biological role of PSH are poorly defined because of the complexity of their reactions, the scarce knowledge of relationships with other antioxidant systems, and most important the difficulty to categorise a change of a PSH portion into a specific biological effect.

This review is an attempt to answer some of these questions concerning mechanisms of protein *S*-thiolation and *S*-nitrosation during phenomena of oxidative and nitrosative stress, and their biological relationships.

The antioxidant potential of thiols: interaction with ROS and RNS and formation of disulfides and nitrosothiols

The antioxidant system of living organisms is constituted by a variety of enzymes, binding proteins, micronutrients, catabolites, vitamins and chemical compounds etc, that control redox reactions elicited by the oxygen consumption and other oxidants (Sies, 1993). A wide array of interconnected equilibria forming the antioxidant/oxidant balance exists in every biological compartment even though the control mechanisms are scarcely known. The possible shifts in the balance towards a prevalence of pro-oxidant situations increase the risk for diseases. Pathologies depending on oxidative stress, also called free radical diseases, may occur in premature newborns, and are a recurrent characteristic of various pathological states, ranging from atherosclerosis, stroke, carcinogenesis, and of neurodegenerative and inflammatory disorders as well as of ageing processes (Saugstad, 1998; Floyd, 1990; Floyd, 1999; Berlett and Stadtman, 1997; Ashok and Ali, 1999; McCall and Frei, 1999; Merkesbery and Carney, 1999). Since ROS also possess important physiological roles, for example as redox regulators of cell signaling (Schreck et al., 1991; Sen and Packer, 1996; Suzuki et al., 1997; Powis et al., 1997; Flohe et al., 1997), the knowledge of mechanisms of loss of reversibility of the system is of primary importance.

NO and its by-products (RNS), like ROS, are radical and toxic agents that fulfill mediator functions, acting in tissues as autocrine paracrine agents (Moncada et al., 1991; Bredt, 1999; Feldman et al., 1993).

The NO metabolism is more complex than that of oxygen. For example, whereas ROS are products of oxygen reduction, RNS can be formed by NO reduction (e.g. NO^-) or oxidation (e.g. NO^+). Moreover, the NO reactions change in the presence of various factors (e.g., heavy metal ions or oxygen itself).

The complex biological activities of RNS (and ROS) are in general governed by chemistry laws (Wink and Mitchell, 1998). In particular, since ROS and RNS are potent electrophiles they react with nucleophilic centers, such as –OH, $–NH_2$ and –SH groups, of small compounds and macromolecules, such as DNA, proteins, and polisaccarides with irreversible consequences, as cell death via apoptosis or necrosis, if damages are not promptly repaired.

The thiol group (SH), for the particular position of sulfur in the Periodic Scale, is the best nucleophile. Consequently, RSH are more easily oxidized or conjugated than compounds bearing –OH or $–NH_2$ moieties.

The relative concentrations of RSH (PSH plus NPSH) at cellular level are nearly equal, whereas pronounced differences exist between tissues and plasma. Inside the cells, levels of NPSH (mainly GSH) are much lower than the highly heterogeneous population of PSH. By contrast, in extracellular milieu (plasma) PSH are essentially represented by albumin (that has only one SH group), while NPSH is comprised of nearly equal concentrations of various species such as CSH, CGSH, GSH and Hcy.

RSSR are relatively low inside the cells, whereas they are relatively rich in plasma. Consequently, the thiol/disulfide ratio (GSH/GSSG), an index of the redox status,

is relatively high at intracellular level (range of GSH/GSSG variation: 30–300), whereas in plasma its value is nearly 1. The RSH/RSSR of other thiols ranges between 1–10 at intracellular levels (Giustarini et al., 2000), whereas is low in plasma (lower or equal to 1) (Di Giuseppe et al., 2003); for example, the CSH/CSSC ratio of human plasma is less than 0.2, whereas that of CGSH/GCSSCG is less than 0.4. The reason why the thiol population is so different inside and outside the cells is unknown. However, it is generally accepted that plasma, having a relatively poor antioxidant potential in terms of thiols, uses other strategies of defence against ROS.

RSSR and RS-NO are generally formed from the interaction of RSH with RNS (Becker et al., 1998, Stamler and Hausladen, 1998). The rate and the relative proportion of RSSR and RS-NO may vary in dependence of the oxygen presence in the system (Kharinotov et al., 1995; Wink et al., 1994; Hoog, 2002). In fact, the relatively slow reaction between NO and RSH:

$$NO + RSH = RSSR + 2NO^- + H^+ \quad (1)$$

becomes accelerated in the presence of oxygen for the formation of the very reactive intermediate, N_2O_3 (dinitrogen trioxide):

$$2NO + 1/2O_2 = N_2O_3 + RSH = RS-NO + 2NO_2^- + 2H^+ \quad (2)$$

In the presence of oxygen, other mechanisms have been proposed that involve the contemporaneous formation of RS-NO and $ONOO^-$ from the intermediate nitrosyldioxyl radical (Gow et al., 1997). According to these authors (Gow et al., 1997), the mechanism could explain the biosynthesis of RS-NO at relatively low NO levels in *in vivo* conditions.

NO is endogenously generated by the enzyme of NO synthase (Stuehr, 1999). However, under appropriate conditions of substrate deficiency (arginine or BH_4, bound to the core region containing reductase and oxygenase domains of NOS) these enzymes may also produce ROS (O_2^-) (Schmidt et al., 1996; Maxwell and Cooke, 2001). NO is extremely reactive with O_2^-, and their combination will form peroxynitrite ($ONOO^-$), a potent oxidant, that is supposed to be a source of hydroxyl radical (Beckman et al., 1990; Crown and Beckman, 1996). Alternatively, $ONOO^-$ may be formed also by reaction of NO^- with oxygen (Hughes, 1999):

$$NO^- + O_2 = ONOO^-$$

$ONOO^-$ is scavenged by NPSH (and PSH) with formation of disulfides (Arteel et al., 1999; Groves, 1999; Murphy et al., 1998).

Thus, uncontrolled oxidations or antioxidant deficiency may be events that prime oxidative stress that under particular circumstances of NO production, may be further worsened by the combination of NO and oxygen. Metabolic consequences of it are the RSSR (reaction 1) and RS-NO (reaction 2) formation that in turn may easily exchange with RSH, forming mixed disulfides or other nitrosothiols, according to the following SH/SS exchange reactions:

$$RSSR + R'SH = RSSR' + RSH$$

$$RSNO + R'SH = R'SNO + RSH$$

In the presence of PSH, protein *S*-thiolation or *S*-nitrosation may occur as illustrated by the following reactions of GSSG and GS-NO:

$$GSSG + PSH = GSSP + GSH \quad (3)$$

$$GSNO + PSH = PSNO + GSH \quad (4)$$

GS-SP or PSNO may also be generated by other mechanisms (Hauslanden and Stamler, 1998). Since a recent study supports the occurrence of various exchange reactions of PSH (Sengupta et al., 2001), we can assume that protein *S*-thiolation or *S*-nitrosation may occur by multiple pathways.

Biological responses elicited by RS-NO or RSSR are linked to *S*-thiolation and *S*-nitrosation of specific PSH or receptors. However, as established by equilibria (3) and (4), the possibility of having functional *S*-thiolated or *S*-nitrosated proteins is modulated by the action of NPSH and non-essential PSH, that compete with essential PSH. Since reactions (3) and (4) are easy reversible, being the corresponding equilibrium constants nearly 1 (Gilbert, 1995; Hogg, 1999), a crucial point for any biological effect mediated by PSH is certainly linked to protein structures and the possibility of conformation change after formation of thiol- or nitroso-adducts. In this context, it is interesting to note that the rate constants of PSH with disulfides are characterised by a very high range of variation, equal to 10^6 (Snyder et al., 1981), a feature that prepares a hierarchy of possible reactions inside the PSH pool.

Interactions between GSH and PSH

Mammalian tissues are relatively rich in small (GSH, 2–6 mM) and/or high molecular weight thiols (PSH 20–40 mM) (Gilbert, 1984; Torchinsky, 1981). In this

context GSH, together with ascorbic acid and NAD(P)H, is the most important biological reductant operating in cellular aqueous phase. A reducing power (GSH, $E'^{\circ} = -0.23$ V), intermediate between NAD(P)H ($E'^{\circ} = -0.32$ V) and ascorbate ($E'^{\circ} = +0.08$ V), and a relatively high concentration render GSH the best reductant inside the cells, being utilised for the metabolism of xenobiotics, chelation of heavy metals, interception of free radicals and restoration of endogenous reductants (ascorbate) consumed in intracellular redox processes (regeneration in membranes of α-tocopherol). This potential is further enhanced by a variety of ubiquitous enzymes that in situations of oxidative and nitrosative stress guarantee an efficient GSH regeneration by GSH-recycling or biosynthetic pathways (Di Simplicio et al., 1998a; Giustarini et al., 2000; Kondo et al., 1999; Moellering et al., 1999).

In theory reactions of thiols (PSH and GSH) with electrophilic agents in situations of oxidative and nitrosative stress should be characterised by rather similar rates. Instead, for sterical, lipophilic or charge reasons, rates of the PSH reactions are highly variable (Snyder et al., 1981) so that reactants are sometimes totally impeded to reach PSH (buried PSH), or react with PSH much faster (fast reacting PSH) or slower (slow reacting PSH) than with NPSH.

The RSH reactivity is generally related to their ability to dissociate to the thiolate anion. This tendency, measured by the pKa value, is dependent on the RSH structure. Whereas rate constants of NPSH are strictly related to corresponding pKa values, this is not always true for PSH whose reaction rates are conditioned by steric factors (the PSH exposure to the milieu). The examples of albumin and human hemoglobin, whose pKa are respectively lower (about 5–7) or similar to GSH (about 9), are paradigmatic. In fact, the reaction rate of albumin with DTNB is much lower than that of GSH and is accelerated by detergents (Di Simplicio et al., 1985) that increase the PSH exposure to the milieu. The importance of the PSH exposure to the milieu has more recently been investigated for albumin by Christodoulou et al. (1995) and Sengupta et al. (2001).

Human hemoglobin is characterised by only one reactive cysteinyl residue (at position β93), that, in spite of the similar pKa to GSH, is much less reactive than GSH (Di Simplicio et al., 1998a).

By contrast, rat hemoglobin, equipped with three titrable cysteine residues (α13, β93 and β125) of which one (β125) fast reacting and with a pKa value of 6.9 (Rossi et al., 1998), exhibits a reactivity much higher than GSH (of about thirty times). This property confers to rat hemoglobin an antioxidant capacity greater than GSH. In rat RBC exposed to oxidants or conjugating agents, PSH of hemoglobin with fast feature rather than GSH are preferentially consumed (Di Simplicio et al., 1998a; Rossi et al., 2001a).

Steric reasons may influence protein reactions. In fact, the formation of intramolecular protein disulfides may be impeded if the topology costraints prohibit it. This is the reason why PSH are more easily transformed into RS-SP rather than PSSP when subjected to oxidations. Albumin is known to be unable to form dimers (PSSP) when exposed to oxidant agents. This lack of dimerization may reflect its rapid reaction, with GSH, cysteine, cysteinylglycine and homocysteine to form RS-SP (Mansoor et al., 1992; Di Giuseppe et al., 2003).

Several proteins of relatively very low pKa exist and under conditions of physiological pH they are considered easy targets of electrophilic compounds (RNS). Consequently, cGMP-independent biological effects of NO are attributed to non-enzymatic chemistry probably mediated by thiols (Stamler, 1994; Ahern et al., 2002; Hogg, 2002). The relatively great acidity of PSH seems to be dependent on the close proximity of positively charged groups of basic amino acid residues, such as histidine, lysine or arginine, as observed in papain, thioredoxin, protein phosphatase 1B and other proteins (Snyder et al., 1981; Lohse et al., 1997; see for references Di Simplicio et al., 1998b).

Experiments of oxidative stress, in RBC of species characterised by hemoglobins with different SH reactivity, have confirmed the correspondence between reactivity and scavenging properties of PSH (Di Simplicio et al., 1998a). It is as yet unknown whether this correspondence exists and how is used with oxidants that evoke physiological responses. For example O_2^- or H_2O_2 are considered cell mediators of the redox signaling and, at least in theory, PSH could be regarded as possible functional targets. In such cases, it remains to be established how the PSH escape from the interception of other RSH or enzymes (such as SOD and catalase).

Studies in RBC models on the interaction of GSH and PSH with electrophilic substances have contributed to some extent to the resolution of these questions. RBC are equipped with a rather simple PSH population, in which hemoglobin accounts for more than 95% of total proteins, and are characterised by a very good antioxidant system (van Asbeck et al., 1985). Consequently, since the reaction of electrophilic agents with PSH is non-enzymatic (Thomas et al., 1995), possible PSH interference on the GSH metabolism in each model should be related to the

corresponding PSH reactivity. In RBC subjected to oxidative stress, the GSH metabolism is strictly linked to the composition of the PSH population and very reactive PSH were preferential targets of oxidants (Di Simplicio et al., 1998a; Giustarini et al., 2000; Rossi et al., 2001a). Thus, the possibility of functional PSH to react with ROS is linked to the competition exerted by other RSH. Nevertheless, successive studies have shown that problems are more complex. For example, when subjected to particular electrophilic agents (menadione), reactive PSH are able to elicit redox cycling phenomena (Lusini et al., 2002). It derives from this that antioxidant PSH (or functional PSH) can even become targets of pro-oxidant actions. Thus high reactivity need is not the only requisite for functional PSH to react with ROS and elicit a physiological response.

The biological role of protein sulfenic acid

RSOH are formed by different mechanisms, as suggested by Stamler and Hausladen (1998), and are considered very reactive intermediates of RSH oxidation. In turn RSOH react with thiols to form mixed disulfides and disulfides (Claiborne et al., 1999).

The –SH can be oxidised to higher oxidation levels depending upon the oxidant capacity (as established by the redox couple of the oxidant in the Nernst' equation) (Schafer and Buettner, 2001). From the biological point of view, the most important and stable oxidation forms of –SH (whose oxidation grade is -2) are disulfides (oxidation grade -1). Higher oxidation grades, sulfinic ($R–SO_2H$, oxidation grade of sulfur $+4$) and sulfonic ($R–SO_3H$ oxidation grade of sulfur $+6$) are less frequent in biology. RSOH (oxidation grade of sulfur is 0) are formed under mild oxidation but are very labile. They are likely candidates that may explain mechanisms why H_2O_2 and PSH are supposed to be involved in more specific functions, as the redox signaling (Lee et al., 1998; Denu and Tanner, 1998; Schmid et al., 1999; Percivall et al., 1999).

Small RSOH are very unstable (Kice, 1980; Hogg, 1990, Claiborne et al., 1993), but their life is prolonged by appropriate steric, electronic, and intramolecular-bonding factors (Claiborne et al., 1993). Although formation of PSOH has been reported (Becker et al., 1998; Schmid et al., 1999; Barret et al., 1999), proofs of their formation is indirect (Claiborne et al., 1999). Under anaerobic conditions, 2 mol of NO reacts with specific PSH to form PSOH and nitrous oxide (N_2O)(De Master et al., 1995). It is important to note that the ability *c-fos* and *c-jun* proteins to bind DNA is associated with PSOH/PSH redox cycle, probably due to sulfenic acid formation (Mc Bride et al., 1992). With Fos and Jun proteins the first demonstration of sulfenic acid formation was indirect (Abate et al., 1990).

The persistence of sulfenic acids at neutral pH is dependent on the acidity of corresponding PSH (the pKa) because at physiological pH (about 7.2) the conjugate sulfenate base is more stable (Claiborne et al., 1993; Claiborne et al., 1999). Cysteine residues responsible for the DNA binding of Fos and Jun proteins are characterized by an highly conserved sequence (Lys-Cys-Arg), invariant among the *fos* and *jun* gene families (Xanthoudakis et al., 1992). This feature is responsible for low pKa of PSH and PSOH, and therefore for the sulfenate stabilization.

RSSP may be formed after reaction of sulfenates (PSO^-) with RSH:

$$PSOH + RSH{=}RS\text{-}SP + H_2O$$

According to Stamler and Hauslanden (1998), a range of cysteinyl modifications of proteins, via sulfenic acid, mixed disulfides or nitrosothiols, might be implicated in regulatory or toxic events. This possibility of discrimination of biological signals, although not totally shared by other authors (Hogg 2002; Claiborne et al., 1999), would be based on the existence of a consensus motif in proteins that allows the recognition specific redox signals (Stamler et al., 1997). In other words, according to this prediction, particular classes of thiols in proteins would be able to discriminate among redox species or to serve different regulatory functions. In this context, although some aspects remain controversial, an increasing role has been attributed to sulfenic acids of proteins. At the moment, there is no doubt that some sulfenic acids of proteins exist, being also proposed rules for their stabilization (Becker et al., 1998; Claiborne et al., 1999; Schmid et al., 1999).

Excessive nitrosylation can result in the loss of cell function under conditions of nitrosative stress. Although GS-NO is considered an important NO carrier involved in redox regulations, its excessive production may be deleterious. For example, GS-NO may nitrosylate Cys63 and/or Cys58 of glutathione reductase causing irreversible inhibition of enzyme activity (Becker et al., 1998). Thus, given the central importance of glutathione reductase and GSH-recycling system in regulating cell levels during oxidative stress (van Asbeck, 1985; Di Simplicio et al., 1998a; Giustarini et al., 2000), the inhibition of glutathione reductase by GS-NO may represent an important toxicity sign elicited by a nitrosative stressor.

The fate of NO and RNS species

NO

NO exhibits a low level of reactivity in reactions with most biological molecules compared to other free radicals and its mode and rate of degradation may change considerably in the gaseous and in the aqueous phase (Kelm, 1999). Even its effects may vary. For example, in spite of its notorious toxicity, predominantly exerted by oxidative reactions, NO is known to possess antioxidant features (Goss et al., 1999) that are particularly evident in lipids (membranes) where NO easily partitions and exerts a potent inhibition of lipid peroxidation (Hogg and Kalyanaraman, 1999).

The major product of NO degradation in aqueous solution phase is nitrite, according to the following reaction (Bonner and Stedman, 1996):

$$4NO + O_2 + 2H_2O = 4HNO_2 \quad (5)$$

whose kinetics and half-life are highly dependent on the NO concentration (Ford et al., 1993).

NO reacts in biological systems with oxygen, superoxide (O_2^-) and transition metals (Stamler et al., 1992a). In addition it may be transformed into nitroxyl anion (NO^-) or nitrosonium ion (NO^+) by respective electron gain or electron loss (Heslop and Jones, 1976; Bonner and Stedman, 1996).

Not all the RNS species generated from the NO chemical reactions seem to have biological relevance. Thus, the importance attributed to NO^+ as a free species is considered very scarce (Hogg, 2002) and even the existence of $ONOO^-$ in tissues has been questioned (Fukuto and Ignarro, 1997).

The half-life of NO is controlled by the oxygen concentration, and the reaction between NO and oxygen is much faster within membranes than in the surrounding aqueous medium (Liu et al., 1998). At increasing partial oxygen pressure, up to atmospheric figures (700 mm Hg), a reduction of half-life of NO (from 6.2 s to 3.8 s from 150 to 700 mm Hg, respectively) has been observed (Kelm et al., 1991). Thus, for its relatively long half-life, approximately 3 sec, and in dependence of the oxygen concentration, NO is assumed to diffuse and reach its biological targets across several cell diameters (Lancaster, 1998). Other authors have estimated a larger range of half-life of NO under physiological conditions (from 9 to 900 min) (Beckman and Koppenol, 1996).

The diffusion capacity of NO in tissues, at long distance from vasculature, has been measured in saline perfused hearts. In absence of hemoglobin that limits a lot the diffusion process, NO is able to reach vascular smooth muscle cells as well as mithocondria of cardiomyocytes (Kelm et al., 1995; Kelm et al., 1997a). However, the entity of diffusion is linked to factors that control the NO decay such as oxygen, ROS, RSH, antioxidant enzymes (SOD) and ligands (hemoglobin). Thus, the extent of diffusion is very plausibly dependent on the microenvironment, the corresponding concentration of bioreactants and the complexity of NO reaction (Kelm et al., 1997b). These facts may explain why differences in NO half-life are observed when NO is estimated with different bioassays (Borland, 1991; Kelm and Schrader, 1990). However, at low concentrations of NO that exists under normal conditions of biosynthesis and diffusion, the most likely reactions are those with the transition metals of proteins (copper and iron) (Cooper, 1999) and free radicals (Beckman et al., 1990; Crow and Beckman, 1996).

Because NO cannot be stored or released by conventional regulatory mechanisms (such as acetylcholine or norepinephrine) the NO synthesis is more important than for other mediators. This is achieved by a well organized machinery of NO synthases to avoid NO overproduction that could cause cell and tissue injury. NOS are the products of three genes, named NOS1 or neuronal (n), NOS2 or inducible (i) and NOS3 or endothelial (e) (Bredt, 1999; Stuehr, 1999; Nathan and Xie, 1994). Eight cysteines are conserved within the core oxygenase domains of NOS. NOS1 may produce nitroxyl anion (NO^-) (Schmidt et al., 1996), whereas all NOSs are able to generate NO, RS-NO and probably small amount of $ONOO^-$ (Stamler, 1999).

The first demonstration of NO biosynthesis was carried out in brain preparations (Knowles et al., 1989). Brain, as any excitable tissue, is relative rich in NOS (Lin et al., 2000). Physiological functions of NO were first demonstrated in the regulation of intestinal function which is mediated by non-adrenergic, non cholinergic nerves and by the NO receptor (the soluble guanylyl cyclase) of adjacent intestinal smooth muscles. The regulation of blood flow, release of neurotrasmitters, penile erections and muscle contractions are other functions elicited by NO (Bredt, 1999). In endothelial cells the NOS system may generate both NO and O_2^- (Schmidt et al., 1996) that can interact to form $ONOO^-$ at a near-diffusion-limited rate ($6.7\,10^9\,M^{-1}s^{-1}$) (Huie and Padmaja, 1993). However the formation of $ONOO^-$ is conditioned by equimolar concentrations of NO and O_2^- because the excess of one radical over the other may

modulate the endogenous $ONOO^-$ formation (Miles et al., 1996).

The steady-state levels of NO depend on the equilibrium between its rate of formation and its rate of decomposition. Under normal conditions NO sources are represented by NOS activities and by RS-NO within a 20 nM–2 μM concentration range (Moncada et al., 1991). However, other possibilities of NO formation exist, for example after disproportionation or reduction of nitrite during ischemia in acid milieu and reducing conditions (Zweier et al., 1999).

Humans excrete nitrates derived from NO biosynthesis. Nitrate excretion is increased in patients with diarrea or inflammation (Hegesh and Shiloah, 1982; Wagner et al., 1983). In macrophages the iNOS form is particularly efficient and mice genetically deficient of these cells display very low excretion of nitrates (Stuehr and Marletta, 1985).

In an effort to simplify the chemistry and the variety of biological reactions of NO and RNS, it was proposed to categorise the NO effects, as direct and indirect (Wink et al., 2000). Thus, direct effects are those supposed to occur at relatively low NO concentrations ($<1\,\mu M$), whereas the indirect ones would occur at higher levels ($>1\,\mu M$). This simplification presumes an hierarchy of reactions that are activated in dependence of the NO concentration. Thiols are presumed to act indirectly and at NO concentrations $>1\,\mu M$ the rate of the reaction would be increased by the presence of oxygen as previously observed (see equation 2). As a consequence of this distinction, low NO doses are considered protecting, whereas higher doses are damaging (Joshi et al., 1999). However not all injuries by NO are mediated by oxidative stress (Eu et al., 2000).

NO^-

NO^- can be generated in the cell milieu by several routes, directly by NOS (Schmidt et al., 1996) or by interaction of NO with Fe(II) heme protein (Hughes, 1999). As noted above, NO^- reacts with O_2 to give $ONOO^-$ and in tissues the reaction would be favoured by the presence of active SOD (Murphy and Sies, 1991; Hughes, 1999). However, according to other authors, NO^- may also be formed in absence of SOD (Sharpe and Cooper, 1998), or by decomposition of RS-NO, as described by Arnelle and Stamler (1995). The parent acid HNO has a pKa of 4.7 and is fully deprotonated at physiological pH.

NO^- is very unstable in solution and via dimerization and dehydration gives nitrous oxide (N_2O). Its stability is increased (in terms of milliseconds) at neutral pH and thiols and metal centres are the best biological targets (Hogg et al., 1996; Wong et al., 1998; Hughes, 1999). However, whereas NO is difficult to oxidise to NO^+, having a reduction potential of 1.2 V, NO^- is more easily formed and is considered an important biochemical entity, having a significant life-time (although shortened by the presence of thiols and oxygen)(Hughes, 1999).

NO^- may exert actions similar to EDRF, but this could be linked to its transformation to $ONOO^-$ (Hughes, 1999).

$ONOO^-$

$ONOO^-$ has been extensively studied as a potent cytotoxic RNS and its action, mediated by $OH^\bullet$ formation, is accompanied by protein tyrosine-nitration in tissues (Beckman et al., 1990; Crown and Beckman, 1996; Beckman and Koppenol, 1996). However a general consensus on protein tyrosine-nitration by $OH^\bullet$ does not exist (Halliwell et al., 1999). Even the toxicity *in vivo* of $ONOO^-$ is rather dubious, as well as the convenience of using the peroxynitrite-mediated nitration of protein tyrosines, as a marker of irreversible damage. In fact, according to recent discoveries, protein-tyrosine nitration, as protein *S*-thiolation or *S*-nitrosation, is subjected to reversible processes (Kamisaki et al., 1998; Kuo et al., 1999). Therefore, more experiments are requested, using $ONOO^-$ donors rather than alkaline bolus, to eliminate possible artefacts (Halliwell et al., 1999), even though the suitability of some $ONOO^-$ donors (SIN-1) has been questioned (Sing et al., 1999).

Many possibilities of $ONOO^-$ generation in tissues have been described, for example in activated macrophages (Ischiropoulos et al., 1992). $ONOO^-$ (and NO^-, see above) may also be generated in mitochondria in absence (Sharpe and Cooper, 1998) or presence of active SOD (Hughes, 1999).

The half-time of $ONOO^-$, of about 1 s, seems sufficient to allow diffusion to the surrounding cells to elicit toxicity that is counteracted by thiols of low and high molecular weight (Hughes, 1999; Radi et al., 1991; Karoui et al., 1996; Quijano et al., 1997; Gatti et al., 1994). Sulfenic acid is a possible intermediate of $ONOO^-$ action, whereas protein-mixed disulfides are possible end-products (Clairborne et al., 1993; Peshenko and Shichi, 2001). However, other authors considered the oxidant action of $ONOO^-$ very potent because the inhibition of creatine kinase that contains an essential SH for substrate binding, was not reversed by thiol reducing agents (Konorev et al., 1998).

The biological consequence of the metabolism of $ONOO^-$ by GSH is rather complex. Although GSH is considered the major protection against $ONOO^-$ toxicity (Karoui et al., 1996), thiols in some circumstances are able to exacerbate the $ONOO^-$ damage (Whiteman and Halliwell, 1997). However, under different experimental conditions the reaction between GSH and $ONOO^-$ may generate NO or GS-NO that represent a further efficient defence mechanism against the potential toxicity of $ONOO^-$ (Moro et al., 1994).

At physiological pH $ONOO^-$ is rather unstable and after isomerization forms nitrate, that originally was considered the best way to scavenge O_2^- (Rubanyi et al., 1991). At alkaline pH $ONOO^-$ is more stable, whereas it decays rapidly when protonated (ONOOH, $pKa = 6.6$) (Beckman and Koppenol, 1996; Beckman et al., 1990; Blough and Zafiriou, 1985). CO_2 is known to have a great role in the decomposition of ONOOH and various metabolic products are generated from it (Uppu et al., 1996).

RS-NO

Ignarro et al. (1981) first demonstrated that the pharmacological effects elicited by various NO donors were mediated by nitrosothiols. About ten year later it was observed that RS-NO exerted bioactivities independent of breakdown to NO (Kowaluch and Fung, 1990). This novel and intriguing observation remained silent until Stamler and coworkers aroused again the attention on SNOs as nitrosonium and nitroxyl donors (Stamler et al., 1992a). Consequently, the general interest in the thiol-NO interaction and in biological activities of RS-NO distinct from those of NO increased and it was demonstrated that RS-NO may modify proteins forming *S*-nitrosylated derivatives and that S-NO proteins exist in mammalian tissues and fluids (Stamler et al., 1992b; Gaston et al., 1993; Jia et al., 1996). Thus NO is supposed to regulate many cell processes by *S*-nitrosation of critical proteins (Hirayama et al., 1999; Xu et al., 1998; Ruiz et al., 1998; Arstall et al., 1998).

Protein *S*-nitrosation may occur more frequently than *N*-nitrosation or *C*-nitrosation of proteins (Simon et al., 1996). Moreover, stable reservoirs of S-NO, but not of N-NO or C-NO compounds, have been detected in tissues (Stamler et al., 1992b; Gaston et al., 1993; Kluge et al., 1997; Giovannoni et al., 1997).

RS-NO, or thionitrites in old chemical literature, are relative stable compounds, if in absence of heavy metal contamination. They are responsible for important biological effects such as, signal transduction, vascular homeostasis, neurotransmission and inflammation and are commonly used as NO donors (Hogg, 2000; Moro et al., 1994; Mayer et al., 1995; Rigacci et al., 1997).

Usually RS-NO are formed by the oxygen-dependent oxidation of NO in the presence of RSH (reaction 2) (Oae and Shinama, 1983; Kharinotov et al., 1995; Hogg et al., 1996; Hogg, 2002). In particular, GS-NO is formed as a minor metabolite during the oxidation of GSH by peroxynitrite (Moro et al., 1994; van der Vliet, 1998). Although the mechanism is still controversial (Zeng et al., 2001), biological effects of GS-NO are ascribed to the NO release.

RS-NO may react differently by three main reactions, that lead to the release of NO, transnitrosation or *S*-thiolation. Thus in the presence of copper ions Cu^+ or Cu^{++} RS-NO decomposes to NO and this is accompanied by generation of RSH or RSSR, respectively (Hogg, 2000).

In the presence of RSH, RS-NO may give transnitrosation (reactions 4) or *S*-thiolation forming a mixed disulfide (Hogg, 2000):

$$RS\text{-}NO + R'SH \rightleftharpoons R'SSR + NO^- \quad (6)$$

Transnitrosation reactions involve the transfer of the nitroso moiety by a nucleophilic attack of the thiolate anion on the nitrogen group (Meyer et al., 1994; Singh et al., 1996; Patel et al., 1999). It has been suggested that transfer of the NO moiety of GS-NO to PSH, represents a potential mechanism for modulation of the protein activity by activation/inactivation (Stamler, 1995). However, since these reactions are readily reversible and the GSH concentration inside the cells is at millimolar range, *S*-nitrosated proteins may be stabilized if the process involves a conformation change that blocks the reverse reaction. These changes may represent a new way of cell signaling and control of homeostasis (Mannick et al., 1999; Ruiz et al., 1998; Dimmeler et al., 1998).

Whereas the tissue concentration of NO is in the low nM range, that of RS-NO (GS-NO) is in the μM range (Gaston et al., 1993; Kluge et al., 1997). GS-NO has been found in various cell types and plasma (Jia et al., 1996; Giovannoni et al., 1997; Gaston et al., 1998; Gordge et al., 1998; Gordge et al., 1996; Clancy et al., 1994; Do et al., 1996), but the real level in plasma is still controversial (Rossi et al., 2001b).

The protein *S*-thiolation process

The importance of protein-mixed disulfide (or thiol-protein mixed disulfide) formation as a functional response to

oxidative modification in living organisms has long been known. This process can take place in all biological compartments as consequence of oxidative/nitrosative stress events or in (patho)-physiological conditions. To cite only few examples, plasma *S*-thiolated proteins have been detected in healthy humans (from newborns to adults) (Di Giuseppe, unpublished results), in patients with cardiovascular diseases (Di Giuseppe et al., 2003; Mansoor et al., 1992), and in several cell types subjected to oxidant exposure (erythrocytes, platelets, neutrophiles, monocytes, macrophages, hepatocytes) (Tsukahara et al., 1987; Chai et al., 1994a; Chai et al., 1994b; Ravichandran et al., 1994; Di Simplicio et al., 1998c; Di Simplicio et al., 1998a; Giustarini et al., 2000). Although increased protein *S*-thiolation is considered an *in vivo* marker of oxidative injury provoked by noxious agents (Park et al., 1998; Lou et al., 1999; Figueiredo-Pereira et al., 1998; Di Giuseppe et al., 2003), the significance of this index in clinical studies has not been well documented.

According to a great variety of studies (reviewed by Gilbert, 1984; Ziegler, 1985; Brigelius, 1985; Gilbert, 1995; Cotgreaves and Jerdes, 1998) the oxidative challenge in tissues or cell cultures results in transient accumulation of protein mixed disulfides, whose prompt recovery is secured by the coordinate action of GSH (and other thiols), the GRX system and the enzyme machinery of GSH regeneration via glucose consumption (Mannervik and Axelsson, 1980; Di Simplicio and Rossi, 1994; Jung and Thomas, 1996; Di Simplicio et al., 1998a; Di Simplicio et al., 1998c; Giustarini et al., 2000). Moreover, cell protein dethiolation may be enzymatic and non enzymatic (Jung and Thomas, 1996; Schuppe-Koistinen et al., 1994b; Di Simplicio et al., 1998c). While the dethiolation by GSH is enzymatically regulated by GRX that by minor thiols (cysteine) is non-enzymatic (Di Simplicio et al., 1998c; Mallis and Thomas, 2000).

The list of enzymes or proteins modified in activity or structure by *S*-glutathionylation is very long and the most relevant examples includes: carbonic anhydrase III (Chai et al., 1991; Lii et al., 1994), creatine kinase (Collison and Thomas, 1987; Reddy et al., 2000), GAPDH (Ravichandran et al., 1994; Schuppe-Koistinen et al., 1994a; Lind et al., 1998; Mohr et al., 1999), actin (Chai et al., 1994b), glycogen phosphorylase *b* (Park and Thomas, 1988; Miller et al., 1990), hemoglobin (Rossi et al., 1998), albumin (Di Giuseppe et al., 2003), metalloproteinases (Okamoto et al., 2001), glutathione *S*-transferase (Terada et al., 1993), c-Jun transcription factor (Klatt et al., 1999a; Klatt et al., 1999b), cathepsin K (Percivall et al., 1999), thrombospondin (Speziale and Detwiler, 1990), caspase-3 (Zech et al., 1999) and aldose reductase (Chandra et al., 1997). According to a more recent study, in which proteins were identified by proteome analysis in oxidant-treated human T lymphocytes, the list of *S*-glutathionylated proteins is further increased (Fratelli et al., 2002).

The protein *S*-nitrosylation process

Sulfhydryl groups of a great variety of proteins, such as hemoglobin (Jia et al., 1996; Di Simplicio et al., 1998b), albumin (Stamler et al., 1992b; Stamler et al., 1992c; Di Simplicio et al., 1998b), ryanodyne receptor (Xu et al., 1998), NMDA receptor (Lipton et al., 1998), clotting factor XIII (Catani et al., 1998), GADPH (Galli et al., 2002; Padgett and Whorton, 1998; Mohr et al., 1999), glutathione-S-transferase (Clark and Debnam, 1988; Lo Bello et al., 2001), several members of the caspase family (Li et al., 1997; Tenneti et al., 1997), ras (Lander et al., 1997), trascription factors (DelaTorre et al., 1997; Morel and Barouki, 1999), papain (Venturini et al., 1998), creatine kinase (Wolokser et al., 1996; Konorev et al., 2000), Janus kinase (Duhé et al., 1998), alcohol dehydrogenase (Gergel and Cederbaum, 1996), isocitrate dehydrogenase (Yang et al., 2002), c-Myb (Brendeford et al., 1998), and proteins involved in the L-arginine transport (Howard et al., 1998) undergo exchange reactions with RS-NO and most of them may be oxidatively modified forming *S*-thiolated proteins.

For example, GS-NO caused *S*-thiolation and inhibition of creatine kinase (Konorev et al., 2000). Similarly, NO inactivates papain by PS-NO formation at cysteine 25 (Venturini et al., 1998), but various RS-NO donors were able to cause protein *S*-thiolation (Xian et al., 2000). Moreover, under more physiological conditions, as in cells exposed to NO, *S*-thiolation of glyceraldehyde-3-phosphate dehydrogenase has been observed (Padgett and Whorton, 1998; Mohr et al., 1999). Thus protein *S*-thiolation seems to be a recurrent phenomenon during protein *S*-nitrosation.

The signal trasduction process may involve nitrosothiols and protein *S*-nitrosation by a guanylyl cyclase-independent mechanism. In the case of $p21^{ras}$, the protein is *in vitro S*-nitrosated at cysteine 118 and the modification is important for the related signaling cascade (Lander et al.,1995; Lander et al., 1997). By a process, specific for the L but not for the D form, involving exchange reactions, *S*-nitrosocysteine is able to increase the palmitate turnover on the oncogenic *H-Ras* in NIH3T3 cells (Baker et al., 2000). This finding seems to be an indirect confirmation of the existence of a consensus motif that

drives specific nitrosation reactions by lowering the pKa of cysteinyl residues in the primary structure of proteins (Stamler et al., 1997). In this connection Mallis and Thomas (2000), in cell cultures exposed to different RS-NO donors, have recently demonstrated that levels of protein *S*-nitrosation and *S*-thiolation may occur depending on the RS-NO concentration. Thus at millimolar RS-NO levels more extensive *S*-nitrosation and *S*-thiolation of protein were observed, whereas under more physiological conditions of exposure these processes were greatly reduced and influenced by the GSH concentration of the cell model (Ji et al., 1999). In addition to the consensus motif of proteins, other authors have stressed the role of protein folding to influence the pKa of PSH (Ascenzi et al., 2000). In our opinion all these studies underscore once more the pivotal importance of the PSH reactivity and exposure, and the competition that other RSH (NPSH + PSH) may exert on the various exchange reactions with endogenous RS-NO.

Mechanisms of protein *S*-thiolation during oxidative and nitrosative stress – the role of reactive nitrogen species

Protein S-thiolation by oxidative stress

The RS-SP increase elicited by the ROS action may occur by various pathways, such as SH/SS exchange reactions between disulfides and PSH (reaction 3), reactions of PSOH with RSH (reaction 6), or interceptions of thiyl radicals by PSH. It is unknown whether all reactions of protein *S*-thiolation are contemporaneous operating in the same cell model and whether the prevalence of one or another mechanism may serve to select specific *S*-thiolated proteins. However some simple predictions, on the role that the antioxidant system may exert to make a selection of *S*-thiolated proteins, can be done.

In our experience and according to literature, the rate of *S*-thiolation of protein models (BSA) via sulfenic acids (H_2O_2 plus GSH):

$$H_2O_2 + PSH{=}PSOH + H_2O$$

$$PSOH + GSH{=}GS\text{-}SP + H_2O \qquad (7)$$

is much higher than via the SH/SS exchange reaction by GSSG (unpublished data). However, PSOH formation is only possible under mild oxidative conditions because at relatively high H_2O_2 concentration PSO_2H or PSO_3H can be formed (not reversed protein *S*-thiolation). Consequently, whether protein *S*-thiolation occurs via GSSG or PSOH mechanisms will be determined by combinations of antioxidant factors.

For example, oxidation in cells having reactive and thiol-permeable PSH may contemporaneously produce increased GSSG and PSOH levels. Since the intermediary formation of PSOH is linked to moderate oxidations, cells requiring GS-SP by this mechanism should control the oxidation process to avoid higher forms of sulfur oxidation. The successive GS-SP formation would be dependent on accessibility of the PSOH for reaction with GSH (reaction 7).

By contrast, a more rigorous control of the oxidation insult and prolongation of the GSSG lifetime is required in order to generate GSSP by the GSSG pathway (i.e., via SH/SS exchange reactions). This can be achieved by appropriate expression of various antioxidant factors such as SOD, CAT and/or GPX.

The above reported speculations are partially supported by results of kinetic measurements of the effects of different oxidant such as *t*-BOOH and diamide on GSH, GSSG, and GSSP levels in RBC of different species (human, calf, turkey and rat) (Di Simplicio et al., 1998a). In all species *t*-BOOH causes a nearly instantaneous (15 sec) total loss of GSH. In turn, GSSG is able to exchange with reactive and well exposed PSH to form GS-SP. In any case, other mechanisms of GS-SP formation via PSOH are plausible, but seem unlikely in view of the relatively high GPX activity (that rapidly metabolises *t*-BOOH) and the pattern of kinetics (see below).

In human and calf species, where the PSH reactivity is extremely low, the possibility of PSH exchange with GSSG is very low and no GS-SP increase is observed. Therefore, differences in PSH reactivity and appropriated expressions of enzyme activities (GPX) are important for the formation of GS-SP from GSSG in rat hemoglobin, whereas for other proteins (BSA, human and calf hemoglobin) this possibility is modest or absent.

Kinetic data of diamide in RBC are interesting because the agent reacts differently from *t*-BOOH and its action is to some extent comparable with the hypothetical one of H_2O_2 in cell models with relatively low GPX and CAT activities. As the diamide metabolism is non-enzymatic, all thiols, NPSH and PSH, compete for it in dependence of corresponding reactivities and concentrations. Diamide causes in human, calf and rat RBC, equal GSH depletions. But in human and calf this is matched by time-dependent formation of GSSG peaks, whereas in the rat GSSG is constant. Parallelly, no GS-SP changes in human and calf RBC are

seen, whereas high GS-SP peaks (at 15–30 sec) are obtained in rat. Interestingly, the GS-SP maximum in the rat (15–30 sec) is earlier than that observed at same *t*-BOOH doses (maximum at 3–5 min). From all these data it is supposed that diamide in the rat reacts first with PSH and then the protein adduct reacts with GSH forming GS-SP. In other terms, the mechanism would be similar to that obtainable with H_2O_2 and mediated by PSOH. Moreover, since the GS-SP peak observed with *t*-BOOH is delayed with respect to that of diamide, it seems unlikely that *t*-BOOH reacts directly with PSH to form PSOH in rat RBC.

In conclusion data on the rat model suggest the possibility of two mechanisms of protein *S*-thiolation depending on the type of oxidant. No massive protein *S*-thiolation is possible in models with relatively low PSH reactivity (human and calf), whereas an intermediate situation between two opposite situations is possible in models with an intermediate PSH reactivity (turkey).

The PSH involvement may be even more complex as is indicated by a comparison of reactions by $ONOO^-$ and H_2O_2. Whereas $ONOO^-$ reacts more rapidly (about three fold) than H_2O_2 with thiols, it prefers undissociated RSH, whereas H_2O_2 reacts more easily with RS^- (Radi et al., 1991). This interesting observation suggests that possible actions of H_2O_2 and $ONOO^-$ could be further modulated by differences in pKa of PSH.

Protein S-thiolation by RNS

In 1988 it was discovered that protein *S*-thiolation was the end-product of complex reactions between GS-NO and PSH of alcohol dehydrogenase (Park, 1988). About ten years later, it was confirmed with another enzyme (aldose-reductase) that treatment with GS-NO led to conversion of the active cysteine site (Cys-298) to its GS-SP derivative with a loss of enzyme activity (Chandra et al., 1997). In the mean time, other authors found that treatment of endothelial cells with GS-NO or other NO-donors leads to *S*-thiolation of proteins, and that this was accompanied by a reversible GSH depletion, likely controlled by an activation of the hexose monophosphate shunt (Padgett and Worthon, 1998), as previously demonstrated in similar experiments by others authors (Clancy et al., 1994).

According to Padgett and Worthon, (1998) the most likely mechanism to generate proteins-thiolation from a NO-donor was the following:

$$GS\text{-}NO + PSH = GS-SP + NO^- + H^+ \quad (8)$$

However, results of a previous study indicates that the interaction of GSNO with GSH may be more complex since in addition to GSSG several other products are formed, including, NH_3, $NO^{\cdot}$, and N_2O (Singh et al., 1996). These authors suggested that, depending on the reaction conditions, the intermediary formation of a *N*-hydroxysulfenamide derivative of GSH (GS-N(OH)-SG) might lead to the observed products. This intermediate could also react with the remaining GSH or undergo homolytic scission to two radicals, which in turn could be involved in other reactions. The various proposed mechanisms did not evoke NO^- as an important intermediate. By contrast, other authors (Wong et al., 1998) confirmed that HNO was generated by reaction (8) and HNO was able to generate some of the products found by Singh et al. (1996). Moreover, according to these authors (Wong et al., 1998), possible end-products could change in dependence of cell variations of the GSH/GS-NO ratio.

Although the molecular mechanism and yield are still unclear, it is now generally accepted that *S*-nitrosation of some proteins is also accompanied by *S*-thiolation. From the biological point of view both these events are equivalent, since changes in activity of *S*-thiolated or *S*-nitrosylated proteins are similar.

Various undetermined mechanisms of protein *S*-thiolation by GSNO may exist, and the question of which mechanism is involved in a given function is matter of ongoing debate. These controversies reflect the inherent complexity of the GSH/NO chemistry as revealed by other workers (Singh et al., 1996). Nevertheless, the most important requisite to have protein *S*-thiolation from processes involving RNS seems to be the PSH nucleophilicity and the possibility to recruit GSH in its proximity (Thomas et al., 1995), but the role of RNS remains controversial. In our opinion crystallographic studies may give important insights on mechanisms by which RNS generate *S*-thiolated proteins, as elegantly demonstrated by Becker et al. (1998).

According to Mohr et al. (1999), cysteine 149 of GAPDH is *S*-thiolated by GS-NO exposure and in endothelial cells the *S*-thiolation by GS-NO is more efficient than with H_2O_2. These differences and the greater efficiency of GS-NO as *S*-thiolating agent was attributed to the relatively high nucleophilicity of cysteine 149 and to a greater weakness of S–N bond in GS-NO relative to the S–S bond of GSSG produced by H_2O_2. However, as discussed above, mechanisms of *S*-thiolation via GS-NO are more complex because GSSG may exchange with PSH only under specific metabolic conditions. Although plausible, to prove that differences in bond strength of S–N and S–S are involved in protein *S*-thiolation, it is

necessary to demonstrate that H_2O_2 does not form GS-SP via PSOH.

Using a different protein model (cathepsin K) and various NO-donors (GS-NO, and also those not containing a sulfur moiety, such as NOR-1 and NOR-3), other authors confirmed the possibility to have *S*-thiolated cathepsin, reversed by DTT, when the protein was exposed to GS-NO, and the formation of sulfinic or sulfonic acid of the cysteine residue when the protein was exposed to NOR-1 or NOR-3 (Percivall et al., 1999). In this case the mechanism of protein *S*-thiolation by GS-NO was attributed to the direct displacement of HNO, according to reaction (8) in agreement with other authors (Stamler et al., 1992c; Sing et al., 1996; Wong et al., 1998).

Exposure of aldose reductase to GS-NO leads to protein *S*-thiolation of Cys-298 at the active site and to corresponding inhibition of enzyme activity (Chandra et al., 1997). According to the interpretation of the authors, the most likely mechanism of protein *S*-thiolation by GS-NO was that of reaction (8), although other possibilities were not excluded (Chandra et al., 1997).

A number of investigators believed that the formation of GS-SP by GS-NO reaction (8) is plausible because GSH and GS-NO should be equally accessibile to PSH (Thomas et al., 1995). Moreover, the energy of the GS-NO bond might favor the exchange (Mirza et al., 1995). However, in view of the multiplicity of products formed during the interaction of GSH with GS-NO (Singh et al., 1996), more complex mechanisms can not be excluded. In any case, in comparison with ROS, the possibility of forming *S*-thiolated protein by RNS must be relatively low because the absolute RNS concentration in all biological environment is very low except in severe cases of nitrosative stress. According to some authors (Figueiredo-Pereira et al., 1998) only 15% of *S*-nitrosated proteins would be *S*-glutathionylated and this is in accord with the limited supply of the substrate.

Conclusions

A growing number of proteins, enzymes, and transcription factors are modulated by changes in redox states of essential PSH. However proteins differ in their sensitivity to oxidation. In particular, the redox susceptibility of cysteinyl residues in essential PSH are dependent upon its localization within the protein, and can be perturbed by the action of non-essential PSH that may compete for the same reactions. Consequently, the role of non-essential PSH may be important in that they may attenuate the oxidant insult, thus reducing the possibility of forming protein sulfinic or sulfonic acids, but they may also cooperate with essential PSH to establish appropriate conditions of conformation or reactivity. In this sense, the experiments of glutathione reductase treated with GS-NO are paradigmatic because they evoke a series of reactions obtainable under physiological conditions (Becker et al., 1998).

Under functional conditions, the ROS- and RNS mediated processes are rapid, reversible and governed only by simple (!) chemistry. In the ROS case, different stimuli, from radiation to inflammation, are able to rapidly elicit the modulation of activity of various genes (Morel and Barouki, 1999) and the logical consequence of it is the activation of various proteins of which a great majority may be *S*-thiolated (Fratelli et al., 2002). More recent studies have indicated possible phenomena of protein *S*-thiolation during *S*-nitrosation processes. This demonstrates the great interconnection between the oxygen and NO metabolism of which protein *S*-thiolation is a further proof. Although various plausible mechanisms of protein *S*-thiolation by ROS and RNS have been suggested more work is necessary to clarify their biological meaning.

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