ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 7 & 8, 2005
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Forum Review

Biochemical and Biological Aspects of Protein Thiolation in Cells and Plasma

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ABSTRACT

Protein thiolation is elicited by oxidation by different mechanisms and is involved in a variety of biological processes. Thiols, protein SH (PSH) and non-protein SH groups (NPSH, namely GSH), are in competition in all biological environments in the regulation of oxidant homeostasis because oxidants thiolate proteins, whereas GSH dethiolates them (*e.g.,* GSSG + PSH → GSSP + GSH). Although poorly investigated, the elimination of disulfides from thiolated proteins to regenerate critical PSH is important. These aspects are poorly known in cells, where glutaredoxin and peroxiredoxin operate as enzymes or potential chaperones to accelerate dethiolation. On the contrary, studies with plasma or albumin have highlighted the importance of protein conformation in dethiolation processes and have clarified the reason why homocysteine (thiol with potential toxicity) is preferentially bound to albumin as protein-thiol mixed disulfide with respect to other NPSH. Here we provide an overview of protein thiolation/dethiolation processes, with an emphasis on recent developments and future perspectives in this field. *Antioxid. Redox Signal.* 7, 951–963.

INTRODUCTION

DURING OXYGEN METABOLISM, the formation of toxic intermediates is controlled by antioxidants in all biological milieus. When the oxidant/antioxidant balance is disrupted, the overproduction of reactive oxygen species (ROS) may damage various cell components and activate specific pathophysiological signaling (37). Numerous antioxidant strategies have evolved to limit the damage from oxidant production. Because of their high nucleophilicity and chemical versatility, thiols play a major role in orchestrating redox homeostasis, which is different in cells and plasma (81).

In cells, reduced thiols of low [nonprotein SH groups (NPSH)] and high molecular weight [protein SH groups (PSH)] prevail over the oxidized forms, which are abundant extracellularly. Indeed, levels of oxidized thiols are relatively high only in certain cell locations, *e.g.,* the endoplasmic reticulum (52, 100), and in spermatozoal mitochondria during the maturation phase (101).

Disulfides exist as symmetrical and asymmetrical compounds and are of low (XSSX and XSSR, respectively) and high molecular weight, *i.e.,* protein-thiol mixed disulfides (XSSP) and protein disulfides (PSSP). Protein thiolation, the posttranslational modification that binds NPSH to PSH to form XSSP, is an antioxidant device that reduces the impact of an oxidative stressor, and at the same time modulates the activities of many enzymes, structural proteins, and protein nuclear factors, or protects them from further oxidation. The control of important biological processes, such as glycolysis, proteolysis, redox regulation, redox signaling and DNA transcription, has been associated with the modulation of protein-thiol mixed disulfides (for reviews, see 20, 32, 56, 95, 108).

Protein thiolation is induced by oxidative and nitrosative stress via the following mechanisms: (a) radical reactions; (b) reactions mediated by protein sulfenic acids (PSOH); (c) exchange reactions between thiols and disulfides (or SH/SS exchange reactions or SH/SS interchange). Other mechanisms have recently been discovered, and some of them are medi-

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ated by free radicals and catalyzed by enzymes (59, 92, 94). However, although enzymes may be involved in these events (92), the phenomenon is generally considered nonenzymatic.

The plasma concentration of various XSSX and XSSP is several times higher than that of the corresponding NPSH (25, 26). XSSP account for 30–40% of total thiols (NPSH + XSSX + XSSP), and the thiol/disulfide ratio is around 1 or less than 1. In plasma, PSH are nearly exclusively represented by albumin (0.6–0.7 m*M*); NPSH range from 15 to 30 μ*M* and glutathione (GSH) from 4 to 8 μ*M*. Conversely, the thiol situation of cells (or tissues) is totally different: PSH and NPSH range from 20 to 30 m*M* and 2 to 6 m*M*, respectively. NPSH are nearly exclusively represented by GSH; glutathione protein mixed disulfide (GSSP) accounts for only a small percentage (1–4%) of total GSH and the thiol/disulfide ratio of GSH ranges from 30 to 300 (81).

A strongly reducing cell environment maintains protein thiolation at relatively low levels via very efficient GSH recycling and prompt activation of the glucose shunt. After a bout of oxidative stress, GSSP increases are rapidly controlled by activation of GSH recycling.

Plasma has prooxidant features, supported by the action of heavy metals bound to proteins (albumin and ceruloplasmin), and thiols added to plasma are easily oxidized (84). For example, injected thioredoxin (TRX) is oxidized in the circulation and loses its antioxidant power (69). Plasma is poor in GSH and the corresponding protein glutathionylation is modest, being lower than the thiolation of other SH compounds, including cysteine, cysteinylglycine, and homocysteine (Hcy) [plasma GSSP ranges from 0.8 to 1.4 μ*M*, plasma cysteine-protein mixed disulfides (CSSP) from 150 to 180 μ*M*]. Moreover, thiols and disulfides are easily exchanged with protein-thiol mixed disulfides and PSH of albumin, respectively (85). Therefore, albumin is a regulator of thiol levels, and this seems to compensate for the lack of GSH recycling in the plasma.

A great increase in cellular PSSP has been found during oxidative stress, and mass spectrometry has shown that these PSSP include peroxiredoxins (PRX), thioredoxin reductase (TR), nucleoside-diphosphate kinase, superoxide dismutase, GADPH, actin, tubulin, and many other proteins (up to 93 in all) (11, 21). More interestingly, cell treatment with various concentrations of hydrogen peroxide (H_2O_2) either promoted or inhibited PSSP formation in a concentration-dependent manner. This suggests that not only protein thiolation, but also PSSP may control multiple physiological processes, such as glycolysis, signal transduction, cell growth, etc. (21), as well as the trafficking to which most cell proteins are subjected. Other authors have demonstrated that, after formation of PSSP between 1-Cys PRX and πGST, 1-Cys PRX acquires its glutathione peroxidase (GPX) activity (61). Interestingly, during oxidative stress, multimeric aggregates of PRX switch their activities from peroxidase to a molecular chaperone function (53).

The biological relevance of the various protein thiolation mechanisms is unknown. To some extent, the question is related to the role of the sulfhydryl, of which protein thiolation is one important aspect. Sulfhydryls have greater reactivity than other nucleophiles, such as amino and hydroxy groups. For example, in conjugating reactions, NPSH react much faster than amino or hydroxy compounds, and thiols, unlike amino compounds (lysine), are able to block the biological effects of potent nucleophiles (8). Moreover, thiols are reductants, a redox property other nucleophiles do not possess. These chemical properties and the diversity of SH proteins make it difficult to understand how PSH are hierarchically organized and how they acquire a functional value in all biological contexts (34). One important issue is to know how proteins defend their integrity and specificity from aspecific attacks. No doubt these features are linked to conformational properties, but how they are further modulated in different biochemical situations is very difficult to predict.

The role played by conformational changes and the variety of PSH reactions has been emphasized during the activation of redox-sensitive transcription factors such as *E. coli* OxyR and *S. cerevisiae* Yap1 (41). We presume that a hidden conformation that reduces access to PSH by the solvent is responsible for the decreased PSH reactivity and the biological specificity against attack by noxious agents. This interpretation seems to be supported by the fact that very many cell proteins (about 2/3 of them) have slow-reacting SH groups, *i.e.,* slower than that of NPSH. Even albumin, a slow-reacting SH protein, is subjected to strong conformational play, which is important for its antioxidant role (45). However, this plausible interpretation of the manner in which conformational play defends PSH contrasts with other features concerning their pK_a values. In fact, various PSH have very low pK_a, which in theory means very high reactivity, because the thiolate anion enhances the chemical properties of the sulfhydryl. Therefore, it is not clear why critical PSH have low pK_a, the case of glutaredoxin (GRX) being paradigmatic ($pK_a = 3.5$) (67, 91). The usual range for NPSH is $pK_a = 7.5$–9.5, and GSH is a very reactive thiol even though only 1–2% of it is a thiolated anion at neutral pH.

Although protein thiolation is considered a complex defensive and functional machinery, it can also be pathological in particular cases. Hcy is generated by methionine and is a precursor of cysteine and GSH. In spite of its antioxidant nature (having a SH group), Hcy is considered a harmful thiol because it behaves as a prooxidant factor in some poorly defined biological circumstances. According to several clinical studies, Hcy is an important risk factor for cardiovascular diseases (43), and hyperhomocysteinemia is a recurrent finding of many other radical diseases (55, 106). However, despite a great wealth of studies, mechanisms of Hcy toxicity are still obscure. One of the various hypotheses of Hcy toxicity is related to the strong tendency to give thiolated albumin, the main endogenous source of Hcy. According to various authors, the homocysteine–albumin adduct may be a risk factor of cell toxicity (19, 46, 60).

Thiolation by the SH/SS exchange reaction:

$$\text{GSSG} + \text{PSH} \rightarrow \text{GSSP} + \text{GSH} \qquad (1)$$

is considered an antioxidant device to reduce GSSG excesses, protect essential PSH, and regenerate GSH. However, because of the different nucleophilicity of PSH and GSH, reaction 1 suggests a possible competition (or cooperation) between GSH and PSH to eliminate noxious agents. Competition among thiols has been poorly investigated; yet it is of great

importance, especially under physiological conditions (*e.g.,* to elicit redox signaling), as it answers the question of how oxidants are beneficial at low doses. There are examples of very reactive PSH that promptly regulate the GSH/GSSG ratio and have high antioxidant power, the best known one being the β125 cysteinyl residue ($pK_a = 6.8$) of rat hemoglobin. These PSH are much more reactive than GSH (~30-fold), and therefore are fast regulators of the GSH/GSSG ratio in red blood cells (RBC) *in vitro* and *in vivo* (30). Moreover, they are optimal scavengers of GSSG (reaction 1) and various electrophilic toxicants (78). Whether reactive PSH are used for other functional purposes is unknown.

These preliminary remarks serve to introduce the most important questions linked to the biochemical features of PSH and to thiolation processes. This review is an attempt to answer some of these questions. Important aspects of the relationship between protein thiolation and nitric oxide (NO) metabolism are not considered, even though they are of great biological relevance, but they have been partly dealt with in a previous review (32).

DETERMINANTS OF PROTEIN THIOLATION

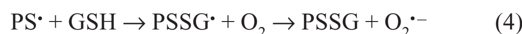
Protein thiolation is the main form of biological oxidation that PSH undergo with minimal redox change (from the sulfur oxidation grade −2 of thiols to −1 of disulfides). The mechanisms of protein thiolation are considered nonenzymatic (97) and characterized by the following molecular determinants: (a) disulfides via SH/SS exchange reactions 1 (or SH/SS interchange), (b) protein sulfenic acids (PSOH) via the reaction:

$$PSOH + GSH \rightarrow GSSP + H_2O \qquad (2)$$

and (c) free radicals via the reaction:

$$PS^{\bullet} + RS^{\bullet} \rightarrow PSSR \qquad (3)$$

As it is rather difficult to know the relative importance of reactions 1–3, we can only hypothesize a few conditions in which they might plausibly operate. For example, as free radicals are very reactive molecules, the corresponding protein thiolation should be limited to particular microenvironments where thiyl radicals are formed. This suggestion is also valid when the protein-thiol mixed disulfide is associated with superoxide anion formation, as suggested by Winterbourn (103):



$$PS^{\bullet} + GSH \rightarrow PSSG^{\bullet} + O_2 \rightarrow PSSG + O_2^{\bullet -} \qquad (4)$$

However, in the presence of GRX, which may prolong the life of free radicals generating GSSP (92), it is possible that protein thiolation can occur at other molecular sites where free radicals are generated.

Protein thiolation is usually obtained by treatment of proteins with GSSG according to reaction 1 (7, 108). Moreover, in oxidative stress experiments, cells exhibit strong GSSG and GSSP increases; in some cases, the GSSG peak precedes that of GSSP (29). These observations have led to the conviction that disulfides (reaction 1) are important for thiolation. Although this may be valid under certain conditions, the reaction of GSSG with PSH is rather unimportant because the rate is *per se* very low for reasons of charge and mass, as shown by the comparison with more lipophilic disulfides (disulfiram) (70) or by the very low yield of GSSG reactions with albumin.

By contrast, protein thiolation via PSOH seems to prevail over reaction 1. In fact, PSH can easily react with ROS, and PSOH with NPSH, because the protein reactants are smaller and more permeable than disulfides. Moreover, protein thiolation via PSOH requires less drastic oxidative stress conditions than protein thiolation via reaction 1, *i.e.,* the presence of GSH (or NPSH). On the other hand, it is also true that, in tissues with a high antioxidant activity of GPX, superoxide dismutase, and catalase, it is rather difficult to identify how ROS bypass the antioxidant control of GSH and enzymes to generate PSOH.

Although it is difficult to prove the existence of PSOH, evidence for its formation has increased in recent years. For example, albumin exposed to NPSH and H_2O_2 can form XSSP (14), even though we have found that the yield is rather low (10–20%) (unpublished observations). Therefore, although NPSH are more reactive than PSH of albumin, H_2O_2 can partly escape from NPSH reduction and form first PSOH and then XSSP according to reaction 2.

Abate *et al.* (1) suggested the importance of PSOH formation to stabilize the binding of proteins to DNA, but the proof of PSOH formation by oxidation (H_2O_2 or NO) and the functional existence of PSOH in proteins came more recently (18, 24, 76).

The possibility of producing PSOH depends on the oxidant control by enzymes and the reducing machinery, whereas the successive thiolation depends on structural restrictions (the accessibility of PSOH to thiols), as demonstrated by DeMaster *et al.* (24). In fact, NPSH exposed to NO under anaerobic conditions very rapidly produce disulfides, whereas PSH that cannot dimerize [such as bovine serum albumin (BSA)], when exposed to NO, produce PSOH first (76) and then protein thiolation if NPSH are present (24).

As reaction 2 is nonenzymatic and driven by structural conditions, the antioxidant properties of cells and tissues are important to regulate the appropriate amount of ROS for functional protein thiolation. However, the conformational characteristics of PSH (*i.e.,* their reactivity) also contribute to this. To underline the complexity of the problem, we should point out that PSH has a very wide range of reactivity (10^{+6}) (87) whose hierarchical rank is fundamental (albeit totally unknown) for an understanding of how protein thiolation is organized in successive steps to serve functional purposes.

The fact that various proteins have critical PSOH (72) raises new questions, *e.g.,* how they are regenerated after their oxidation to PSSP. In fact, the formation of PSOH from PSSP consists in the reduction of PSSP to PSH and then oxidation of PSH to PSOH. In this cyclic pathway, a hidden (or protected) protein conformation is required to avoid aspecific oxidation of PSH to sulfinic or sulfonic acid.

Thiolated proteins in cells have been classified into three broad groups by Thomas *et al.* (95): (a) proteins that are very

easily dethiolated, such as creatine kinase, and thus do not greatly increase the thiolation status during oxidative stress; (b) a small group of unidentified proteins that are always thiolated under normal conditions, in the absence of oxidative stress; and (c) the largest group of proteins (including carbonic anhydrase III, actin, creatine kinase) that become thiolated during oxidative stress via a reversible process. Analysis of the structure of glutathionylated carbonic anhydrase III has clarified the relationship between two reactive cysteines (Cys^{183} and Cys^{188}, which are not critical for the enzyme activity) and their ability to be glutathionylated (95). A common mode of binding of GSH to proteins, recognizable as electrostatic "footprints" that favor the binding of GSH to proteins and the successive protein thiolation during oxidative stress via reaction 4, has been suggested (95).

MECHANISMS OF PROTEIN DETHIOLATION

The process of protein dethiolation can be divided into two successive steps: the formation of disulfides (symmetrical or asymmetrical) and then their diffusion from the original thiolation site (reaction 5) to prevent rethiolation:

$$\mathrm{XSSP + RSH \rightarrow RSSX + PSH} \quad (5)$$

Cell dethiolation is rapid and governed by enzymes. For example, the addition of NADPH to aged cytosolic fractions of mouse liver homogenates, characterized by total GSH depletion and maximum levels of GSSG and GSSP, regenerates the GSH/GSSG ratio and PSH level of fresh samples in half an hour (77). Similar rates are observed in rat tissue homogenates treated with *tert*-butyl hydroperoxide (*t*-BOOH) and then with NADPH to reverse the thiolation process. *In vivo* studies of oxidative stress in rats treated with diamide (a more potent oxidant than *t*-BOOH) show rapid reversible thiolation/dethiolation processes in RBC and tissues via prompt activation of GSH recycling (31).

The enzyme GRX accelerates protein dethiolation more than TRX or protein disulfide isomerase (PDI) (16, 44, 54). Therefore, GRX activity is fundamental in the regulation of redox homeostasis and the protection of PSH against oxidative stress during ageing and various diseases, as well as in the modulation of PSH activity during redox-activated signal transduction (6, 7, 20, 56, 95, 102). After GRX inhibition, virtually all the deglutathionylase activity in mammalian cells is lost (16, 54, 102).

Albumin is a good model to study general aspects of dethiolation. Albumin is characterized by slow-reacting sulfhydryl residue (Cys^{34}). Despite this property, this nucleophilic site is highly thiolated. As the albumin reactivity and conformation are modified by drugs and detergents (17, 27, 45), it is plausible that conformational changes of the protein, as well as mass and charge characteristics of disulfides, may influence BSA dethiolation. For example, studies of BSA dethiolation have clearly indicated that the elimination of cystine (MW 240, two negative charges) from cysteine-BSA is much faster than that of GSSG (MW 612, four negative charges) from glutathione-albumin mixed disulfides (BSA-SSG) (unpublished observations).

The rate of dethiolation of BSA-SSG by a mixture of various thiols (cysteine, GSH, Hcy) is governed by the thiol competition, which depends on their pK_a, and the diffusion of asymmetrical and symmetrical disulfides, which depends on their size and charge. In successive steps of exchange reactions, cysteine forms the asymmetrical disulfide, cysteine-SSG, and then cystine, which diffuses more easily from the protein crevice where the thiolation site is located (Cys^{34}) than GSSG (see below).

It has been observed that deglutathionylation of liver is nearly exclusively carried out by GRX (54): 40% of the thiolated protein is reduced by GRX in 5 min, whereas only 5% of the dethiolation is observed in the absence of GRX. We have also demonstrated the important role of cysteine in dethiolating cells when they are fully GSH-depleted and glutathionylated by oxidative stress (31). As glutathionylation (GSSP) *per se* reduces the thiol (GSH) necessary for the successive dethiolation, the cell organization must compensate to some extent for the excessive loss of GSH by thiolation. GSH is important because it increases the efficiency of GRX more than other thiols (91). Therefore, we speculate that the GPX activity is useful not only to control the excess of ROS, but also to spare GSH in the form of GSSG, thus ensuring that GSH is not totally consumed in protein thiolation.

ENZYMES INVOLVED IN THE THIOLATION/DETHIOLATION PROCESS

GRX, TRX, and PRX are potent antioxidants and multifunctional enzymes with critical PSH; they directly or indirectly control ROS effects and are involved in thiolation/dethiolation processes. GRX, TRX, and PRX are ubiquitous, being present in various cell districts and biological fluids. GRX uses low-molecular-weight thiols (GSH and NPSH) to reduce glutathione-protein mixed disulfides (44, 62), whereas TRX reduces PSSP (48) and PRX uses TRX as a substrate to control ROS (hydroperoxides). During the control of ROS, PRX loses its peroxidase function to become a chaperone via an intricate transformation involving PSSP formation (53).

GRX and TRX have a similar sequence (Cys-X-X-Cys) in their active sites, use the same mechanisms of disulfide (XSSP or PSSP) reduction, and operate at the expense of NADPH using different but related enzymes. The action of GRX is supported by glutathione reductase (GR), which reduces GSSG. Oxidized TRX is regenerated by TR activity. PRX contains critical SH, which once transformed into PSSP after oxidative stress are reduced to PSH by TRX.

Very many stimuli (immunological, physical, chemical) that induce cell disruption increase ROS consumption and thus protein thiolation/dethiolation; in this process, thiolation is associated with oxidation reactions (oxidative or nitrosative stress), whereas dethiolation is associated with reduction reactions (glucose consumption, NADPH regeneration, GSH recycling).

GRX, previously named thioltransferase (62), is a member of the thiol–disulfide oxidoreductase family, with a molecular weight of 12 kDa. It is characterized by the sequence -Cys-Pro-Tyr-Cys- at the active site. Although the GRX system is associated with the control of cell glutathionylation via

reaction 1, it also has other important redox functions, regenerating ascorbate from dehydroascorbate at the expense of GSH.

GRX only recognizes GSSP and mixed disulfides of GSH with low molecular weight (44). The active cysteine of *E. coli* GRX is bound to GSH by mixed disulfide (13) and GSH is more specific than other NPSH in dethiolating GRX and ensuring its efficiency (91). Despite these specificities, GRX can reduce all XSSP and any asymmetrical disulfides on the condition that GSH and the related reducing systems (GR) are present in the system. This explains why cysteine is a good dethiolating agent of diamide-treated rat RBC (31), even though cysteine is a worse substrate than GSH and CSSP is not a substrate of GRX.

Protein glutathionylation can be inactivating or activating. Examples of inactivation are glutathionylation of nuclear factor-κB (NF-κB) (6), protein tyrosine phosphatase-1B (7), and phosphofructokinase (66). Examples of activation are glutathionylation of HIV-1 protease (23), microsomal glutathione *S*-transferase (22), and various glycolytic enzymes (108). In other dubious cases involving signal transduction proteins, it is more difficult to establish whether GSSP is activating or inactivating, although it has been suggested that GRX plays an important regulatory role. Although GRX is unable to reduce PSOH (67) or PSSP (54), it could be involved to some extent in PSSP formation mediated by PSOH.

The role and origin of plasma GRX are unknown. As plasma lacks NADPH and plasma GRX and GSH levels are relatively low (1–3 μ*M*) (25, 69), it is very unlikely that GRX functions as a dethiolating agent.

GRX and TRX may work synergistically to control NPSH and PSH, but GRX is more selective for the reduction of low-molecular weight mixed disulfides, because its efficiency (k_{cat}/K_m) is ~5,000 times greater than that of TRX (16).

TRX is an electron donor with an antioxidant action on PSSP (48, 49). It is a small protein of ~100 amino acid residues (12 kDa) found in both prokaryotes and eukaryotes, and its active site consists of two nearby cysteines in the sequence: -Trp-Cys-Gly-Pro-Cys-Lys-. The human protein (called TRX-1) is characterized by two catalytic site cysteinyl residues (Cys^{32} and Cys^{35}) and three other cysteines (Cys^{62}, Cys^{69}, Cys^{73}) not present in all species. TRX has been found in various cell locations, *i.e.,* cytoplasm, nucleus, and mitochondria, and in plasma (73).

Originally found in platelets (10) and in cultured fibroblasts, human TRX was subsequently identified by various immunologists as a cytokine-like factor (93). A second TRX (TRX-2), identified in pig heart, is mitochondrial; it has the same catalytic site as TRX-1 but not the other cysteinyl residues (73). A third TRX-like cytosolic protein of 32 kDa has been cloned from a human testis cDNA library. The various functions of TRX have recently been reviewed (73).

In addition to serving as a hydrogen donor for ribonucleotide reductase (57) and methionine sulfoxide reductase (12), TRX can also reduce sulfenic acid (35). TRX also reduces hydroperoxides (90) and donates electrons to human plasma GPX (9), although its real plasma function of detoxifying hydroperoxides has been questioned (73).

The reduction of protein disulfides (PSSP) occurs by the formation of a mixed protein disulfide (TRX-protein) at the active site with Cys^{32} via a nucleophilic attack of Cys^{32}. Cys^{35} then forms an internal disulfide with Cys^{32} (TRX-S_2), via a second nucleophilic attack, delivering the protein substrate in a reduced form. TRX-S_2 is reduced by TR at the expense of NADPH (47). Crystallography studies have documented redox-dependent conformational changes in the active site of TRX and have explained the origin of the relatively low pK_a of Cys^{32} (73).

TRX can function as cofactor for the biosynthesis of plasma clotting factors (86) and is an activation factor of DNA binding of a number of transcription factors (NF-κB, glucocorticoid receptor, AP-1, AP-2, estrogen receptor, PEBP2/CBF) (73). For example, under oxidizing conditions, the binding of NF-κB to DNA is inhibited because Cys^{62} of the NF-κB p50 subunit is not maintained reduced by TRX (63, 98). Moreover, TRX activates various enzymes in mammalian cells (35, 39).

TRX forms heterodimers with various redox proteins. This occurs with the p50 NF-κB subunit by means of PSSP between Cys^{32} of TRX and Cys^{62} of NF-κB (73). The active cysteine of human TRX has been shown to bind with a putative protein substrate (74). Other examples of heterodimers of TRX with proteins exist (73).

TRX has been defined as a stress-inducible protein secreted by various cell types after virus exposure, oxidative stress, or proinflammatory stimulation by lipopolysaccharide or phorbol esters (68). It provides extracellular cytoprotection against apoptosis induced by oxidative stress and has a proliferative function as an autocrine growth factor. This "redox regulation" by TRX is considered important for the prevention of several disorders, including viral infections, immunodeficiency, malignant transformation, and degenerative diseases (68). For example, TRX has a co-cytokine activity (83), can induce interleukin-2 receptor activity (93), and protects against tumor necrosis factor cytotoxicity (68). In addition, it can repair oxidative damage; for example, the addition of *E. coli* TRX repairs oxidative damage in *in vitro* systems, as well as in lens epithelial cells (90).

The extracellular activity of TRX increases the cystine uptake and GSH biosynthesis in stimulated BPMC, and both GSH and TRX cooperate in intracellular redox mechanisms, suggesting key roles in the cell-cycle progression of activated lymphocytes (68).

TRX is rapidly secreted and taken up by cells. For example, human TRX is secreted or released by lymphocytes, hepatocytes, and fibroblasts (68, 80). RBC, leucocytes, and platelets contain TRX, which can be released in response to oxidative stress (68). The endothelium and the liver are also possible sources of extracellular TRX (68), whose secretion occurs via a nonclassical pathway because TRX has no signal peptide (80). However, part of the secreted TRX remains bound to the external membrane in a truncated form (104).

The intracellular concentration of TRX is reported to be 2–14 μ*M* (50) and is relatively abundant in the endoplasmic reticulum, Golgi apparatus, and other endosomal compartments (79). The plasma concentration has been estimated at 1–6 μ*M* (69).

The maintenance of a reducing cellular milieu by TRX is ensured by TR activity and NADPH regeneration. Other oxidized proteins are reduced by TR (such as PDI and NK-lysin, a disulfide-containing effector peptide derived from T-lymphocytes and NK cells) (2). Moreover, TR can reduce several

low-molecular-weight substrates, such as selenite, selenodiglutathione, vitamin K, *S*-nitrosoglutathione, and lipoic acid, but even without the cooperation of TRX. For example, lipoic acid (5), vitamin K (89), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) (47), and alloxan (51) are not reduced at the expense of TRX.

TR is a dimer of two identical subunits of 58 kDa, which in human placenta contains a selenocysteine important for its activity in the penultimate COOH terminus. All TR are homologous to GR, but with the catalytically active COOH-terminal elongation -Gly-Cys-SeC-Gly- (48). Other TR have been found, *i.e.,* TR2, TR3 (located in mitochondria), and TRβ (73).

Humans lack the ability to produce vitamin C, which must be obtained from exogenous sources. For the maintenance of cellular stores, vitamin C must be regenerated from its oxidized forms. This occurs very actively in RBC (and other cells), which are known to have a very high antioxidant capacity (40). Dehydroascorbate reduction in blood is carried out by GRX (71, 107) and TR (65). May *et al.* (64) have calculated that cells can renew the dehydroascorbate content of blood every 3 min. Thus, the GRX and TRX systems operate on different types of disulfides (GSSP and PSSP), but their functions converge for vitamin C regulation.

TR expression has been found in several mammalian cell types, such as skin keratinocytes, placental cells, liver cells, secretory cells, leucocytes, and monocytes (the cells with the highest concentration) (79, 82, 88). There is no TR activity, or a negligible amount, in platelets (88). Moreover, TR is secreted by normal and neoplastic cells, its secretion being induced by physiological stimuli (88). The normal plasma concentration of TR is ~18 ng/ml, corresponding to ~0.31 n*M* (88). The cell secretion of TR, unlike that of TRX, occurs by the classical Golgi pathway.

TR has a very reactive SH residue, which is much more reactive than GSH; indeed, it has been estimated that 1-chloro-2,4-dinitrobenzene (CDNB) reacts with the cysteinyl residue of TR 10,000 times faster than GSH (4). This raises the question of how the enzyme is protected by inactivation, because GSH seems theoretically unable to protect it. It has also been proposed that this inactivation confers NADPH oxidase activity on the protein, as its ability to oxidize NADPH increases ~30-fold (4). Moreover, TR is more reactive than GR, because its inhibition with CDNB is 100 times faster than that of GR (4).

TR contains vicinal critical thiols, and its activity is decreased after treatment with agents specific for vicinal or nearby thiols (49).

PRX is a 25-kDa enzyme without a prosthetic group, which in yeast exists as a homodimer, with two monomers arranged in a head-to-tail manner, each subunit containing two essential cysteine residues, Cys^{47} (the primary site of oxidation, also called peroxidatic Cys) and Cys^{170} (15, 21) (also called resolving cysteine) (105). After oxidation, an intermolecular disulfide is formed between Cys^{47} and Cys^{170} of different subunits, and this PSSP can then be reduced by TRX. Unlike other peroxidases that have selenocysteine or heme at their active sites, PRX has cysteine.

This family of enzymes can use TRX as an electron donor (also classified as PRX1–4) or other electron donors such as GSH (PRX5–6). PRX that require only one cysteine on account of their peroxidase activity, such as PRX5 and PRX6, are called 1-Cys PRX, whereas those requiring two cysteines (PRX1–4) are called 2-Cys PRX. The peroxidatic cysteinyl center is usually near residue 50 and is conserved. If the other cysteine, the resolving one located near position 170, is conserved, we have typical 2-Cys PRX (the largest class), whereas in atypical 2-Cys PRX this position is not conserved (105).

PRX is primarily located in cytosol, but is also found in other organelles and is probably exported (105). PRX is considered the second or third most abundant protein in erythrocytes, but it is also relatively abundant in other cells (0.1–0.8%) (105). Other redox functions (cell proliferation, differentiation, apoptosis) have been associated with PRX activity via cytokine production mediated by H_2O_2 (105).

The catalytic mechanism of PRX enzymes is well established and is divided into two steps. In the first step (the same for all PRX), the peroxidatic cysteine (by virtue of its lower pK_a because of the proximity to an arginine residue) is transformed into sulfenic acid by peroxides. The next step, the resolution of sulfenic acid via formation of a disulfide, is different in the three PRX classes. In typical 2-Cys PRX, the resolving cysteine (Cys^{170}, conserved or nonconserved) reacts with the peroxidatic cysteine and a disulfide between different subunits (PSSP intermolecular) is formed. In atypical 2-Cys PRX, the resolving cysteine reacts with Cys^{50} of the same subunit, with the formation of intramolecular PSSP. As 1-Cys PRX does not have a resolving cysteine, any thiol (PSH or NPSH) can form the disulfide as PSSP or XSSP (protein thiolation). GSH, lipoic acid, and cyclophilin have been suggested as possible thiols (38, 58).

PRX has the special characteristic of being oxidized upon reaction with the peroxide and being transformed into various oligomeric forms with the loss of enzymatic activity. To some extent, this is similar to the peroxidases that are functionally related to sperm maturation (101). Although the precise relationship between the peroxidase activity and the oligomeric state of PRX is still unclear, they are reciprocally linked. Typical 2-Cys PRX tend to form a decameric structure when reduced or overoxidized, and structural considerations suggest that, during peroxide decomposition, the peroxidatic cysteine (Cys^{50}, conserved) is transformed into a sulfenate group that becomes more accessible to the solvent (105). In this regard, it is interesting that reducing conditions are necessary to maintain the sulfenate condition; otherwise the sulfenate might be transformed into sulfinic or sulfonic acid. However, paradoxically, it should also be protected against the attack of thiols; otherwise PSOH would be transformed into XSSP. Overoxidation of the PRX active site has recently been reported in *in vivo* experiments (75). As reducing conditions inside the cell are ensured by thiols (NPSH or PSH, GSH or TRX), it is not clear how they preserve PRX integrity. Therefore, the chaperone function of PRX may be essential to reduce PSH reactivity and protect the protein from undesired oxidation or thiolation.

THE ROLE OF PROTEIN CONFORMATION

The cellular response to oxidative stress involves the activation of genes that repair and eliminate toxic compounds

and reduce protein modification. The process of transcriptional activation in response to oxidative stress, also called redox sensing, is activated by transcription factors via cysteine modifications. The switch between activation and inactivation of these factors generates strong changes in protein conformation. The best example of a sequence of conformational events mediated by cysteine modifications by oxidation is the activation of *E. coli* transcription Oxy R (41).

Specific factors, *e.g.,* steric hindrance, proximity of appropriate amino acids, lipophilic microenvironment, and hydrogen bonding, characterize the protein conformation and thus the reactivity of PSH, which is usually lower than that of NPSH. For example, the rate of reaction of tubulin PSH with an SH reagent is much higher in the presence of Ca^{2+} (a depolymerizing agent) than in its absence, whereas the PSH reaction rate in the presence of Mg^{2+} (a polymerizing agent) is much lower than in its absence (28) (Fig. 1).

The biological properties of albumin change according to modifications of its conformation state due to chemical compounds bound to Cys^{34} and His^{3} (45). As copper is bound to His^{3}, changes in protein conformation via the communication between Cys^{34} and His^{3} may increase the plasma autooxidizability of thiols and thus protein thiolation (17).

PSH of receptors are important for the modulation of a biological response. αIIbβ3 platelet integrin belongs to the integrin family of receptors, which regulate platelet function. Platelet stimulation is accompanied by complex conformational changes, from the cytoplasmic domains of integrins to the extracellular ligand binding site. αIIbβ3 integrin is rich in PSSP located in a cysteine-rich region, but also has free PSH. PDI belongs to the TRX family, is abundant in endoplasmic reticulum, and is present on the cell surface, where it is functionally active. In platelets, PDI mediates aggregation (34) via complex changes of the PSSP population and possible conformational changes.

As GSH and other NPSH have a proaggregation effect on human platelets in the presence of subthreshold levels of agonist (34), this action might be mediated by protein thiolation and changes in protein conformation in αIIbβ3 integrin.

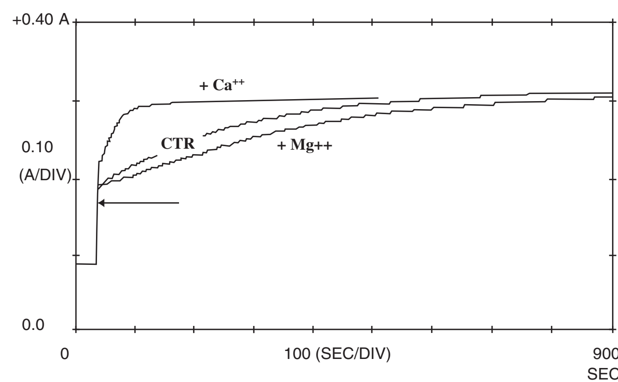


FIG 1. Curve profiles of the SH/SS exchange reaction between tubulin and DTNB alone (CTR) and in the presence of $MgCl_2$ (0.2 m*M*) or $CaCl_2$ (0.2 m*M*). The conditions were as follows: tubulin, 44 μg/ml; DTNB, 200 μ*M*; medium, 0.1 *M* Tris-Hcl buffer, pH 7.4. The spectrophotometric tracings of each experiment were superimposed in the figure; the arrow indicates the absorbance increase due to the addition of DTNB.

PROTEIN THIOLATION IN CELLS

The protein thiolation process of cells is rapidly reversible, as it is sustained by GRX activity and by GSH regeneration via glucose consumption (30, 32, 54, 62, 95).

Protein thiolation can differ according to the cell metabolism and type of oxidant. For example, diamide reacts rapidly nonenzymatically with any thiols, but the observed protein glutathionylation differs according to the nature of the PSH that can be fast- and slow-reacting.

RBC of different species have hemoglobin with different SH reactivity; depending on the relative abundance of fast PSH, their protein glutathionylation may be accompanied by GSSG increases or not (30).

Rat RBC have fast and slow PSH, human and calf RBC have only slow PSH, whereas PSH in turkey RBC are intermediate in reactivity. After treatment with diamide, rat RBC do not form GSSG but only GSSP, human and calf RBC form only GSSG with no GSSP increase, whereas turkey RBC form both GSSG and GSSP at the same time. When RBC are treated with hydroperoxide (*t*-BOOH), which is immediately scavenged by GPX activity with the formation of maximum GSSG levels, *S*-thiolation is always present in the rat after the GSSG peak; there is no formation of GSSP in human and calf RBC, whereas GSSG formation prevails over GSSP formation in turkey RBC. In the rat, thiolation with *t*-BOOH is always reversible in a great range of doses and even after repeated exposure; in contrast, thiolation with diamide may become totally irreversible at relatively low doses when all GSH is consumed for protein thiolation.

New thiol probes (biotinylated thiols) have been used to demonstrate protein thiolation in cells and isolated organs exposed to oxidants or oxidative stress (33). These probes hold great potential, because they could serve as a new tool to investigate the accessibility of thiolated proteins and protein disulfides, as well as the mechanism of dethiolation (see below).

Exposure to Cd for a relatively short time also leads to increased GSSP production in HT4 neuronal cells. This is interesting because Cd is not an oxidant (36) and it causes irreversible glutathionylation of proteins, acting as an inhibitor of GRX activity (16). This clearly suggests that glutathionylated proteins are continuously formed and reduced under basal conditions. The inhibition of cells by heavy metals that do not act as direct oxidants can also lead to apoptosis (16). More recently, increased GSSP was observed after treatment of HL60 with Kathon (a toxic preservative widely used in cosmetology), which causes apoptosis (3) with modest increases in GSSG (unpublished observations).

It is also possible that thiolation of dimethyl arginine dimethyl amino hydrolase by Hcy is involved in the toxicity of asymmetrical dimethylarginine, reducing its metabolism and increasing the risk of damage to vessels (19). Another example of toxicity by glutathionylation could be the activity of GRX in HIV-1 virus, because GRX can regulate the activity of glutathionylated HIV-1 protease (23).

PROTEIN THIOLATION/DETHIOLATION IN PLASMA

Plasma has prooxidant characteristics, and this influences the levels of NPSH depending on their pK_a because their oxidation rate is inversely related to the pK_a of the sulfhydryls. Thus, Hcy with pK_a ~ 9.5 has much lower oxidizability than cysteine (pK_a ~ 8.3) or cysteinylglycine (pK_a ~ 7.9), whereas GSH (pK_a ~ 8.8) has an intermediate oxidation rate, slightly higher than that of Hcy (84). The oxidizability of thiols is confirmed when they are added to plasma or albumin solutions (84), but their oxidation rates are not related to the distribution of the various redox forms in normal individuals, indicating that plasma thiols have different metabolic fates (25, 26). In particular, the plasma thiol/disulfide ratios of cysteine (0.152), Hcy (0.206), cysteinylglycine (0.381), and GSH (1.96) in healthy individuals (25) are only partly related to their oxidizability, and that of Hcy does not agree with its oxidation rate, which is the lowest of all compounds (84).

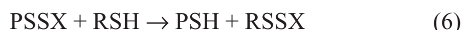
When the percentages of reduced thiols with respect to total thiols (sum of all redox forms) are analyzed for each compound in plasma of individuals of various ages, those of cysteine and cysteinylglycine are similar (4–6%) at all ages and higher than those of Hcy (3–4%). In turn, the GSH/total GSH (GSH, GSSG, and GSSP) percentage ranges from 15% (newborns) to 60% (adults, 50–70 years) (26). The larger difference in the GSH/total GSH percentage than that of cysteine or cysteinylglycine can be explained by differences in the oxidation rates (84). However, this does not explain why Hcy and GSH, which have similar redox properties (*e.g.,* oxidizability), differ greatly in the concentration of reduced forms in plasma.

The plasma levels of protein-thiol mixed disulfides of cysteine and Hcy are minimal at birth (~60 nmol/ml), strongly increase at 1–2 years (~110 nmol/ml), and then continue to increase into adulthood (~150 nmol/ml at 50–70 years) (26). The increase in protein-bound Hcy by age is proportionally lower, being ~3–4 nmol/ml at birth, ~4–5 nmol/ml at 1–2 years, and ~8 nmol/ml at 50–70 years.

In radical pathologies associated with hyperhomocysteinemia, the plasma levels of Cys and Hcy bound to proteins and the corresponding disulfides increase, whereas corresponding increases of GSH and cysteinylglycine are sporadic or absent (25, 26).

These data confirm that, with respect to other thiols, Hcy is preferentially bound to albumin (for example, 70–80% of Hcy is bound to protein versus 50% of cysteine) as explained by Sengupta *et al.* (85).

Thiolation and dethiolation of albumin via reaction 1 are relatively fast even in the absence of enzymes (85). Two types of reaction may be involved in dethiolation whose amount depends on the nucleophilic nature of the reactants:



$$PSSX + RSH \rightarrow PSH + RSSX \quad (6)$$

$$PSSX + RSH \rightarrow PSSR + XSH \quad (7)$$

However, reaction 6 should be considered protein dethiolation, whereas reaction 7 is protein thiol substitution. The possibility of having net protein dethiolation (reaction 6) or thiolation substitution (reaction 7) depends on several factors,

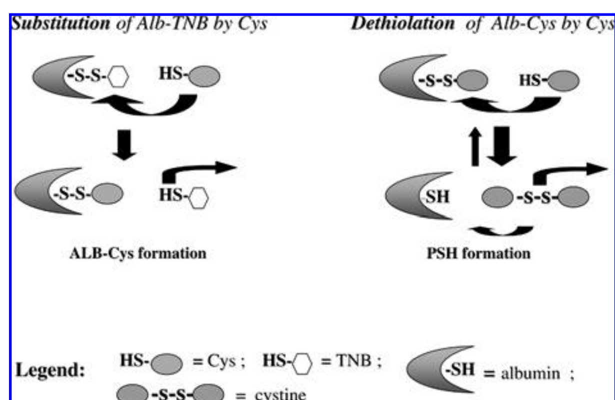
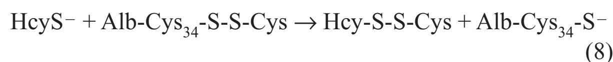


FIG 2. Thiol substitution and dethiolation of thiolated albumin by thiols.

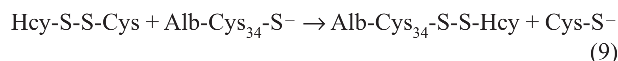
such as the nucleophilicity of the attacking thiol and the pK_a of the leaving thiol, and thiols with the lowest pK_a are encouraged to leave the mixed disulfide (42, 85) (Fig. 2).

Plasma homocysteinylation with albumin are of great scientific interest because it could be involved in the putative toxicity of Hcy, because albumin is the best storage of Hcy in humans and a possible vehicle of Hcy transportation inside endothelial cells after binding with proteins that transcytose albumin (96).

Plasma Hcy tends to replace Cys bound to albumin via two successive reactions:



$$HcyS^- + Alb\text{-}Cys_{34}\text{-}S\text{-}S\text{-}Cys \rightarrow Hcy\text{-}S\text{-}S\text{-}Cys + Alb\text{-}Cys_{34}\text{-}S^- \quad (8)$$



$$Hcy\text{-}S\text{-}S\text{-}Cys + Alb\text{-}Cys_{34}\text{-}S^- \rightarrow Alb\text{-}Cys_{34}\text{-}S\text{-}S\text{-}Hcy + Cys\text{-}S^- \quad (9)$$

The substitution of Cys by Hcy via reactions 8 and 9 is favored by the pK_a difference between Hcy and Cys and by the fact that the albumin-thiolated anion (Alb-Cys$_{34}$-S$^-$) has the lowest pK_a value and is thus a good leaving group.

As demonstrated by Sengupta *et al.* (85) in *in vitro* experiments and in agreement with *in vivo* results in humans after Hcy (97) or methionine administration in healthy and pathological subjects (unpublished observations), plasma homocysteinylation increases over time, and this is associated with decreases in Cys bound to albumin and with PSH increase.

The question concerning the type of reaction that is preferentially activated [i.e., dethiolation (reaction 6) or substitution (reaction 7)] is not marginal, because these alternatives may have a different physiological meaning when applied to PSH of enzymes. In fact, critical PSH are restored after reaction 6, but not after reaction 7, and this is conditioned by the rate of diffusion of the asymmetrical disulfide (RSSX) from the thiolation site.

We have further investigated these particular aspects of dethiolation of albumin by thiols (unpublished observations). First, we prepared thionitrobenzoic acid (TNB)-thiolated albumin (bovine), where TNB is the thiolate anion of DTNB. We then treated TNB-albumin with an excess of various NPSH (GSH, cysteine, and Hcy, molar ratio 1:5 albumin/thiol) and measured the TNB dethiolation by each thiol. In other experi-

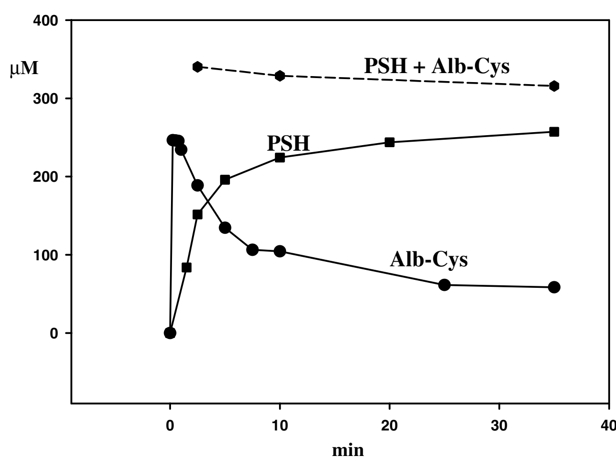


FIG 3. TNB substitution of albumin-TNB and dethiolation of albumin-Cys by Cys. TNB-thiolated albumin (bovine) at 400 µ*M* was treated with 7.5 m*M* Cys at room temperature in 0.05 *M* phosphate buffer, pH 7.4, and reactions were stopped by trichloroacetic acid (TCA) precipitation. The pellet was extensively washed with 1.5% TCA and utilized for PSH and protein-thiol mixed disulfides measurements. PSH was assayed by DTNB at 0, 1.5, 2.5, 5, 10, 20, and 35 min. Protein-thiol mixed disulfide at cysteine (Alb-Cys) was measured after dithiothreitol reduction and monobromobimane derivatization at 0, 0.25, 0.5, 0.75, 1, 2.5, 5, 7.5, 10, 25, and 35 min. Within 45 s, nearly all TNB was delivered from Alb-TNB by substitution. The dethiolation of Alb-Cys was equally fast (see PSH value at 1.5 min and Fig. 2 for further details).

ments, we treated TNB-albumin with a mixture of all thiols together, maintaining the same molar ratio (1:5 albumin/all thiols). We observed that TNB-thiolated albumin was totally and rapidly replaced by each thiol (reaction 7). This phase was followed by rapid dethiolation, sustained by the remaining free thiol (reaction 6) (Fig. 3). However, there were different dethiolation rates depending on the structural characteristics of each compound: cysteine was the best dethiolating agent and GSH the worst. Similar results were obtained when TNB dethiolation was followed by the thiol mixture.

Despite the complexity, we can interpret the dethiolation processes as follows. On the basis of the empirical rule that in SH/SS exchange reactions the nucleophile with the lowest pK_a is most likely to leave the reaction, TNB with $pK_a = 4.4$ (99) is the first product to leave albumin, and albumin binds to the other thiol (thiol substitution). The excess of thiols then forms symmetrical or asymmetrical disulfides, dethiolating albumin ($pK_a = 6$), which leaves the system. The PSH levels increase, as confirmed by *in vivo* experiments (methionine administration). Subsequently, the increased levels of disulfides (asymmetrical or symmetrical) lead to rethiolation of albumin, and a new equilibrium is reached. In this process, Hcy (with the highest pK_a) thiolates the proteins, whereas cysteine tends to leave the system.

CONCLUDING REMARKS

Very many proteins are reversibly regulated in cells and the extracellular environment by protein thiolation during oxidative and nitrosative stress and by free radicals. The best examples are proteins of the glycolytic pathway, which are functionally activated or inactivated during oxidative stress.

Interest in the literature on protein thiolation, and more recently on protein disulfides, is very great and increasing. Nevertheless, our knowledge is still observational, and research is oriented toward the discovery of new thiolated proteins rather than toward an understanding of mechanisms. For example, little attention has been dedicated to proteins that only have an antioxidant action and to their relationship with GSH. These ancillary proteins can probably support GSH for more physiological functions, *e.g.,* to control the appropriate ROS dose for redox signaling.

In addition to being a potent antioxidant device, thiolation and dethiolation might be of great importance in the regulation of the internal circulation, regulation, and binding of proteins, but there are no data on this topic. Studies of relatively simple models (albumin) have been useful to clarify the mechanisms of thiolation and dethiolation. For example, they have clearly indicated that GSSG is a very poor thiolation factor, in that it is a slow and poorly penetrating agent. This notion can be transferred to the great majority of cell proteins with a partially hidden conformation. In addition, as GSSG is not a substrate of GRX, but is the most abundant disulfide generated during dethiolation, the mechanism by which GRX acts to dethiolate proteins remains uncertain in that it does not clarify how GSSG leaves proteins. Studies on dethiolation of albumin have indicated that GSSG is not a good leaving group during dethiolation, as it is impeded for structural and charge reasons, whereas asymmetrical disulfides with GSH (which are a substrate of GRX) are more easily eliminated from thiolation sites. This is an interesting feature that reinforces the hypothesis by which other thiols (cysteine) may cooperate in protein dethiolation.

More recently, it has been discovered that a very impressive number of proteins are involved in protein disulfide formation during oxidative stress. This complex scenario deserves further investigation, as it suggests new relationships between thiolated proteins and their possible reactions with PSH to form protein disulfides, whose functional role (if it exists) is totally unknown.

ABBREVIATIONS

t-BOOH, *tert*-butyl hydroperoxide; BSA, bovine serum albumin; BSA-SSG, glutathione-albumin mixed disulfides; CDNB, 1-chloro-2,4-dinitrobenzene; CSSP, cysteine-protein mixed disulfides; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); GPX, glutathione peroxidase; GR, glutathione reductase; GRX, glutaredoxin; GSH, glutathione; GSSG, glutathione disulfide; GSSP, glutathione-protein mixed disulfide; Hcy, homocysteine; H_2O_2, hydrogen peroxide; NF-κB, nuclear factor-κB; NO, nitric oxide; NPSH, nonprotein SH groups; PDI, protein disulfide isomerase; PRX, peroxiredoxin; PSH, protein SH groups; PSOH, protein sulfenic acid; PSSP, protein disulfides; RBC, red blood cells; ROS, reactive oxygen species; TNB, thionitrobenzoic acid; TR, thioredoxin reductase; TRX, thioredoxin; XSSP, protein-thiol mixed disulfides; XSSR, asymmetrical disulfides; XSSX, symmetrical disulfides.

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Received for publication October 15, 2004; accepted February 2, 2005.

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