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Comprehensive Invited Review

Molecular Mechanisms and Potential Clinical Significance of *S*-Glutathionylation

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ABSTRACT

Protein *S*-glutathionylation, the reversible binding of glutathione to protein thiols (PSH), is involved in protein redox regulation, storage of glutathione, and protection of PSH from irreversible oxidation. *S*-Glutathionylated protein (PSSG) can result from thiol/disulfide exchange between PSH and GSSG or PSSG; direct interaction between partially oxidized PSH and GSH; reactions between PSH and *S*-nitrosothiols, oxidized forms of GSH, or glutathione thiyl radical. Indeed, thiol/disulfide exchange is an unlikely intracellular mechanism for *S*-glutathionylation, because of the redox potential of most Cys residues and the GSSG export by most cells as a protective mechanism against oxidative stress. *S*-Glutathionylation can be reversed, following restoration of a reducing GSH/GSSG ratio, in an enzyme-dependent or –independent manner. Currently, definite evidence of protein *S*-glutathionylation has been clearly demonstrated in few human diseases. In aging human lenses, protein *S*-glutathionylation increases; during cataractogenesis, some of lens proteins, including α- and β-crystallins, form both mixed disulfides and disulfide-cross-linked aggregates, which increase with

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cataract severity. The correlation of lens nuclear color and opalescence intensity with protein *S*-glutathionylation indicates that protein–thiol mixed disulfides may play an important role in cataractogenesis and development of brunescence in human lenses. Recently, specific PSSG have been identified in the inferior parietal lobule in Alzheimer's disease. However, much investigation is needed to clarify the actual involvement of protein *S*-glutathionylation in many human diseases. *Antioxid. Redox Signal.* 10, 445–473.

I. INTRODUCTION

Mammalian cells generally function in a reduced state, though they can produce low (*i.e.*, subtoxic) amounts of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in a regulated way and use them as signaling molecules. Under physiological (basal) conditions, ROS/RNS, particularly hydrogen peroxide, are important intracellular signal transducers of growth factors and extracellular matrix receptors and, therefore, second messengers to activate many downstream signaling molecules, and numerous cellular processes, including gene expression, can be regulated by subtle changes in cellular redox balance/homeostasis. Examples of this include the activation of certain redox-dependent nuclear transcription factors, such as nuclear factor κB (NF-κB), p53, and activator protein-1, by which cells may transduce redox signaling into an inducible expression of a wide variety of genes implicated in cellular changes such as proliferation and differentiation, as well as the determination of fate of certain cell types, that is their tendency to undergo programmed cell death (*i.e.*, apoptosis) or necrosis (176).

Cellular redox state is maintained by an array of both enzymatic and nonenzymatic antioxidant systems to restore and control the normal reducing environment, so that overall reducing conditions prevail. The cellular thiol redox state is a crucial mediator of multiple metabolic, signaling, and transcriptional processes in cells, and a fine balance between oxidizing and reducing conditions is essential for the normal function and survival of cells. Low molecular mass thiols and protein thiols, by virtue of their ability to be reversibily oxidized, are recognized as key components involved in the maintenance of redox state. Increasing evidence suggests that thiol groups located on various molecules act as redox-sensitive switches, thereby providing a common trigger for a variety of ROS/RNS-mediated signaling events (42, 73, 93, 101, 124, 151, 176).

Excessive production of ROS/RNS and/or impairment of cellular antioxidant defense(s), or an imbalance between ROS/RNS and antioxidants, can lead to a common pathophysiological condition termed oxidative stress, a situation in which the cellular redox homeostasis is altered. However, such a limiting definition of oxidative stress as a global imbalance of prooxidants and antioxidants (as first defined in 1985 by Helmut Sies; 237) has been recently updated. Indeed, deficient concentrations of vitamins C and E, as well as conditions that limit glutathione and other antioxidant systems, are associated with disease processes that can benefit from antioxidants. Therefore, an updated and improved definition of oxidative stress has recently been introduced by H. Sies and D. P. Jones in the "Encyclopedia of Stress", 2nd ed., G. Fink, Ed. (2007) as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" (240) (Fig. 1).

Oxidative stress can cause damage to cellular macromolecules such as lipids, proteins, and nucleic acids (238), possibly leading to subsequent cell death by necrosis or apoptosis. Under normal physiological conditions, an efficient antioxidative defense system constantly buffers the oxidative action of ROS/RNS, thus minimizing oxidative damage. Antioxidants include the enzymes superoxide dismutases, catalases, glutathione peroxidase, and glutathione reductase, the vitamins α-tocopherol (vitamin E), β-carotene (vitamin A), and ascorbic acid (vitamin C), uric acid, and the tripeptide glutathione (GSH). A major constituent of the cellular redox buffer are protein cysteinyl thiols, which—when taken together—provide thiols in the 10–30 m*M* range (229). The "two-faced" character of ROS/RNS is clearly substantiated. For example, a growing body of evidence shows that ROS/RNS within cells act as secondary messengers in intracellular signaling cascades that induce and maintain the oncogenic phenotype of cancer cells, however, ROS/RNS can also induce cellular senescence and apoptosis and can therefore function as antitumorigenic species (93).

Oxidative stress has been acknowledged to be a pathogenic and/or etiological factor of numerous human diseases including cardiovascular disease, atherosclerosis, cancer, neurodegenerative diseases, asthma, diabetes mellitus, ischemia/reperfusion, just to name a few (12, 51, 54, 57, 109, 142, 248, 264). Oxidative/nitrosative stress—and the resulting damage to all of the major classes of macromolecules in cells—is involved in physiological aging and degenerative processes occurring in age-related diseases (12, 22, 26, 49, 77, 153). Whether uncon-

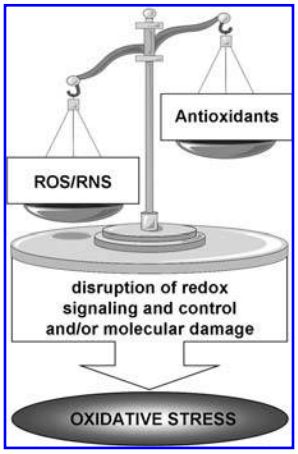

FIG. 1. Updated definition of oxidative stress. H. Sies and D. P. Jones (240) have recently re-defined oxidative stress as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage."

trolled excessive formation of ROS/RNS is a primary cause, or merely a downstream consequence of the neurodegenerative process, is still an open question. More generally, whether ROS/RNS activity has a causal, initiating, or propagating role in human diseases associated with oxidative stress remains generally unresolved at present (6, 248). Indeed, an increasing amount of evidence suggests that oxidative/nitrosative stress is linked to either the primary or secondary pathophysiologic mechanisms of multiple acute and chronic human diseases (51, 54, 111, 142, 248). However, definitive evidence for this association has often been lacking because of recognized shortcomings with methods available to assess oxidative stress status *in vivo* in humans (54).

The study of the role of oxidative stress in disease involves the measurement of biomarkers of oxidative damage that reflect damage induced from an attack by ROS/RNS or secondary by-products of oxidative/nitrosative stress. Biomarker is a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or responses to a therapeutic intervention. Biomarkers of oxidative stress/damage are isolated from tissues and biological fluids, such as saliva, cerebrospinal fluid, bronchoalveolar lavage fluid, urine, serum, and plasma. Currently, there is an overwhelming need to validate more specific oxidative damage biomarkers that can be easily measured in human biological fluids, in order to overcome limitations of invasive monitoring. A number of biomarkers of oxidative stress/damage, including products of lipid peroxidation such as F_2-isoprostanes, glutathione, and glutathione disulfide (GSSG), as well as oxidized proteins, are validated and commonly used (54). However, limited human data are available on the levels of specific biomarkers of protein damage in controlled clinical studies. Consequently, at present, it is unknown if some oxidative stress-induced protein modifications may be an important tool in clinical trials as biomarkers of oxidative stress and, more importantly, have diagnostic (particularly early and possibly presymptomatic) and therapeutic uses.

Oxidative stress may cause reversible and/or irreversible oxidative modifications on sensitive proteins that may lead to a change in the activity or function of the oxidized protein (77). Reversible modifications, usually occurring at cysteine or methionine residues, may have a dual role: protection from irreversible damage and modulation of protein function (redox regulation). Irreversible modifications induced by ROS/RNS, such as protein–protein cross-linking or protein carbonylation, are generally associated with permanent loss of function and may lead to either the unfolding and degradation of the damaged proteins by the proteasomal system, or to their progressive accumulation into cytoplasmic inclusions or extracellular aggregates, as observed in most age-related neurodegenerative disorders (16, 53, 153, 244). On the other hand, redox reversible protein modification is involved in redox signaling and is also an early cellular response to mild oxidative stress (93, 101, 127).

In mammalian cells, a significant amount of glutathione may be reversibly bound to proteins, by a mechanism called *S*-glutathionylation, a post-translational modification resulting in the formation of mixed disulfides between glutathione and protein sulfhydryl groups to form *S*-glutathionylated proteins. Protein *S*-glutathionylation is a dynamic process thought to be involved in redox regulation of protein and to contribute to the modulation of cell shape, signaling, ion transport, vascular tone, metabolism, mitochondrial function, and transcription factors. For example, physiological (low) levels of ROS/RNS reversibly *S*-glutathionylate the small GTPase transforming protein, p21ras (44, 148), and sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) (1, 45), which play crucial roles in regulating redox-sensitive signaling and vasodilation.

The oxidative/nitrosative modification of proteins has become an important field of investigation in human disease because of the potential of modified proteins to regulate or disrupt cell signaling pathways, particularly those involving cell death cascades.

II. INTRACELLULAR REDOX HOMEOSTASIS AND THE GSH/GSSG REDOX COUPLE

Glutathione is a water-soluble tripeptide ubiquitously distributed, consisting of glycine, cysteine, and glutamic acid (L-γ-glutamyl-L-cysteinylglycine, GSH) (Fig. 2). In mammalian cells, it is the predominant intracellular low molecular mass thiol, being present at concentrations of ~1–10 m*M* in most cells. Glutathione is less prone to oxidation than cysteine, making it an ideal compound for maintaining intracellular redox potential. The sulfhydryl group on its cysteinyl portion accounts for GSH's strong electron-donating character. As electrons are lost,

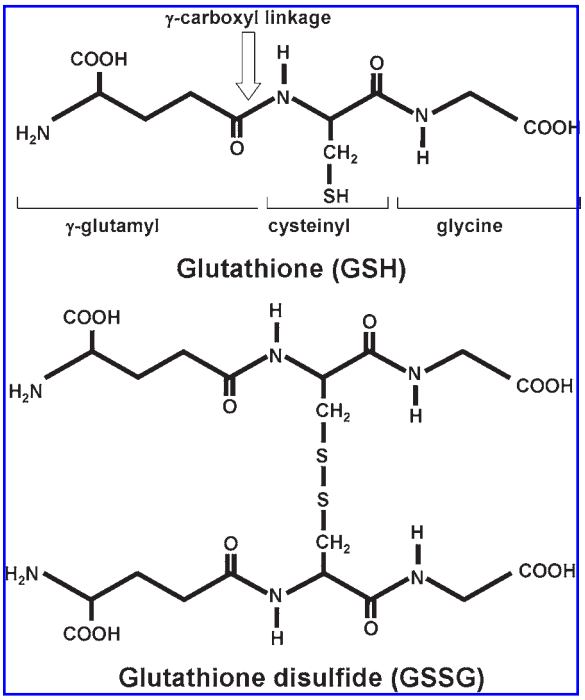

FIG. 2. Structure of the thiol form (GSH) and disulfide form (GSSG) of glutathione. In both forms, the peptide bond linking the amino-terminal glutamate and the cysteinyl residue is through the γ-carboxyl group and protect glutathione from degradation by serum aminopeptidases or intracellular proteases.

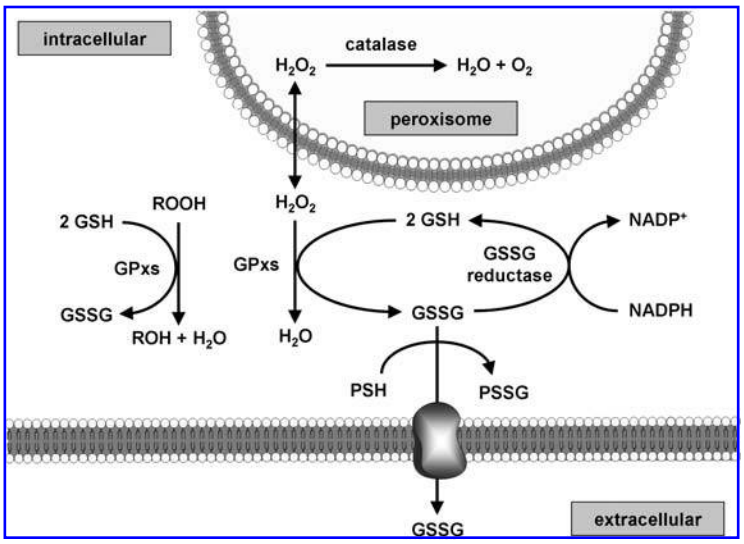

FIG. 3. Antioxidant functions of GSH. Glutathione can directly scavenge free radicals or act as a substrate for glutathione peroxidases (GPxs) and, to a smaller extent, for some glutathione *S*-transferases during the cytoplasmic detoxification of hydrogen peroxide, lipid hydroperoxides, and electrophilic compounds. Hydrogen peroxide, which is generated as a result of aerobic metabolism, can also be metabolized by catalase in the peroxisome as well as by peroxiredoxins, which are distributed within cytosol, mitochondria, peroxisome, endoplasmic reticulum, and plasma membrane, and also reduce lipid hydroperoxides. The production of GSSG by GPxs can lead to the formation of mixed disulfides in cellular proteins (*S*-glutathionylated proteins, PSSG), the release of GSSG excess by the cell to maintain the intracellular GSH/GSSG ratio, and the back-reduction to GSH by GSSG reductase utilizing NADPH as a reductant, thereby forming a redox cycle. The resulting depletion of cellular GSH can be replaced by a *de novo* synthesis through two sequential ATP-dependent reactions catalyzed by glutamate cysteine ligase and glutathione synthase.

the molecule becomes oxidized, and two such molecules become linked (dimerized) by a disulfide bridge to form glutathione disulfide (GSSG) (Fig. 2). This linkage is reversible upon reduction. Glutathione disulfide is derived from GSH following oxidation of the cysteinyl thiol by direct interaction either with ROS/RNS or, more often, with enzymes, primarily with glutathione peroxidases (Fig. 3). GSSG is reduced to GSH by the enzyme glutathione reductase in a reaction requiring NADPH as a cofactor (120) (Fig. 3). Within mammalian cells, glutathione exists mainly (>98%) in the thiol-reduced form (GSH) and the disulfide content is usually <1% of GSH, but the redox ratio can drastically shift during severe oxidative stress (229). Some is also present in the thioether, mercaptide, or other thioester forms (GSH *S*-conjugates) (239).

With the exception of bile, extracellular concentrations of GSH are relatively low. In humans, the usual range of plasma GSH is 1–5 μM (94, 95).

Glutathione is ubiquitously distributed in different cellular compartments, which results in different subcellular redox environments (Fig. 4), GSH being usually the predominant form. Almost 90% of glutathione is in the cytosol (1–10 m*M*), ~10% in the mitochondria (5–10 m*M*), and a small percentage in the endoplasmic reticulum and, possibly, the nucleus (163). But all cellular GSH is synthesized within the cytoplasm. Furthermore, cellular GSH/GSSG redox state varies in cells in association with proliferation, differentiation, and apoptosis (177). Mitochondrial GSH is generally considered more important than cytoplasmic GSH levels for cell survival. Moreover, mitochondrial GSH concentration may be higher than in the cytosol and there may also be variations in GSH content between mitochondria (242).

Normal cells maintain a specific redox homeostasis, such that multiple, interrelated redox couples are maintained within physiologic ranges for oxidation and reduction, all contributing to the intracellular redox environment. Among the most important of these redox couples are the $NADPH/NADP^+$, GSH/GSSG, reduced thioredoxin/oxidized thioredoxin ($Trx(SH)_2$/TrxSS), and cysteine/cystine couples (130, 132, 166, 229). When these redox couples are stimulated to move out of the physiologic range, biologically adverse consequences often result. For example, high reduction states for $NADPH/NADP^+$, besides diverse pathophysiological stimuli (*e.g.*, inflammatory signals and

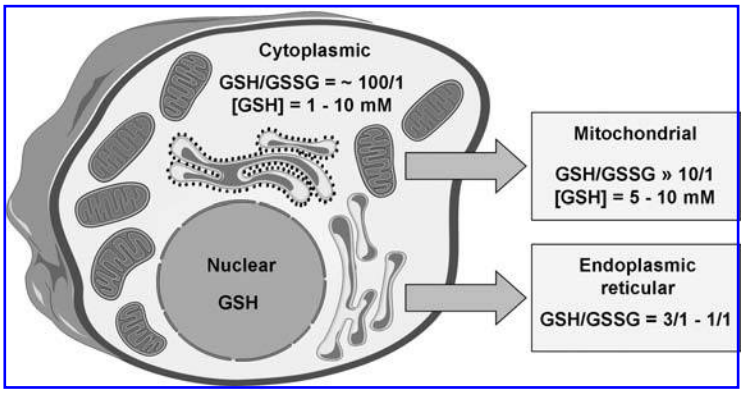

FIG. 4. Subcellular distribution of the glutathione pool. Glutathione is widely distributed in different cellular compartments, which may result in different subcellular redox environments. Almost 90% of glutathione is in the cytosol (1–10 m*M*), about 10% in the mitochondria (5–10 m*M*) and a small percentage in the endoplasmic reticulum and, possibly, the nucleus. Modified from Ref. 113.

growth factors), can activate the ubiquitous plasma membrane NADPH oxidase to generate superoxide, a process physiological for macrophage, neutrophil, and eosinophil activation as well as for noninflammatory cells (*e.g.*, endothelial cells) that produce ROS constitutively as signaling molecules, but toxicological in other cases (27, 126).

The intracellular redox homeostasis (or redox buffering) capacity is determined primarily by GSH/GSSG and thioredoxin$_{red}$/thioredoxin$_{ox}$ redox couples. The high ratios of reduced to oxidized glutathione and thioredoxin are maintained by the activity of glutathione reductase and thioredoxin reductase, respectively. Generally, a more reducing environment (maintained by elevated levels of GSH and reduced thioredoxin) of the cell stimulates proliferation and a slight shift towards a mildly oxidizing environment is associated with cell differentiation. A further shift towards a more oxidizing cellular environment leads to apoptosis or necrosis. Whereas apoptosis is induced by moderate oxidizing stimuli, necrosis is induced by an intense oxidizing effect (72, 267). Both glutathione and thioredoxin redox buffering thiol systems counteract intracellular oxidative stress. Cellular GSH/GSSG redox state, measured under conditions identical to those used to measure thioredoxin redox state, shows that the GSH and thioredoxin systems function independently (114, 188). For instance, during differentiation of Caco2 cells, GSH/GSSG redox state was oxidized while that of thioredoxin remained unchanged (188).

Redox regulation has also been shown to be an important component of malignant cell survival (69, 72). Hence, preclinical and clinical studies are currently testing novel therapeutic agents, including buthionine sulfoximine, ascorbic acid, and motexafin gadolinium, both as single agents and in combination, that modulate the cellular redox system. Among these, motexafin gadolinium, an expanded porphyrin, is a tumor-selective redox mediator that reacts with many intracellular reducing metabolites, including GSH. The mechanism of cytotoxicity was related to induction of apoptosis that was accompanied by depletion of intracellular GSH and increased ROS production (69, 72). These studies provide a rationale for clinical investigation of this novel redox-mediating agent in patients with multiple myeloma and related disorders.

The normal/physiological intracellular GSH/GSSG ratio is far higher (~100–10,000 greater) than most redox active compounds, thus constituting the major redox buffer in cytosol (92, 130, 132, 139, 166), but its value can be affected by other redox couples, including NADPH/NADP$^+$ and thioredoxin$_{red}$/thioredoxin$_{ox}$ (133). Maintaining optimal GSH/GSSG ratios in the cell is critical to cell survival, and is important in regulating the redox status of protein thiols, thus influencing protein function and activity (229). Changes in GSH/GSSG ratios can potentially influence a number of target proteins by causing oxidation and/or disulfide exchange reactions at specific protein cysteinyl residues. However, minor changes in GSH/GSSG ratio could unlikely lead to substantial *S*-glutathionylation of proteins according to simple thiol–disulfide exchange involving GSSG and protein –SH, because the redox potentials of most protein cysteines are such that they would only be 50% *S*-glutathionylated at a very low GSH/GSSG ratio of 1 (*i.e.*, a very substantial and usually unlikely change in GSSG concentration; see Refs. 89, 92, 236, 278). An assessment of the GSH/GSSG ratio therefore provides a reliable estimation of cellular redox status in cells and is thus frequently measured as a representative indicator for the redox environment of the cell (229).

The Nernst equation can be used to determine the redox potential (E) of GSH in cells (229). At 25°C and pH 7, the redox potential (E) of the GSH/GSSG redox couple can be defined as follows:

$$E = -240 - \left(\frac{59.1}{2}\right)\log\left(\frac{[GSH]^2}{[GSSG]}\right)\text{mV}$$

Since two GSH molecules are needed to form one GSSG molecule, the reaction is second order for GSH and, therefore, [GSH] is squared in the Nernst equation. Thus, any changes in the absolute concentration of [GSH] will change the redox potential, even without changes in the GSH/GSSG ratio. For example, a hepatocyte that has 0.01 *M* GSH and a GSH/GSSG of 100:1 would have a potential of –240 mV. A cell with just 0.001 *M* GSH with a similar GSH/GSSG of 100:1 would have a potential of about –210 mV, even though the GSH/GSSG ratio remained the same. This would suggest that cells with more GSH, such as hepatocytes, have a greater reducing capacity than cells with lower GSH levels, such as neurons (112).

The GSH/GSSG ratio is normally closely regulated. Disruption of this ratio results in several cellular reactions involved in signal transduction and cell cycle regulation under conditions of oxidative stress; the GSH/GSSG ratio tends to decrease either through an increase in the level of GSSG or a decrease in GSH (229). Under pathological conditions, the redox state can be altered to lower or higher values. The redox balance can be maintained, however, even in the face of an oxidative stress by increasing glutathione reductase activity or via elimination of GSSG from cells (Fig. 3). Export of GSSG from cells would prevent the shift of GSH/GSSG ratio and protect cells and tissues from oxidative stress. Numerous members of the multidrug resistance protein family serve as export pumps that prevent the accumulation of glutathione-*S*-conjugates and GSSG in the cytoplasm, and play, therefore, an essential role in detoxification and defense against oxidative stress, contributing to the control of the intracellular GSSG level (123, 145, 147). Multidrug resistance proteins are members of the subgroup ABCC of the superfamily of ATP-binding cassette (ABC) transporters (147). Although these proteins are low affinity GSSG transporters, they can play essential role in response to oxidative stress when the activity of GSSG reductase becomes rate limiting.

Because GSSG is not taken up by cells, but is rather degraded extracellularly, loss of GSSG from cells under conditions of oxidative stress increases cellular requirements for *de novo* synthesis of GSH (105). The availability of GSH during oxidative stress is ensured by GSH recycling (*i.e.*, GSH degradation and resynthesis) (Fig. 5), which can be upregulated in situations of oxidative stress (105).

It has been established that a decrease in GSH levels and/or a decrease in GSH/GSSG ratio, indicating a decrease in the antioxidant capacity of the cell, may be correlated with aging and a variety of human diseases including diabetes mellitus, cardiovascular disease, rheumatoid arthritis, amyotrophic lateral sclerosis, HIV infection, Alzheimer's disease (AD), alcoholic liver disease, cystic fibrosis, cataract genesis, and Down's syndrome (54, 121, 149, 200, 228). Given the role of glutathione

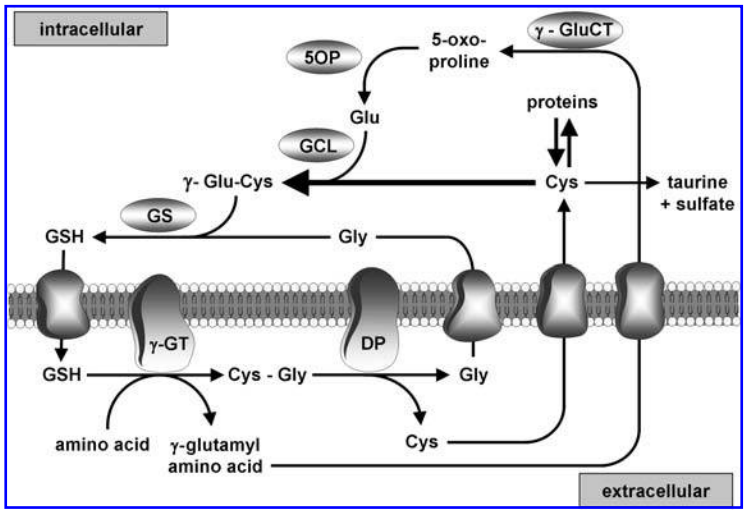

FIG. 5. Glutathione synthesis and degradation: the γ-glutamyl cycle. One of the most important functions of the γ-glutamyl cycle is to provide a continuous source of cysteine, which is taken up readily by most cells. Once entered the cell, most cysteine is incorporated into GSH, some is incorporated into newly synthesized proteins, and some is broken down into sulfate and taurine. GSH is exported from the cell by carrier-mediated transporters, and the ecto-enzyme γ-GT then transfers the γ-glutamyl moiety of GSH to an amino acid (the best acceptor being cystine), forming γ-glutamyl amino acid and cysteinylglycine (Cys–Gly). The γ-glutamyl amino acid can then be transported back into the cell to complete the cycle. Once inside the cell, the γ-glutamyl amino acid can be further metabolized to release the amino acid and 5-oxoproline, which can be converted to glutamate. Cysteinylglycine is broken down by dipeptidase (DP) to generate cysteine and glycine, which are then transported back into the cell to be reincorporated into GSH. CT, cyclotransferase; DP, dipeptidase; GCL, glutamate cysteine ligase; GS, glutathione synthase; γ-Glu-Cys, γ-glutamylcysteine; γ-GT, γ-glutamyl transpeptidase; Glu, L-glutamic acid; Gly, L-glycine; GSH, glutathione; 5OP, 5-oxo-prolinase.

in protection against oxidative stress, its availability in the reduced form may be a key factor in the maintenance of human health. Glutathione depletion could cause damage to protein, DNA, and/or membrane lipids, and thus potentially lead to cellular dysfunction. Oxidative stress that results in GSH depletion promotes a conformational change in GCL, the first enzyme involved in glutathione synthesis, which increases the catalytic activity of this enzyme, thus stimulating the synthesis of GSH (243). On the contrary, physiological GSH concentrations reduce GSH synthesis through feedback inhibition mechanisms (243). Therefore, decreased GSH level and/or decreased GSH/GSSG ratio might contribute to cellular dysfunction only when oxidative stress becomes prolonged, and cellular defense systems are not sufficient to counteract the oxidative stress-mediated insult.

III. OXIDATIVE MODIFICATIONS OF PROTEIN CYSTEINYL THIOLS

The concentration of protein sulfhydryl groups in cells and tissues is much greater than that of GSH (229). Protein sulfhydryl groups can be present as thiols, oxidized to sulfenic, sulfinic, or sulfonic acids, as well as intramolecular or intermolecular disulfides or mixed disulfides with low-molecular-mass thiols such as homocysteine, cysteinylglycine, cysteine, and glutathione, as a whole defined as *S*-thiolated proteins (Fig. 6) (19, 67, 97). These modifications can alter the function of numerous proteins that contain cysteines of structural importance, within their catalytic centers or as part of protein–protein interaction interfaces. Since free cysteine and GSH are the most abundant low-molecular-mass thiols, *S*-glutathionylated (Fig. 7) and *S*-cysteinylated proteins will be the main mixed disulfides that are not equally distributed between extracellular and intracellular settings/compartments (see below) (253).

Thomas and colleagues first reported that the process of *S*-thiolation can occur under physiologic circumstances, during the respiratory burst in mouse macrophages (211, 212) or human neutrophils (39), and the absence of any detectable increase in GSSG in both cellular settings suggested that *S*-thiolation of the proteins did not occur by thiol/disulfide exchange. They also provided the first evidence that protein *S*-thiolation/dethiolation is a dynamic process that occurs under physiologic conditions

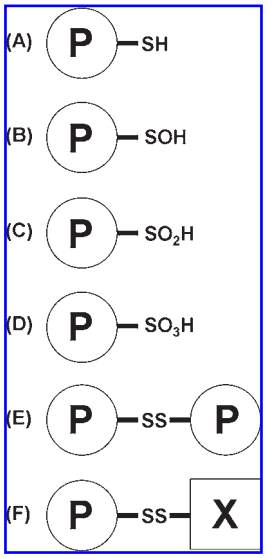

FIG. 6. Modifications of protein thiol groups. Protein sulfhydryl groups can be present as (**A**) reduced thiols (P-SH), oxidized to (**B**) sulfenic (P-SOH), (**C**) sulfinic (P-SO_2H), or (**D**) sulfonic acids (P-SO_3H), as well as (**E**) intra- or intermolecular disulfides (P-SS-P) or (**F**) mixed disulfides (P-SS-X) with low-molecular-mass thiols (X) such as homocysteine, cysteinylglycine, cysteine, and glutathione, as a whole defined as *S*-thiolated proteins and, respectively, *S*-homocysteinylated, *S*-cysteinylglycinylated, *S*-cysteinylated, and *S*-glutathionylated proteins.

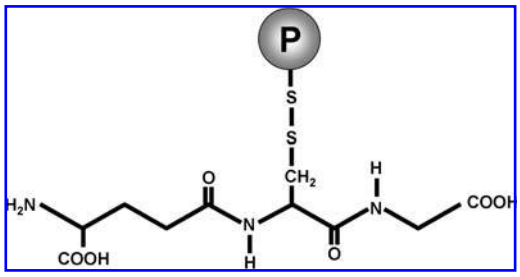

FIG. 7. Scheme of *S*-glutathionylated proteins (PSSG).

in human cells, during stimulation of the respiratory burst in human monocytes. They showed that the process is reversible, and the dynamic events of *S*-thiolation/dethiolation occur at different rates with different proteins (233). GSH was determined to be the most abundant low molecular mass thiol bound to *S*-thiolated (actually, *S*-glutathionylated) proteins, but γ-glutamylcysteine and cysteine were also bound (233).

The two major determining factors of the susceptibility of cysteinyl residues to redox reactions are the accessibility of the thiol within the three-dimensional structure of the protein and the cysteine reactivity, which is influenced by the neighboring amino acids. Most thiol modifications are unstable and can easily be reversed or replaced by other, more stable ones. The bulk of cysteine sulfhydryls within cytoplasmic proteins is characterized by a pK_a (acid dissociation constant) value >8.0; therefore, because of the reducing environment of the cytoplasm, they remains essentially in the protonated state at physiological pH, and thus are not sensitive to oxidation. However, redox-sensitive proteins have specific Cys residues localized in a basic environment, being flanked by basic (*i.e.*, positively charged) amino acid residues, which lowers their pK_a values so that the cysteines are in the thiolate form at neutral pH, making them susceptible targets for oxidative modifications (Fig. 8) (209). Protein tyrosine phosphatases (PTPs) are well-documented examples in which the conserved cysteine in their active sites is susceptible to reversible oxidation to sulfenic acid because a nearby arginine lowers its pK_a so that the cysteine is in the thiolate form, which enhances its nucleophilic properties but renders it susceptible to oxidation. Oxidation of the active site Cys abolishes its nucleophilic properties, thereby inhibiting PTP enzymatic activity, and can be reversed by cellular thiols (209).

The oxidation of sulfhydryl groups of cysteinyl residues to form –S–S– crosslinks (*i.e.*, protein disulfides) can occur between two proteins (intermolecular) or within a protein (intramolecular), causing changes in protein aggregation and conformation; protein disulfides can be readily reduced by disulfide exchange reactions catalyzed by thiol–disulfide oxidoreductases, such as thioredoxins and glutaredoxins (see below), ensuring the transient nature of the modification. Intermolecular disulfides are also formed in the cytoplasm upon exposure of cells to oxidative stress (18, 48) and can also have regulatory function (17).

The highly reactive protein sulfenic acids, the direct products of the reaction of cysteine thiolates with H_2O_2, although alkyl hydroperoxides, peroxynitrite, and hypochlorous acid too may play a role in sulfenic acid formation, are very unstable, being frequently susceptible, unlike protein disulfides, to irreversible oxidation to cysteine sulfinic and sulfonic acids (the latter also known as cysteic acids), or readily reacting with vicinal thiols to form intra- or intermolecular disulfides (227). Thus, sulfenic acids may play an important role in protein *S*-glutathionylation (see below). Nonetheless, recent evidences demonstrate that, in spite of their reactivity, some sulfenic acids are more stable, and these can be identified in proteins also under physiological conditions (34, 35, 227). Finally, protein sulfenate formation has important roles in redox signaling pathways, which are thought to involve oxidation of those particular cysteines that are located in an environment promoting dissociation of thiols (205).

Strong oxidants (or oxidative stress conditions), besides oxidizing protein thiols or thiolates to sulfinic and sulfonic derivatives, which are essentially irreversible modifications *in vivo* and associated with oxidative injury, can lead to excessive disulfide bonding, protein misfolding, and aggregation (241). Excessive disulfide bonding may lead to covalent aggregates that are difficult to reduce even when intracellular redox conditions are restored to normal.

Major differences between cellular and extracellular compartments exist both in terms of the concentrations of sulfhydryl/disulfide systems and their relative redox states (132, 177). The intracellular concentrations of free Cys, GSSG, and cystine are lower (micromolar) than those (~1–10 m*M*) of GSH, while extracellular free Cys is more abundant than GSH: actually, the cysteine/cystine redox couple quantitatively represents the largest pool of low-molecular-mass thiols and disulfides in plasma and the extracellular compartment on the whole.

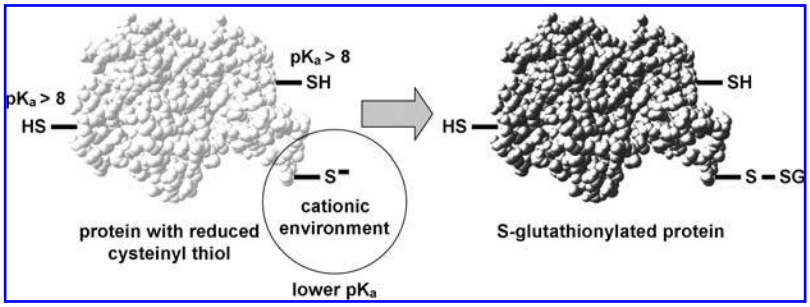

FIG. 8. Susceptibility to *S*-glutathionylation. Redox-sensitive cytoplasmic proteins have specific Cys residues that exist as thiolate anions at neutral pH, due to a lowering of their pK_a values as a result of electrostatic interactions with neighboring positively charged amino acid residues, becoming "active cysteines", which can then react to form a mixed disulfide with GSH or a protein disulfide. Therefore, a cationic environment renders the protein thiol group highly reactive and particularly susceptible to *S*-glutathionylation. Remarkably, Cys374 of actin, which has a typical pK_a value (*i.e.*, ~8.5) (268), represents a notable exception to the concept that low thiol pK_a is associated with propensity for *S*-glutathionylation. See text for further details.

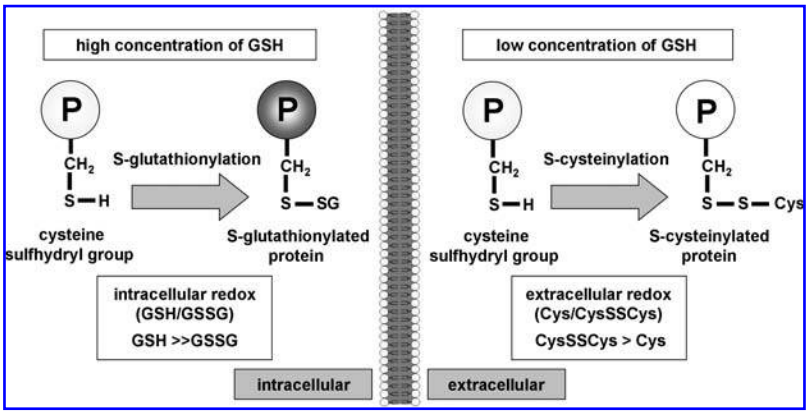

FIG. 9. *S*-Glutathionylation and *S*-cysteinylation. The major low-molecular-mass sulfhydryl/disulfide system in cells, GSH/GSSG, is principally in the reduced form, while that in the extracellular compartment, cysteine/cystine, is principally in the disulfide form, cystine. Thus, extracellular proteins may be prevalently *S*-cysteinylated, while intracellular proteins may be prevalently *S*-glutathionylated.

The major low-molecular-mass sulfhydryl/disulfide system in cells, GSH/GSSG, is principally in the reduced form, while that in the extracellular compartment, cysteine/cystine, is principally in the disulfide form, cystine. Thus, extracellular proteins may be prevalently *S*-cysteinylated while intracellular proteins may be prevalently *S*-glutathionylated, as first reported by Thomas's group (253) (Fig. 9). For example, while the small fraction of *S*-thiolated hemoglobin in red blood cells is only *S*-glutathionylated (96, 172, 225), plasma proteins such as albumin are mainly *S*-cysteinylated (13, 97, 191), possibly following formation of an intermediate sulfenic acid (34, 35). Nevertheless, although GSH is usually the predominant bound thiol, minor amounts of cysteine, cysteinylglycine, and/or homocysteine were also detected as protein-bound thiols in cytoplasmic proteins in several cell types, for instance in both human neutrophils and monocytes and mouse macrophages (*e.g.*, 39, 207, 233) as well as rat cardiocytes (64, 65).

IV. MECHANISMS AND REVERSIBILITY OF PROTEIN *S*-GLUTATHIONYLATION

Protein *S*-glutathionylation is a post-translational modification that occurs also under basal (physiological) conditions (usually referred to as constitutive *S*-glutathionylation) (40, 66, 154, 208), as demonstrated, for instance, in hemoglobin in red blood cells (96, 171), γ-crystallin from human lens (46), and actin in human fibroblasts (198) and human epidermal A431 cells (268). Protein *S*-glutathionylation under basal conditions suggests the possible involvement of this post-translational modification in cellular signaling and redox regulation of protein functions (87, 89, 101).

In addition to a potential regulatory role, *S*-glutathionylation may serve as a means of storing glutathione and of protection, under conditions of oxidative stress, by preventing the irreversible oxidation of protein thiols (*e.g.*, 152, 166) (Fig. 10), often at the expense of temporary loss in protein activity (139, 168, 207, 229). In fact, if the modified protein sulfhydryl group is functionally critical, *S*-glutathionylation will also render the protein inactive, and eventually compromise cellular functions (*e.g.*, 66, 190). Moreover, this reaction can effect a change in conformation and/or charge that may alter protein function. In fact, by adding the glutathione tripeptide to a target protein, an additional negative charge is introduced (as a consequence of the Glu residue), and a change in protein conformation is made likely. For instance, the *S*-glutathionylation of γ-glutamyl transpeptidase appears to protect this membrane-bound enzyme from the irreversible oxidative damage by hydrogen peroxide produced during γ-glutamyl transpeptidase-mediated metabolism of GSH (60), whereas the *S*-glutathionylation of α-ketoglutarate dehydrogenase in response to alterations in the mitochondrial GSH status produces reversible inactivation of the enzyme (190).

The formation of mixed disulfides between glutathione and protein sulfhydryl groups (*i.e.*, the formation of *S*-glutathionylated proteins) can occur by several mechanisms (Fig. 11) (87, 89, 101, 127, 139). Mixed disulfides can be formed in response to changes in the GSH/GSSG ratio, through thiol/disulfide exchange reactions between a protein sulfhydryl group and GSSG (Fig. 11, mechanism A), a reaction that both removes GSSG and generates GSH, thus restoring the physiological redox conditions (101, 229). Thiol/disulfide exchange reactions can also occur between a reduced protein thiol and a *S*-glutathionylated protein. In experimental models of oxidative stress, transient shifts in the GSH/GSSG ratio from 100 to 10 or even 1 have been described and found to correlate with the amount of *S*-glutathionylated proteins formed (92). For example, a pronounced change in the GSH/GSSG ratio in a model of oxida-

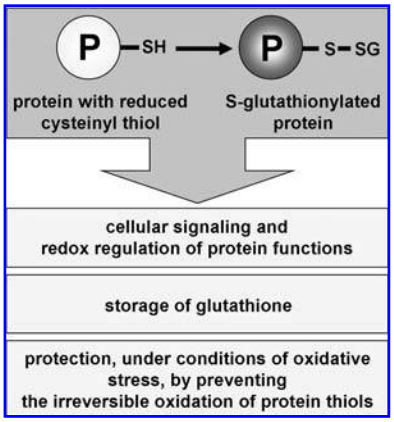

FIG. 10. Proposed roles of protein *S*-glutathionylation. See text for details.

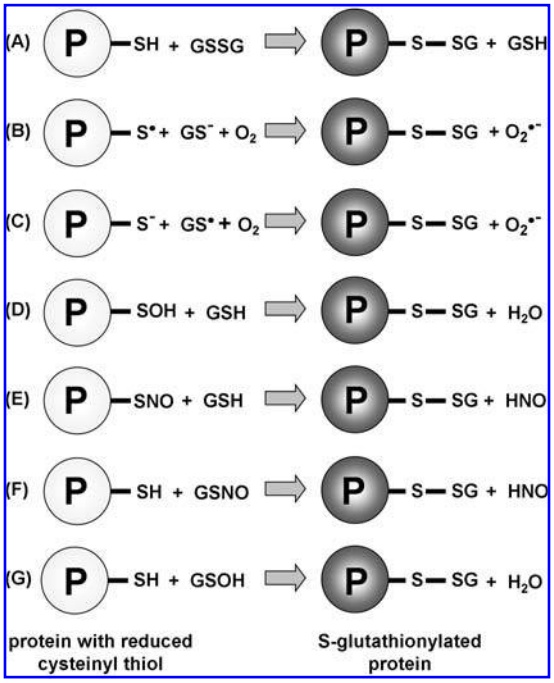

FIG. 11. Proposed mechanisms of formation of *S*-glutathionylated proteins. Mixed disulfides can be formed in response to changes in the GSH/GSSG ratio, through thiol/disulfide exchange reactions between a protein sulfhydryl group and GSSG (mechanism **A**). Thiol/disulfide exchange reactions can also occur between a reduced protein thiol and a *S*-glutathionylated protein. *S*-Glutathionylated proteins can also be formed following activation (*i.e.*, partial oxidation) of the protein sulfhydryl group or the sulfhydryl group on the cysteinyl portion of GSH. This can occur either by a radical reaction, by one electron oxidation of the protein thiol or the thiol on GSH to give the respective thyil radical (mechanisms **B** and **C**), which further reacts with GSH or a reduced protein thiol, respectively, to give a *S*-glutathionylated protein (mechanisms **B** and **C**), or from two electron oxidation of the protein thiol to sulfenic acid (PSOH), followed by reaction with GSH (mechanism **D**). Furthermore, *S*-glutathionylated proteins can arise from reactions between GSH and *S*-nitrosated proteins (PSNO) (mechanism **E**) or from reactions between protein cysteinyl thiols and *S*-nitrosothiols such as GSNO (mechanism **F**) or other oxidized forms of GSH such as glutathione sulfenic acid (GSOH) (mechanism **G**) or glutathione disulfide *S*-monoxide (GS(O)SG).

tive stress in human red blood cells induced protein 4.2 and spectrin *S*-glutathionylation (215).

However, large shifts in the intracellular redox potential are believed to be unlikely to occur under normal physiological condition (55, 101), because of the capability of most cell types to actively export GSSG as a protective mechanism against oxidative stress (123, 145). Consequently, substantial protein *S*-glutathionylation according to simple thiol–disulfide exchange reaction between a protein sulfhydryl group and GSSG is unlikely to occur, also because the redox potential of many protein cysteinyl residues does not favor (see also subheading II) this type of *S*-glutathionylation mechanism (mechanism A in Fig. 11). In fact, *S*-glutathionylation may occur in intact cells in various experimental models of ROS generation without any detectable changes in the GSH/GSSG ratio (39). *S*-Glutathionylated proteins can also be formed following activation (*i.e.*, partial oxidation) of the protein sulfhydryl group (194) or the sulfhydryl group on the cysteinyl portion of GSH. This can occur either by a radical reaction, by one electron oxidation of the protein thiol or the thiol on GSH to give the respective thiyl radical (Fig. 11, mechanisms B and C), which further reacts with GSH or a reduced protein thiol, respectively, to give a *S*-glutathionylated protein (Fig. 11, mechanisms B and C), or from two electron oxidation of the protein thiol to sulfenic acid (PSOH), followed by reaction with GSH (Fig. 11, mechanism D). The reaction of glutathione thiyl radical with proteins to generate *S*-glutathionylated proteins is catalyzed by glutaredoxin (246), an enzyme normally acting as a reductant (see below and Fig. 12). Furthermore, *S*-glutathionylated proteins can arise from reactions between GSH and *S*-nitrosated proteins (Fig. 11, mechanism E) or from reactions between protein cysteinyl thiols and *S*-nitrosothiols such as GSNO (Fig. 11, mechanism F) or other oxidized forms of GSH such as glutathione sulfenic acid (GSOH) (Fig. 11, mechanism G) or glutathione disulfide *S*-monoxide [GS(O)SG] (98, 253).

Critical in ascribing any regulatory function to *S*-glutathionylation is the reversibility of this process, which is also a key element of a signaling response, by small-molecule, cysteine-rich proteins. *S*-Glutathionylation of protein cysteinyl thiols can be reversed, in a process called deglutathionylation (*i.e.*, the detachment of glutathione from mixed disulfides) as a whole, via direct thiol/disulfide exchange reactions with GSH, once an appropriate GSH/GSSG ratio (*i.e.*, the reducing intracellular redox balance) has been restored (229), or frequently by means of reactions catalyzed by thiol–disulfide oxidoreduc-

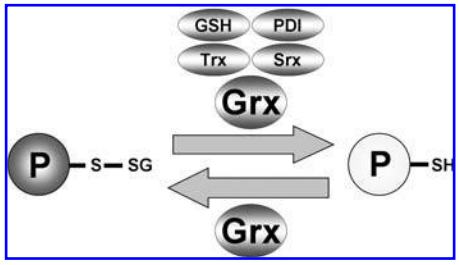

FIG. 12. Thiol-disulfide oxidoreductases implicated in addition and removal of GSH to/from proteins. Protein deglutathionylation (*i.e.*, the detachment of glutathione from protein mixed disulfides) can occur via direct thiol/disulfide exchange reactions with GSH, once an appropriate intracellular GSH/GSSG ratio has been restored (229), or, more frequently, by means of reactions catalyzed by thiol-disulfide oxidoreductases, chiefly by glutaredoxins (also known as thioltransferases)/glutaredoxin reductase (75, 236) and, to a minor extent, by thioredoxins/thioredoxin reductase (122), protein disulfide isomerase (134, 253), and sulfiredoxin (76). Glutaredoxin non only deglutathionylates specific proteins (75), but also catalyzes the *S*-glutathionylation of several proteins in the presence of a glutathione thiyl radical generating system (104, 246). Hence, glutaredoxin is capable of catalyzing both *S*-glutathionylation and deglutathionylation of proteins via distinct mechanisms (273). Grx, glutaredoxin; PDI, protein disulfide isomerase; Srx, sulfiredoxin; Trx, thioredoxin.

tases, mainly by glutaredoxins (also known as thioltransferases)/glutaredoxin reductase (75, 236) and, in some cases, by thioredoxins/thioredoxin reductase (122), protein disulfide isomerase (134, 253), and sulfiredoxin (76) (Fig. 12). Glutaredoxin, which is associated with a specific glutathionyl-mixed disulfide oxidoreductase activity (104, 246), not only deglutathionylates specific proteins (75) but also catalyzes the *S*-glutathionylation of several proteins in the presence of a glutathione-radical generating system (246). Hence, glutaredoxin is capable of catalysing both *S*-glutathionylation and deglutathionylation of proteins via distinct mechanisms (236, 273).

V. SPECIFICITY OF PROTEIN *S*-GLUTATHIONYLATION

Protein sulfhydryls exhibit a striking differential susceptibility to *S*-glutathionylation (141). Thomas and colleagues, for instance, showed that H-ras can be *S*-glutathionylated on multiple reactive cysteines *in vivo* and that at least one of these thiols is normally lipid-modified (167). In NIH-3T3 cells treated with *S*-nitrosocysteine, evidence for both *S*-nitrosated and *S*-glutathionylated H-ras was obtained and *S*-nitrosation was the predominant modification. Thomas and colleagues also provided evidence that H-ras can be oxidatively modified simultaneously on four separate cysteinyl residues *in vitro*. They further showed that, while *S*-nitrosation may modify all four of these residues, *S*-glutathionylation occurs primarily on two of four possible reactive cysteinyl residues (167). Furthermore, H-ras can be both *S*-nitrosated and *S*-glutathionylated more efficiently than most cytosolic proteins within NIH-3T3 cells treated with *S*-nitrosocysteine (167). Nevertheless, at present, it is not fully definite what features contribute to the sensitivity of a given cysteine residue to *S*-glutathionylation and the question about the factors that facilitate and confer site specificity on such modification is still debated. The identity of surrounding residues in the primary sequence or the tertiary structure may play a role in making a thiol more or less reactive.

Actually, not all cysteinyl residues are equally susceptible to reaction with ROS/RNS and/or glutathione. Indeed, the reaction rate of most protein cysteines with ROS/RNS and/or GSH is too slow to be of physiological relevance under the cellular conditions and concentrations. This situation changes drastically when cysteine is bound to a metal ion (271), such as Mg^{2+}, Zn^{2+}, or Ca^{2+}, which can function as an allosteric effector to control protein thiol reactivity, or is in the thiolate anion ($-S^-$) form (209). For instance, most protein Cys residues are not sensitive to oxidation by a hydroperoxide, such as hydrogen peroxide, at about neutral (*i.e.*, physiological) pH, since hydrogen peroxide selectively oxidizes Cys residues with a low acid constant (pK_a) (138). Because hydrogen peroxide reacts rapidly with the cysteine thiolate anion (Cys-S^-) but not with the Cys sulfhydryl group (Cys-SH) (138, 155), and because the pK_a values of cysteinyl residues of the great majority of cytoplasmic proteins is around 8.5—similar to that for cysteine in GSH, which, at physiological ionic strength (160 m*M*), is 8.9 (229)—thus remaining almost completely protonated at physiological pH in the normally reducing environment of the cytoplasm, few proteins would be expected to possess a Cys residue that, being in the thiolate form as a result of its lower pK_a value, is readily susceptible to oxidation by hydrogen peroxide and/or to *S*-glutathionylation in cells (Figs. 8 and 11). Thus, a protein thiol does not react at physiologically significant rates with hydrogen peroxide, unless the reaction is catalyzed. As only those proteins with cysteines in the thiolate form significantly react with low concentrations of ROS/RNS and/or glutathione, one can postulate that the number of potential targets is limited, thereby providing specificity. However, additional features are important, as thiolates (S^-) react with hydroperoxides at rates varying from $\sim 10\ M^{-1}\ s^{-1}$ for small molecule thiols and PTPs to $\sim 10^6\ M^{-1}\ s^{-1}$ for cysteine-dependent peroxidases and the transcription factor OxyR, depending on their local environment (79, 205, 271). This protein "environment" effect contributes to specificity. Protein Cys residues exist as thiolate anions at (about) neutral pH often because the pK_a values of these Cys residues are lowered as the result of the electrostatic interaction between the negatively charged thiolate and the positively charged amino acid residues nearby (Fig. 8). Other possible factors contributing to lower cysteine pK_a values are helix–dipole effects, thought to lower the pK_a of cysteine residues, for instance, in thioredoxins, and hydrogen bonding of Cys residues with charged residues such as Ser or His (269). Hydrogen bonds involving charged His residues have been implicated, for instance, in low cysteine pK_a values (below 6) in papain (203) and thiosubtilisin (202).

Studies on a protein model system showed that introducing positively charged (*i.e.*, basic) amino acid residues around cysteines increases *S*-glutathionylation rates by favoring electrostatic interaction with the overall negative charge of glutathione disulfide and by lowering the pK_a of the cysteine (115). Such results show that the electrostatic milieu of protein cysteine thiols can have substantial effects on the rates of the thiol–disulfide exchange reactions. Thus, formation of a cysteine thiolate anion (*i.e.*, an "active cysteine"), which can then react to form a mixed disulfide with glutathione or a protein disulfide (48, 209), is favored by basic amino acids in its vicinity, while acidic vicinal amino acids will have the opposite effect. Therefore, a cationic environment renders the thiol group highly reactive and particularly susceptible to *S*-glutathionylation (Fig. 8) (140, 169). This provides a basis for specificity in protein *S*-glutathionylation. Proteins with low-pK_a Cys residues include protein disulfide isomerase (pK_a, 6.7) (83, 119, 136), protein tyrosine phosphatase (pK_a, 4.7–5.4) (249), creatine kinase (pK_a, 5.5 ± 0.1) (84, 86, 269), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (pK_a, 5.5) (174), and peroxiredoxin (pK_a, 4.6) (43). In addition, a few proteins contain selenocysteine (Cys–SeH) residues, which are readily oxidized at neutral pH because their pK_a values are 5.7 (14). Similarly, the cysteines in the regulatory site of some protein kinase C isoforms are bound to zinc in such a way that the negative character of their sulfur atoms renders them susceptible to hydrogen peroxide oxidation, thereby altering the regulation of these important signaling proteins (103).

Although the low pK_a of a protein thiol greatly influences its reactivity, other factors are likely to have some limitations in the susceptibility of a given cysteine to *S*-glutathionylation, such as redox-active motifs based on primary amino acid sequence, as the three-dimensional structure of the protein will influence sulfhydryl reactivity and its accessibility to ROS/RNS

and/or glutathione. In addition, the subcellular localization of the protein target and the proximity of the site of reactive species production as well as whether glutathione can actually make contact with a potentially reactive protein cysteinyl residue are expected to play a role in specificity, although this has not yet been studied extensively. Therefore, also Cys residue accessibility in the three-dimensional structure provides a basis for *S*-glutathionylation specificity. For instance, solvent exposure of the Cys73 residue is a major determinant in the *S*-glutathionylation of human thioredoxin (38).

The hypothesis that electrostatic interactions are involved in *S*-glutathionylation (201) is supported by the observation that covalent binding of creatine kinase, glyceraldehyde-3-phosphate dehydrogenase, glycogen phosphorylase b, and c-Jun to GSNO-Sepharose by mixed disulfide formation is impaired when the negatively charged carboxylic groups of the GSNO moiety are eliminated by esterification (141). An elegant study has recently analyzed the susceptibility towards *S*-glutathionylation of the four Cys residues of cyclophilin A (Cys52, Cys62, Cys115, and Cys161)—an ubiquitous intracellular protein, target of the immunosuppressive drug cyclosporin A, with multiple actions, including protein folding and chaperone activity—considering both the solvent exposure through molecular dynamics simulation and the influence of structural neighboring amino acids through electrostatic calculations (88). Cys52 and Cys62 were identified as targets of glutathionylation in T lymphocytes, although molecular dynamic simulation showed that Cys52 and Cys161 exposed a larger surface of their side chains than Cys62 and Cys115. Therefore, a correlation between the solvent accessibility of Cys residues of cyclophilin A and *S*-glutathionylation cannot be made (88). Electrostatic energy computations made to further define the nucleophilic reactivity of the various Cys residues in cyclophilin A showed that the changes in electrostatic energy involved in the conversion of a sulfhydryl to the corresponding thiolate anion follow the order: Cys52 < Cys62 < Cys115 < Cys161, meaning that Cys52 and Cys62 form the thiolate anion with greater ease than Cys115 and Cys161, and can therefore be expected to be more reactive. Hence, the experimental observations, together with the theoretical calculations on the susceptibility of cyclophilin A Cys residues to glutathionylation, suggest that, even when a Cys residue has a small surface exposure, as in the case of cyclophilin A Cys62, electrostatic interactions can lead to susceptibility to *S*-glutathionylation (88).

The local pH and hydrophobic compartmentalization could be other important factors that render certain Cys residues highly reactive and particularly susceptible to *S*-glutathionylation (140, 201). Actin and carbonic anhydrase III well exemplify how *S*-glutathionylation may depend on the solvent accessibility of the Cys residues. Actin contains five cysteinyl residues existing in the reduced form. Native actin exposes one fast reacting sulfhydryl group, that of Cys374, next to the C-terminus and easily accessible to sufhydryl reactive agents. The exposed Cys374 residue is the most likely glutathionylation site both *in vivo* and *in vitro* (52, 268), notwithstanding its typical pK_a value (*i.e.*, near 8.5) (268). This notable exception to the concept that low thiol pK_a is associated with propensity for *S*-glutathionylation would be explained by both a significant accessible surface and a noteworthy polarity of the S–H bond of Cys374 of actin (5). The three-dimensional structure of *S*-glutathionylated mammalian carbonic anhydrase III reveals that glutathione binds to Cys181 and Cys186, the two highly surface-exposed of its five cysteinyl residues (169, 253). Cys181 and Cys186 are located in a rather neutral environment; nonetheless, *S*-glutathionylation can be achieved by specific interactions between the GSH moiety and solvent-accessible Cys residues. Although both surface-exposed Cys181 and Cys186 are susceptible to *S*-glutathionylation, Cys186 is more readily modified both *in vitro* and *in vivo*. Lys211 appears to be primarily responsible for the lowering of the pK_a of Cys186, making its thiol more reactive (137).

VI. TECHNIQUES FOR STUDYING *S*-GLUTATHIONYLATED PROTEINS

Several different methodologies have been applied to detect *S*-glutathionylated proteins. In most cases, their quantification is performed by measuring the amount of GSH released after reduction of the disulfide bond (Fig. 13). Sample proteins are first separated by acidification/centrifugation or by gel filtration to remove soluble GSH, and the disulfide bonds within *S*-glutathionylated proteins are then cleaved by reducing agents. Once released, GSH can be measured using colorimetric or fluorescent reagents, sometimes after chromatographic (HPLC) separation.

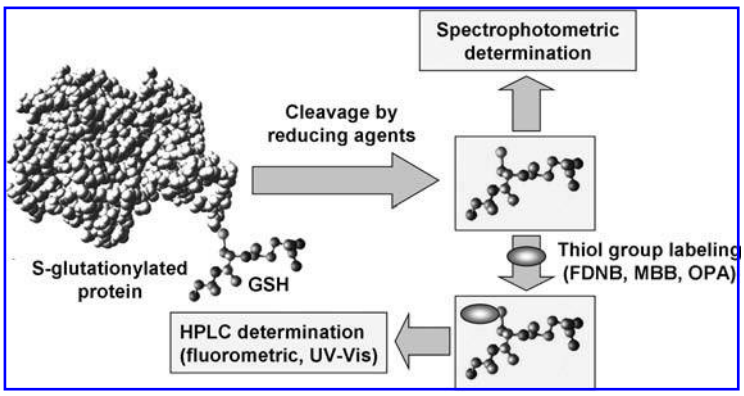

FIG. 13. Diagram of the main techniques that can be applied to detect *S*-glutathionylated proteins. In most cases, quantification of *S*-glutathionylated proteins is performed by measuring the amount of GSH released after reduction of the disulfide bond. See text for further details.

A. Pre-analytical concerns

The choice of the most reliable reagent to cleave disulfide bond in *S*-glutathionylated proteins in order to release GSH is a critical step and many different molecules have been used for this purpose. Several reducing agents containing thiol groups, such as dithiothreitol, mercaptoethanol, and dithioerythritol, can be employed to release GSH from the *S*-glutathionylated protein. These molecules are quite efficient in reducing the disulfide bond, but can also react with molecules used for successive GSH titration. This problem can be overcome by using an excess of the titrating molecule and HPLC separation. Alternatively, other reductants, such as sodium borohydride or trialkylphosphines, have been used to cleave the disulfide bond. However, these reductants present some drawbacks that limit their application. The reaction of sodium borohydride is frequently accomplished by sample heating with formation of gas foam during the process (74). Additionally, since the use of this reagent can also interfere with GSH revealing agents, its extraction from the sample is frequently needed (175). Trialkylphosphines under use for *S*-glutathionylated protein determination comprise different molecules such as tri-*n*-buthylphosphine and tris-(2-carboxyethyl-phosphine). It was observed that the reducing properties of such molecules are influenced by temperature and concentration of the reducing agent, tris-(2-carboxyethyl-phosphine) giving more reproducible and robust results (146). Some years ago, we developed a methodology utilizing the protein sulphydryl group itself as an endogenous reductant to cleave the disulfide bond in alternative to the use of the above mentioned molecules (213). According to this procedure, acidified protein pellets are adjusted to slight alkaline pH and kept under rotary shaking. Acidification itself, leading to protein denaturation, exposes protein thiols normally buried within the native protein conformation. At slight alkaline pH, *S*-glutathionylated proteins undergo thiol disulfide exchange reactions releasing GSH that can be measured spectrophotometrically.

The use of strong reductants to reduce the disulfide bond can have some common drawbacks. The quantification of *S*-glutathionylated proteins in different mammalian tissues performed in the early eighties indicated their physiological levels as ~20% of total GSH pool (2, 135, 175). In those studies, it was assumed that the acid-soluble thiols released from cellular proteins upon treatment with strong reductants (such as sodium borohydride) and then precipitated with strong acids were mainly GSH. Differently, it was demonstrated that GSH accounts for only a very small fraction of such "thiols" whereas the most part was constituted by protein fragments cleaved by borohydride (20). Thus, the high concentration of *S*-glutathionylated proteins measured in different mammalian tissues was due to experimental artifacts (278).

Another critical step for the analysis of *S*-glutathionylated proteins in the presence of hemoproteins is represented by sample acidification, which is required in most procedures to separate proteins and remove the pre-existing GSH. We have demonstrated that, during sample acidification in the presence of hemoglobin, thiol groups can be rapidly oxidized, because denaturation of hemoglobin leads to a burst of ROS from the oxygenated heme moiety, thus artifactually increasing the amount of *S*-glutathionylated proteins (95, 214, 217). To avoid this problem, sulfhydryl groups must be masked, for example, by reaction with *N*-ethyl maleimide (NEM), before sample acidification. Since this step has been neglected in some of the applied procedures for *S*-glutathionylated protein detection, this could be another reason why erroneously high physiological levels are measured when acidifying samples that contain a high amount of oxygenated hemoglobin (or other hemoproteins). On the other hand, in the absence of hemoproteins, oxidation of protein thiols is inhibited by low pH. For example, we found 2–3 μM *S*-glutathionylated hemoglobin in human blood from healthy people (96, 214), whereas others reported largely higher values, such as 50–200 μM (170, 183). Furthermore, a common precaution to prevent artefactual formation of *S*-glutathionylated proteins when handling samples rich in protein thiols and GSH (*e.g.*, cells and tissues) should be that of immediately blocking all free thiols, both protein and low-molecular-mass ones, through their alkylation or arylation. It is evident that leaving these free thiols as such during sample handling makes the artifactual formation of *S*-glutathionylated proteins a drawback of some of the applied procedures.

B. Spectrophotometric assays

The analysis of *S*-glutathionylated proteins by spectrophotometry generally consists in the quantification of GSH after reduction of the protein mixed disulfide. In this case, molecules that contain SH groups cannot be used to reduce the *S*-glutathionylated protein, but methods utilizing borohydride or endogenous protein thiols as reductans have been applied (20, 213). Colorimetric reagents such as 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and 1-chloro-2,4-dinitrobenzene (CDNB) are routinely used for GSH detection by spectrophotometer. DTNB reacts not specifically with all thiols, whereas CDNB needs catalysis of GSTs and is specific for GSH, thus it is to be preferred. These procedures have been applied to detect *S*-glutathionylated proteins both in blood and other tissues after treatments with oxidants (20, 213). An alternative method to measure GSH released by *S*-glutathionylated proteins is the GSH recycling assay (144, 183). This procedure is specific for GSH detection and has also a low detection limit with respect to the other spectrophotometric procedures. Anyway, by all these methodologies basal levels of *S*-glutathionylated protein have been found to occur in the range 100–200 μM but, since NEM (or any other thiol blocking agent) was not used to prevent thiol oxidation, most of this amount is likely due to procedural artefacts.

C. HPLC assays

Most of the procedures applied for the detection of *S*-glutathionylated proteins rely on high performance liquid chromatography (HPLC) separation to increase the sensitivity of the assay and to decrease the detection limit. The chromatographic run is generally coupled to spectrophotometric or fluorometric detection of GSH, which has been tagged with revealing molecules. HPLC-based methods have the advantage that GSH can be measured also in the presence of reducing agents that bear SH groups, in that chromatographic separation ensures the necessary selectivity. GSH released from proteins [by treatment with reducing agents, such as dithiothreitol (DTT)] has been re-

ported to be measured by several methods: HPLC with UV-Vis detection of the GSH-2,4-dinitrobenzene conjugate has been applied for detection of GSH deriving from *S*-glutathionylated proteins in different tissues, showing a good reproducibility and being only limited by the sensitivity (175). Different fluorescence-based methods for detection of GSH released from *S*-glutathionylated proteins have also been developed. These analytical procedures have the advantage of great sensitivity and low detection limit. *o*-Phthalaldheyde (OPA) and monobromobimane (mBrB) are the fluorescent reagents more often used for such GSH determinations. OPA becomes fluorescent after its reaction with a primary amine in the presence of a thiol group, cyanide, or sulfite. It has been used to quantify *S*-glutathionylated proteins in blood, tissues, and plasma after GSH cleavage with DTT (195). Anyway, some doubts have been posed about the occurrence of interfering molecules for GSH determination in some tissues by using this regent (78); therefore, some precautions should be taken into consideration to perform this analysis (232). mBrB is routinely used in our laboratory for the quantification of *S*-glutathionylated proteins in different tissues after GSH cleavage with DTT. By this procedure, we are able to measure *S*-glutathionylated protein levels as low as 1 μM both in plasma and red blood cells (RBCs). The method is very rapid, reproducible, and, if necessary, allows us to discriminate among different thiols physiologically bound to proteins (96, 97). Alternatively, dansyl chloride can be used as a fluorescent label to quantitate *S*-glutathionylated proteins (129). In this procedure, the S–S bond is reduced by adding DTT, proteins are removed by sample acidification, the free thiol group of the released GSH is alkylated by iodoacetic acid, and, finally, the amino group of GSH is dansylated with dansyl chloride for fluorimetric detection. This methodology was successfully applied to measure *S*-glutathionylated (and, more generally, *S*-thiolated) proteins in human plasma.

An alternative HPLC procedure has been proposed, in which the sample is directly measured for its *S*-glutathionylated protein content without GSH cleavage. The sample is analyzed by a cation-exchange HPLC with UV detection, thus avoiding any step for its processing (196). This method has been applied to measure levels of *S*-glutathionylated hemoglobin in hemolysates. The analytical procedure is very fast, easy to perform, and reproducible, but it has been applied only to RBCs, where a single protein (hemoglobin) represents about 95% of the whole protein content, and its possible application for more complex protein samples appears difficult. Moreover, the authors report a value for *S*-glutathionylated hemoglobin in human blood that is largely higher than that we measured in RBCs (96, 214).

D. Liquid chromatography–mass spectrometry

The occurrence of *S*-glutathionylated proteins can be revealed by conventional mass spectrometry (MS) procedures; specifically, the mass difference due to GSH binding can be ascertained by direct electrospray ionization (ESI) measurements in intact proteins. Some methods have been proposed to measure *S*-glutathionylated proteins by HPLC–ESI–MS, thus coupling the high selectivity and sensitivity of chromatographic separation to the MS detector. In particular, a method has been proposed for determination of *S*-glutathionylated hemoglobin in human erythrocytes, which requires only the direct injection of the hemolysate into the instrument (187). *S*-Glutathionylated hemoglobin is separated by a reverse phase column and quantified by ESI–MS analysis. This methodology has been applied only to detect *S*-glutathionylated hemoglobin and its utility for quantification of *S*-glutathionylated proteins in other biological samples is questionable. Additionally, the measured concentration of *S*-glutathionylated hemoglobin in human blood is largely higher in comparison with other reports (96, 102, 214), thus indicating some drawbacks of this procedure.

In alternative to HPLC–ESI–MS analyses, an innovative technique based on linear mode matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been developed for the quantitative determination of *S*-glutathionylated hemoglobin in blood samples (15). Also in this case the hemolysed sample is directly analyzed by the mass spectrometer without any pretreatment. The method may be promising but, due to its recent publication, no data are available about its application, at least searching on PubMed. Notwithstanding, it is conceivable that, also the use of this procedure will be limited to blood samples.

As a general consideration, we can infer that MS techniques are employed for the measurement of *S*-glutathionylated proteins by considering the native molecular weight of the specific protein and its increase due to the GSH moiety. A clear advantage of MS techniques is that they allow the identification of the specific cysteinyl residue to which GSH is bound in the *S*-glutathionylated protein.

E. Detection of S-glutathionylation at single protein level

The above described techniques allow the quantification of the total content of *S*-glutathionylated proteins in the sample. However, the assessment of *S*-glutathionylation at single protein level has greatly attracted the attention of researchers in the field. The detection of the total amount of *S*-glutathionylated proteins in cells or tissues may be an index, even sensitive, of oxidative stress (214), but the identification of the proteins undergoing *S*-glutathionylation under normal/physiological conditions as well as after mild to severe oxidative stress is a key step in order to evaluate whether and/or to what extent this kind of reversible post-translational modification is an important regulator of cellular functions. For years, the lack of suitable analytical approaches has hampered the identification of *S*-glutathionylated proteins. However, more recently, both the burst of improvements in proteomic techniques and the commercial availability of a monoclonal antibody for glutathione–protein complexes (see below) have led to rapid progresses in the field.

The first analytical approach to identify *S*-glutathionylated proteins at single protein level was developed >20 years ago. It was essentially based on the intracellular radioactive ^{35}S-labeling of the GSH pool (106). This is achieved by incubation of cells with ^{35}S-cysteine, which is utilized to synthesize GSH. Cysteine is used instead of GSH because cells are not permeable to the tripeptide. Proteins are then separated by electrophoresis, in the absence of reducing agents, and *S*-glutathionylated proteins revealed by autoradiography and sequence analysis. Recently, the method has also been coupled

to two-dimensional (2D) electrophoresis and MALDI-TOF analysis of the excised spots and applied to identify *S*-glutathionylated proteins in oxidatively stressed human T lymphocytes (80), human hepatoma cells, and primary rat hepatocytes (81). This method is based on the incorporation of radioactive glutathione ([^{35}S]GSH) into proteins, as originally described by Grimm *et al*. (106). Cells are preincubated with cycloheximide to block protein synthesis, incubated with L-[^{35}S]-cysteine to radiolabel their GSH pool, and exposed to oxidative stress in comparison with control culture conditions. Proteins are separated by 2D gel electrophoresis under nonreducing conditions, and *S*-glutathionylated proteins are located by autoradiography and identified by mass spectrometry after tryptic digestion. Thus, this technique allows the identification of proteins undergoing *S*-glutathionylation in various cell types. An advantage of this method is that it permits testing on living cells, in contrast to studies where recombinant proteins are used. Identification of the proteins undergoing *S*-glutathionylation in living cells is an important step in defining the molecular targets involved in the regulation of cell function by the redox state. However, this method has several drawbacks. First, the method is applicable only to identification of redox modification of proteins during oxidative stress (154), in that the specific activity of the ^{35}S-labeled cellular GSH pool is generally too low to reveal constitutively modified proteins in gels. Therefore, the basal state of *S*-glutathionylation in tissues or cells cannot be assessed. Second, cells should be simultaneously treated with radiolabeled cysteine and cycloeximide to inhibit protein synthesis and consequent incorporation of ^{35}S-cysteine; this can interfere with normal cell functions. Third, ^{35}S-Cys can form *S*-cysteinylated proteins that are evidenced by autoradiography, thus the method is not able to discern between *S*-glutathionylated and *S*-cysteinylated proteins. Nevertheless, this technique primarily detects *S*-glutathionylation, and not *S*-cysteinylation, since labeling was decreased by 80% by the addition of the GSH synthesis inhibitor buthionine sulfoximine (80). Fourth, it has limited sensitivity in the detection of low abundance proteins.

Several alternative analytical approaches to discriminate *S*-glutathionylated proteins at single protein level have been developed. A rapid thin-gel isoelectric focusing (IEF) method (252) was developed for the analysis of *S*-thiolated proteins (*i.e*., mixed disulfides with low molecular weight thiols) both in isolated proteins and in tissue homogenates. This method seems able to provide a quantitative analysis of each different *S*-thiolated form of a protein and also to give some information on the molecular nature of the thiol modification involved. This goal is achieved by treating each protein sample with both iodoacetamide and iodoacetic acid, making possible to determine if the protein contains a free thiol or disulfides with either charged or uncharged thiols on the basis of the net charge introduced and the protein mobility in IEF gels. The method looks to work very well with purified proteins, however its application in more complex mixtures is quite limited since protein bands can easily overlap.

Sullivan and colleagues (250) utilized the membrane-permeable biotinylated glutathione ethyl ester. During oxidative stress, proteins undergo protein biotin–glutathionylation and the tagged ones can be purified by affinity chromatography on streptavidine–agarose beads and separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE). Bands are then excised, proteins eluted and charged onto HPLC–MS for peptide mapping and protein identification. The main drawback of this method is the fact that cellular proteins are not glutathionylated by the cellular GSH itself but by an "exogenous" molecule (biotin–GSH) that is chemically different and is also characterized by a greater steric hindrance. A similar approach was described by Eaton and collegues (66), who used biotinylated GSH (biotin-GSH) to probe for protein *S*-glutathionylation during the oxidative stress of ischemia and reperfusion in isolated perfused rat hearts, using nonreducing Western blots and streptavidin–HRP. Streptavidin–agarose was then used to isolate *S*-glutathionylated proteins, thus demonstrating that many proteins, including GAPDH, are targets for *S*-glutathionylation during cardiac oxidative stress. The same authors also synthesized *N*,*N*-biotinyl GSSG (biotin–GSSG–biotin), a compound that carries the biotin tag attached to the amine group of L-glutamates on the GSSG molecule. This molecule undergoes thiol–disulfide exchange reactions with proteins and was reported to be a useful tool to evidence proteins prone to be *S*-glutathionylated by this mechanism in tissue homogenates from different rat organs (17).

An interesting approach (Fig. 14) is represented by the procedure described by Lind and colleagues (154). Cellular proteins are first alkylated with NEM to block all free thiols, NEM excess is then removed, and *S*-glutathionylated proteins are specifically reduced by action of a mutant of glutaredoxin-3 from *Escherichia coli*. This mutant enzyme exhibits preference for the reduction of *S*-glutathionylated proteins. Stripped proteins are then alkylated with NEM–biotin and subjected to affinity purification on avidin agarose. Purified proteins are finally analyzed by proteomic analysis using 2D gel electrophoresis and MALDI-TOF-based identification of individual protein spots. One limitation of this method is the possibility that the *E. coli* enzyme may be specific for some proteins while does not reduce others or may reduce protein disulfides too (82).

In recent years, the analysis of *S*-glutathionylated proteins has been improved by commercial availability of monoclonal anti-GSH antibodies (Virogen, Watertown, MA). This allows researchers to detect *S*-glutathionylation at protein level in cells or tissues without pretreatments with radiolabeled GSH precursors or tagged GSH analogues, thus making experimental conditions more close to physiological ones. *S*-Glutathionylated proteins can be revealed by Western blot after 1D or 2D gel electrophoresis separation, in the absence of reducing agents. In addition, anti-GSH antibodies can be used to reveal *S*-glutathionylated proteins in tissue sections through immunocytochemistry. Several *S*-glutathionylated proteins have been revealed by this procedure, such as actin, hemoglobin, heat shock cognate 70 kDa protein, and α-enolase (50, 125, 182, 186, 198). The use of anti-GSH antibodies looks promising, however, being essentially based on the specific recognition of the GSH moiety of *S*-glutathionylated proteins, the method may suffer from sensitivity.

An alternative to anti-GSH antibodies is represented by GST from *Schistosoma japonicum*, which was found to bind specifically to the glutathione moiety of *S*-glutathionylated proteins (41). In this method, GST is previously biotinylated, thus *S*-glutathionylated proteins may be detected after electrophoretic separation and Western blotting using biotinylated GST and

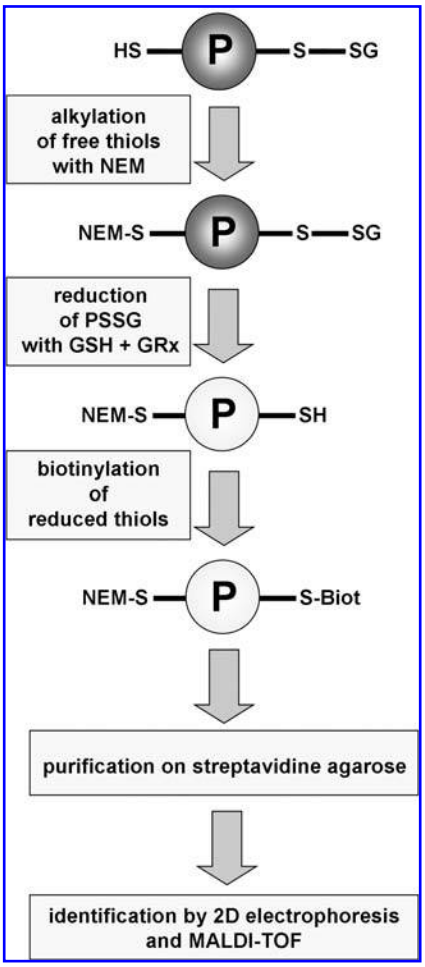

FIG. 14. Scheme of the procedure described by Lind and colleagues (154) to detect *S*-glutathionylated proteins. See text for further details.

anti-biotin antibodies. *S*-Glutathionylated proteins from HeLa cells under basal conditions or after induction of *S*-glutathionylation by diamide or GSNO were found to be specifically detected by this procedure. The method was also applicable for the histochemical detection of *S*-glutathionylated proteins *in situ* (41).

VII. GLUTATHIONE AND *S*-GLUTATHIONYLATED PROTEINS IN HUMAN BLOOD

Albumin reaches plasma concentrations of 0.5–0.6 m*M* and, thus, is an important regulator of the oncoosmotic pressure of the blood plasma. Albumin contains a free thiol group at Cys34, which can be oxidized to a mixed disulfide. Therefore, albumin is also a quantitatively important redox buffer of the human blood (108, 110). However, low-molecular-mass thiols are critical blood components that play important roles in metabolism and homeostasis and their concentrations in blood could possibly serve as important redox buffers. Reduced/thiol, disulfide and protein-bound forms of cysteine, cysteinylglycine, homocysteine, and glutathione comprise the plasma thiol redox status (97, 171). The thiol forms occur in the 0.1–20 μM range (171, 270). The cysteine/cystine redox couple quantitatively represents the largest pool of low-molecular-mass thiols and disulfides in human plasma. The predominant form of cysteine in the blood plasma is the disulfide one, cysteine being in the range 8–10 μM, while cystine is found at concentrations >40 μM (129). Cysteinylglycine, a product of the extracellular enzymatic degradation of GSH, is the second most abundant thiol in plasma after cysteine. Homocysteine is a sulfur-containing amino acid that is formed from methionine, an essential amino acid derived from dietary protein. More than 80% of homocysteine is found in plasma, mostly conjugated to proteins through disulfide bonding or as homocystine disulfide, as mixed disulfide Hcy–Cys, or as a free thiol (<2%) (277). Interestingly, elevated plasma homocysteine is a strong, independent risk factor for the development of AD (234) and elevated brain homocysteine has been reported in AD (71).

Although only a minor component compared to other thiols, glutathione is the most extensively studied extracellular thiol/disulfide component in the human plasma. During oxidative stress and detoxification reactions involving GSH, the concentration of GSH may decrease and that of GSSG may increase in the affected cells. This results in a decreased export of GSH and an increased export of GSSG to plasma, thereby altering the GSH redox state of the plasma pool. The altered plasma GSH and GSSG concentrations can, thus, reflect GSH/GSSG status and oxidative stress in other less accessible cells/tissues, both in healthy subjects and in patients suffering from different oxidative stress-related diseases (131, 133, 224). Therefore, measurement of both GSH and GSSG concentrations in plasma has been considered essential as an index of whole body glutathione and a useful indicator of disease risk in humans (133). Plasma GSH/GSSG redox is more reduced than all other measured thiol/disulfide couples in human plasma (131). In human blood, GSH is mainly located inside RBCs (about 3 m*M*) (Fig. 15), as white blood cells, which are loaded with 2–4 m*M* GSH,

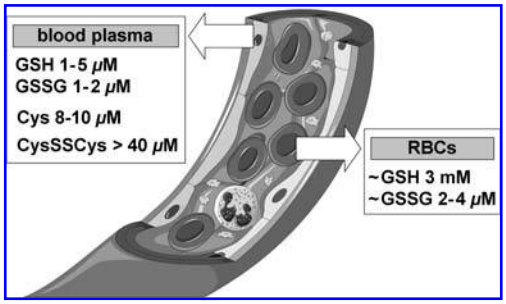

FIG. 15. Distribution of GSH and GSSG in red blood cells (RBCs) and plasma. In human blood, GSH is mainly located inside RBCs (about 3 m*M*), as white blood cells, which are loaded with 2–4 m*M* GSH, represent only a small volume as compared with RBCs, whereas plasma contains only ~1–5 μM GSH and even less GSSG and *S*-glutathionylated proteins. The predominant form of Cys in the blood plasma is the disulfide form, cystine (CysSSCys). Cys is in the range of 8–10 μM in the plasma, while CysSSCys is found at concentrations >40 μM.

represent only a small volume as compared with erythrocytes, whereas plasma contains only ~1–5 μM GSH and even less GSSG and *S*-glutathionylated proteins (97, 129, 131, 171, 223).

The mature erythrocyte is unique in many ways among cells that constitute the human body. One of the characteristics of the erythrocyte is the presence of large amounts of the oxygen-transport protein, hemoglobin. Moreover, human erythrocytes are the "cells" most exposed to oxidative damage by ROS/RNS. During their life, RBCs, whose principal role is to carry oxygen to tissues and organs, come in close contact with ROS/RNS from various sources: (i) the normal oxidation of hemoglobin, which ultimately produces the very reactive superoxide; (ii) oxidation-reduction of drugs or xenobiotics that are transported by blood; (iii) metabolism in various organs, especially the liver, which generates other ROS/RNS that can also be transported by blood (109).

Glutathione is the main intracellular thiol redox system in erythrocytes (63), where, with a high GSH concentration and lower level of GSSG, it is predominantly responsible for the protection of both hemoglobin and the RBC membrane against oxidative stress. Erythrocytic GSH has a rapid turnover rate (60–100% per day) and arises from *de novo* synthesis from its constituent amino acids glutamate, cysteine, and glycine (164, 165, 275).

The extent of protein *S*-glutathionylation in RBCs of healthy subjects is very low and is mainly due to glutathionyl-hemoglobin, formed by thiol–disulfide exchange between the Cys-β93 residue and glutathione (197). However, under particular conditions (*e.g.*, diabetes mellitus, Friedreich's ataxia, and hyperlipidemia) glutathionyl-hemoglobin concentration increases (96, 184, 185, 187, 200, 225).

VIII. SAMPLE COLLECTION, PREPARATION, STORAGE, AND ARTIFACTUAL FORMATION OF GSSG THAT INVALIDATE MEASUREMENTS OF GSH, GSSG, AND *S*-GLUTATHIONYLATED PROTEINS

The blood glutathione redox status (*i.e.*, [GSH], [GSSG], and/or GSH/GSSG ratio in plasma and/or RBCs) has been measured in hundreds of studies because they are considered important for assessing the possible role of oxidative stress in disease onset and/or progression. However, as we discuss successively in this section, many of those measurements were not appropriately done. As for any laboratory-based assay that is used in human research, methodological issues such as reproducibility, inter- versus intra-person variability, limits of detection, and specificity, need to be considered. There are certain methodological issues, however, that are unique to research studies using biomarkers of oxidative stress status. The first of such issues concerns the potential for artifacts in estimates of baseline levels of oxidation markers. That is, oxidation of samples can occur during normal sample handling, processing, and analysis, such that measured levels are not at all reflective of the levels encountered *in vivo*. This issue has plagued investigations of many different biomarkers of oxidation. For example, the extremely great variability in the levels of circulating *S*-nitrosothiols and 3-nitrotyrosine in plasma of healthy humans at the basal state is an impressive indicator of existing methodological problems in this field of research (99, 100, 216, 257–262).

Large differences in glutathione redox status have been found also for control values in healthy people (32, 37, 196, 217, 223, 279). For example, the levels of GSH and, even more, of GSSG and *S*-glutathionylated proteins measured by different laboratories span over >100-fold in healthy humans (217), thus suggesting that biomarkers of oxidative stress are often unreliable indicators of disease if not correctly measured (214).

A major concern in evaluation of blood glutathione redox status is sample collection, preparation, and storage, as well as artifactual formation of GSSG that invalidate GSH, GSSG, and *S*-glutathionylated protein measurements (32, 37, 196, 214, 217, 222, 223, 247, 280). In particular, sample manipulation for GSSG determination is easily susceptible to methodological mistakes, depending on: (a) oxidation of thiols in acidified samples; (b) oxidation of thiols after restoring neutral-alkaline pH; (c) oxidation of thiols during acid deproteinization; (d) shift in the GSH/GSSG equilibrium because of irreversible blocking of free thiols; (e) reaction of electrophiles with amino groups (217). In our experience, very little GSSG and *S*-glutathionylated proteins have been found (217) in whole blood, with concentrations in healthy humans that span in the 1–5 μM range (94, 95).

For blood samples, deproteinization, acidification, addition of organic solvent (acetonitrile, acetone, methanol), or filtration can be used. Acids used for deproteinization are trichloroacetic, trifluoroacetic, 5-sulfosalicylic, perchloric, or metaphosphoric acid (32, and citations therein). The best results were obtained with acetonitrile or 5-sulfosalicylic acid precipitation in GSH identification upon derivatization with a fluorescent tag. Even if protein precipitation by acidification is the most effective, it has the disadvantage that a neutral to slightly alkaline pH must be restored prior to derivatization of thiols with a chromophore or fluorophore. Organic solvents are preferable over acids when a MS is used as a detector (33). The separation between most proteins and low-molecular-mass compounds like GSH and GSSG through filters with low molecular cut-off is a valid (though time consuming and expensive) method to remove proteins because it does not require the addition of acids or organic solvents that can affect separation, derivatization, and detection (32).

The measurement of plasma GSH requires caution in order to avoid assay artifacts or data misinterpretation, the main sources of errors being storage at room temperature and hemolysis of erythrocytes; in addition, γ-glutamyl transpeptidase can degrade GSH if the enzyme is not inhibited during sample preparation. Storage at room temperature leads to underestimation of GSH since autoxidation and proteolysis are not repressed and GSH is consumed (143). Despite using efforts to minimize hemolysis, lysed RBCs cause overestimation of GSH in plasma (94), since GSH is mainly located inside RBCs (~3 mM), whereas it is present in low micromolar range in plasma (94, 129, 131, 171, 217, 222, 223). In addition, some pathological conditions exist, besides anemias, in which RBC hemolysis can occur (reviewed in Ref. 99). RBC hemolysis is increased concomitantly to hemodialysis, in diabetes, epilepsy,

hypercholesterolemia, low platelet count (HELLP) syndrome, sickle cell disease, in subjects suffering from acute post-streptococcal glomerulonephritis, following cardiopulmonary bypass procedures, and treatment with some nonsteroidal anti-inflammatory drugs (*e.g.*, Na-salicylate, naproxen, and ketorolac) at therapeutic concentrations, resulting in an increase in plasma GSH pool.

The quantification of GSSG in human plasma is difficult because its concentration is very low (low μM range: ~0.9-2 μM; 97) and GSSG does not possess specific detection properties, so that sensitive methods are required. Usually, GSSG is measured in plasma as GSH after reduction of disulfide and thiol labeling; however, since GSH circulates mainly as disulfide with cysteine, direct measurement of GSSG yields even lower values (129, 131). GSSG exists in both RBCs and plasma at about the same concentration (2 and 1 μM, respectively) (171, 217). Since the human hematocrit value is near 50%, the blood GSSG concentration is also ~2 μM. In contrast to erythrocytic GSH, plasma GSH is rapidly oxidized to the disulfide form in a few seconds after blood withdrawal (173). A comprehensive work elucidated this problem with time course data of plasma GSH measured within a few seconds after drawing the blood sample (7).

To avoid blood GSSG overestimation due to plasma GSH oxidation, some precautions should be taken. We suggest that a rapid sample derivatization is necessary to reduce GSH oxidation. According to previous data (7), our experiments show that plasma GSH is mainly oxidized to GSSG (94). Therefore, if blood samples are not rapidly derivatized, this phenomenon can result in GSSG overestimation.

The quantification of GSH, GSSG, and *S*-glutathionylated proteins in human RBCs is difficult because of the very low concentration of GSSG and *S*-glutathionylated proteins under physiological conditions. Furthermore, the case of RBCs is even more complicated by the presence of hemoglobin, which undergoes autoxidation to produce superoxide (272). Since the RBC concentration of oxygenated hemoglobin is 5 mM, even a small rate of autoxidation can produce substantial levels of ROS/RNS and, because RBCs make up 40% of the blood volume, ROS/RNS escaping from RBCs have the potential to damage other components of the circulation. Sample acidification, in the presence of oxygenated hemoglobin, leads to a large production of ROS, which in turn increase of more than one order of magnitude the levels of GSSG and *S*-glutathionylated proteins, lowering GSH by 20–30% (217). Oxidation of blood GSH to GSSG may be observed after treatment of sample with acids (*e.g.*, trichloroacetic or perchloric acid), which is one of the most widely used procedures for deproteinization (32, 33, 36, 217). Therefore, the physiological amount normally present in blood and that deriving from artefactual GSH oxidation may be responsible for blood GSSG overestimation (214). The stability of GSH and its possible oxidation to GSSG during the period between collection and analytical determination have been questioned and analyzed in detail (214, 217, 280). We previously observed that these pitfalls can be overcome by sample pretreatment with the thiol blocking agent NEM a few seconds after blood collection and its successive extraction after sample acidification (217). Our data suggest that such a rapid thiol blocking prevents blood GSH oxidation and, consequently, the related blood GSSG overestimation (94). Consistently, we have developed methods for the quantitative determination of GSH, GSSG and *S*-glutathionylated proteins in order to avoid these pitfalls (95, 96, 214, 217).

IX. GLUTATHIONE AND *S*-GLUTATHIONYLATED PROTEINS IN HUMAN DISEASES

Several human disease processes have been associated with decreased blood GSH. Depletion of blood GSH has been documented in severe burn injury (275), human immunodeficiency virus (HIV) infection (24, 228) due to a systemic decrease in synthesis, Gaucher disease (219), cirrhosis (158), type 1 diabetes mellitus (59, 68), as well as in septic, critically ill pediatric patients (164). Decreased plasma GSH has also been found in patients with cystic fibrosis (218), type 2 diabetes mellitus (224), age-related macular degeneration (178, 224), and early atherosclerosis in healthy adults (8). In the latter condition, plasma glutathione redox state has been proposed as an independent predictor for identifying healthy (asymptomatic) individuals at risk for early atherosclerosis, independent of traditional risk factor assessment and presence of inflammation (8).

In whole blood measurements, where erythrocytes account for almost all of the whole blood GSH, the concentrations of GSH and total thiols have been found to be lower in patients with rheumatoid arthritis (128) and type 2 diabetes (224). Patients with untreated, uncomplicated essential hypertension were found to have decreased levels of erythrocytic GSH and increased levels of GSSG, which resulted in decreased ratio of GSH/GSSG as compared to controls (179). Furthermore, erythrocytic GSH correlated inversely with systolic blood pressure in untreated hypertensive patients (179). The blood GSH/GSSG ratio was found to be significantly decreased also in patients with colon and breast cancer compared to healthy subjects (196).

GSH content was found to be significantly decreased in the RBCs from male patients afflicted with Alzheimer's disease, which was associated with decreases in GCL and glutathione synthase activities (157). Altered thiol status was demonstrated also in peripheral lymphocytes from AD patients, which showed significantly decreased GSH levels and corresponding significantly increased GSSG levels (31). These changes significantly decreased the GSH/GSSG ratio in AD lymphocytes compared with controls.

Studies on patients afflicted with multiple sclerosis (MS) evidenced a link between nitrosative stress and thiol concentration and homeostasis (29, 30). Increased levels of RNS are present in the cerebrospinal fluid (CSF) of MS patients and this is associated with increased *S*-nitrosation of sulfhydryl moieties (*i.e.*, RSNO formation). Western blot analysis showed in MS patients increased iNOS expression, responsible for increased NOS activity, and increased protein-bound nitrotyrosine, associated with a significant decrease in both protein thiol groups and GSH, and with increased levels of GSSG and *S*-nitrosothiols (29). In particular, the content of total GSH (*i.e.*, GSH + GSSG) and the GSH/GSSG ratio in the CSF and in plasma of MS patients were determined as a measure of the antioxidant

status and compared with the levels of control group. MS-affected patients exhibited a significant decrease (~38% of control values) in the CSF levels of total GSH (*i.e.*, GSH + GSSG) and GSH, which was associated with a significant reduction in the GSH/GSSG ratio (to 45% of control GSH/GSSG ratio) and with a significant increase in GSSG. Conversely, plasma levels of GSH or the ratio GSH/GSSG were higher in MS patients than in controls, whereas no significant changes were observed in the plasma level of GSSG (29, 30). A decrease in sulfhydryl groups was previously measured in the CSF and plasma of MS patients (28). Interestingly, treatment of MS patients for 6 months with acetylcarnitine led to decreased CSF levels of NO reactive metabolites and protein nitration and significantly higher CSF levels of both GSH and the GSH/GSSG ratio and to a lower GSSG content than in MS alone, whereas the plasma GSH/GSSG ratio was similar to control values (29).

In diabetes, increased oxidative stress is known to play a decisive role in the pathogenesis of vascular complications (23). In addition, GSH depletion may adversely affect insulin sensitivity, since oxidative stress impairs insulin-sensitive glucose transport in adipocytes (220), potentially through activation of NF-κB (192). Subjects with impaired glucose tolerance, including early hyperglycemics, had reduced blood GSH (266). In type 2 diabetes, whole blood, plasma, and erythrocytic GSH is decreased (224, 226, 274), plasma total GSH pool (*i.e.*, GSH, GSSG, and other disulfide forms obtained following treatment with DTT) is decreased (224), whereas whole blood GSSG is increased (224).

Immune cell functionality and proliferation rely on adequate intracellular GSH. Glutathione is essential for the activation of T-lymphocytes and polymorphonuclear leukocytes as well as for cytokine production, and therefore for mounting successful immune responses when the host is immunologically challenged (255).

The immunologic hallmark of HIV infection is a numerical and functional decline in $CD4^+$ T cells, which over time leads to the development of acquired immunodeficiency syndrome (AIDS). Several factors seem to be involved in the pathogenesis of HIV infection, and some data suggested that oxidative stress may play an important role in this process (193). Several reports have suggested that decreased antioxidant defense due to disturbed glutathione homeostasis and enhanced spontaneous generation of ROS play a role in the immunopathogenesis of HIV infection (9, 11, 24, 180, 245). In particular, ROS act as intracellular second messengers for the activation of NF-κB, which augments the replication of HIV (231).

Intracellular levels of GSH are lowered in advanced HIV disease (210), suggesting a role for oxidative stress in the pathogenesis of HIV disease. The importance of this role is underscored by evidence demonstrating that T cell function is impaired in HIV disease when intracellular GSH levels are low and is restored by treatments that replenish GSH (210). In detail, by fluorescence-activated cell sorter (FACS) analysis using monochlorobimane, authors revealed that T cells are subdivided into high-GSH cells and low-GSH cells in healthy individuals and found that high-GSH T cells are selectively lost early during the HIV infection, and are effectively missing in AIDS patients (210). Therefore, the intracellular GSH levels determined by FACS-measured GSH-*S*-bimane fluorescence in T cells decrease during the disease progression of AIDS (245).

In addition, HIV infection lowers GSH in plasma (91, 193), also in children with HIV (107, 159), erythrocytes, and monocytes (193). Clinical reports have shown that HIV-infected individuals with lower intracellular GSH levels have a poor survival rate. In fact, the probability that HIV-infected subjects will die within 2–3 years is dramatically higher when $CD4^+$ T cells have low GSH levels (121). However, a study in which blood samples collected from HIV patients and healthy subjects were immediately stabilized and quantified using HPLC with dual electrochemical detection showed that the erythrocytic GSH levels were the same in healthy subjects and in HIV patients regardless of their $CD4^+$ lymphocyte level. Only those with the lowest $CD4^+$ level plus opportunistic infections had supranormal GSH concentrations; furthermore, GSH plus GSSG levels also were normal in patients (150).

Highly active antiretroviral therapy was accompanied by both an improvement of glutathione redox status and an increase in levels of antioxidant vitamins in patients with HIV infection (10). Moreover, it was shown that supplementation with GSH or antioxidants may improve immunologic and virologic indexes in HIV-infected persons (181). For instance, oral *N*-acetylcysteine (NAC) administration for 8 weeks safely replenishes whole blood GSH and T cell GSH in HIV-infected individuals (58).

Since the blood concentration of *S*-glutathionylated proteins may reflect alterations in redox signaling and oxidative stress status even in hardly accessible tissues and compartments of the body (95), *S*-glutathionylated hemoglobin has been analyzed in some human diseases as a marker of whole-body oxidative stress. GSH can form complexes specifically with Cys-93 of the β chain of hemoglobin *in vitro*, and this moiety is a likely site of *S*-glutathionylation *in vivo*. While the physiological role of hemoglobin *S*-glutathionylation has not yet been identified, binding of GSH to hemoglobin *in vitro* increases its oxygen affinity and decreases the Hill coefficient (187) and *S*-glutathionylation may play an important role in maintaining hemoglobin structure and function (144). Furthermore, since *S*-glutathionylated proteins appear to be fairly stable, unlike GSSG, the blood concentration of *S*-glutathionylated hemoglobin has been proposed as a useful biomarker of blood oxidative stress in humans (25, 144).

Friedreich's ataxia is an autosomal recessive neurodegenerative disease due to a GAA expansion in the gene coding for the mitochondrial protein frataxin, implicated in the regulation of iron metabolism, resulting in the deficiency of frataxin. Oxidative stress and mitochondrial dysfunction have long been considered to play a role in Friedreich's ataxia. A significant increase in *S*-glutathionylated hemoglobin, accompanied by a significant decrease in free GSH, and *S*-glutathionylated actin, accompanied by significant decrease in the GSH/GSSG ratio, has been found in the blood and fibroblasts, respectively, of patients with Friedreich's ataxia (198, 200). But, also in this case, the pathophysiological role of protein *S*-glutathionylation is at present unknown, though *S*-glutathionylated hemoglobin was suggested as a useful clinical marker for oxidative stress in Friedreich's ataxia (200).

S-Glutathionylated hemoglobin is also increased in patients suffering from type I (4, 96) and type II diabetes mellitus (187),

hyperlipidemia (187), and uremia associated with hemodialysis or peritoneal dialysis (251). *S*-Glutathionylated hemoglobin in RBC lysates from diabetic subjects with and without microangiopathy was analyzed by using LC/ESI-MS (225). The positivity for glutathionyl-hemoglobin in diabetic patients with microangiopathy was significantly higher compared to diabetics without microangiopathy and control subjects. *S*-Glutathionylated hemoglobin levels were significantly associated with the duration of diabetes, glycosylated hemoglobin and thiobarbituric acid substances (TBARS) levels, whereas GSH levels were negatively correlated with *S*-glutathionylated hemoglobin in diabetic subjects. Thus, since diabetic subjects also exhibited increased lipid peroxidation and decreased GSH levels in erythrocytes, it appears that enhanced oxidative stress may account for the increased glutathionyl-hemoglobin concentrations (225).

Differently, a decrease in the normal (basal) concentration of *S*-glutathionylated hemoglobin has been measured in children with Down's syndrome (199).

The levels of *S*-glutathionylated serum proteins, estimated using biotinylated GST, are increased in patients suffering from arteriosclerosis obliterans, a chronic peripheral arterial disease in which risk factors, such as smoking, obesity, hypertension, diabetes, and hypercholesterolemia, create oxidants that damage endothelial cells (189). Unfortunately, there are currently no specific and sensitive markers for arteriosclerosis obliterans. Serum levels of *S*-glutathionylated proteins, including apolipoprotein B100, were elevated even in the earlier stages of the disease. *In vitro*, levels of *S*-glutathionylated proteins were decreased strongly in the presence of the GSH/glutaredoxin system, suggesting that the increase in the serum levels of *S*-glutathionylated proteins reflects a decreased redox regulation in patients with arteriosclerosis obliterans. As a whole, the data suggest that serum levels of *S*-glutathionylated proteins are a risk marker for the diagnosis of arteriosclerosis obliterans at an early stage (189).

Increased levels of *S*-glutathionylation were also found in proteins from plasma and whole blood in smokers, for both men and women (183).

Here, it is especially relevant to discuss the oxidative modification of human eye lens proteins since these long-lived proteins have a large number of sulfhydryl groups. The lens is an avascular cellular tissue in the middle of the eye. Its function is to focus light on to the retina and, hence, it should have a high refractive index and a high degree of transparency. The unique feature of the lens, besides the high content of GSH, is the unusually high protein concentration. A large percentage of these proteins are structural proteins called crystallins, which make up 90% of the total protein and contain a high level of thiol groups that are necessary to be in the reduced state to maintain clarity of the lens. α-Crystallin is a heteromultimer composed of two different subunits, αA and αB, each having a molecular mass of ~20 kDa. β-Crystallins form a multi-gene family of basic and acidic polypeptides of 23–35 kDa and combine to form 40–200 kDa dimers, trimers, tetramers, and higher oligomers with high polydispersity. γ-Crystallins have a mass of 20 kDa and do not usually form oligomers. The lower turnover of these proteins makes it likely that irreversible oxidative damage may accumulate in the lens.

In aging human lenses, there are well-documented oxidation of protein methionine residues (230) and increases in the *S*-thiolation (either as protein-glutathione or protein-cysteine or protein-γ-glutamylcysteine mixed disulfides) of protein sulfhydryls (156, 161, 162). Glutathione is present in very high concentrations in the lens, which is believed to protect thiols in structural proteins and enzymes for proper biological functions. It acts as an essential antioxidant, vital in maintaining the transparency of this tissue, as the lens depends on a balanced redox state for maintaining its transparency (90). Human lenses contain 2–4 m*M* GSH in 19–21-years-old and $<$2 m*M* in clear lenses above 60 years old. There is a concentration gradient of GSH with the highest level present in the outer layer and a gradual decrease toward the center of the lens (161, 162). The GSH pool diminishes as the lens ages, because the *de novo* synthesis and the recycling system for GSH become less efficient, or in lenses under oxidative stress, and loss of GSH appears to be influential in the development of cataract (161, 162).

Cataractous lenses show a decrease in the GSH/GSSG ratio compared with clear lenses. During cataractogenesis, especially in nuclear cataract, the lens proteins unfold and thiols that were buried become reactive (116). Some of these thiols then react to form both mixed disulfides with glutathione and cysteine and disulfide-cross-linked aggregates in the lens (117, 118). With increasing severity of cataract, there is a decrease in total protein thiols with a concurrent increase in protein disulfide content (256). These changes occur mostly in the nucleus, where the oldest proteins are present. Protein *S*-thiolation in human lenses was correlated with nuclear color and opalescence (160). In this study, the intensities of nuclear opacity and pigmentation (brunescence) were compared with the changes in free GSH and the three species of protein–thiol mixed disulfides: protein–glutathione, protein–cysteine, and protein-γ-glutamylcysteine. It was found that nuclear GSH decreased as the nuclear color increased from yellow to dark brown and as the nuclear opalescence increased, and that levels of both *S*-glutathionylated and *S*-cysteinylated proteins progressively increased as the nuclear color intensified. Concentration of protein-γ-glutamylcysteine mixed disulfides progressively increased with increases in both nuclear pigmentation and nuclear opacity. The correlation of lens nuclear color and opalescence intensity with nuclear protein *S*-thiolation indicates that protein–thiol mixed disulfides may play an important role in cataractogenesis and development of brunescence in human lenses (160). *S*-Glutathionylated proteins produced *in vivo* in human clear and cataract lenses were identified by Western blot analysis using anti-GSH antibody, followed by MS (46). The results show that even young 24-year-old lenses have glutathione bound to α- and β-crystallins. Several different *S*-glutathionylated proteins were observed, and a 47 kDa band was of particular interest: it was found principally in the outer part of the lens, the cortex, but not in the lens nucleus where older proteins are present. The 47 kDa component was composed of βB1-, βB2-, and γS-crystallin, with the γS-crystallin having glutathione bound at Cys82 and at Cys22, Cys24, or Cys26. Binding of the anti-GSH antibody to the 47 kDa protein decreased with cataract formation and with increasing severity of cataract, as did α-crystallin binding. The authors conclude that, when glutathione becomes bound to γS-crystallin, it causes it to bind in turn to the β-crystallin polypeptides to form a dimer (46).

Protein expression profiling has been increasingly used to discover and characterize biomarkers that can be used for diagnostic, prognostic, or therapeutic purposes. Most proteomic studies published to date have identified relatively abundant host response proteins as candidate biomarkers, which are often dismissed because of an apparent lack of specificity. A 503-patient study to identify biomarkers that could be used to distinguish patients with early stage ovarian cancer from control individuals (benign disease or healthy women) was recently completed (276). Three markers, transthyretin, apolipoprotein A1, and a fragment of inter-α-trypsin inhibitor heavy chain 4 (ITIH4), were identified to comprise a multimarker panel with higher diagnostic accuracy than existing serum biomarkers such as CA-125. Although these proteins represent abundant host response proteins, two components of the three-marker panel, transthyretin and ITIH4, were fragments of their mature counterparts. This raised the interesting possibility that diagnostic specificity of these analytes would be conferred by the relative amounts of the modified and unmodified forms of these proteins, in addition to their use in a multimarker panel. A more recent study (85) demonstrated that two out of three host response proteins previously identified as candidate markers for early stage ovarian cancer, transthyretin and ITIH4, are actually post-translationally modified following proteolytic truncation, *S*-cysteinylation, and *S*-glutathionylation. Immunologic and chromatographic assays based on Surface Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS) may provide a means to confer specificity to these proteins because of their ability to detect and quantitate multiple post-translationally modified forms of these proteins in a single assay. Quantitative measurements of these modifications using chromatographic and antibody-based SELDI ProteinChip assays reveal that proteolytic truncation, *S*-cysteinylation, and *S*-glutathionylation occur to different extents in different cancers (breast, colon, ovarian, and prostate cancer). In particular, these novel SELDI chromatographic assays for transthyretin variants demonstrate that, among these cancers, the *S*-cysteinylated and *S*-glutathionylated forms are decreased to a significant level only in ovarian cancer. Because each cancer demonstrates a different combination of differentially expressed post-translational modified forms of the proteins, multivariate analysis may be used to improve the classification of these cancers. Authors have termed this process "host response protein amplification cascade," since the process of synthesis, post-translational modification, and metabolism of host response proteins amplifies the signal of potentially low-abundant biologically active disease markers such as enzymes (85). These results demonstrate that relatively abundant serum proteins can in fact be specifically associated with a disease, when their post-translational modifications are taken into consideration and when used in combination.

The total level of sulfhydryl groups was not significantly altered in the hippocampus and cerebellum of AD patients. Differently, the level of protein-bound SH groups was decreased in the AD hippocampus compared with controls. RT-PCR analysis of the expression of key glutathione redox system genes demonstrated the induction of glutathione reductase and glutathione peroxidase messages in the AD hippocampus, whereas mRNA levels of the two brain isoforms of glutathione transferase were unchanged. The decrease in free SH groups in proteins extracted from the hippocampus of AD patients provided additional evidence for increased oxidative damage of proteins in a vulnerable region of the AD brain. This study also suggested that insufficient induction of protective antioxidant gene responses may contribute to the accumulation of oxidatively modified proteins in the AD brain (3). However, a reductive compensation to oxidative stress in AD was hypothesized on the basis of an upregulation of neuronal glucose-6-phosphate dehydrogenase, the key enzyme in reestablishing steady-state levels of GSH/GSSG ratio at the intracellular level, associated with increased sulfhydryl levels that were found solely in large pyramidal neurons of AD brain, the same type of neurons to which is limited oxidative stress/damage in AD (221).

Specific targets of protein *S*-glutathionylation have recently been identified in the inferior parietal lobule from subjects afflicted with AD by using a redox proteomics approach (186). Deoxyhemoglobin, α-crystallin B, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and α-enolase were identified as significantly *S*-glutathionylated relative to these brain proteins in age-matched control inferior parietal lobule. GAPDH and α-enolase were also shown to have reduced activity in the inferior parietal lobule from AD patients (186). The exact function of this reversible oxidative modification of distinct proteins in the brain afflicted with AD is at present unknown. Further studies investigating the specific *in vivo* effects of *S*-glutathionylation in oxidative stress conditions could be important to determining the role of *S*-glutathionylation in the brain plagued by AD.

X. CONCLUSIONS AND PERSPECTIVES

Under pathological conditions, abnormally large concentrations of ROS/RNS may lead to permanent changes in signaling mechanisms and gene expression. Aberrant signaling mechanisms are related to various disease states (21). Since one of the most fundamental processes regulated through signal transduction mechanisms is cell growth, alterations in the normal regulatory processes of cells may lead to cancer. The abnormal behavior of neoplastic cells can often be traced to an alteration in cell signaling mechanisms, such as receptor or cytoplasmic tyrosine kinases, altered levels of specific growth factors, intracellular processes for conveying membrane signals to the nucleus, portions of the transcription apparatus, and genes involved in the cell cycle and the regulation of DNA replication. It has been clearly demonstrated that ROS interfere with the expression of a number of genes and signal transduction pathways and are thus instrumental in the process of carcinogenesis (204, 265). The activation of transcription factors, including mitogen-activated protein kinase (MAPK)/activator protein-1 and NF-κB pathways, has a direct effect on cell proliferation and apoptosis (265). Abnormalities in growth factor receptor functioning are closely associated with the development of many forms of cancers (62). Many signaling molecules and transcription factors fundamental for cell growth, differentiation, and apoptosis, appear to be regulated by *S*-glutathionylation (70, 139). *S*-Glutathionylation plays a key role in the regulation of the kinase activity of MEKK1 (MAPK/ERK kinase kinase 1; MAP3K), an upstream activator of the SAPK/JNK (stress-activated pro-

tein kinase/c-Jun N-terminal kinase) pathway, in response to oxidative stress (47). At least some of the critical redox-responsive signaling proteins contain redox-sensitive cysteine moieties, which activate or inactivate their regulatory function upon *S*-glutathionylation. This conversion into mixed disulfide formation is typically mediated by ROS, but it is also facilitated by an oxidative shift in the thiol/disulfide redox status of the microenvironment. An oxidative shift in redox status may thus also lead to dysregulation of signaling processes. The pathological consequences of oxidative stress (ROS/RNS are increased or the level of antioxidants is decreased) may involve direct oxidative tissue damage (irreversible protein modification) or the dysregulation of signaling processes by anomalous protein *S*-glutathionylation (reversible protein modification), thus contributing to cellular and tissue damages. However, antioxidant protection therapy, in particular in cancer patients, should be used with caution (61, 263). Since apoptosis is caused by elevated levels of free radicals, decreased concentrations of free radicals due to the excessive administration of antioxidants might actually stimulate survival of damaged cells and proliferation into neoplastic state and thus rather promote process of carcinogenesis than interrupt it. In addition, antioxidant therapy during the progression stage of cancer might actually stimulate growth of tumors through the enhanced survival of tumor cells. It has also been demonstrated that free radical scavengers blocked oxidants-mediated neuroprotection evoked by preconditioning (206). Thus, a major concern ought to be given to intentions of attenuating toxic effects of ROS/RNS by their scavenging, since that should also affect some essential signaling processes.

Owing to its reversibility, *S*-glutathionylation has also been proposed to play a protective role by preventing the irreversible oxidation of protein cysteine residues, buffering the effects of oxidative stress (139, 229). In this case, protein *S*-glutathionylation must be considered as an adaptive response to oxidative stress. Thus, *S*-glutathionylation can serve the dual purpose of redox signaling in physiological conditions, as well as protecting protein from irreversible oxidative modifications during mild oxidative stress.

Overproduction or underscavenging of ROS/RNS can irreversibly oxidize reactive protein thiols, causing sulfination/sulfonation of protein cysteines. These irreversible oxidations can eliminate thiol-dependent signaling mediated by *S*-glutathionylation. These irreversible protein thiol oxidations can occur in atherosclerosis, neural diseases (*e.g.*, AD, Parkinson's disease, and stroke) and possibly with various cardiovascular diseases associated with aging, hypertension, and angiogenesis. For example, *S*-glutathionylation may play crucial roles in the signaling mechanisms of vascular smooth muscle cells to modulate vascular tone and hypertrophy, which can be altered in disease (1). Sulfonation of multiple thiols on sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) in atherosclerosis with loss of free thiols was found (1). In association with the oxidation of thiols, NO-induced *S*-glutathionylation and activation of SERCA, and SERCA-dependent relaxation were almost eliminated in diseased arteries. Thus, chronic enhancement of ROS/RNS irreversibly oxidized key reactive thiols and prevented thiol-dependent regulation of SERCA by physiological ROS/RNS generation.

Because formation of *S*-glutathionylated proteins is among the earliest cellular responses to an increase in ROS/RNS production, and can serve as an adjustable mechanism for the activation/inactivation of proteins, levels of *S*-glutathionylated proteins may indicate the degree of oxidative stress within cells and tissues. However, the potential role of protein *S*-glutathionylation in physiological and pathological conditions, as well as the issue of whether this post-translational modification is protective or detrimental, remain to be determined. Thus, much investigation is needed to clarify the actual involvement of protein *S*-glutathionylation in human diseases.

Currently, direct measurement of ROS/RNS in biological samples is very difficult, requiring sophisticated apparatus; therefore, it is important to develop as wide a range of biomarkers as possible for oxidative/nitrosative stress in order to validate their diagnostic and prognostic significance in humans. In order for this to be carried out, methods of biomarker analysis need to be rigorously examined and validated in well-controlled studies by multiple laboratories with facilities to conduct reliable biomarker analysis of oxidative damage to proteins, lipids, and/or DNA. Protein oxidation is one of the most important oxidative/nitrosative stress-induced damaging events in humans, due to high physiological protein concentration. Therefore, in clinical studies investigating oxidative damage, the measurement of protein oxidative damage is an important biomarker of oxidative/nitrosative stress and tissue damage and also reflects the overall balance of endogenous antioxidant defenses. The measurement of protein oxidation products would provide convenient and quantitative biomarkers for the formation of different ROS/RNS *in vivo*. Biomarkers of damage may be used to aid diagnosis and therapy of disease and potentially lead to further research on the role of antioxidants in combating disease.

Blood concentrations of GSH, GSSG, and *S*-glutathionylated proteins (mainly hemoglobin) have been proposed as useful biomarkers of whole-body oxidative/nitrosative stress in humans (25, 144, 187, 196, 214, 217). However, the process of translation to clinical laboratory use is very complex, and, to date, few new biomarkers have progressed to clinical laboratory use. This delay may reflect the many steps in the translation of a newly identified biomarker into a useful clinical laboratory test. Analogous to drug development, discovery may be one of the quickest and easiest steps in the test development process. Validation of markers is a challenging process requiring multisite analytical and clinical studies about test ordering and interpretation. Testing in the clinical environment requires extensive standardization and development of detailed quality assurance processes. As recently suggested by Tsikas about the determination of free and protein-associated 3-nitrotyrosine and *S*-nitrosothiols, the method validation is one of the measures universally recognized as a necessary part of a comprehensive system of quality assurance in analytical chemistry (262). Application of this principle to blood GSH, GSSG, and *S*-glutathionylated proteins means that only validated analytical methods should be used for the quantitative determination of these biomarkers in human blood. Guiding principles for validation of analytical methods, previously provided for the quantitative determination of drugs in animals and humans (235), can be adopted with minor modifications for physiological molecules including glutathione. Minimum recommendations on procedures that should be employed to ensure adequate validation of single-laboratory analytical methods have been reported

(254). Furthermore, measurements of *S*-glutathionylated proteins will also need to be compared with currently accepted and validated markers of oxidative stress, such as protein carbonyls, nitrated and halogenated tyrosine residues, as well as F_2-isoprostanes (53, 54, 57). Furthermore, and very importantly, before attributing a pathological or clinical significance to *S*-glutathionylated proteins, their physiological levels should be clearly and uniformly defined by different research groups. Much care must be taken to define and establish references or baseline profiles from normal tissues, cells, or body fluids, because of the potential for artifacts in estimates of basal levels of oxidatively-modified proteins. That is, oxidative/nitrosative modifications of biological samples can occur during normal sample handling, processing, and analysis such that measured levels are not at all reflective of the levels encountered *in vivo*. This issue has plagued investigations of many different biomarkers of oxidative stress status (57).

The key question when addressing the significance of protein *S*-glutathionylation in human diseases is whether it has some substantive consequence on protein function that impacts on tissue injury and/or disease progression, or is simply a biomarker for the presence of an oxidative stress status; in other words, whether *S*-glutathionylation could be a cause or a result of a particular disease process. To achieve this, it is important to show that *S*-glutathionylation is related to altered function, and not simply a parallel event, and that a positive correlation exists between altered protein function and development of disease. Some studies suggest that oxidized proteins could be a link between increased oxidative stress and diseases (56). However, most of findings obtained so far arise from *in vitro* treatment of cells/tissues or purified proteins with oxidants, thus showing only that a protein can be prone to *S*-glutathionylation having a reactive sulfhydryl group, and that such modification can alter protein function(s). A direct evidence of *S*-glutathionylation occurrence *in vivo* has been clearly demonstrated only in a few reports (see above). Further studies will hopefully answer these questions.

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ABBREVIATIONS

AD, Alzheimer's disease; AIDS, acquired immunodeficiency syndrome; CDNB, 1-chloro-2,4-dinitrobenzene; CSF, cerebrospinal fluid; Cys, cysteine; DTNB, 5′,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent; DTT, dithiothreitol; ECD, electrochemical detector/detection; ESI, electrospray ionization; ESI–MS, electrospray ionization–mass spectrometry; FACS, fluorescence-activated cell sorter; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, gas chromatography; GCL, glutamate-cysteine ligase; γ-GT, γ-glutamyl transpeptidase; GPx, glutathione peroxidase; GSH, glutathione; GSNO, *S*-nitrosoglutahione; GSSG, glutathione disulfide; GST, glutathione transferase; HIV, immunodeficiency virus; HPLC, high performance liquid chromatography; ITIH4, inter-α-trypsin inhibitor heavy chain 4; LC, liquid chromatography; MALDI-TOF MS, matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry; MAPK, mitogen-activated protein kinase; MRP, multidrug resistance-associated protein; mBrB, monobromobimane; MS, mass spectrometry; NAC, *N*-acetylcysteine; NEM, *N*-ethyl maleimide; NK-κB, nuclear factor-κB; OATP, organic anion-transporting polypeptide; OPA, *o*-phthaladehyde; PSSG, protein-glutathione mixed disulfide (*S*-glutathionylated protein); PTPs, protein tyrosine phosphatases; RBCs, red blood cells; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SELDI-TOF, surface enhanced laser desorption/ionization time-of-flight; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; TBARS, thiobarbituric acid substances.

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