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Invited Review

S-Glutathionylation: from redox regulation of protein functions to human diseases

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Abstract

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) play an integral role in the modulation of several physiological functions but can also be potentially destructive if produced in excessive amounts. Protein cysteinyl thiols appear especially sensitive to ROS/RNS attack. Experimental evidence started to accumulate recently, documenting that *S*-glutathionylation occurs in a number of physiologically relevant situations, where it can produce discrete modulatory effects on protein function. The increasing evidence of functional changes resulting from this modification, and the growing number of proteins shown to be *S*-glutathionylated both *in vitro* and *in vivo* support this contention, and confirm this as an attractive area of research. *S*-glutathionylated proteins are now actively investigated with reference to problems of biological interest and as possible biomarkers of human diseases associated with oxidative/nitrosative stress.

Keywords: protein thiols - *S*-glutathionylated proteins - oxidative/nitrosative stress - redox proteomics

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Introduction

Proteins are ubiquitous in all cells and tissues, constituting more than 50% of the dry weight of cells, and are susceptible to oxidative/nitrosative modifications. When reactive oxygen species (ROS) and reactive nitrogen species (RNS) levels exceed the cellular antioxidant capacity, a deleterious condition known as oxidative/nitrosative stress occurs. It describes a status in which cellular antioxidant defences are insufficient to keep the levels of ROS/RNS below a toxic threshold. This may be either due to excessive production of ROS/RNS, loss of antioxidant defences or both. Unchecked, excessive ROS/RNS generation can lead to the destruction of cellular components including proteins, and ultimately cell death *via* apoptosis or necrosis [1].

Protein modification is induced either directly by ROS/RNS or indirectly by reaction with secondary by-products of oxidative stress. It can lead to oxidation of amino acid side chains, formation of cross-linked protein aggregates, and cleavage of the polypeptide chain. The latter is rarely used to quantify protein oxidation in complex systems because of the presence of other proteins and the potential activity of proteases. Differently, the use of stable products of protein side chain oxidation as potential markers for assessing oxidative / nitrosative damage *in vivo* is amply diffused in the study of human diseases [2–5].

Irreversible modifications induced by ROS/RNS (Fig. 1), such as protein carbonylation, are generally associated with permanent loss of protein function and may lead to either the progressive accumulation of the damaged proteins into cytoplasmic inclusions, as observed in age-related neurodegenerative disorders [6] or their degradation [7, 8]. In cells, the level of the progressive accumulation of modified proteins reflects the balance between the rate of protein oxidation and the rate of oxidised protein degradation, and is dependent on a multitude of factors that influence the rates of ROS/RNS generation, the ability of cells to scavenge ROS/RNS, and the levels and activities of proteases that catalyse the degradation of oxidised proteins [7, 8].

Under conditions of moderate oxidative stress, Cys oxidation can lead to the reversible formation of mixed disulphides between protein thiol groups (PSHs) and low-molecular-mass thiols (*S*-thiolation), particularly with glutathione (*S*-glutathionylation). Differently from irreversible modifications, reversible modifications at Cys residues (Fig. 1) may have a dual role of protection from irreversible (terminal) oxidation and modulation of protein function (redox regulation). However, if the modified Cys residue is functionally critical, *S*-glutathionylation will also render the protein inactive and eventually compromise cellular functions [9–13]. On the other hand, as *S*-glutathionylation is reversible, when normal cellular redox status is recovered, so is protein function. A number of functionally critical proteins within the cell possess accessible cysteine residues, liable to undergo redox changes depending on variations of the intra- as well as extracellular conditions. Glutathione (γ-glutamyl-cysteinyl-glycine, GSH) is capable of affecting the redox status of such critical thiols in proteins, which makes cellular GSH a crucial modulating factor for an ever increasing number of proteins including transporters, receptors, protein kinases and phosphatases, transducers, proteases, and cytoskeletal proteins.

In the first part of this review, we will describe the chemical basis of the oxidative modifications of PSHs, before briefly reviewing the currently available methodologies for the study of *S*-glutathionylation. In the subsequent sections, the impact of *S*-glutathionylation on protein function will be examined in detail, specifically focussing on those studies where oxidation has been related to protein redox regulation, altered function, and human disease.

Oxidative modifications of protein thiols

Protein thiol groups can be modified by ROS/RNS to form a cysteine sulphenic derivative (PSOH), which can undergo further oxidation to sulphinic (PSO_2H) and sulphonic (PSO_3H) acids (Fig. 2). The formation of PSOH is always reversible under physiological conditions, though it can readily oxidise to the irreversible oxidation products PSO_2H or PSO_3H or can react with glutathione to form a mixed disulphide. However, it has recently been demonstrated that the sulphinic inactive form of

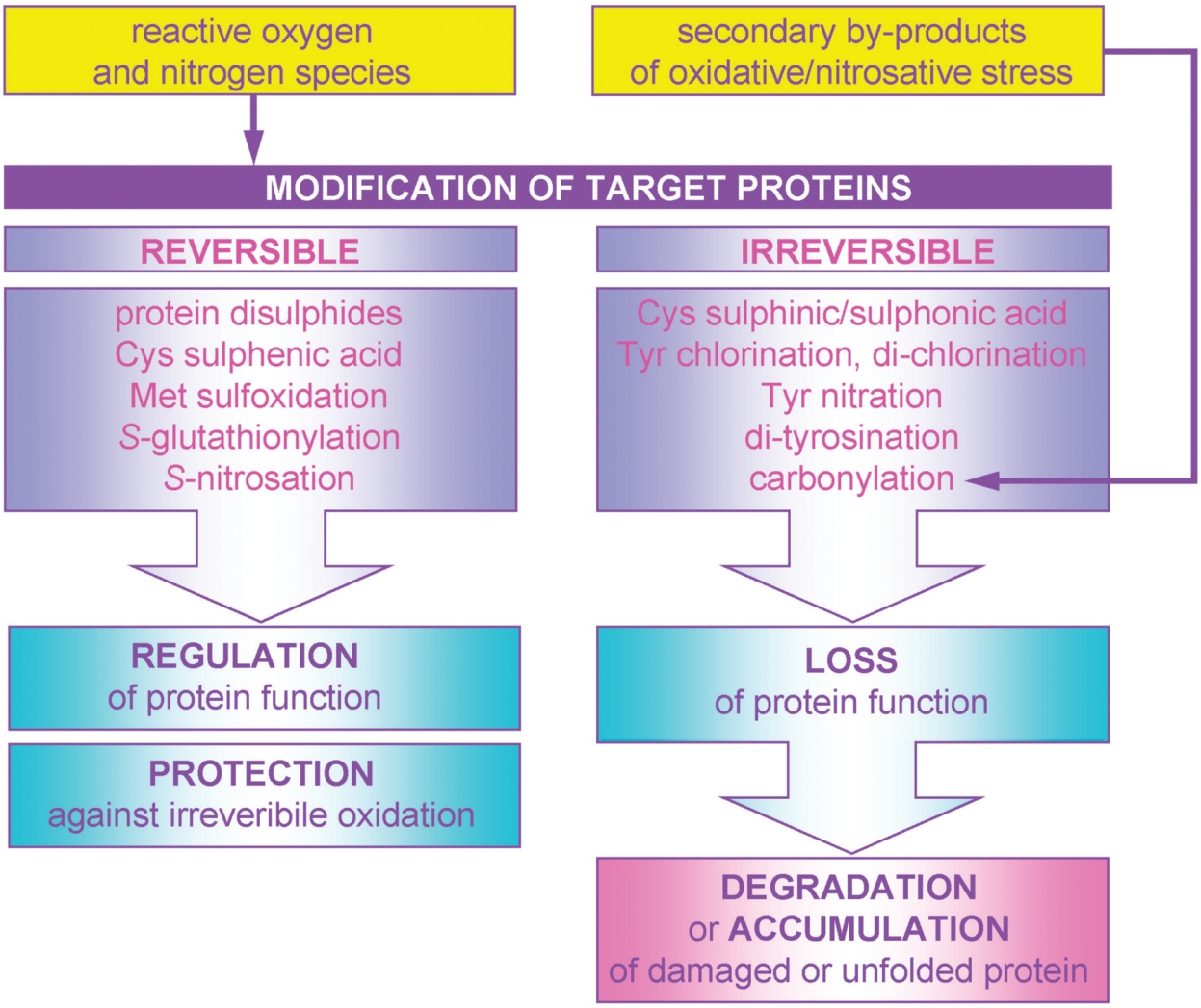

Fig. 1 Consequences of ROS/RNS and oxidative/nitrosative stress on protein function and fate. ROS/RNS may cause oxidative/nitrosative modifications on sensitive target proteins. Reversible modifications, usually at Cys and Met residues, may have a dual role of modulation of protein function and protection from irreversible modification. Irreversible modifications are usually associated with permanent loss of protein function and may lead to the degradation of the damaged proteins by proteasome and other proteases or to their progressive accumulation.

both the cytosolic enzymes peroxiredoxin I and II and the mitochondrial enzyme peroxiredoxin III is rapidly reduced to the catalytically active thiol form through an unknown conversion process [14, 15].

One major means of protein redox regulation is mediated by the formation, or reduction, of disulphides, including mixed disulphides with low-molecular-mass thiols (Fig. 2). The formation of disulphide bonds within a protein (intra-molecular cross-linking) or between two proteins (inter-molecular cross-linking) can cause aggregation of proteins. Both these modifications can be reversed by the enzyme protein disulphide isomerase, which reverses the formation of non-native disulphide bridges, by the thioredoxin system (protein disulphide oxidoreductase), and by reducing agents.

The formation of the disulphide bond between protein cysteine thiol and low-molecular-mass thiols such as cysteine, homocysteine, and GSH, generally referred to as *S*-thiolation, generates so-called mixed (protein/non-protein) disulphides, generally described as *S*-thiolated proteins. Since GSH is the dominant ligand in this reaction, being the most abundant low-molecular-mass thiol given

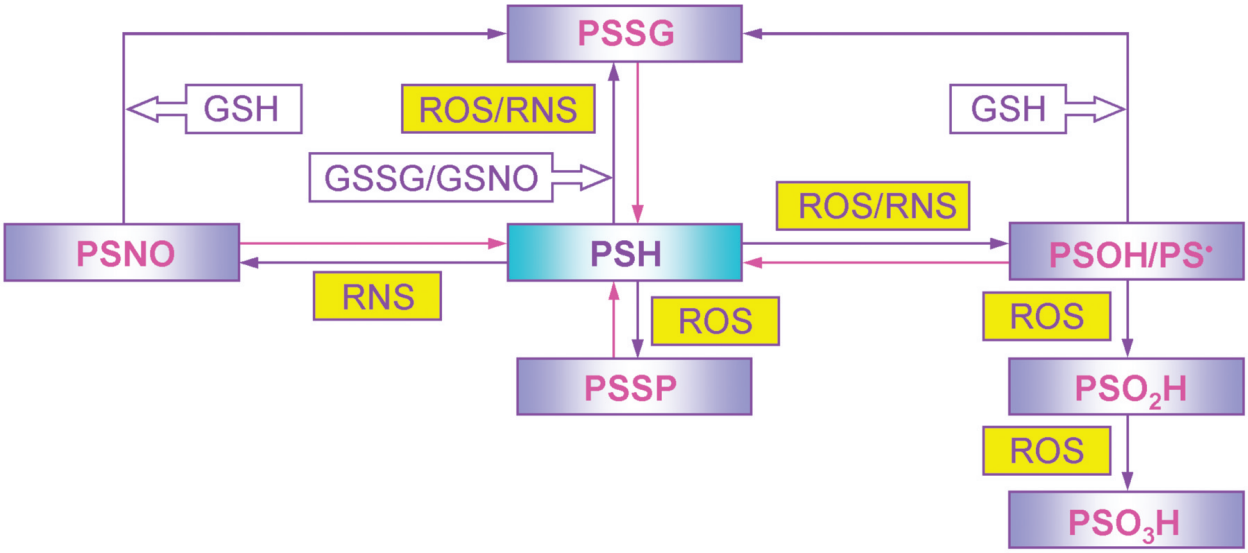

Fig. 2 Oxidative/nitrosative modifications of protein Cys residues. ROS/RNS may induce the formation of mixed disulphides between protein thiol groups (PSH) and GSH to form *S*-glutathionylated proteins (PSSG) by essentially two pathways. PSH may be initially "activated" by oxidative/nitrosative modifications to give thyil radical (PS·), sulphenic acid (PSOH), or protein *S*-nitrosothiol/*S*-nitrosated protein (PSNO). These modifications may be either stabilised as such or react with GSH to the mixed disulphide (PSSG). All these modifications are reversible and can be reduced back by increases in the GSH/GSSG ratio, reduced thiols, or enzymatic reactions. Otherwise, PSSG may be generated by thiol/disulphide exchange reaction with GSSG or by reaction with other "reactive" intermediates of GSH, such as GSNO. PSOH may also be irreversibly oxidised by ROS/RNS to form sulphinic (PSO_2H) and sulphonic (PSO_3H) derivatives, leading to irreversible loss of biological activity. PSH may also be oxidised to disulphide both within and between proteins (PSSP). PSSP can be reversed by enzymes (protein disulphide isomerase and thioredoxin/thioredoxin reductase) or reducing agents.

its high concentration (0.5-10 mM) in mammalian cells, most studies focus on *S*-glutathionylation and *S*-glutathionylated proteins (PSSGs).

The intracellular reducing environment during normal cellular homeostasis is assumed to inhibit oxidation of PSHs to either protein disulphides (PSSPs) or mixed disulphides (Fig. 2). While PSSP generation can be hampered by steric hindrance, PSHs can easily react with low-molecular-mass thiols in response to an oxidative/nitrosative insult producing *S*-thiolated proteins. As the intracellular environment is highly reducing while the extracellular one is highly oxidising, cytoplasmic proteins are rich in free cysteine thiols and secreted proteins are rich in disulphide bonds. As a result of this prevalence of free thiol groups in cytoplasmic proteins, in many cases targets of redox regulation identified so far are cytoplasmic proteins that can undergo oxidation, while cases of targets of redox regulation that are normally in the oxidised state and become reduced are less frequent [*e.g.*, 16].

Several mechanisms (reactions 1–3) have been proposed for PSSG formation: by reaction of GSH with partially oxidised reactive PSHs (thiyl radical or sulfenic acid intermediates) or by thiol/disulphide exchange reaction [9]:

(1) $PS\cdot + GSH \rightarrow PSSG^{-\cdot} + O_2 \rightarrow PSSG + O_2\cdot + H^+$

(2) $PSOH + GSH \rightarrow PSSG + H_2O$

(3) $PSH + GSSG \leftrightarrow PSSG + GSH$

Glutathione-thiyl radical, which may be continuously produced at a low level when a redox signalling pathway is activated, has also been proposed as a potential alternative mediator of PSSG formation; a recent study supports this hypothesis, demonstrating that glutaredoxin catalyses *in vitro* formation of specific PSSG adducts, including actin, *via* transfer of the glutathione-thyil radical [17]. Furthermore, PSSG formation can be promoted by NO, through various mechanisms including the formation of *S*-nitrosoglutathione (GSNO) and thiyl radicals [9, 18]. *S*-Glutathionylated proteins accumulate under oxidative/nitrosative stress con-

ditions, but they can be readily reduced to free SH groups by the enzyme glutaredoxin (thioltransferase) or by reducing agents.

Detection and identification of *S*-glutathionylated proteins

The relevance of protein *S*-glutathionylation in physiological and pathological conditions as well as the issue of whether this modification is protective or detrimental can be determined by the identification of the proteins that are *S*-glutathionylable.

Total *S*-glutathionylated proteins, as well as GSH and GSSG, can be detected by reversed phase HPLC with fluorescence detection after thiol derivatisation with monobromobimane or 1-fluoro-2,4-dinitrobenzene [19–21]. Recently, the detection of PSSGs has been facilitated by the availability of specific monoclonal anti-GSH antibodies that allow detecting of *S*-glutathionylated proteins by slot immunoblotting or by 1D- or 2D-PAGE followed by Western blot [16, 20, 22, 23].

Conventional approaches for the identification of PSSGs are based on the labelling of the intracellular GSH pool with ^{35}S-Cys in a state of cellular quiescence (protein synthesis inhibition), with subsequent protein extraction, separation by SDS-PAGE, and identification of *S*-glutathionylated proteins by autoradiography and sequence analysis.

Several approaches involve various kinds of affinity purification of PSSGs. By an approach that utilises the affinity of GSNO for PSHs to immobilize cellular proteins on a solid matrix, several cellular and nuclear extract proteins were shown to form mixed disulphides with the matrix [24]. The sensitivity and specificity of mixed disulphide detection can be increased by immunoprecipitation of modified proteins from cellular extracts and subsequent analysis of proteolytic digests of the isolated protein by liquid chromatography/mass spectrometry techniques [25].

Proteomics methods for the detection of PSSGs have been devised using either [^{35}S]-labelled GSH for the specific detection of [^{35}S] incorporation into proteins resolved on 2D-PAGE [26] or biotinylated GSH ester for the selective immunoaffinity purification of *S*-glutathionylated proteins [11, 27-29]. In the first case, PSSGs are identified by incubation of cells with radiolabelled Cys in the absence of protein synthesis, exposure to oxidants, isolation, and detection of radiolabelled (*i.e.*, glutathionylated) proteins. This methodology applied to the study of stressed T lymphocytes has revealed that numerous proteins are targets for redox-dependent modification [26]. In the second approach, the amino terminus of cysteine is tagged with biotin and loaded into cells or tissues. When oxidising changes occur, formation of a disulphide bond between redox-sensitive protein cysteines and biotin-cysteine is induced. These oxidised proteins carrying a biotin tag are detected using non-reducing Western blotting and streptavidin-HRP or purified by streptavidin-agarose affinity chromatography. The efficiency of purification can be improved with the use of gel-filtration chromatography for separating proteins from free biotin-cysteine in homogenates, which otherwise competes with *S*-thiolated proteins for column binding. These proteins are then separated by SDS-PAGE and stained by unspecific compounds. Methods based on labelled GSH possess several drawbacks [30]. Briefly, only proteins that are present in high quantities may be identified; proteins that are constitutively (*i.e.*, under basal conditions) *S*-glutathionylated may not be identified as they may not incorporate labelled GSH. Furthermore, some technical details have to be considered, including the necessity of blocking free SH (*e.g.*, with *N*-ethylmaleimide) to prevent thiol-disulphide exchange between protein thiols.

The impact of *S*-glutathionylation on protein function

Oxidative modifications of PSHs can have a variety of effects on protein function. They may render the protein inactive and thus compromise cellular function. On the other hand, reversible post-translational modifications of Cys residues, such as *S*-glutathionylation, may have a dual role of protection against irreversible protein thiol oxidation and modulation of protein function (redox regulation) (Fig. 1). Indeed, reversible modifications of signal transduction proteins can initiate signalling events that lead to adaptive responses that combat injury.

S-Glutathionylation will render the protein inactive, thus contributing to cellular dysfunction during oxidative stress, if the Cys residue involved in the mixed disulphide is functionally critical, as in the case of protein tyrosine phosphatase 1B (PTP1B), cAMP-dependent protein kinase, tyrosine hydroxylase, α-ketoglutarate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), protein kinase C, and creatine kinase [9-11, 13, 31-35]. Creatine kinase (CK), an important source of ATP in myocytes, is completely inhibited by *S*-glutathionylation at Cys^{283}. Interestingly, GSNO modifies the acidic cysteine in CK predominantly by *S*-glutathionylation rather than *S*-nitrosation (a covalent modification describing the incorporation of NO moieties at protein Cys residues forming a nitrosothiol group, or thionitrite ester) [36]. Moreover, the position of the cysteine undergoing modification is important in determining the effect of *S*-glutathionylation. For instance, the activity of the human immunodeficiency virus (HIV) type 1 protease can be inhibited or increased and stabilised depending on which cysteine is involved [37], whereas matrix metalloproteinases are activated upon *S*-glutathionylation of the autoinhibitory domain [18].

While *S*-glutathionylation is often considered a modification occurring in response to oxidative stress, a number of PSSGs have been observed under basal conditions [26, 38, 39], suggesting their possible involvement in signalling and redox regulation of protein function.

In cells, *S*-glutathionylation could result from thiol disulphide exchange reactions involving GSSG or from direct partial oxidation of PSHs (thyil radicals and sulphenic acid intermediates) followed by reaction with GSH. In either case, a relative specificity could be generated from protein secondary and tertiary structure by promoting the ionization of an exposed thiol to a relatively nucleophilic thiolate anion. Regulatory cysteines are generally located in protein regions rich in positively charged amino acids, as these areas favour the formation of reactive thiolated ions, which are sensitive to oxidative modification. These reactive Cys residues could be significantly oxidised at ROS concentrations below those inducing irreversible damage to the cell. Reversal of *S*-glutathionylation (dethiolation or deglutathionylation) could be achieved by either changes in the intracellular redox status (increases in the GSH/GSSG ratio), reduced thiols, or *via* enzymatic reduction by protein disulphide isomerase, thioredoxin system, and mainly glutaredoxin (also known as thioltransferase), which selectively dethiolates PSSGs by a GSH-dependent mechanism [31]. Therefore, PSH oxidation involving *S*-glutathionylation seems to contain many of the main elements of a regulatory system: sensitivity, specificity, and reversibility. However, further data are necessary to confirm this aspect, since other chemical species besides ROS/RNS and GSSG, *e.g.*, electrophilic molecules, can react with PSHs with a rate depending on the capability of the PSH to form the thiolate anion [40].

In addition to its regulatory role, *S*-glutathionylation may serve as a means of storing GSH and of protection, when the cell experiences oxidative / nitrosative stress, by preventing the irreversible oxidation of critical Cys residues, often at the expense of temporary loss in protein activity [9, 41]. Examples supporting this hypothesis come from the *S*-glutathionylation of the γ-glutamyl transpeptidase, which appears to protect this membrane-bound enzyme from irreversible oxidation [42], the *S*-glutathionylation, causing reversible inactivation, of α-ketoglutarate dehydrogenase in response to alterations in the mitochondrial GSH status [13], and the *S*-glutathionylation of the active site Cys^{215} of PTP1B, which regulates its activity [31].

Many proteins are targets for, and can be regulated by, *S*-glutathionylation. It has been shown to affect the activity of a growing list of enzymes (Table 1), including antioxidant enzymes such as 1-CYS peroxiredoxin [43] and glutaredoxin [17], a member of the thiol-disulphide oxidoreductase family, which has a binding site for GSH and uses GSH as its cofactor to catalyse specifically the reduction of protein-GSH mixed disulphides to free sulfhydryl groups. It has also been suggested that the action of the homodimeric HIV-1 protease may be regulated by redox changes to its cysteines by *S*-glutathionylation [37, 44]. *S*-Glutathionylation of Cys^{67}, a solvent exposed residue, increases activity severalfold and also stabilises it. However, *S*-glutathionylation of Cys^{95}, located at the dimer interface, abolishes protease activity, which can be restored with the use of thioltransferase [37, 44]. Moreover, human T-cell leukemia virus type 1 pro-

tease, a transforming retrovirus, can be similarly regulated through reversible *S*-glutathionylation of its two conserved cysteine residues at the dimer interface [45].

Furthermore, *S*-glutathionylation has been shown to affect H-ras, a low molecular weight G-protein, which is critical to activation of several signal transduction pathways, including the extracellular signal-regulated kinases (Erk1 and Erk2). These pathways are activated when several different cell types are exposed to ROS. H-Ras was found to be *S*-glutathionylated in cells exposed to *S*-nitrosocysteine [46].

A number of proteins involved in transcription, translation, and degradation, which are key processes of cellular adaptation as they control protein expression levels, can also be directly regulated by *S*-glutathionylation. This modification inhibits DNA binding activity of transcription factors c-Jun and NF-κB [47, 48]. The chymotrypsin-like activity of 20 S proteasome extracted from both mammalian cells and yeast is modulated by *S*-glutathionylation, though in different ways [12, 49]. In the case of mammalian 20 S proteasome, both GSH and GSSG at micromolar concentrations activate the chymotrypsin-like activity, whereas it is inhibited by millimolar concentrations [49]. Differently, the yeast 20 S proteasome is inhibited strongly by GSH millimolar concentrations and slightly by GSSG at all concentrations tested; in addition, it is *S*-glutathionylated *in vivo* when cells are submitted to oxidative challenge [12].

Also the cytoskeletal proteins tubulin [50] and actin can be *S*-glutathionylated. Actin has been identified as a target for *S*-thiolation in several cells under varying conditions of oxidative stress: during reperfusion of the ischaemic rat heart [51], in human T cell blasts exposed to oxidative stress or HIV infection, used as a model of an infectious disease associated with oxidative stress [26, 52]. Interestingly, in ECV 304 endothelial-like cells cytoplasmic β-actin is *S*-glutathionylated under oxidative stress, and also under constitutive cellular metabolism [38]. The importance of reversible *S*-glutathionylation of actin Cys^{374} as a physiologically relevant regulatory mechanism of G-actin polymerisation has been evidenced in human A431 cells undergoing oxidant insult and in studies on isolated actin [16, 22, 23, 53]. Also the F-actin-binding phosphoprotein annexin is regulated by *S*-glutathionylation of A2 subunit at Cys^{8} and Cys^{132} [54].

Table 1. Selected enzymes whose activities are modulated by *S*-glutathionylation.

Enzyme	References
1-CYS peroxiredoxin	[43]
Aconitate hydratase	[51]
Alpha-ketoglutarate dehydrogenase	[13]
cAMPdependent protein kinase	[35]
Creatine kinase	[34, 36]
Glyceraldehyde-3-phosphate dehydrogenase	[32]
Mitochondrial complex I (NADH-ubiquinone oxidoreductase)	[68]
Nucleoside diphosphate kinase B	[51]
Protein kinase Cα	[33]
Protein phosphatase 2A	[69]
Protein tyrosin phosphatase 1B	[25, 31, 70]
Thioredoxin	[17, 71]
Thioredoxin peroxidase II	[27]
Triosephosphate isomerase	[51]
Tyrosine hydroxylase	[10]

Some members of the heat shock protein (HSP) family are a target for *S*-glutathionylation. Under basal conditions, small stress protein HSP27 exists as a high molecular weight non-phosphorylated complex that is broken down following phosphorylation by stress-activated kinase pathways, such as p38 MAPK, which activates MAPKAPK2, which directly phosphorylates HSP27. The phosphorylation-induced breakdown of the macromolecular structure correlates with the loss of molecular chaperone activity [55]. Eaton and colleagues have shown that cardiac HSP27 is *S*-thiolated at Cys^{141} during oxidant stress, and this modification, independently of phosphorylation, disaggregates multimeric HSP27 [28]. The oligomeric state of HSP27 regulates its ability to act as a molecular chaperone and also controls its ability to inhibit polymerisation of actin [reviewed in 56],

Table 2. Human diseases associated with *S*-glutathionylated proteins.

Disease	References
Friedreich's ataxia	[39, 58]
Human immunodeficiency virus	[52]
Hyperlipidemia	[61]
Renal cell carcinoma	[72]
Type I diabetes	[20, 62]
Type II diabetes	[61]
Uremia and hemodialysis or peritoneal dialysis	[63]

which itself is also a target of *S*-glutathionylation, also during brief periods of cardiac reperfusion following ischaemia [28, 51]. Recently, reversible *S*-glutathionylation of HSP70 has been shown as a mechanism for post-translation regulation of chaperone activity [57].

S-Glutathionylated proteins in human diseases

S-Glutathionylated proteins have been investigated as possible biomarkers of oxidative stress in correlation with disease. The increase or decrease in PSSGs, caused by patho-physiological conditions, can make them mediators of important functions or parameters of clinical significance. It is possible that the variation in the levels of PSSGs may serve as an indicator of the evolution of the disease, with the consequent attribution of diagnostic/prognostic importance.

Significant increases in PSSGs have been found in human diseases such as hyperlipidemia, chronic renal failure, and diabetes mellitus (Table 2).

A significant increase in glutathionyl-haemoglobin and glutathionyl-actin has been found in the blood and fibroblasts, respectively, of patients with Friedreich's ataxia [39, 58], the most common of the hereditary ataxias, caused by severely reduced levels of frataxin, a protein implicated in iron metabolism, characterised by degeneration of the large sensory neurons and spinocerebellar tracts, cardiomyopathy, and increased incidence in diabetes.

Abundant free radicals in cigarette smoke cause increases in blood PSSG concentration. The mean concentration of blood PSSGs was found 32% higher in cigarette smokers and 43% higher when standardised by haemoglobin concentrations; plasma PSSG levels were also 20% higher in smokers than non-smokers. The relationship was dose-dependent, with blood PSSG levels significantly correlated with cigarettes smoked per day, plasma cotinine (the major metabolite of nicotine), and plasma thiocyanate (a metabolite of cyanide in tobacco smoke) [59].

There is an extensive literature reporting low GSH levels in lymphocytes of HIV patients [60]. Using an *in vitro* model of HIV, Ghezzi and coworkers found that HIV-infected cells have decreased ability to dethiolate PSSGs [52]. Interestingly, *N*-acetyl cysteine (NAC) restored dethiolation in infected cells to the level of that in uninfected cells. This is in agreement with previous data showing that NAC raises GSH levels in lymphocytes from AIDS patients [60]. Similar alterations in PSSG metabolism, and consequently in overall cell metabolism, can be expected in other infectious and inflammatory diseases, since oxidative stress is a common accompaniment in these kinds of diseases. Alterations in protein *S*-glutathionylation can be expected under any circumstances in which GSH is seriously depleted, such as treatment with GSH-depleting drugs, and excessive ethanol consumption.

Since the blood concentration of PSSGs may reflect alterations in the oxidative stress status even in hardly accessible tissues and compartments of the body, haemoglobin has been analysed in some human diseases as a marker of whole-body oxidative stress. Glutathionyl-haemoglobin is increased in patients suffering from type I and type II diabetes, Friedreich's ataxia, hyperlipidemia, and uremia associated with haemodialysis or peritoneal dialysis [20, 58, 61-63]. Differently, a decrease in the normal (basal) concentration of glutathionyl-haemoglobin has been measured in children with Down's syndrome [64]. Further, the blood concentration of glutathionyl-haemoglobin has been proposed as a useful clinical marker of oxidative stress in humans [61, 65, 66], and recent data indicate that at least three different types of *S*-glutathionylation

of haemoglobin can exist in human erythrocytes [67]. However, we have shown that inappropriate blood sample manipulation can lead to an artifactual increase in PSSGs, because many variables may affect the PSSG measurement: for instance, used reagents, incubation times with different substances, sample freezing, acid precipitation of proteins [20, 40]. Thus, before attributing a pathological or clinical significance to glutathionylhaemoglobin, its physiological levels should be clearly and uniformly defined by different research groups working on this field, possibly following an accurate revision of applied methods.

Conclusions

The key question when addressing the significance of ROS/RNS-induced protein modifications is whether they are mechanisms of protection from irreversible damage, mechanisms for modulation of protein function (redox regulation), simply biomarkers for the presence of oxidative stress, or have some substantive consequence on protein function that impacts on cell injury and/or disease progression.

In the last few years, there has been a significant shift away from the measurement of protein oxidation products simply to establish the presence of oxidative stress, to a focus on identifying specific proteins sensitive to oxidation, and establishing the functional consequences of these modifications. In addition, the identification of specific enzyme systems to repair these oxidative modifications has led to the belief that protein function may be regulated through these oxidation reactions.

Phosphorylation/dephosphorylation is a well known example of modification that controls protein functions. The modulation of protein function by ROS/RNS (redox regulation) may be in many ways analogous to phosphorylation, except that protein modification no longer occurs on specific serine, threonine, or tyrosine residues, but instead on redox-sensitive Cys residues. Although both *S*-glutathionylation and phosphorylation are covalent modifications of protein residues, the crucial difference is that phosphorylation is enzyme-driven, whereas *S*-glutathionylation can be achieved through the non-catalysed chemical modification of a protein Cys residue. Thus, in *S*-glutathionylation, the reaction specificity does not rely on the recognition of a target structure by an enzyme. Instead it depends solely on the chemical reactivity between the oxidating agent and the target cysteine.

In recent years, *S*-glutathionylation is emerging as a redox-sensitive post-translational modification that potentially plays a key regulatory role in signal transduction. The increasing evidence of functional changes resulting from this modification, and the growing number of proteins shown to be *S*-glutathionylated *in vitro* and, though to a lesser extent, *in vivo* support this contention, and confirm this as an attractive area of research. However, protein *S*-glutathionylation/deglutathionylation is a relatively new scientific field, and much remains to be learned about its role in cell signalling, including if it is a mechanism of signal transduction regulation as ubiquitous as phosphorylation/dephosphorylation. Anyway, recent studies have begun to demonstrate how redox signalling could be accomplished. Examples include the reversible activation of the 1-CYS peroxiredoxin [43], and the reversible inactivation of α-ketoglutarate dehydrogenase [13]. In both cases, oxidation-reduction of PSHs provides the on-off switch.

Key questions are whether altered functions of PSSGs contribute to disease development - in this case protein *S*-glutathionylation should occur at an early stage of disease - and whether this Cys modification is a detrimental or adaptive component of injury during disease, or whether this Cys modification is merely a consequence of the oxidative damage of cells and tissues, reflecting the presence of disease. Basically, the answer to these questions requires identification of specific *S*-glutathionylated proteins, and a positive correlation between altered protein function and development of disease. Some recent data indicate that aberrant *S*-glutathionylation is associated with some human diseases (Table 2) and suggest that protein *S*-glutathionylation may be a link between increased oxidative stress and diseases. The finding of increased oxidative stress in conditions such as diabetes and hyperlipidemia has suggested the use of antioxidant supplementation as a potentially useful therapy in preventing or delaying the development of complications such as atherosclerosis and heart disease. Consequently, the availability of clinical markers that can provide an accurate assessment of the degree of oxidative stress will become impor-

tant in clinical trials aimed at investigating the effectiveness of antioxidant therapy for preventing or reducing the risk of complications as well as in ameliorating such complications in diseases associated with increased oxidative/nitrosative stress.

Nevertheless, much work remains to be done to elucidate whether, and how, *S*-glutathionylated proteins are involved in (at least) some human diseases associated with oxidative stress. For many years, much of the work in research on protein oxidation has been descriptive, because the tools needed to ask discrete molecular questions were not available. However, there has been a dramatic and recent transformation in our ability to conduct qualitative and quantitative analysis of protein oxidative modifications. The rapid development of redox proteomics in recent years has made possible the study of oxidative/nitrosative stress-induced modifications in the proteome [5], even under conditions very close to the physiological one, *e.g.*, during the oxidant stress that occurs during reperfusion of the heart following ischemia [11]. Thus, it is reasonable to hypothesise that the next few years will witness the identification and characterisation of a more and more growing number of novel protein targets for ROS and a more precise definition of the events that lead from chemical protein modification to altered cellular function and, possibly, disease. This application of the tools of chemistry to biology will provide a strong experimental basis for the development of new diagnostic, therapeutic, and preventive strategies.

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