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REVIEW

Regulation of protein function by glutathionylation

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Abstract
The main function of reduced glutathione (GSH) is to protect from oxidative stress as a reactive oxygen scavenger. However, in the context of redox regulation, the ratio between GSH and its oxidized form (GSSG) determines the redox state of redox-sensitive cysteines in some proteins and, thus, acts as a signaling system. While GSH/GSSG can catalyze oxido-reduction of intra- and inter-chain disulfides by thiol-disulfide exchange, this review focuses on the formation of mixed disulfides between glutathione and proteins, also known as glutathionylation. The review discusses the regulatory role of this post-translational modification and the role of protein disulfide oxidoreductases (thioredoxin/thioredoxin reductase, glutaredoxin, protein disulfide isomerase) in the reversibility of this process.

Keywords: *Glutathione, cysteines, thiol-disulfide exchange, dethiolating agent*

Glutathione: From oxidative stress to redox regulation

Glutathione (GSH), the tripeptide γ-glutamyl-cysteinyl-glycine, is one of the most important antioxidants in the cell. In the non-scientific literature, and commercial brochures (glutathione is present in many nutraceuticals preparations and health food supplements), it is described as a major defense molecule that can prevent ageing and virtually all diseases, and is sometimes referred to as "superfood", "master antioxidant", "ultimate antioxidant", "your body's most powerful protector", "your body's most powerful healing agent" and, finally, "life extension molecule".

In fact, the scientific literature on glutathione has pointed out the antioxidant role of GSH. Since GSH is the non-protein thiol present in highest concentration in the cell, it plays a major role in maintaining a reduced state of protein thiols in the cytoplasm [1–3]. Its antioxidant, protective role is in part a direct effect as a free radical scavenger, a term that defines a molecule acting to trap reactive oxygen species that would otherwise react with protein sulfhydryls or other potential targets, thus behaving as a suicidal, decoy molecule. Glutathione also serves as an antioxidant through GSH peroxidases to catalyze the overall reaction:

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \quad (1)$$

$$2GSH + ROOH \rightarrow GSSG + ROH + H_2O \quad (2)$$

In these reactions, whether spontaneous or enzyme-catalyzed, GSH is not lost, since GSSG (glutathione disulfide) can subsequently be reduced back to GSH. This underlines the importance of the GSH-regenerating system represented by GSSG reductase (glutathione reductase) at the expenses of NADPH, catalyzing the reaction:

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \quad (3)$$

The key role of GSH as an antioxidant is demonstrated by the worsening effect that GSH

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depletion, achieved by different means, has on the toxicity of several compounds, both *in vitro* and *in vivo*.

In the past, with a few exceptions, the oxidation of protein cysteines by ROS was pertinent to the field of toxicology as it was, in the vast majority of the scientific literature, the concept of oxidative stress. We think that the concept of redox regulation evolved from that of oxidative stress in the early 90s, after several papers have shown that ROS or antioxidants oppositely regulated transcription factors, such as NF-κB [4,5], and cytokine-mediated signaling. Those data suggested that ROS did not merely damage cell macromolecules, either proteins or lipids or DNA, but might actually change the cellular metabolism and gene expression.

The difference between oxidative stress and redox regulation is often viewed as a quantitative one (e.g. low ROS levels are regulatory; high levels induce oxidative stress and toxicity) or qualitative (reversible oxidation of cysteines to mixed disulfides represents redox regulation; irreversible oxidation to sulfonic acids is toxic oxidative stress). Clearly, of the many known forms of protein oxidation (including nitration and carbonylation) thiol oxidation to disulfides and S-nitrosothiols are the most interesting candidates as means of redox regulation, due to their reversibility. Despite the fact that, with respect to redox regulation, protein glutathionylation has received particular attention in recent years, this is not a recent discovery. Pioneering works by Brigelius et al. [6,7] had shown that in normal liver, around 1% of total glutathione is consistently found as mixed disulfides with proteins (in the range of 30 nmol/g). Depending on the cellular redox state, the amount of such mixed disulfides may rise up to 20–50% of the total glutathione content [8].

Formation of glutathionylated proteins

Formation by thiol-disulfide exchange with GSSG

The overall reaction of thiol-disulfide exchange is given below, where PSH indicates a protein with a free cysteine:

$$PSH + GSSG \rightarrow PSSG + GSH \quad (4)$$

This reaction implies that the GSH/GSSG ratio is a key determinant in the glutathionylation status of proteins [8]. Glutathionylation is often thought to occur primarily by this mechanism.

Formation by direct oxidation

A direct oxidation of 2 SH compounds, e.g. by oxygen is not likely relevant physiologically, since it would require a ternary collision of 2 SH and the oxidant. Instead, this overall reaction:

$$GSH + PSH \rightarrow PSSG \quad (5)$$

can be catalyzed by many oxidants. A rather specific thiol oxidizing agent widely used in biochemistry is diamide [9], that will oxidize sulfhydryls to disulfides (including GSSG and PSG) by the following reaction (R and R′ can be glutathione or a protein):

$$(CH_3)_2NCON{=}NCON(CH_3)_2 + RS^- + H^+ \rightarrow (CH_3)_2NCON(SR)NHCON(CH_3)_2 \quad (6)$$

$$(CH_3)_2NCON(SR)NHCON(CH_3)_2 + R'S^- + H^+ \rightarrow RSSR' + (CH_3)_2NCONHNHCON(CH_3)_2 \quad (7)$$

Formation via sulfenic acid intermediates

Cysteines can form other oxidation products, including sulfenic (Cys-SOH), sulfinic (Cys-SO_2H) and sulfonic acids (Cys-SO_3H). Of these, the latter are quite stable and should be considered irreversible forms of oxidation. Sulfenic acid can react with GSH or with any other RSH. This has been shown to occur, for instance, with protein tyrosine phosphatase-1B [10], according to the following reactions where H_2O_2 can be derived from the dismutation of $O_2^{\cdot -}$:

$$PSH + H_2O_2 \rightarrow PSOH + H_2O \quad (8)$$

$$PSOH + GSH \rightarrow PSSG + H_2O \quad (9)$$

Alternatively, oxidation of protein SH followed by disulfide formation can also be achieved by peroxynitrite [11].

$$PSH + ONOO^- \rightarrow PSOH + NO_2^- \quad (10)$$

Formation via S-nitrosothiols

Glutathionylated proteins can also be formed by the intermediate production of S-nitrosoglutathione, GSNO. GSNO (as well as, by analogy, protein S-nitrosothiols PSNO) can be produced by NO^+ which is derived from $NO^{\cdot}$ and O_2 via N_2O_3 and subsequent decomposition into NO^+ and NO_2^-. The formation of glutathionylated proteins by GSNO or PSNO can take place in various ways (reactions 11–13).

$$GS^- + NO^+ \rightarrow [GSNO] \quad (11)$$

$$PSNO + GSH > PSSG + HNO \quad (12)$$

$$PSH + GSNO > PSSG + HNO \quad (13)$$

Of note, GSNO can form GSSG by reaction with GSH, and GSSG can then glutathionylate proteins by thiol disulfide exchange.

Thus, several mechanisms may account for the formation of glutathionylated proteins and it is not clear which one is prevalent *in vivo*. While thiol-disulfide exchange is often thought to be a major one, it should be noted that the concentrations of GSSG are very low compared to those of GSH. Evidences against a role of GSSG in the formation of PSSG have been published. Specifically, increase in protein glutathionylation due to the oxidative burst in human neutrophils stimulated with phorbol esters is observed in the absence of an increase in GSSG concentrations [12]. A recent paper by Dalle Donne et al. [13] has shown that actin is not glutathionylated by physiological levels of GSSG and even not by an up to 100-fold molar excess of GSSG over actin.

Formation via thiyl radicals

Thiyl radical of GSH or PSH can be formed by reaction with hydroxyl radicals ($OH^{\cdot}$). We are giving below the example of formation of protein thiyl radical, but the same reaction can take place starting from a glutathione thiyl radical, although with low efficacy:

$$PSH + HO^{\cdot} \rightarrow PS^{\cdot} + H_2O \quad (14)$$

$$PS^{\cdot} + GS^{\cdot} \rightarrow PSSG \quad (15)$$

It is of interest to note that there are evidences that glutaredoxin (Grx) can catalyze the latter reaction, suggesting that this enzyme not only acts as a dethiolating agent but can function in both ways [14].

Specificity of glutathionylation

Very little is known on the specificity of protein glutathionylation. Are all free -SH groups equally susceptible? Many papers suggest that two factors determine the susceptibility of a given cysteine. The first is its accessibility in the 3D structure, since GSH is not a small molecule, and even an alkylating agent like iodoacetamide (IAM) preferentially labels accessible cysteines. For instance, studying the susceptibility of the different cysteines of human thioredoxin (Trx) for glutathionylation, we observed that when human Trx is kept in its native conformation, IAM will, apart from the N-proximal cysteine in the CXXC motif also alkylate the same cysteine (Cys72) that is susceptible to glutathionylation [15]. The other determinant is the reactivity of the cysteine, largely determined by the surrounding amino acids. For instance, formation of a cysteine thiolate ($Cys\text{-}S^{-}$), which can then react to form a mixed disulfide with GSH, is favoured by basic amino acids in its vicinity, while acidic vicinal amino acids will have the opposite effect [16]. Again, in the case of Trx, a GSH adduct on Cys72 might also be favored by the primary structure where a basic amino acid, Lys71, could stabilize the protein-glutathione adduct by interacting electrostatically with the γ-glutamyl group of GSH [17].

Other specific factors determine the susceptibility of a specific cysteine to glutathionylation. In particular, a neighbouring thiol can reduce a mixed disulfide once formed. Accordingly, Trx has a CXXC motif and none of the two cysteines in the active site forms a (stable) mixed disulfide with glutathione [15].

Reversibility of glutathionylation and the role of protein disulfide oxidoreductases

The other side of the coin is how reversible is the process. As mentioned above, some cysteines may form more stable mixed disulfides than others. In fact, the main feature that makes glutathionylation a possible regulatory mechanism is its reversibility. It should be noted that most of the studies on dethiolation focus more on the damaging, oxidative stress-related enzyme inactivation effects of glutathionylation, hence use the term protein regeneration for dethiolation, rather than on what we call redox regulation. Clearly, mixed disulfides are unstable and reversible by the action of other thiols according to the general reaction of thiol disulfide exchange shown above (reaction 4). The process of dethiolation can also be catalyzed by glutaredoxins (Grx, also known as thioltransferases) and other enzymes of the family of protein disulfide oxidoreductases. These enzymes include Grx, the thioredoxins (Trx) /thioredoxin reductase (TR) system, and protein disulfide isomerases (PDI), and are characterized by a redox-active CXXC motif [17–19]. The two cysteines in the active site undergo reversible oxidoreduction to form a monothiol or a dithiol intermediate. It is generally thought that Trx/TR and Grx are "antioxidant", reducing enzyme systems that catalyze reduction of protein disulfides and GSH-protein mixed disulfides, respectively, whereas PDI is acting as an oxidant by forming intra-chain disulfide bonds and, thus, assists in protein folding. This is generally true, and is confirmed by the localization, where PDI is mainly located in the endoplasmic reticulum and often has a chaperone function. On the other hand, there are many evidences that Grx and Trx have a function in the antioxidant defense system. They catalyze reduction of disulfide bonds and become concomitantly oxidized by forming an intramolecular disulfide in the CXXC active site. The oxidized enzyme is then reduced by Trx reductase, in the case of Trx, or by GSH, in the case of Grx (Figure 1). A particularly important example of such protein disulfide reduction is the role of Trx as substrate of peroxiredoxins, many of which are therefore addressed to as 'thioredoxin peroxidases' and are implicated in redox regulation [20]. These reductive

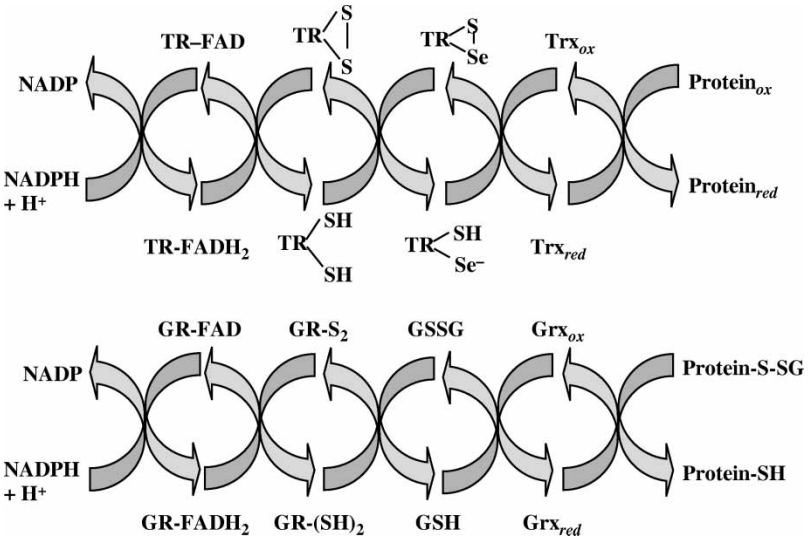

Figure 1. Redox cycle of Trx and Grx. TR, Trx reductase; GR, glutathione reductase. The figure shows that TR undergoes two reactions: The reduction by $FADH_2$ of the disulfide between Cys59 and Cys94, and subsequent reduction of the disulfide between Cys497 and selenoCys498. Sources: [52,53].

processes are important under physiological conditions. Beyond, Trx in particular is an essential cofactor of ribonucleotide reductase and, thus, is required for DNA synthesis [17]. From the perspective of this review, they have been implicated in the repair of oxidatively damaged proteins, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or phosphofructokinase where oxidative stress has caused oxidation of thiols to disulfides [21–24].

The overall reaction catalyzed by Grx will be:

$$PSSG + GSH \rightarrow PSH + GSSG \qquad (16)$$

The relative importance of Grx-catalyzed versus non-enzymatic dethiolation by thiol-disulfide exchange is not known. However, a recent paper using RNA interference to silence endogenous Grx in 3T3 fibroblasts has established a key role of this enzyme in catalyzing the deglutathionylation of actin and consequently regulating its polymerization and reorganization [25].

In vitro studies using glutathionylated hemoglobin have shown that Grx is over 1000-fold more efficient that dithiothreitol in catalyzing dethiolation, and that GSH (even in the presence of a GSH-regenerating system using GSSG reductase) was unable to carry out the dethiolation in the absence of Grx [21,26]. It should be noted that although Grx regenerates the proteins inactivated by mixed disulfide formation more efficiently than Trx, also Trx can dethiolate glutathionylated proteins [26,27]. Trx also efficiently regenerates glyceraldehyde 3-phosphate dehydrogenase and phosphofructokinase proteins inactivated by monothiol oxidation to sulfenic or sulfinic acid [23].

We have mentioned above the role of Grx in thiolation mediated by thiyl radicals. It is important to remind that although Trx/TR and Grx probably work mainly as reducing systems and PDI as an oxidizing one, like most enzymes they can work both ways. For instance, Trx can become an oxidizing protein under certain conditions [28,29]. Likewise, also PDI was implicated in the regeneration of oxidized proteins, thus, acting in a reducing direction [30].

Effects of protein glutathionylation

Glutathionylation modifies a protein in many ways. In most cases this leads to an inhibition of its activity, which is particularly evident in case of enzymes (see the section of glycolytic enzymes below). Also transcription factors, like Jun and NF-κB, are inhibited by glutathionylation [31,32], and evidences for this have also been published for activating transcription factor/cyclic AMP-responsive element binding protein (ATF/CREB) [33]. In the case of transcription factors, inhibition likely occurs through inhibition of DNA binding as the glutathionylation adds the two negative charges of GSH (that of γ-glutamic acid and the C-terminal one of glycine) to the positively charged DNA-binding domain of the protein, which in turn impairs binding to the negatively charged DNA [32,33].

It is important to note that glutathionylation is not equal to the blocking of an active-site cysteine with an alkylating agent or mutating the target cysteine. For instance, c-Jun mutants lacking the cysteine undergoing glutathionylation can still bind to DNA, but they are not responsive to thiol oxidation. In fact, unlike blocking a cysteine, binding of the GSH

tripeptide to a protein affects its activity due to steric hindrance, or changes in its isoelectric point, as mentioned above. In this way, glutathionylation is different from other forms of S-thiolation. For instance, glutathionylation inhibits several protein kinase C isozymes, while cysteinylation does not affect their activity [34]. Glutathionylation may have other effects on enzymes, and in the case of HIV-1 protease glutathionylation of Cys 95 inhibits the activity but glutathionylation of Cys 67 stabilizes the enzyme [35]. Recently, it was shown that the SERCA transporter is activated by glutathionylation [36].

Glutathionylation may have other functions than those which can be viewed as generally protective or antioxidant. If a cysteine is glutathionylated, it is not available for other oxidative reactions. In this respect, glutathionylation is often considered a way to protect sensitive cysteines from other, possibly irreversible, forms of oxidation, thus allowing the cell to restore the cognate function of the protein when oxidative stress conditions are overcome. Finally, glutathionylation may serve as a way of storing GSH in the cell during oxidative stress. In fact, when GSH is oxidized to GSSG, the latter is then exported from the cell [37].

An example of redox regulation: Glycolytic enzymes

The inverse regulation of glycolysis by oxygen is the basis of the well-known Pasteur effect described in 1861. The genes coding for glycolytic enzymes often are regulated by oxygen [38], but it is likely that direct inhibition of glycolytic enzymes by thiol oxidation, including glutathionylation, may represent a rapid mechanism to inhibit glycolysis before changes in gene expression result in a change in the amount of the respective proteins. In fact, a number of reports have indicated that glycolytic enzymes are targets for S-thiolation or glutathionylation [39–41].

In 1984 Gilbert et al. [8] already pointed out that thiol-disulfide exchange regulates the activity of several glycolytic enzymes. We have summarized in Figure 2 the data reviewed by Gilbert et al. [8] and the data of a more recent picture from Shenton and Grant [39] showing the activity of which enzymes is increased or decreased upon oxidation, and which enzymes are susceptible to glutathionylation or, more generally, to S-thiolation based on ours and others' work [39,40]. It can be seen that there is not a complete match, but there is an overall inhibitory effect of thiol oxidation. Many of the enzymes previously known to be inhibited by GSSG [8], including hexokinase and phosphofructokinase, were not shown to be glutathionylated, at least up to know. This could possibly mean that the inhibitory effect of GSSG occurs by catalyzing formation of intrachain disulfides. However, it is also likely that the redox proteomics approaches do not have a very good "recovery", i.e. they only pick up a few enzymes undergoing glutathionylation. It would be interesting to complete the picture investigating whether glutathionylation of specific enzymes affects other steps of the glycolytic pathway.

Conclusions and perspectives

As mentioned in the introduction, the layman often uses antioxidants drugs or supplement in the absence of any evidence-based medicine or approval by a regulatory agency, as a consequence of an over simplification of the concept of oxidative stress and of the fact that this concept is a fashionable one. We must admit that the concept of redox regulation being fashionable, helps us scientists to publish our findings "better". In the past, the description that oxidants, including H_2O_2 or GSSG, inactivated an enzyme was part of the characterization of the enzyme. Today, the observation that an enzyme is inhibited by H_2O_2 or GSSG through oxidation of a cysteine can be published in a nice journal as an example of redox regulation. Looking at a database such as the "Enzyme" database of Expasy (http://www.expasy.org/enzyme) or the Brenda database at the University of Koeln, Germany (http://www.brenda.uni-koeln.de) many enzymes can be found that are inhibited by thiol reagents, oxidants or GSSG. As in the case of glycolysis, it would be important to directly address the question of which of these enzymes can actually be regulated by reversible glutathionylation. Revisiting the old concepts of oxidant susceptibility with the knowledge of redox regulation might represent a gold mine for biochemists.

However, I would suggest that the term "redox regulation" should be used in a more restrictive manner to define proteins whose redox state, or, in the case of cysteines, the redox state of its thiols and disulfides, may vary under physiological or pathological conditions. Clearly, all disulfides can be reduced (for instance by treating with DTT or mercaptoethanol) and all SH can be oxidized by high concentrations of H_2O_2. However we should not aim at identifying all proteins whose -SH can be oxidized by H_2O_2, which can probably be done *in silico*. We should identify those -SH groups that, under physiological conditions, or anyway in conditions compatible with cell viability, can exist both in oxidized and reduced forms. Vice versa, we should identify those disulfides that are reduced by physiological concentrations of thiols, or by low concentrations of thiol antioxidants which are required to fine-tune biological processes.

We often try to describe the potential regulatory role of protein glutathionylation in analogy to that of phosphorylation. Clearly, protein phosphorylation has several levels of complexity, in terms of specificity, as several protein kinases and phosphatases with different specificities exist. Furthermore, the chemistry of

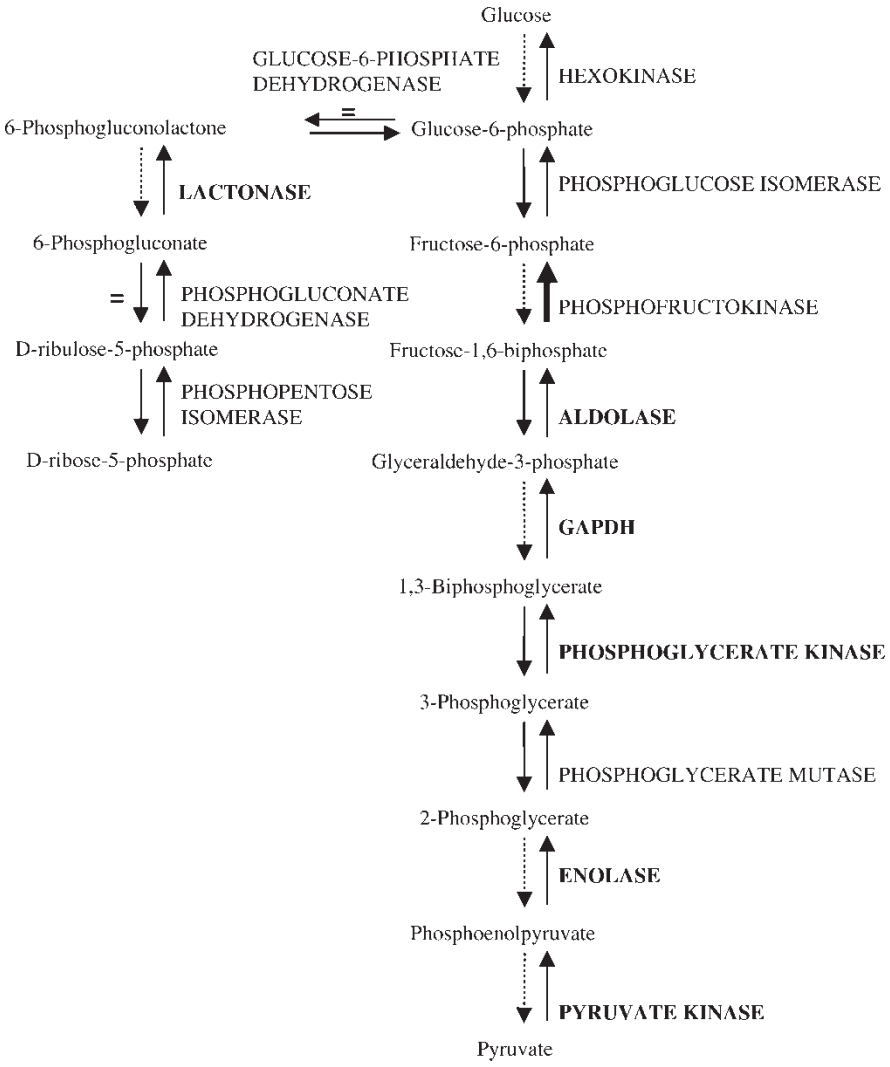

Figure 2. Redox regulation of glycolytic and hexose monophosphate pathways. Enzymes in bold denote those undergoing glutathionylation; dashed lines, pathways inhibited by thiol oxidation; thick lines, pathways up-regulated by oxidation; =, demonstrated to be unaffected by thiol oxidation. Sources: [8,39,40].

protein phosphorylation is easier to investigate due to the high stability of phosphoamino acids, and the availability of specific inhibitors of the various kinases and phosphatase and of antibodies that recognize specific phosphoproteins.

If the hypothesis that glutathionylation is a posttranslational modification used as a signalling mechanism is true, then GSH is not only an essential antioxidant but glutathione (both GSH and GSSG) is also an essential signalling molecule. In this case, one should wonder whether the deleterious consequences that GSH-GSSG depletion has in many models of oxidative stress is not only due to the lack of an antioxidant, thiol-sparing molecule, but also due to the blockade of glutathionylation-dependent signalling mechanisms similar to the findings that ATP depletion can affect phosphorylation-mediated signalling [42]. This would imply that manipulating GSH levels with oxidants that convert it to GSSG will have a different effect from inhibitors of GSH synthesis that would also deplete GSSG levels.

To date, however, at least in our opinion, there are not conclusive evidences that glutathionylation might be implicated in signalling, and particularly in the response to stress. But there are many indications suggesting this, in addition to the ones mentioned above involving glycolytic enzymes and transcription factors. One further example is the chaperone activity of heat shock proteins. A series of elegant studies have shown that Hsp33 is activated by formation of disulfide bonds [43,44]. A recent paper has reported that glutathionylation converts Hsp70 to an active chaperone [45]. Other observations in the literature might be explained by a regulation of protein function through glutathionylation, although the issue has not been addressed directly. Another example is NF-κB. This transcription factor was shown to be inhibited by

antioxidants and activated by hydrogen peroxide [4,5]. Along this line, Droge et al. came up with the intriguing observation that GSSG is required for optimal activation of NF-κB [46]. At the time it was postulated that GSSG could inhibit protein phosphatases thus potentiating the kinase pathways implicated in NF-κB activation [46]. However, it would be interesting to revise that early observation in the light of a recent report showing that NF-κB subunit p50 can be glutathionylated [47]. Of note, also hypoxia-inducible factor (HIF)-1 alpha activation by hypoxia is inhibited by GSH depletion [48].

Clearly, the challenge of identifying glutathionylated proteins implicated in redox regulation has intrinsic technical difficulties associated with the instability of thiol-disulfide chemistry. The term "redox proteomics", which we coined in a 2001 paper [41], has been used to identify proteins undergoing various types of oxido-reductive post-translational modifications. Our own paper, to be precise, detected only proteins whose cysteines where in a reduced state and able to form mixed disulfides with radioactive GSH, since we labeled the glutathione pool [41], not the steady-state glutathionylated proteins. Other methods relay on principles all based on the reactivity of free cysteines to bind radioactive or biotinylated GSH, or GSH immobilized onto an affinity column. Using these techniques, one could pick up only proteins that are not basally glutathionylated, as in the latter case the cysteine will not be available. Development of other methods, such as those based on binding to Grx mutants [49] or based on anti-GSH antibodies [50,51] might lead to different results, and possibly help in defining the concept and mechanisms of redox regulation by glutathione.

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