Oxidoreduction of protein thiols in redox regulation

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Abstract
Protein cysteines can undergo various forms of oxidation, some of them reversible (disulphide formation, glutathionylation and S-nitrosylation). While in the past these were viewed as protein damage in the context of oxidative stress, there is growing interest in oxidoreduction of protein thiols/disulphides as a regulatory mechanism. This review discusses the evolution of the concept of redox regulation from that of oxidative stress and the redox state of protein cysteines in different cellular compartments.

Protein oxidation in oxidative stress
Aerobic organisms reduce molecular oxygen (O$_2$) to water during respiration. However, the interaction with biological systems also generates intermediate steps in the reduction of O$_2$, such as superoxide radicals, H$_2$O$_2$, hydroxyl radical, singlet oxygen etc., which are very reactive. Since these ROS (reactive oxygen species) are toxic, the consequence of excessive production – since low levels of ROS are presumably produced and quickly detoxified by various antioxidant enzymes – is termed ‘oxidative stress’. This is of great toxicological and pathological importance, and is implicated in the toxicity of radiation, oxygen toxicity [1] and other such events. It has aroused popular interest in other fields too, including cosmetics, nutriceuticals and alternative medicine.

The molecular mechanism of the toxicity of ROS is that they damage several cellular components, including lipids, nucleic acids and proteins. Proteins can undergo different types of oxidation. These include carbonylation (an irreversible process that targets different amino acids including lysine, arginine, proline and threonine [2]), nitration of tyrosine and oxidation of methionine to methionine sulphoxide.

From the viewpoint of this review, protein cysteines can be oxidized to form different products, listed in Table 1. Intraprotein disulphide bonds are viewed, in classical textbooks, as part of the tertiary structure of the protein and their formation is an important step in protein folding. Similarly, many disulphide bonds are important in the quaternary structure of proteins, in the formation of homo- or hetero-multimers. The other forms of oxidation, in the past, have been mainly considered as protein damage induced by oxidative stress. Many enzymes have essential thiols and are thus inactivated during oxidative stress, including that induced by oxygen poisoning or radiation (reviewed in [1]).

Many forms of thiol oxidation are reversible. Clearly, these are the modifications of interest from the point of view of redox regulation, not only oxidative stress. From the angle of oxidative damage, this reversibility has often been referred to as ‘regeneration’, as discussed below.

Protein glutathionylation as the main form of protein S-thiolation
This term indicates the formation of mixed disulphides between proteins and glutathione. Glutathionylation of protein is not only observed after oxidative stress, and Brigelius et al. [3,4] showed that, in normal liver, substantial amounts of glutathione (in the range of 30 nmol/g) are found as mixed disulphides with protein; this can reach up to 20–50% of the total glutathione content under oxidative stress [5].

Glutathionylated proteins can be formed by various mechanisms and all can have a role in vivo. A first possibility is direct oxidation:

\[ X + \text{PSH} + \text{GSH} \rightarrow \text{PSSG} + \text{XH}_2 \]

where PSSG stands for protein-SS-glutathione (mixed disulphide). The reaction is catalysed by an oxidant, X: for instance the diazenecarbonyl derivative diamide, a widely used thiol-specific oxidant originally described by Kosower and Kosower [6]. A second mechanism by which proteins can be glutathionylated is thiol/disulphide exchange:

\[ \text{PSH} + \text{GSSG} \rightarrow \text{PSSG} + \text{GSH} \]

In this reaction, a thiolate anion is probably the reacting species in the nucleophilic attack of a thiol [7]. An additional mechanism is through the formation of S-nitrosoglutathione [8], thus linking protein glutathionylation and the NO pathway.

The thiol radical of GSH, generated by reaction with hydroxyl radicals (OH$^*$), can also form glutathionylated proteins. The reaction of glutathione thiol radicals with proteins to generate PSSG is catalysed by glutaredoxin [9], an enzyme normally acting as a reductant.

It is not clear whether any one of the mechanisms listed above is prevalent in the formation of glutathionylated proteins under physiological conditions. The thiol/disulphide
Table 1 | Oxidation forms of protein cysteines

<table>
<thead>
<tr>
<th>Oxidation product</th>
<th>Notes</th>
<th>Reversibility</th>
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<tbody>
<tr>
<td>Free thiol (SH)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intraprotein disulphides (SS)</td>
<td></td>
<td></td>
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<tr>
<td>Interprotein disulphides (SS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mixed disulphides (SS)</td>
<td>Glutathionylation (PSSG); cysteinylation</td>
<td></td>
</tr>
<tr>
<td>S-nitrosothiols (SNO)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sulphenic acids (SOH)</td>
<td></td>
<td></td>
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<tr>
<td>Sulphinic acids (SO$_2$H)</td>
<td></td>
<td></td>
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<tr>
<td>Sulphonic acids (SO$_3$H)</td>
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</table>

 exchange reaction is often considered the most ‘physiological’ and would directly relate the glutathionylation state of proteins to the GSSG/GSH ratio (the ‘redox state’ of the cell). However, there is experimental evidence of protein glutathionylation that is not dependent on this mechanism. For instance, the oxidative burst in neutrophils induces marked protein glutathionylation with no detectable increase in the formation of GSSG [10]. More recently, Dalle-Donne et al. [11] have shown that actin, a major glutathionylated protein, is not glutathionylated by physiological levels of GSSG, even with a 100-fold molar excess of GSSG over actin.

Other forms of S-thiolation: cysteinylation

Since GSH is the most abundant intracellular non-protein thiol, glutathionylation is the main form of S-thiolation. However, outside the cell, for instance in plasma, GSH is virtually absent and the predominant low-molecular-mass thiol is cysteine. Thus the predominant extracellular form of S-thiolation is cysteinylation. In fact, whereas haemoglobin in red blood cells is glutathionylated [12], plasma proteins such as albumin [13] and transthyretin [14] are mainly cysteinylated.

Reversibility of protein thiolation and the role of ‘antioxidant’ systems

A number of enzymatic and non-enzymatic systems can protect proteins from oxidation or regenerate oxidized proteins. In many cases, these are the same enzymes or molecules that are part of the cell’s ROS-detoxifying machinery. However, to ‘regenerate’ S-thiolated proteins (in case of glutathionylated or cysteinylated proteins, the term ‘dethiolation’ is used), these systems act as reducing agents, rather than as free radical scavengers. In the context of thiol/disulphide-mediated redox regulation, these systems are key regulatory molecules and should be viewed in analogy with the role played by protein kinases and phosphatases in phosphorylation-mediated regulation. The different roles of thiol-reducing agents in these contexts are outlined in Table 2.

The enzymes catalysing protein thiol disulphide oxidation-reduction (protein disulphide oxidoreductases) include glutaredoxin, thioredoxin and protein disulphide-isomerase, and have a CXXC motif in the active site. Oxidoreduction of these cysteines, by either mono- or di-thiol mechanisms, is part of their catalytic cycle [15–17].

Glutathione and the glutaredoxin system; thioredoxin and the thioredoxin system

GSH is the major low-molecular-mass thiol in the cytoplasm. Its role as the main thiol antioxidant in the cells is demonstrated by the dramatic effect of its depletion in many toxicological conditions.

GSH acts as a free radical scavenger, trapping ROS that would otherwise interact with cellular thiols. It does so through enzyme-catalysed reactions. GSH peroxidases use it as an electron donor in the reduction of peroxides, including lipid peroxides. However, GSH does more than just trap ROS and is also involved in the ‘regeneration’ of oxidized proteins. GSH can reduce sulphenic acids and, via thiol/disulphide exchange, mixed disulphides:

\[
PSSG + GSH \rightarrow PSH + GSSG
\]

Significant dethiolation of oxidized proteins, and subsequent reactivation of enzymes inactivated by thiolation, can also be achieved by GSH alone [18,19]. However, this reaction

Table 2 | Thiol oxidation and thiol antioxidants in oxidative stress and redox regulation

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<tr>
<th>Protein thiol oxidation</th>
<th>Oxidative stress</th>
<th>Redox regulation</th>
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</thead>
<tbody>
<tr>
<td>Oxidants</td>
<td>Damage</td>
<td>Regulation</td>
</tr>
<tr>
<td></td>
<td>‘Evil’ molecules</td>
<td>Neither good nor bad</td>
</tr>
<tr>
<td>Thiol antioxidants</td>
<td>Free radical scavengers and antioxidants</td>
<td>Reducing agents</td>
</tr>
<tr>
<td>(e.g. GSH, NAC, Grx)</td>
<td></td>
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is very efficiently catalysed by glutaredoxin/thioltransferase [20,21] and, to a lesser extent, by thioredoxin [21]. The role of glutaredoxin is demonstrated, for instance, by the finding that suppressing endogenous glutaredoxin using RNA interference causes overglutathionylation of actin [22]. Although glutaredoxin is often described as a mere catalyst in this thiol disulphide exchange reaction, the mitochondrial enzyme (Grx2) can also reduce mixed disulphides in the absence of GSH, using electrons from thioredoxin reductase and NADPH [23]. In both mechanisms, glutaredoxin will use the reducing power provided by NADPH, through either GSSG reductase or thioredoxin reductase.

**Topography of protein thiols**

Aerobic organisms maintain a reduced state in the cell despite the highly oxidizing environment (21% oxygen, at sea level) where they live. The redox state of protein thiols is dependent on the cellular location. In the cytoplasm, the environment is highly reduced, mainly due to the high intracellular concentration of GSH, and the GSH/GSSG ratio is 30–100. For this reason, the cysteines of cytoplasmic proteins are mainly present as free thiols, both in mammalian cells and bacteria [24,25]. It is generally thought that the only disulphide bonds in the cytoplasm are transient ones formed as a part of the oxidoreduction reactions of enzymes, particularly protein disulphide oxidoreductases where the active-site cysteines in the CXXC motif form a transient intramolecular disulphide [25].

In contrast, extracellular proteins are mainly disulphide proteins, because of the oxidative extracellular environment [24], and globular proteins exemplify this, where some of these bonds are structurally important while others can be reduced with no major effect on the protein [26]. Structural disulphides are formed during the folding process in the endoplasmic reticulum, and according to a widely accepted view, this intracellular compartment is different from the cytosol in that it is highly oxidizing, with a GSH/GSSG ratio of approx. 1 [27]. The other possible exceptions are the structural disulphide bonds in the cytoplasm of extreme thermophiles, probably to stabilize thermostable proteins [25].

Recent studies of redox proteomics have challenged the belief that cytosolic proteins only have free cysteines, showing that many disulphide bonds are formed in a large number (~100) of cytoplasmic proteins in cardiomyocytes and neuronal cells exposed to oxidants [28,29]. Formation of disulphide bonds has also been described in proteins acting as redox sensors, such as Hsp33 (heat-shock protein 33) [30] and OxyR [31]. Evidence that a substantial amount of glutathione is present as mixed disulphide with proteins also points in this direction. However, most of these disulphides are different from those described in classical biochemistry textbooks, which are those important for the structural integrity of proteins. Probably, these non-structural disulphides differ from structural ones in that the former are reversible, and probably both reduced and oxidized forms of these proteins coexist. Thus reversibility can be used not only to classify different types of cysteine oxidation but also different types of disulphides.

Proteins on the plasma membrane are at the interface between an oxidizing and a reducing environment. Several papers have described the presence of exofacial protein thiols (surface thiols) and they appear to be kept in the reduced state by protein disulphide isomerase [32]. Since membrane proteins include several key proteins such as receptors, ion channels, ectoenzymes etc., this is clearly another possible target for redox regulation. Several biological processes may be regulated by the redox state of surface thiols, including entry of HIV-1 [33], platelet aggregation [34], integrin-mediated adhesion [35] and receptor shedding [36].

Exofacial thiols, due to their accessibility, might offer a preferential target for oxidants, but also for reducing agents. For instance, low concentrations of N-acetylcysteine, far below those reported to inhibit NF-κB (nuclear factor κB) activation, have a dramatic effect on the expression of surface thiols, suggesting that many effects of this thiol ‘antioxidant’ may not require any action on intracellular GSH or transcription factors [35].

**Conclusions**

Clearly, the concept of oxidative stress is currently suffering from excessive popularity, caused, in most cases, by us scientists when, with different levels of awareness, we try to ‘sell’ the idea that it is involved in almost all pathological conditions and, with different levels of financial involvement, try to ‘sell’ antioxidant molecules as life prolongers or healing molecules for all diseases.

On the other hand, studying the differences – if there really are any – between the concepts of ‘oxidative stress’ and ‘redox regulation’ also poses interesting scientific and technological challenges. The difference between those changes taking place during redox regulatory processes and oxidative stress is in the amount of oxidants generated, and redox regulation should imply oxidoreductive processes that do not result in cell death or malfunction. The application of proteome analysis to this field is blooming and is rapidly defining the molecular targets of redox regulation. The fact that a thiol can be oxidized, or a disulphide reduced, is obvious, and the latter is of course done routinely when we reduce protein samples with mercaptoethanol before electrophoresis. This will, for instance, reduce all disulphide bonds in a globular protein like albumin. But these disulphide bonds are unlikely to be reduced in vivo under experimental conditions compatible with survival. The key to understanding redox regulation is that there are many disulphide bonds that can be reduced without killing a cell and free thiols that can be oxidized.

Finally, we need to investigate what exactly redox changes signal. The most obvious possibility is that redox regulation orchestrates the responses to oxidative stress. But thiol antioxidants influence many biological responses, including those associated with inflammation and immunity, suggesting that they may in fact be a more widely used signalling mechanism.
References


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