Cysteine-based redox regulation and signaling in plants

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INTRODUCTION

The production of reactive oxygen (ROS) and nitrogen (RNS) species is unavoidable under aerobic conditions, especially at the level of electron transfer chain reactions for ROS or via enzymatic processes for RNS. Moreover, their generation is often exacerbated in plants facing abiotic and biotic constraints (Moller et al., 2007). Another category of molecules, called reactive sulfur species (RSS), is currently the subject of intense investigation as the major physiological signaling molecules such as nitrogen dioxide (NO2), dinitrogen trioxide (N2O3) and peroxynitrite (ONOO−), is formed via a reaction of NO with O2·−. Several of these molecules have dual functions since they damage cellular components such as proteins, DNA, and lipids but, at sub-toxic concentrations, they constitute messengers. Owing to the reactivity of their thiol groups, some protein cysteine residues are particularly prone to oxidation by these molecules. In the past years, besides their recognized catalytic and regulatory functions, the modification of cysteine thiol group was increasingly viewed as either protective or redox signaling mechanisms. The most physiologically relevant reversible redox post-translational modifications (PTMs) are disulfide bonds, sulfenic acids, S-glutathione adducts, S-nitrosothiols and to a lesser extent S-sulfinyl-amides, thiosulfinates and S-persulfides. These redox PTMs are mostly controlled by two oxidoreductase families, thioredoxins and glutaredoxins. This review focuses on recent advances highlighting the variety and physiological roles of these PTMs and the proteomic strategies used for their detection.

Keywords: cysteine, disulfide bond, glutathionylation, nitrosylation, redox regulation, sulfenic acid, thiolate

MULTIPLE CYSTEINE OXIDATION FORMS FOR DISTINCT REDOX SIGNALS

Due to their unique physico-chemical properties, cysteinyl residues participate in catalytic reactions, serve as metal ligands and are also susceptible to various PTMs. Whereas free cysteines have an ionization constant (pKa) of about 8.3, in proteins, some cysteines defined as reactive cysteines, possess lower pKa, ranging from three to seven. In thiol-oxidoreductases as thioredoxins (Trxs) and glutaredoxins (Grxs), the lowering of the pKa results in stabilization of cysteine disulfide bonds, sulfenic acids, S-glutathione adducts, S-nitrosothiols and to a lesser extent S-sulfinyl-amides, thiosulfinate and S-persulfides. These redox PTMs are mostly controlled by two oxidoreductase families, thioredoxins and glutaredoxins. This review focuses on recent advances highlighting the variety and physiological roles of these PTMs and the proteomic strategies used for their detection.

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Thiol groups via ROS, RNS, and RSS, particularly H$_2$O$_2$, NO, and H$_2$S. There is an interesting parallel here because these three molecules, which were initially thought to be exclusively toxic, may in fact represent key regulators for various biological processes and in particular for signaling purposes, considering their relative stability, their capacity to diffuse across membranes (NO and H$_2$S are gases and the uncharged H$_2$O$_2$ is channeled via aquaporins) and their propensity to react with thiols (Bienert et al., 2007).

**H$_2$O$_2$-mediated modification of protein thiols**

With methionines, cysteines are the most H$_2$O$_2$-sensitive residues. The two-electron oxidation of a cysteine thiolate (–S$^-$) by H$_2$O$_2$ forms a sulfenic acid (–SOH; Figure 1). Due to its unstable and highly reactive nature, the sulfenic acid will be further modified (Reddie and Carroll, 2008). In the absence of other proximal thiols, sulfenic acid can further react with one or two additional peroxides, forming sulfenic (–SO$_2$H) and sulfonic (–SO$_3$H) acids (Figure 1). These two modifications are usually considered as irreversible reactions, except for the SO$_2$H in the specific case of the peroxiredoxin (Prx) catalytic cycle. Alternatively, sulfenic acids can react with the main chain nitrogen of a neighboring residue to form a sulfenyl-amide or condensate with another sulfenic acid leading to thiosulfinate but the physiological relevance of these modifications is unclear (Figure 1).

Most of the time, the sulfenic acid will react with another...
thiolate from a protein cysteine or glutathione, leading to the formation of an intra- or inter-molecular disulfide bond. The coverage of the interaction of glutathione to a protein thiol group constitutes an important redox PTM known as glutathionylation (Figure 1).

**NO-MEDIATED MODIFICATION OF PROTEIN THIOLS**

NO is a relatively unreactive gas radical and should in principle not react directly with protein thiol groups to form S-nitrosylated proteins. The primary cellular targets include other radical species such as O$_2^*$ to form peroxynitrite, oxygen to form NO$_3^-$ and metalloproteins as those containing hemes and iron-sulfur clusters. Several mechanisms of S-nitrosylation, i.e., the covalent binding of an NO group to a cysteine thiol group, have been proposed, but what one or operate in plant cells is not yet elucidated. S-nitrosothiols may first be formed by the reaction of NO with thiol radicals generated by a one-electron oxidation of a thiolate, for example via NO$_2^-$. Alternatively, NO$_2^-$ could combine first with NO, forming N$_2$O$_3$ which might subsequently react with cysteine thiolate. An additional possibility would be the transfer of haem-bound NO to a free thiol group. Once formed de novo, another physiologically relevant mechanism for S-nitrosothiol formation is trans-nitrosylation, i.e., the transfer of an NO moiety from a S-nitrosylated protein to another (Wang et al., 2006). Incidentally, the NO moiety can also be transferred to glutathione forming nitrosoglutathione, a possible transport and/or reservoir form (Lee et al., 2008). In support of an important role of nitrosoglutathione in plants, mutants for the nitrosoglutathione reductase gene exhibit important growth defects and modified responses to abiotic and biotic constraints (Vecch et al., 2005; Lee et al., 2008; Kwon et al., 2012).

**H$_2$S-MEDIATED MODIFICATION OF PROTEIN THIOLS**

The involvement of H$_2$S as a signaling molecule is only emerging both in plants and animals. Like NO, H$_2$S can play regulatory roles and modulate protein activity by binding to some protein haems. Besides, H$_2$S can promote the formation of persulfide groups, a process known as S-sulfhydration, through several potential mechanisms. H$_2$S could perform a nucleophilic attack on oxidized protein cysteine residues either as sulfenic acid, disulfide bond or as glutathione (Sunkel, 2012). Another possibility is that the sulfenyl-amide intermediate, as shown for human protein tyrosine phosphatase 1B (PTP1B), can also react with H$_2$S, the resulting persulfide being reduced by Trx (Krsihan et al., 2011). These reaction mechanisms are uncertain considering that H$_2$S is a poor reductant compared to glutathione and that it is also less abundant and reactive. Another potential mechanism involves oxidation of H$_2$S into H$_2$S$_2$ by reaction with ROS and subsequent nucleophilic attack by a protein thiolate. Similarly, to trans-nitrosylation, S-sulfinyls could eventually react with another thiol, forming either a disulfide or more likely transferring its sulfur to an acceptor protein in a trans-sulfhydration reaction. Such persulfide transfer is documented in the case of the sulfurtransferase/rhodanese and cysteine desulfurase protein families (Poin-Smits et al., 2002; Papenbrock et al., 2011).

**FUNCTIONAL SIGNIFICANCE OF REDOX PTMs**

Numerous cellular processes are dependent on thiol-dependent mechanisms. Besides the purely structural role of some disulfide bonds, various effects can be ascribed to redox changes, as they can participate in catalytic, regulatory, protective and signaling mechanisms by promoting conformational changes and influencing protein-protein interactions or subcellular localization which in fine affect the biological activity of the modified proteins. The principal redox PTMs considered here (S-nitrosylation, S-glutathionylation, S-sulfenylation, S-sulfhydration, and disulfide bond formation) are mostly regulated by the Trx and Grx families.

An interesting and well-characterized example showing how changes in the protein oligomeric state can regulate localization is represented by the pathogen-responsive NPR1 (non-expresser of pathogenesis-related genes 1) protein. In the absence of the pathogen, NPR1 is retained in the cytosol by forming covalent disulfide-bridged oligomers. Upon reduction, NPR1 becomes monomeric and is translocated into the nucleus where it activates plant immune responses (Mou et al., 2003). While S-nitrosylation of NPR1 is likely involved in this oligomerization change, depending on the physiological context, it could either promote cytosolic retention (Tada et al., 2008) or nuclear translocation (Lindermayr et al., 2010). Other well-known examples in plants of disulfide-regulated proteins concern the dark/light-dependent oxidation/reduction of critical disulfide bonds in several enzymes of the carbon metabolism including many enzymes of the Calvin–Benson cycle (Chibani et al., 2010). By definition, the so-called regulatory cysteines are not involved in catalysis but owing to their strategic position, they modify active site access or surfaces involved in protein–protein or protein–DNA interactions.

In terms of catalysis, several enzyme families, as cysteine proteases, phosphatases, antioxidant enzymes such as Prxs, glutathione peroxidases and methionine sulfoxide reductases and many oxidoreductases, use critical reactive cysteines during the catalytic act. The functioning and regeneration of some Prxs is an interesting example as it involves the formation of several cysteine oxidation forms that divert the catalytic role into H$_2$O$_2$ signaling functions. The first step of the catalytic cycle is the formation of a sulfenic acid. For isoforms possessing only the peroxidatic cysteine, this species is likely reduced by the glutathione/Grxs couple (Noguera-Mazon et al., 2006). For enzymes possessing a recycling cysteine, the formation of a sulfenic acid constitutes an intermediate for the formation of a disulfide usually reducible by Trx (Chae et al., 1994). For the so-called 2-Cys Prx subgroup, two sequence motifs surrounding the active site and specific to sensitive eukaryote isoforms, were shown to delay the formation of the intermolecular disulfide which allows, depending on H$_2$O$_2$ concentrations, the formation of a S$_2$O$_4^-$H. It is now accepted that this overoxidation leads to the transient inactivation of the peroxidase activity allowing the local accumulation of H$_2$O$_2$ which can then promote appropriate signaling pathways (Wood et al., 2005; D’Autreaux and Toledoano, 2007). While SO$_2$H is mostly irreversible oxidation forms, an enzyme called sulfiredoxin (Srx) is able to catalyse the ATP-dependent reduction of the SO$_2$H formed on these sensitive 2-Cys Prxs (Bitinas et al., 2003). This mechanism is likely valid for plants as a Srx, dual-targeted to chloroplasts...
and mitochondria, can specifically reduce SO₂H formed on C-Cys Prx but also on plant PrxII or mammalian PrxV (Rey et al., 2007; Iglesias-Barrera et al., 2010, 2011). Interestingly, the SO₂H reduction proceeds via the formation of a phosphoryl intermediate on the sulfinyl moiety, attacked by the catalytic cysteine of Srx finally forming a thiosulfinate intermediate between Prx and Srx (Jenenson et al., 2009; Roussel et al., 2008).

To further illustrate cysteine-based signaling mechanisms in response to peroxides, it is worth mentioning that the DNA binding activity of numerous transcription factors in microbes (bacteria or fungi), such as OxyR, OhxR, API, or CreI is regulated by the primary formation of a sulfenic acid which is often transformed into a disulfide bond (D’Autreaux and Toledano, 2007; Cheng et al., 2012). To date, few examples of such signaling functions are known in plants.

The biochemical characterization of recombinant proteins coupled to redox proteome studies often led to the observation that a single protein, either a metabolic enzyme or a signaling protein can undergo a plethora of redox PTMs. In this context, it is not trivial to differentiate between protective and signaling purposes. This is well exemplified in mammals for glycerolaldehyde-3-phosphate dehydrogenase (GAPDH), PTP1B or the p65 subunit of the NF-kB transcription factor. In these proteins, the critical cysteine can react with H₂O₂, NO and H₂S derived forms, forming sulfenic acid, nitrosotiothiol or persulfide intermediates, respectively, that can eventually form sulfinyl-amide or glutathione adduct or interchange with each other. Whereas most of these reversible modifications lead to protein inactivation, S-sulfhydration can be interchanged with each other. Whereas muscles use these reversible modifications to freeze a covalent interaction with their targets or could promote trans-nitrosylation reactions, this approach may have detected most reversible redox PTMs (Mitchell and Martinez, 2005; Benhar et al., 2008; Krishnan et al., 2011; Bedhomme et al., 2012).

Most of the current methods designed to detect reactive cysteines and redox PTMs are indirect and are based on the differential alkylation of reduced and oxidized thiols (Figure 2). They require an initial step of thiol alkylation of free unreactive cysteines using generally N-ethylmaleimide (NEM), iodoacacetamide (IAM) for irreversible modifications or MMTS (methyl methane-thiosulfonate) for reversible modifications. In a second step, the various types of PTM are reduced by general tris(2-carboxyethyl)phosphine, dithiothreitol (TCEP, DTT) or specific chemical compounds or enzymes. Finally, nascent thiols are labeled by derivation with biotinylated- or fluorescent- forms of these alkylation reagents. The biotinylated proteins are recovered on avidin columns and identified preferentially using a gel-free method by LC-MS-MS. Until recently, this was rather a qualitative inventory but quantitative thiol-trapping techniques (namely OxIS-CAT, isotope-coded affinity tag for the identification of Oxidized cysteines, isoTOP-ABPP, isotopic tandem orthogonal proteolysis-activity-based protein profiling), essentially based on the use of isotopic light¹²C- and heavy¹³C-forms of IAM, have been developed to identify the site(s) of modifications and assess the degree of modification and reactivity (Hagglund et al., 2008; Leichert et al., 2008; Marino et al., 2010; Werrapana et al., 2010).

For sulfenic acids, reduction was initially achieved through arsenite but other probes derived from dimedone, namely DAZ-2 and DYN-2, have recently been engineered (Leonard and Carroll, 2011; Ross and Mersel, 2011). For S-glutathionylated and S-nitrosylated proteins, reduction is usually performed using Grx and ascorbate, respectively. The latter strategy was called biotin switch method (Jeffrey and Snyder, 2001). For instance, a modified biotin switch omitting ascorbate was used for isolating S-sulfhydrated proteins (Mustafa et al., 2009). Some alternative methods for assessing redox PTMs or recent improvement of these
Besides the wish for detecting basal redox PTMs, experiments are usually designed to assess in vivo redox changes after applying an oxidative stress treatment. Most current approaches to detect redox PTMs rely on the same three-step strategy. The first step consists of blocking free thiol groups with alkylating agents such as N-ethylmaleimide (NEM), iodoacetamide (IAM) and its isotopically light \(^{12}\)C-derivative or methyl methanethiosulfonate (MMTS) (1). The second step consists of the reduction of reversibly oxidized cysteine residues (2). General reductants as dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine hydrochloride (TCEP) are used to identify all types of oxidized cysteines. In contrast, selective chemical agents (ascorbate, arsenite, dimedone) or enzymes (glutaredoxins, thioredoxins) are used for the reduction of specific redox PTMs. Finally, the third step corresponds to the labeling of liberated thiol groups by reaction with biotinylated-, fluorescent- or isotopically heavy \(^{13}\)C-derivatives of alkylating reagents mentioned previously, keeping in mind that fluorescent reagents are rather devoted to the detection of modified proteins, whereas biotinylated reagents are devoted to protein enrichment for subsequent mass spectrometry analyses (3). Readers interested by the qualitative iCAT approach can obtain more details in a recent review (Leonard and Carroll, 2011). Interestingly, because MMTS does not seem to reduce persulfide groups, Mustafa et al. (2009) demonstrated that these persulfide groups could be detected without the reduction step if MMTS is used for the first alkylation step (4).

differential alkylation methods can be found in (Gao et al., 2009; Wang and Xian, 2011; Astier and Lindermayr, 2012). The proteins identified through the different methods show only partial overlap, indicating that a significant portion of modifications are specific, being dependent on the protein local environment and/or on the applied oxidizing conditions. Long lists of plant proteins undergoing various redox PTMs, especially glutathionylation and nitrosylation, and cellular processes affected were recently published (Gao et al., 2009; Astier et al., 2012; Paul and Snyder, 2012; Zaffagnini et al., 2012).

All these methods are usually subject to the same limitations. Alkylation, reduction and labeling are usually performed on cell lysates and though protein extraction is performed using acidic thioredoxin, or sometimes anaerobic conditions, it does not entirely preclude that modifications of the cysteine redox state occur during the procedure or that the modifications are insufficiently trapped. To circumvent this problem, cell permeable probes were developed, in particular for sulfenic acids (Leonard and Carroll, 2011). As explained above, another major drawback of such studies concerns the specificity of a given reductant for a given PTM as also discussed for S-nitrosothiol in (Wang and Xian, 2011). This will clearly need to be addressed in the future. Moreover, protein abundance is often a limiting factor, mostly for gel-based methods. Possibilities to circumvent this problem are to perform pre-fractionation or to use biotin affinity for avidin in order to increase the amount of modified proteins and decrease
complexity of the sample. Another very important question that often remains unanswerd is to determine the site of modification and to what extent a given cysteine is modified. Answering this question requires ICAT-derived gel strategies that were used only in rare cases (Hagglund et al., 2008; Leichert et al., 2008).

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