# Protein Oxidative Modifications

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**Abstract** Proteins are molecules especially susceptible to oxidative modifications owing to their abundance and reactivity to radicals. Amongst the protein oxidative (redox) changes, carbonylation of the molecules represents an irreversible process that leads to the lost of protein functionality. The bulk of carbonylated proteins are produced as a result of metal oxidative stress induction in plants. In addition, metal ions-catalyzed oxidation (MCO) systems have been used especially for the introduction of carbonyl groups in the protein molecules in vitro. The mechanism underlying protein carbonylation for redox active metals is the direct catalysis of reactive oxygen species (ROS) generation, while metals considered redox inactive act in decreasing the antioxidant defence system. Despite the fact that protein carbonylation is associated with general and random processes; recent advances indicate a great degree of selectivity in the protein oxidation process. In turn, there are proteins, such as catalase, that respond to metal-induced oxidative stress by regulating the translation of isoforms and thus inducing the synthesis of new subunits less sensitive to oxidation. Further, the intracellular level of oxidized proteins is the product of a balance between the rate of oxidation and the rate of degradation of proteins. Metals can alter plant cell capacity for removing damaged proteins. As part of the proteolytic system, the 20S proteasome is responsible for the proteolysis of the carbonylated proteins. The 20S proteasome activity is regulated through oxidative modification of the proteasome itself, where a moderate 20S protein oxidation increases its activity, but a severe oxidative condition decreases it, concomitantly producing oxidized protein accumulation. The widespread occurrence of protein modifications and regulated proteolysis, as well as the existence of regenerative mechanisms of oxidative modifications, is presented.

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## 1 Proteins as Molecular Targets of Oxidative Reactions

Proteins are one of the main cellular macromolecules susceptible to oxidative modifications. One of the reasons for this consideration is the abundance of proteins in living organisms. Proteins comprise the major, non-water, component in biological systems, at the tissue, cellular or biological fluid levels. For example, in a typical plant cell, proteins represent about 30% of the total dry weight (DW), but they are between 60 and 70% of DW when the cell wall and the starch are excluded (Taiz and Zeiger 2010). Moreover, proteins are found ubiquitously in the cell, not only in soluble forms but attached to or forming part of biological membranes. On the other hand, a further indication of the importance of proteins as targets for oxidants is the rate constants for the reaction of a range of reactive radicals with proteins with respect to other biological macromolecules (Davies 2005; Xu and Chance 2007). Oxidised proteins accumulation has been considered a cause of cellular damage (Berlett and Stadtman 1997). However, considering that there are many types of protein oxidative modifications and proteins play a variety of functions in the cell, ranging from catalytic activities, structural features or regulation of several processes, it is possible to assume that protein oxidation can directly modify cell structure, signaling and metabolism.

### *1.1 Protein Oxidative Products*

Protein oxidation is defined as the covalent modification of a polypeptide induced either directly by reactive oxygen species (ROS) or indirectly by reaction with secondary by-products of oxidative stress (Møller et al. 2007). One of the most common oxidative protein modifications is the formation of carbonyl derivatives, in which the amino-acid side chains, mainly histidine, arginine, lysine, proline, threonine, and tryptophan residues, are converted by the action of ROS to aldehyde or keto groups (Møller et al. 2007). The majority of the ROS-mediated reactions are hydrogen atom abstraction from α carbon position of the protein or radical transfer from side chains (Davies 2005; Xu and Chance 2007). In addition to the direct action of ROS on amino acids, protein carbonyl derivatives can be formed indirectly on lysine, cysteine, and histidine by forming adducts with reactive carbonyl compounds on carbohydrates (glycoxidation products), lipids, and advanced glycation/lipoxidation end products (Madian and Regnier 2010). The rise in the number of carbonyl group per protein molecule is called protein carbonylation. Protein carbonylation is considered an irreversible process because damaged proteins are unable to be enzymatically repaired in the cell (Nyström 2005). The classical method developed for the detection and quantification of protein carbonyl groups involves derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine (2,4-DNPH) and the subsequent immunodetection of the resulting hydrazone using monoclonal or polyclonal antibodies (Levine et al. 1990, 1994; Yan et al. 1998).

Detectable protein carbonyl content by assessment with 2,4-DNPH is one of the most common assays used to quantify oxidative stress in vivo. Tryptophan oxidation is another apparently irreversible aminoacid modification that involves the formation of *N*-formylkynurenine, a breakdown product of tryptophan caused by dioxygenation and ring breakage (Finley et al. 1998; Møller and Kristensen 2006). Polypeptides with sulfur-containing residues suffer oxidative modifications that involve cross-linking or the occurrence of sulfur-containing derivatives like sulfenic, sulfinic, sulfonic, or sulfoxide forms (Davies 2005). The oxidation of cysteine thiol group to disulfide is considered a reversible modification where the reduced form can be regenerated by the thioredoxin (Trx) or glutaredoxin systems. In the former, the enzyme thioredoxin reductase transfers electrons from NADPH to Trx via a flavin carrier. Glutaredoxin is also able to reduce disulfide bonds, but using GSH as an electron donor (Møller et al. 2007; Rey et al. 2007; Rouhier et al. 2008). Further oxidation of cysteine to cysteic acid ($R\text{-}SO_3H$) appears to be irreversible and damaging to the protein (Ghezzi and Bonetto 2003). Like cysteine, methionine belongs to the most easily oxidized amino acids owing to the presence of sulfur. The first stage of oxidation leads to methionine sulfoxide ($R\text{-}SOCH_3$, abbreviated as MetSO) a biologically occurring product (Vogt 1995; Hong and Schöneich 2001). Oxidation of methionine to MetSO, which results in modification of activity and structure for many proteins, is reversed by an enzyme present in most organisms named methionine sulfoxide reductase (MSR; EC 1.8.4.11). This enzyme catalyzes the thioredoxin-dependent reduction of MetSO back to the correct Met residue. Two types of MSR has been isolated, MSRA specific to the MetSO S-enantiomer, and MSRB, which catalytically reduces the MetSO R-enantiomer. Both enzymes are required, since the cell oxidation of Met residues at the sulfur atom results in a racemic mixture of the two stereoisomers (Rouhier et al. 2008). *MSRA* and *MSRB* genes encode different MSR isoforms and are found to be relatively ubiquitous, with homologues found in many different organisms from bacteria and yeast to insects and mammals (Rouhier et al. 2006). Oxidation of Met residues can block phosphorylation-induced regulation of proteins (Hardin et al. 2009). The highest level of Met oxidation, $R\text{-}SO_2CH_3$ (sulfone) appears to be irreversible.

## 2 Metals as Responsible of Protein Oxidation

Different methods are used to generate ROS capable of producing protein oxidation in vitro (Xu and Chance 2007). Among them, one of the most common and potent mode of inducing protein oxidation derives from the metal ions-catalyzed oxidation (MCO) systems. In the same sense, the natural presence of metal in many protein structures can increase the molecule susceptibility to suffer oxidative modifications.

## 2.1 Metals Ions-Catalyzed Oxidation Systems

A metal ions-catalyzed oxidation (MCO) system comprises the presence of transition metals, such as Fe (III) or Cu (II), and $H_2O_2$. In a number of metal-catalyzed oxidation (MCO) systems, $H_2O_2$ is formed by the catalysis of different electron donors in the presence of $O_2$ and Fe (III) or Cu (II). Reducing agents include nonenzymatic autooxidizable substrates, like ascorbate, or sulfhydryl compounds, and enzymatic systems, e.g., NAD(P)H dehydrogenases, xanthine oxidase, and cytochrome P450 reductases (Stadtman 1993). Some metal−chelator complexes, e.g., ethylenediaminetetraacetate (EDTA)–Fe(II) are more effective than the unchelated metals in MCO systems, producing greater amounts of radicals and at fast rates (Stadtman and Berlett 1991). EDTA also increases the solubility of the metal ions thus allowing the reaction to be carried out at neutral pH. The action of MCO systems on amino acids like Arg, Pro, His, and Lys were reported to result in the formation of a carbonyl derivative which provides a means for monitoring the protein oxidation process (Berlett and Stadtman 1997; Schöneich 2000; Temple et al. 2006). The ability of MCO systems to catalyze protein carbonylation is attributable to the hydroxyl radicals ($HO^{\cdot}$) generation via Fenton-type chemistry. The Fenton reaction is comprised in the Haber–Weiss reaction (Fig. 1). Fe (II) and Cu (I) ions bind to a specific metal binding site within the protein and react with $H_2O_2$ to generate $HO^{\cdot}$, which then attacks the amino acid residues near the metal binding site and in turn leads to the production of a carbon radical (Stadtman 1993). The quantitatively most important carbonyl products of the metal-catalyzed oxidation reaction are glutamic semialdehyde from arginine and proline, and aminoadipic semialdehyde from lysine (Requena et al. 2001, 2003). Metal ions-catalyzed oxidation system also oxidizes tyrosine residues with the concomitant formation of dityrosine (Kato et al. 2001). In this case, since Cu(I)/$H_2O_2$ oxidative conditions did not lead to the formation of dityrosine, the MCO system dityrosine production, it is unlikely to occur via Fenton chemistry (Ali et al. 2004).

## 2.2 Metalloproteins Susceptibility to Oxidative Stress

Metals are natural components of many proteins. The main biological roles played by metals in living organisms are carried out in relationship to proteins. Almost half

$$
\begin{array}{rl}
O_2^{\cdot} + Fe^{3+} \rightarrow Fe^{2+} + O_2 & \\
Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^{-} & \text{(Fenton Reaction)} \\
\hline
O_2^{\cdot} + H_2O_2 \rightarrow O_2 + HO^{\cdot} + OH^{-} & \text{(Haber-Weiss Reaction)}
\end{array}
$$

**Fig. 1** The Haber–Weiss reaction generates $HO^{\cdot}$ from $H_2O_2$ and $O_2^{-}$. In the presence of metals such as iron or copper, hydrogen peroxide ($H_2O_2$) can be converted into a highly reactive species hydroxyl radical ($HO^{\cdot}$) in a chemical reaction called Fenton reaction. The oxidized metal can undergo a re-reduction in a subsequent reaction with superoxide anion radical ($O_2^{-}$)

of all proteins are associated with metal ions to perform their specific functions, and the majority of these metalloproteins contain transition metals as part of their structure (Dudev and Lim 2003). The natural selection of proteins metal cofactors has been based on both their unique physicochemical properties and their bioavailability in the Earth's crust (Williams 1997). The presence of the metal ion adds new functionality to proteins and helps proteins to catalyze some of the most difficult biological reactions. Protein reactivity is finely tuned by using different metal ions, different redox states of the same metal ion, or different ligands and geometric arrangements. For example, metalloenzymes perform functions such as redox reactions that cannot easily be carried out by the limited set of functional groups found in amino acids. Thus, metalloproteins participate in the most important biochemical processes including respiration, nitrogen fixation and oxygenic photosynthesis. The available data demonstrate that metal cofactors interact with the host protein mainly in two different ways: some bind to a well-structured cavity in a folded protein, in this case protein largely directed the final structure of the metalloprotein; others bind to the unfolded polypeptide and assist in folding, in this case metal cofactors largely directed the final structure of the metalloprotein (Kharenko and Ogawa 2004; Wilson et al. 2004). Nowadays, there are considerable interests in creating metal binding sites in designed proteins to understand the structural roles of metal ions and to design new metalloproteins with useful functions (Dudev and Lim 2008). Metal-catalyzed oxidation of proteins binds a cation capable of redox cycling to a metal-binding site on the protein. At present, the evidence suggests that metalloprotein oxidative modifications occur in functional groups of amino acid residues at or near the metal-binding site (Hong and Schöneich 2001; Sharp et al. 2003; Bridgewater et al. 2006). For example, amino acid residues nearby of the metal center(s) in Cu,Zn-superoxide dismutase (Cu,Zn-SOD; EC 1.15.1.1) can be selectively oxidized by addition of $H_2O_2$, which generates reactive oxygen species via a Fenton-like reaction upon reaction with Cu ions (Stadtman 1993; Kurahashi et al. 2001; Bridgewater and Vachet 2005). The combination of MCO system with mass spectrometry (MS) constitutes an interesting method for determining the coordination structure of metalloproteins (Bridgewater et al. 2006; Sadineni et al. 2006). Moreover, the amino acid of the metal coordination in enzymes active sites can be detected using as strategy the exchange of the metal by Cu (Miyazaki et al. 2009).

## 3 Metal Stress in Plants Is Associated to an Increase in Protein Carbonylation

The excess of essential metals, or the presence of those considered not essential for plant survival – even though in low concentration – has been associated with an imbalance between ROS production and antioxidant defence system capacity in the cell with the concomitant oxidative stress generation (Gallego et al. 1996, 2002;

Sharma and Dietz 2009). In this context, since carbonylation is an irreversible protein modification, it is widely used as oxidative stress biomarker. Protein oxidation constitutes a post-translational modification (PTM) observed in different parts of the plant as result of metal toxicity. The bulk of protein carbonylation is produced as a result of the metal-induced oxidative stress in plants as shown in Table 1, which lists those metals that produce protein carbonylation on different plant species. It is important to highlight that protein carbonylation can be produced to different extents in the same plant species subjected to metal stress. This effect

**Table 1** Different plants species subjected to metal stress with marked increase on carbonyl group content

| Plant specie | Part of the plant | Metal | Exposure time (d) | Concentration (μM) | Reference |
|---|---|---|---|---|---|
| *Cucumis sativus* | Leaves | $Al^{3+}$ | 10 | 1–2,000 | Belmonte Pereira et al. (2010) |
| | Seedlings | $Cd^{2+}$ | 10 | 400–1,000 | Gonçalves et al. (2007) |
| | Seedlings | $Hg^{2+}$ | 10–15 | 250–500 | Cargnelutti et al. (2006) |
| *Hordeum vulgare* | Leaves | $Cu^{2+}$ | 5 | 150–1,500 | Demirevska-Kepova et al. (2004) |
| | Leaves | $Mn^{2+}$ | 5 | 183–18,300 | |
| *Pisum sativum* | Leaves | $Cd^{2+}$ | 14 | 50 | Romero-Puertas et al. (2002) |
| *Zea mays* | Leaves | $Cd^{2+}$ | 1 | 100 | Pena et al. (2007) |
| | Shoot/root | $Cd^{2+}$, $Hg^{2+}$ | 7 | 6–30 | Rellán-Álvarez et al. (2006) |
| *Helianthus annuus* | Callus | $Cd^{2+}$ | 28 | 150 | Gallego et al. (2005) |
| | Cotyledons | $Cd^{2+}$ | 4 | 100–200 | Gallego et al. (1999) |
| | Cotyledons | $Cd^{2+}$, $Cu^{2+}$, $Al^{3+}$, $Co^{2+}$, $Pb^{2+}$, $Cr^{3+}$, $Ni^{2+}$, $Hg^{2+}$ | 4 | 100 | Pena et al. (2006a) |
| | Leaves | $Cd^{2+}$ | 4 | 100–300 | Pena et al. (2006b) |
| *Medicago sativa* | Seedlings | $Cd^{2+}$, $Hg^{2+}$ | 7 | 30 | Ortega-Villasante et al. (2005) |
| *Allium cepa* | Roots | $Al^{3+}$ | 0.6 | 200 | Murali Achary et al. (2008) |
| *Triticum durum* | Roots | $Cd^{2+}$ | 3–7 | 20–40 | Paradiso et al. (2008) |
| *Triticum aestivum* | Shoots | $Cu^{2+}$, $Cd^{2+}$, $Ni^{2+}$ | 7 | 75 | Gajewska and Skłodowska (2010) |
| *Arabidopsis thaliana* | Leaves | $Cd^{2+}$ | 6 | 50 | Polge et al. (2009) |
| *Solanum lycopersicum* | Leaves | $Cd^{2+}$ | 3–10 | 300 | Djebali et al. (2008) |

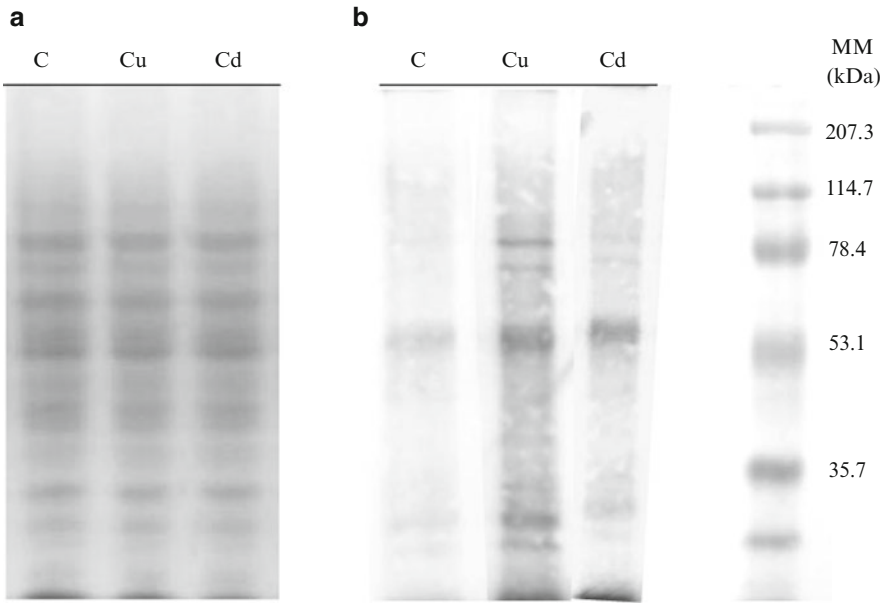


**Fig. 2** One-dimensional PAGE of carbonylated proteins from wheat roots of control and metal-treated plants. The same protein amount was loaded per lane. (**a**) Coomassie Brilliant Blue (protein stain) and (**b**) anti-DNP immunoassay (carbonyl groups) are shown. *C* control, *MM* molecular mass marker

could be attributable to differences in the studied organs, the age of the plant, the metal concentration and the exposure time. The qualitative pattern obtained by immunodetection of carbonyl residues on plants treated with toxic metal concentrations shows a general protein oxidation profile, including proteins from low to high molecular weight (Romero-Puertas et al. 2002; Pena et al. 2008). As an example, Fig. 2 shows a typical immunoblot where proteins were tagged with 2,4-dinitrophenylhydrazine (DNPH) and detected with anti-DNP antibodies.

## *3.1 Metals Catalyze Reactive Oxygen Species Generation Inside the Cell*

The mechanism involved on protein carbonylation process for redox active metals, like Fe and Cu ions, is related to the capacity of these metals to directly catalyze ROS generation (Stohs and Bagchi 1995). Other metals considered non-redox active, like Cd, are able to alter the redox cell status mainly by modifying the antioxidant defence system and thus increasing ROS cell level (Schützendübel et al. 2001; Romero-Puertas et al. 2004; Garnier et al. 2006). It could be inferred that ROS accumulation produced by metal stress is responsible for protein oxidation

since the same targeted proteins were detected in pea (*Pisum sativum*) plants treated with Cd and treated with $H_2O_2$ (Romero-Puertas et al. 2002). Protein carbonylation is a non-enzymatic process and has been suggested to be rather non-specific and would be expected to be at random. However, new evidence has recently been found in relation to the selectivity of the protein oxidation process inside the cell. In this sense, a higher degree of protein oxidation linked to cellular compartments associated to ROS production would be expected. In plants, chloroplasts and peroxisomes are the main sources of ROS in autotrophic tissues under light conditions (Foyer and Noctor 2003; Gill and Tuteja 2010). On the other side, ROS come mostly from mitochondria in heterotrophic tissues or in green cells on darkness (Foyer and Noctor 2003; Gill and Tuteja 2010). In plants under metal stress, proteins from organelles appear to be particularly susceptible to oxidative modification. For example, in Cd-treated pea plants, the degree of protein carbonylation was proportionally higher in isolated peroxisomes compared to the whole-plant extracts (Romero-Puertas et al. 2002). Analysis of the soluble matrix fraction of rice (*Oryza sativa*) leaf mitochondria showed that proteins markedly affected by MCO system in vitro were particularly prone to oxidation in vivo (Kristensen et al. 2004). These authors further identified a group of mitochondrial proteins that are particularly susceptible to mild oxidation in vitro (Kristensen et al. 2004). The molecular basis for the apparent sensitivity of some proteins to carbonylation is not well understood, but it is likely that MCO is an intrinsic problem for proteins containing transition metals (Nyström 2005). In this sense, it has recently been demonstrated a strong correlation among the sets of immobilized metal affinity chromatography-interacting proteins, proteins predicted to contain metal-binding motifs, and protein sets known to be oxidized or degraded during abiotic stress in isolated *Arabidopsis thaliana* mitochondria (Tan et al. 2010).

## 4 Metals Can Alter Cell Metabolism by Mediating Protein Carbonylation

Proteins serve vital roles in the cells by catalyzing process, regulating structure or participating in signaling processes. Thus, protein oxidative modification can therefore rapidly affect plant metabolism. Analysis of the effect of MCO system on plant proteins allowed to study the oxidative modification of proteins in vitro, its implication on protein functionality and to assume the consequences of the oxidative stress in the whole plant. For example, the large subunit (LSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) purified from wheat (*Triticum aestivum*) was broken down in the catalytic site by exposition to MCO system into two polypeptides (Ishida et al. 1999). Identical fragmentation of the protein was described in intact leaves of cucumber (*Cucumis sativus* L.) under chilling-light conditions (Nakano et al. 2006).

Moreover, different cell processes can be altered by metal oxidative inactivation of enzymes. For example, Kranner and Colville (2011)) proposed that metals can compromise seed germination by inducing oxidative damage to hydrolytic enzymes and storage proteins. Another metabolic process that has been greatly studied is the inactivation by MCO system of the enzymes involved in the nitrogen assimilation cycle. In the assimilation pathway, ammonium is incorporated into glutamine by glutamine synthetase (GS; EC 6.3.1.2), which is then converted to glutamate by glutamate synthase using 2-oxoglutarate (GOGAT; EC 1.4.1.13) (Lancien et al. 2000). Moreover, the GS/GOGAT cycle connects nitrogen and carbon metabolism in cells. Glutamine synthetase from *Escherichia coli* has been shown to be regulated by MCO, inducing an inactivation of the enzyme that precedes selective degradation by specific proteases (Kim et al. 1985). Besides GS from *E coli*, diverse glutamine synthetases have been described to undergo inactivation by some kind of oxidative modification produced by MCO system, as the enzymes of the green alga *Monoraphidium braunii* (Humanes et al. 1995) or the marine oxyphotobacterium *Prochlorococcus* (Gómez-Baena et al 2001). Ortega et al. (1999) demonstrated that GS from soybean (*Glycine max*) root extract subjected to MCO systems was not only inactive but more susceptible to degradation than non-oxidized GS. A decrease of both GS and GOGAT activities concomitant with the oxidative stress generation has been shown in the GS/GOGAT cycle in nodules and roots of soybean plants after Cd exposure (Balestrasse et al. 2001, 2003). In this case, the inactivation of the nitrogen assimilation cycle was closely related to the increase in the carbonylation level of GS and GOGAT proteins (Balestrasse et al. 2006).

## *4.1 Regulation of the Translation of Isoforms: The Catalase*

In many cases, there is a lack of information not only about the effect of oxidation on protein activity or function but also in the form that plant metabolism copes with protein oxidative modification. In this regard, an interesting mechanism consisting of increasing the synthesis of protein subunits less sensible to oxidation has been described in plants for the enzyme catalase. Catalase (CAT; EC 1.11.1.6) is one of the main antioxidant enzymes that catalyzes the conversion of hydrogen peroxide to $O_2$ and $H_2O$, which in plants is localized inside peroxisomes. In plants, catalase activity shows a great degree of susceptibility to metal stress (Gallego et al. 1996, 2002; Balestrasse et al. 2001; Pandey and Sharma 2002; Singh et al. 2006). Moreover, catalase has been involved in Cd tolerance enhancement in plants, as was demonstrated in transgenic tobacco over-expressing a Cd-induced catalase cDNA from the hyperaccumulator species *Brassica juncea* (BjCAT3) (Guan et al. 2009). In contrast, catalase deficiency has been related to cadmium toxicity in tobacco plants (Iannone et al. 2010).

Catalase enzyme is encoded by a small unlinked nuclear gene family, and heterotetramers of CAT are formed when polypeptides encoded by distinct genes are simultaneously expressed in the same plant cell. In sunflower (*Helianthus*

*annuus* L.), at least eight isoforms have been identified: CAT1 to CAT8 (Eising et al. 1990), where the biogenesis of the subunits is controlled by four different genes (*CATA1* to *CATA4*). Studies of CAT activity, protein expression, state of protein oxidation and CATA transcripts accumulation showed that inactivation of catalase produced by cadmium in sunflower was due to a mechanism involving the oxidation of CAT protein (Azpilicueta et al. 2007, 2008). Therefore, damaged CAT might cause the augmentation of free radical-mediated oxidative damage to other proteins, as has been observed under $H_2O_2$ treatment in peroxisomal enzymes of castor bean (*Ricinus communis*) endosperm (Anand et al. 2009). Interestingly, under redox stress conditions generated by metal ions, sunflower plant cells maintain catalase activity by regulating isoforms translation. The induction of transcription of *CATA3* and *CATA4* genes derived in CAT isoforms enriched in the subunit less sensitive to the oxidative damage (Grotjohann et al. 1997). Thus, differential expression of catalase genes under metal treatment in sunflower leads to the synthesis of enzyme isoforms less sensitive to oxidation, which prevent enzyme inactivation (Azpilicueta et al. 2007, 2008). Amino acid substitutions that occur in strictly conserved positions of *CATA* gene products can be expected to contribute to catalase ability to resist oxidative stress conditions (Engel et al. 2006).

## 5 Carbonylated Protein Degradation

Oxidative modifications of proteins result in physical changes in the protein structure that lead to dysfunction, inactivation, cross-linking of polypeptide chains or chemical fragmentation of the protein. The accumulated oxidized proteins tend to form insoluble high molecular weight aggregates, also known as inclusion bodies or plaques, all of which occur as a result of the increased level of hydrophobic bonds, ionic and covalent bonds that are potentially cytotoxic and can actively influence cellular metabolism (Grune et al. 2004; Davies 2005). Thus, oxidative stress conditions trigger an increase in protein turnover and degradation. The bulk of carbonylated proteins created must be degraded to prevent accumulation of the unfolded protein forms which are highly toxic for the cell. Proteolysis in plants not only regulates protein processing and intracellular protein levels, but removes abnormal or damaged proteins from the cell (Buchanan et al. 2000). So, the intracellular level of oxidized protein is the product of the balance between the rate of protein oxidation and the rate of oxidized protein degradation. The proteolytic process depends on many variables that determine the concentrations and/or activities of the proteases that degrade oxidatively damaged proteins, and many other factors like the presence of metal ions, endogenous inhibitors and regulatory proteins that modify the proteolytic activity (Berlett and Stadtman 1997). Moreover, a rise in the protein degradation could be due either to an increase in proteolytic enzyme activities or to modifications in the protein that make it a more suitable substrate for proteases already present. In this sense, protein carbonylation initially makes the molecule more susceptible to proteolytic attack, though,

accumulated oxidized proteins tend to form high molecular weight aggregates with increasing resistance to proteolysis (Grune et al. 2004; Davies 2005). At the cellular level, protein breakdown is a complex process that includes proteases mainly localized in vacuole and other cell organelles, and the ubiquitin-proteasome system (UPS) active in both the cytoplasm and nucleus.

## *5.1 Role of Proteases*

Proteolytic enzymes are classified as exopetidases or endopeptidases depending on the site of the hydrolytic cleavage of the peptide chain. Exopeptidases can be classified on the basis of the reaction that they catalyze. Depending on the hydrolyzed substrate, they are classified as dipeptidases (EC 3.4.13) and tripeptide aminopeptidase (EC 3.4.11.4), or can be characterized by the terminal group attacked as aminopeptidases (EC 3.4.11), carboxypeptidases (3.4.16−18) and omega-peptidase (EC 4.3.19). Exopeptidases are also classified according to the number of amino acid residues released as peptidyl-dipeptidase (EC 4.3.15), dipeptidyl-peptidase or tripeptidyl-peptidases (EC 3.4.14) (Dalling 1986). Endopeptidases, also known simply as proteases, are classified according to their catalytic mechanism. Different classes of proteases found in plants are serine endopeptidases (EC 3.4.21), cysteine endopeptidases (EC 3.4.22) and aspartic endopeptidases (EC 3.4.23) according to the amino acid in their active sites. On the other hand, there are the metalloendopeptidases (EC 4.3.24) which contain a metal such as $Zn^{2+}$, $Co^{2+}$ or $Mn^{2+}$ in the reaction center (Dalling 1986; Palma et al. 2002). The proteolytic system of plants comprises a large number of proteases. For example, in the genome of *Arabidopsis thaliana* more than 650 nucleotide sequences encoding proteases of different catalytic classes have been identified, but only some of them have a known function for the life of the plant. Moreover, 41 sequences have been described as known or putative protease inhibitors (Rawlings et al. 2010). The most common assays for determining total endoproteolytic activity use non-specific substrates such as gelatin, hemoglobin, casein or albumin, and then measure the amino acids released by hydrolysis. The use of chromogenic substrates, like azocasein, enables direct measurement of diazotized fragments that are soluble in trichloroacetic acid. Moreover, the immobilization of the protein substrate in SDS-polyacrylamide gels (SDS-PAGE) can be used to detect endoprotease activity and provide an estimated molecular weight. In this case, protease either has not to be sensitive to the presence of SDS, or has to be renatured after denaturation by SDS (Simpson 2001).

There are two important subjects to be highlighted with respect to the response of the proteolytic system to metal stress in plants: the effect of metals on protease activity and the relationship between the proteolytic activity and the level of carbonylated proteins. The action of metals on the proteolytic activity in sunflower has been shown to be independent of metal redox capacity or type of metal (Pena et al. 2008). Metal stress affected plant endoproteolytic activity but their effects

could not be generalized. Moreover, the response of the proteolytic activity and the accumulation of carbonylated proteins lead to contrasting observations depending on the metal, plant or organ. In sunflower plants subjected to cadmium stress, oxidized protein accumulated in cotyledons and leaves even though protease activity increased (Pena et al 2006a, b, 2007). Carbonyl groups contents, global endopeptidase activity, specific protease activities and their transcript levels increased in the leaves of *Arabidopsis* plants exposed to cadmium (Polge et al. 2009). However, in axes and seeds of *Sorghum bicolor,* increasing Cd concentrations had a negative effect on protease activity, although additional new isozymes were induced (Kuriakose and Prasad 2008). Cadmium-treated pea plants did not show any significant change in the total endoproteolytic activity in leaf extracts, but increased protease activity in leaf peroxisomes as a consequence of the overall increase in the activity of the endopeptidases isozymes inside the organelle (Romero-Puertas et al. 2002). In spite of the results mentioned above for *S. bicolor* (Kuriakose and Prasad 2008) and pea plants (McCarthy et al. 2001; Romero-Puertas et al. 2002), cadmium produced an increase in protein carbonylation in both species. Cadmium treatment decreased protease activity without accumulation of carbonyl group content in roots of tomato (*Solanum lycopersicon*) plants, but both parameters increased in leaves of Cd-treated plants (Djebali et al. 2008). Copper-exposed wheat roots and shoots showed an enhanced protein carbonylation that corresponded to the induction of protease activity. While no activation of proteolysis was observed in wheat plants treated with Cd and Ni, accumulation of carbonylated proteins was only detected in shoots (Gajewska and Skłodowska 2010). The presence of putative proteases involved in degradation of the oxidatively damaged proteins was suggested in isolated mitochondria of *Arabidopsis* plants using $H_2O_2$ as a model stress (Sweetlove et al. 2002) whereas in root mitochondria of cucumber (*Cucumis sativus* L.) mutant lines (MSC16) with different mitochondrial genome rearrangement, low carbonyl group content was accompanied with a high protease activity (Juszczuk et al. 2008). Thus, different behaviors of the key elements of protein turnover machinery in response to metal stress has been observed, and although unspecific proteases could recycle oxidative proteins, their activities were not strong enough to diminish oxidized protein content in plant cells. Proteolysis susceptibility to metals should involve more than one mechanism, including direct effects on enzyme structure and/or functionality, as well as indirect mechanisms, such as increased levels of protein oxidation (Pena et al. 2007). Moreover, metals could have an impact on protease inhibitors that regulate endogenous proteolytic activity, a topic extensively surveyed under biotic stress conditions (van der Hoorn and Jones 2004; Farrokhi et al. 2008).

## *5.2 Role of 20S Proteasome*

The selective removal of proteins in both the cytoplasm and the nucleus of eukaryotes involve the covalent attachment of multiple ubiquitin (Ub) molecules

to protein substrates targeted for breakdown. The binding of the core protease 20S with the regulatory particles 19S forms the 26S proteasome, a 2-MDa ATP-dependent multi-subunit protease complex, which is responsible for the degradation of ubiquitinated proteins, releasing Ub molecules for recycling (Smalle and Vierstra 2004; Jung et al. 2009). The 20S proteasome core has a molecular weight of about 700 kDa and is arranged as a cylindrical stack of four heptameric rings with two central rings composed by of seven β-type subunits, and two peripheral rings composed of seven related α subunits. In the center of the barrel there is channel with an active site for protein degradation. Three of the β subunits are proteolytically active: β1 has cysteine protease with peptidylglutamyl peptide-hydrolyzing-like activity (PGPH), β2 presents serine protease with trypsin-like activity, and β5 is also a serine protease with chymotrypsin-like activity (Smalle and Vierstra 2004). The active sites of the plant 20S proteasome are very sensitive to the 26S proteasome inhibitors, MG115, MG132, lactacystin, and epoxomycin (Yang et al. 2004). Thus, the assay to determine proteasome peptidase activities is performed by monitoring cleavage of three different peptide substrates linked to the fluorescence reporters: Ala-Ala-Phe-7-amido-4-methylcoumarin, Boc-Leu-Ser-Thr-Arg-7-amido-4-methylcoumarin and N-Clz-Leu-Leu-Glu-β-nafthylamide for chymotrypsin-, trypsin-, and PGPH-like activities, respectively, either in the absence or the presence of a proteasome inhibitor. Besides its function as the proteolytic core of the 26S complex, 20S proteasome has a key role in degradation of mildly oxidatively modified proteins without non-ATP and ubiquitin requirement in mammalian cells (Shringarpure et al. 2003). The major recognition motif of the substrates by the 20S proteasome seems to be hydrophobic surface patches formed by partial unfolding and exposure of hydrophobic amino acid residues during oxidation. However, heavily oxidized proteins form covalent cross-links that decrease their susceptibility to proteolysis by the 20S proteasome (Grune et al. 2003). One of the effects exerted by metal stress condition is the inhibition of the 20S proteasome activity on sunflower leaves (Pena et al. 2008). But the mechanism underlying this inhibition seems to be dependent on the metal tested. Inactivation produced by $Cd^{2+}$ is associated with the oxidative damage of the proteasome (Pena et al. 2006a, b), in a similar way as that of $Cu^{2+}$ and $Hg^{2+}$ (Pena et al. 2008). Cadmium decreased 20S proteasome activity, and induced the accumulation not only of the oxidized but also of the ubiquitinated proteins (Pena et al. 2006a, b). On the other hand, metals like $Co^{2+}$ and $Ni^{2+}$ inhibit in vitro proteasome activity (Pena et al. 2008). In this sense, Amici et al. (2002) suggested a direct effect of metals on proteasome activity that might result from the displacement of $Mg^{2+}$ from its binding sites inside the catalytic chamber that decrease its functionality.

Interestingly, protein 20S oxidation might not be entirely detrimental to 20S proteasome function. Maize (*Zea mays*) leaf segments subjected to low cadmium treatment increased the 20S proteasome activity together with its level of protein carbonylation (Pena et al. 2007). The moderate oxidative chemical modification of 20S proteasome acts to relax the structure of the proteasome and can activate its proteolytic activity (Shringarpure et al. 2001), thus avoiding the accumulation of carbonylated proteins in Cd-treated maize segments (Pena et al. 2007). Treatments

with low metal concentrations together with MG132 or higher cadmium concentrations decreased 20S proteasome activity with a concomitant accumulation of oxidized and ubiquitinated proteins. Impairment of proteasome functionality under high Cd concentration was associated with severe oxidation of 20S protein (Pena et al. 2007). Similar observations were described for proteasome isolated from maize roots submitted to a mild oxidative treatment through MCO system in vitro (Basset et al. 2002). Polge et al. (2009) demonstrated that RNAs encoding subunits of the 20S proteasome were up-regulated in response to cadmium in the leaves of *A. thaliana*. The increase in proteasome structural and catalytic subunit transcripts was followed by increases in proteasome quantity and chymotrypsin-like activity. Further, mutations of one of the subunits of the 19S regulatory particle resulted in an enhanced accumulation of the 20S versus 26S proteasome and a higher tolerance to oxidative stress in *Arabidopsis* (Kurepa et al. 2008). So far, 20S proteasome plays a major role in the degradation of moderately oxidized proteins during oxidative stress produced by metals in plants, and it is regulated at both transcriptional and post-translational level. Moreover, metal effects on proteasome functionality may be additional to other mechanisms previously demonstrated to be involved in metal toxicity in plants.

## 6 Conclusion

Metal stress alters the plant cell redox state causing serious reversible and irreversible oxidative changes in the proteome. Nowadays, the information about the content and distribution of metals (Salt et al. 2008) together with the cell redox proteomics (Rinalducci et al. 2008) is beginning to shed light about the impact that biological and environmental relevant metal concentrations have on plant cells. The widespread occurrence of protein modifications, the selectivity in the oxidation, the existence of regenerative mechanism of oxidative modifications and the regulated control of proteolysis are indicating a key role of the process of protein oxidation in normal plant cell physiology and in the response to stress conditions. The idea is emerging that protein oxidative modifications, together with other post-translational modifications like protein glutathionylation, nitrosylation, or even ubiquitination, could have signalling ramifications (Møller and Sweetlove 2010).

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