Oxidative Modifications to Cellular Components in Plants

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Abstract

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced in many places in living cells and at an increased rate during biotic or abiotic stress. ROS and RNS participate in signal transduction, but also modify cellular components and cause damage. We first look at the most common ROS and their properties. We then consider the ways in which the cell can regulate their production and removal. We critically assess current knowledge about modifications of polyunsaturated fatty acids (PUFAs), DNA, carbohydrates, and proteins and illustrate this knowledge with case stories wherever possible. Some oxidative breakdown products, e.g., from PUFA, can cause secondary damage. Other oxidation products are secondary signaling molecules. We consider the fate of the modified components, the energetic costs to the cell of replacing such components, as well as strategies to minimize transfer of oxidatively damaged components to the next generation.

Contents

INTRODUCTION

ROS: reactive oxygen species

RNS: reactive nitrogen species

The production of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($HO^{\bullet}$), and singlet oxygen ($^1O_2$), is an unavoidable consequence of aerobic metabolism (41). Reactive nitrogen species (RNS), such as the hormone nitric oxide ($NO^{\bullet}$) and peroxinitrite ($ONOO^-$), are also formed. In some cases, ROS are produced in plants as products of mainstream enzymatic reactions, e.g., glycolate oxidase in the peroxisomes during photorespiration. In other cases, ROS production appears to be an unavoidable accident, e.g., the $O_2^{\bullet -}$ produced by the mitochondrial electron transport chain (ETC). Hypoxia and anoxia are special cases where most of the ROS production occurs during the reoxygenation phase (12). ROS can also be produced to perform certain tasks, e.g., production of $O_2^{\bullet -}$ on the outer surface of the plasma membrane by the NADPH oxidase following pathogen recognition and in a variety of other processes (105). It is generally observed that ROS production and ROS-induced damage increase during abiotic and biotic stress and ROS are important signaling molecules (5, 33, 36, 67).

The emphasis on the role of ROS in signaling has led to the suggestion that the expression "oxidative stress" should be replaced by "oxidative signaling" (33). However, being reactive molecules, ROS oxidize all types of cellular components. To some extent the cell has learned to live with that and some modifications can be used in metabolic regulation, e.g., the oxidation of protein cysteines. In other cases, the modification prevents the molecule from performing its original function, i.e., it is damaging. Typically, more damage is observed under stress conditions when the ROS levels are increased (**Figure 1**). At the same time the oxidized products can be important secondary signaling molecules, and in such cases damage and signaling are two sides of the same story (**Figure 1**).

In this review we focus on the specific oxidative modifications caused by ROS without losing sight of ROS signaling. First, we briefly summarize the current knowledge about ROS turnover in plant cells. We then consider the type and extent of oxidative modifications in plant cells. Finally, we look at the possible repair processes and estimate the energetic cost of replacing modified components. Throughout the review we compare and

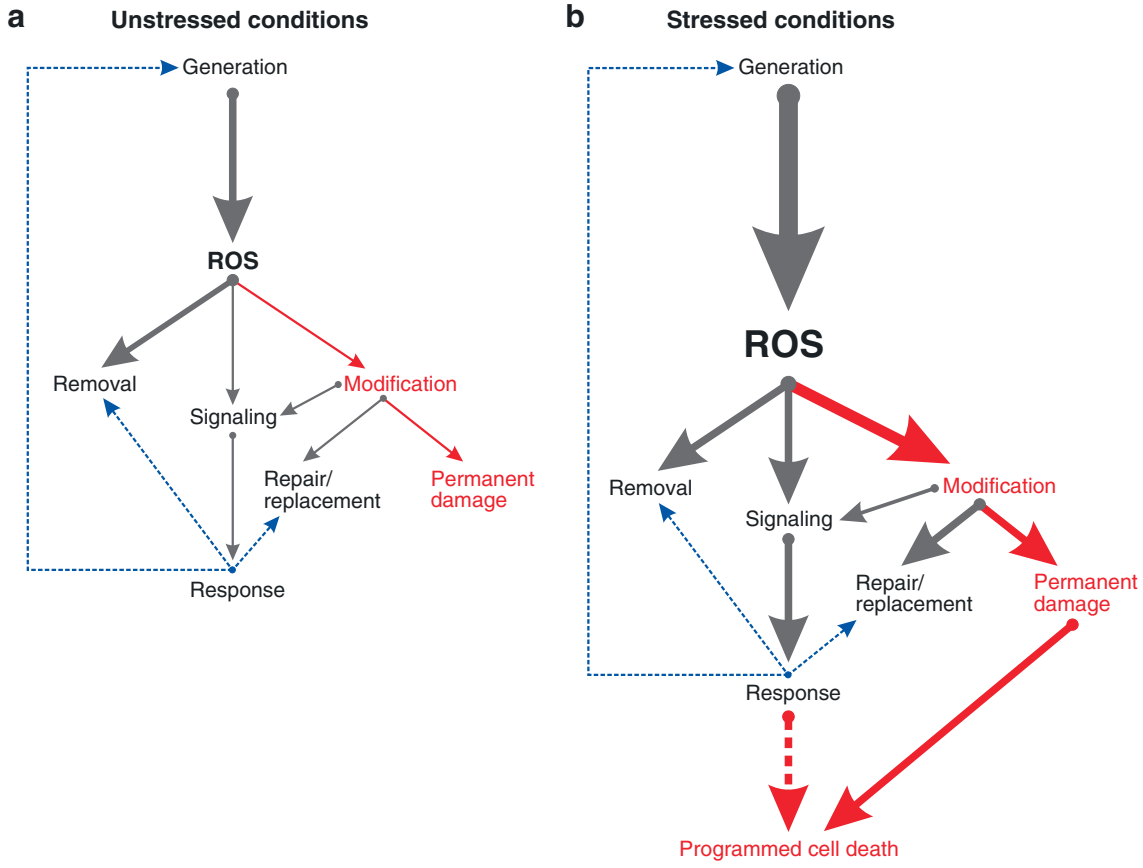


Figure 1

The relationship between reactive oxygen species (ROS) production, removal, modification, signaling, and damage in plant cells under (*a*) unstressed and (*b*) stressed conditions. The arrow from "Modification" to "Signaling" indicates that some of the modified molecules are secondary signal molecules.

contrast our knowledge about oxidative modifications in plants with the greater knowledge gained from experiments on fungi and animals.

ROS PRODUCTION AND REMOVAL

The response of the plant and its components to ROS depends on the ROS in question as well as on its concentration, site of production, and interaction with other stress molecules. It also depends on the developmental stage and prehistory of the plant cell (e.g., previous stress encounters) (36). In this section we look at the types of ROS produced and the mechanisms for regulating the rate of production. In the following section we look at processes removing ROS and then analyze the modifications and/or damage that cellular components can sustain when interacting with ROS.

ROS Production

In green plant parts in the light, the chloroplasts and peroxisomes (through photorespiration) are the main ROS producers (32).

NO•: nitric oxide (or nitrogen monoxide), a volatile signaling molecule, is an RNS and a radical

ONOO⁻: peroxinitrite, an RNS formed by the condensation of $O_2^{\bullet -}$ and NO•. It is very reactive, especially in the protonated state.

ETC: electron transport chain

$^1O_2$: singlet oxygen, an ROS that is oxygen in a higher energy state, is mainly produced in the chloroplasts

PSI and II: photosystem I and II

$O_2^{\bullet-}$: superoxide, an ROS that is both a radical and an anion

SOD: superoxide dismutase

HO•: hydroxyl radical, the most reactive of all the common ROS

In nongreen plant parts or in darkness, the mitochondria appear to be the main ROS producers (64, 68). The chloroplasts produce $^1O_2$ at photosystem II (PSII) and $O_2^{\bullet-}$ at photosystem I (PSI) (6) and PSII (84) as byproducts. The mitochondria produce $O_2^{\bullet-}$ at complexes I and III, also as byproducts. An estimated 1–5% of the oxygen consumption of isolated mitochondria results in ROS production (68). The peroxisomes produce $O_2^{\bullet-}$ and H_2O_2 in several key metabolic reactions (26). And, finally, the NADPH oxidase in the plasma membrane produces $O_2^{\bullet-}$, which participates in several physiological processes (105).

$O_2^{\bullet-}$ can be converted into H_2O_2, e.g., by superoxide dismutase (SOD), and H_2O_2 can give rise to HO• through the Fenton reaction, which is catalyzed mainly by free transition metal ions. The different ROS have very different properties (**Table 1**). H_2O_2 is relatively stable and its concentration in plant tissues is in the micromolar to low millimolar range, probably depending on the compartment (14, 41, 86). The other ROS have very short half-lives and are probably present at very low concentrations. They also have different reactivities: Whereas HO• reacts rapidly with all types of cellular components, $O_2^{\bullet-}$ reacts primarily with protein Fe-S centers and $^1O_2$ is particularly reactive with conjugated double bonds as found in polyunsaturated fatty acids (PUFAs). This means that they leave different footprints in the cell in the form of different oxidatively modified components.

H_2O_2 is already an established messenger in bacteria, yeast, and mammals where transcription factors are sensors (56) (see also below), as they may also be in plants (66). Its relative stability and ability to cross membranes possibly through aquaporins (9, 10) makes H_2O_2 a good messenger molecule. However, all the ROS forms can, in principle, act as messengers either directly or by using an oxidized product as a secondary messenger. Using a secondary messenger, e.g., transcription factors, depends on the distance the ROS molecule travels before reacting with cellular components (**Figure 2**; see also **Table 1**).

Table 1 Important ROS and RNS in plant tissues and their basic properties[a]

Property	Singlet oxygen ($^1O_2$)	Superoxide ($O_2^{\bullet-}$)	Hydrogen peroxide (H_2O_2)	Hydroxyl radical (HO•)	Peroxynitrite ($ONOO^-$)[e]
Half-life[b]	1 μs	1 μs	1 ms	1 ns	?
Distance traveled[c]	30 nm	30 nm	1 μm	1 nm	?
Cellular concentration	?	?	μM-mM[f]	?	?
Reacts with					
Lipids	PUFA	Hardly	Hardly	Rapidly	Yes
DNA	Mainly guanine	No	No	Rapidly	Especially guanine
Carbohydrates[d]	No	No	No	Rapidly	?
Proteins	Trp, His, Tyr, Met Cys[g]	Fe-S centers	Cysteines	Rapidly[h]	Tyr, Trp, Phe, Met

[a]Where no source is indicated the information is from Halliwell & Gutteridge (41).
[b]In biological systems (9, 85, 99).
[c]Distance traveled in one half-life if the diffusion coefficient is assumed to be 10^{-9} m^2s^{-1}.
[d]Reference 34.
[e]Peroxynitrite is included instead of NO•. It is formed by the condensation of NO• with $O_2^{\bullet-}$, and it is the protonated form (ONOOH) that is most reactive (41).
[f]References 14, 41, 86.
[g]Reference 22.
[h]Order of reactivity for all the amino acids given by Xu & Chance (114), the most reactive being Cys, Met, Trp, Tyr, and Phe.

The presence of relatively high concentrations of H_2O_2 in plant tissues (14, 41, 86) has consequences. To limit the inadvertent formation of the very reactive $HO^{\bullet}$ in the Fenton reaction, the cell must keep the concentration of free metal ions (mainly Fe, Cu, and Mn) extremely low by binding to metallochaperone molecules. This means that there is much less than one free copper ion in the average unstressed yeast cell (31, 87).

Nitrate reductase and the mitochondrial ETC appear to be major sources of $NO^{\bullet}$ in plant cells (38, 83). Since $O_2^{\bullet-}$ is also produced in all mitochondria (68), the reactive fusion product $ONOO^-$ will probably also be formed there.

In addition to producing ROS themselves, plants may also be exposed to anthropogenic ROS (and RNS) in the environment. Thus, ozone (O_3), sulfur dioxide (SO_2), and nitrogen dioxide (NO_2) are all major air pollutants that can cause oxidative modifications to many cellular components, and can even induce programmed cell death (41, 81).

Regulating ROS Production

In mitochondria, ROS production is generally caused by an over-reduction of the ETC. The mitochondria have several strategies for preventing or limiting such an over-reduction (68, 70). The stimulation of proton leakage in potato mitochondria by $O_2^{\bullet-}$, probably through a stimulation of the uncoupling protein, provides an interesting feedback mechanism for lowering the reduction level of the mitochondrial ETC, thus preventing further $O_2^{\bullet-}$ production (18).

The photosynthetic apparatus is constantly challenged by ROS generation during the light reactions of photosynthesis. Changes in light intensity and other environmental stresses can result in the absorption of more light energy than can be utilized for photosynthesis and plants have several mechanisms for dissipating this excess and preventing over-reduction of the photosynthetic ETC (52, 77).

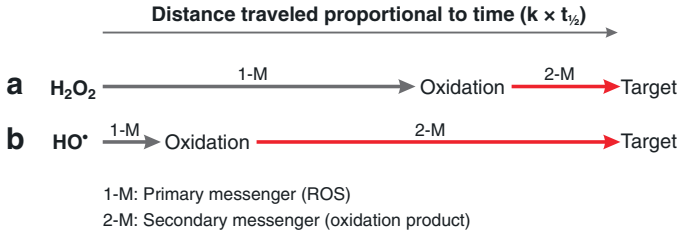


Figure 2

Signal transduction involving reactive oxygen species (ROS) as a primary messenger and a secondary messenger that is an oxidation product of a cellular component. The ultimate target is a gene or a group of genes. (*a*) The relatively long-lived H_2O_2 brings the signal close to the target, where it oxidizes an H_2O_2 sensor, which could be a transcription factor (56). (*b*) A short-lived ROS, in this case $HO^{\bullet}$, reacts with a cellular component ($HO^{\bullet}$ sensor) close to the site of production. The oxidation product, the second messenger, brings the signal to the gene(s) or perhaps to other transcription factors.

Removal of ROS

The plant cell and its organelles—peroxisomes (26), chloroplasts (6), and mitochondria (68, 70, 75)—contain multiple enzymes or enzyme systems for removing ROS (5, 13, 67). The reducing power for all of these systems (except SOD and catalase, which use internal oxidation/reduction of their substrate) derives directly or indirectly from NADPH or ferridoxin. It is outside the scope of this review to discuss their properties in detail, but **Table 2** contains an overview.

Several of the ROS-removing systems mentioned in **Table 2** use the reversible oxidation of peptide- or protein-bound cysteines to reduce ROS, thus illustrating the high reactivity of that amino acid with ROS (see below).

The balance between ROS production and the activities of these ROS-removing systems determines the type and concentration of ROS present and thus to what extent signaling and/or damage will occur.

PUFA: polyunsaturated fatty acid

MODIFICATIONS TO POLYUNSATURATED FATTY ACIDS

The polyunsaturated fatty acids (PUFAs) linoleic acid (18:2) and linolenic acid (18:3)

Table 2 ROS-removal mechanisms in plant cells[a]

Mechanisms	Removes (product)	Cellular location[b]	Useful references
SOD	$O_2^{\bullet -}$ (H_2O_2)	Chl, Cyt, Mit, Per	(26, 67)
Catalase	H_2O_2 (H_2O)	Mit?, Per	(26, 67, 68)
Peroxidases	H_2O_2 (H_2O)	Many locations	(82)
Ascorbate/glutathione cycle	H_2O_2 (H_2O)	Chl, Cyt?, Mit, Per	(26, 67, 70, 78)
Glutathione peroxidases	H_2O_2 (H_2O)	Chl, Cyt, ER, Mit	(67, 88)
	Lipid hydroperoxides		
	Other hydroperoxides		
Peroxiredoxin system	H_2O_2 (H_2O)	Chl, Cyt, Mit, Nucl	(27, 67)
	Alkyl hydroperoxides		
	Peroxinitrite		
Thioredoxin system[c]	H_2O_2 (H_2O)[c]	Chl, Cyt, Mit	(13, 67)
Glutaredoxin system[c]	H_2O_2 (H_2O)[c]	Chl, Cyt, Mit, Sec	(13, 67, 91)
	Hydroperoxides		
Carotenes and tocopherol	$^1O_2$ (O_2)	Chl	(6)

[a]Most of these are enzymes or enzyme systems. A good general reference is Mittler et al. (67).
[b]Chl, chloroplasts (plastids); Cyt, cytosol; ER, endoplasmic reticulum; Mit, mitochondria; Nucl, nucleus; Per, peroxisomes; Sec, secretory pathway.
[c]These systems are mainly involved in regulating the sulfhydryl/disulfide ratio in proteins. Their importance in removing H_2O_2 is uncertain.

are the major fatty acids in the plant membrane galactolipids (thylakoid membrane) and phospholipids (all other membranes). PUFAs are particularly susceptible to attack by $^1O_2$ and HO•, giving rise to complex mixtures of lipid hydroperoxides (**Figure 3**) (41, 73). Extensive PUFA peroxidation decreases the fluidity of the membrane, increases leakiness, and causes secondary damage to membrane proteins (see below) (40). Several aldehydes, e.g., 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), as well as hydroxyl and keto fatty acids, are formed as a result of PUFA peroxidation (**Figure 3**). The aldehyde breakdown products can form conjugates with DNA and proteins (see below). Aldehydes formed in the mitochondria may be involved in causing cytoplasmic male sterility in maize because a restorer gene in this species encodes a mitochondrial aldehyde dehydrogenase (62, 69).

In animals, oxidized fatty acids are selectively released by certain phospholipases, but such enzymes have not yet been identified in plants. In plant cells, some of the PUFA oxidation products function as secondary messengers either directly or after enzymatic modification (**Figure 3**) (73).

Glutathione peroxidases comprise an enzyme family using glutathione to reduce H_2O_2, lipid hydroperoxides, and other hydroperoxides (**Table 2**) (41). One member of this family is the phospholipid-hydroperoxide glutathione peroxidase, which can act directly on lipid hydroperoxide without the need to release the hydroperoxy fatty acid (88).

Membrane lipids appear to decrease initial radiolytic oxidative modifications (mostly by HO•) to membrane proteins (25). This is energetically advantageous to the cell because a damaged lipid molecule or a PUFA is cheaper to remove and replace than a protein molecule (see below).

Case Story

Lipid peroxidation in the *flu* mutant. To assess the role of $^1O_2$ relative to those of

HNE: 4-hydroxy-2-nonenal

MDA: malondialdehyde

Radiolysis: here, the formation of ROS (mainly HO•) from water by a pulse of high-energy electrons

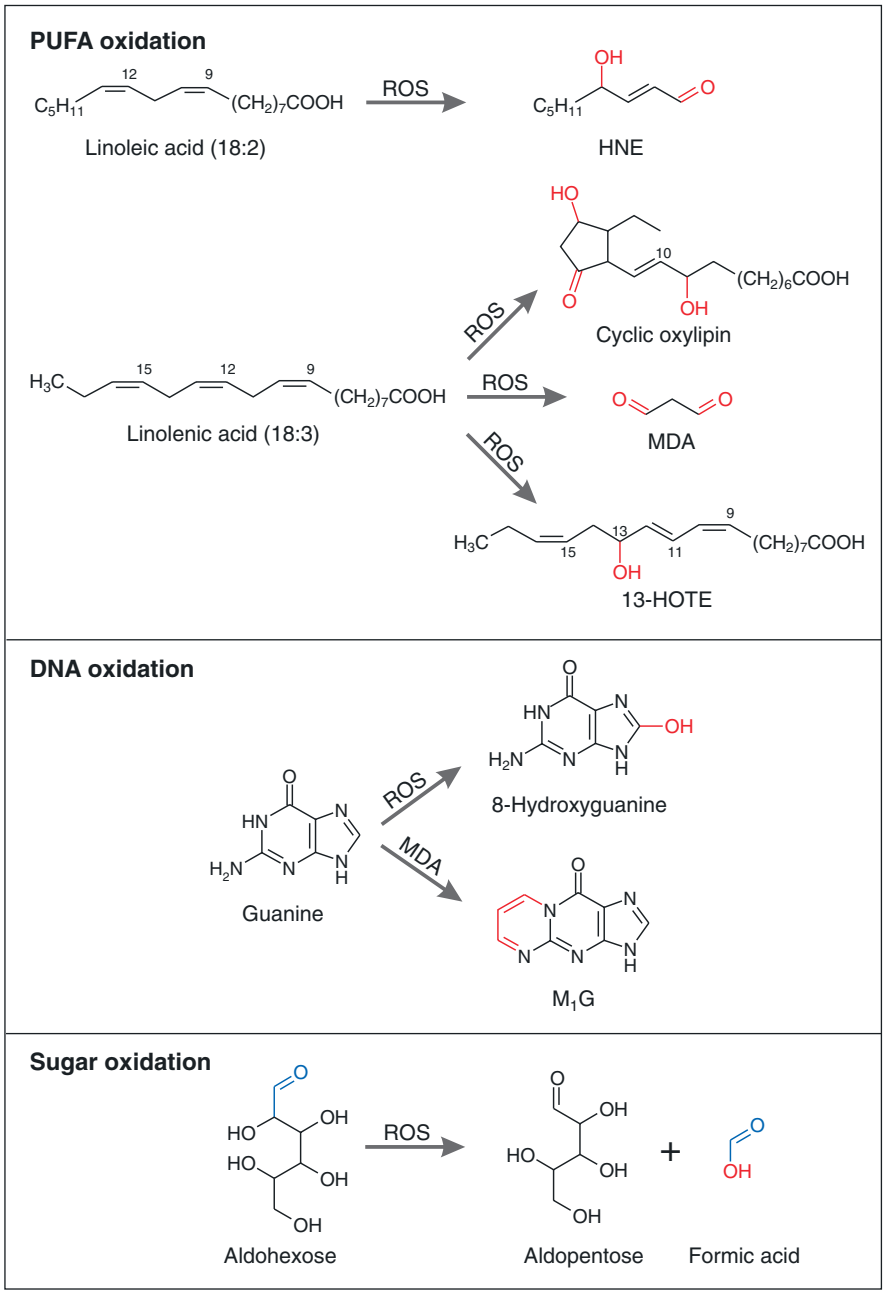


Figure 3

Commonly observed oxidative modifications of polyunsaturated fatty acids (PUFAs), DNA, and carbohydrates. In plant cells, 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) are formed mainly by oxidation of linoleic acid (18:2) and linolenic acid (18:3), respectively (41). The 13-HOTE and cyclic oxylipins are only examples of the wide range of similar products arising from peroxidation of linolenic acid (73). Similar products would be created by oxidation of linoleic acid. Both PUFA and guanine are drawn as free although in the cell they would normally be oxidized while attached to a lipid and DNA, respectively. Modifications caused by reactive oxygen species (ROS) are highlighted in red. The group oxidized in the sugar (aldohexose) is shown in blue. M_1G, pyrimido[1,2-a]-purin-10(3H)-one.

other ROS, op den Camp et al. (80) took advantage of the Arabidopsis *flu* mutant. In darkness, this mutant accumulates a specific chlorophyll precursor, protochlorophyllide, in its chloroplasts. Because protochlorophyllide is a photosensitizer that specifically generates $^1O_2$, this system provided highly localized production of $^1O_2$ without generating other oxidizing species. The results clearly indicate that the chloroplasts generated $^1O_2$

HOTE: hydroxyoctadecatrienoic acid

selectively and rapidly activated distinct sets of nuclear genes that were different from those induced by $O_2^{\bullet-}/H_2O_2$. At the same time a transient and selective accumulation of a stereospecific isomer of free hydroxyoctadecatrieonic acid (HOTE) (**Figure 3**), which is an oxidation product of linolenic acid, was observed. The observation of stereospecificity was interpreted to be indicative of an enzymatic (lipoxygenase-mediated) oxidation rather than direct chemical oxidation by $^1O_2$. The authors concluded that $^1O_2$ does not primarily act as a toxin but rather as a signal activating several stress-response pathways (80). This conclusion was later supported by the identification of EXECUTER1, a hitherto unknown nucleus-encoded chloroplastic protein. Although the double mutant *flu/executer1* accumulates the same amount of free protochlorophyllide generating the same amount of $^1O_2$ as the *flu* mutant, the double mutant did not initiate any stress response (110).

The oxidation of linolenic acid reported by op den Camp et al. (80) presumably occurred while it was still part of the membrane galactolipids and the oxidized product(s) was subsequently released by enzymatic action. Thus, it cannot be excluded that a lipase or perhaps enzymes degrading other peroxidation products generated the stereospecificity bias observed (only one stereoisomer of HOTE was detected).

The primary oxidation event in the above study would probably take place within 0.1–0.3 μm of the site of $^1O_2$ generation as this is as far as $^1O_2$ can diffuse in the few microseconds it exists (**Table 1**). The HOTE formed is a possible secondary messenger (73).

MODIFICATIONS TO DNA

DNA can be modified by ROS in many different ways, mainly on the nucleotide bases. $HO^\bullet$ is the most reactive, $^1O_2$ primarily attacks guanine, and H_2O_2 and $O_2^{\bullet-}$ do not react at all (112). 8-Hydroxyguanine is the most commonly observed modification (**Figure 3**). Given that mtDNA and ctDNA are close to major sites of ROS production and that these DNA forms have no histones and no chromatin structure, one might expect high rates of modification and equally high repair rates and this appears to be the case for mammalian mitochondria (104). No comparable information is available for plant cells. However, the presence of multiple copies of mtDNA and ctDNA enables the cell to select against negative mutations.

ROS damage to both mtDNA and nDNA is not completely random and mutation clusters at hot spots have been observed (41). However, no gene has been identified as being particularly susceptible to ROS damage.

In addition to direct DNA oxidation, ROS can also indirectly modify DNA. A common type of damage involves conjugation of the PUFA breakdown product MDA with guanine, which creates an extra ring (**Figure 3**) (47). In addition to mutations, oxidative DNA modifications can lead to changes in the methylation of cytosines, which is important for regulating gene expression (40).

A number of mechanisms are available for repairing DNA damage both in the nucleus and in the mitochondria. These include direct reversal of the damage, replacement of the base, and replacement of the whole nucleotide (55, 106). An accumulation of DNA damage is observed in several human diseases characterized by ROS accumulation, implying that the repair mechanisms are overwhelmed (112).

MODIFICATIONS TO CARBOHYDRATES

$HO^\bullet$ reacts with free carbohydrates, such as sugars, and polyols (100). Transgenic tobacco accumulating mannitol in the chloroplasts shows increased resistance to oxidative stress possibly because mannitol removes $HO^\bullet$ before it reacts with more vital cellular components (97). The oxidation of sugars with $HO^\bullet$ often releases formic acid as the main breakdown product (44). This may be the long-sought-after source of substrate for the

enigmatic enzyme formate dehydrogenase (17, 42, 50).

Plant cell wall polysaccharides are susceptible to oxidative scission mediated by $HO^{\bullet}$ in vitro under physiologically relevant conditions (34). Cell extension during elongation growth in plants is mediated by auxin, which promotes apoplastic ROS production. Cell wall–bound peroxidases use the ROS to generate $HO^{\bullet}$ close to the site of scission (95). Support that $HO^{\bullet}$ actually acts as a wall-loosening agent in vivo comes from chemical features, which are diagnostic of an $HO^{\bullet}$ attack, that can be found in cell wall polysaccharides of ripening fruit (35). These oxidative modifications are not damaging as they are not detrimental to the modified component or the cell.

MODIFICATIONS TO PROTEINS

Protein oxidation is defined here as covalent modification of a protein induced by ROS, RNS, or byproducts of oxidative stress. Most types of protein oxidations are essentially irreversible whereas a few involving sulfur-containing amino acids are reversible (37). Protein oxidation is widespread and often used as a diagnostic marker for oxidative stress. The most common oxidative protein modifications are shown in **Figure 4**. A number of excellent reviews have been written about protein oxidation in mainly mammalian tissues (see 21, 22, 25, 41, and 96 as well as **http://www.medicine.uiowa.edu/FRRB/VirtualSchool/Virtual.html**). Protein oxidation in plants has been reviewed for mitochondria (45, 70, 71), but until now not for the whole plant cell.

Sulfur-Containing Amino Acids

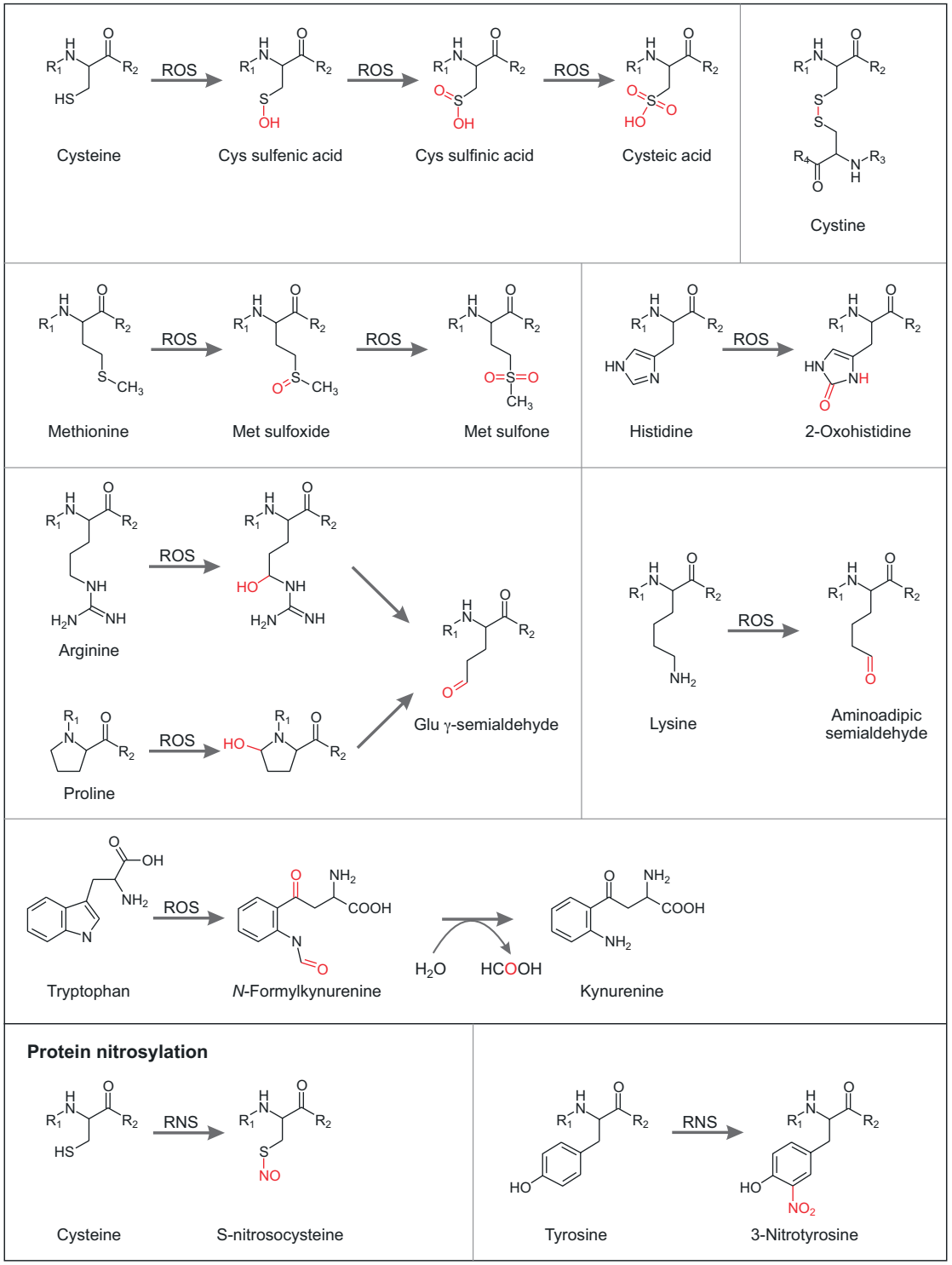
Cys and Met are quite reactive, especially with $^1O_2$ and $HO^{\bullet}$ (**Table 1**). The oxidation of thiol to disulfide (R_1-S-S-R_2, cystine) (**Figure 4**), which can be caused by several different ROS, is a very important metabolic redox regulation mechanism. Intra- or intermolecular disulfide bonds can be formed between cysteine side chains and the reduced form can be regenerated by the thioredoxin (Trx) or glutaredoxin systems (**Table 2**). A large number of potential Trx-regulated proteins have been identified in different cellular compartments including chloroplasts and mitochondria (13). The further oxidation of cysteine via cysteine sulfenic acid (R-SOH) to cysteine sulfinic acid ($R\text{-}SO_2H$) (**Figure 4**) is also enzymatically reversible and probably involved in signaling pathways (11). The highest level of cysteine oxidation, cysteic acid ($R\text{-}SO_3H$) (**Figure 4**), appears to be irreversible and damaging (37).

Trx: thioredoxin

Cysteine can also form mixed disulfides primarily with the cysteine-containing tripeptide glutathione and this might serve to protect the cysteine group against further oxidation. The mixed disulfides are re-reduced by glutaredoxins again involving glutathione (37). Glutathione can also be used to remove H_2O_2 (**Table 2**) and is the main nonprotein thiol in the cell (78).

Oxidation of methionine to methionine sulfoxide (**Figure 4**) is another reversible modification. An interesting example is the small heat shock protein in chloroplasts, which is inactivated by methionine sulfoxidation, but reactivated by reduction catalyzed by the enzyme peptide methionine sulfoxide reductase using Trx as the reductant (39). In Arabidopsis, a null mutation in a gene encoding a cytosolic isoform of the enzyme showed increased ROS content, lipid peroxidation, and protein oxidation at the end of a long night, which was clearly stressful to the plant (8).

It has been suggested that some peripheral methionine residues act as endogenous antioxidants protecting the active site and other sensitive domains in the protein while helping to remove ROS (58). It is quite likely that reversible methionine sulfoxidation will turn out to be an important regulatory mechanism (101). Further oxidation of Met to the sulphone (**Figure 4**) appears to be irreversible and damaging to the protein.



Carbonylation

Apart from the reactions involving the sulfur-containing amino acids, carbonylation is the most commonly occurring oxidative protein modification. There are no indications that carbonylation is reversible (96). The oxidation of a number of protein amino acids—particularly Arg, His, Lys, Pro, Thr, and Trp—give free carbonyl groups (**Figure 4**). The detection of carbonyl groups is relatively simple, e.g., by conjugation with dinitrophenylhydrazine followed by antibody detection of the conjugate (59). Protein carbonylation has therefore been studied extensively.

In mammalian cells, protein carbonylation is found at a basic level of 1 nmol/mg protein increasing to 8 nmol/mg in samples from diseased tissue. This is, on average, equivalent to 0.05–0.40 carbonyl group per 50 kDa protein molecule, although these values may be overestimates (25). Thus, in stressed tissues a significant proportion of all proteins are carbonylated. An analysis of plant proteins gave an estimated 4 nmol carbonyl groups per milligram of protein (76, 89). Values as high as 60–700 nmol/mg have also been reported (107), but these are likely serious overestimates.

An in vivo treatment of pea plants with Cd^{2+} (89) or a strong in vitro oxidative treatment (76) raised the carbonylation level from 4 to 5.6 and 34 nmol/mg or about 0.3 and 1.7 carbonyl groups per 50 kDa protein molecule, respectively. In Arabidopsis, protein carbonylation in total protein extracts increased during the vegetative phase, but decreased sharply at the start of the reproductive phase, staying relatively low until and during senescence. It was suggested that a low degree of protein carbonylation during the reproductive phase could be part of a strategy to limit the transfer of oxidatively damaged components to the offspring (49) (see also below).

In wheat leaves, protein carbonylation was higher in the mitochondria than in chloroplasts and peroxisomes (7). This indicates that the mitochondria are more susceptible to oxidative damage and/or the removal of modified proteins is less efficient in the mitochondria. A number of carbonylated proteins have been identified in the mitochondrial matrix, 20 of which were probably carbonylated in vivo and a further 31 were oxidized by an in vitro treatment with $HO^{\bullet}$ (53) (follow the Supplemental Material link from the Annual Reviews home page at **http://www.annualreviews.org** to see **Supplemental Table 1**). Many of these oxidized proteins are Krebs cycle enzymes, other redox-active enzymes, or chaperones, or are involved in ROS detoxification (SOD). None of them are located immediately adjacent to the primary sites of ROS production in the ETC, so migration of ROS from the ETC to the matrix is implied.

The level of protein carbonylation in isolated pea leaf peroxisomes rose from 6.9 to 16.3 nmol per mg of peroxisomal protein as a result of Cd^{2+} treatment of the intact plant. These values are higher than for whole plant extracts from control and Cd^{2+}-treated plants, respectively, which could be the result of a higher local ROS concentration in the peroxisomes (89).

As mentioned above, Nguyen & Donaldson (76) exposed extracted proteins from castor bean peroxisomes to $HO^{\bullet}$ and monitored protein carbonylation and

Figure 4

Commonly observed oxidative modifications of protein amino acids. With the exception of Trp, which is shown as the free amino acid for convenience, all the amino acids are shown as part of a polypeptide chain. However, the names shown are those of free amino acids (i.e., proline rather than prolyl). Modifications caused by reactive oxygen species (ROS) or reactive nitrogen species (RNS) are highlighted in red. Cysteic acid is also known as cysteine sulfonic acid.

enzyme activities. The degree of carbonylation increased strongly over the first 90 min without a detectable change in the overall protein pattern. This indicates that the oxidative treatment did not lead to chain breakage or degradation. In response to the oxidative treatment, the activities of malate synthase, isocitrate lyase, catalase, and malate dehydrogenase decreased by 50–100%. Catalase was the protein with the most carbonylation and its activity was reduced by 80%. Carbonylated catalase has also been identified in other investigations (53, 89) (**Supplemental Table 1**).

Sweetlove et al. (102) identified shortened products of several proteins in mitochondria isolated from Arabidopsis cell cultures exposed to H_2O_2. Presumably, these smaller forms had been formed by oxidative breakage of peptide bonds in the proteins. This is surprising given that an in vitro treatment of peroxisomal proteins with $HO^{\bullet}$ did not cause substantial chain breakage, as judged by one-dimensional gel electrophoresis (76). The greater separation power of two-dimensional gel electrophoresis employed by Sweetlove et al. (102) likely made it possible to detect relatively small amounts of breakdown products.

In dry Arabidopsis seeds most of the carbonylation was found in the storage proteins, but carbonylation of a number of other proteins increased strongly during seed germination. The oxidized proteins derived from several cellular compartments including the cytosol, chloroplasts, and mitochondria (48) (**Supplemental Table 1**).

Tryptophan Oxidation

Tryptophan oxidation is another apparently irreversible protein modification (96). Mass spectrometric analysis of mitochondrial proteins permitted the identification of numerous proteins containing oxidized Trp in the form of N-formylkynurenine (**Figure 4**) as well as the specific site(s) of modification (72). With the exception of the oxidation-sensitive aconitase (a Krebs cycle enzyme that contains an Fe-S center), all of these proteins were either redox active or subunits in redox-active enzyme complexes (including the ETC). The same site was modified in (*a*) several adjacent spots containing the P-protein of the glycine decarboxylase complex, (*b*) two different isoforms of the mitochondrial processing peptidase in complex III, and (*c*) the same Trp residues in Mn-SOD in both rice and potato mitochondria. This indicates that Trp oxidation is not a random process. It is possible that exposed Trp residues act as intramolecular antioxidants, as suggested for Met residues (58).

Nitrosylation

The covalent attachment of the messenger $NO^{\bullet}$ to cysteine thiol (**Figure 4**) is a post-translational modification that potentially regulates the function of proteins. Protein (and glutathione) thiols can react with $NO^{\bullet}$ derivatives to produce a range of products including disulfides, sulfenic, sulfinic and sulfonic acids, as well as S-nitrosothiols (19).

The most common $NO^{\bullet}$ derivative is peroxynitrite ($ONOO^-$), which is formed through the condensation reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$ ($NO^{\bullet} + O_2^{\bullet-} \rightarrow ONOO^-$) (**Table 1**) (41). In this way, $NO^{\bullet}$ is intimately linked with ROS, but $NO^{\bullet}$ does not cause S-nitrosylation on its own; it seems to involve S-nitrosothiols (20). However, peroxynitrite can cause depletion of SH groups and other antoxidants, oxidation of lipids, deamination of DNA bases, nitration of aromatic amino acid residues in proteins (**Figure 4**), and oxidation of methionine to its sulfoxide (41).

In a proteomic study of proteins from Arabidopsis cell suspension cultures or leaves treated with an $NO^{\bullet}$-donor or gaseous $NO^{\bullet}$, respectively, 63 proteins from cell cultures and 52 proteins from leaves that represent candidates for S-nitrosylation

were identified. Among these were stress-related, redox-related, signaling/regulating, structural, photosynthetic, and metabolic proteins, indicating that NO• is involved in the regulation of all of these processes (61) (**Supplemental Table 1**).

Interaction with Products of PUFA Oxidation

In vitro treatment of isolated mitochondria or enzyme complexes with HNE, one of the products of PUFA peroxidation (**Figure 3**), causes an inhibition of the decarboxylating enzyme complexes, e.g., pyruvate dehydrogenase, in mitochondria, probably by forming an adduct with their lipoamide moiety (65, 102, 103). HNE also reacts with the regulatory Cys (and other amino acids) in the alternative oxidase, thereby inhibiting its activity (111).

In vivo treatments of Arabidopsis cell cultures or pea plants, which increase the production of $O_2^{\bullet -}$ (and consequently other ROS), also increase the amount of PUFA breakdown as measured by MDA formation. At the same time, the amount and number of proteins conjugated with HNE increase dramatically while glycine oxidation by isolated mitochondria is inhibited (103, 111).

What Makes a Protein Susceptible to Oxidative Damage?

Cherry et al. (15) attempted to answer the question using the method of directed evolution. A fungal peroxidase was subjected to multiple rounds of directed evolution and an enzyme that had 100 times the oxidative stability (measured as the half-life at 40°C, pH 10.5 in the presence of 0.2 mM H_2O_2) of the wild-type enzyme was obtained. Changing three oxidizable amino acids near the active site heme group only improved the stability fivefold. The other changes required to increase the stability a further 20-fold changed the overall structure of the active site and probably reduced the accessibility of susceptible groups in the interior of the protein to H_2O_2 (15).

Case Stories of Protein Oxidation

Here we outline several case stories to illustrate the interactions between ROS and proteins.

Ribulose-1,5-bisphosphate carboxylase/oxygenase. Rubisco is located in the stroma of chloroplasts where it catalyzes the primary reactions of CO_2 assimilation and photorespiration. ROS trigger Rubisco degradation and the large subunit of Rubisco is site-specifically cleaved into five major fragments in light-treated leaf discs under chilling temperatures (74). In this case, the fragmentation was completely inhibited by n-propyl gallate or 1,2-dihydrobenzene-3,5-disulfonic acid, suggesting the involvement of HO• and $O_2^{\bullet -}$, respectively. The fragmentation was stimulated by $FeSO_4$ and suppressed by an iron-specific chelator (deferoxamine), indicating a role for free Fe-ions in the generation of ROS. The results indicate that in situ formation of HO• via the Fenton reaction between iron and H_2O_2 in vivo causes the fragmentation of the large subunit of Rubisco.

Transition metal ions like Fe^{2+} and Cu^{+} bound to proteins can generate ROS that can potentially cleave the polypeptide backbone. The ROS generated probably only diffuse relatively short distances from the generation site, suggesting that proximity is a very important parameter for the cleavage reaction of proteins. Therefore, it is likely that metals bound to the protein prior to damage are the source of ROS. An in vitro study with the Rubisco large subunit identified specific cleavage at six residues (Gly, Ala, Asp) located within a 1.2-nm radius around a metal-binding site. They all had the $C_\alpha H$ group of their peptide backbone completely or partially exposed to the bound metal (63).

Rubisco: ribulose-1,5-bisphosphate carboxylase/oxygenase

Photosystem I. PSI is degraded in intact leaves under chilling-light conditions, resulting in destruction of the Fe-S centers and detectable degradation of the PSI-B reaction center subunit (94). The data indicate the involvement of ROS, probably $HO^{\bullet}$ produced by the Fenton reaction between photoreduced Fe-S centers and H_2O_2. PSI photodestruction results in the release of free iron from the damaged Fe-S centers. Iron ions leaking from the thylakoid membrane into the stroma of the chloroplast could lead to the generation of $HO^{\bullet}$, which can attack stromal proteins such as Rubisco.

A similar case is the release of Fe^{2+} from damaged Fe-S centers in mitochondrial aconitase in mammalian cells, e.g., as a result of heat stress, and the subsequent collateral damage (43). Aconitase in plant mitochondria is also sensitive to oxidative damage (70, 72) (**Supplemental Table 1**).

D1 protein (PSII). Under illumination D1 has the highest turnover rate of all thylakoid membrane proteins. The cause of D1 fragmentation is likely $^1O_2$ produced by the reaction center chlorophyll of PSII (P680), because in vitro treatment with $^1O_2$-generating substances results in a similar fragmentation pattern of the D1 protein, as observed under in vivo photoinhibitory conditions (79). However, it is not the $^1O_2$, per se, that causes cleavage of the peptide backbone, because the fragmentation is not observed in a mutant lacking an FtsH protease. Rather, the accumulation of oxidized amino acids in the D1 protein triggers a conformational change that renders the D1 protein susceptible to the FtsH protease (98).

Superoxide dismutase. Two groups of proteins must be able to function in the presence of ROS: (*a*) ROS-detoxifying enzymes and (*b*) enzymes that produce ROS as part of their normal catalytic cycle. SOD, three forms of which are found in different compartments, qualifies on both of these counts. This raises several questions: Are these enzymes more resistant to ROS-induced damage? And, if yes, how is this resistance achieved? Does oxidation of these enzymes lead to activation, which would be an interesting auto-regulatory mechanism? Do these enzymes have a more rapid turnover than other proteins? Carbonylation of several ROS-metabolizing enzymes including SOD and catalase has been identified (**Supplemental Table 1**).

Mammalian Cu,Zn-SOD found in the cytosol and the mitochondrial intermembrane space is inactivated by oxidation of the Cu^{2+} His ligand in the catalytic site (54). In a proteomic study of the role of SOD in neurodegenerative diseases, several spots were identified as Cu,Zn-SOD isoform 1. One spot contained a carbonylated form, which could be caused by His oxidation (**Figure 4**), but the amino acid modified was not identified. Another spot contained a cysteine sulfonic acid (**Figure 4**), a modification in vitro caused by high concentrations of H_2O_2, the SOD reaction product (16).

The inactivation of human Mn-SOD, which removes $O_2^{\bullet-}$ in the mitochondrial matrix, by nitration of a tyrosine (**Figure 4**) near the Mn in the active site has been reported (115).

Transcription factors. The transcription factor PerR in *Bacillus subtilis* contains two His residues coordinating bound Fe^{2+}. Upon exposure to low levels (<10 μM) of H_2O_2 one or both of the His are oxidized presumably by $HO^{\bullet}$ generated by a Fenton reaction involving the bound Fe^{2+}. The oxidized transcription factor derepresses the PerP regulon, encoding enzymes acting to detoxify peroxides. The His oxidation is probably irreversible and leads to degradation of the protein. Thus, the transcription factor is sacrificed to help minimize damage from Fenton reactions elsewhere in the cell (56). Several other transcription factors have been identified in bacteria, yeast, and mammals, which use the reversible oxidation of cysteines to sense H_2O_2 (56).

WHAT HAPPENS TO OXIDIZED PROTEINS?

It is generally thought that oxidized proteins are degraded relatively rapidly, probably because a change in conformation exposes more hydrophobic residues, which are recognized by proteases. However, massive protein damage can lead to the formation of protein aggregates that apparently cannot be degraded (23, 25) or are degraded by autophagy (113).

A number of proteases are found in all the major cellular compartments: cytosol (109), chloroplast (1, 93), and mitochondria (46, 71). It is unlikely that specific proteases are dedicated to the removal of oxidatively modified proteins because there are so many types of oxidative modifications. It is more likely that the proteases recognize proteins with a more open conformation. However, an unfolded conformation does not appear to be a requirement for degradation (90). We need to know more about the function and specificity of the proteases before we can understand the turnover of oxidized proteins.

Peptides deriving from mitochondrial matrix proteins have been found outside the mitochondria (51). Such peptides or even individual oxidized amino acids released from oxidized proteins are second messenger candidates.

THE COST OF ROS-INDUCED MODIFICATIONS OF CELLULAR COMPONENTS

The fate of oxidized proteins can be graded by the energetic cost to the cell (**Table 3**). The cheapest method, one used extensively in metabolic regulation, is to reverse the modification, i.e., reduce the oxidized sulfur-containing amino acid side chain back to its original form. At the second level, the oxidatively modified polypeptide is removed, degraded, and replaced by a new polypeptide. Presumably, the degradation goes to the amino acid level, where the vast majority of unmodified amino acids are recycled, whereas the modified amino acids are further metabolized. If a damaged polypeptide is part of a protein complex, the complex might dissociate, the damaged peptide be replaced, and the complex reassembled. The most expensive solution (short of programmed cell death) is to remove the entire organelle by autophagy, e.g., mitochondria by mitophagy (57).

Table 3 Estimated costs of replacing damaged cellular components[a]

Component	Size	Cost[e]
One amino acid molecule	100–200 Da	10^{f}
One PUFA molecule[b]	250–300 Da	4×10^{1}
One phospholipid molecule (with 2 PUFAs)	700–800 Da	10^{2}
One average protein molecule[c]	50,000 Da	2×10^{3}
One average protein molecule imported across a membrane	50,000 Da	10^{4}
One mitochondrion[d]	1 pg protein	2×10^{10}–10^{11}

[a] All values are very approximate.

[b] This is an estimate of the cost of synthesizing the PUFA from acetyl-CoA and does not consider the potential cost of excising and degrading the oxidized PUFA.

[c] An estimate of the cost of synthesizing the protein from amino acids. The cost of RNA turnover, e.g., mRNA, has not been considered, but it would probably not change the estimate significantly.

[d] Calculated assuming that 1 mg mitochondrial protein (consisting of 50 kDa proteins) has a matrix volume of 1 μl and a mitochondrion is a 2 μm cylinder with a diameter of 0.5 μm. The cost of replacing the ca 25% (w/w) phospholipids (28) is about half the cost. The cost of DNA and RNA turnover has not been considered, but it would probably not change the estimate significantly.

[e] In ATP equivalents/molecule or unit.

[f] This is only to give an order of magnitude.

The replacement for oxidatively damaged proteins will often have to cross membranes and/or be inserted into membranes. It is difficult to estimate the total energy consumption of this translocation and insertion, but it appears to be costly. Estimates of 10^3—10^4 ATP per protein molecule translocated have been obtained for secretion of bacterial proteins using the secretory pathway (29, 60) and translocation of proteins that use the chloroplastic twin arginine (cpTat) pathway (3).

An Arabidopsis null mutation in a gene encoding a cytosolic peptide methionine sulfoxide reductase (see above) showed increased ROS content, lipid peroxidation, and (curiously) protein carbonylation at the end of a long night. This coincided with increased protein turnover and respiration. It was interpreted to mean that increased protein turnover caused by oxidative damage was so extensive that it required increased respiration to produce the necessary extra ATP (8). Let us therefore estimate the energetic costs of replacing all the proteins in a plant mitochondrion. The cost of synthesizing and degrading 1 mg mitochondrial protein containing 20 nmol protein with an average size of 50 kDa is 20–200 μmol ATP (see **Table 3**). Assuming that the mitochondria are oxidizing Krebs cycle intermediates such as malate at 100 nmol oxygen min^{-1}mg $protein^{-1}$ (typical rates under in vitro conditions), they will produce around 400 nmol ATP min^{-1}mg $protein^{-1}$. It will therefore take approximately 1–10 h for the mitochondria to produce enough ATP to replace all their proteins.

What is the half-life of mitochondrial proteins? Estimates range from 20 h to >100 h for mammalian mitochondria (see 71 and references therein). Thus, if we assume that the average lifespan is 50 h in plant mitochondria, then mitochondrial protein turnover will consume 2–20% of all the ATP the mitochondria can make working at their maximum rate. However, this may be an underestimate because mitochondria generally do not respire at their maximum rate in situ and because the activity of energy-wasteful enzymes in the mitochondrial ETC, such as the alternative oxidase, can lower the ATP yield (e.g., 30). We must also keep in mind that the relative contribution of oxidative modifications to total protein turnover remains to be established. A significant part of cellular protein turnover is regulated to suit metabolic flexibility and that will also contribute to mitochondrial protein turnover.

HOW TO PREVENT THE TRANSFER OF OXIDATIVE DAMAGE TO THE NEXT GENERATION

It is important to prevent, or at least limit, the transfer of damaged components, especially DNA and DNA-containing organelles, to the next generation. Several very different strategies have been described for plants, mammals, and yeast.

The biochemical activity is very high in the stamen where pollen are produced and the mitochondria appear to be working close to their maximal capacity because most mutations in genes encoding mitochondrial proteins lead to dramatically lowered pollen production (e.g., 92, 108). High activity generally means high ROS production, although direct evidence for this in stamen is lacking. However, mitochondria (and plastids) are generally maternally inherited in plants. Therefore, organelles derive from the relatively quiescent egg cell, thus minimizing oxidative damage to the fertilized egg cell (4).

Another possible strategy has been proposed for mammalian cells where a small, biochemically relatively inactive subpopulation of "breeding" mitochondria in each cell is dividing while the biochemically active, and therefore relatively damaged, mitochondria do not divide (57). Finally, a third strategy has been observed in yeast where carbonylated proteins, regardless of subcellular localization, are prevented from entering the daughter cell by an as-yet-unknown mechanism (2).

SUMMARY POINTS

1. In each compartment of the plant cell, ROS formation and removal are tightly regulated.
2. Specifically, the formation of $HO^{\bullet}$ from H_2O_2 in the Fenton reaction is limited by keeping the concentration of free metal ions extremely low.
3. Oxidation of PUFA generates many products, some of which are secondary signaling molecules in plants whereas others, including MDA and HNE, can form adducts and damage DNA and proteins.
4. Nitrosylation and reversible oxidation of cysteine and methionine serve regulatory purposes.
5. Many other types of protein oxidations, e.g., carbonylation, are irreversible and probably mainly damaging. There is a relatively high level of protein carbonylation in plant tissues, which increases during stress. Many well-known proteins are oxidized, but little is known about the percentage of the molecules modified or the effect of the modifications on the properties of the proteins. Oxidized proteins are normally degraded rapidly by a number of proteases present in all plant cell compartments.
6. The cost of replacing all the proteins in a plant mitochondrion is estimated to be 2–20% of the ATP produced by the mitochondrion.
7. Yeast, mammals, and plants have mechanisms for minimizing the transfer of oxidatively modified components, especially DNA, to the next generation.
8. Many oxidative modification products are involved in metabolic regulation and signal transduction. Thus, oxidative damage and signaling are often two sides of the same story.

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