

## Role of Oxidant Species in Aging

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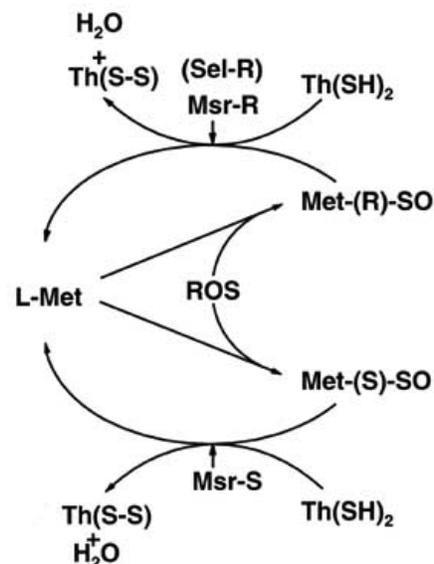
**Abstract:** Organisms are constantly exposed to many different forms of reactive oxygen species and reactive nitrogen species that damage proteins, nucleic acids, and lipids, leading to loss of biological function. The possibility that reactive oxygen/nitrogen-mediated protein damage contributes to the aging process is supported by results of many studies showing that aging is associated with the accumulation of such protein damage. Summarized here are results of studies, showing that the accumulation of protein damage is a complex function of a multiplicity of factors that govern the intracellular levels of reactive oxygen/nitrogen species, on the one hand, and a multiplicity of factors that govern the degradation and/or repair of damaged proteins, on the other. Basic mechanisms involved in the modification of proteins by various forms of reactive oxygen/nitrogen species are also discussed.

### INTRODUCTION

The possibility that oxidative damage to nucleic acids, lipids, and proteins is implicated in the aging process has been at the forefront of numerous studies in many laboratories, and has been the subject of many reviews [1-11]. The present review is focused on the possible implication of protein oxidation in aging. As summarized in Table 1 and in Fig. (1A), biological systems are frequently exposed to reactive oxygen species (ROS) and reactive nitrogen species (RNS) that: (a) are present as pollutants in the atmosphere; (b) are generated during irradiation by UV light, X-rays, gamma-rays; (c) are by-products of mitochondria-catalyzed electron transport reactions; (d) are products of oxidase-catalyzed reactions; (e) are generated by metal-catalyzed reactions; (f) are products of arginine metabolism; and (g) are produced by neutrophils and macrophages during inflammatory conditions. In their defense against ROS-mediated oxidative damage, organisms possess a battery of antioxidant systems (enzymes, vitamins, metabolites) that can either prevent the formation of these ROS/RNS or convert them to inactive derivatives [Fig. (1A)]. Nevertheless, maintenance of a low steady-state level of many of these ROS (NO,  $O_2^{\bullet S}$ ,  $H_2O_2$ ) may serve important biological functions [12] and are activators of diverse cell signaling events [12-14]. However, as shown in Fig. (1B), at high concentrations, ROS can oxidize nucleic acids, lipids, and proteins. If unrepaired, the oxidized forms of DNA and RNA can lead to transcription/translation errors, and therefore to synthesis of abnormal proteins that are more sensitive to oxidation by ROS [15,16]. Significantly, the ROS-mediated oxidation of proteins marks them for proteolytic degradation, Fig. (1B) [17-38].

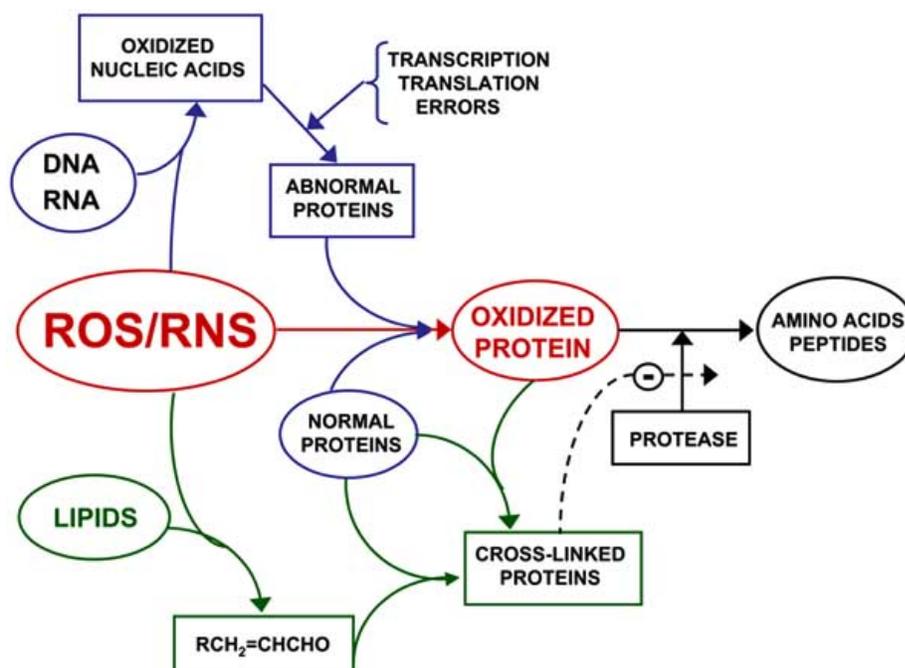
**Table 1. Origin of Reactive Oxygen Species**

Source	ROS
Atmosphere Pollution	CO, Ozone, NO <sub>2</sub> , N <sub>2</sub> O <sub>2</sub>
By-products of electron transport	$O_2^{\bullet -}$
Irradiation (X-, $\gamma$ -, UV)	$O_2^{\bullet -}$ , $\bullet OH$ , $^1O_2$
Metal-catalyzed oxidation	$\bullet OH$ , $H_2O_2$ , Ferryl ion
Inflammation (neutrophils, macrophages)	$OCl^-$ , $H_2O_2$ , $O_2^{\bullet -}$ , NO, ONOO <sup>-</sup>
Oxidases	$H_2O_2$
Arginine metabolism	NO



**Fig. (1A). Generation of reactive oxygen/nitrogen species (ROS/RNS).** Abbreviations: SOD, superoxide dismutase; CAT, catalase; Gpx, glutathione peroxidase; GST, glutathione sulfur transferase; GR, glutathione reductase; Msr, methionine sulfoxide reductase; RSH-Px, thiol-specific peroxidase.

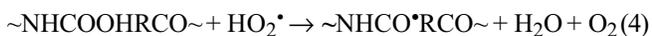
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**Fig. (1B).** Interrelationship between reactive oxygen/nitrogen-dependent modifications of lipids, DNA, and RNA and proteins on protein degradation.

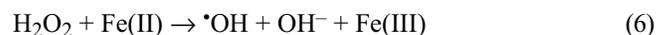
### Mechanisms of Protein Oxidation

Basic mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, peptides, and proteins were exposed to ionizing radiations under conditions where  $\cdot\text{OH}$  or a mixture of  $\cdot\text{OH}$  and  $\text{O}_2\cdot\text{S}$  are formed [39-43]. In these studies, it was demonstrated that reactions with  $\cdot\text{OH}$  leads to abstraction of a hydrogen atom from the protein polypeptide backbone to form a carbon-center radical (reaction 1), which, under aerobic conditions, reacts readily with molecular oxygen to form peroxy radicals (reaction 2). The peroxy radical is then converted to the alkyl peroxide by reaction with the protonated form of superoxide (reaction 3). Further reactions of the alkylperoxide with superoxide yield an alkoxy radical (reaction 4) and the hydroxyl derivative (reaction 5).



Alternatively: the carbon-centered radical formed in reaction 1 can react with another carbon-centered protein derivative to form a -C-C- cross-linked protein derivative; the peroxy radical derivative formed in reaction 2 can abstract a hydrogen atom from another amino acid residue in the same or another protein molecule to form another carbon-centered radical derivative; the alkoxy radical formed in reaction 4 may also undergo peptide bond cleavage (see below). Whereas the reactions described above were elucidated in the course of studies using ionizing radiation to generate the superoxide and the hydroxyl radicals, the

same series of reactions can be initiated by hydroxyl radical produced *in vivo* by the Fenton reaction (reaction 6).



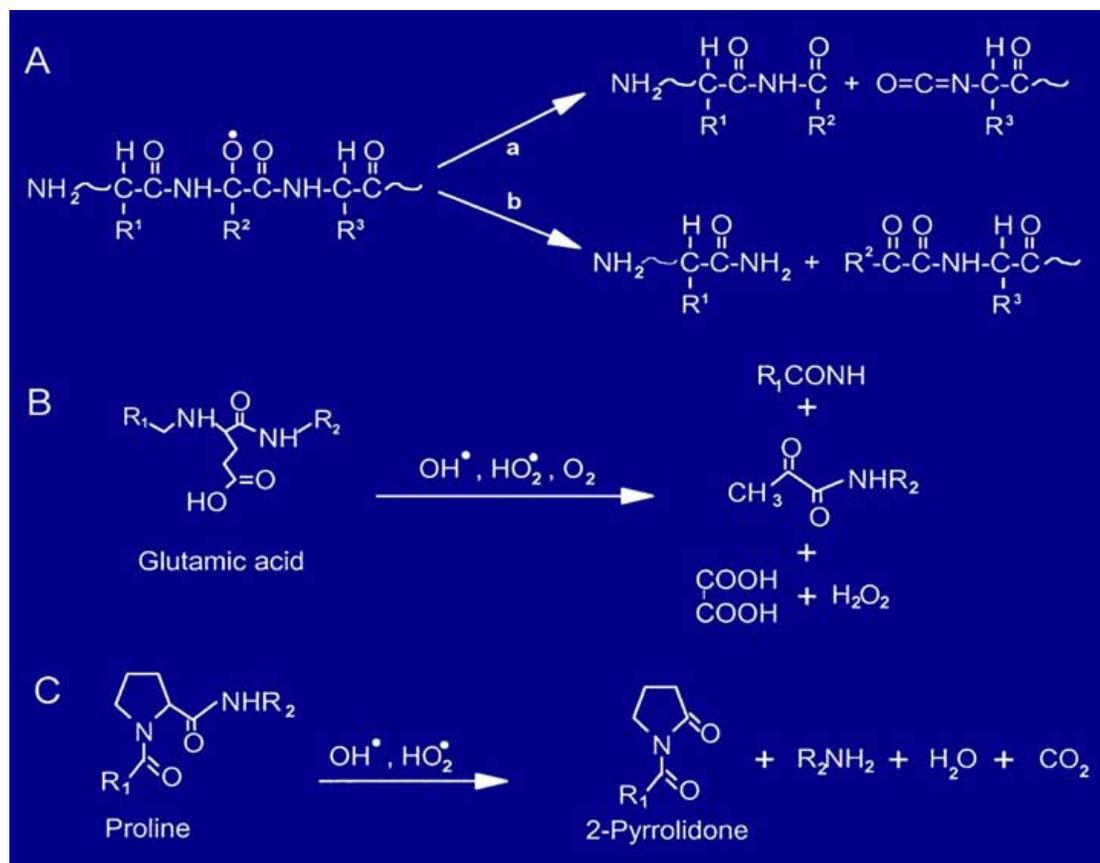
Furthermore,  $\text{Fe(II)} + \text{H}^+$  can replace  $\text{HO}_2\cdot$  in reactions 3, 4, and 5, illustrated in [44].

### Peptide Bond Cleavage

In addition to a role in hydroxylation of proteins (reaction 5), the alkoxy radical derivatives of proteins are capable of undergoing peptide bond cleavage by either of two mechanisms [40]. As shown in Fig. (2A), route a, cleavage by the diamide pathway leads to two peptides; the N-terminal amino acid residue of the peptide fragment obtained from the C-terminal portion of the protein exists as a isocyanate derivative, whereas the C-terminal amino acid residue of the peptide derived from the N-terminal portion of the protein exists as a diamide derivative. However, when cleavage occurs by the  $\alpha$ -amidation pathway, Fig. (2A), route b, the N-terminal amino acid residue of the peptide derived from the C-terminal portion of the protein exists as an  $\alpha$ -ketoacyl derivative, and the C-terminal amino acid residue of the peptide derived from the N-terminal portion of the protein exists as an amide derivative. Peptide bond cleavage can occur also by hydroxyl radical-initiated attack of the glutamic acid [40] and proline [45] residues of proteins to form a mixture of products as illustrated in Figs. (2B) and (2C).

### Oxidation of Amino Acid Residue Side Chains

The side chains of all amino acid residues of proteins are susceptible to oxidation by ionizing radiation. The amino



**Fig.(2).** Free-radical mediated cleavage of the poly peptide backbone.

acid residues that are most vulnerable to attack by various ROS/RNS, along with their reaction products, are shown in Table 2.

**Table 2. Amino acid Residue Modifications**

Amino acid residue	Products formed
Arginine	Glutamic semialdehyde
Cysteine	Disulfides: Cys-S-S-Cys, Cys-S-S-R
Glutamate	4-Hydroxy-glutamate, Pyruvate, $\alpha$ -Ketoglutarate
Histidine	2-Oxo-histidine
Leucine	3- and 4-Hydroxy-leucine
Lysine	2-Amino adipic-semialdehyde, 3-,4-, and 5-hydroxy-lysine
Methionine	Methionine sulfoxide, Methionine sulfone
Phenylalanine	2-, 3-, and 4-hydroxy-phenylalanine
Proline	Glutamic semialdehyde, Pyroglutamic acid, 2-Pyrrolidone 4-hydroxy-proline
Threonine	2-Amino-3-keto-butyric acid
Tryptophan	N-Formyl-kynurenine, kynurenine, 2-, 4-, 5-, 6-, and 7- Hydroxy-tryptophan
Tyrosine	3-4-dihydroxy phenylalanine (DOPA), Tyr-Tyr cross-linked proteins, 3-Nitro-tyrosine, 3,5-Dichloro-tyrosine
Valine	3-, 4-Hydroxy-valine

### Generation of Protein Carbonyl Derivatives

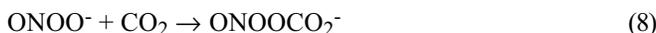
Lysine, arginine, proline, and threonine residues of proteins at metal binding sites are highly susceptible to site-specific oxidation by metal-catalyzed reactions (Table 2) [46, 47]. As noted above [Fig. (2A, 2B)], protein carbonyl derivatives are also formed in cleavage of the poly peptide chain by the  $\alpha$ -amidation and glutamic acid oxidation pathways. Carbonyl groups can also be introduced in proteins by Michael addition of amino acid side chains (histidine imidazole groups, lysine amino groups, and cysteine sulfhydryl groups) to  $\alpha$ - $\beta$ -unsaturated aldehydes formed in the peroxidation of lipids [48-50], and by reactions of lysine amino groups with another lipid peroxidation product, malondialdehyde [51]. Carbonyl groups are also generated as a result of glycation/glycoxidation reactions [52-53]. In addition, interactions of protein lysine residues with lipid peroxidation and glycation/glycoxidation products can lead to formation of *N*-carboxymethyl-lysine (CML) derivatives [54]. Because CML is a strong metal ion chelator [55], it is able to promote the generation of carbonyl groups by metal-catalyzed reactions [56].

In view of the fact that protein carbonyl groups are generated by so many different mechanisms, it is not surprising that the concentration of protein carbonyl groups is orders of magnitude greater than any other kind of protein oxidation [57]. Because a number of highly sensitive methods have been developed for the assay of protein carbonyl groups [58-65], the concentration of carbonyl

groups has become the most widely used measure of ROS-mediated protein oxidation.

### Modification of Proteins by Peroxynitrite

Nitric oxide produced in the metabolism of arginine serves as a second messenger in the regulation of various cellular functions [66-69]. However, it reacts rapidly with superoxide anion to form the highly toxic peroxynitrite derivative [70-72] (reaction 7), which is able to nitrosate cysteine sulfhydryl groups [73-75], nitrate tyrosine and tryptophan residues of proteins, and oxidize methionine residues to methionine sulfoxide (see [76,77] for reviews). However, its ability to mediate these reactions is strongly influenced by the presence of physiological concentrations of CO<sub>2</sub>. In the presence of CO<sub>2</sub>, peroxynitrite reacts rapidly with CO<sub>2</sub> to form the ONOOCO<sub>2</sub><sup>-</sup> (reaction 8) [78,79].

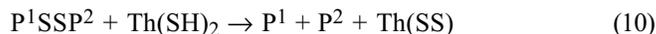
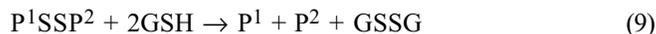


The ability of peroxynitrite to oxidize methionine residues of proteins is strongly inhibited by physiological concentrations of CO<sub>2</sub>, and its ability to nitrate tyrosine residues is almost completely dependent upon the presence of CO<sub>2</sub> [80-82]. The importance of tyrosine nitration in metabolism is underscored by the demonstration that nitration prevents the phosphorylation [83] or adenylation [80, 84] of tyrosine residues of regulatory proteins, and in some cases can mimic the effects of these regulatory processes [84]. This could seriously compromise these regulatory processes since the nitration of tyrosine residues is irreversible. Based on these observations and results of other studies not discussed, here it is evident that the toxicity of peroxynitrite is linked to its ability to generate various radical intermediates ( $\cdot\text{OH}$ ,  $\text{O}_2^{\cdot-}$ ,  $\text{NO}_2^{\cdot}$ ,  $\cdot\text{CO}_2^{\text{S}}$ ), possibly by mechanisms illustrated in Fig. (3). However, there is still considerable uncertainty regarding the mechanisms of these reactions [69,76].

### Oxidation of Sulfur-Containing Amino Acids

Cysteine and methionine residues of proteins are particularly susceptible to oxidation by ROS [85-87]. In contrast to other ROS-mediated oxidations, oxidation of the sulfur amino acids is reversible. Oxidation of cysteine

sulfhydryl groups of proteins leads to the production of either intra-molecular (P<sup>1</sup>SSP<sup>1</sup>) or inter-molecular (P<sup>1</sup>SSP<sup>2</sup>) protein cross-linked derivatives and reactions with oxidized forms of glutathione (GSSG) yields the mixed disulfide (PSSG). These disulfide derivatives can be repaired by disulfide exchange reactions catalyzed by thiol transferases that catalyze reactions between glutathione (GSH) or thioredoxin [Th(SH)<sub>2</sub>] to regenerate the protein sulfhydryl groups (reactions 9, 10, 11).



The GSSG and Th(SS) generated in these reactions can be reduced back to their sulfhydryl forms by specific reductases that use NADPH as an electron donor (for reviews see [89,90]).

It is now well established that the oxidation of methionine (Met) residues of proteins leads to the formation of a mixture of the S- and R- isomers of methionine sulfoxide [Met-(S)-SO and Met-(R)-SO] [91]. Most organisms contain two different methionine sulfoxide reductase activities; one (Msr-S, sometimes referred to as MsrA) that is specific for reduction of the S- isomer [93-96] and another (Msr-R, sometimes referred to as MsrB) that is specific for reduction of the R-isomer [93,94,96,97]. Msr-R contains a selenocysteine moiety at the catalytic site, whereas Msr-S contains a cysteine moiety at the catalytic site [93,98]. Substitution of cysteine for selenocysteine at the catalytic site of Msr-B leads to considerable decrease in its activity [98]. As illustrated in Fig (4), both reductases utilize Th(SH)<sub>2</sub> as a source of electrons for reduction of the sulfoxides back to methionine. From the metabolic point of view, it is important to note that the oxidized form of thioredoxin [Th(SS)] can be reduced back to Th(SH)<sub>2</sub> by NADPH in a reaction catalyzed by another seleno-protein, thioredoxin reductase [100,101]. Accordingly, the overall cyclic oxidation and reduction of methionine residues is described by reactions 12-15, in which the term "products" refers to the derivatives of the ROS species involved in Met oxidation.

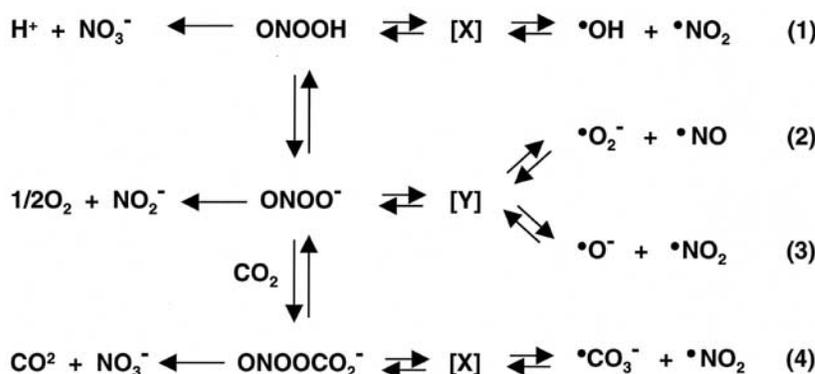
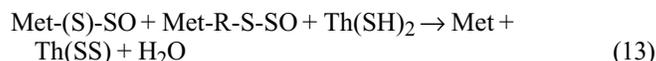
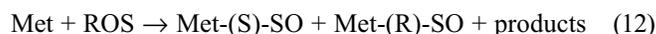
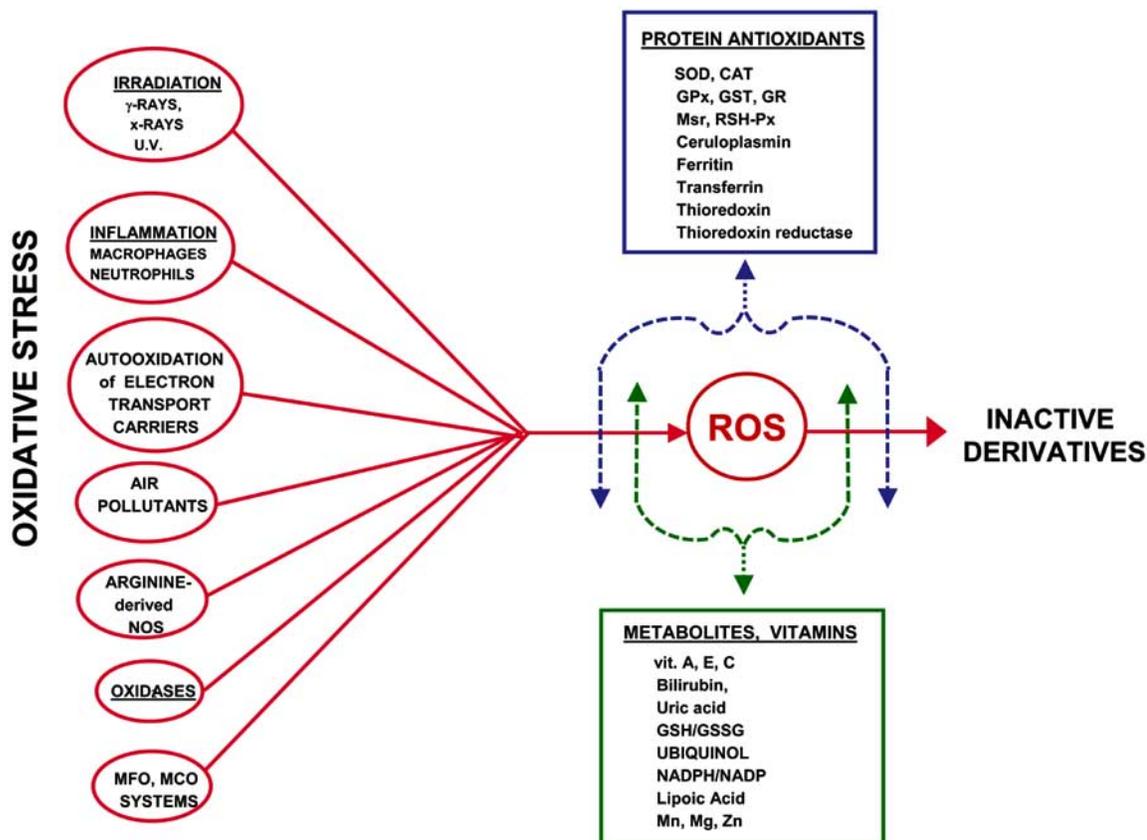


Fig. (3). Possible mechanisms of free radical generation by peroxynitrite. X, Y, and Z represent "caged radical pairs" as indicated in [81].



**Fig. (4). Cyclic oxidation-reduction of methionine residues of proteins.** Abbreviations: L-Met, methionine residues of proteins; Met-(R)-SO, and Met-(S)-SO, the R- and S-isomers of methionine sulfoxide, respectively; ROS, reactive oxygen species; Msr-R<sub>1</sub>(Sel-R) and Msr-S<sub>1</sub>, the methionine sulfoxide reductases that are specific for reduction of the R- and S-isomers of methionine sulfoxide, respectively; Th(SH)<sub>2</sub> and Th(S-S), the reduced and oxidized forms of thioredoxin, respectively.



Because almost all forms of ROS are able to oxidize methionine residues of proteins to methionine sulfoxide, it was proposed that the cyclic oxidation and reduction of methionine residues of proteins serves an important antioxidant (ROS-scavenger) function to protect cells from oxidative damage [102]. This proposal is supported by results of studies showing that mutations leading to a decrease in the Msr activities of bacteria, yeast, and mice lead also to a loss in their resistance to oxidative stress and to an increase in the levels of oxidized proteins [95,104,105]; whereas overproduction of Msr in yeast and *Drosophila* leads to an increase in resistance to oxidative stress [104,105].

#### Oxidation-Dependent Generation of Protein-Protein Cross-Links

Protein oxidation is implicated in the generation of many different kinds of inter- and intra-protein cross-linkages, including those formed: (a) by addition of lysine amino groups to the carbonyl group of an oxidized protein; (b) by interaction of two carbon-centered radicals obtained by the

<sup>•</sup>OH-dependent abstraction of hydrogen atoms from the polypeptide back bone or amino acid side chains (in the absence of oxygen) to form -C-C- protein cross links; (c) by Michael additions of histidine, lysine, and cysteine side chains to the double bonds of unsaturated aldehydes formed in the oxidation of polyunsaturated fatty acids (*viz.*, 4-hydroxy-2-nonenal); (d) the oxidation of sulfhydryl groups of cysteine residues to form -S-S- cross-links; (e) the reaction of lysine amino groups with carbonyl groups of glycated/glycoxylated proteins; and (f) the oxidation of tyrosine residues to form -tyr-tyr- cross-links (see [107] for review). Whereas some cross-linked proteins are susceptible to degradation by the 20S proteasome, other cross-linked proteins are not only resistant to proteolytic degradation by the proteasome but in addition they are potent inhibitors of the proteolytic degradation of other oxidatively modified proteins by the proteasome [21,36].

#### Role of Protein Oxidation in Aging

A role of protein modification in aging was highlighted by results of studies showing that many different enzymes isolated from young animals were catalytically more active and more heat stable than the same enzymes isolated from old animals [108-111]. Because exposure of enzymes from

young animals to metal-catalyzed oxidation led to changes in activity and heat-stability similar to those observed during aging [21,26,36,112,113], it was proposed that ROS-mediated protein damage is involved in the aging process [114]. In the meantime, this concept is supported by studies in many different animals showing that aging is often associated with the accumulation of oxidized forms of proteins [3, 5-11,113,115-122]. Moreover, mutations and dietary or environmental regimens that lead to an increase in animal life-span lead also to a decrease in the tissue levels of oxidized proteins, and *vice versa* [116,123-125].

It is evident from an analysis of the schemes presented in Figs. (1A) and (1B) that the age-related accumulation of oxidized proteins is dependent upon the balance between many different processes, including: (a) the rate of ROS synthesis by any one of the numerous mechanisms; (b) the ability of various antioxidants to scavenge ROS; (c) the ability to repair nucleic acid damage leading to the generation of altered proteins that are highly sensitive to oxidation; (d) the concentrations of proteases that degrade oxidized forms of proteins; (e) the generation of cross-linked proteins that inhibit the proteolytic degradation of oxidized proteins; and (f) the ability to repair oxidation of sulfur-containing amino acid residues of proteins (discussed below). Because the level of oxidized protein depends on the balance between so many different factors, it is obvious that the observed age-related increases in the level of oxidized protein in one individual may be due to a different defect than that in another individual. For example, the accumulation of oxidized proteins in one individual may be due to an elevation in the steady level of ROS or RNS or to a decrease in the antioxidant capacity, whereas the accumulation in another individual may be caused by a decrease in the ability to degrade oxidized proteins due either to a decrease in the protease concentrations or to an increase in the levels of protease inhibitors. Regardless of the mechanism, the overall result could be the same, *i.e.* an age-related loss of biological functions. Significantly, the level of oxidized protein increases exponentially as a function of animal age [114]. In a recent study, it was shown that the observed age-related increase in protein oxidation is consistent with a simple auto-catalytic model based on the assumption that free radicals randomly oxidize proteins and thereby targets them for proteolytic degradation [126].

Failure of some studies to detect an age-dependent increase in the level of oxidized protein [127,128] does not exclude a role of protein oxidation in aging. As noted above, the intracellular steady-state level of oxidized proteins is determined by both the rate of protein oxidation and the rate of proteolytic degradation of oxidized proteins. Because aging is often associated with a decrease in the level of the 20S proteasome, it is likely that the age-related increase in the level of oxidized protein is due in part to the loss of proteasome activity. However, this may not always be the case. In the absence of an age-related loss of proteasome activity, an age-related increase in the rate protein oxidation may go undetected because the rate of protein oxidation may be equal to the rate of oxidized protein degradation. Under these conditions, the steady-state levels of enzymes and regulatory proteins that are most susceptible to protein oxidation would be expected to decrease and seriously compromise biological function. Furthermore, because there

are so many different mechanisms of protein oxidation, the failure to observe an age-related increase in the protein carbonyl content in one individual does not preclude an age-dependent increase in the level of other forms of protein oxidation; *viz.*, Tyr-Tyr, -S-S- cross-links, tyrosine nitration, methionine sulfoxide formation, carboxymethyl lysine formation, etc.

### Protein Oxidation in Diseases

Although not the subject of this review, it should be noted that oxidation of proteins is associated with a number of age-related diseases, including (but not limited to) Alzheimer's disease, rheumatoid arthritis, amyotrophic lateral sclerosis, cataractogenesis, Parkinson's disease, Progeria, Werner's syndrome, systemic amyloidosis, muscular dystrophy, and respiratory distress syndrome. For reviews, see [9, 11, 44, 64, 115, 117, 129].

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