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Minireview

Metal-catalyzed Oxidation of Proteins

PHYSIOLOGICAL CONSEQUENCES

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Detailed studies of the oxidative modification of glutamine synthetase (GS), in extracts of Escherichia *coli* **(1, 3)** and *Klebsiella* aerogenes **(2, 4)** established that the oxidation is dependent upon NAD(P)H, O_2, and Fe(III) or Cu(II). The requirements for O_2 and an auxiliary electron donor (*i.e.* a donor other than GS itself) indicated that the oxidation is promoted by a mixed function oxidation (MFO)' type mechanism. In the meantime, it was found that many enzymes are readily inactivated by MFO systems **(5–7)** and that this renders them susceptible to proteolytic degradation by a variety of exogenous and endogenous proteinases **(1, 8–12).** It became evident also that the oxidation of proteins is likely implicated in the killing of bacteria by neutrophils (13), and in the generation of altered (inactive or less active) forms of enzymes that accumulate during animal aging (6, 14–16) and under several pathological conditions **(17–23).** It is the intent of this review to summarize the results of studies on the mechanism of protein oxidation by MFO systems and the biological implications of such oxidations

The Mechanism of Metal Ion-catalyzed Protein Oxidation

The current view of how MCO[2] systems mediate protein oxidation is illustrated in Fig. 1. In this representation, the electron donor system is needed only to catalyze the reduction of O_2 to H_2O_2 and of Fe(III) to Fe(II). Depending upon the electron donor system used, the reduction of O_2 can proceed by a two-electron mechanism yielding H_2O_2 directly, or by way of two sequential one-electron transfer processes leading first to superoxide anion followed by its dismutation to H_2O_2 and O_2. Similarly, the reduction of Fe(III) to Fe(II) can occur directly or via the intermediate formation of O_2^-, which can react directly with Fe(III) to form Fe(II) and O_2. It is believed that the Fe(II) then binds to a metal binding site on the protein (P) after which the protein.Fe(II) complex reacts with the H_2O_2 to generate in *situ* an activated oxygen species (OH, ferryl ion), which reacts with the side chains of amino acid residues at the metal binding site. Among other modifications, some amino acid residues are converted to carbonyl derivatives **(25, 26).** After oxidative modification, the protein becomes highly sensitive to proteolytic degradation, and in the case of enzymes they are converted to catalytically inactive or less active, more thermolabile forms (14).

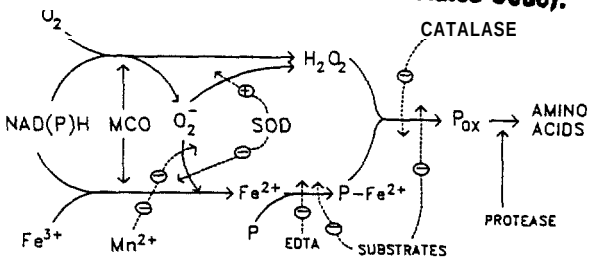

FIG 1. [illegible] oxidation and [illegible]. *SOD*, superoxide; *P*, protein; P_{ox}, oxidized protein. The symbols ⊕ and ⊖ indicate stimulation and inactivation respectively

Site-specific Nature of the MCO-catalyzed Reactions

According to Fig. 1, the MCO-catalyzed oxidation of proteins is a site-specific process involving the interaction of H_2O_2 and Fe(II) at metal binding sites on the protein. The site-specific nature of the reaction is indicated by the following facts. (*a*) The inactivation of enzymes by MCO systems is relatively insensitive to inhibition by free radical scavengers (formate, ethanol, and mannitol) **(5, 6, 27).** (*b*) Only one or at most only a few amino acid residues in a protein can be modified by MCO systems **(25, 26)** whereas almost all amino acid residues can be modified when proteins are subjected to free radicals obtained by radiolysis **(28–30).** (*c*) Most of the enzymes that are highly sensitive to modification by MCO systems require metal ions for catalytic activity. Therefore, they must contain a metal ion binding sire. (*d*) In the case of *E.* coli glutamine synthetase, the loss of catalytic activity correlates with the loss of a single histidyl and a single arginyl residue per subunit, both of which are situated in close proximity to one of two divalent metal binding sites on the enzyme **(31, 32).**[3]

A plausible mechanism for the site-specific modification of a lysyl residue at the metal binding site of a protein is illustrated in Fig. **2.** In this mechanism the reduction of Fe(III) (step *a*) is followed by binding of the Fe(II) to the enzyme (step *b*) to form a coordination complex in which the c-amino group of a lysyl residue at the metal binding site Serves as one of several ligands to which the Fe(II) is bound. The H_2O_2 produced by the reduction of O_2 (step *c*) may react with Fe(II) in the complex to form OH, OH^-, and a Fe(III)·enzyme complex (step *d*). The OH thus formed abstracts a hydrogen atom from the carbon atom bearing the **c-amino** group to form a carbon-centered radical (step e), which then donates its unpaired electron to Fe(III) in the complex to regenerate Fe(II) and coincidently converts the c-amino group to an imino derivative (step *f*). Finally, the imino derivative undergoes spontaneous hydrolysis whereby NH_3, Fe(II) are released and an aldehyde derivative of the lysyl residue is generated (step g). The derivatized protein is thus rendered susceptible to proteolytic degradation. Upon acid hydrolysis, 2-aminoadipic semialdehyde would be one of its products. In this representation, the reaction of H_2O_2 with the Fe(II)·enzyme complex is shown to yield OH as a primary product. Similar

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[1] The abbreviations used are: MFO. mixed function oxidation: MCO. metal-catalyzed oxidation: PBN, *N-tert*-butyl-α-phenylnitrone; LDL. low density lipoprotein.

[2] To avoid confusion with mixed-function *oxidases* (24), MFO systems are now referred to as metal-catalyzed oxidation MCO systems.

[3] I. Climent and R. L. Levine, personal communication.

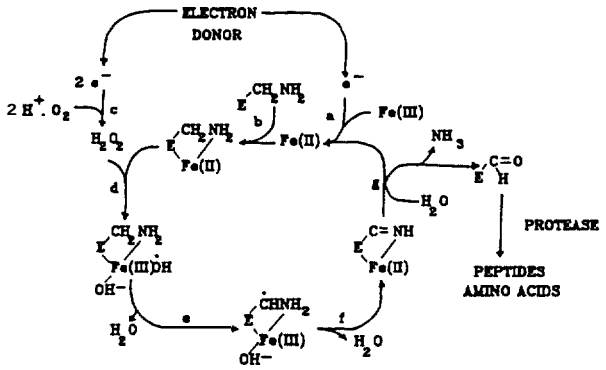

FIG. 2. Site-specific metal ion-catalyzed protein oxidation.

TABLE I

MCO systems that *catalyze* protein *modification*

1.	NAD(P)H oxidase/NAD(P)H/O_2/Fe(III)
2.	Xa[illegible] idase/xanthine, III)/C
3.	Cytochrome P-450/cytochrome P-450 reductase/Fe(III)/(
4.	Cy[illegible] P 450, 'P tid[illegible] /P [illegible])H/(
5.	[illegible] (II
6.	Fe(II)/O_2
7.	Fe(II)/H_2O_2
8.	RSH/Fe(III)/O_2

mechanisms in which $(FeO)^{2+}$ or $(Fe(OH)_2)^{2+}$, or $(FeOH)^{3+}$ + OH– is the active intermediate might also be considered. Nevertheless, the reaction is perceived as a "caged" reaction in which the activated oxygen species does not escape but reacts preferentially with functional groups at the metal binding site of the enzyme. This would account for the failure of radical scavengers to inhibit the reaction.

It follows from the mechanisms depicted in Figs. 1 and 2 that any system capable of producing H_2O_2 and of reducing Fe(III) or Cu(II) can provoke site-specific modifications of proteins possessing a metal binding site. Several enzymic and nonenzymic systems which have been shown to catalyze the oxidative modification of enzymes are listed in Table I. Perhaps the most physiologically relevant of these are those for which NADH or NADPH serves as the auxiliary electron donor, *e.g.* the NAD(P)H oxidases and the cytochrome **P-450** systems.

Of the nonenzymic MCO systems, those in which ascorbate and mercaptans serve as electron donors are particularly important. The ascorbate system, originally developed by Udenfriend *et al.* (33), has been considered a model for mixed function oxidases that catalyze the O_2-dependent hydroxylation of aromatic compounds (drugs) **(24).** In the meantime, it was found to promote the oxidation of lipids (34), nucleic acids (35), and proteins **(25–27, 36).** Indeed, studies of Levine and co-workers on the oxidation of glutamine synthetase **(25, 27)** and amino acid homopolymers **(26)** by the ascorbate MCO system have contributed greatly to **our** understanding of the mechanism of site-specific metal ion-catalyzed oxidation of proteins. The biological significance of the $RSH/Fe(III)/O_2$ system seems assured by the discovery of a protein in yeast and in rat tissues that protects proteins from damage by this MCO system **(37,** 38).

Oxidation Marks Proteins for Degradation

The proposition that metal-catalyzed oxidation marks proteins for degradation stemmed from the observations that the oxidized form of glutamine synthetase is degraded by cell-free extracts of *E.* coli more rapidly than is the native enzyme **(1,** 3). Confirmation of this hypothesis was obtained by Rivett **(9,** 10) and by Roseman and Levine **(11)** showing that highly purified preparations of neutral alkaline proteinases from rat liver and *E.* coli catalyze rapid degradation of the oxidized forms of glutamine synthetase but have little or no ability to degrade the unoxidized enzymes. The concept gained additional support from the studies of Davies and co-workers showing that the degradation of endogenous proteins in *E.* coli (39), red blood cells (40, 41), and liver or heart mitochondria **(42)** is greatly enhanced following exposure of the cells to oxygen radicals or H_2O_2, and also by their studies showing that prior exposure of highly purified proteins to oxygen radical generation systems in *vitro* makes them highly susceptible to degradation by proteases in extracts of *E.* coli (39) or red blood cells **(40).**

The discovery that carbonyl derivatives of some amino acid residues are among the products of oxygen radical damage to proteins provides a means of assessing the extent of such damage under various physiological conditions. To this end, several methods have been developed for the quantitation of protein carbonyl groups (43).

Protein Oxidation and Aging

Catalytically inactive or less active, more thermolabile forms of enzymes accumulate in cells during aging **(6, 44).** To test the possibility that the age-related changes are due in part to metal-catalyzed oxidation reactions, the concentrations of protein carbonyl groups were measured in three different aging models, *viz.* in human erythrocytes, in cultured human dermal fibroblasts, and in rats of different ages. In the erythrocyte model advantage is taken of the fact that the density of erythrocytes increases with cell age. Therefore, cells of different ages can be separated from a single batch of blood by means of density gradient sedimentation. **As** shown in Fig. 3A, ***upper panel,*** the level of protein carbonyl groups in various fractions increased with cell density, *i.e.* with cell age **(14).** Moreover, the levels of glyceraldehyde-3-P dehydro-

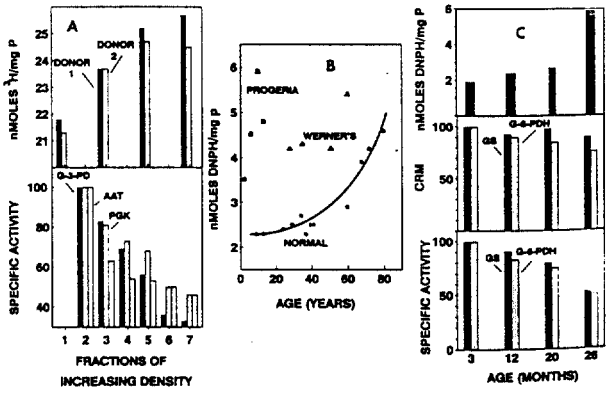

FIG. 3. Age-dependent accumulation of oxidized protein and enzyme inactivation. *A.* the human erythrocyte model: upper *panel.* the *black* and *shaded bars* refer to the protein carbonyl content of red cells from human donors 1 and 2. respectively: *lower panel,* the *black, shaded,* and *open bars* refer to the relative catalytic activities of glyceraldehyde-3-P dehydrogenase (*G-3-PD*), aspartate aminotransferase (*AAT*), and phosphoglycerate kinase (*PGK*), respectively. of human red cells *B,* the cultured fibroblast model shows the carbonyl content of protein from cultured human fibroblasts. *C,* the rat model: *upper panel.* the *bars* refer to the amount of carbonyl groups in protein from rat liver extracts, *middle panel,* the *black* and *shaded bars* refer to the relative amount of matenal in rat liver extracts (*CRM*) that cross-reacts with antibodies to purified glutamine synthetase (*GS*) and glucose-6-P dehydrogenase (*G-6-PDH*), respectively, *lower panel,* the *black* and *shaded bars* refer to the relative catalytic activities of glutamine synthetase and glucose-6-P dehydrogenase, respectively The data in A and *B* are from experiments as described in Oliver *et al.* (14) The data in *C* are from Starke-Reed and Oliver (16). The carbonyl content per mg of protein (*P*) is expressed either as ^3H per mg of protein obtained by reduction of carbonyl groups with [^{3}H]BH_4 or DNPH per mg of protein, where DNPH refers to the carbonyl content as a measured reaction with DNPH.

genase (G-3-PD), aspartate aminotransferase (ATT), and phosphoglycerate kinase (PGK) declined with cell density, in confirmation of earlier studies showing the activities of these enzymes decrease with cell age. In the fibroblast model, the carbonyl content of protein in cultured fibroblasts from individuals of different ages and from individuals with premature aging diseases (patients with progeria or Werner's syndrome) was measured using the 2,4-dinitrophenylhydrazone assay procedure **(14).** As shown in Fig. 3*B*, there was an almost exponential increase in the level of protein carbonyl groups with donor age over the range of 10–80 years. Moreover, the carbonyl content of protein in fibroblasts from individuals with either progeria or Werner's syndrome was very much higher than that in fibroblasts of normal individuals of the same age. In fact, the levels in fibroblasts from young donors with these aging diseases was about the same as that found 'n cultured fibroblasts from normal 80-year-old donors. Finally, as shown in Fig. 3*C*, upper panel, the carbonyl content of protein in hepatocytes from rats was found to increase gradually with animal age over the range of 3–20 months and then increased rapidly over the range of 20–26 months (16). Data in Fig. 3*C*, *lower* panel, show also that the activities of GS and glucose-6-P dehydrogenase (Glc-6-PD) decreased with animal age to values only about **50%** of that found in young animals; moreover, the loss in enzyme activity was not associated with a comparable loss of antibody-specific immunoreactive protein (Fig. 3*C*, middle panel). Furthermore, he level of neutral alkaline protease(s) activity in hepatocytes lso declines with animal age **(Fig.4)** suggesting that the age-dependent accumulation of altered enzyme forms is due partly to a loss of those proteases that degrade oxidized proteins. This possibility is supported further by an analysis of the data in Fig. **4** which shows a replot of some of the data in Fig. 3C. The shaded area in Fig. **4** represents the difference between catalytically active Glc-6-P dehydrogenase and total Glc-6-P dehydrogenase protein as measured by specific antibody titration. This shaded fraction therefore is a measure of inactive Glc-6-PD. As shown in the inset there is a linear inverse elationship between the level of inactive Glc-6-PD and the evel of neutral alkaline protease activity. Taken together the data in Figs. 3C and **4** show that the level of oxidized protein increases with animal age and that the concomitant accumulation of altered Glc-6-PD is related to a loss of the proteinases that are responsible for the degradation of altered (oxidized?) protein.

It should be emphasized that the 2–3-fold increase in levels

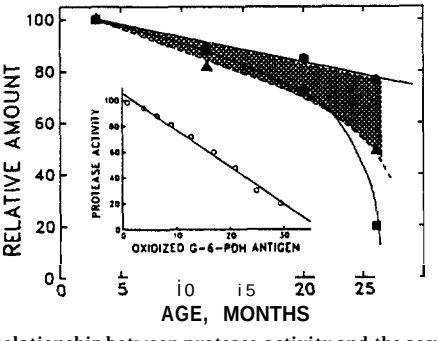

FIG. 4. **Relationship between protease activity and the accumulation of catalytically inactive glucose-6-P dehydrogenase protein. The data are replots of data presented by Starke-Reed and Oliver (16). The *curves* are as follows: ■, neutral alkaline protease activity; A, Glc-6-PD (*G-6-PDH*) activity: ●, protein that reacts with antibodies against highly purified Clc-6-PO. The *shaded area* is a measure of the catalytically inactive Glc-6-PD antigen which accumulated. The inset represents the relationship between the relative amounts of observed neutral protease activity and the amount of inactive Glc-6-PD as determined by the difference between the total immunologically reactive Glc-6-PD protein and the catalytically active Glc-6-PD enzyme.**

of oxidized proteins which occurs during aging (Fig. 3, *B* and *C*) is by **no** means trivial. It represents a substantial increase in the fraction of total protein that is oxidized. Based **on** the observation that on the average only one carbonyl group **is** introduced per enzyme subunit and the assumption that **the** average molecular weight of enzyme subunits is **50,000,** it can be calculated that the amount of oxidized protein ranges from 10% of the total protein in young animals to between 23 and 30% in old animals **(16).** These are clearly minimal values since not all metal-catalyzed oxidation reactions yield carbonyl derivatives, *viz.* histidyl, prolyl, and methionyl residues yield asparaginyl, pyroglutamyl, and methionyl sulfoxide residues, respectively.

Protein Oxidation and Disease

Inflammatory Diseases—It is becoming apparent that MCO-mediated protein oxidation is an early indicator of tissue damage and that the formation of protein carbonyl derivatives is associated with pathological conditions both in humans and in animal model systems. Studies in this laboratory **(45)** have shown that activated neutrophils generate diffusible products (*viz.* H_2O_2, O_2^-, etc.) which in the presence of Fe(III) will cause inactivation of enzymes within intact bacterial cells by an endocytotic independent mechanism. Moreover, during periods of oxidative burst some endogenous neutrophil proteins are converted to carbonyl derivatives and endogenous enzymes are inactivated. This may account for the fact that neutrophils lose activity and undergo lysis after activation. These results raise the possibility that recruitment and activation of neutrophils at extravascular sites may provoke protein oxidation and thus contribute to the tissue damage that occurs during chronic inflammation. Indeed, Chapman et *al.* (21) have shown that the levels of protein carbonyl groups in the synovial fluid of patients with rheumatoid arthritis are higher than in patients with osteoarthritis.

Atherosclerosis—It is **now** generally believed that metal ion-catalyzed oxidation of low density lipoprotein (LDL) by endothelial cells and the subsequent uptake of the oxidized LDL by monocytes/macrophages to form foam cells are important events in atherogenesis (for review see Steinberg et *al.* **(46)).** Most attention has been focused **on** the oxidation of the lipid moieties of LDL **(47, 48).** However, the likelihood that protein oxidation is also involved is implied by the fact that **modifi-**cations of apoprotein B include fragmentation and the loss of histidyl, lysyl, and prolyl residues; such changes are characteristic of MCO reactions **(49).**

Ischemia *Reperfusion* ***Tissue*** Damage—The proposal of McCord and co-workers **(50)** that oxygen free radicals are involved in ischemia reperfusion injury is supported by results of more recent studies with spin trapping techniques showing that free radicals are generated during the reperfusion phase (51, 52).

A role of protein oxidation in ischemia reperfusion injury was verified by the finding that during reperfusion for 60 min following ischemia in the gerbil brain the level of protein carbonyl groups increased from **6** to 13 nmol/mg protein; coincidentally, the specific activity of glutamine synthetase declined to about one-half of its original value (52). That free radicals are involved in these changes is indicated by the **fact** that prior treatment of the animals with the **spin** trap *N-tert*-butyl-a-phenylnitrone (PBN) partially protected them against protein oxidation and loss of glutamine synthetase provoked by ischemia reperfusion.

Neurologic Disorders—A role of protein oxidation in ***some*** neurological disorders is indicated by (a) the studies of Konat

and Wiggins (53) showing that myelin proteins are lost when rat myelin preparations are incubated with Cu(II) and H_2O_2; (*b*) subsequent studies showing that when myelin preparations are incubated with MCO systems, protein carbonyl groups are generated and the myelin is rapidly degraded by myelin proteases'; and (*c*) the studies of Mickel *et* al. (23) showing that rapid correction of vasopressin-induced hyponatremia in rats is associated with increased protein carbonyl content and myelinolysis. In addition, Murphy and Kehrer (19) have reported that the level of protein carbonyl groups is elevated in the pectoralis muscle of dystrophic chickens.

Catarmtogenesis—Finally,Garland and co-workers (54, 55) showed that there is a small but significant age-dependent increase in the protein carbonyl content of human lens, and that the level of oxidized proteins in cataractous lenses reaches values several times that of normal lenses. They showed also *that* exposure of bovine crystallins to MCO systems *in vitro* produces physical changes similar to those observed in human crystallins *in viuo.*

Conclusion

It would appear from the above that the accumulation of oxidized proteins may be an early indication of oxygen radical-mediated tissue damage, and in some pathological states may account for 50–70% of the total cellular protein (16). Since the intracellular level of oxidized proteins reflects the balance between the rates of oxidation and the rate of degradation of oxidized protein, the accumulation of oxidized protein is a complex function of the numerous factors that govern the synthesis and oxidation of proteins and the activities of various proteases that selectively degrade the oxidized forms. Except under special conditions (*viz.* smoking or excessive exposure to high energy radiation), MCO reactions are likely responsible for most of the free radical damage. The processes that govern the levels of H_2O_2 and the availability of Fe(III) and Cu(II) may be fundamental to the etiology of various metabolic disorders. The level of H_2O_2 is dictated by the availability of O_2 and the activities of numerous enzymic and nonenzymic MFO systems that generate H_2O_2 on the one hand, and of those enzymes that catalyze the decomposition of H_2O_2 (peroxidases, catalase) on the other. Factors that control the availability of Fe(III) and Cu(II) are still poorly understood, but the release of Fe(II) from ferritin and the release of Fe(III) from Fe(III)·enzyme complexes by the degradation of hemoglobin as occurs under certain physiological and pathological conditions are the most likely sources. Factors that govern the levels and activities of multicatalytic proteases and other proteases that degrade oxidized protein are even less well understood. It is likely, however, that the loss of these protease activities is largely responsible for the accumulation of oxidized enzymes during aging and oxidative stress. The possibility that the loss of protease activity is also due to oxidative free radical damage deserves attention.

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