⊣ℛ· Original Contribution

METAL ION-CATALYZED OXIDATION OF PROTEINS: BIOCHEMICAL MECHANISM AND BIOLOGICAL CONSEQUENCES

EARL R. STADTMAN

Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892

Abstract—In the presence of O_2, Fe(III) or Cu(II), and an appropriate electron donor, a number of enzymic and nonenzymic oxygen free radical-generating systems are able to catalyze the oxidative modification of proteins. Whereas random, global modification of many different amino acid residues and extensive fragmentation occurs when proteins are exposed to oxygen radicals produced by high energy radiation, only one or a few amino acid residues are modified and relatively little peptide bond cleavage occurs when proteins are exposed to metal-catalyzed oxidation (MCO) systems. The available evidence indicates that the MCO systems catalyze the reduction of Fe(III) to Fe(II) and of O_2 to H_2O_2 and that these products react at metal-binding sites on the protein to produce active oxygen (free radical?) species (viz; OH, ferryl ion) which attack the side chains of amino acid residues at the metal-binding site. Among other modifications, carbonyl derivatives of some amino acid residues are formed; prolyl and arginyl residues are converted to glutamylsemialdehyde residues, lysyl residues are likely converted to 2-amino-adipylsemialdehyde residues; histidyl residues are converted to asparagine and/or aspartyl residues; prolyl residues are converted to glutamyl or pyroglutamyl residues; methionyl residues are converted to methionylsulfoxide residues; and cysteinyl residues to mixed-disulfide derivatives.

The biological significance of these metal ion-catalyzed reactions is highlighted by the demonstration: (i) that oxidative modification of proteins "marks" them for degradation by most common proteases and especially by the cytosolic multicatalytic proteinase from mammalian cells; (ii) protein oxidation contributes substantially to the intracellular pool of catalytically inactive and less active, thermolabile forms of enzymes which accumulate in cells during aging, oxidative stress, and in various pathological states, including premature aging diseases (progeria, Werner's syndrome), muscular dystrophy, rheumatoid arthritis, cataractogenesis, chronic alcohol toxicity, pulmonary emphysema, and during tissue injury provoked by ischemia-reperfusion. Furthermore, the metal ion-catalyzed protein oxidation is the basis of biological mechanisms for regulating changes in enzyme levels in response to shifts from anaerobic to aerobic metabolism, and probably from one nutritional state to another. It is also involved in the killing of bacteria by neutrophils and in the loss of neutrophil function following repeated cycles of respiratory burst activity.

Keywords—Protein modification, Protein turnover, Aging, Metal ion catalysis, Protein carbonyl groups, protein oxidation and diseases, Site-specific radical formation, Oxygen free radicals

INTRODUCTION

The ability of oxygen free radicals to damage biological molecules was clearly established by results of pioneering studies in which proteins, nucleic acids, lipids, and various metabolites were exposed to high energy radiation (for review, see Swallow[1]). Exposure of proteins to free radicals produced by radiolysis led to modifications of the side chains of amino acid residues, to the formation of protein aggregates via protein–protein cross-linking reactions, and to cleavage of some peptide bonds.[1,2,3] By varying the conditions, it was possible to specify the kinds of radicals formed during radiolysis and thereby gain insight into the mechanisms of the reactions involved. Nevertheless, it is unlikely that radiolysis is a major source of the oxygen free radicals produced in vivo, except under extreme circumstances; namely, exposure to high X-ray doses or to high levels of radioactivity.

The high capacity for free radical generation in cells by nonradiation-based mechanisms is evident from the large increases in radical-mediated tissue damage that occurs during brief exposure to high oxygen tensions,[4] during reperfusion following ischemia,[5–8] and under
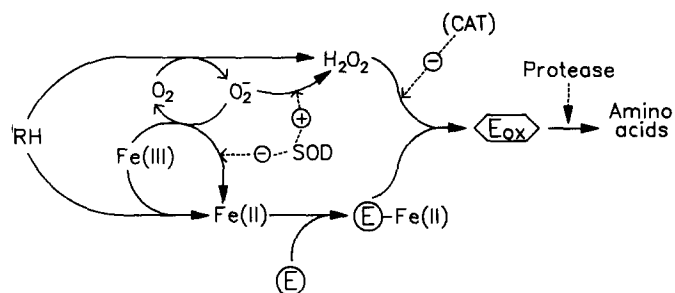
Fig. 1. Role of iron-catalyzed oxidation of enzymes in enzyme degradation.‡ ‡Abbreviations: E—native enzyme; E$_{ox}$—oxidized enzyme; SOD—s;uperoxide dismutase; CAT—catalase; RH—electron donor; ⊕—activation; ⊖—inhibition.

conditions of inflammatory stress.[9–10] Most prominent of these alternative mechanisms are probably those involving site-specific metal ion-catalyzed reactions of the Fenton and Haber–Weiss types. It is the purpose of this review to summarize the results of studies showing that metal ion (Cu(II) or Fe(III))-catalyzed oxygen radical-generating systems damage proteins and that they are partly responsible for the alterations of proteins in vivo, and are implicated in one pathway of intracellular protein turnover as well as the accumulation of altered forms of enzymes during aging.

Metal ion-catalyzed modification of proteins

Our studies on the metal-catalyzed denaturation of proteins were an outgrowth of investigations on the regulation of protein turnover in bacteria.[11–13] These studies led eventually to the discovery that the intracellular

Table 1. Systems That Catalyze Protein Modification

No.	System	References
1.	NAD(P)H oxidase/NAD(P)H/O$_2$/Fe(III)	15
2.	Xanthine oxidase/xanthine/Fe(III)/O$_2$	16, 17
3.	P450/P450 reductase/Fe(III)/NADPH/O$_2$	11, 15, 18
4.	P450$_{cam}$/putida redoxin/redoxin reductase/NADH/O$_2$	11
5.	Asborbate/Fe(III)/O$_2$	12, 19
6.	Ascorbate/Cu(II)/O$_2$	20, 21, 22
7.	Ascorbate/Fe(III)/O$_2$/EDTA	14, 23, 24
8.	Fe(II)/O$_2$	23
9.	Fe(II)/H$_2$O$_2$	15, 23, 25
10.	Cu(II)/H$_2$O$_2$	26, 27, 28
11.	Myeloperoxidase	29
12.	Peroxidase/ferredoxin/H$_2$O$_2$	16
13.	Glucose oxidase/ferredoxin/glucose/O$_2$	16
14.	Peroxidized lipids/Cu(II)/O$_2$	30
15.	Peroxidized lipids/Fe(III)/O$_2$	30
16.	RSH/O$_2$/Fe(III)	31
17.	Microsomes/O$_2$/NADPH	32
18.	Microsomes/O$_2$/Fe(III)/EDTA	32
19.	Hemin/RSH/O$_2$	33
20.	H$_2$O$_2$ (metal assumed to be present)	34
21.	Horseradish peroxidase/O$_2$/glucose/glucose oxidase	35

degradation of glutamine synthetase (GS) in cell-free extracts of *Klebsiella aerogenes* or in *Escherichia coli* is a two-step process in which the enzyme is first inactivated and then is degraded.[12,13,14] Detailed studies of the inactivation step in crude bacterial extracts established that it is dependent on NAD(P)H, O$_2$, and metal ions (Fe(III), Cu(II)), and is inhibited by either EDTA, catalase or Mn(II), and by anaerobiosis.[12,15] It was therefore assumed that inactivation of the enzymes was catalyzed by a mixed-function oxidation (MFO) system. This was confirmed by the demonstration that GS is readily inactivated by any one of the several different enzymic and nonenzymic metal ion-catalyzed oxidation (MCO) systems.*[11–16] Table 1 lists several MCO systems that have been used by various investigators to catalyze the modification of proteins. Table 2 contains a list of proteins that have been shown to be modified by one or more of these MCO systems, and indicates also the kind of modifications that have been observed. The rates of inactivation of enzymes by the MCO systems is greatly enhanced by the presence of catalytic levels of one-electron carriers such as menadione or proteins having nonheme iron sulfur centers (viz; *Putida* redoxin, ferredoxin).[16] It is noteworthy that enzyme inactivation by most of the MCO systems studied is dependent on the presence of added free metal (Fe(III) or Cu(II)). However, for inactivation of GS by the horseradish peroxidase/H$_2$O$_2$ system and the glucose/glucose oxidase/O$_2$ system, the requirement for a nonheme iron sulfur protein (redoxin or ferredoxin) could not be satisfied by Fe(III).[16]

Mechanism of the metal ion-catalyzed reaction

From an in-depth study of the inactivation of GS by

*In earlier reports, the MCO systems were referred to as mixed-function oxidation (MFO) systems because, in addition to O$_2$, they require a cosubstrate (NAD(P)H, ascorbate) which serves as an electron donor for the reduction of O$_2$. To avoid confusion between reactions catalyzed by these mixed-function oxidation systems and those catalyzed by mixed-function oxidases, we now refer to these systems as metal ion-catalyzed oxidation (MCO) systems.

Table 2. Proteins That Are Modified by Metal Ion-Catalyzed Oxidation Systems

Protein	Source	Radical-Generating System[a]	Observed Modification[b]	References
Acetyl CoA hydrolase	Rat liver	5, 6	a	36
Acetylcholine esterase	Horse serum	6	a	20
Albumin	Bovine serum	1	a	37
Albumin	Bovine Serum	14, 15	i, l	38
Albumin	Bovine serum	5, 7, 8	c	39
Alkaline phosphatase	Bovine kidney	6	a	40, 41
Alcohol dehydrogenase	Yeast	1, 3	a	15
Alcohol dehydrogenase	Horse liver microsome	17, 18	a	42
Carbamoyl-P synthetase	Rabbit liver	5	a, c	21
Catalase		6	a	22, 43
Chymotrypsin	Bovine pancreas	21	b	35
Chymotrypsinogen	Bovine pancreas	8	c	26
Creatine kinase	Rabbit muscle	1, 3	l, l	23
Crystallins	Bovine lens	5, 6	i, j, d, h	44, 45
Crystallins	Bovine lens	8	j	46
Enolase	Yeast	1, 3, 5	a, l, c	15, 23
Fructose-1,6-bisphosphatase	Yeast	1, 7	a, c	47
Glucose-6-P dehydrogenase	Bovine adrenals	7	a, c, f	48
Glucose-6-P dehydrogenase	Rat liver microsomes	17	a	32
Glutamine synthetase	E. coli	1, 2, 3, 4, 12, 13, 16	a, b, c, d, f, i, l	11–16, 18,23
Glutamine synthetase	Rat liver	1, 3	a, c	15
Glutamine synthetase	N. crassa	5, 9	a	49
Glutamine synthetase	A. crococum	9	a	25
Glutamic dehydrogenase	N. crassa	7	a	50
Glyceraldehyde dehydrogenase	Rabbit muscle	1, 3	a, l	15, 51
Glyceraldehyde-3-P acyltransferase	Liver microsomes	14, 15	a	30
Hexokinase	Rabbit reticulocytes	5	a	52
Heme binding protein	Rabbit serum	19	i	33
Lactic dehydrogenase	Rabbit muscle	1, 3, 5	a	15
Lactoperoxidase	Bovine	20	a	34
Leucyl-t-RNA synthetase	Rat liver	5, 7	a, f, k	54
Lysozyme	Egg white	5, 8	a, c	39
Mucus glycoprotein	Overian cysts	10	i, g, b	28
Myelin				54a
Myoglobin	Whale	19	i	33
Myosin	Muscle	10	b, i, g	26
Ornithine decarboxylase	Rat heart	2	a	30
6-Phosphogluconate dehydrogenase	Rat liver	5	a	55
Phosphoglucomutase	Rabbit muscle	1, 5	a	19
Phosphoglycerate kinase	Yeast	1, 3, 5	a, c, f, l	15, 23
Phosvitin	Hen yolk	8	c	26
α-1-Proteinase inhibitor	Human plasma	2, 7, 11	a, e	29, 56
Pyruvate dehydrogenase	Rabbit muscle	1, 3, 5, 6	a	15, 23, 21
Superoxide dismutase	Bovine erythrocytes	20	a, b	53
Ribonuclease A	Bovine pancreas	1, 7, 8	a, c	39
Ribonuclease B	Bovine pancreas	1, 7, 8	a, c	39
Tryosyl-t-RNA-synthetase	Rat liver	5	a, f	54

[a]The numbers refer to the MCO systems listed in Table 1.

[b]The letters refer to the following types of modification: (a) loss of catalytic activity; (b) amino acid modification; (c) carbonyl group formation; (d) increase in acidity; (e) conversion of methionine residues to methionine sulfoxide residues; (f) decrease in thermal stability; (g) change in viscosity; (h) change in fluorescence; (i) fragmentation; (j) formation of protein-protein cross-links; (k) formation of -S–S-beridges; (l) increase in proteolytic susceptibility.

various MCO systems, it was concluded that the MCO system is concerned solely with the production of H_2O_2 and Fe(II).[12,14,15] As illustrated in Fig. 1, depending on the MCO system used, H_2O_2 can arise by direct transfer of reducing equivalents to O_2 or by a one-electron transfer to O_2 to form $O_2^{\cdot-}$, which, upon dismutation,

Table 3. Effect of EDTA on Protein Oxidation[a]

Protein	Carbonyl Groups Formed nmol/mg Protein	
	−EDTA	+EDTA
Glutamine synthetase	13.9	2.7
Glusose-6-P dehydrogenase	30.0	75.0
RNA'ase-A	1.0	6.5
Lysozyme	1.5	3.9
Collagen (calf skin)	8.0	19.0
Bovine serum albumin	0	26.6

[a]Reaction mixtures contained: phosphate buffer, 50 mM; KCl, 90 mM; $MgCl_2$, 10 mM; ascorbate, 25 mM; Fe(III), 100 μM; 1.0 mg protein/mL; and, where indicated, 1.0 mM EDTA. After gentle shaking in air for 30 min at 30°C, the carbonyl content was measured.

will yield H_2O_2. Similarly, the reduction of Fe(III) to Fe(II) can occur either by direct one-electron transfer from the cosubstrate (RH) or indirectly by reduction with $O_2^{\cdot}$. Whatever the mechanism, it is believed that the Fe(II) binds to a metal-binding site on the enzyme and that the Fe(II)-enzyme complex reacts with H_2O_2 to yield an active oxygen species (ÖH, ferryl ion, etc.) at the metal-binding site on the protein. The active oxygen species preferentially attacks the side chains of amino acid residues at the metal-binding site. This leads, in the case of some enzymes, to conversion of some amino acid residues to carbonyl derivatives, to loss of catalytic activity, and to increased susceptibility of the protein to proteolytic degradation. The role of H_2O_2 in this process is evident from the observation that inactivation of enzymes by any one of the MCO systems listed in Table 1 is completely inhibited by catalase.[12,14–16] The role of Fe(II) is inferred by the fact that all MCO systems studied catalyze the reduction of Fe(III) to Fe(II) and the demonstration that mixtures of H_2O_2 and Fe(II) together, but neither one by itself, will cause inactivation of the enzymes, in the absence of any other MCO system. Moreover, Mn(II), which inhibits the reduction of Fe(III) to Fe(II) by the MCO system, will inhibit also the oxidative modification of proteins by all MCO systems tested in which Fe(III) is supplied as the metal ion catalyst.[12,15,14] According to this mechanism, there is no need for direct contact between components of the MCO system (i.e., the electron donor or the enzymes involved) and the proteins which are modified. This was confirmed in a study showing that GS is inactivated by the P450/P450 reductase MCO system even when it is separated from the P450/P450 reductase proteins by a semipermeable membrane, and was protected from inactivation by this system when catalase was added to either membrane compartment.[18]

As shown in Table 3, EDTA may either inhibit or stimulate the oxidation of proteins as measured by the generation of carbonyl groups. The inhibition by EDTA

is likely due to sequestration of iron and copper ions, thereby preventing their binding to the protein metal-binding sites. Hydroxyl radicals produced by the interaction of H_2O_2 with the free EDTA-metal ion complex will have little opportunity to attack a specific amino acid residue of the protein. In those cases where EDTA stimulates the protein modification, it is presumed that the protein lacks a high affinity site for the metal ions but can bind the EDTA-metal ion complexes; thus, EDTA serves as a carrier of the metal ion to the enzyme and interaction of the EDTA-metal ion–protein complex with H_2O_2 will lead to a site-specific generation of radical species.

The effects of EDTA on the MCO-dependent loss of catalytic activity are more pronounced than on the MCO-dependent generation of carbonyl groups. The metal-catalyzed oxidative inactivation of GS,[12,15,16] pyruvate kinase,[15,23] and phosphoglycerate kinase[15] are almost completely inhibited by EDTA, whereas the inactivation of either creatine kinase[12] or adrenal glucose-6-phosphate dehydrogenase (unpublished data) is greatly stimulated by the presence of EDTA.

Metal ion-catalyzed oxidation of proteins is a site-specific process

Results obtained with the ascorbate/O_2/Fe(III) MCO system are similar if not identical in every important respect to those obtained with the enzymic MCO system.[12,23] Therefore, the ascorbate system has been the model of choice for detailed mechanistic studies. Using this system, Levine and coworkers[58,59] showed that the metal ion-catalyzed inactivation of GS is associated with the conversion of a single histidine residue to an asparagine residue and of a single arginine residue to a glutamic semialdehyde residue. After limited degradation of GS, two small polypeptide fragments, one containing the sensitive histidine residue[58] and one containing the sensitive arginine residue (cited in Chevalier et al.),[60] were isolated and their amino acid sequences determined. From comparisons of the amino acid sequences of these peptides with the known three-dimensional structure of native GS,[61] it was established that the amino acid residues which are modified by the ascorbate MCO system are His^{269} and Arg^{344}, both of which are situated at one of two manganese-binding sites on the enzyme. It is noteworthy that the His^{269} -containing peptide isolated from GS has an unusual palindromic amino acid sequence, -Met-His*-Cys-His-Met-. Curiously, all of the amino acid residues in this sequence are among those shown to be particularly susceptible to oxidation by oxygen free radicals; nevertheless, only one residue, His^{269}, at the N-terminal side (designated His*) is modified in the metal ion-catalyzed reaction. These

NOTE: $Fe^{2+} + H_2O_2 \rightleftarrows \left[Fe(OH)_2\right]^{2+} \rightleftarrows (FeOH)^{3+} + OH^- \rightleftarrows Fe^{3+} + \dot{O}H + OH^-$
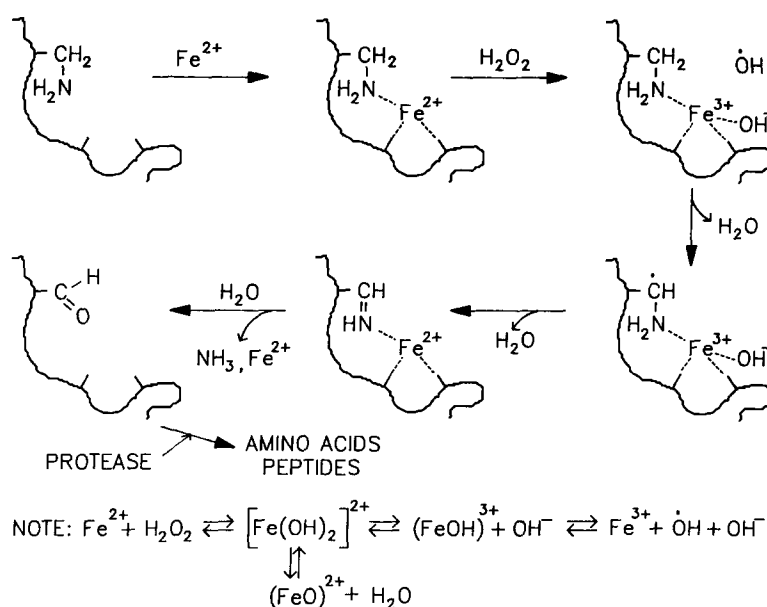
$\Updownarrow$

$(FeO)^{2+} + H_2O$

Fig. 2. One possible mechanism for the site-specific metal ion-catalyzed oxidation of proteins (see text for discussion).

results and the earlier observation that metal-catalyzed inactivation of GS and other enzymes is insensitive to inhibition by various free radical scavengers[12,15] and the fact that only one or at most only a few amino acid residues of a given protein are modified, have led to the proposition that the metal ion-catalyzed oxidative modification of proteins is a site-specific process.[12,15,62] A plausible mechanism for the site-specific oxidation of a protein is illustrated in Fig. 2. In this mechanism, it is assumed that the ε-amino group of a lysyl residue serves as one of several ligands to which Fe(II) is bound to form a Fe(II)-protein coordination complex. Reaction of H_2O_2 with Fe(II) in this complex leads to the formation of $\dot{O}H$, OH^-, and the Fe(III)-protein complex. The $\dot{O}H$ then abstracts a hydrogen atom from the carbon bearing the ε-amino group to form a carbon-centered radical, which immediately donates its unpaired electron to Fe(III) of the coordination complex to regenerate Fe(II)-protein. Coincidentally, the lysyl residue would be converted to an amino derivative, which upon spontaneous hydrolysis would yield an aldehyde derivative of the lysyl residue and NH_3. This would lead to destruction of the metal-binding site and therefore to dissociation of the Fe(II) from the protein. The overall process is thus visualized as a "caged" reaction, accounting for its insensitivity to inhibition by radical scavengers. The scheme depicted in Fig. 1 is but one of several mechanisms that might be involved. Similar schemes in which $(FeO)^{2+}$, $[Fe(OH)_2]^{2+}$, $(FeOH)^{+3} + OH^-$ are the active intermediates could also be envisioned. The possibility that $\dot{O}H$ abstracts a hydrogen atom from the amino group of

lysine form a hydronitroxide radical[63] also deserves consideration, in view of the fact that this radical is formed during the oxidation of free amino acids by an MCO system.[63] Because metal ion-catalyzed oxidation of amino acid residues of a protein leads, in some cases, to the generation of carbonyl derivatives, the level of protein carbonyl groups can be used as a measure of oxygen radical-mediated protein damage under various physiological conditions. To this end, several sensitive methods for the determination of protein carbonyl groups have been developed.[64] These involve: i) the spectrophotometric measurement of 2,4-dinitrophenylhydrazone derivatives formed by reactions with 2,4-dinitrophenylhydrazine (reaction 1);[65,64,66] ii) measurement of the radioactivity of protein following reduction of the carbonyl groups to [³H]-labeled alcohol derivatives by treatment with [³H]NaBH₄ (reaction 2);[66,67,39] fluorescence measurements of the hydrazones produced by reaction with fluorescein hydrazide (reaction 3);[68] spectrophotometric, measurement of stable secondary amines produced by reaction of the carbonyl groups with fluoresceinamine to form Schiff bases followed by reduction of the Schiff base with NaCNBH₃ (reaction 4).[59]

$$\underset{R}{E-C=O} + H_2NNH\overset{NO_2}{\underset{}{\bigcirc}}-NO_2 \rightarrow \underset{R}{E-C=NNH}\overset{NO_2}{\underset{}{\bigcirc}}-NO_2 \quad (1)$$

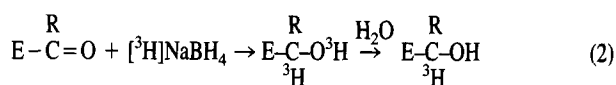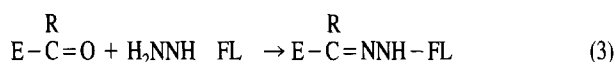$$\underset{R}{E-C=O} + [^3H]NaBH_4 \rightarrow \underset{^3H}{E-C-O^3H} \overset{H_2O}{\rightarrow} \underset{^3H}{E-C-OH} \quad (2)$$
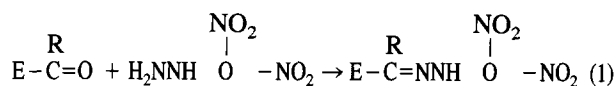
Table 4. Metal Ion-Catalyzed Protein Modification[a]

			References
Arginyl residues	------>	Glutamylsemialdehyde residues	39, 59
Prolyl residues	------>	Glutamylsemialdehyde residues	39
Lysyl residues	------>	α-Aminoadipylsemialdehyde residues	[a]
Histidyl residues	------>	Asparaginyl/aspartyl residues	28, 34, 58
Prolyl residues	------>	Pyroglutamyl/glutamyl residues	28, 39
Prolyl residues	------>	Cis/trans-4-hydroxy prolyl residues	71
Cysteinyl residues	------>	-S–S-protein–protein cross-links; or mixed-disulfide protein-S-S-R	1, 2
Methionyl residues	------>	Methionylsulfoxide residues	1, 2
Tyrosyl residues	------>	Tyrosyl–tyrosyl cross-links	71
RCONHCHCONHR' R[2]	------>	RCONH$_2$ + R[2]COCONHR'	2, 69, 70

[a] D. G. Miller, B. S. Berlett, and E. R. Stadtman, unpublished results.

$$\underset{E-C=O}{\overset{R}{}} + H_2NNH\ FL \rightarrow \underset{E-C=NNH-FL}{\overset{R}{}} \quad (3)$$

$$\underset{E-C=O}{\overset{R}{}} + NH_2-FL \rightarrow \underset{E-C=N-FL}{\overset{R}{}} \xrightarrow[NaCNBH_3]{} \underset{E-CHNH-FL}{\overset{R}{}} \quad (4)$$
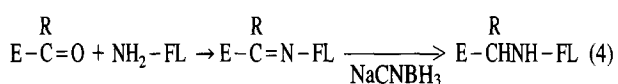
Reaction 2 has been used also in the identification of some amino acid residues in proteins that are particularly sensitive to metal ion-catalyzed oxidation. Acid hydrolysis of the oxidized forms of amino acid homopolymers, following reduction of their carbonyl groups with [^{3}H]NaBH$_4$, yields a multiplicity of [^{3}H]-labeled amino acids which can be separated by ion exchange chromatography. Since each oxidized homopolymer yields a different set of labeled amino acids, the elution profiles of each can be used as a reference standard (i.e., a "finger print") with which the profiles of identically treated proteins can be compared. Such comparisons have shown that proline, histidine, arginine, and lysine residues in proteins are highly sensitive to metal ion-catalyzed oxidation.[39] As shown in Table 4, it is now known that prolyl and arginyl residues are converted to glutamylsemialdehyde residues; lysyl residues are likely converted to 2-amino adipylsemialdehyde residues; histidyl residues are converted to asparaginyl or to aspartyl residues; prolyl residues are converted to pyroglutamyl or glutamyl residues; methionyl residues are converted to methionine sulfoxide residues, and cysteinyl residues to protein–protein disulfide conjugates or to mixed-disulfide derivatives. In addition, metal ion-catalyzed oxidative cleavage of the polypeptide chain may occur by an α-amidation mechanism, that is, by oxidative cleavage of the α–carbon–nitrogen bond of a given amino acid residue. This leads to the formation of one peptide fragment containing a C-terminal amide group and another fragment in which the N-terminal residue is blocked by an α-ketoacyl group (Table 4). Acid hydrolysis of the latter peptide will yield a free α-ketoacid which would be identical to the one that would be obtained by oxidative deamidation of the amino acid from which it was derived. For example, cleavage of alanyl
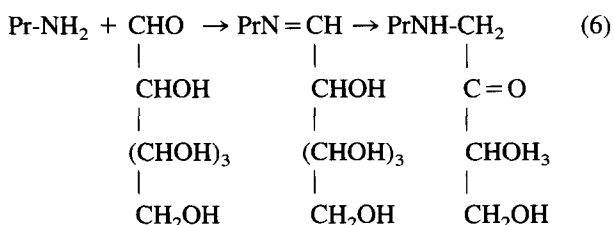
and glutamyl residues would yield pyruvic and α-ketoglutaric acids, respectively. It has been suggested that the oxidation of prolyl residues may also lead to peptide bond cleavage[3,72,73] in which case the N-terminal residue of one peptide fragment would be blocked by a pyroglutamyl residue, which upon hydrolysis would yield glutamic acid.[39]

The conversion of basic amino acid residues (Arg, Lys, His) to aldehydes as well as the further oxidation of the aldehyde products to carboxylic acid derivatives (viz; prolyl → glutamylsemialdehyde → glutamyl residues) would lead to a decrease in the isoelectric point of the protein. This has, in fact, been observed. During exposure of GS to the ascorbate/Fe(III)/O$_2$ MCO system, there is a progressive decrease in the isoelectric point.[97]

It should be noted that carbonyl groups may also be introduced into protein by mechanisms that do not involve oxidation of amino acid residues. Thus, α, -β unsaturated alkenals such as 4-hydroxynonenal produced during the peroxidation of polyunsaturated fatty acids have been shown to react with the sulfhydryl groups of proteins to form stable covalent thiolether adducts carrying a carbonyl function (reaction 5).[74]

$$Pr\text{-}SH + R\text{-}CHOH\ CH=CHCHO \rightarrow RCHOHCH\text{-}CH_2CHO \quad (5)$$
$$\underset{SPr}{\mid}$$
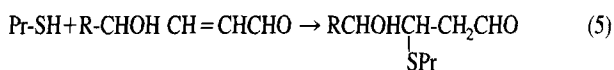
Also, Schiff bases obtained by reaction of reducing sugars with the ε-amino group of lysyl residues in proteins (the glycation reaction) may, upon Amadori rearrangement, yield ketoamine protein conjugates (reaction 6).[75]

$$Pr\text{-}NH_2 + CHO \rightarrow PrN=CH \rightarrow PrNH\text{-}CH_2 \quad (6)$$

CHOH	CHOH	C=O
(CHOH)$_3$	(CHOH)$_3$	CHOH
CH$_2$OH	CH$_2$OH	CH$_2$OH

Wolff et al.[76,77] have presented evidence that α-ketoaldehydes produced in the metal ion-catalyzed oxidation of glucose may undergo analogous reactions with protein lysyl groups to yield α-ketocarbinol amines, which, upon enolization, would yield protein ketoimine derivatives. It is argued that these carbonyl derivatives are likely produced under conditions of hyperglycemia, as, for example, in patients with diabetes. The role of this dicarbonyl pathway in the generation of protein carbonyl derivatives is under debate.[78,79] There is clearly a need for the development of sensitive methods that can distinguish between these various kinds of protein carbonyl derivatives.

Oxidation "marks" enzymes for degradation

Studies to determine how cells regulate the degradation of one enzyme with respect to another led eventually to the discovery that oxidative modification of enzymes by MCO systems renders them highly susceptible to proteolytic attack. It was therefore proposed that oxygen radical-mediated oxidation of enzymes is a "marking" step in protein turnover.[11-14] In the meantime, this concept has been supported by the following observations:

1. Neutral-alkaline proteases that degrade the oxidized forms of GS but have little or no ability to degrade the native, unoxidized forms have been isolated from rat liver cytosol,[51,80,81,82] E. coli extracts,[83,84] and red blood cells;[85]
2. a number of common proteases, including trypsin, chymotrypsin, pepsin, subtilisin, cathepsin D, and calpains will degrade oxidized proteins more rapidly than unoxidized forms;[86,51,87,38]
3. the rate of endogenous protein degradation in E. coli cells,[88] liver and heart mitochondria,[89] and red blood cells[90] is greatly increased following their exposure to oxygen radicals or H_2O_2;
4. prior exposure of pure proteins to oxygen radicals in vitro makes them more susceptible to degradation by ATP-independent proteases in extracts of E. coli,[88] liver and heart mitochondria,[89] and red blood cells;[90,91]
5. following their exposure to oxygen radicals, 17 different enzyme proteins were degraded up to 50 times more rapidly than their unmodified counterparts by ATP- and lipid-independent mechanisms when they were incubated with cell-free extracts of human or rabbit reticulocytes, or E. coli.[91]

It should be noted that in some of these studies,[88-91] γ-irradiation with ^{60}CO was used to generate oxygen free radicals. By carrying out radiolysis in the presence of various supplements (O_2, HCOOH, or N_2O) it is possible to specify which kinds of oxygen radicals the pro-

tein is exposed to. However, protein damage provoked by these so-called "clean" systems may be significantly different from the damage caused by site-specific metal ion-catalyzed mechanisms. Whereas all amino acid residues of a protein are subject to attack by oxygen radicals produced during radiolysis, the tyrosine, phenylalanine, tryptophan, histidine, methionine, and cysteine residues are preferred targets.[1,92] Moreover, exposure of proteins to radiolysis leads, in the absence of oxygen, to appreciable protein aggregation due to $\dot{O}H$-facilitated tyrosine–tyrosine and -S–S- cross-linking reactions, and in the presence of O_2 to appreciable fragmentation of the polypeptide chain, presumably via a peroxyl radical mediated α-amidation mechanism.[1,92] In contrast, only one or at most only a few amino acid residues in a protein are modified by MCO systems, with histidine, proline, arginine, lysine, cysteine, and less often, methionine residues as the preferred targets.[39] Significantly, there is, to date, no evidence that the aromatic amino acid residues are modified by MCO systems. Moreover, protein fragmentation and aggregation are only minor events. Major differences are observed also in the oxidation of free aromatic amino acids by the metal ion-catalyzed and radiolysis pathways. For example, exposure of phenylalanine to radiolysis leads mainly to hydroxylation of the aromatic ring;[1] however, hydroxylation accounts for less than 5% of the products found when phenylalanine (in bicarbonate buffer) is oxidized by the $Fe(II)/H_2O_2$ MFO system; indeed, phenylpyruvate, phenylacetaldehyde, phenylacetic acid, CO_2, and NH_3 account for over 90% of the products formed in the metal ion-catalyzed reaction.[93]

Basis of proteolytic susceptibility

Whereas it is evident that oxygen radical-mediated modification of a protein makes it more susceptible to degradation, the structural features of the modified protein that are recognized by various proteinases are poorly understood. In view of the marked differences in peptide bond substrate specificities exhibited by diverse proteases, it is unlikely that all oxidized proteins would have in common a unique feature that is recognized by all of the proteases that preferentially degrade oxidized proteins. The existence of multiple recognition parameters in oxidized proteins is evident from studies on the degradation of oxidized forms of E. coli GS by highly purified preparations of the multicatalytic proteinase from rat liver[82,97] and a neutral protease from E. coli.[83] Among other changes, the oxidation of GS by the ascorbate/$Fe(II)/O_2$ MCO system leads to the modification of two histidine residues[95,97] and sequential conversion of the enzyme first to a more hydrophilic form, and then to a more hydrophobic form.[94] Degradation of the oxi-
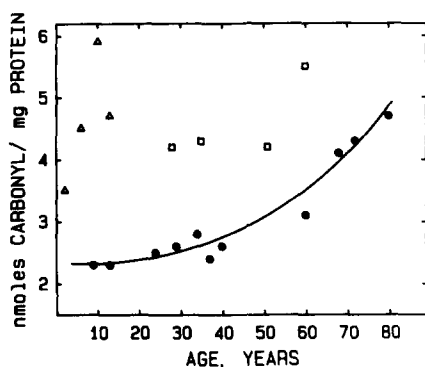
Fig. 3. Relationship between levels of oxidized protein in cultured fibroblasts and the age of fibroblast donor. Symbols are as follows: ●, cultures from normal individuals; △, cultures from individuals with progeria; □, cultures from individuals with Werner's syndrome. From Oliver et al.[48]

dized enzyme by the multicatalytic proteinase is correlated with modification of the two histidine residues and is independent of the changes in hydrophobicity,[96,97] whereas degradation by the E. coli protease is correlated with the generation of the more hydrophobic form and not with the histidine modification.[94] Variations in protease recognition parameters of oxidized proteins are illustrated also by the studies of Davies and co- workers.[87,98] In their studies on the oxidation of bovine serum albumin (BSA) by radiolytically generated oxygen radicals, they noted that the proteolytic susceptibility of proteins damaged by ȮH radicals alone is attributable to an increase in hydrophobicity of the protein, as judged by the decrease in solubility in concentrated salt solution. It is evident that other parameters may determine proteolytic susceptibility, since the degradation of BSA preparations oxidized by an ȮH + O_2^--generating system in the presence of O_2 did not correlate with decreased solubility in saline solutions.[98]

Protein oxidation and aging

It is well established that inactive or less active, more heat labile forms of several enzymes accumulate in cells during aging.[14,99–102] Many of these enzymes are among those that have been shown to be sensitive to modification by MCO systems.[14,100] This led to the proposition that some of the age-related enzyme alterations are caused by oxygen free radical-mediated modifications.[14,15] In the meantime, using the level of protein carbonyl groups as a measure of oxygen radical damage, it has been demonstrated that:

1. The amount of oxidized protein in human erythrocytes increases with the age (i.e., cell density) of the cell;[48,57]

2. the level of protein carbonyl groups in cultured hu-

man fibroblasts increases, almost exponentially, as a function of the age (9–80 years) of the fibroblast donor (Fig. 4), and is independent of the cell passage number (at least over the intermediate range of passages);

3. the level of protein carbonyl groups in cultured fibroblasts of individuals with premature aging diseases (Werner's syndrome, progeria) is very much higher than that in proteins in fibroblasts of age and sex-matched controls (Fig. 3);[48,57]

4. the level of protein carbonyl groups in rat hepatocytes[103] and human lenses[104] increases with age of the animal;

5. the age-related changes in heat stability and other structural features of several enzymes (GS,[97] glucose-6-P dehydrogenase,[48,57] tyrosyl-and leucyl-tRNAs,[53] crystallins[44,45]) can be mimicked by exposure of these enzymes to the ascorbate/Fe(III)/O_2 MFO system.

Age-related increases in the levels of rat liver protein carbonyl groups can be simulated by short time exposure (0–48 h) of rats to 100% oxygen.[4,103]

The age-related increase in the level of oxidized protein may be due in part to a decrease in the rate of oxidized protein degradation. The level of rat liver neutral protease declines with animal age.

Other physiological implications

Besides its role in protein degradation and aging, oxidative damage to protein has been associated with a number of pathological processes including: ischemia-reperfusion tissue injury,[105] muscular dystrophy,[106,107] pulmonary emphysema,[29,56] bronchopulmonary dysplasia,[108] cataractogenesis,[45] rheumatoid arthritis,[109] chronic alcohol toxicity,[42] atherosclerosis,[110] rapid correction of hyponatremia,[111] and in chronic oxygen toxicity.[4,103] It is also likely implicated in the killing of bacteria by neutrophils as well as the loss of neutrophil function following cycles of oxidative burst,[112] and probably also in cancer and in some neurological diseases, namely, those involving demyelination.[113]

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