QUANTIFICATION AND SIGNIFICANCE OF PROTEIN OXIDATION IN BIOLOGICAL SAMPLES *

EMILY SHACTER
Food and Drug Administration
Center for Biologics Evaluation and Research
Building 29A, Room 2A-11, HFM 535
29 Lincoln Drive
Bethesda, Maryland 20892-4555

---

* This paper was refereed by Jason D. Morrow, M.D., Vanderbilt University, Nashville, TN 37232.

ABSTRACT

Protein oxidation is defined here as the covalent modification of a protein induced either directly by reactive oxygen species or indirectly by reaction with secondary by-products of oxidative stress. Oxidative modification of proteins can be induced experimentally by a wide array of prooxidant agents and occurs in vivo during aging and in certain disease conditions. Oxidative changes to proteins can lead to diverse functional consequences, such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity. There are numerous types of protein oxidative modification and these can be measured with a variety of methods. Protein oxidation serves as a useful marker for assessing oxidative stress in vivo. There are both advantages and disadvantages to using proteins for this purpose compared to lipids and DNA. Finally, it is important to monitor the degree of oxidative modification of therapeutic proteins manufactured for commercial use. This review will examine various aspects of protein oxidation, with emphasis on using proteins as markers of oxidative stress in biological samples.

Key Words: Protein oxidation; Disease; Oxidative stress; Markers; Methods; Therapeutic proteins.

I. INTRODUCTION

Oxidants are reactive oxygen species (ROS) which have the ability, either directly or indirectly, to damage all biomolecules, including proteins, lipids, DNA, and carbohydrates. ROS such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH·) are generated under numerous conditions in vivo. They are formed in the course of normal metabolism through leakage of electrons from the electron-transport chain and by the activities of oxidoreductase enzymes such as NADPH oxidase, myeloperoxidase, xanthine oxidase, glucose oxidase, the cytochrome P450 enzymes, and cyclooxygenases. Because of the potential of ROS to induce significant biological damage, cells and tissues have an abundance of antioxidant systems to scavenge or otherwise eliminate them. These include antioxidant enzymes such as catalase, superoxide dismutase, peroxiredoxins, and glutathione peroxidase as well as low-molecular-weight compounds such as vitamins C and E and reduced glutathione (GSH). Under normal circumstances, there is a well-managed balance between formation and neutralization of ROS so that there is minimal modification of biomolecules. ''Oxidative stress'' occurs when the balance of formation of oxidants exceeds the ability of antioxidant systems to remove ROS, such as occurs when inflammatory phagocytes (e.g., neutrophils and macrophages) are activated to undergo an oxidative burst by exposure to a foreign agent or when prooxidant xenobiotics such as CCl_4 and paraquat are introduced into the body. Under these conditions, biomolecules become subjected to attack by excess ROS and significant molecular and physiological damage can occur. This review will examine various aspects of oxidative damage to proteins, with emphasis on using proteins as markers of oxidative stress. Other reviews that describe mechanisms and consequences of protein oxidative modification can be found elsewhere [1–3].

II. TYPES OF PROTEIN OXIDATIVE MODIFICATION

Protein oxidation is defined here as the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary by-products of oxidative stress. Agents that lead to protein oxidation include reagents such as H_2O_2 [4] and HOCl [5–9], xenobiotics such as paraquat [10], CCl_4 [11], and acetaminophen [12], cigarette smoke [13], reduced transition metals such as Fe^{2+} [14] or Cu^+ [15,16], γ-irradiation in the presence of O_2 [4,17–19], activated neutrophils [20], ultraviolet (UV) light [21–23], ozone [24,25], oxidoreductase enzymes [26], and by-products of lipid and free amino acid oxidation [27–29] (reviewed in Refs. 2 and 14). Because there are so many mechanisms for induction of protein oxidation and because all of the amino acyl side chains can become oxidatively modified, there are numerous different types of protein oxidative modification. These are summarized in Table 1 and discussed briefly below. The two amino acids that are perhaps the most prone to oxidative attack are Cys [30,31] and Met [32], both of which contain susceptible sulfur atoms. All oxidizing species can induce modification of Cys and Met. In the case of Cys, oxidation leads to the formation of disulfide bonds, mixed disulfides (e.g., with glutathione), and thiyl radicals [33,34]. With Met residues, the major product under biological conditions is Met sulfoxide [32]. Other amino acids may require more stringent conditions for oxidative modification. A potent mode of direct oxidative attack on a protein derives from site-specific metal-catalyzed oxidation (MCO), in which the reduced form of a protein-bound transition metal (e.g., Fe^{2+}, Cu^+) reduces

TABLE 1

Oxidative Modifications of Proteins

Modification	Amino acids involved	Oxidizing source[a]
Disulfides, glutathiolation	Cys	All, $ONOO^-$
Methionine sulfoxide	Met	All, $ONOO^-$
Carbonyls (aldehydes, ketones)	All (Lys, Arg, Pro, Thr)	All
Oxo-histidine	His	γ-Ray, MCO, $^1O_2$
Dityrosine	Tyr	γ-Ray, MCO, $^1O_2$
Chlorotyrosine	Tyr	HOCl
Nitrotyrosine	Tyr	$ONOO^-$
Tryptophanyl modifications (N-formyl)kynurenine	Trp	γ-Ray
Hydro(pero)xy derivatives	Val, Leu, Tyr, Trp	γ-Ray
Chloramines, deamination	Lys	HOCl
Lipid peroxidation adducts (MDA, HNE, acrolein)	Lys, Cys, His	γ-Ray, MCO (not HOCl)
Amino acid oxidation adducts	Lys, Cys, His	HOCl
Glycoxidation adducts	Lys	Glucose
Cross-links, aggregates, fragments	Several	All

[a] MCO-metal catalyzed oxidation; All = γ-ray, MCO, HOCl, ozone, $^1O_2$.

TABLE 2

Metal-Catalyzed Oxidation of Amino Acid Residues in Proteins

Amino acid residue oxidized	Product(s)
Histidine	Aspartate, asparagine, oxo-histidine
Proline	Hydroxyproline, glutamate, γ-glutamylsemialdehyde
Arginine	γ-Glutamylsemialdehyde
Lysine	Amino-adipicsemialdehyde
Threonine	Amino-ketobutyrate
Tyrosine	Tyr-Tyr (dityrosine)
Cysteine	—S—S— (disulfide cross-links)

H_2O_2 to form a reactive intermediate (e.g., OH·, ferryl radical) in the immediate proximity of amino acid side chains [1,10,35,36]. Examples of products of metal-catalyzed protein oxidation are listed in Table 2. The chelating amino acids in proteins, such as histidine, are most susceptible to oxidative attack due to their proximity to the radical formed [37,38]. Metal-catalyzed oxidation of histidine generally causes formation of oxo-His [39] or Asp [25]. Other amino acyl moieties, especially Lys, Arg, Pro, and Thr [1,40], incur formation of carbonyl groups (aldehydes and ketones) on the side chains. A discussion of mechanisms of induction of protein carbonyls by metal-catalyzed oxidation can be found in Refs. 41 and 42. Another major endogenous oxidizing species is myeloperoxidase-derived HOCl. Interaction of this molecule with Tyr, Trp, Lys, and Met residues leads to formation of chlorotyrosine, chloramines, aldehydes, and Met sulfoxide [8,43–45]. In a recent series of studies, HOCl was also shown to modify free amino acids to reactive products [46]. One well-characterized product of free tyrosine oxidation by HOCl is *p*-hydroxyphenylacetaldehyde (pHA), which has been shown to attach to lysyl residues in proteins to form a covalent adduct [47]. Peroxynitrite, generated from the interaction of nitric oxide and superoxide, causes nitrosylation of tyrosine residues and oxidative modification of other amino acid residues, including Cys, Trp, Met, and Phe [48], but it is a poor inducer of protein carbonyls [49]. Gamma-irradiation, which causes direct formation of hydroxyl radical in addition to inducing metal-catalyzed oxidation, may cause more generalized protein damage, which is not restricted to the metal-binding site in a protein [4,38]. For this reason, γ-irradiation may cause a higher degree of protein aggregation and chemical degradation than metal-catalyzed oxidation. Hydrophobic amino acyl residues such as Val, Leu, and Tyr are oxidized to hydroxy and hydroperoxy derivatives upon exposure to γ-irradiation in O_2 [19,50]. Radical attack on Tyr residues leads to formation of dityrosyl cross-links by several different mechanisms [51–53].

Indirect oxidative modification of protein amino acyl side chains occurs through the formation of adducts with products of oxidatively modified lipids, amino acids, sugars, and glutathione. For example, lipid peroxidation breakdown products such as hydroxynonenal (HNE), malondialdehyde (MDA), and acrolein bind covalently to Lys, His, and Cys residues, leading to the addition of aldehyde moieties to the protein [27,29,54–56]. As noted earlier, products of free amino acid oxidation can also form covalent attachments to proteins [47]. Glutathiolation of Cys residues [31,57] also occurs under conditions of oxida-

tive stress. Carboxymethyllysine represents a form of protein modification generated by the oxidation products of sugars [14,58,59].

III.   BIOLOGICAL CONSEQUENCES
OF PROTEIN OXIDATIVE MODIFICATION

Because proteins have many different and unique biological functions, oxidative modifications to proteins can lead to diverse functional consequences. For example, oxidative modification of enzymes has been shown to inhibit a wide array of enzyme activities [1,26]. Enzymes that are particularly sensitive to inhibition by metal-catalyzed oxidation generally have a metal in or near the active site [1,60]. Oxidative modification of enzymes can have either mild or severe effects on cellular or systemic metabolism, depending on the percentage of molecules that are modified and the chronicity of the modification. Several groups have demonstrated that certain enzymes become oxidatively modified during aging [61]. Enzymes that have been identified as being increasingly oxidized during aging include glutamine synthetase [62], mitochondrial aconitase [63], adenine nucleotide translocase [64], and carbonic anhydrase [65]. The first three have shown increases in protein carbonyls; the last incurs an increase in glutathiolation [57].

Modification of structural proteins can also lead to a loss of function. For example, when the plasma protein fibrinogen is oxidized either by treatment with an iron/ascorbate radical-generating system or with ionizing radiation, it loses its ability to form a solid clot [66]. The degree of clotting inhibition correlates with the extent of carbonyl formation in the protein. Oxidation of synovial fluid immunoglobulins causes aggregation, which may contribute to the etiology of rheumatoid arthritis [67,68], although a correlation between the extent of oxidation of immunoglobulin molecules and severity of disease has not been established. When protease inhibitors such as α-1 antitrypsin become oxidatively modified, severe physiological consequences may ensue. This plasma protein has primary responsibility for inhibiting proteolysis in tissues such as lung and cartilage. Modification of a critical Met residue in α-1 antitrypsin causes a loss of function, which seems to contribute to the tissue destruction seen in emphysema [69]. The primary oxidant responsible for this modification may be HOCl produced by inflammatory neutrophils [70]. Plasma low-density lipoprotein (LDL) has been demonstrated to undergo several different types of oxidative modification [15,27,29,44,71–74]. Exposure of LDL to Cu^+ causes oxidation of the LDL protein, leading to the formation of carbonyl groups [16,43,75], aggregation [43], and increased cellular uptake by tissue macrophages through the scavenger receptor. Importantly, oxidatively modified LDL has been found in atherosclerotic tissues by several groups, lending strong support for the idea that oxidation of LDL may play a significant role in the etiology of atherosclerosis [55,74,76–78]. Similarly, oxidation of crystallin proteins in the lens of the eye probably plays a role in cataractogenesis [19,79–82]. Generalized increases in oxidized proteins have been found in other disease states, but, in many cases, the specific proteins have not been identified. Table 3 contains a partial list of some conditions under which oxidized proteins have been identified in vivo.

The question often arises as to whether there are repair mechanisms for oxidized proteins, akin to repair systems that exist for reversal of oxidative DNA damage. So far, the only true enzymatic repair mechanisms that have been identified are for restoration

TABLE 3

**Forms of Protein Oxidative Modification That Have Been Found in
Biological Tissues**

Modification	Disease/tissue
Carbonyls	
Glutamine synthetase	Aging, Alzheimer's, ischemia/reperfusion
IgG	Rheumatoid arthritis
Kidney proteins	Chronic estrogen exposure
Lung proteins	Hyperoxia
Brain proteins	Ischemia/reperfusion
Eye lens proteins	Cataracts
Muscle proteins	Muscular dystrophy (chicken)
Unidentified proteins	Aging
Unidentified proteins	Parkinson's disease
Unidentified proteins	Neonatal lung fluids/ventilators
Methionine sulfoxide	
α-1-proteinase inhibitor	Smoker, bronchitis lung fluids, synovial fluid
Lipid peroxidation adducts	
LDL	Atherosclerosis
Glutathiolation (SH oxidation)	
Carbonic anhydrase III	Aging
Muscle proteins	Muscular dystrophy (chicken)
Unidentified proteins	Activated monocytes
3-Chlorotyrosine, dityrosine	
LDL	Atherosclerosis
Hydro(pero)xyleucine	
Unidentified proteins	Cataracts
Nitrotyrosine	
Unidentified proteins	Acute inflammatory lung tissue
	Atherosclerosis
	Rheumatoid arthritis
Aggregates	
IgG	Rheumatoid arthritis

of Met from Met sulfoxide and for restoration of Cys sulfhydryls from disulfides. The former is catalyzed by Met sulfoxide reductase [83]; the latter is accomplished by cellular reducing equivalents such as GSH. At present, there are no other known mechanisms for reversal of oxidative damage to proteins. On the other hand, oxidized proteins can be removed from the cell or tissue by proteolysis. Specific removal of oxidized proteins is carried out primarily by a multicatalytic protease, proteasome, which distinguishes between oxidized and native forms of a protein [80,84]. Defects in proteasome activity may be responsible for the accumulation of damaged proteins in some disease conditions and during aging [61,80,85]. Additional protein oxidation repair mechanisms may yet be dis-

covered. It may be worth noting that some oxidative protein changes will go unnoticed by any repair system. This may occur when one amino acid is converted to another, such as occurs in the metal-catalyzed conversion of proline to hydroxyproline or glutamate and the conversion of histidine to asparagine and aspartate. Unless these changes result in an overtly recognizable conformational change, they will likely go unnoticed by proteolytic or other repair systems. Note that such amino acid changes are equivalent to a mutation, only the effects are not permanent.

IV.   METHODS OF DETECTION OF PROTEIN OXIDATION

As outlined earlier, there are numerous types of protein oxidative modification. Consequently, there are at least as many different methods for detection and quantification of those modifications. A summary of methodological approaches and at least one reference for each method is given in Table 4. The most commonly measured products of protein oxidation in biological samples are the protein carbonyls (reviewed in Ref. 86). Examples of carbonyl derivatives of Pro, Arg, Lys, and Thr are shown in Table 2. These moieties are chemicaly stable, which is useful for both their detection and storage. They are present in most protein preparations at low levels of approximately 1 nmol/mg protein, which is equivalent to 0.05 mol carbonyl/mol of a 50-kDa protein. At this background level, roughly 1 in 3000 amino acids is estimated to contain a carbonyl group. Although this estimate may vary depending on the assay employed to measure the carbonyls [87], it is, by all estimates, low enough that biologically meaningful increases can be detected under physiological conditions. Increases of twofold to eightfold have been detected under conditions of oxidative stress in vivo [3]. Protein carbonyls are induced in vitro and in vivo by a diverse range of agents (see Table 1), including metal-catalyzed oxidation [40], ozone [24], HOCl [16,44,45], singlet oxygen [88], and ionizing radiation [66]. As a result, they serve as markers of oxidative stress for most types of ROS. Highly sensitive assays are available for detection of carbonyls (reviewed in Ref. 86). Many of the assays involve derivitization of the carbonyl group with dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenyl hydrazone product [89]. This then can be detected by various means. The DNP group itself asorbs ultraviolet (UV) light at 370 nm, with a molar extinction coefficient of 22,000, so that the total carbonyl content of a protein or mixture of proteins can be quantified by the ratio of the absorbance at 370 nm to that at 280 nm [89]. The spectrophotometric DNPH assay can be coupled to protein fractionation by high-performance liquid chromatography (HPLC) to give greater sensitivity and specificity than measuring total carbonyls in a protein mixture [90]. Other DNPH-associated assays utilize anti-DNP antibodies to quantify carbonyl content. Total carbonyls can be measured by enzyme-linked immunosorbent assay (ELISA) [91], slot blotting [92], or immunohisto-chemistry [93]; the carbonyl content in individual proteins is assessed by one-dimensional [94–96] or two-dimensional [97] sodium dodecyl sulfate (SDS) gel electrophoresis followed by immunoblotting (Western blotting). These latter two methods have significantly more sensitivity and specificity than all other total carbonyl assays, but they are only semiquantitative. The Western-blot assays can detect as little as 1 pmol carbonyl in a protein sample and require as little as 50 ng protein oxidized to the extent of 0.5 mol carbonyl/mol protein. Detailed procedures have been described in earlier publications [86,90,94,98,99]. The sensitivity of the method for detecting minor oxidized proteins in

TABLE 4

Methods for Detection of Oxidative Protein Modifications

Modification	Methods of detection	Ref.[a]
Disulfides	SDS–gel electrophoresis ($\pm\beta$-ME); DTNB	30
Thiyl radicals	Electron spin resonance spectroscopy	34
Glutathiolation	RP-HPLC/mass spectroscopy; IEF; ^{35}S–Cys/Chx/SDS-PAGE	31, 57, 122, 123
Methionine sulfoxide	CNBr cleavage/amino acid analysis	105, 106
Carbonyls	DNPH-coupled assays: Western blot/ELISA/immunocyto-chemistry/HPLC/A$_{370}$	89–91, 93, 94, 97
	Reduction with NaB^{3}H$_3$	100, 101
2-Oxo-His	Amino acid analysis	39
Dityrosine	Fluorescence; proteolysis or hydrolysis/HPLC	51, 74
Chlorotyrosine	Hydrolysis/nitroso-napthol/HPLC; HBr hydrolysis-GC/MS	44, 45
Nitrotyrosine	Immunoassay; hydrolysis/HLPC; HPLC/electrochemical detection	124–128
Tryptophanyl	Fluorescence; amino acid analysis (alk. hydrolysis); proteolysis/ MS	129
Hydroperoxides	KI/I$_3^-$/spectroscopy; NaBH$_4$/ hydrolysis/OPA–HPLC	19
Lipid peroxidation adducts	NaBH$_4$/hydrolysis/OPA–HPLC; hydrolysis–GC/MS; DNPH; immunoassays	28, 29, 54, 55
Amino acid oxidation adducts	NaCNBH$_3$ reduction/hydrolysis/ H^1–NMR/MS	47
Glycoxidation adducts	Derivitization—GC/MS	58
Cross-links, aggregates, fragments	SDS–gel electrophoresis; HPLC	43, 130, 131

Note: DTNB: dithiobisnitrobenzoate; IEF: isoelectric focusing; DNPH: dinitrophenylhydrazine; CNBr: cyanogen bromide; Chx: cyclohexamide; OPA: *o*-phthaldialdehyde.

[a] References for some methods currently in use. Other methods are also available.

a cell can be greatly enhanced by preparing subcellular fractions prior to derivatization [63,64]. The carbohydrate groups in glycoproteins have no apparent interaction in the assay and are not, themselves, readily oxidizable by metal-catalyzed systems [99]. Hence, they neither interfere nor contribute significantly to protein carbonyl measurements. A commercial kit for the Western-blot method published in 1994 [94] is currently marketed

by Intergen, Inc. Two general schemes for measuring protein carbonyls using DNPH are given in Fig. 1.

As an alternative to measuring carbonyls using DNPH, the carbonyls can be reduced and detected using sodium borotritide either in solution [100] or prior to gel electrophoresis [101]. The radioactive hydrogen can then be detected by standard methods. Protein carbonyl assays offer the advantage that no special or expensive equipment is required. As a result, they can be performed in any normally equipped biochemistry laboratory.

It is important to note that protein carbonyl assays generally measure any carbonyl in a protein, regardless of the source. Hence, introduction of a carbonyl through adduction of a lipid peroxidation by-product such as malondialdehyde, 4-HNE [54], or acrolein [13] will give a positive result in a carbonyl assay as long as the adduct is stable. If the adduct has to be reduced with $NaBH_4$ in order to stabilize the lipid–protein bond, it will not be detected by the carbonyl assay because this procedure also reduces and eliminates carbonyls. In order to establish whether the carbonyl came from direct or indirect modification of the amino acid side chain, additional assay methods must be employed. For example, lipid peroxidation adducts can be detected using specific antibodies [28,76,102].

Other approaches for the measurement of protein oxidation are listed in Table 4. Tyrosine is a target of oxidative attack by various agents, leading to the formation of dityrosine, chlorotyrosine, and dihydroxyphenylalanine (DOPA [103]). Assays for these products are very sensitive and specific [5,51,104] and may, in some cases, shed light on the oxidizing species [44]. Tyrosine oxidation products do not appear to be as abundant in biological samples as protein carbonyls [2]. For example, the level of 3-chlorotyrosine in atherosclerotic lesions reaches an average of 424 μmol/mol Tyr, which is equivalent to 1 modified residue per 2500 Tyr residues. Dityrosine levels may be even lower [2]. This relatively low degree of modification may result from the lower presence of Tyr in proteins compared to the sum of the several amino acids that form carbonyls (Lys, Arg, Pro, Thr). Nevertheless, tyrosine oxidation products can be measured, are highly specific, and show biologically meaningful increases under conditions of oxidative stress in vivo. Met is one of the most susceptible amino acids to oxidative modification and, hence, is an excellent marker for protein oxidative modification [32]. Unfortunately, there is no
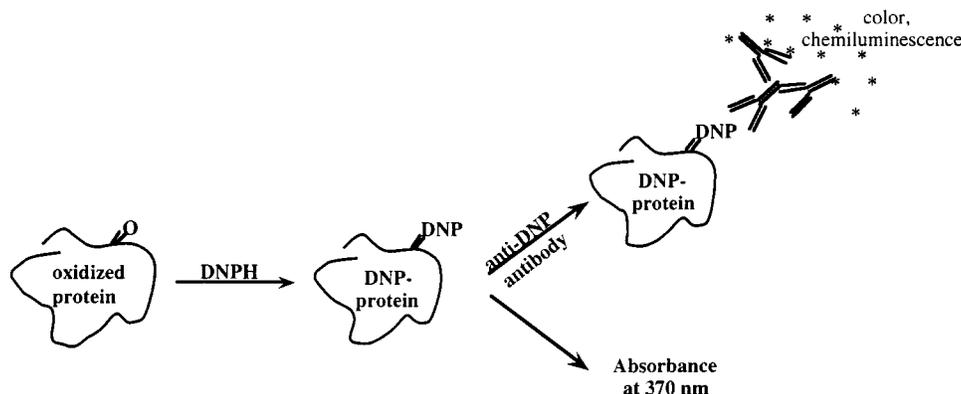
FIG. 1. Common approaches for using DNPH to test for protein carbonyls: immunoassay and spectrophotometric assay.

information available on the levels of oxidized Met under conditions of oxidative stress in vivo. Current methods for measurement of Met sulfoxide for in vitro samples employ amino acid analysis following CNBr cleavage [105,106]. To date, there are no antibodies available which can specifically detect Met sulfoxide, although some laboratories have tried to generate them. It is not clear whether Met sulfoxide is immunogenic.

V. PROTEIN OXIDATION AS A MARKER OF OXIDATIVE STRESS: ADVANTAGES AND DISADVANTAGES

One of the greatest challenges in oxidation research today is the determination of oxidative stress in vivo. Because proteins are ubiquitous in all cells and tissues and are susceptible to oxidative modification, they can serve as useful markers of oxidative stress. As shown in Table 3, oxidized proteins have been detected in numerous disease conditions and in aging. In some cases, the sources of the oxidative stress are known (e.g., activated neutrophils and monocytes in arthritis, atherosclerosis, respiratory distress syndrome, and emphysema). In others, the source of the oxidants is still being sought.

Compared to measuring products of lipid peroxidation [107] and DNA oxidative base modifications [108], proteins offer some advantages as markers of oxidative stress. Proteins have unique biological functions, so there are unique functional consequences resulting from their modification (e.g., loss of clotting from oxidation of fibrinogen [66], impaired ATP synthesis by oxidation of G3PDH [109]). Products of protein oxidative modification are relatively stable and there are sensitive assays available for their detection; thus, from a purely technical perspective, they serve as suitable markers for oxidative stress. Importantly, the nature of the protein modification can give significant information as to the type of oxidant involved in the oxidation process. For example, chlorotyrosyl moieties and amino acyl adducts on lysine residues are probably specific markers of oxidation by HOCl and, hence, reflect neutrophil and/or monocyte involvement in the oxidative stress [104,110]. Both of these types of oxidation products have been found in human atherosclerotic lesions (see Sec. IV). Similarly, the presence of nitrotyrosyl residues in proteins indicates that nitric oxide and superoxide (and, hence, peroxynitrite) were generated at the site of the damaged protein (reviewed in Ref. 111). Carbonyls can be induced by almost all types of ROS and, hence, do not shed significant light on the source of the oxidative stress. In addition, carbonyls are relatively difficult to induce compared to Met sulfoxide and cysteinyl derivatives and may, thus, be reflective of more severe cases of oxidative stress. Indeed, detection of elevated levels of protein carbonyls is generally a sign not only of oxidative stress but also of a disease-associated dysfunction (reviewed in Refs. 38 and 41). On the other hand, although Met and Cys are the two amino acyl residues that are most highly susceptible to oxidative attack, modification of these residues may not aways result in an effect on protein function [112,113]. In fact, Met residues may, in some cases, serve as internal scavengers which protect critical amino acyl residues from oxidative attack [106].

The highly specific nature of protein oxidation also leads to one of the disadvantages of using these macromolecules as markers of oxidative stress; that is, there is no single universal marker for protein oxidation. Because so many different protein oxidation products can be formed, it may be necessary to set up several different assays in order to find the most appropriate assay for the type of oxidative stress involved. For example, there

is minimal point in measuring chlorotyrosine if neither neutrophils nor monocytes are involved in the oxidative stress (except possibly to use it as a negative control). Similarly, because singlet oxygen is not a particularly good inducer of protein carbonyls, then when this ROS is the source of the oxidative stress, it might be more useful to assay products of Met, His, Tyr, and Trp oxidation because these are more susceptible to attack by singlet oxygen [21]. In determining whether to use proteins, lipids, or DNA as a marker of oxidative stress, the nature of the oxidative stress will play a significant role. For example, as noted earlier, HOCl is an excellent inducer of protein oxidative modification but causes little or no modification of lipids or DNA. Hence, when HOCl is the predominant oxidizing species, proteins must be used as the marker. On the other hand, other ROS are more effective at inducing lipid peroxidation or oxidative DNA damage than they are at modifying proteins. For example, treatment of cultured cells with reagent H_2O_2 induces extensive DNA single-strand break formation [114,115] and DNA base modifications [116], but it is a relatively poor inducer of protein carbonyls unless the iron level in the cells is restored by adding extracellular iron [109]. The F_2 isoprostanes have proven to be sensitive and specific markers of lipid peroxidation for a range of disease conditions in vivo [107]. Overall, the selection of a suitable marker of oxidative stress in vivo will require some knowledge of the physiological conditions being explored as well as some exercise of trial and error.

VI.   RAMIFICATIONS OF PROTEIN OXIDATION FOR THERAPEUTIC PROTEINS

Therapeutic proteins are major products of the biotechnology industry. Recombinant proteins include the interferons, interleukins, hematopoietic growth factors, clotting inhibitors, thrombolytics, and chemokines. Other therapeutic proteins, such as clotting factors, are isolated from human and animal tissues. It is essential to ensure minimal lot-to-lot variation in the manufacture of a therapeutic protein so that there are no significant differences in the effective dose being administered and to ensure that no unexpected side effects occur. This requires maintenance of a highly controlled and reproducible manufacturing process. Unfortunately, biotechnology manufacturing processes are generally more difficult to control than chemical syntheses because of the innate variability of biological systems and the reactivity of amino acid side chains. Covalent changes in a protein molecule can affect both bioactivity and pharmacokinetic/pharmacodynamic parameters, both of which affect drug efficacy and potential side effects. In addition, some protein modifications can lead to increased immunogenicity of a protein [117], leading possibly to development of a neutralizing immune response to the protein. This would cause a permanent loss of efficacy for the protein for the immunized individual. Other undesirable immunological consequences may also occur, such as cross-reactivity to and neutralization of endogenous homologs of the protein, leading to autoimmunity. One source of protein variability comes from oxidative protein modification. This can occur during both manufacture and storage of the protein. Manufacturing methods designed to minimize protein oxidative modification include lowering of oxygen tension [118] or carrying out of certain manufacturing steps under inert gases, utilization of a pH that minimizes redox reactions (e.g., performing certain steps at acidic pH to minimize sulfhydryl reactivity), and inclusion of low-molecular-weight antioxidants in the processing solutions. Oxidized variants

that are formed in the course of protein synthesis can be removed from the final product by downstream purification techniques [119]. The most commonly used marker of protein oxidative modification in manufactured proteins is Met sulfoxide. In some cases, oxidation of Met residues can have a profound effect on biological activity, as is seen following oxidation of *Escherichia coli*-derived stem-cell factor [120]. In other cases, such as for interferon-α-2b, oxidation of a single Met residue has no effect on bioactivity but may affect antigenic properties of the protein [113]. Oxidative modification of Met residues in recombinant coagulation factor VIIa had a differential effect on bioactivities of the molecule compared to its binding properties [121]. The redox state of cysteine residues usually determines correct folding of a protein and must also be monitored as part of the manufacturing process. Deamidation is induced by several mechanisms, including oxidation, and this is generally followed as well. Other potential protein oxidative modifications are not routinely screened by commercial protein manufacturers, although, if necessary, this could be easily accomplished. Dityrosine cross-linking can be detected by various techniques employed to characterize the bulk drug substance (e.g., peptide mapping, mass spectrometry), and amino acid analysis should reveal changes to Trp, Tyr, and His residues if appropriately performed. Protein carbonyl groups could easily be measured by several different assays. A comprehensive analysis of different protein oxidative modifications in commercial proteins may be merited to determine whether additional markers should be monitored on a routine basis.

ACKNOWLEDGMENT

REFERENCES

1. E. R. Stadtman, Metal ion-catalyzed oxidation of proteins: Biochemical mechanism and biological consequences, *Free Radical Biol. Med*., *9*, 315–325 (1990).
2. R. T. Dean, S. Fu, R. Stocker, and M. J. Davies, Biochemistry and pathology of radical-mediated protein oxidation, *Biochem. J*., *324*, 1–18 (1997).
3. E. R. Stadtman and B. S. Berlett, Reactive oxygen-mediated protein oxidation in aging and disease, *Drug Metab. Rev*., *30*, 225–243 (1998).
4. W. M. Garrison, M. E. Jayko, and W. Bennett, Radiation-induced oxidation of protein in aqueous solution, *Radiat. Res*., *16*, 483–502 (1962).
5. J. W. Heinecke, W. Li, H. L. Daehnke, and J. A. Goldstein, Dityrosine, a specific marker of oxidation, is synthesized by the myeloperoxidase-hydrogen peroxide system of human neutrophils and macrophages, *J. Biol. Chem*., *268*, 4069–4077 (1993).
6. I. U. Schraufstatter, K. Browne, A. Harris, P. A. Hyslop, J. H. Jackson, O. Quehenberger, and C. G. Cochrane, Mechanisms of hypochlorite injury of target cells, *J. Clin. Invest*., *85*, 554–562 (1990).
7. G. J. Handelman, Z. D. Nightingale, G. G. Dolnikowski, and J. B. Blumberg, For-

mation of carbonyls during attack on insulin by submolar amounts of hypochlorite, *Anal. Biochem.*, *258*, 339–348 (1998).

8. C-Y. Yang, Z-W. Gu, H-X. Yang, M. Yang, A. M. Gotto, and C. V. Smith, Oxidative modifications of APOB-100 by exposure of low density lipoproteins to HOCl in vitro, *Free Radical Biol. Med.*, *23*, 82–89 (1997).

9. L. J. Yan, M. G. Traber, H. Kobuchi, S. Matsugo, H. J. Tritschler, and L. Packer, Efficacy of hypochlorous acid scavengers in the prevention of protein carbonyl formation, *Arch. Biochem. Biophys.*, *327*, 330–334 (1996).

10. P. Korbashi, R. Kohen, J. Katzhendler, and M. Chevion, Iron mediates paraquat toxicity in Escherichia coli, *J. Biol. Chem.*, *261*, 12,472–12,476 (1986).

11. D. P. Hartley, D. J. Kroll, and D. R. Petersen, Prooxidant-initiated lipid peroxidation in isolated rat hepatocytes: Detection of 4-hydroxynonenal- and malondialdehyde-protein adducts, *Chem. Res. Toxicol.*, *10*, 895–905 (1997).

12. M. A. Tirmenstein and S. D. Nelson, Acetaminophen-induced oxidation of protein thiols. Contribution of impaired thiol-metabolizing enzymes and the breakdown of adenine nucleotides, *J. Biol. Chem.*, *265*, 3059–3065 (1990).

13. J. P. Eiserich, A. Van der Vliet, G. J. Handelman, B. Halliwell, and C. E. Cross, Dietary antioxidants and cigarette smoke-induced biomolecular damage: A complex interaction, *Am. J. Clin. Nutr.*, *62*, 1490S–1500S (1995).

14. B. S. Berlett and E. R. Stadtman, Protein oxidation in aging, disease, and oxidative stress, *J. Biol. Chem.*, *272*, 20,313–20,316 (1997).

15. U. P. Steinbrecher, J. L. Witztum, S. Parthasarathy, and D. Steinberg, Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL, *Arteriosclerosis*, *7*, 135–143 (1987).

16. L. J. Yan, J. K. Lodge, M. G. Traber, S. Matsugo, and L. Packer, Comparison between copper-mediated and hypochlorite-mediated modifications of human low density lipoproteins evaluated by protein carbonyl formation, *J. Lipid Res.*, *38*, 992–1001 (1997).

17. K. J. A. Davies, Protein damage and degradation by oxygen radicals, *J. Biol. Chem.*, *262*, 9895–9901 (1987).

18. K. J. A. Davies, M. E. Delsignore, and S. W. Lin, Protein damage and degradation by oxygen radicals. II. Modification of amino acids, *J. Biol. Chem.*, *262*, 9902–9907 (1987).

19. S. L. Fu and R. T. Dean, Structural characterization of the products of hydroxyl-radical damage to leucine and their detection on proteins, *Biochem. J.*, *324*, 41–48 (1997).

20. C. N. Oliver, Inactivation of enzymes and oxidative modification of proteins by stimulated neutrophils, *Arch. Biochem. Biophys.*, *253*, 62–72 (1987).

21. D. Balasubramanian, X. Du, and J. S. Zigler, The reaction of singlet oxygen with proteins, with special reference to crystallins, *Photochem. Photobiol.*, *52*, 761–768 (1990).

22. H. R. Shen, J. D. Spikes, P. Kopecekova, and J. Kopecek, Photodynamic crosslinking of proteins. Model studies using histidine- and lysine-containing *N*-(2-hydroxypropyl)methacrylamide copolymers, *J. Photochem. Photobiol. B*, *34*, 203–210 (1996).

23. M. L. Hu and A. L. Tappel, Potentiation of oxidative damage to proteins by ultraviolet-A and protection by antioxidants, *Photochem. Photobiol.*, *56*, 357–363 (1992).

24. C. E. Cross, A. Z. Reznik, L. Packer, P. A. Davis, Y. J. Suzuki, and B. Halliwell, Oxidative damage to human plasma proteins by ozone, *Free Radical Res. Commun.*, *15*, 347–352 (1992).

25. B. S. Berlett, R. L. Levine, and E. R. Stadtman, Comparison of the effects of ozone on the modification of amino acid residues in glutamine synthetase and bovine serum albumin, *J. Biol. Chem.*, *271*, 4177–4182 (1996).

26. L. Fucci, C. N. Oliver, M. J. Coon, and E. R. Stadtman, Inactivation of key metabolic enzymes by mixed-function oxidation reactions: Possible implication in protein turnover and ageing, *Proc. Natl. Acad. Sci. USA*, *80*, 1521–1525 (1983).

27. M. E. Haberland, D. Fong, and L. Cheng, Malondialdehyde-altered protein occurs in atheroma of Watanabe heritable hyperlipidemic rabbits, *Science*, *241*, 215–218 (1988).

28. J. G. Kim, F. Sabbagh, N. Santanam, J. N. Wilcox, R. M. Medford, and S. Parthasarathy, Generation of a polyclonal antibody against lipid peroxide-modified proteins, *Free Radical Biol. Med.*, *23*, 251–259 (1997).

29. J. R. Requena, M. X. Fu, M. U. Ahmed, A. J. Jenkins, T. J. Lyons, J. W. Baynes, and S. R. Thorpe, Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein, *Biochem. J.*, *322*, 317–325 (1997).

30. R. Radi, K. M. Bush, T. P. Cosgrove, and B. A. Freeman, Reaction of xanthine oxidase-derived oxidants with lipid and protein of human plasma, *Arch. Biochem. Biophys.*, *286*, 117–125 (1991).

31. C-K. Lii, Y-C. Chai, W. Zhao, J. A. Thomas, and S. Hendrich, S-Thiolation and irreversible oxidation of sulfhydryls on carbonic anhydrase III during oxidative stress: A method for studying protein modification in intact cells and tissues, *Arch. Biochem. Biophys.*, *308*, 231–239 (1994).

32. W. Vogt, Oxidation of methionyl residues in proteins: Tools, targets, and reversal, *Free Radical Biol. Med.*, *18*, 93–105 (1995).

33. M-L. Hu, Measurement of protein thiol groups and glutathione in plasma, *Methods Enzymol.*, *233*, 380–385 (1994).

34. B. Kalyanaraman, Thiyl radicals in biological systems: Significant or trivial? *Biochem. Soc. Symp.*, *61*, 55–63 (1995).

35. G. Czapski, On the use of OH radical scavengers in biological systems, *Israel J. Chem.*, *24*, 29–32 (1984).

36. A. Samuni, J. Aronovitch, D. Godinger, M. Chevion, and G. Czapski, On the cytotoxicity of vitamin C and metal ions. A site-specific Fenton mechanism, *Eur. J. Biochem.*, *137*, 119–124 (1983).

37. J. M. Farber and R. L. Levine, Sequence of a peptide susceptible to mixed-function oxidation. Probable cation binding site in glutamine synthetase, *J. Biol. Chem.*, *261*, 4574–4578 (1986).

38. E. R. Stadtman and C. N. Oliver, Metal-catalyzed oxidation of proteins, *J. Biol. Chem.*, *266*, 2005–2008 (1991).

39. S. A. Lewisch and R. L. Levine, Determination of 2-oxohistidine by amino acid analysis, *Anal. Biochem.*, *231*, 440–446 (1995).

40. A. Amici, R. L. Levine, L. Tsai, and E. R. Stadtman, Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions, *J. Biol. Chem.*, *264*, 3341–3346 (1989).

41. E. R. Stadtman and B. S. Berlett, Reactive oxygen-mediated protein oxidation in aging and disease, *Chem. Res. Toxicol*., *10*, 485–494 (1997).

42. E. R. Stadtman, Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions, *Annu. Rev. Biochem*., *62*, 797–821 (1993).

43. L. J. Hazell, J. J. M. van den Berg, and R. Stocker, Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather than lipid oxidation, *Biochem. J*., *302*, 297–304 (1994).

44. S. L. Hazen and J. W. Heinecke, 3-Chlorotyrosine, a specific marker of myeloperoxidase-catalyzed oxidation, is markedly elevated in low density lipoprotein isolated from human atherosclerotic intima, *J. Clin. Invest*., *99*, 2075–2081 (1997).

45. A. J. Kettle, Neutrophils convert tyrosyl residues in albumin to chlorotyrosine, *FEBS Lett*., *379*, 103–106 (1996).

46. S. L. Hazen, F. F. Hsu, A. d'Avignon, and J. W. Heinecke, Human neutrophils employ myeloperoxidase to convert alpha-amino acids to a battery of reactive aldehydes: A pathway for aldehyde generation at sites of inflammation, *Biochemistry*, *37*, 6864–6873 (1998).

47. S. L. Hazen, J. P. Gaut, F. F. Hsu, J. R. Crowley, A. d'Avignon, and J. W. Heinecke, *p*-Hydroxyphenylacetaldehyde, the major product of L-tyrosine oxidation by the myeloperoxidase–H_2O_2–chloride system of phagocytes, covalently modifies e-amino groups of protein lysine residues, *J. Biol. Chem*., *27*, 16,990–16,998 (1997).

48. H. Ischiropoulos and A. B. Al-Mehdi, Peroxynitrite-mediated oxidative protein modifications, *FEBS Lett*., *364*, 279–282 (1995).

49. M. Tien, B. S. Berlett, R. L. Levine, P. B. Chock, and E. R. Stadtman, Peroxynitrite-mediated modification of proteins at physiological carbon dioxide concentration: pH dependence of carbonyl formation, tyrosine nitration, and methionine oxidation, *Proc. Natl. Acad. Sci. USA*, *96*, 7809–7814 (1999).

50. J. A. Simpson, S. Narita, S. Gieseg, S. Gebicki, J. M. Gebicki, and R. T. Dean, Long-lived reactive species on free-radical-damaged proteins, *Biochem. J*., *282*, 621–624 (1992).

51. C. Giulivi and K. J. A. Davies, Dityrosine: A marker for oxidatively modified proteins and selective proteolysis, *Methods Enzymol*., *233*, 363–371 (1994).

52. J. W. Heinecke, W. Li, G. A. Francis, and J. A. Goldstein, Tyrosyl radical generated by myeloperoxidase catalyzes the oxidative cross-linking of proteins, *J. Clin. Invest*., *91*, 2866–2872 (1993).

53. S. Fu, M. J. Davies, R. Stocker, and R. T. Dean, Evidence for roles of radicals in protein oxidation in advanced human atherosclerotic plaque, *Biochem. J*., *333*, 519–525 (1998).

54. K. Uchida and E. R. Stadtman, Quantitation of 4-hydroxynonenal protein adducts, *Methods Enzymol*., *233*, 371–380 (1994).

55. M. E. Rosenfeld, J. C. Khoo, E. Miller, S. Parthasarathy, W. Palinski, and J. L. Witztum, Macrophage-derived foam cells freshly isolated from rabbit atherosclerotic lesions degrade modified lipoproteins, promote oxidation of low-density lipoproteins, and contain oxidation-specific lipid-protein adducts, *J. Clin. Invest*., *87*, 90–99 (1991).

56. K. Uchida, M. Kanematsu, Y. Morimitsu, T. Osawa, N. Noguchi, and E. Niki, Acrolein is a product of lipid peroxidation reaction. Formation of free acrolein and

its conjugate with lysine residues in oxidized low density lipoproteins, *J. Biol. Chem.*, *273*, 16,058–16,066 (1998).

57. E. Cabiscol and R. L. Levine, The phosphatase activity of carbonic anhydrase III is reversibly regulated by glutathiolation, *Proc. Natl. Acad. Sci. USA*, *93*, 4170–4174 (1996).

58. T. P. Degenhardt, S. R. Thorpe, and J. W. Baynes, Chemical modification of proteins by methylglyoxal, *Cell. Molec. Biol.*, *44*, 1139–1145 (1998).

59. M. M. Anderson, J. R. Requena, J. R. Crowley, S. R. Thorpe, and J. W. Heinecke, The myeloperoxidase system of human phagocytes generates Nepsilon–(carboxymethyl)lysine on proteins: A mechanism for producing advanced glycation end products at sites of inflammation, *J. Clin. Invest.*, *104*, 103–113 (1999).

60. C. N. Oliver, B. Ahn, M. E. Wittenberger, and E. R. Stadtman, Oxidative inactivation of enzymes: Implication in protein turnover and aging, in *Cellular Regulation and Malignant Growth* (S. Ebashi, ed.), Japan Science Society Press, Tokyo, 1985, pp. 320–331.

61. C. N. Oliver, R. L. Levine, and E. R. Stadtman, A role of mixed-function oxidation reactions in the accumulation of altered enzyme forms during aging, *J. Am. Geriat. Soc.*, *35*, 947–956 (1987).

62. J. M. Carney, P. E. Starke-Reed, C. N. Oliver, R. W. Landum, M. S. Cheng, J. F. Wu, and R. A. Floyd, Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity, and loss in temporal and spatial memory by chronic administration of the spin-trapping compound *N-tert*-butyl-*a*-phenylnitrone, *Proc. Natl. Acad. Sci. USA*, *88*, 3633–3636 (1991).

63. L. J. Yan, R. L. Levine, and R. S. Sohal, Oxidative damage during aging targets mitochondrial aconitase, *Proc. Natl. Acad. Sci. USA*, *94*, 11,168–11,172 (1997).

64. L. J. Yan and R. S. Sohal, Mitochondrial adenine nucleotide transferase is modified oxidatively during aging, *Proc. Natl. Acad. Sci. USA*, *95*, 12,896–12,901 (1998).

65. E. Cabiscol and R. L. Levine, Carbonic anhydrase III. Oxidative modification in vivo and loss of phosphatase activity during aging, *J. Biol. Chem.*, *270*, 14,742–14,747 (1995).

66. E. Shacter, J. A. Williams, and R. L. Levine, Oxidative modification of fibrinogen inhibits thrombin-catalyzed clot formation, *Free Radical Biol. Med.*, *18*, 815–821 (1995).

67. H. E. Jasin, Generation of IgG aggregates by the myeloperoxidase-hydrogen peroxide system, *J. Immunol.*, *130*, 1918–1923 (1983).

68. J. Lunec, D. R. Blake, S. J. McCleary, S. Brailsford, and P. A. Bacon, Self-perpetuating mechanisms of immunoglobulin G aggregation in rheumatoid inflammation, *J. Clin. Invest.*, *76*, 2084–2090 (1985).

69. H. Carp, F. Miller, J. R. Hoidal, and A. Janoff, Potential mechanism of emphysema: Alpha 1-proteinase inhibitor recovered from lungs of cigarette smokers contains oxidized methionine and has decreased elastase inhibitory capacity, *Proc. Natl. Acad. Sci. USA*, *79*, 2041–2045 (1982).

70. N. R. Matheson, P. S. Wong, and J. Travis, Enzymatic inactivation of human alpha-1-proteinase inhibitor by neutrophil myeloperoxidase, *Biochem. Biophys. Res. Commun.*, *88*, 402–409 (1979).

71. H. Esterbauer, G. Striegl, H. Puhl, and M. Rotheneder, Continuous monitoring of in vitro oxidation of human low density lipoprotein, *Free Radical Res. Commun.*, *6*, 67–75 (1989).

72. K. Uchida, M. Kanematsu, K. Sakai, T. Matsuda, N. Hattori, Y. Mizuno, D. Suzuki, T. Miyata, N. Noguchi, E. Niki, and T. Osawa, Protein-bound acrolein: Potential markers for oxidative stress, *Proc. Natl. Acad. Sci. USA*, *95*, 4882–4887 (1998).

73. L. J. Hazell and R. Stocker, Oxidation of low-density lipoprotein with hypochlorite causes transformation of the lipoprotein into a high-uptake form for macrophages, *Biochem. J.*, *290*, 165–172 (1993).

74. C. Leewenburgh, J. E. Rasmussen, F. F. Hsu, D. M. Mueller, S. Pennathur, and J. W. Heinecke, Mass spectrometric quantification of markers for protein oxidation by tyrosyl radical, copper, and hydroxyl radical in low density lipoprotein isolated from human atherosclerotic plaques, *J. Biol. Chem.*, *272*, 3520–3526 (1998).

75. C. Y. Yang, Z. W. Gu, H. X. Yang, M. Yang, A. M. Gotto, Jr., and C. V. Smith, Oxidative modifications of apoB-100 by exposure of low density lipoproteins to HOCl in vitro, *Free Radical Biol. Med.*, *23*, 82–89 (1997).

76. W. Palinski, M. E. Rosenfeld, S. Yla-Herttuala, G. C. Gurtner, S. S. Socher, S. W. Butler, S. Parthasarathy, T. E. Carew, D. Steinberg, and J. L. Witztum, Low density lipoprotein undergoes oxidative modification in vivo, *Proc. Natl. Acad. Sci. USA*, *86*, 1372–1376 (1989).

77. L. J. Hazell, L. Arnold, D. Flowers, G. Waeg, E. Malle, and R. Stocker, Presence of hypochlorite-modified proteins in human atherosclerotic lesions, *J. Clin. Invest.*, *97*, 1535–1544 (1996).

78. D. Steinberg, S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum, Modifications of low-density lipoprotein that increase its atherogenecity, *N. Engl. J. Med.*, *320*, 915–924 (1989).

79. D. Garland, Role of site-specific, metal-catalyzed oxidation in lens aging and cataract: A hypothesis, *Exp. Eye Res.*, *50*, 677–682 (1990).

80. K. J. A. Davies, Protein oxidation and proteolytic degradation. General aspects and relationship to cataract formation, *Adv. Exp. Med. Biol.*, *264*, 503–511 (1990).

81. K. Murakami, J. H. Jahngen, S. W. Lin, K. J. Davies, and A. Taylor, Lens proteasome shows enhanced rates of degradation of hydroxyl radical modified alpha-crystallin, *Free Radical Biol. Med.*, *8*, 217–222 (1990).

82. S. Fu, R. Dean, M. Southan, and R. Truscott, The hydroxyl radical in lens nuclear cataractogenesis, *J. Biol. Chem.*, *273*, 28,603–28,609 (1998).

83. J. Moskovitz, B. S. Berlett, J. M. Poston, and E. R. Stadtman, Methionine sulfoxide reductase in antioxidant defense, *Methods Enzymol.*, *300*, 239–244 (1999).

84. J. A. Sahakian, L. I. Szweda, B. Friguet, K. Kitani, and R. L. Levine, Aging of the liver: Proteolysis of oxidatively modified glutamine synthetase, *Arch. Biochem. Biophys.*, *318*, 411–417 (1995).

85. A. J. Rivett, J. E. Roseman, C. N. Oliver, R. L. Levine, and E. R. Stadtman, Covalent modification of proteins by mixed-function oxidation: Recognition by intracellular proteases, *Prog. Clin. Biol. Res.*, *180*, 317–328 (1985).

86. E. Shacter, Protein oxidative damage, *Methods Enzymol.*, *319*, 428–436 (2000).

87. P. Evans, L. Lyras, and B. Halliwell, Measurement of protein carbonyls in human brain tissue, *Methods Enzymol.*, *300*, 145–156 (1999).

88. J. A. Silvester, G. S. Timmins, and M. J. Davies, Protein hydroperoxides and carbonyl groups generated by porphyrin-induced photo-oxidation of bovine serum albumin, *Arch. Biochem. Biophys.*, *350*, 249–258 (1998).

89. R. L. Levine, D. Garland, C. N. Oliver, A. Amici, I. Climent, A. Lenz, B. Ahn,

   S. Shaltiel, and E. R. Stadtman, Determination of carbonyl content in oxidatively modified proteins, *Methods Enzymol*., *186*, 464–478 (1990).

90. R. L. Levine, J. Williams, E. R. Stadtman, and E. Shacter, Carbonyl assays for determination of oxidatively modified proteins, *Methods Enzymol*., *233*, 346–357 (1994).

91. H. Buss, T. P. Chan, K. B. Sluis, N. M. Domigan, and C. C. Winterbourn, Protein carbonyl measurement by a sensitive ELISA method, *Free Radical Biol. Med*., *23*, 361–366 (1997).

92. C. E. Robinson, A. Kashavarzian, D. S. Pasco, T. O. Frommel, D. H. Winshop, and E. W. Holmes, Determination of protein carbonyl groups by immunoblotting, *Anal. Biochem*., *266*, 48–57 (1999).

93. M. A. Smith, L. M. Sayre, V. E. Anderson, P. L. R. Harris, M. F. Beal, N. Kowall, and G. Perry, Cytochemical demonstration of oxidative damage in Alzheimer disease by immunochemical enhancement of the carbonyl reaction with 2,4-dinitrophenylhydrazine, *J. Histochem. Cytochem*., *46*, 731–735 (1998).

94. E. Shacter, J. A. Williams, M. Lim, and R. L. Levine, Differential susceptibility of plasma proteins to oxidative modification. Examination by Western blot immunoassay, *Free Radical Biol. Med*., *17*, 429–437 (1994).

95. R. J. Keller, N. C. Halmes, J. A. Hinson, and N. R. Pumford, Immunochemical detection of oxidized proteins, *Chem. Res. Toxicol*., *6*, 430–433 (1993).

96. L. J. Yan, W. C. Orr, and R. S. Sohal, Identification of oxidized proteins based on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, immunochemical detection, isoelectric focusing, and microsequencing, *Anal. Biochem*., *263*, 67–71 (1998).

97. A. Nakamura and S. Goto, Analysis of protein carbonyls with 2,4-dinitrophenyl hydrazine and its antibodies by immunoblot in two-dimensional gel electrophoresis, *J. Biochem*., *119*, 768–774 (1996).

98. E. Shacter, J. A. Williams, E. R. Stadtman, and R. L. Levine, Determination of carbonyl groups in oxidized proteins, in *Free Radicals*: *A Practical Approach* (N. Punchard and F. Kelly, eds.), Oxford University Press, Oxford, 1994, pp. 159–170.

99. Y-J. Lee and E. Shacter, Role of carbohydrates in oxidative modification of fibrinogen and other plasma proteins, *Arch. Biochem. Biophys*., *321*, 175–181 (1995).

100. A. Lenz, U. Costabel, S. Shaltiel, and R. L. Levine, Determination of carbonyl groups in oxidatively modified proteins by reduction with tritiated sodium borohydride, *Anal. Biochem*., *177*, 419–425 (1989).

101. L. J. Yan and R. S. Sohal, Gel electrophoretic quantitation of protein carbonyls derivatized with tritiated sodium borohydride, *Anal. Biochem*., *265*, 176–182 (1998).

102. G. Waeg, G. Dimsity, and H. Esterbauer, Monoclonal antibodies for detection of 4-hydroxynonenal modified proteins, *Free Radical Res*., *25*, 149–159 (1996).

103. S. P. Gieseg, J. A. Simpson, T. S. Charlton, M. W. Duncan, and R. T. Dean, Protein-bound 3,4-dihydroxyphenylalanine is a major reductant formed during hydroxyl radical damage to proteins, *Biochemistry*, *32*, 4780–4786 (1993).

104. A. J. Kettle, Detection of 3-chlorotyrosine in proteins exposed to neutrophil oxidants, *Methods Enzymol*., *300*, 111–120 (1999).

105. K. L. Maier, A. G. Lenz, I. Beck-Speier, and U. Costabel, Analysis of methionine sulfoxide in proteins, *Methods Enzymol*., *251*, 455–461 (1995).

106. R. L. Levine, L. Mosoni, B. S. Berlett, and E. R. Stadtman, Methionine residues

as endogenous antioxidants in proteins, *Proc. Natl. Acad. Sci. USA*, *93*, 15,036–15,040 (1996).

107. J. D. Morrow, Y. Chen, C. J. Brame, J. Yang, S. C. Sanchez, J. Xu, W. E. Zackert, J. A. Awad, and L. J. Roberts, The isoprostanes: Unique prostaglandin-like products of free-radical-initiated lipid peroxidation, *Drug Metab. Rev*., *31*, 117–139 (1999).

108. M. K. Shigenaga, E. N. Aboujaoude, Q. Chen, and B. N. Ames, Assays of oxidative DNA damage biomarkers 8-oxo-2′-deoxyguanosine and 8-oxoguanine in nuclear DNA and biological fluids by high-performance liquid chromatography with electrochemical detection, *Methods Enzymol*., *234*, 16–33 (1994).

109. R. L. Levine and H. P. Ciolino, Modification of proteins in endothelial cell death during oxidative stress, *Free Radical Biol. Med*., *22*, 1277–1282 (1997).

110. S. L. Hazen, F. F. Hsu, J. P. Gaut, J. R. Crowley, and J. W. Heinecke, Modification of proteins and lipids by myeloperoxidase, *Methods Enzymol*., *300*, 88–105 (1999).

111. J. S. Beckman and W. H. Koppenol, Nitric oxide, superoxide, and peroxynitrite: The good, the bad, and ugly, *Am. J. Physiol*., *271*, C1424–C1437 (1996).

112. V. Y. Reddy, P. E. Desorchers, S. V. Pizzo, S. L. Gonias, J. A. Sahakian, R. L. Levine, and S. J. Weiss, Oxidative dissociation of human alpha 2-macroglobulin tetramers into dysfunctional dimers, *J. Biol. Chem*., *269*, 4683–4691 (1994).

113. G. Gitlin, A. Tsarbopoulos, S. T. Patel, W. Sydor, B. N. Pramanik, S. Jacobs, L. Westreich, S. Mittelman, and J. N. Bausch, Isolation and characterization of a monomethioninesulfoxide variant of interferon alpha-2b, *Pharm. Res*., *13*, 762–769 (1996).

114. H. C. Birnboim, DNA strand breaks in human leukocytes induced by superoxide anion, hydrogen peroxide and tumor promoters are repaired slowly compared to breaks induced by ionizing radiation, *Carcinogenesis*, *7*, 1511–1517 (1986).

115. E. Shacter, E. J. Beecham, J. M. Covey, K. W. Kohn, and M. Potter, Activated neutrophils induce prolonged DNA damage in neighboring cells, *Carcinogenesis*, *9*, 2297–2304 (1988).

116. K. Frenkel, K. Chrzan, W. Troll, G. W. Teebor, and J. J. Steinberg, Radiation-like modification of bases in DNA exposed to tumor promoter-activated polymorphonuclear leukocytes, *Cancer Res*., *46*, 5533–5540 (1986).

117. W. Chen, J. W. Yewdell, R. L. Levine, and J. R. Bennink, Modification of cysteine residues in vitro and in vivo affects the immunogenicity and antigenicity of major histocompatibility complex class I-restricted viral determinants, *J. Exp. Med*., *189*, 1757–1764 (1999).

118. P. J. Berti, I. Ekiel, P. Lindahl, M. Abrahamson, and A. C. Storer, Affinity purification and elimination of methionine oxidation in recombinant human cystatin C, *Protein Exp. Purif*., *11*, 111–118 (1997).

119. C. V. Olson, D. H. Reifsnyder, E. Canova-Davis, V. T. Ling, and S. E. Builder, Preparative isolation of recombinant human insulin-like growth factor 1 by reversed-phase high-performance liquid chromatography, *J. Chromatog. A*, *675*, 101–112 (1994).

120. Y. R. Hsu, L. O. Narhi, C. Spahr, K. E. Langley, and H. S. Lu, In vitro methionine oxidation of *Escherichia coli*-derived human stem cell factor: Effects on the molecular structure, biological activity, and dimerization, *Protein Sci*., *5*, 1165–1173 (1996).

121. T. Kornfelt, E. Persson, and L. Palm, Oxidation of methionine residues in coagulation factor VIIa, *Arch. Biochem. Biophys*., *363*, 43–54 (1999).

122. D. A. Davis, K. Dorsey, P. T. Wingfield, S. J. Stahl, J. Kaufman, H. M. Fales, and

R. L. Levine, Regulation of HIV-1 protease activity through cysteine modification, *Biochemistry*, *35*, 2482–2488 (1996).

123. J. A. Thomas, Y-C. Chai, and C-H. Jung, Protein *S*-thiolation and dethiolation, *Methods Enzymol*., *233*, 385–395 (1994).

124. M. A. Smith, P. L. R. Harris, L. M. Sayre, J. S. Beckman, and G. Perry, Widespread peroxynitrite-mediated damage in Alzheimer's disease, *J. Neurosci*., *17*, 2653–2657 (1997).

125. Y. Z. Ye, M. Strong, Z. Q. Huang, and J. S. Beckman, Antibodies that recognize nitrotyrosine, *Methods Enzymol*., *269*, 201–209 (1996).

126. H. Ischiropoulos, L. Zhu, J. Chen, M. Tsai, J. C. Martin, C. D. Smith, and J. S. Beckman, Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase, *Arch. Biochem. Biophys*., *298*, 431–437 (1992).

127. J. P. Eiserich, C. E. Cross, A. D. Jones, B. Halliwell, and A. Van der Vliet, Formation of nitrating and chlorinating species by reaction of nitrite with hypochlorous acid, *J. Biol. Chem*., *271*, 19,199–19,208 (1996).

128. M. K. Shigenaga, H. H. Lee, B. C. Blount, E. T. Shigeno, H. Yip, and B. N. Ames, Inflammation and NO(X)-induced nitration: assay for 3-nitrotyrosine by HPLC with electrochemical detection, *Proc. Natl. Acad. Sci. USA*, *94*, 3211–3216 (1997).

129. E. L. Finley, J. Dillon, R. K. Crouch, and K. L. Schey, Identification of tryptophan oxidation products in bovine alpha-crystallin, *Protein Sci*., *7*, 2391–2397 (1998).

130. K. J. A. Davies and M. E. Delsignore, Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure, *J. Biol. Chem*., *262*, 9908–9913 (1987).

131. R. E. Pacifici and K. J. A. Davies, Protein degradation as an index of oxidative stress, *Methods Enzymol*., *186*, 485–502 (1990).