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Review

The Importance of Proteins in Defense Against Oxidation

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ABSTRACT

Free radicals are a normal feature of cellular oxygen metabolism. However, free radical-associated damage is an important factor in many pathological and toxicological processes. For a long time, lipid peroxidation, mediated by oxygen-derived free radicals, was probably the most extensively investigated process. From more recent studies, it has become evident that proteins are also the targets of free radicals, and this has important implication for their activity, unfolding, and degradation, as well as in cell functioning. After giving a brief overview of the key role of proteins in the overall antioxidant defense, this review examines their role as targets of oxidation reactions, taking into account the reactivity of amino acid residues and some of their oxidation products. In light of recent data, we then consider the specific role of sulfur-containing amino acids in protein degradation and their possible interplay with the reversal of limited oxidative lesions. The participation of proteins in the overall antioxidant defense is also discussed, specifically the role of metallothionein as an intracellular antioxidant and that of albumin as a circulating antioxidant. Antioxid. Redox Signal. 3, 293–311.

INTRODUCTION

REACTIVE OXYGEN SPECIES (ROS) is a collective term for oxygen radicals and nonradicals that are oxidizing agents and/or are easily converted into free radicals. Because ROS can be generated *in vivo*, recent advances in our understanding of the biochemistry of these compounds have led to the recognition that ROS are likely implicated in the pathogenesis of a number of diseases, including cancer and cardiovascular and neurodegenerative diseases, as well as in certain physiological processes such as aging. Oxidative stress occurs when endogenous antioxidant defenses are inadequate to scavenge ROS completely. The resulting oxidative damage to lipids, DNA, proteins, and other molecules may contribute to the development of the aforementioned diseases (58). It follows that there is a growing interest in additional research on biomarkers (31) and mechanisms of antioxidant protection with potential relevance for therapeutic use (32, 57).

In this review, we discuss the role of proteins as targets of free radical attack, as well as their role as part of the antioxidant defense in plasma and various intracellular compartments. With a special focus on the oxidation ability of amino acids, we shall highlight the importance of sulfur-containing amino acid residues. As examples, the specific roles of serum albumin and metallothionein (MT) will be especially described.

THE ANTIOXIDANT DEFENSE

ROS are produced either endogenously, from normal metabolic reactions, or exogenously as components of tobacco smoke and air

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pollutants (21, 88, 100) as well as indirectly through the metabolism of drugs, solvents, pesticides, and exposure to radiation (UV light) (5, 57–59, 76). The cytochrome *c* oxidase-catalyzed reaction involves the transfer of electrons to oxygen and results in partially reduced oxygen species. Other enzymes, especially flavin enzymes, also generate partially reduced oxygen species. However, numerous natural defenses exist either to prevent ROS formation or to neutralize them after they are generated (26, 57). They include enzymes and other low-molecular-weight compounds. The discovery of superoxide dismutase (SOD) provided understanding of the importance of free radicals in biological systems (49, 60). Three distinct forms have been described: CuZn-SOD, which is cytosolic; Mn-SOD, which is located in mitochondria; and a third form, also a CuZn-SOD, which is immunologically distinct from the others and is located extracellularly. SOD catalyzes the conversion of superoxide anion ($O_2^{-\bullet}$) to hydrogen peroxide (H_2O_2). In the presence of transition metals, H_2O_2 is rapidly converted to the potent $HO^{\bullet}$ through the Fenton reaction. Fortunately, two enzymes are able to neutralize H_2O_2: catalase and glutathione peroxidase. The latter enzyme also has the properties to reduce peroxides, including lipid peroxides. It has been shown, however, that increased activity/expression of only one antioxidant enzyme could be deleterious and that it is the balance between CuZn-SOD and the peroxide-removing enzymes catalase and glutathione peroxidase that is of prime importance in the antioxidant defense. An additional antioxidant defense mechanism is to keep transition metal ions safely sequestered by metal binding proteins, such as ferritin, transferrin, ceruloplasmin, MT, and possibly albumin.

Aside from these enzymes and proteins, an array of antioxidant molecules are present to scavenge free radicals (2). The most notable are vitamins C and E, various carotenoids (β-carotene, lycopene), glutathione, ubiquinol, uric acid, and bilirubin. It is interesting to note that several dietary micronutrients contribute greatly to the protective mechanism. This is particularly true for vitamins, oligominerals and perhaps also phytochemicals such as polyphenols (flavonoids, resveratrol), which are currently the subject of extensive research (3).

Another defense system that has been often neglected also exists (83). It consists of various enzymes involved in the repair of ROS-induced damage to biomolecules. It includes the following: (i) phospholipases (phospholipases A_2, platelet-activating factor-acetyl hydrolase), which remove the oxidized products of fatty acids because generally the latter have to be in a nonesterified form to be detoxified (80, 110, 118, 123); (ii) nucleases, also named redoxyendonucleases (33), which repair lesions occurring in DNA either via endogenously generated oxidant or following exposure to ionizing radiations (83); and (iii) proteases generated from latent proteolytic systems (26, 95) activated when cells have been challenged with ROS for the removal of oxidatively modified proteins (see below).

ROLE OF PROTEINS IN THE ANTIOXIDANT ACTIVITY OF SERUM

Endogenous ROS include those that are produced intracellularly as well as those that are released into the surrounding area. Defenses against ROS are generally located close to the site of production. However, circulating fluids not only bring the nutrients necessary for cellular metabolism, they also help to neutralize cell-borne ROS and to improve cellular defenses. Previous work has shown that blood serum has strong antioxidant properties (124). This has been demonstrated by incubations of tissue homogenates or lipid emulsions in free radical-producing systems. Surprisingly, most of the serum's protecting activity has not been attributed to vitamin E or C, but to the presence of urate and proteins (17, 75, 124). Our laboratory has obtained similar results using 2,2′-azo-*bis*(2-amidinopropane) dihydrochloride (AAPH)-mediated blood hemolysis (8, 35, 51). We found that diluted serum strongly inhibited the free radical-induced hemolysis of washed red blood cells (Fig. 1). The contribution of various components of serum were analyzed using different experimental conditions as shown in Table 1. These data showed that

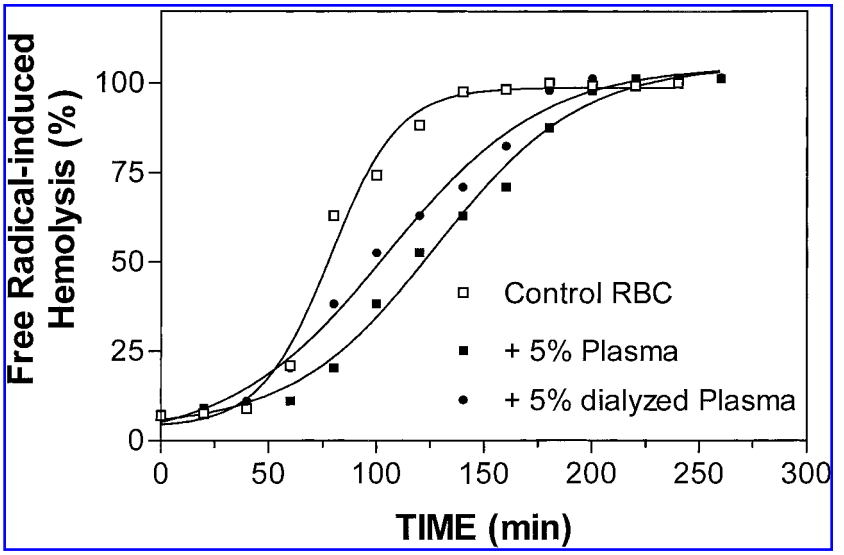


FIG. 1. Antioxidant effect of plasma. Washed red blood cells (RBC) were incubated in the presence of an azo-typed free radical generator as described in the text (34, 51). Plasma or dialyzed plasma was added in a final 5% volume, and the time course of the hemolysis (in %) was plotted and fitted by computer analysis. Curves were characterized by the time corresponding to 50% hemolysis and are from one representative experiment carried out with the same plasma in Table 1. For the purpose of clarity, standard deviations have not been represented. Results ($n = 3$) are 78.3 ± 2.8, 127.7 ± 3.8, and 104.8 ± 4.8 for control RBC, 5% plasma, and 5% dialyzed plasma, respectively.

20–25% of the total antioxidant activity of serum was due to urate, as assessed by either removal of urate (and other small molecules) by dialysis or addition of urate to levels found in lipoprotein-deficient serum. The contribution of vitamins E and C was obtained by the difference in antioxidant activity between total serum and lipoprotein-deficient serum because these were removed during preparation of lipoprotein-deficient serum. Consequently, most of the total antioxidant activity of whole serum can be attributed to proteins, especially albumin. Similar results were obtained with dialyzed lipoprotein-deficient serum and equiv-

TABLE 1. FREE RADICAL-INDUCED HEMOLYSIS AND SERUM ANTIOXIDANTS

	HT 50% (min)	*%*
Red blood cells (control)	74	100
+ 5% Serum	129	174
+ lipoprotein-deficient serum (dialyzed)	109	147
+ lipoprotein-deficient serum + ascorbic acid (2 μM)	112	151
+ lipoprotein-deficient serum + urate (15 μM)	130	175
+ HSA (2.5 g/L)	115	155
+ urate (15 μM)	96	130
+ HSA (2.5 g/L) + urate (15 μM)	126	170

Washed human red blood cells were incubated with an azo derivative as free radical generator, and samples were analyzed for the released hemoglobin as in Fig. 1. Results are expressed as half hemolysis time (HT 50%) expressed in min. Various additions, such as 5% diluted serum, ascorbic acid, uric acid, and serum albumin (HSA), were performed and compared with control conditions (red blood cells alone). Antioxidants were added in concentrations equivalent to those found in 5% diluted serum. Data (average values, with standard deviations $< 7\%$) are representative of several experiments carried out with the same serum having the following characteristics: HSA, 48 g/L; ascorbic acid, 34 μM; uric acid, 300 μM. Dialyzed serum has no detectable ascorbic acid and uric acid.

alent solutions of human serum albumin against free radical-induced hemolysis (Table 1). These data confirm earlier findings on the measurement of individual antioxidant and radical trapping activity of plasma, using oxygen uptake (18, 19, 124). It was also concluded that the thiol content of plasma could account for 52–80% of the total radical trapping activity.

OXIDATIVE MODIFICATION OF AMINO ACIDS AND PROTEINS

A molecule that could intercept the oxidation reaction is regarded as an antioxidant if this interception interrupts the propagation reaction. Proteins may be considered as such because, although the prime targets for ROS are polyunsaturated fatty acids, proteins are also very sensitive to direct peroxidative damage. Proteins may work as sacrificial antioxidants. The consequences are that these free radical-mediated modifications may alter the metabolism and the function of the target proteins.

It has largely been shown that isolated amino acids can be oxidatively modified (Table 2). The quantitative significance of the overall oxidative process, involving either ionizing radiations or metal-catalyzed oxidations, is dependent upon the complexity of the amino acids examined (aliphatic, aromatic), the availability of oxygen, and the strength of the oxidation reaction (109). The resulting products are α-keto acids, hydroxyamino acids accompanied by a mixture of cross-linked derivatives. Formylkynurenine is a major product of tryptophan oxidation, whereas *o*-tyrosine and other hydroxy derivatives are formed from phenylalanine. Tyrosine oxidation may also lead to dimerization and to formation of 3,4-dihydroxyphenylalanine (DOPA). Among the various amino acids, sulfur-containing ones such as methionine and cysteine are considered as preferential targets. Methionine is almost completely recovered as methionine sulfoxide or sulfone (Fig. 2) (122).

The oxidative modifications of amino acid residues may lead to important consequences for the protein concerned. It may vary from slight modification of the protein structure to large denaturation accompanied by fragmentation. The most relevant studies have established that histidine, arginine, lysine, proline, methionine, and cysteine residues are among the most common sites of oxidation by metal-catalyzed processes, whereas all amino acid residues are susceptible to oxidation by ROS generated during radiolysis. Because histidine preferentially works as a ligand for divalent metals, the resulting oxidation product, 2-oxohistidine, may be used as a marker for metal-catalyzed oxidative modifications occurring in the vicinity of the binding site of the protein (71). Figure 3 shows a proposed mechanism for the iron-catalyzed site-specific oxidation of the ϵ-amino group of a lysine residue in a protein (109). The immediate environment of proteins influences the nature and extent of their reactions with ROS. The radical-induced modifications depend on protein concentration and on radical flux and specificity (30). Lipid oxidation products such as aldehydes can react with

Table 2. Some Oxidation Products of Amino Acids

Amino acids	*Major products*
Arginine	Glutamic semialdehyde
Cysteine	-S-S- disulfide cross-links
Histidine	2-Oxohistidine, aspartate, asparagine
Leucine	Hydroxyleucine
Lysine	2-Aminoadipic semialdehyde
Methionine	Methionine sulfoxide
Phenylalanine	*o*-Tyrosine
Proline	Glutamate, pyroglutamate, *cis/trans*-4-hydroxyproline, 2-pyrrolidone, glutamic semialdehyde, γ-aminobutyric acid
Threonine	2-Amino-3-ketobutyric acid
Tryptophane	*N*-Formylkynurenine
Tyrosine	Tyr-Tyr cross-links

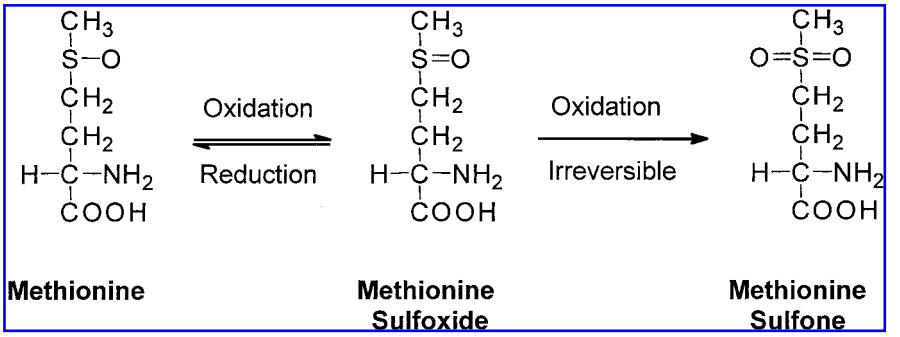


FIG. 2. Structure of methionine and its oxidation products.

sulfhydryl (cysteine) or basic amino acids (histidine, lysine) (42, 116). Increased exposure of proteins and lipoproteins to lipid-derived aldehydes may be representative of oxidant stress in various pathologies. Only a few studies have directly addressed the quantitative damage in complex systems. However, oxidized amino acids in proteins are emerging as attractive candidate markers of oxidant stress (63).

ROS interaction with nitric oxide (NO) results in the generation of a number of radical and nonradical reactive nitrogen species (RNS). These aspects are the subject of important developments in cell signaling and protein modifications (6, 25, 30). NO itself and peroxynitrite ($ONOO^-$) are also able to modify proteins and amino acid residues oxidatively. The interaction of NO with $O_2^{-\bullet}$ seems to attenuate the beneficial actions of NO, such as stimulation of soluble guanylate cyclase and effects on mitochondrial respiration. The resulting oxidation products, such as 3-nitrotyrosine, may have important biological functions (39). The most potent actions of $ONOO^-$ and RNS appear to be

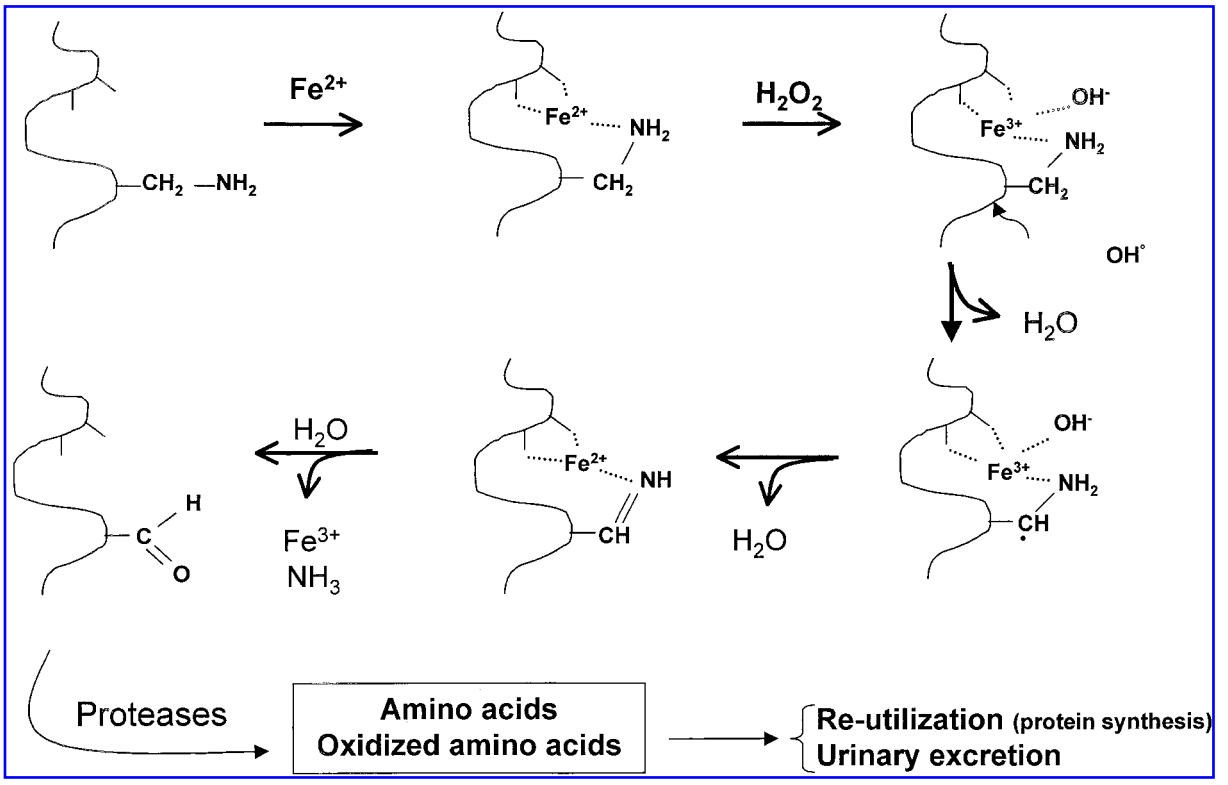


FIG. 3. Proposed mechanism for the metal-catalyzed oxidation of an ϵ-amino group of a lysine residue in a protein and its degradation (redrawn from 109).

thiol modifications. RNS may also participate in cellular signaling processes through interactions with prostaglandins. An example can be illustrated by the inactivation of prostaglandin GI_2 synthase as a result of nitration by $ONOO^-$ of a tyrosine residue (128). HOCl produced by neutrophils to kill foreign organisms can also be regarded as a reactive species. Hypochlorite-oxidized proteins that contain 3-chlorotyrosine probably originating from oxidized apolipoprotein B after interaction with myeloperoxidase have been detected in atherosclerotic plaques (62).

BIOLOGICAL SIGNIFICANCE OF PROTEIN OXIDATION

The biological significance of ROS-induced damage to proteins has important physiological implications. Numerous studies have shown that such modifications are relevant for protein activity, cell functioning, receptor-mediated processes, and degradation (30). These modifications can be related to induced changes in electronegativity, loss of tryptophan, bityrosine formation (Fig. 4), or the appearance of disulfide cross-links. These modifications may occur at the intra- or intermolecular level and may favor protein aggregation. Other modifications may also result from indirect alterations by lipid oxidation products.

Various biological roles have been proposed for ROS-mediated damage to proteins. Some are key elements of physiological processes like protein turnover and regulation, whereas others are implicated in pathological disorders. The precise metabolic fate of oxidized proteins requires specific studies. However, these types of studies are difficult to conduct because oxidative modifications of proteins might be complex. Indeed, experiments carried out in simple oxidative systems, in which only the amino acid residues are oxidized, indicated that oxi-

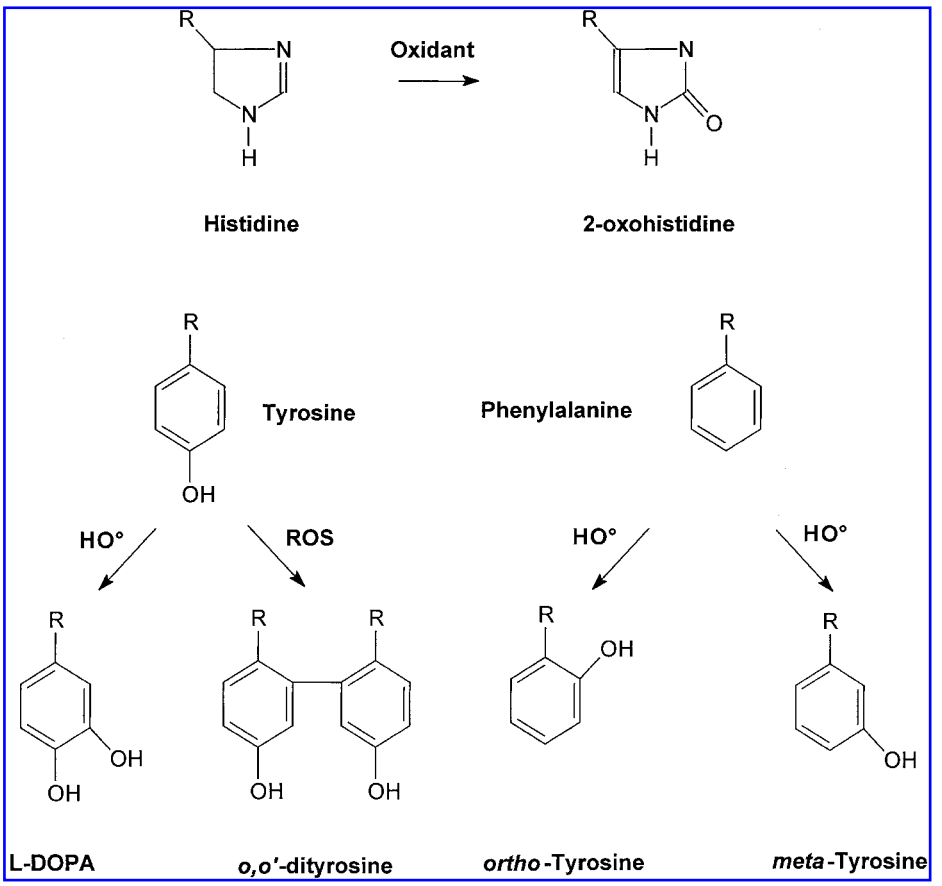


FIG. 4. Some examples of attack by ROS on amino acids in proteins. R represents $C(NH_2)COOH$.

dized proteins are usually sensitive to proteolytic attack by most proteases (27, 30, 83). However, in more complex systems, in which proteins might be modified by products of lipid and carbohydrate oxidation, the range of modifications is wider (42, 116, 120). In these latter situations, which are more analogous to what is encountered in biology, proteins are more heavily modified and show decreased susceptibility to proteases (27). This has been described for aldehyde-modified apolipoprotein B found in oxidized low-density lipoproteins (LDL), which was poorly degraded compared with native LDL. Various aldehydes originate from lipid oxidation products, including reactive aldehydes (malondialdehyde, 4-hydroxynonenal) (42). Other protein-modifying agents are produced by nonenzymatic glycation, defined as the chemical union of glucose to protein. Several not entirely defined products are formed beginning with a Schiff base and undergoing a series of subsequent reactions and/or rearrangements until formation of advanced species corresponding to brown or fluorescent pigments called advanced glycation end products (AGE) (15). These products accumulate in long-life proteins, such as basal membrane proteins (collagen or lens proteins). Some examples include alkyl-formyl-diglycoxylpyrrole (a pentose-derived cross-link between arginine and lysine), pyrroline, carboxymethyllysine, and pentosidine. AGE elicit a wide range of cell-mediated responses that are thought to be largely induced through an AGE-specific cell-surface receptor (121). Although precise information about the rates of catabolism, pool sizes, and half-lives of native and oxidized/glucoxidized proteins is still needed, accumulation might be the fate of highly oxidized proteins (54, 73). It has been suggested that macroxyproteinases or proteasomes are part of the selective degradation of oxidatively modified proteins (83, 95). Actually, the various data available show that mild oxidation of proteins leads to an increased sensitivity to proteases, whereas extensive oxidation is associated with resistance to proteolysis. These differences could be explained by the fact that proteases might have a limited access to strongly oxidized proteins because of marked conformational changes due to denaturation and/or aggregation (30). Elevated levels of protein carbonyls and various compounds resulting from oxidative modification of amino acids have been reported in aging, neurodegenerative diseases, atherosclerosis, and diabetes (7, 83). However, the increased susceptibility of mildly oxidized proteins to proteolytic degradation may explain an increased urinary excretion of oxidized amino acids (69). As a matter of fact, normal amino acids are known to be reutilized but it is still not known whether oxidized amino acids are processed for protein biosynthesis at a normal rate. Alternatively, when oxidatively induced changes are still reversible, damaged proteins may undergo direct repair and thus be spared from proteolytic degradation.

SPECIFIC ROLE OF SULFUR CONTAINING AMINO ACID RESIDUES IN PROTEINS

Thiols

As discussed above, cysteine and methionine belong to the most easily oxidized amino acids. Conversion of SH groups into disulfides is one of the early steps of protein oxidation (81, 109, 115). Within a protein, oxidation of two nearby cysteine residues results in the formation of a disulfide bridge producing a more rigid protein. Disulfide bonds can also form between two proteins, and it can result in a very complex structure at the supramolecular level with several proteins cross-linked together. It is easily understandable that the dynamic properties of cell membranes may strongly be altered when such processes occur. The changes can be monitored using the sulfhydryl-selective spin label MAL-6 (2,2,6,6-tetramethyl-4-maleimidopiperidine-1-oxyl), which covalently binds only to SH groups on the proteins (108). The difference in spin-label motion indicates modifications of protein interactions. Other modifications may also exist when low-molecular-weight thiols, such as reduced free SH-containing amino acids or glutathione, are linked to proteins. This process is called protein thiolation (115) and appears to be favored by oxidant stress (Fig. 5). Not every SH group

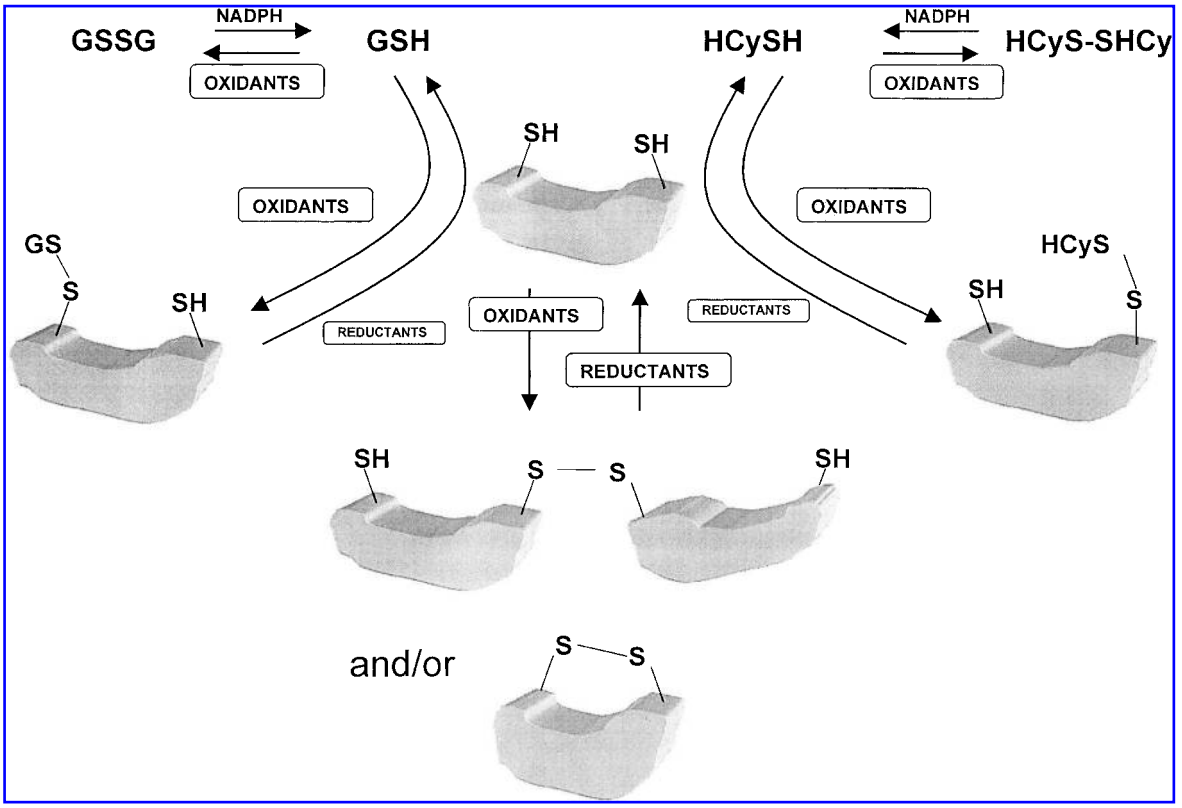


FIG. 5. Various proposed oxidation and reduction processes resulting in protein thiolation/dethiolation. GSH and GSSG: reduced and oxidized glutathione, respectively; HCySH and HCyS-SHCy: homocysteine and homocystine, respectively; Reductants: GSH, glutaredoxin, thioredoxin. Oxidants: $O_2^{-\bullet}$, H_2O_2, $HO^\bullet$, HOCl, $RO^\bullet$, $ROO^\bullet$, $NO^\bullet$, $ONOO^-$.

of a protein may react with the same efficacy and, obviously, the reactivity depends on the local environment and/or whether the cysteine residue is buried or exposed. An important feature of the oxidation of thiol groups is its reversibility in the presence of appropriate reductants. Reduced glutathione, exogenous dithiothreitol (*in vitro*), or enzymes are involved in this dethiolation process. Much of our current understanding of the thiolation/dethiolation process as an early response to oxidative stress arose from experiments carried out with reactive thiol groups (96). Such a limited oxidation could be transient, and it could be involved in the regulation of enzyme activity and in the maintenance of protein folding/conformation. One example can be illustrated by the complete inactivation of creatine kinase by a single *S*-thiolation of each subunit (115). However, there is no evidence that this process could be an enzyme-driven process, suggesting that direct reaction with oxidizing materials prevails. Moreover, oxidation-induced changes of protein conformation may bring a formerly distant thiol group in the proper vicinity to react quickly. For the dethiolation reaction, available data suggest that dithiol redox proteins, such as thioredoxin or glutaredoxin, are involved (55, 93, 115).

Homocysteine (Hcy)

Among the sulfhydryl amino acids, it is of importance to consider specifically the case of Hcy. Hyperhomocysteinemia, caused by the accumulation of this amino acid in plasma, is recognized as a strong and independent risk factor for cardiovascular disease, as well as venous and arterial thrombosis (11, 20, 38, 53, 97). Experimental evidence has established that iatrogenic or nutritional folate deficiency is also related, through hyperhomocysteinemia, to the thrombotic side of vascular disease (35–37). In rats, it has been recently demonstrated that long-term folate deficiency favors the occurrence of a prethrombotic state consequent to an

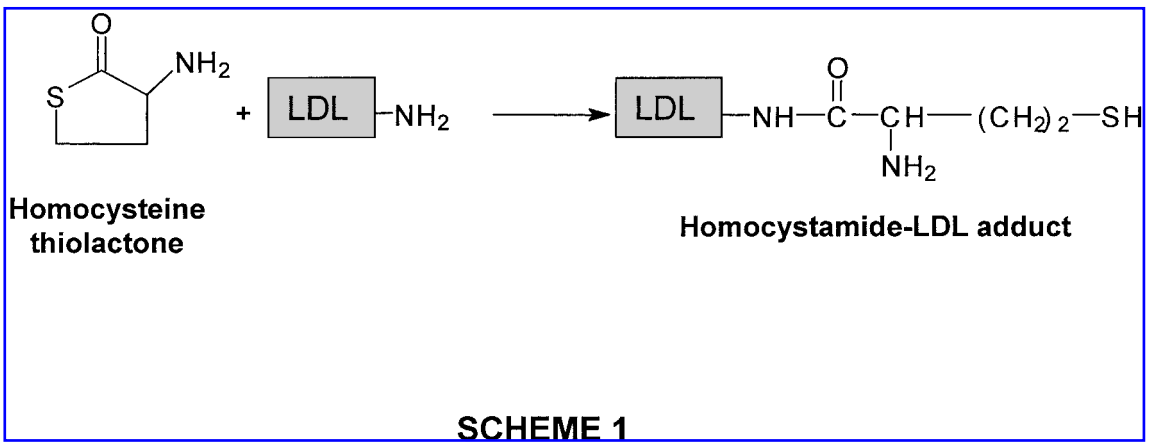
oxidative stress (35). The imbalance of the redox status mediated by folate deficiency has been characterized by various indices of oxidation, such as an increase in end products of lipid oxidation (thiobarbituric acid reactive substances, conjugated dienes, hydroperoxides), changes in fatty acid composition with a decrease in polyunsaturated fatty acids (mostly n-3 fatty acids), and a lower antioxidant defense as shown using our free radical-mediated erythrocyte hemolysis test (8, 34, 87). The prothrombotic and prooxidant effects of folate deficiency have been linked to a moderate increase in circulating Hcy levels. When methionine loading is used as the sole source of Hcy, plasma Hcy was demonstrated to be correlated both to lipoperoxidation markers and to platelet aggregation, as well as to the biosynthesis of the potent proaggregant thromboxane (36).

A high proportion of HcySH (>50%) is found bound to plasma proteins either by disulfide bonds to cysteine residues or by peptide bonds to lysine residues. Experimental evidence is still needed to increase our knowledge of the consequence of thiolation of proteins by Hcy. However, it has been proposed that in aging tissues the oxidative condition of proteins and the loss of catalytic activity of enzymes may be related to changes in Hcy metabolism (74, 82). Hcy, or its derivatives, may combine with lysine groups of elastin, preventing desmosine formation. The thiolation of the apolipoprotein B of LDL by Hcy can promote oxidation of the lipoprotein. It is now well documented that oxidized LDL strongly enhances cholesterol accumulation in macrophages and that this results in atherogenesis (43, 111). These effects may depend on the type of adduct formed. When the reaction involves the thiol group of Hcy and protein, the result is a decrease in the antioxidant properties of the thiolated protein. Conversely, it has been also demonstrated that when Hcy reacted in its thiolactone form with LDL, the resulting homocystamide–LDL adduct was more resistant to oxidation, possibly due to an increase in thiols (47) according to Scheme 1. However, the precise conditions leading to thiolation or homocystamide adduct are unknown.

Methionine oxidation

As mentioned above, methionine can be oxidized by a great variety of oxidants such as H_2O_2, hydroxyl radicals, hypochlorite, chloramine, and $ONOO^-$. The resulting oxidation product obtained in the first step is methionine sulfoxide (12). Oxidation to sulfone, which is the next step, is only obtained under drastic conditions not usually occurring in biological systems. It is noteworthy that methionine sulfoxide is quickly reversed back to methionine with mild reductants, whereas sulfone formation is biologically irreversible.

There are very few studies reporting on the occurrence of methionine sulfoxide in proteins during oxidation-related diseases. This could be due in part to three main factors all related to the difficulty of analyzing this modified amino acid. First, routine procedures, such as amino acid analysis and automated Edman se-



SCHEME 1

quencing, usually do not identify oxidized methionine because of its reduction back to methionine. Second, oxidation of methionine residues does not lead to formation of a carbonyl group that could react with the widely utilized carbonyl assay for oxidatively modified proteins. Finally, the only technique available at present is rather cumbersome. As CNBr cleaves peptide bonds on the carboxyl side of methionine only, yielding homoserine, and does not cleave at methionine sulfoxide, the technique of simultaneous sequencing of the CNBr peptides allows determination of the status of most of the methionine residues (48).

Several examples of oxidation of methionine residues in proteins have been reported. These data confirm the importance of this process in protein function (12, 122). Some studies concern the H_2O_2-mediated oxidation of methionine residues in L12 ribosomal protein resulting in impaired protein synthesis (12). This activity can be reactivated by reduction with mercaptoethanol or by methionine sulfoxide reductase (12, 13, 16). Other examples come from studies on activity of antiproteases. It has been reported that the antiproteolytic activity of human α_2-macroglobulin was inhibited by oxidation and that methionine and tryptophan play critical roles in controlling its structure and function (94). Similar observations were reported for α_1-proteinase inhibitor present in blood plasma and other body fluids, which inhibits a variety of serine proteases. This protein contains eight methionyl residues. Treatment with oxidants leads to oxidation of two surface-exposed methionine to methionine sulfoxide, resulting in a drastic loss of the inhibition of elastase activity (for review, see 44). No cleavage occurs, and no other amino acid residue is affected. The biological significance of the impairment of the activity of α_1-proteinase inhibitor has been related to the frequent occurrence of emphysema in cigarette smokers due to the oxidative stress induced by cigarette smoke and activated leukocytes in the lungs (9). Another example comes from data dealing with activation of the fifth component of human complement (C5), which normally occurs through the action of convertases formed from other complement components (122). It was found that oxidants lead to oxidation of methionine without peptide cleavage. Whereas the physiological activation of C5 is restricted to targets, oxidant-activated C5 bearing oxidized methionine is able to diffuse and might attack cells far from its origin. This results in subsequent release of anaphylatoxin activity.

Others examples of the importance of methionine oxidation may also be found in proteins from various origins. It has been shown that the content of the oxidized residue measured in lens proteins increases with age (64, 122). Apolipoprotein AI, which is a main protein component of high-density lipoproteins, is involved in the clearance of cholesterol back to the liver (reverse cholesterol transport; see 98 for review). Although other components, such as phospholipids and oxysterols, are known to be of importance (50), it has been shown that oxidation of apolipoprotein AI by H_2O_2 leads to reduced binding capacity for lipids. It has been proposed that the introduction of oxidized methionine residues in apolipoprotein AI resulted in change in secondary structure due to modification of its hydrophobicity (1). All these selected examples clearly show that the integrity of methionine has to be maintained for an optimal cell functioning and that oxidation of methionine may be related to the onset of pathological diseases.

Methionine sulfoxide reductase

Until recently, the occurrence of oxidized methionine residues in proteins was merely considered as part of their denaturation process. Oxidation of methionine residues in proteins, which can be reversed enzymatically, may suggest that oxidation of methionine and its reversal may serve to regulate the various functions of proteins (12, 122). Although only found so far at the intracellular level, this widespread enzyme appears capable of reducing either the free or the protein-bound form of methionine sulfoxide. This enzyme has been cloned from bacteria (14, 91), plants (99), and bovine adrenal gland (79). Thus, the hypothesis concerning the role of methionine sulfoxide reductase as part of a repair system resulting in free radical detoxification was recently put

forward in several articles from the groups of Brot and Stadtman (see Scheme 2) (70, 78, 79). This idea has recently been strengthened by the finding that overexpression of methionine sulfoxide reductase in yeast and human T cells provided them with high resistance to H_2O_2-mediated oxidative stress (78). Recent findings also suggest that methionine oxidation might be a regulator of cellular excitability. The functional properties of a transient A-type potassium channel expressed in *Xenopus* oocytes were impaired by oxidizing a critical methionine residue (22). The channel protein was protected from oxidation by antioxidants, but most importantly by coexpression of methionine sulfoxide reductase (23).

MT AS AN INTRACELLULAR ANTIOXIDANT

MTs are ubiquitous, low-molecular weight proteins (6000–7000 Da) with still unclear functions (66, 113, 119). They were characterized by their unusually high content in thiols. All vertebrates contain two or more distinct MT forms designed MT-1 to MT-4. In mammals, MT-1 and MT-2 are found in all organs, whereas MT-3 is most abundant in brain. First identified as cadmium-binding proteins, MTs are known to form high-affinity complexes with other trace metals, including mercury, silver, zinc and copper. As these metals are also able to induce MT expression (mostly MT-1 and MT-2), a critical role has been proposed for MTs in intracellular metal ion homeostasis. The 20 cysteines of a total of 61 amino acids of the MT molecules form two thiolate clusters involved in metal binding. Most of the past studies indicated that extracellular Cd-MT complexes are toxic, especially for the kidney. These effects are in contrast with the induction of MT, which allows cells to sequester intracellular Cd in a nontoxic form (113). Thus intracellular MT appears to be protective, whereas extracellular MT is harmful especially during metal exposure. The large number of free thiols in MT suggests that these proteins might be subjected to sulfhydryl oxidation cycles. It has been demonstrated that oxidation with H_2O_2 and various free radicals induces metal release that might become available for Fenton reactions (45, 89). These findings allowed a role for MT in antioxidant defense to be proposed (102). This idea is in full agreement with previous findings that MT is a redox-sensitive gene whose expression is elevated by oxidative stress. Furthermore, an enhanced sensitivity to oxidant injury has been shown in MT-null cells and mice sensitive to Cd and oxidant exposure (67). Conversely, overexpression of MT decreases sensitivity to oxidant injury (86, 104).

The precise mechanism of the antioxidant activity of MT is still unclear. Data resulting from *in vitro* experiments clearly indicated that MT scavenges $O_2^{-\bullet}$ and various types of radicals

$$Met_{Protein} + H_2O_2 \longrightarrow Met(O)_{Protein} + H_2O$$

$$Met(O)_{Protein} + NAD(P)H + H^+ \xrightarrow{\text{reductase}} Met_{Protein} + NAD(P)^+ + H_2O$$

$$H_2O_2 + NAD(P)H + H^+ \xrightarrow[\text{overall}]{} NAD(P)^+ + 2\,H_2O$$

SCHEME 2

such as hydroxyl, phenoxyl, and NO radicals (102–104). It is likely that this activity could be due to the high content of cysteine residues involved in thiolate clusters. It is considered that MT is indeed an expandable target for free radicals and that it may work in conjunction with an unknown regenerating system. However, the capability of MT to inhibit both metal-dependent and -independent lipid peroxidation would suggest that the antioxidant role of MT does not rely only on its interaction with metals.

ALBUMIN AS A CIRCULATING ANTIOXIDANT

Albumin

With 585 amino acids and a molecular weight of 69 kDa, this highly soluble protein that does not contain carbohydrate is present in human plasma at normal concentrations between 35 and 50 g/L. Albumin has a number of important physiological and pharmacological functions, such as a transporter for metals, fatty acids, cholesterol, NO, bile pigments, and drugs. It is a key element of the regulation of osmotic pressure and fluid distribution between different compartments. In normal conditions, its half-life is ~20 days and its plasma concentration represents an equilibrium not only between its synthesis by the liver and its catabolism, but also its transcapillary escape. As indicated above, albumin may actually represent the major and predominant antioxidant in plasma, a body compartment known to be exposed to continuous oxidative stress.

Epidemiological data

Most of the epidemiological studies have established an inverse relationship between serum albumin levels and mortality risk. In diseased populations, as well as in the general population, it has been estimated that the odds of death increase by ~50% for each 2.5 g/L decrement in the initial albumin level (for review, see 52). This association holds also for cardiovascular disease after adjustment for the usual risk factors (72, 85). Variation in albumin concentration may reflect variation in nutritional state. In fact, only a small number of factors are known to result in variation in serum albumin concentration. Beside analbuminemia, a rare congenital disease (24, 77), the main pathological situation known to lower albumin concentration is the nephrotic syndrome, which is the subject of many studies (61, 125). In addition, it has been reported that serum albumin decreases with age and cigarette smoking (85).

Modifications of albumin structure

Alteration of the structure of albumin may result in modifications of its biological properties. As albumin is capable of reacting with most oxygenated species, this may lead to some oxidative-induced changes, which can explain why albumin exists in an equilibrium between its reduced and oxidized form. Although in normal conditions the reduced form predominates, this equilibrium could shift depending on pathological states and during aging (41). Products of lipid peroxidation, such as reactive aldehydes, may also react with lysine or arginine residues, resulting in albumin alteration. These modifications, together with structural and functional alterations due to increased glycation, could occur in insulin-dependent diabetes mellitus, which is one of the pathological conditions associated with early occurrence of vascular complications (126). The binding of glucose to albumin typically occurs *in vivo* and is known to involve the nonenzymatic covalent attachment of glucose to a lysine side chain. Approximately 6–10% of the albumin in normal human serum is modified by nonenzymatic glycosylation (106). This proportion typically increases between two and three times in hyperglycemia (127). Moreover, diabetic patients exhibit elevated levels of iron and copper ions, which, in the presence of glycated proteins, have been shown *in vitro* to generate free radicals (29). These highly reactive species are able to induce oxidative degradation of protein *in vitro* (83). If the biosynthesis process is fully functional, damaged albumin may be degraded by proteolysis and quickly replaced. It has been calculated that in normal conditions, ~3 g of albumin must be synthesized daily just to keep the basal concentration (56).

In a recent work, the antioxidant activity of

bovine serum albumin (BSA) has been addressed (10). By using different approaches, it has been found that first, albumin markedly delayed the copper-initiated oxidation of LDL as monitored by the appearance of conjugated dienes (Fig. 6), by changes in electrophoretic mobility and by formation of end products of fatty acid oxidation, such as thiobarbituric acid reactive substances. Second, albumin offered a significant protection against free radical-mediated hemolysis. These antioxidant effects were found to be concentration-dependent, which is consistent with the idea of a beneficial effect of high albumin levels in humans. However, besides its concentration, the integrity of the albumin molecule might be a key determinant of its activity. When BSA was preincubated in the presence of various glucose concentrations, it progressively lost its antioxidant properties as regards measured indices of the propensity of LDL to oxidize. Under our conditions, data suggest that only minor modifications of the BSA molecule and no large damage occurred following incubation with glucose and with free radicals. Oxidation of BSA by free radicals decreased its thiol content. Of the 35 cysteines of the BSA molecule, 34 are engaged in 17 S-S-bonded cystines, leaving only Cys^{34} available for reactions (84). As isolated from plasma, about one-third of the albumin molecules carry a mixed disulfide picked up in plasma (cysteine, reduced glutathione, Hcy) on this cysteine. When this cysteine residue has been selectively blocked by *in vitro* treatment with *N*-ethymaleimide, albumin lost most of its thiol groups, and this resulted in a significant reduction in its antioxidant properties as assessed by free radical-mediated hemolysis (Fig. 7). However, the significant antioxidant activity remaining in the thiol-blocked BSA indicated that other amino acid residues (Tyr, Trp, Met) participated in the total activity. As suggested by the group of Davies (28, 65) using electron paramagnetic resonance spin trapping with BSA (and other proteins), thiols may work either as a sink for radicals, thus protecting the protein from entire denaturation, or as agents transferring the damage to other sites, such as the peptide backbone. These data obtained with albumin as an experimental model confirm that protein-to-protein damage transfer and protein-oxidation reactions may readily occur. This may explain why it was found that, in some instances, BSA not only lost its antioxidant effect, but became prooxidant (Fig. 6).

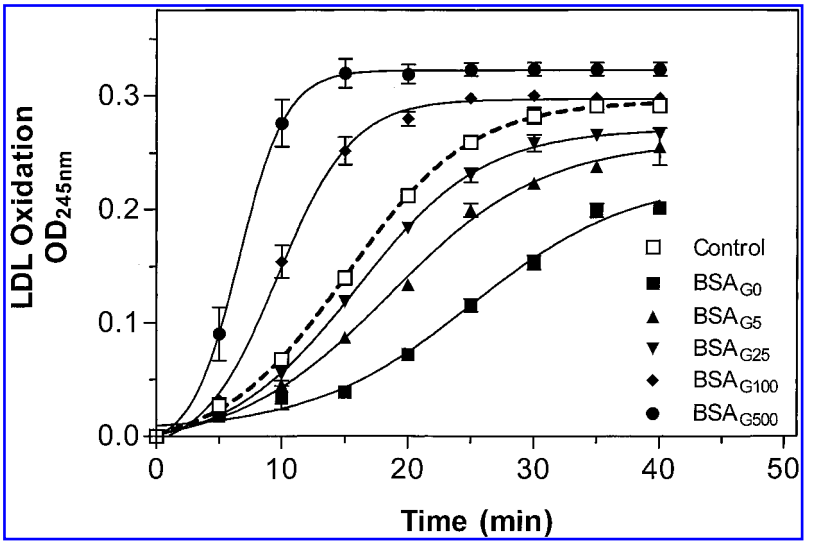


FIG. 6. Effects of glucose- and oxidation-modified BSA on the Cu^{2+}-induced LDL oxidation. Oxidation of LDL was started by adding 40 μM $CuSO_4$ and monitored by the absorbance of the conjugated dienes in the absence (control, dashed line) or presence of various BSA preparations (2 mg/ml). $BSAG_x$ refers to BSA incubated for 60 days with x m*M* glucose as described (10). This figure is reproduced with the permission of *FASEB J.*

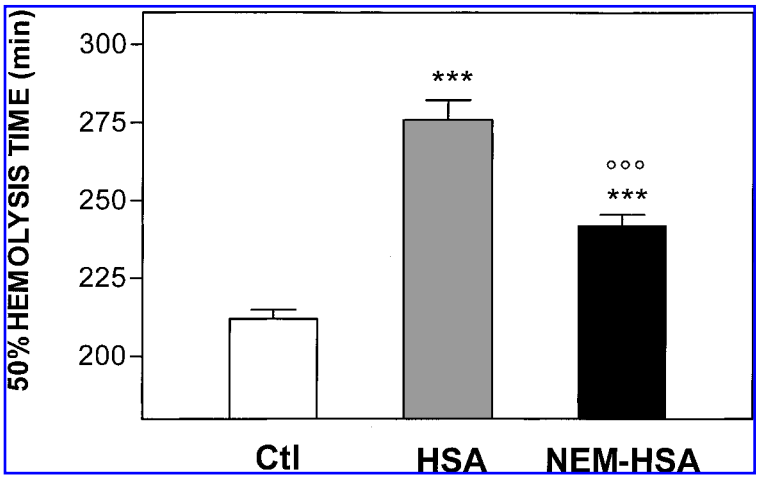


FIG. 7. Effects of modified albumin on free radical-induced hemolysis. Experiments, performed as in Fig. 1, were carried out with native human serum albumin (HSA) and with *N*-ethylmaleimide (NEM)-modified HSA (2 mg/ml), which blocked accessible thiols measured as described (10) using Ellman's assay (40). Corresponding thiol values (n = 3) were 4.3 ± 1.0 and 1.3 ± 0.4 pmol/mg of protein for native HSA and NEM-HSA, respectively. Results are expressed as 50% hemolysis time in minutes.

CONCLUDING REMARKS

Oxidative damage to proteins may be of particular importance for severeal reasons. First, it affects the normal function of proteins, *e.g.*, receptor and enzyme activities, transport proteins, coagulation process, peptide hormones (4, 46, 92, 101, 105, 107, 112, 114, 117). Second, the appearance of ROS-induced epitopes on proteins may contribute to the generation of new antigens that can provoke cell-mediated and immune responses. Finally, the loss of the normal function of proteins may result in secondary damage to other biomolecules, *e.g.*, inactivation of DNA repair enzymes, changes in the rates of biosynthesis of mediators (cyclic nucleotides, prostaglandins), and receptor recycling. The important role of oxidized proteins is supported by the growing number of reports indicating that tissue concentrations of oxidized amino acids are increased in pathological situations and in experimental models (30, 68, 69). The important role of oxidized proteins is further reinforced by the fact that thiol repletion achieved with albumin infusion to patients has been suggested to restore the antioxidant balance (90). However, further work is needed to precisely document *ex vivo* the occurrence of oxidized proteins using standardized techniques and to confirm the validity of specific markers, in particular after action of antioxidants. The specific role of methionine, which could act not only as an endogenous antioxidant, but also as a regulatory molecule, has to be more thoroughly considered due to the reversibility between its reduced and oxidized forms. The functionality of methionine sulfoxide reductase needs to be clearly established in future research. Mild oxidant stress can result in up-regulation of certain antioxidants to restore the balance between free radical attack and defense; however, repeated severe oxidant stress produces major interdependent alterations that can lead to chronic disease.

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ABBREVIATIONS

AGE, advanced glycation end products; BSA, bovine serum albumin; Hcy, homocysteine; H_2O_2, hydrogen peroxide; LDL, low-density lipoproteins; MT, metallothionein; NO, nitric oxide; $O_2^{-\bullet}$, superoxide anion; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase.

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