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Histidine and lysine as targets of oxidative modification

Review Article

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Summary. Histidine and lysine are two representative targets of oxidative modifications. Histidine is extremely sensitive to a metal-catalyzed oxidation, generating 2-oxo-histidine and its ring-ruptured products, whereas the oxidation of lysine generates carbonyl products, such as aminoadipic semialdehyde. On the other hand, both histidine and lysine are nucleophilic amino acids and therefore vulnerable to modification by lipid peroxidation-derived electrophiles, such as 2-alkenals, 4-hydroxy-2-alkenals, and ketoaldehydes, derived from lipid peroxidation. Histidine shows specific reactivity toward 2-alkenals and 4-hydroxy-2-alkenals, whereas lysine is a ubiquitous target of aldehydes, generating various types of adducts. Covalent binding of reactive aldehydes to histidine and lysine is associated with the appearance of carbonyl reactivity and antigenecity of proteins.

Keywords: Oxidative modification of protein – Lipid peroxidation – Reactive aldehydes

Abbreviations: HNE, 4-hydroxy-2-nonenal; HHE, 4-hydroxy-2-hexenal; MDA, malondialdehyde; ONE, 4-oxo-2-nonenal; iso-LGs, isolevuglandins; CML, carboxymethyllysine; GLD, glyoxal-derived lysine-lysine dimer; FDP-lysine, *N*ε-(3-formyl-3,4-dehydropiperidino)lysine; DHP-lysine, 1,4-dihydropyridine-3,5-dicarboxaldehyde-lysine; EMP-lysine, *N*ε-(5-ethyl-2-methylpyridinium)lysine; DNPH, 2,4-dinitrophenylhydrazine

Introduction

Several lines of evidence indicate that oxidative modification of protein and the subsequent accumulation of oxidized proteins, which could be an early indication of oxygen radical-mediated tissue damage, have been found in cells during aging, oxidative stress, and in various pathological states including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis (Stadtman, 1992). There is ample evidence to support the notion that the most important mechanism of oxidative damage to proteins is metal-catalyzed oxidation (Stadtman and Berlett, 1998; Berlett and Stadtman, 1997). Results of the mechanistic studies are consistent with the view that metal ion and hydroperoxides undergo site-specific Fenton reactions at the metal-binding sites on the proteins followed by generation of free radical species. This highly reactive free radical species attacks neighboring amino acid residues, some of which are converted to carbonyl-containing derivatives (Stadtman and Berlett, 1998; Berlett and Stadtman, 1997). Metal-catalyzed oxidation of proteins has been modeled *in vitro* by using a variety of electron donors and often results in loss of enzymatic activity and alteration of protein structure (Rivett and Levine, 1990; Fucci et al., 1983).

On the other hand, the most sensitive cellular target of free radical reactions may represent polyunsaturated fatty acids. The lipid peroxidation mediated by a free radical chain reaction mechanism yields lipid hydroperoxides as primary products, and subsequent decomposition of the lipid hydroperoxides generates a large number of reactive aldehyde species. The lipid peroxidation-derived reactive aldehydes have been implicated as causative agents in cytotoxic processes initiated by the exposure of biological systems to oxidizing agents (Esterbauer et al., 1991). Compared to free radicals, the aldehydes are relatively stable and can diffuse within or even escape from the cell and attack targets far from the site of the original event. Therefore, they are not only end products and remnants of lipid peroxidation processes but also may act as ''second cytotoxic messengers'' for the primary reactions. Some of these aldehydes have been shown to exhibit facile reactivity with various biomolecules, including proteins,

DNA, and phospholipids, generating stable products at the end of a series of reactions that are thought to contribute to the pathogenesis of many diseases.

In the present review, I provide an overview of the oxidative modification of histidine and lysine by metal-catalyzed oxidation and lipid peroxidation. In addition, the involvement of histidine-and lysine-bound reactive aldehydes in the appearance of protein carbonyl and antigenecity is also described.

Histidine and lysine as the targets of metal-catalyzed oxidation

Oxidative modification of proteins involves the conversion of amino acids to their oxidized forms (Stadtman, 1995). Histidine is one of the most vulnerable amino acids to oxidation reactions. In the study of sensitized photooxidation of a histidine derivative, Tomita et al. (1969) isolated a series of oxidation products during the photooxygenation of an imidazole compound by singlet oxygen ($^1O_2$) and proposed a total oxidation mechanism, in which the imidazole group was oxygenated via the 1,4-cycloaddition of $^1O_2$. Formation of asparagine and aspartate has been demonstrated from the degradative reactions of the imidazole side chain with a lipid hydroperoxide (Yong and Karel, 1978). Amici et al. (1989) have also identified aspartate in the acid hydrolysate of poly-L-histidine treated with O_2/Fe^{2+}/ascorbate. On the basis of these findings, oxidative modification of histidine seemed to be a more or less random process. However, 2-oxo-histidine was discovered as the major oxidation product upon incubation with the metal-catalyzed oxidation system (O_2/Cu^{2+}/ascorbate) (Uchida and Kawakishi, 1986). It was also shown that 2-oxo-histidine further underwent oxidation reaction to generate the ring-opened products, such as aspartate, aspartylurea, and formylasparagine (Uchida and Kawakishi, 1989). Moreover, the use of HPLC with electrochemical detection enabled sensitive and specific detection of 2-oxo-histidine in the oxidized proteins *in vitro* (Uchida and Kawakishi, 1993). So far, 2-oxo-histidine has been detected in the *in vitro* oxidation of proteins, such as Cu,Zn-superoxide dismutase (Uchida and Kawakishi, 1994; Kurahashi et al., 2002), human relaxin (Li et al., 1995), vanadium bromoperoxidase (Meister et al., 1996), human growth hormone (Zhao et al., 1997), oxidized low density lipoprotein (Retsky et al., 1999), and prion protein (Requena et al., 2001a). The mechanism of the formation of 2-oxo-histidine remains unclear, while the detection of dioxygenated histidine in the H_2O_2-oxidized Cu,Zn-superoxide dismutase

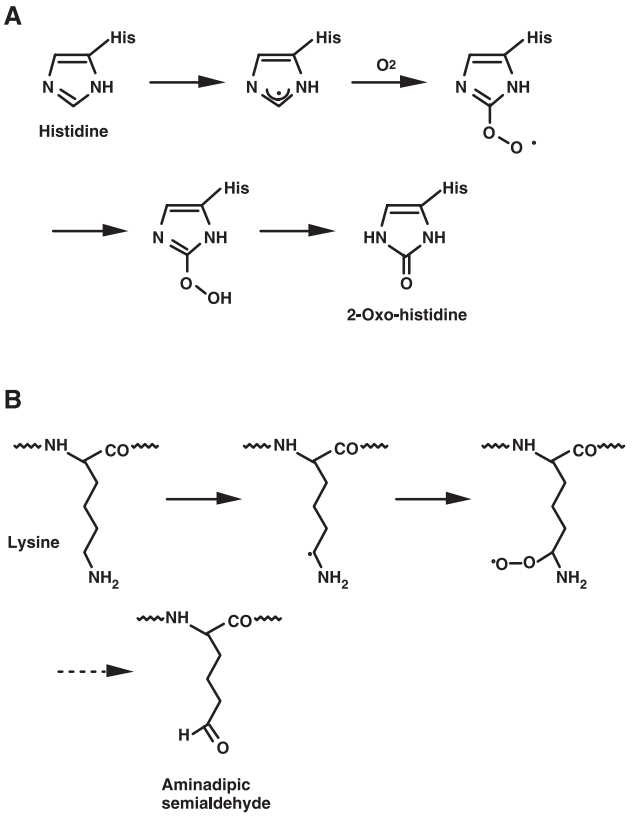

Fig. 1. Metal-catalyzed oxidation of histidine (**A**) and lysine (**B**) residues

suggests an involvement of 2-hydroperoxy-histidine as a possible intermediate (Fig. 1A) (Kurahashi et al., 2002).

Lysine is also susceptible to free radical attack but is much stabler than histidine. Requena et al. (2001b) have shown that lysine is converted to aminoadipic semialdehyde in the metal-catalyzed oxidation system (O_2/Cu^{2+}/ascorbate) (Fig. 1B). In addition, they have also demonstrated evidence for its formation in biological samples (Requena et al., 2001b).

Reactive aldehydes derived from lipid peroxidation

The polyunsaturated fatty acids in cholesterol esters, phospholipids, and triglycerides are subject to free radical-initiated oxidation and can participate in chain reactions that amplify damage to biomolecules. A key feature of lipid peroxidation is the breakdown of these polyunsaturated fatty acids to yield a broad array of smaller fragments, 3–9 carbons in length. The important fragments that give rise to the modification of a protein may be represented by reactive aldehydes, such as 2-alkenals,

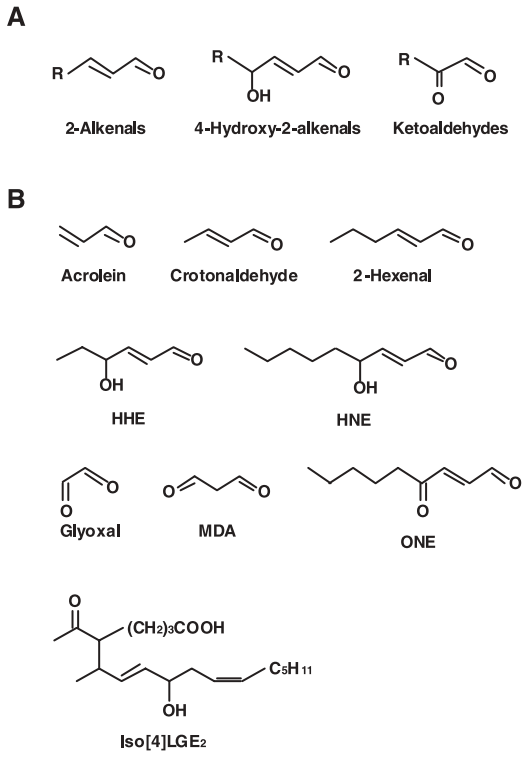

Fig. 2. Structures of reactive aldehydes. **A**, general structures of reactive aldehydes. **B**, structures of reactive aldehydes generated from lipid peroxidation

4-hydroxy-2-alkenals, and ketoaldehydes (Fig. 2A) (Uchida, 2000).

2-Alkenals

2-Alkenals represent a group of highly reactive aldehydes containing two electrophilic reaction centers. A partially positive carbon 1 or 3 in such molecules can attack nucleophiles, such as protein. 2-Hexenal is one of the most well established 2-alkenals generated from lipid peroxidation (Fig. 2B). Acrolein and its methyl derivative, crotonaldehyde, represent the most potent electrophilic 2-alkenals commonly detected in mobile source emissions, cigarette smoke, and other products of thermal degradation (International Agency for Research on Cancer, 1995). Thus, they had been considered as the ''unnatural'' environmental pollutants; however, recent studies revealed that these aldehydes were endogenously produced under oxidative stress (Uchida et al., 1998a, 1999; Kondo et al., 2001; Ichihashi et al., 2001).

4-Hydroxy-2-alkenals

4-Hydroxy-2-alkenals, which contain two electrophilic reaction centers like 2-alkenals, represent the most prominent aldehyde substances generated during peroxidation of ω6 polyunsaturated fatty acids (Esterbauer et al., 1991). Of these, 4-hydroxy-2-nonenal (HNE) has achieved the status of one of the best recognized and most studied of the cytotoxic products of lipid peroxidation (Fig. 2B) (Esterbauer et al., 1991). It has been suggested that HNE can be causally involved in many of the pathophysiological effects associated with oxidative stress in cells and tissues. In addition to studies on its bioactivity, HNE is commonly used as a biomarker for the occurrence and/or the extent of lipid peroxidation. In an elegant study using the 9- and 13-hydroperoxides of linoleic acid as starting material, Brash and his colleagues have recently established two distinct mechanisms, leading to the formation of HNE via 4-hydroperoxy-2-nonenal (Schneider et al., 2001). Peroxidation of ω3 polyunsaturated fatty acids generates a closely related compound, 4-hydroxy-2-hexenal (HHE).

Ketoaldehydes

Other important reactive aldehydes originated from lipid peroxidation include ketoaldehydes, such as malondialdehyde (MDA), glyoxal, 4-oxo-2-nonenal (ONE), and isolevuglandins (iso-LGs) (Fig. 2B). Glyoxal, a well-established α-ketoaldehyde intermediate in the glycation reaction, has been demonstrated to be the product of lipid peroxidation reaction (Fu et al., 1996). The β-ketoaldehyde MDA is the most abundant individual aldehyde resulting from lipid peroxidation, and its determination by 2-thiobarbituric acid is one of the most common assays in lipid peroxidation studies. ONE, representing the γ-ketoaldehydes, has been recently established as a lipid peroxidation product (Lee and Blair, 2000). The iso-LGs, including iso[4]LG_2, also represent the reactive γ-ketoaldehydes, which are formed by the isoprostane pathway (Salomon et al., 1999; Roberts II et al, 1999; Brame et al., 1999).

Modification of histidine by reactive aldehydes

Formation of thiol-derived Michael adducts was initially considered to consititute the main reactivity of aldehydes, such as 2-alkenals and 4-hydroxy-2-alkenals (Esterbauer et al., 1991). However, other studies led to the realization that these aldehydes could also form Michael adducts with the imidazole moiety of histidine residues. The modification of histidine by 2-alkenals and 4-hydroxy-2-alkenals primarily consists of a Michael-type addition of the imidazole nitrogen atom of histidine to the α,β-unsaturated

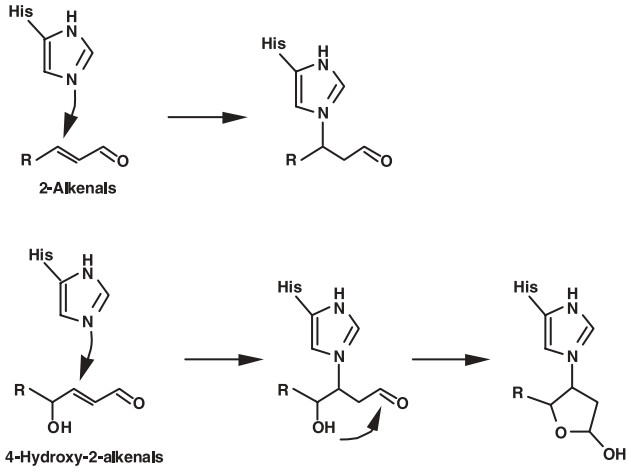

Fig. 3. Reaction of histidine residue with 2-alkenals and 4-hydroxy-2-alkenals

bond, while the primary 4-hydroxy-2-alkenal-histidine Michael adducts, possessing a free aldehyde group, further undergo cyclization to form cyclic hemiacetal derivatives (Uchida and Stadtman, 1992, 1993) (Fig. 3). Because oxo-cyclo equilibrium favors hemiacetal formation, the free aldehyde moiety of the primary product may react with the 4-hydroxyl group to form the hemiacetal derivative (Esterbauer et al., 1991). It has also been suggested that the 4-hydroxy-2-alkenal-histidine Michael adduct may be stabilized toward a retro-Michael reaction, because of the poorer leaving group ability of imidazole under neutral condition. The structures of the histidine adduct with 4-hydroxy-2-alkenals have been well characterized with HNE. It was first speculated that the HNE-histidine adduct was a mixture of the isomeric form of the N^{π}- and N^{τ}-substituted adducts of the imidazole ring; however, on the basis of the NMR spectral analysis of the adducts, it appeared that the reaction exclusively occurs at one position (N^{τ}-alkylation) (Nadkarni and Sayre, 1995). Because HNE generated in lipid peroxidation is a racemic mixture of 4(*R*)- and 4(*S*)-isomers, the HNE cyclic hemiacetal adducts contain chiral centers at C-1, C-3, and C-4 of the HNE moiety. Accordingly, the cyclic hemiacetal adducts are composed of at least eight isomers. However, due to the complicating ^{1}H-NMR diastereoscopic splittings by the three chiral centers, the structural nature of HNE-histidine isomers in solution has not been characterized.

Modification of lysine by reactive aldehydes

Lysine is a ubiquitous target of aldehydes. The reactive aldehydes, 2-alkenals, 4-hydroxy-2-alkenals, and ketoaldehydes, indeed show the highest reactivity with lysine residues of protein. Apart from the histidine modification, the modification of lysine by reactive aldehydes is quite diversified (Fig. 4).

2-Alkenals

The reactions of lysine with 2-alkenals have been mainly studied with acrolein, crotonaldehyde, and 2-hexenal. Like other α,β-unsaturated aldehydes, acrolein selectively reacts with cysteine, histidine, and lysine residues of proteins. Of these, lysine generates the most stable product. The β-substituted propanals and Schiff's base crosslinks had been suggested as the predominant acrolein-lysine adduct; however, the major product formed on reaction of acrolein with protein was identified to be a novel lysine product, *N*ε-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine), which requires attachment of two acrolein molecules to one lysine side chain (Uchida et al., 1998b). This and the fact that crotonaldehyde also forms a similar FDP-type adduct, *N*ε-(2,5-dimethyl-3-formyl-3,4-dehydropiperidino)lysine (dimethyl-FDP-lysine) (Ichihashi et al., 2001), suggest that this type of condensation reaction is characteristic of the reaction of 2-alkenals with primary amines (Fig. 4A). Indeed, upon reaction with a lysine derivative, other 2-alkenals, such as 2-pentenal and 2-hexenal, generate diethyl-FDP-lysine and dipropyl-FDP-lysine, respectively, while the formation of FDP adducts is inversely proportional to the length of the alkyl groups of the 2-alkenals (Ichihashi et al., 2001). A recent study by Furuhata et al. (2002) has shown that the FDP adducts are not the end products but the electrophilic intermediates that potently react with thiol compounds. On the other hand, lysine adduction with 2-alkenals generates another class of condensation adducts possessing a pyridinium ring. The pyridinium adduct, *N*ε-(5-ethyl-2-methylpyridinium)-lysine (EMP-lysine), has been identified as minor products upon reaction of lysine with crotonaldehyde (Fig. 4A) (Ichihashi et al., 2001), while the formation of the pyridinium adducts is a dominant pathway for modification of the primary amine with 2-alkenals, such as 2-hexenal and 2-octenal (Alaiz and Barragán, 1995; Baker et al., 1998; Baker et al., 1999). The formation of the lysine-pyridinium species in proteins results in the placement of a fixed, positive charge on the ε-amino group.

4-Hydroxy-2-alkenals

With regard to the modification of protein-based lysine ε-amino groups by 4-hydroxy-2-alkenals, most of the data

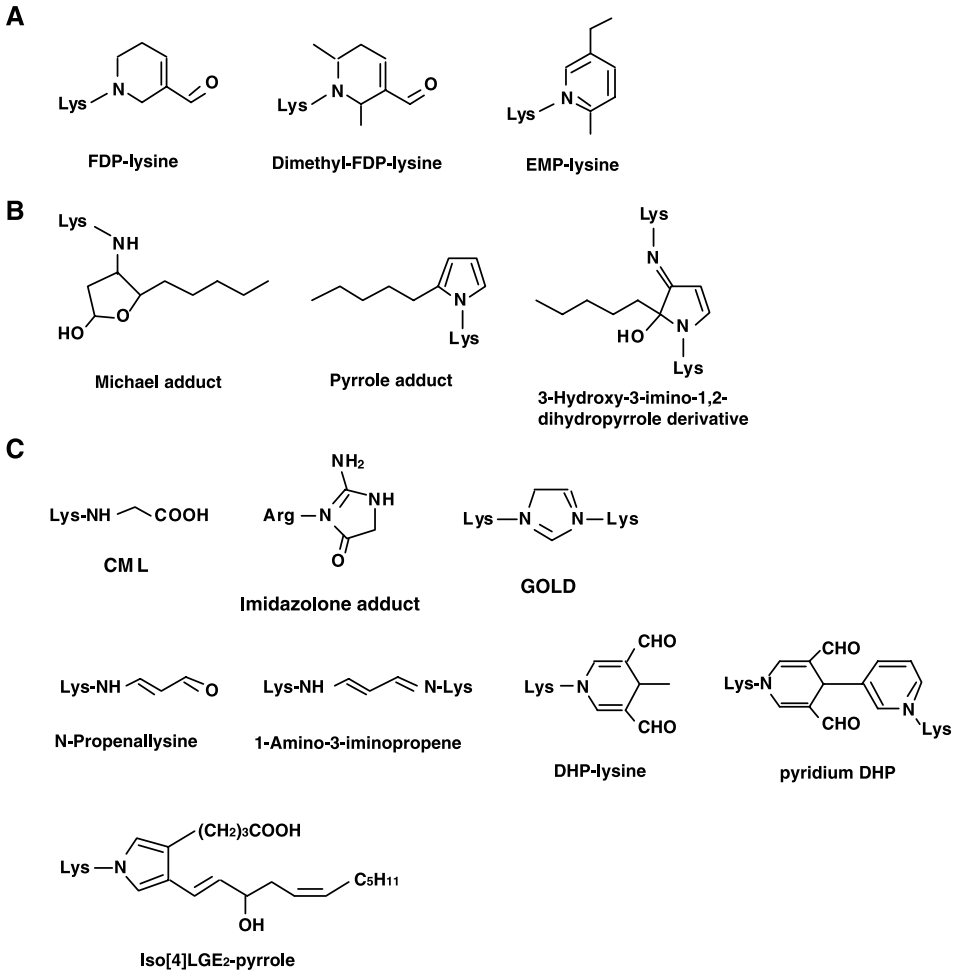

Fig. 4. Reaction products of lysine residue with reactive aldehydes. **A**, 2-alkenals. **B**, 4-hydroxy-2-alkenals. **C**, ketoaldehydes

has been obtained from the reaction with HNE. In a manner similar to the modification of histidine, HNE mainly forms a Michael adduct with lysine (Fig. 4B). Sayre et al. (1993) later reported a novel HNE-lysine adduct possessing a pyrrole structure. Although the yield of the pyrrole from HNE is very low, the pyrrole adduct is apparently very stable and represents the most stable end-product (Fig. 4B). On the other hand, Esterbauer and his colleagues demonstrated that treatment of LDL with HNE generates the same lipofuscin-like fluorescence properties as seen in the Cu^{2+}-oxidized LDL (Esterbauer et al., 1986). This finding suggested that HNE could be the major contributor to the fluorescence generated in the oxidized LDL. The chemical nature of the fluorophore arising from HNE protein modification had remained elusive; however, Itakura et al. (1998) have identified for the first time the major lipofuscin-like fluorophore derived from HNE and lysine to be the 3-hydroxy-3-imino-1,2-dihydropyrrole derivative (Fig. 4B) and found that the fluorescent properties of this pigment are similar to those of the oxidized LDL. The same adduct was later reported by Xu and Sayre (1998) and Tsai et al. (1998). A pathway for the formation of the fluorophore that has been proposed is that the ε-amino group of lysine reacts readily with C-1 and C-3 of HNE via Schiff base formation and Michael addition, respectively, to form the initial 1:2 HNE-amine intermediate, which is subsequently converted into the fluorophore via two oxidation steps and intermolecular cyclization. Mechanistic studies on the HNE-derived fluorophore formation have proposed an alternative mechanism, involving two 2e oxidations following the initial Schiff base formation (Xu et al., 1999).

Ketoaldehydes

Glyoxal is a common intermediate in the formation of carboxymethyllysine (CML) (Fu et al., 1996) and a glyoxal-derived lysine-lysine dimer (GOLD) (Wells-Knecht et al.,

1995) during the oxidation of both carbohydrates and lipids followed by reaction with lysine residues (Fig. 4C). Glyoxal also reacts with arginine residues to form an imidazolone adduct (Schwarzenbolz et al., 1997). MDA specifically modifies lysine residues of proteins. The major reaction of MDA comprises addition to primary amines, generating *N*ε-(2-propenal)lysine (Fig. 4C) (Chio and Tappel, 1969a, 1969b). This adduct has been detected as the major form in which endogenous MDA is excreted in rat and human urine (McGirr et al., 1985; Draper et al., 1988). MDA also forms fluorescent products with primary amino compounds, and it had long been considered that the MDA-derived fluorophores might be responsible for the fluorescence of lipofuscin. In early studies, Chio and Tappel (1969a, 1969b) proposed the aminoenimines (R–NH–CH=CH–CH=N–R) as the MDA-derived fluorophores and suggested that the fluorescence of lipofuscin was due to these cross-links. In addition, the aminoenimine-derived cross-linkings were quantified in native and oxidized low density lipoproteins (Requena et al., 1997). In both cases, however, the structural confirmations were conducted after reduction of the aminoenimines to the non-fluorescent R–NH–CH_2–CH_2–CH_2–NH–R forms with $NaBH_4$. Recently, Itakura et al. (2001) succeeded in isolating the aminoenimine formed from MDA and lysine residues and found that this cross-link did not contribute to the fluorescence formation of lipofuscin. On the other hand, the 1,4-dihydropyridine-3,5-dicarboxaldehydes (DHP adducts) have also been identified as the major fluorophore generated upon reaction of MDA with primary amines (Fig. 4C) (Kikugawa et al., 1981; Kikugawa and Ido, 1984). Itakura et al. (1996) have recently reported another fluorescent MDA-lysine adduct (pyridium DHP), which has dihydropyridine and pyridinium rings (Fig. 4C). ONE, upon reaction with lysine, forms a 3-hydroxy-3-imino-1,2-dihydropyrrole derivative, which was originally identified as the fluorescent HNE-lysine cross-linking adduct (Fig. 4B) (Itakura et al., 1998). Based on the fact that ONE generates this fluorophore much more efficiently than HNE, ONE rather than HNE may represent a major source of this cross-linking adduct. Iso-LGs, which have remarkable reactivity with primary amines, forms oxidized pyrrole adducts (lactams and hydroxylactams) with lysine (Fig. 4C) (Brame et al., 1999).

Oxidatively modified histidine and lysine as the origins of protein carbonyls

Protein carbonyls represent a putative marker of oxidatively modified proteins and can be conveniently measured by sensitive methods, particularly those using 2,4-dinitrophenylhydrazine (DNPH), which reacts with carbonyl groups to generate dinitrophenylhydrazones with characteristic absorbance maxima at 360–390 nm (Levine et al., 1994). It has been established that protein carbonyls accumulate on tissue proteins during aging and disease development (Stadtman, 1992). Increased levels of protein carbonyls are associated with Alzheimer's disease (Smith et al., 1991), progeria and Werner's syndrome (Oliver et al., 1987), amyotrophic lateral sclerosis (Bowling et al., 1993), and respiratory distress syndrome (Gladstone and Levine, 1994), among others. Although the experimental evidence is so far mostly correlative, it lends strong support to the hypothesis that the protein carbonyl content of tissues reflects the fraction of oxidatively damaged protein with impaired function and might therefore be at the root of disease and aging-related functional losses (Berlett and Stadtman, 1997). The origin of protein carbonyl was initially ascribed to the metal-catalyzed oxidation of amino acid residues of proteins, and the oxidized amino acids, such as glutamic and aminoadipic semialdehydes, were indeed identified as the main carbonyl products of the metal-catalyzed oxidation of proteins (Fig. 5A) (Amici et al., 1989; Requena et al., 2001b). However, Yang et al. (1999) have recently shown that oxidation of cysteine residues of LDL apoB by hypochlorous acid also results in the formation of a product, which can be derivatized with DNPH. Upon oxidation of histidine and lysine residues of protein, aminoadipic semialdehyde, which is generated through the oxidation of lysine residue, has been reported to contribute to the generation of protein carbonyls, whereas no DNPH-positive products have been identified from the oxidation of histidine.

On the other hand, a carbonyl-containing amino acid can be generated more widely through the reaction of proteins with reactive aldehydes than through the oxidation of amino acids (Fig. 5B). The incorporation of acrolein into the protein is accompanied by selective loss of amino acid residues and concomitant formation of the protein-linked carbonyl derivative, which can be ascribed to the formation of FDP and N-propanal adducts, possessing a carbonyl function (Uchida et al., 1998a). In a manner similar to acrolein, crotonaldehyde generates the butanal derivatives of histidine and lysine and an FDP-type lysine adduct (dimethyl-FDP-lysine), which react with DNPH to generate their dinitrophenylhydrazone derivatives (Ichihashi et al., 2001). Thus, both 2-alkenal Michael-type and FDP-type adducts represent potential DNPH-reactive carbonyl derivatives. The hemiacetal moiety of 4-hydroxy-2-alkenal-derived Michael adducts also

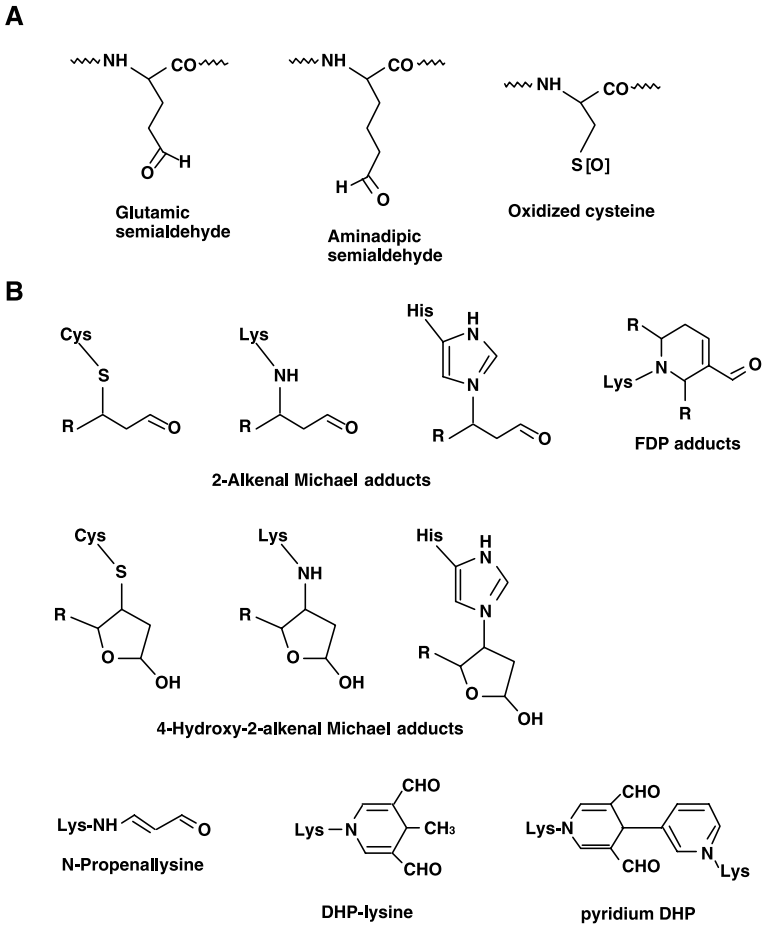

Fig. 5. Origins of protein carbonyls. **A**, oxidation of amino acids. **B**, modification of histidine and lysine residues with lipid peroxidation-specific reactive aldehydes

contain a reactive aldehydic function, and their derivatizations with DNPH or with ^{3}H-labeled sodium borohydride (NaB[^{3}H]H$_4$), which converts the adducts into ^{3}H-labeled dihydroxy derivatives, have been used as a measure of protein-bound 4-hydroxy-2-alkenals (Uchida and Stadtman, 1992a, 1993). MDA also has been reported to form a DNPH-positive protein adduct (Burcham and Kuhan, 1996), which may be ascribed to *N*ε-(2-propenal)-lysine. In addition, due to the presence of free aldehyde groups, both DHP-lysine and pyridium DHP adducts may also react with the carbonyl reagent. Thus, these aldehyde-modified amino acids, rather than oxidized amino acids, may represent the major carbonyl derivatives generated in the oxidatively modified protein *in vivo*.

Histidine- and lysine-bound reactive aldehydes as the antigenic sites

Protein modification by reactive aldehydes enables production of specific antibodies for the detection of reactive aldehydes as the protein-bound forms *in vivo*. The selectivity and sensitivity of this procedure have been established by the results of many immunological studies on aging and disease development. In agreement with the facts that both histidine and lysine represent the targets of reactive aldehydes, the histidine- and lysine-bound reactive aldehydes constitute the major antigenic sites in the modified proteins (Table 1). The monoclonal antibody (mAb5F6) raised against acrolein-modified protein was found to recognize FDP-lysine (Uchida et al., 1998), whereas the monoclonal antibody (mAb82D3) raised against crotonaldehyde-modified protein was specific to the Schiff base-derived crotonaldehyde-lysine adduct, EMP-lysine (Ichihashi et al., 2001). Polyclonal antibodies, raised against HNE-modified proteins, recognize the HNE Michael adducts of histidine, lysine, and cysteine as the epitopes (Uchida et al., 1993), while the monoclonal antibody (mAbHNEJ2) exclusively recognizes the HNE-histidine adduct (Toyokuni et al., 1995). Several antibodies, which recognize the non-Michael addition-type HNE-lysine adducts, have also been raised. Sayre

et al. (1996) have raised and epitopically characterized the specific polyclonal antibodies recognizing the lysine-based 2-pentylpyrrole adducts. The polyclonal and monoclonal antibodies against the HNE-lysine (or ONE-lysine) fluorophore (3-hydroxy-3-imino-1,2-dihydropyrrole) have also been prepared against the fluorophore-conjugated protein (Tsai et al., 1998; Itakura et al., 2000). Yamada et al. (2001) have raised a monoclonal antibody (mAb1F83), which specifically recognizes the fluorescent MDA-lysine adduct, DHP-lysine, as the major epitope.

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