Malondialdehyde, a Lipoperoxidation-Derived Aldehyde, Can Bring About Secondary Oxidative Damage To Proteins

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Lipoperoxidation-derived aldehydes, for example malondialdehyde (MDA), can damage proteins by generating covalent adducts whose accumulation probably participates in tissue damage during aging. However, the mechanisms of adduct formation and their stability are scarcely known. This article investigates whether oxidative steps are involved in the process. As a model of the process, the interaction between MDA and bovine serum albumin (BSA) was analyzed. Incubation of BSA with MDA resulted in rapid quenching of tryptophan fluorescence and appearance of MDA protein adduct fluorescence; transition metal ion traces interfered with the latter process. MDA induced generation of peroxides in BSA, which was preventable with the antioxidant 2,6-di-tert-butyl-4-methylphenol (BHT). MDA-exposed BSA underwent aggregation, degradation, and BHT-sensitive “gel retardation” effects. Phycoerythrin fluorescence disappearance, a marker of damage mediated by reactive oxygen species, indicated synergism between MDA and metal ions. The interaction between reactive aldehydes and proteins is likely to occur in several steps, some of them oxidative in nature, giving rise to advanced lipoperoxidation end-products, which could participate, with advanced glycation end-products, in the generation of tissue damage during aging.

Oxidative damage to proteins can occur through an indirect mechanism involving the production of lipoperoxidation-derived aldehydes such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) (1). These aldehydes are able to bind spontaneously to proteins, modifying them and generating fluorescent adducts (2). The structures of these adducts are not completely known, although several models have been proposed (3–6); moreover, it is not clear whether these structures are stable or can undergo further spontaneous modifications (7).

The onset of glycation is similar. Glycation is another spontaneous protein modification, which starts with the nonenzymatic binding of reducing sugars to free amino groups; but in this case, this reaction is recognized as the initial step in a very complex phenomenon, the Maillard cascade, which gives rise to several final products that are responsible for protein alterations (such as fluorescence development, functional deficit, fragmentation, or aggregation) (8). During protein glycation, reactive oxygen species (ROS) are generated; complex interactions between glycation and oxidation occur and are considered crucial in protein modifications (9–11).

Oxidation, glycation, and their interactions (the “glycoxidation” process) are thought to play an important role in the pathogenesis of aging and aging-associated and aging-accelerating diseases (12–15); in particular, specific roles of aldehydic adducts to proteins have been suggested in the accumulation of fluorescent pigments during aging and in the formation of atherosclerotic plaques (16–18).

While advanced glycation steps have been widely studied (19), less is known about protein modification by aldehydes derived from lipoperoxidation. This article deals with MDA-mediated modification of bovine serum albumin (BSA) as a model of aldehyde-protein interaction; particular attention has been focused on possible oxidative mechanisms involved. Analyses included tryptophan fluorescence, as a sign of tryptophan integrity and an index of protein conformation (20,21); specific MDA-adduct fluorescence, as an index of MDA-protein binding (22,23); protein peroxide generation, which indicates oxidative damage of proteins (24); and SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) separation, which can show structural protein modifications. The possible interference of metal ions and ROS in MDA-mediated protein modification was studied by adding ion chelators, copper ions, or antioxidants in some experiments. Moreover, the potential role of MDA in ROS generation was studied by analyzing the disappearance of phycoerythrin fluorescence, which is considered a marker of ROS-mediated damage (25).

This study suggests that, like glycation, the interaction between MDA and BSA is not a simple binding process but it probably results in further oxidative stress for the protein, and that metal ions can play a relevant role in the process.
is to be underlined as a caution that the MDA concentrations used in this study were not physiological; however, they were in the range used by many other researchers in analogous studies (26–28).

**METHODS**

Bovine serum albumin was used as a model protein. It was incubated (1.5 mg/ml) in PBS (phosphate-buffered saline) (10 mM, pH 7.4) at 37°C with 0–10 mM MDA sodium salt for 0–6 hours. BHT (2,6-di-tert-butyl-4-methylphenol) (up to 500 μM), CuSO₄ (20 μM), or diethylenetriaminepentaacetic acid (DETAPAC, 1 mM) were added to some incubation mixtures in order to investigate the role of ROS and metal ions in the process. MDA sodium salt was prepared according to Nair and colleagues (29); briefly, malondialdehyde bis(dimethylacetel) was hydrolyzed in 1 N HCl for 24 hours, and then treated with 5 N NaOH up to pH = 8; the solvent was removed under reduced pressure. The residue was slurried with methanol and Norit A (Aldrich, Milan, Italy) filtered, and evaporated under reduced pressure. The residue was washed in ethanol/absolute ethanol and finally crystallized twice from ethanol/ether and dried. Quality control was done in the elemental analysis and the ultraviolet spectrum (2%). MDA concentration in solution was checked spectrophotometrically at 245 nm in 1% H₂SO₄ with an extinction coefficient of 13700 M/cm (30).

Protein fluorescence modifications were registered both at the wavelengths typical for tryptophan (280 nm ex/340 nm em) (9,20) and in the spectrum area related to the aldehyde-protein adducts (390 nm ex/460 nm em) (22,23,31). Protein peroxides were evaluated by the ferrous ions oxidation-xylalen 1 method (FOX1), which has high sensitivity enhanced by sorbitol (32); the assay was calibrated with hydrogen peroxide and the results are expressed as H₂O₂-equivalents (33,34). A study on the structural modification of the aldehyde-exposed protein was performed by SDS-PAGE in denaturing conditions (35), with a 3% stacking gel and an 8% resolving gel; 20 μg of protein were applied per lane. Gels were stained with Coomassie brilliant blue R250.

A series of incubations of B-phycoerythrin (1.5 nM in 10 mM PBS, pH 7.4, at 37°C) were carried out with MDA concentration ranging from 0 to 100 mM, in the absence or presence of 100 μM ethylenediaminetetraacetic acid (EDTA) in order to chelate the traces of free metal ions (36). Phycoerythrin fluorescence was evaluated at 541 nm ex/576 nm em (25).

Statistical analyses included calculation of mean, standard deviation, and standard error of the mean (SEM) and evaluation of significance by two-tailed Student’s t-test; as usual, p values < .05 were considered significant. All data are presented as mean ± SEM of three separate experiments, performed in triplicate.

**RESULTS**

**Protein Fluorescence**

MDA produced a dramatic decrease in tryptophan fluorescence during the first hour of incubation. Each MDA treatment reached significance versus control (0 mM MDA) at 1 hour (p < .001); the process was dependent on MDA concentration: both 10 mM MDA and 5 mM MDA treatments reached significance versus 2.5 mM MDA treatment at 1 hour (p < .01) (Figure 1A). As expected, incubation of BSA with MDA gave rise to fluorescence development at 390 nm ex/460 nm em, which are specific wavelengths for MDA-protein adducts (22,23,31). Each MDA treatment reached significance versus control (0 mM MDA) at 3 hours (p < .05); again, the process was dependent on MDA concentration: both 10 mM MDA and 5 mM MDA treatments reached significance versus 2.5 mM MDA treatment at 6 hours (p < .05) (Figure 1B).
Minimal effects on tryptophan fluorescence quenching due to MDA were caused by the addition of either copper ions or DETAPAC; no significant difference resulted among these treatments (MDA $\pm$ Cu$^{2+}$ $\pm$ DETAPAC) (Figure 2A). In contrast, the development of MDA-adduct fluorescence was markedly enhanced by the presence of DETAPAC: the combined treatment with MDA + DETAPAC reached significance versus the simple MDA treatment at 6 hours ($p < .01$); the addition of copper ions caused a statistically insignificant augmentation of this fluorescence (Figure 2B). BHT was not able to interfere with either tryptophan fluorescence quenching or aldehydic adduct fluorescence development (not shown).

**Protein Peroxides**

Protein peroxides appeared in BSA incubated with MDA (Figure 3). Higher MDA concentrations produced higher levels of protein peroxides. Peroxide concentration reached its peak after 1 hour, and then decreased, probably owing to the instability of protein peroxides at 37°C. At each time, each MDA treatment reached significance versus control (0 mM MDA) ($p < .01$ at least); at 1 hour, 10 mM MDA treatment had the highest significance versus the other treatments ($p < .001$). BHT was able to reduce the production of protein peroxides (see insert, Figure 3).

FOX assay failed to give accurate peroxide evaluation in proteins incubated with copper ions or DETAPAC, since these substances interfere with the FOX reagent. Precipitation of the incubated proteins with cold acetone before the FOX assay eliminated the interference but decreased the peroxide content. However, DETAPAC seemed able to protect against the MDA-induced formation of protein peroxides, while copper ions increased their production only slightly (not shown).

**SDS-PAGE Pattern**

SDS-PAGE (Figure 4) revealed that incubation with MDA induced: a) “retardation” of the main band of BSA; b) degradation of the main band; c) appearance of a new band with a higher molecular weight than the main band; d) accumulation of very high molecular weight material unable to enter the gel. Higher MDA concentrations accentuated the gel retardation effect. The antioxidant BHT did not influence the formation of aggregates but attenuated the retardation effect in a dose-dependent way. Copper ions did not alter the pattern of MDA-modified BSA, while DETAPAC provided protection against the gel retardation effects (not shown).

**Phycocerythrin Fluorescence Disappearance**

The oxidative potential of MDA-protein interaction and the possible role of metal ions were also studied by evaluating phycocerythrin fluorescence disappearance, a marker of ROS-mediated damage (25), during incubation of this protein with MDA, in the presence or absence of EDTA (Figure 5). When copper ion traces were chelated by EDTA, low MDA concentration (1–10 mM) induced a mild reduction in phycocerythrin fluorescence; in the presence of copper ion traces (i.e., without EDTA), the phenomenon was significantly accentuated. Higher MDA concentrations (50–100 mM) induced a more marked reduction in phycocerythrin fluorescence, which became virtually insensitive to EDTA. When copper ions were added in excess in the MDA-phycocerythrin mixture, fluorescence was almost completely suppressed and no further MDA-mediated damage was assessable (not shown).

**DISCUSSION**

The disappearance of tryptophan fluorescence in the BSA incubated with MDA could be attributed either to the destruction of tryptophan residues (37) or to a change in the microenvironment in which tryptophan residues are located (21,38), due to the binding of the aldehyde to other sites of the protein. The expected development of the fluorescence related to the MDA adduct did not parallel the decrease in...
tryptophan fluorescence, being slow during the first hour of incubation and more rapid thereafter; this indicates that the two phenomena are not directly correlated (Figure 1).

Metal ions seem to influence tryptophan alteration only mildly, if at all, while the generation of the aldehydic adducts is markedly affected by metal ion chelation by DETAPAC (Figure 2); we suggest that DETAPAC may enhance accumulation of "early" adducts by inhibiting their possible further modification by metal ion-dependent oxidative mechanisms. Indeed, we observed that tryptophan incubation with aldehydes caused the generation of adduct-specific fluorescence. After a few hours of incubation, a shift of the fluorescence peak occurred (not shown); DETAPAC prevented this shift, while copper ions caused it to occur earlier. These data seem to indicate that the initial adduct is prone to further modifications, leading to other molecular structures, and that metal ions play an important role in this process.

The inability of BHT to interfere with either tryptophan fluorescence quenching or aldehydic adduct fluorescence development during incubation of BSA with MDA seems to indicate that ROS are not involved in these processes; however, keeping in mind that DETAPAC enhanced the generation of the adduct-specific fluorescence, we believe that oxidative phenomena can be involved but only at metal-binding sites of proteins. Soluble antioxidants such as BHT are unlikely to be able to interfere with this damage (39,40).

The formation of protein peroxides indicates oxidative damage to BSA during the interaction with MDA (Figure 3). The ability of the antioxidant BHT to limit the formation of protein peroxides suggests that they could be generated through ROS-mediated mechanisms. Moreover, in preliminary experiments, catalase was able to prevent the formation of protein peroxides (not shown); therefore, we think that, like "autoxidative glycosylation" of proteins (9), the interaction of MDA with BSA also could produce ROS, which, in turn, would generate protein peroxides (41). Considering the effects of DETAPAC and copper ions on the development of protein peroxides, it is possible to suggest that traces of metal ions are sufficient to enhance MDA-mediated peroxide generation in proteins, while an excess of such ions causes little additional effect. It is to be kept in mind that protein peroxides can give rise to further oxidative stress since they are not stable and, in turn, can be a source of secondary free radicals (42).

As far as the SDS-PAGE pattern is concerned (Figure 4), the "retardation" effect of the main band of BSA could...
phenomena (9). Therefore, the interaction of aldehydes with metal ions in an autoxidative way, inducing the generation of oxidative species and triggering oxidative damage to other proteins induced by MDA or other reactive aldehydic adducts; it includes different phenomena and, in part, dependent on oxidative mechanisms.

MDA is able to induce oxidative damage to phycocerythrin in a dose-dependent way, and traces of metal ions seem able to enhance the phenomenon in the low range of MDA concentrations used (1–10 mM) (Figure 5). We hypothesize that, at physiological MDA concentrations, which are much lower than those used in this study, this phenomenon could still occur, and therefore might be relevant in vivo.

It is to be kept in mind as a precaution that the MDA concentrations used in this study were not in the physiological range; however, this procedure is common in the scientific literature when an in vitro protein modification is to be analyzed. The concentrations used here were in the range used by several other researchers in analogous studies (26–28). Furthermore, rather high MDA concentrations can be present in cooked food (45), which might represent an exogenous source of MDA adducts as it has been shown for advanced glycation end-products (46).

Conclusion
The interaction between MDA and BSA seems to be far more complex than the simple formation of covalent aldehydic adducts; it includes different phenomena and probably evolves through various steps, some of which are influenced by the presence of metal ions, while others, parallel or subsequent, are oxidative in nature. This suggests similarities and possible interactions between protein glycation and MDA-mediated protein modification. These interactions may be considered part of the complex phenomenon of glycoxidation, which is recognized as one of the main causes of molecular aging. The mechanisms of damage to other proteins induced by MDA or other reactive aldehydes may or may not be similar to those evidenced in this article; however, preliminary studies showed similar results as far as the interactions MDA–ovalbumin and fumaraldehyde–BSA are concerned.

An interesting question is how the oxidative potential of MDA (and maybe of other aldehydes) is generated. Comparison with glycation (47) suggests that the binding of aldehydes with proteins may give rise to the production of “early” and relatively unstable products that are able to interact with metal ions in an autoxidative way, inducing the generation of oxidative species and triggering oxidative phenomena (9). Therefore, the interaction of aldehydes with proteins should give rise to a series of different products, some early and unstable, some late and irreversible. During these transformations, various different reactive species may be generated. Again, comparison with glycation suggests that especially the end-stage products should be responsible for molecular functional damage, and so should be considered involved in the molecular basis of aging.

These observations should be borne in mind when analyzing biological samples for the presence of aldehydic adducts as markers of oxidative damage (7,48). Like Amadori compounds in the glycation process, the initial MDA-protein adducts (or other aldehydic adducts) might only constitute early products prone to further spontaneous modifications leading to still unknown molecular structures. Researching the presence of the early adducts gives only limited information. The knowledge of the structure of end-stage products will allow more accurate evaluation of the role of aldehydic modifications of proteins during aging and in aging-associated diseases.


47. MDA ACTS AS A SOURCE OF OXIDATIVE DAMAGE

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