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Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems

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ABSTRACT **Aging and some pathological conditions are associated with the accumulation of altered (inactive or less active) forms of enzymes. It was suggested that these age-related alterations reflect spontaneous changes in protein conformation and/or posttranslational modifications (e.g., oxidation). Because changes in protein conformations are often associated with changes in surface hydrophobicity, we have examined the effects of aging and oxygen radical-dependent oxidation on the hydrophobicity of rat liver proteins. As a measure of hydrophobicity, the increase in fluorescence associated with the binding of 8-anilino-1-naphthalene-sulfonic acid to hydrophobic regions on the proteins was used. By this criterion, the hydrophobicity of liver proteins of 24-month-old rats was 15% greater than that of 2-month-old animals. Exposure of liver proteins to a metal-catalyzed oxidation system (ascorbate/Fe(II)/H_2O_2) or a peroxyl radical generating system, 2,2′-azobis(2-amidino-propane) dihydrochloride (AAPH) led to increases of 2% or 30% in surface hydrophobicity, respectively. Treatment of liver proteins with the metal-catalyzed oxidation system led to a significant increase in reactive carbonyl content and to conversion of methionine residues to methionine sulfoxide residues. Treatment with AAPH led also to oxidation of methionine, tyrosine, and tryptophan residues and to the precipitation of some proteins. Dityrosine was detected in AAPH-treated protein, both the precipitate and supernatant fraction. The oxidation-dependent increase of hydrophobicity was correlated with an increase in the levels of methionine sulfoxide and dityrosine. These results suggest that oxidative modification of proteins may be responsible for the age-related increase of protein surface hydrophobicity *in vivo*, and that the oxidation of methionine by an oxidative system may be an important event for the change of protein conformation.**

Covalent modification of proteins by oxidative systems have been implicated in various physiological and pathological conditions, such as aging, ischemia repurfusion and inflammatory disorders [for reviews, see Oliver *et al.* (1, 2), and Stadtman (3, 4)]. These disorders are often associated with the accumulation of altered (inactive or less active) forms of various enzymes [for reviews, see Rothstein (5) and Oliver *et al.* (6)]. The age-related alterations may reflect spontaneous changes in protein conformations (7, 8) or in protein oxidative modifications by reactive oxygen species (ROS) [for reviews, see Stadtman (9)]. The latter possibility is consistent with the facts that (*i*) many enzymes that accumulate as altered forms during aging are highly susceptible to modification by ROS; (*ii*) the ROS-mediated modifications of an enzyme can provoke changes in thermostability (10) and susceptibility to proteolytic degradation (10) similar to those that occur during aging (11); (*iii*) the level of oxidatively damaged protein, as measured by the presence of reactive carbonyl groups, increases with age (2). By whatever mechanism, the age-related accumulation of altered enzymes is thought to involve either an age-related increase in the rate of change in protein conformation or to a decrease in the activities of proteases that preferentially degrade the altered enzymes. These considerations and the further observations that the oxidation of glutamine synthetase by a metal-catalyzed oxidation (MCO) system (12) lead to an increase of surface hydrophobicity and susceptibility to proteolytic degradation prompted the present study. This study was designed to determine if there is an age-dependent change in the hydrophobicity of rat liver proteins and if so whether comparable changes can be enhanced by exposure of rat liver proteins to MCO system or to oxidation by a metal ion independent, ROS-generating system (2,2′-azobis(2-amidino-propane) dihydrochloride; AAPH) (13, 14)

We report here that the surface hydrophobicity of rat liver proteins increases with animal age, and that *in vitro* exposure of rat liver proteins to a MCO system or to AAPH leads to an increase in surface hydrophobicity, protein carbonyl content, and conversion of methionine residues to methionine sulfoxide (MeSOX) residues. In addition, treatment with AAPH, but not the MCO system, leads to oxidation of tryptophan residues, precipitation of some proteins, and formation of dityrosine derivatives. The increase in surface hydrophobicity correlated with an increase in dityrosine and MeSOX formation.

MATERIALS AND METHODS

Materials. Salts (KCl, $MgCl_2$, and NaCl) were from Mallinckrodt. Constant boiling HCl, PeroXOquant Quantative Peroxide Kit, *o*-phaldialdehyde were obtained from Pierce. Cyanogen bromide and 8-anilino-1-naphthalene-sulfonic acid (ANSA) were purchased from Sigma. Azo compound, AAPH, was purchased from Waco Pure Chemical (Osaka). DTT and Hepes were obtained from ICN. Carbonyl derivatization reagent, 2,4-dinitro-phenylhydrazine, was from Fluka. Bradford protein assay reagents and Chelex-100 resins were purchased from Bio-Rad.

Protein Extraction. Fisher 344 rats were killed and the livers were excised as described (15). Liver from an individual rat was homogenized in 10 mM Mops and 150 mM NaCl (pH 7.4) with a Potter–Elvehjem glass homogenizer fitted with a Teflon pestle (15) and was immediately frozen by dropping into liquid nitrogen. The liver homogenates were stored at −80°C until use. Liver proteins were extracted according to Oliver *et al.* (11). Protein supernatant from centrifugation (100,000 × *g* for 3 min) was incubated with 1% streptomycin sulfate for 20 min

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Abbreviations: AAPH, 2,2′-azobis(2-amidino-propane) dihydrochloride; ANSA, 8-anilino-1-naphthalene-sulfonic acid; MeSOX, methionine sulfoxide; MCO, metal-catalyzed oxidation; ROS, reactive oxygen species.

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to remove nucleic acid contamination as described (16). The protein supernatant was applied to a PD-10 column (Pharmacia Biotech) to remove low-molecular-weight compounds. Protein concentration was determined using Bradford reagents.

Determination of Protein Surface Hydrophobicity. Protein surface hydrophobicity was determined as described (10). Briefly, 100 μg of protein was incubated with ANSA at 37°C in 50 mM Hepes/100 mM KCl (pH 7.8) for 30 min. The change in fluorescence associated with the binding of ANSA to protein surface hydrophobic regions was monitored at 490 nm (excitation, 370 nm) using a LS 100 device from Photon Technology International (Princeton). Under our experimental conditions, the fluorescence intensity at 490 nm obtained by addition of ANSA to the untreated liver protein fraction (1.0 mg/ml) from 2-month-old rat was taken as a point of reference (we have arbitrarily set this value as 1.0). For purposes of comparison, surface hydrophobicity of all other samples is expressed as increase or decrease in ANSA-dependent fluorescence intensity with respect to the 2-month-old reference value unless otherwise indicated.

Treatment of Proteins with MCO or AAPH. Proteins (1 mg/ml) were incubated with 100 μM $FeSO_4$/25 mM ascorbate and indicated concentrations of H_2O_2 in 100 mM KCl, 100 mM $MgCl_2$, and 50 mM Hepes (pH 7.2) or incubated with indicated concentrations of AAPH in Chelex-100-treated PBS at 37°C for 5 hr. After 5 hr of reaction, proteins were concentrated with Centricon 10 (Amicon) microconcentrator to remove excess low-molecular-weight compounds (e.g., reagents). For AAPH treatment, samples were either centrifuged in a bench-top centrifuge (Eppendorf 5415C) at 14,000 rpm for 10 min before Centricon 10 treatment to sediment protein precipitates, or directly applied to Centricon 10 microconcentrator for concentrating. Protein concentration after treatment was determined as described previously. The protein supernatant or precipitate was used for further characterization. The concentration of peroxide generated during the decomposition of AAPH was determined using a PeroXOquant Quantitative Peroxide Assay from Pierce.

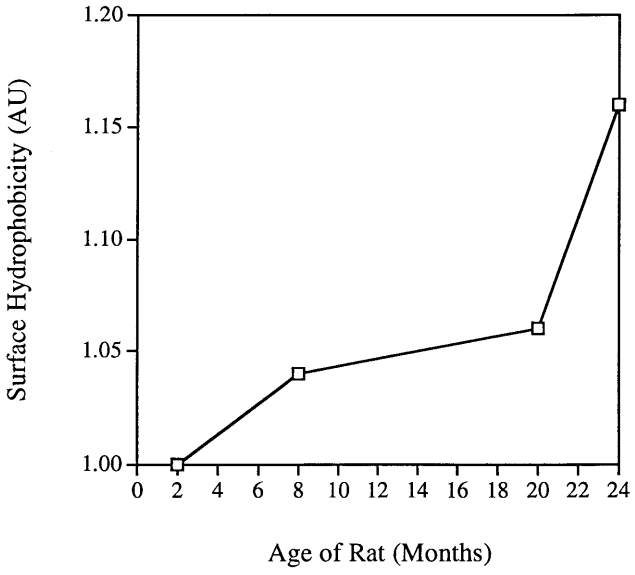


FIG. 1. Age-related increase of protein surface hydrophobicity. Liver proteins of different rats were extracted and incubated as described. The ANSA-dependent fluorescence intensity of 2-month-old rat liver proteins is used as a point of reference [arbitrary unit (AU) is set as 1.0]. The surface hydrophobicity (arbitrary value) of all other samples is expressed as values with respect to the 2-month-old reference value.

Amino Acid Analysis and Carbonyl Determination. For amino acid analysis the concentrated protein samples (10 μg) were hydrolyzed at 155°C for 45 min after addition of 200 μl of 6 M constant boiling HCl containing 1 mM DTT as described (17). Amino acid composition of the acid hydrolysate was determined by precolumn *o*-phaldialdehyde derivatization and HPLC, and analyzed with fluorescence detection, as described (18). The MeSOX content of the protein was determined by measuring the level of methionine (Met) both before and after treatment with 5 mM cyanogen bromide (BrCN) for 12 hr prior to the acid hydrolysis step (19). Under these conditions BrCN leads to conversion of Met to homoserine or homoserine lactone. The yield of Met obtained in the

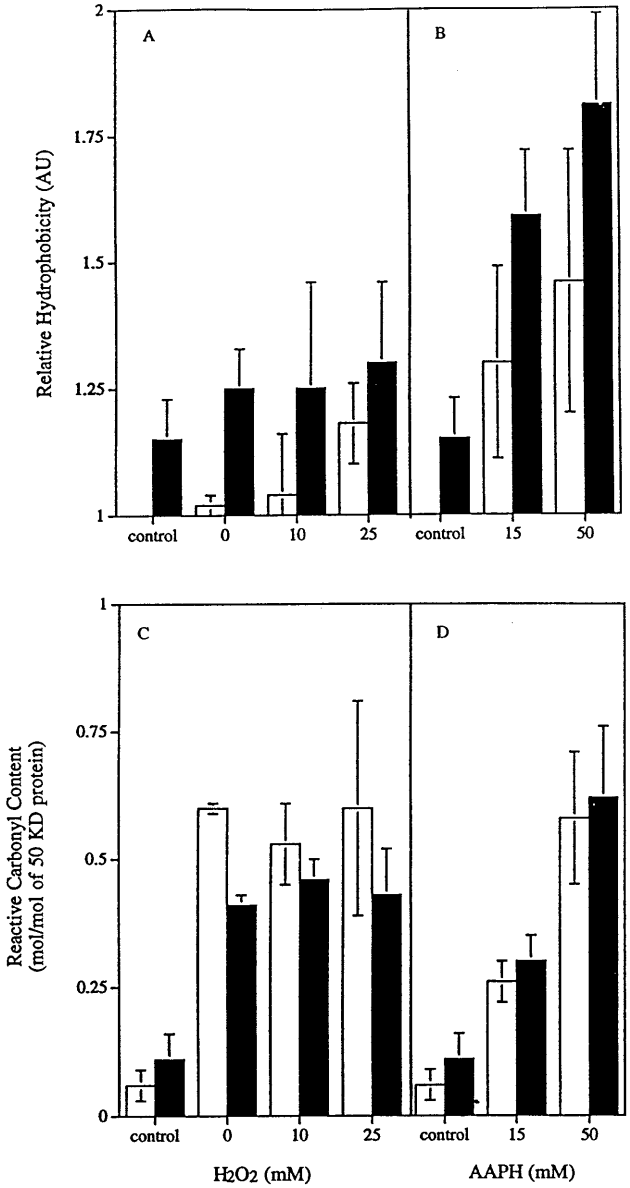


FIG. 2. Effect of MCO or AAPH treatment on protein surface hydrophobicity (*A* and *B*) or reactive carbonyl content (*C* and *D*). Proteins were treated with MCO ($FeSO_4$/ascorbate with the addition of indicated concentrations (10 and 25 mM) of H_2O_2 or AAPH (15 and 50 mM) at 37°C for 5 hr, protein surface hydrophobicity and the formation of reactive carbonyl content were determined as described. Protein surface hydrophobicity was normalized as described in legend to Fig. 1. Open bars represent the results of 2-month-old rat liver proteins, and solid bars represent those of 24-month-old rat liver proteins. Results are presented as the average of at least three independent experiments. AU, arbitrary unit.

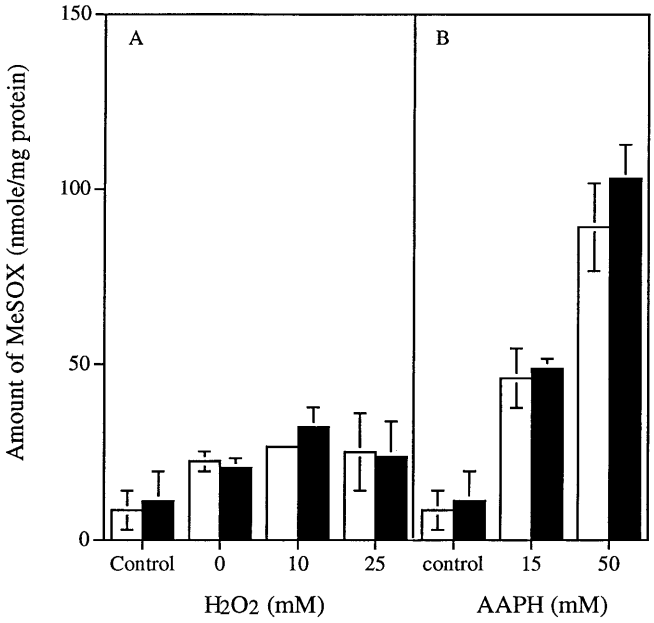


FIG. 3. Effect of MCO (*A*) or AAPH (*B*) treatment on methionine oxidation. Proteins were treated with MCO or AAPH at 37°C for 5 hr, and the amount of MeSOX (nmol/mg protein) in each samples was determined and calculated as described. Open bars represent the results of 2-month-old rat liver proteins and solid bars represent those of 24-month-old rat liver proteins. Results are presented as the average of at least three independent experiments.

absence of BrCN treatment is a measure of both Met and MeSOX, whereas the Met measured after BrCN treatment is a measure of MeSOX only.

Dityrosine Determination. The amount of dityrosine and/or tyrosine in protein was determined as described by Giulivi and Davies (20). We are grateful to Lin Tsai (Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health) for making available an authentic sample of dityrosine, which he synthesized with the procedure of Tew and Ortiz de Montellano (21). The identity of dityrosine was confirmed by Mass Spectroscopy (Joel SX-102 Mass Spectrometer) and its characteristic second-derivative UV spectrum. The concentration of purified dityrosine was determined spectrophotometrically (Hewlett–Packard HP 8452A diode array spectrophotometer).

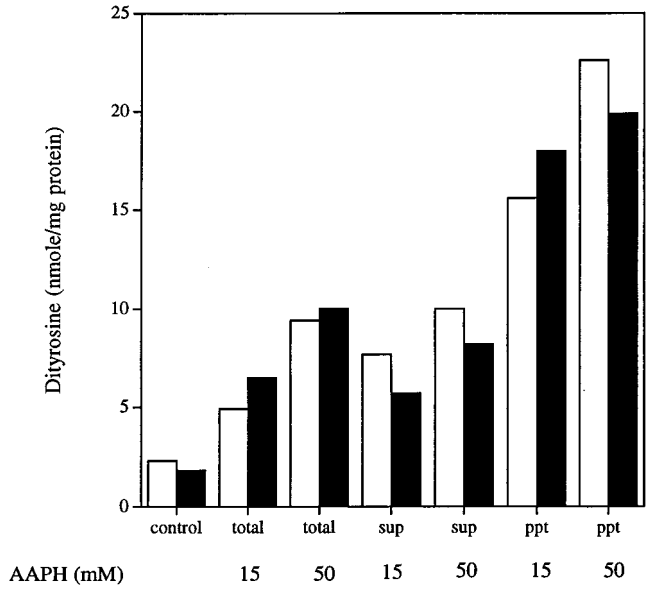


FIG. 4. Effect of AAPH treatment on the formation of dityrosine in different protein fractions. Proteins were treated with indicated concentrations of AAPH at 37°C for 5 hr. Dityrosine (nmol/mg protein) in three different fractions of AAPH-treated proteins were determined and quantitated as described. Total represents total protein fraction after AAPH treatment, sup represents supernatant fraction of AAPH-treated proteins after separation of precipitate, and ppt represents precipitate fraction of AAPH-treated proteins. Open bars represent the results of 2-month-old rat liver proteins, and solid bars represent those of 24-month-old rat liver proteins. Results are presented as the average of two independent experiments.

RESULTS

Age-Related Increase of Protein Hydrophobicity. Changes in protein conformation are often associated with changes in surface hydrophobicity. Therefore, to test the hypothesis that accumulation of inactive or less active forms of enzymes during aging is due to conformational changes, we measured the hydrophobicity of liver proteins in rats of different ages. As shown in Fig. 1, there was a modest increase in hydrophobicity over the range of 2–20 months of age, followed by a dramatic increase between 20 and 24 months. A 15% increase of surface hydrophobicity from 24-month-old rat liver proteins was observed in comparison with that of 2-month-old rat liver proteins.

Effect of Oxidative Modification on Protein Hydrophobicity and Carbonyl Formation. To determine if the age-related increase in hydrophobicity could have been caused by oxidative modifications, rat liver proteins were exposed *in vitro* to (*i*) a MCO system consisting of $FeSO_4$/ascorbate/O_2 in the presence and absence of 10 or 25 mM H_2O_2, or (*ii*) to AAPH (15 or 50 mM), which in aerated solution decomposes slowly to form carbon-centered radicals, peroxyl radicals, and alkylperoxides (13). The results, summarized in Fig. 2*A*, show that treatment with the MCO system led to a significant increase in hydrophobicity of proteins from both 2- and 24-month-old animals. Curiously, the further addition of H_2O_2 to the MCO reaction mixture had only a slight effect (4% increase) in the

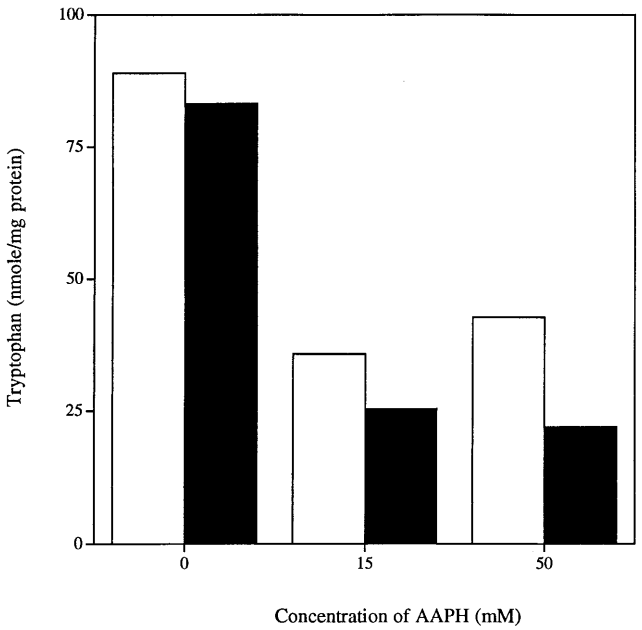
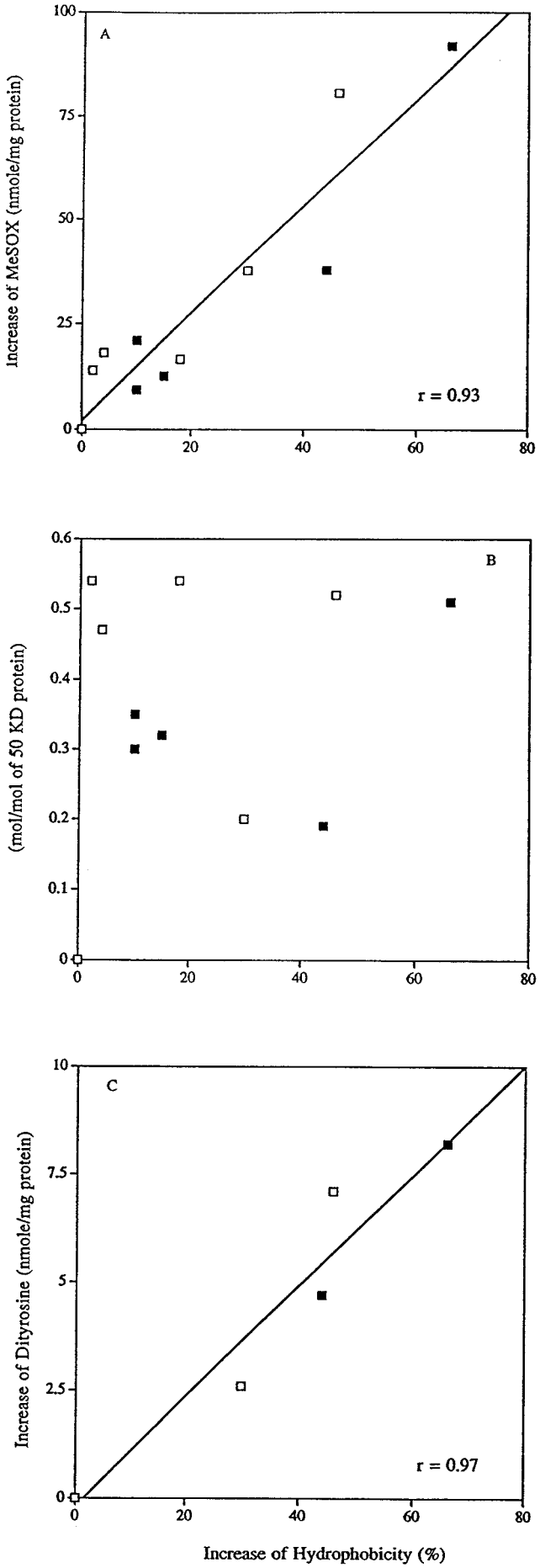


FIG. 5. Effect of AAPH treatment on tryptophan oxidation. Proteins were treated with indicated concentrations of AAPH at 37°C for 5 hr. The amount of oxidized tyrptophan was determined as described. Open bars represent the results of 2-month-old rat liver proteins, and solid bars represent those of 24-month-old rat liver proteins. Results are presented as the average of two independent experiments.



hydrophobicity of the 24-month-old animal but had a marked effect (18% increase) in the hydrophobicity of protein from the 2-month-old animals. Exposure of rat liver untreated, control proteins to 50 mM AAPH caused a 46% and 81% increase in the hydrophobicity of proteins from 2- and 24-month old animals, respectively (Fig. 2*B*). Treatment of the rat liver proteins with the ROS generating systems led also to substantial increases in the protein carbonyl content. As illustrated in Fig. 2*C*, exposure to the MCO system led to 8-fold and 4-fold increases in the carbonyl content of proteins from 2- to 24-month-old animals, respectively. The MCO-dependent formation of carbonyl groups was not significantly affected by addition of 10 or 25 mM H_2O_2 to the MCO reaction mixtures. Similar results were obtained by the AAPH treatment. When tested at a concentration of 50 mM, AAPH led to 9-fold and 5.6-fold increases in the carbonyl content of rat liver proteins from 2- and 24-month-old animals, respectively (Fig. 2*D*)

Modification of Amino Acid Residues by the MCO and AAPH System. *Methionine oxidation.* Upon exposure to the MCO system, conversion of methionine residues to MeSOX was increased about 3-fold and 2-fold in liver protein from both 2- and 24-month-old rats. Addition of H_2O_2 to the reaction mixtures had no effect on the extent of methionine oxidation (Fig. 3*A*). By comparison, the treatment with AAPH was more effective. At concentrations of 15 and 50 mM, treatment with AAPH led to 5-fold and 10-fold increase in oxidation of the methionine residues to MeSOX, respectively. (Fig. 3*B*). As with the MCO system, there was no significant difference in the susceptibility of methionine residues in liver proteins from young and old animals to modification by AAPH.

Dityrosine formation. Treatment of rat liver protein fractions with AAPH led to precipitation of about 30% of the soluble protein and to a significant increases in the level of dityrosine. After exposure to AAPH, the level of dityrosine residues were 6–10 nmol/mg protein, representing a 3- to 4-fold increase over that found in the untreated samples (Fig. 4). It is likely that the dityrosine formation is due in part to some intermolecular cross-linking reactions because the fraction of protein that precipitated during the AAPH treatment contained about two times as much dityrosine as was present in the soluble fraction. We could not detect any difference in the dityrosine content of liver proteins from 2- and 24-month-old animals, either before or after treatment with AAPH (Fig. 4). In contrast, treatment of liver protein with the MCO system did not cause protein precipitation nor did it produce a detectable increase in the amount of dityrosine formed (data not shown).

Tryptophan. There was little difference in the tryptophan content of proteins from young and old rat livers. However, treatment with 50 mM AAPH led to a loss of between 50% and 75% of the tryptophan residues in 2- and 24-month-old animals, respectively (Fig. 5). In contrast treatment with the MCO system had no detectable effect on the tryptophan content (data not shown).

Correlation of Protein Modification and Hydrophobicity in Proteins Treated with MCO or AAPH. To determine which types of protein modification observed in these experiments

Fig. 6. Correlation of methionine oxidation (*A*), carbonyl (*B*), or dityrosine (*C*) with protein surface hydrophobicity. Proteins were treated with MCO or AAPH as described; methionine oxidation, carbonyl, or dityrosine formation in treated proteins was determined as described. The increase of hydrophobicity (expressed as percentage) was calculated by subtracting the ANSA-dependent fluorescent intensity of 2- or 24-month-old control, untreated proteins from those of treated 2- or 24-month-old proteins, respectively, then multiplying by 100. The increase of methionine oxidation, carbonyl, and dityrosine formation was calculated by subtracting values from treated samples by values from their corresponding control, untreated samples. Data (□, 2 months; ■, 24 months) are plotted as increase of hydrophobicity (%) against increase of MeSOX (nmol/mg protein), carbonyl (mol/mol of 50-kDa protein), and dityrosine (nmol/mg protein).

may be primarily responsible for the increase of hydrophobicity, the correlation of various modifications with protein surface hydrophobicity was analyzed. The results in Fig. 6*A* showed a linear correlation between increase of MeSOX and percentage increase of surface hydrophobicity from various treatments ($r = 0.93$), suggesting that methionine oxidation may be responsible for the increase of hydrophobicity. There was no correlation between increase of carbonyl content and hydrophobicity in treated 2- or 24-month-old rat liver proteins (Fig. 6*B*). The correlation between percentage increase of hydrophobicity and dityrosine formation in AAPH-treated proteins (Fig. 6*C*) was also linear ($r = 0.97$), indicating that dityrosine formation may also contribute to increase of hydrophobicity.

DISCUSSION

The demonstration that surface hydrophobicity of rat liver proteins increases with animal age suggests that changes in hydrophobicity along with the levels of protein carbonyl groups may serve as a marker of aging. Whether the age-related changes in hydrophobicity are due to spontaneous changes in protein conformation as suggested by Rothstein (8) or to posttranslational oxidative modifications (6) remains unsettled. However the demonstration that *in vitro* treatment of purified proteins with radiolytically generated ·OH leads to an increase in hydrophobicity (22–24), and the results presented here showing that treatment of rat liver proteins with AAPH leads to a substantial increase in hydrophobicity, support the possibility that the age-related increase in hydrophobicity might be due to radical-mediated oxidative reactions. This view is supported further by the results of studies by Zhou and Gafni (25) showing that age-related changes in phosphoglycerate kinase are associated with the oxidation of protein sulfhydryl groups, and that treatment of the enzyme from young animals with an MCO system converts the enzyme to a configuration that is indistinguishable from the enzyme from old animals.

The finding that treatment of rat liver proteins with AAPH leads to aggregation and formation of dityrosine derivatives of some proteins is analogous to the results of earlier studies (20, 26–28) showing that insoluble aggregates and dityrosine derivatives are formed by treatment of purified proteins with radiolytically generated ·OH. However, the mechanisms involved may be quite different since dityrosine formation and aggregation of proteins by the ·OH generating system occurs only in the absence of oxygen (26), whereas similar changes induced by the AAPH system occur only in the presence of oxygen. The ability of O_2 to inhibit cross-linking reactions by ·OH is understandable since reaction of O_2 with carbon-centered radicals prevents the formation of carbon–carbon cross-linkages, and reaction of $O_2^{\cdot-}$ (produced during radiolysis in presence of O_2) with tyrosyl radicals leads to regeneration of the unmodified form of tyrosine (29). The requirement for O_2 in the AAPH system is also understandable because the reaction of O_2 with the primary product of AAPH decomposition (a carbon-centered radical) leads to the generation of alkyl peroxyl radicals and ultimately also to alkoxy radicals, both of which can abstract hydrogen atoms from proteins to form protein radical derivatives capable of undergoing cross-linking reactions. It is not clear, however, why in this case the presence of O_2 would not also interfere with the cross-linking reactions as described above for the ·OH-mediated reactions.

In any case, the formation of protein–protein cross-linkage may contribute to the age-related accumulation of altered ("oxidized") forms of proteins because cross-linked proteins are more resistant to proteolytic degradation by the multicatalytic protease (10, 23, 28, 30, 31). In fact, some kinds of cross-linked proteins inhibit the ability of the multicatalytic protease to degrade other oxidized proteins (10). The possibility that a metal ion, such as iron, is implicated in the oxidation-mediated aggregation of liver proteins by AAPH is discounted by the demonstration that desferrioamine did not prevent formation of precipitated protein and addition of iron to the AAPH incubation mixtures had no effect on the amount precipitate formed.

The observation that the oxidation of Met residues leads to an increase in surface hydrophobicity is unexpected, since as noted by Vogt (32) the conversion of Met to MeSOX would be expected to decrease hydrophobicity. Therefore the observed change in hydrophobicity might be due to the formation of dityrosine which also correlates with the increase in hydrophobicity, or as suggested by Vogt (32) the formation of MeSOX may induce conformational changes in protein structure leading to an exposure of hydrophobic residues. The latter possibility is supported by results of recent studies showing that the oxidation of methionine residues in *Escherichia coli* glutamine synthetase leads to extensive changes in protein conformation (33). The fact that the 15% age-related increase in surface hydrophobicity was not associated with a comparable increase in MeSOX does not preclude a role of Met oxidation in the observed age-related increase in hydrophobicity because the method for determining MeSOX in crude extracts is not sufficiently sensitive to detect such small differences between proteins in animals of different ages. Lack of sensitivity of amino acid analysis of protein hydrolysates may also explain the failure to detect age-related differences in the oxidation of other amino acid residues in liver proteins following their treatment with MCO or AAPH systems.

The fact that H_2O_2 had little if any effect on the oxidation modification of proteins in liver extracts was explained by the demonstration that the added H_2O_2 was completely destroyed within seconds after its addition to reaction mixtures (data not shown).

In summary, we have demonstrated that there is an age-related increase in the surface hydrophobicity of rat liver proteins, and have presented evidence that the age-related changes could be due to oxidation by ROS. There is a linear correlation between protein hydrophobicity and the conversion of Met residues to MeSOX and the conversion of tyrosine residues to dityrosine cross-linked derivatives.

We thank Dr. Lin Tsai for his synthesis and characterization of dityrosine as well as his many helpful suggestions and discussions throughout this study.

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