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Polylysines Modified with Malonaldehyde, Hydroperoxylinoleic Acid and Monofunctional Aldehydes

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The interaction of components of peroxidized lipids with polylysine, as a model of protein, was investigated by evaluating the fluorescence and cross-links produced. Treatment of polylysine with 1/3 molar excess malonaldehyde at pH 7.5 gave modified polylysines containing 1,4-dihydropyridine-3,5-dicarbaldehyde residues that fluoresced at 398 nm (excitation maximum) and 470 nm (emission maximum). The amount of the fluorescent residues was estimated to be less than 0.2% of the ε-amino groups. Most of the malonaldehyde was incorporated into the ε-amino groups as non-fluorescent aminopropenal residues (22%) which exhibited an absorption maximum at 280 nm and were reactive to 2-thiobarbituric acid. These residues are unstable and might produce cross-links by reacting with unmodified ε-amino groups. Reaction of polylysine with hydroperoxylinoleic acid, acetaldehyde or *n*-hexylaldehyde produced cross-linked polylysines which exhibited much weaker fluorescence with excitation maxima at 340—360 nm and emission maxima at 410—430 nm. Studies of the characteristics of fluorescence of these modified polylysines revealed that the fluorophore in the hydroperoxide-modified polylysine was distinguishable from that of the malonaldehyde-modified polylysine. It is likely that the fluorophores and cross-links in the hydroperoxide- and the monofunctional aldehyde-modified polylysines were formed by different mechanisms from those involved in the case of malonaldehyde.

Keywords——polylysine; malonaldehyde; hydroperoxylinoleic acid; acetaldehyde; *n*-hexylaldehyde; fluorescence; cross-link; 1,4-dihydropyridine-3,5-dicarbaldehyde; conjugated Schiff base; aminopropenal

Introduction

It has been demonstrated that aging tissues contain lipofuscin or ceroid pigments containing characteristic fluorescent components.[1,2] The formation of these pigments is supposed to be related to lipid oxidation. Unsaturated fatty acids are initially oxidized to produce hydroperoxides, which are in turn degraded into a complex mixture of secondary products including malonaldehyde and monofunctional aldehydes.[3,4] There have been several studies on non-enzymatic reaction between oxidized lipids and proteins, and although amino acid residues in proteins were shown to be destroyed or to form cross-links by interaction with hydroperoxides, the mechanisms were not well elucidated.[3] Malonaldehyde, a typical secondary product bearing a bifunctional aldehyde grouping, also produced fluorescence and cross-links in proteins. Tappel and his associates[5–7] obtained fluorescent conjugated Schiff bases I between malonaldehyde and amino acids, and suggested that the fluorescent components in lipofuscin were these conjugated Schiff bases. They also suggested that the Schiff bases formed cross-links between protein molecules. On the other hand, we demonstrated that reaction of malonaldehyde with amino compounds in the neutral pH region gave strongly fluorescent 1,4-dihydropyridine-3,5-dicarbaldehydes II,[8–11] and we suggested that the fluorescent components were II, if malonaldehyde was produced during

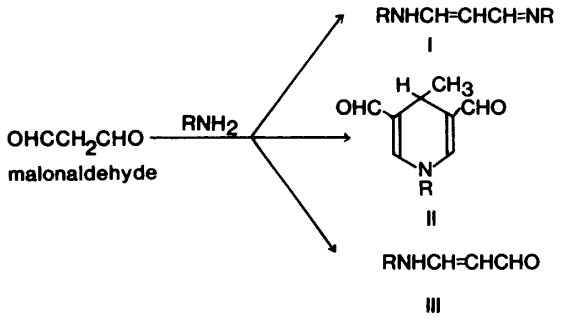

Chart 1

lipid peroxidation (Chart 1).

It is important to compare the properties of proteins modified with lipid hydroperoxides and their secondary degradation products, in order to elucidate the mechanisms of aging of tissues. This time, we investigated the reaction of polylysine with malonaldehyde, hydroperoxylinoleic acid and monofunctional aldehydes to compare the properties of the modified polylysines. Polylysine is a simple protein model which contains mainly ε-amino groups, and the modification of the ε-amino groups can be well characterized since all the reaction products of the ε-amino groups are recoverable in a non-dialyzable polymer.

Materials and Methods

Materials——Poly-DL-lysine hydrobromide (type VIIB; degree of polymerization, 170) and DL-lysine monohydrochloride were the products of Sigma Chemical Company, St. Louis. Malonaldehyde was prepared by acid hydrolysis of malonaldehyde bis (dimethylacetal) (Aldrich Chemical Company, Inc., Milwaukee) according to the method described previously.[12] While this preparation was free from the starting acetal,[13] it may contain small amounts of β-methoxyacrolein, 3,3-dimethoxypropylaldehyde[14] and some polymers.[15,16] Since malonaldehyde is unstable, it was prepared just prior to use. A portion of the acidic solution was diluted with water and adjusted to pH 7.5 with 1 N NaOH to prepare neutral 400 mM malonaldehyde solution for use. 3,5-Diformyl-4-methyl-1,4-dihydropyridine-1-acetic acid IIa and *N*-(2-formylvinyl)glycine IIIa were prepared as described.[9] 13-Hydroperoxylinoleic acid was prepared according to the method of Gardner[17] by the use of linoleic acid (Sigma Chemical Company, St. Louis) and soybean lipoxygenase (Lipoxidase type I, Sigma Chemical Company). The purity of the hydroperoxide was 95% when estimated based on a molecular extinction coefficient of ε (233 nm): 25300.[18] Acetaldehyde and *n*-hexylaldehyde were reagent-grade products of Kanto Chemical Company, Ltd., Tokyo. 2-Thiobarbituric acid (TBA) was from Sigma Chemical Company.

Analytical Methods——Gel filtration of the reaction products was performed on a column (1.1 × 63 cm) of LKB Ultrogel AcA 44 (fractionation range from 10000 to 130000 daltons) by elution with 0.1 M phosphate buffer (pH 7.0) containing 1.0% NaCl. The column was first calibrated using molecular weight standards (Pharmacia Fine Chemicals, Sweden): Ribonuclease A, 13700 daltons; chymotrypsinogen A, 25000 daltons; ovalbumin, 43000 daltons; and bovine serum albumin, 67000 daltons. The void volume of the column was estimated to be 20 ml from the elution position of blue dextran (2000000 daltons).

Ultraviolet absorption spectra were measured by the use of a UV-200S Shimadzu double beam spectrophotometer. Fluorescence spectra were measured by the use of a Hitachi 650—40 fluorescence spectrometer and a Perkin-Elmer 650—10S fluorescence spectrometer. The spectra were measured in 0.1 M phosphate buffer (pH 7.0).

TBA Test——Sample solution (0.3 ml) was mixed with 1.0 ml of 20 mM TBA solution and 1.0 ml of glacial acetic acid, and the mixture was heated at 100°C for 20 min.[12] The absorption spectrum at 400—600 nm was measured after the mixture had been diluted 50—500 fold with water. Standard solutions (2 mM) of malonaldehyde bis(dimethylacetal) and IIIa were similarly tested as controls.

Reaction of Poly-DL-lysine with Malonaldehyde——Poly-DL-lysine hydrobromide (50 mg; 0.24 mmol of ε-amino groups) was dissolved in 2.4 ml of 0.1 M phosphate buffer (pH 7.5) containing 0.075 mmol of malonaldehyde. The mixture was agitated under air in an open vessel and incubated at 37°C. A portion was withdrawn from the reaction mixture at various times, and dialyzed against distilled water to remove low-molecular-weight contaminants. The concentration of ε-amino groups in the dialysate was adjusted to 8.0 mM by addition of water, and the fluorescence and ultraviolet absorption spectra of the dialysate were measured. The TBA test was perfomed on the dialysate. Incubation of malonaldehyde alone under the same conditions did not produce any non-dialyzable polymers which exhibited fluorescence or ultraviolet absorption.

Reaction of Polylysine with Hydroperoxylinoleic Acid, Acetaldehyde and *n*-Hexylaldehyde——Poly-DL-lysine hydrobromide (10 mg; 0.05 mmol of ε-amino groups) dissolved in 10 ml of 0.1 M phosphate buffer (pH 7.5) was mixed with 4.0 mg of 13-hydroperoxylinoleic acid (0.013 mmol) [A], 0.1 ml of 1 M acetaldehyde solution (0.1 mmol) [B], 0.1 ml of 1 M *n*-hexylaldehyde solution in methanol (0.1 mmol) [C], 0.025 ml of 400 mM malonaldehyde solution (0.01 mmol) [D], a mixture of 0.025 ml of 400 mM malonaldehyde solution (0.01 mmol) and 0.1 ml of 1 M acetaldehyde solution (0.1 mmol) [E], or a mixture of 0.025 ml of 400 mM malonaldehyde solution (0.01 mmol) and 0.1 ml of 1 M *n*-hexylaldehyde in methanol (0.1 mmol) [F]. The mixture was agitated under air in an open vessel at 37 °C for 24 h. The homogeneous reaction mixture was dialyzed against water to remove low-molecular-weight impurities. Each dialysate was made up to 20 ml (concentration of ε-amino groups: 2.25 mM), and the fluorescence spectra of the modified polylysines in the dialysate were measured. An aliquot of the dialysate (0.1 or 1.0 ml) was applied to a column of Ultrogel AcA 44 for fractionation according to molecular size.

Results

It is known that three types of compounds I, II and III are produced by the reaction of malonaldehyde and amino acids (Chart 1). The amorphous conjugated Schiff bases I exhibit fluorescence with excitation maxima of 370 nm and emission maxima of 450 nm, and have complex ultraviolet absorption spectra showing maxima at 256 and 435 nm in an aqueous solution.[5] These compounds I liberate malonaldehyde in the TBA-reaction.[19] Crystalline 1,4-dihydropyridine-3,5-dicarbaldehydes II, whose structure was unambiguously established,[9] exhibit fluorescence with excitation maxima of 400 nm and emission maxima of 465 nm, and absorption spectra with maxima at around 235, 265 and 400 nm.[10,11] They do not produce malonaldehyde in the TBA-reaction.[10] Aminopropenals III, which are non-fluorescent and show absorption maxima at around 280 nm,[9,20,21] are unstable and liberate malonaldehyde in the TBA-reaction.[19,20]

When lysine monomer was reacted with malonaldehyde at neutral pH, the fluorescence of the reaction mixtures showed excitation maxima at 395 nm and emission maxima at 466 nm. The spectrum of the reaction mixture with lysine in excess [b] was the same as that with malonaldehyde in excess [a]. The fluorophore produced in the reactions must be the 1,4-dihydropyridine-3,5-dicarbaldehyde structure since the spectra of the reaction mixtures were very similar to those of II. The structure of the fluorescent product of the reaction of α-*N*-acetyl-L-lysine and malonaldehyde,[6] which showed an excitation maximum at 395 nm and an emission maximum at 470 nm, must be II, although the authors assigned the structure as I.

When polylysine was reacted with an 8-fold molar excess of malonaldehyde at pH 7.5 and 37 °C for 12 h, the reaction gave a yellow precipitate which could not be dissolved in any solvents tested. Polylysine was treated with 1/3 molar excess of malonaldehyde, and a portion of the clear reaction mixture was removed after selected periods and dialyzed. Modified polylysines with different degrees of modification were obtained. The fluorescence spectra of the malonaldehyde-modified polylysines obtained at any reaction period showed excitation maxima at 398 nm and emission maxima at 470 nm, indicating that the fluorophore was 1,4-dihydropyridine-3,5-dicarbaldehyde II (Fig. 1). The time course of the relative molar

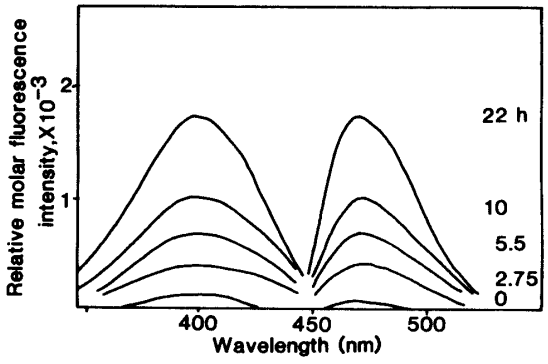

Fig. 1. Fluorescence Spectra of the Malonaldehyde-Modified Polylysines

Poly-DL-lysine (0.24 mmol of ε-amino groups) was treated with 0.075 mmol of malonaldehyde at pH 7.5 and 37 °C for the indicated periods. Relative molar fluorescence intensity was expressed with respect to IIa.

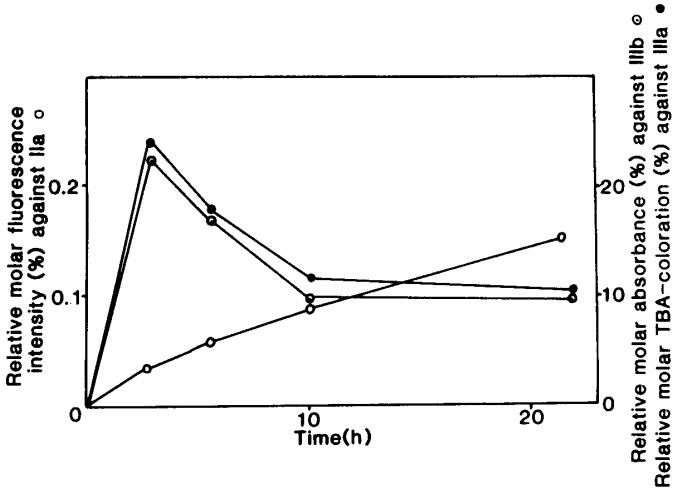

Fig. 2. Time Course of Fluorescence Intensity, Ultraviolet Absorption and TBA-Coloration of the Malonaldehyde-Modified Polylysines

The reaction conditions were the same as described in Fig. 1.

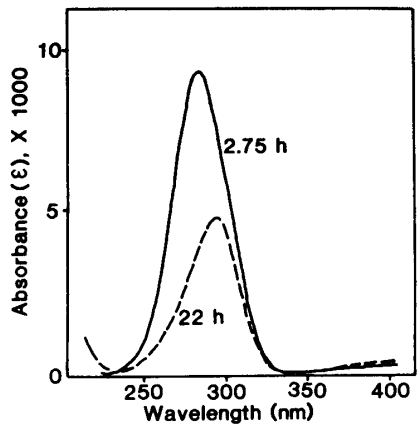

Fig. 3. Ultraviolet Absorption Spectra of the Malonaldehyde-Modified Polylysines

The malonaldehyde-modified polylysines were prepared as described in Fig. 1.

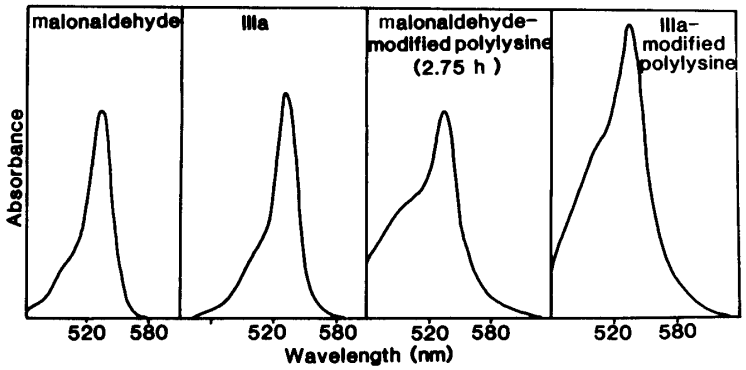

Fig. 4. Absorption Spectra of TBA-Reaction Mixtures of the Modified Polylysines

The malonaldehyde-modified polylysine was prepared as described in Fig. 1. The IIIa-modified polylysine was prepared by reaction of poly-DL-lysine (0.024 mmol) with 0.24 mmol of IIIa at pH 7.5 and 37 °C for 14 h.

fluorescence intensity of the modified polylysine against that of the standard 3,5-diformyl-4-methyl-1,4-dihydropyridine-1-acetic acid IIa is shown in Fig. 2. Formation of the fluorophore progressively increased, but the extent of its formation was less than 0.2% of the ε-amino groups after 22 h. The ultraviolet absorption spectra of 2.75 h modified polylysine showed an absorption maximum at 283 nm (Fig. 3), indicating that the major chromophore was aminopropenal III. The extent of this modification was calculated by the use of the extinction coefficient of the standard aminopropenal of α-acetyllysine methyl ester IIIb, log ε 4.53 (282 nm),[21] and it was found that 22% of the ε-amino residues were modified as aminopropenal III. This indicates that most of the malonaldehyde was trapped as aminopropenal residues in polylysine. As the modification proceeded, the spectrum altered slightly and the maximum shifted to 293 nm after reaction for 22 h (Fig. 3), and the amount of aminopropenal residues decreased to 10% (Fig. 2). Aminopropenal is unstable, and may be readily transformed. The TBA test of the modified polylysines showed absorption maxima at 532 nm similar to those of the standard malonaldehyde and *N*-(2-formylvinyl)glycine IIIa (Fig. 4). The time course of the relative molar TBA-coloration of the modified polylysines was in good agreement with that of aminopropenal residues determined by ultraviolet absorption

measurement (Fig. 2).

In order to obtain information on the reaction of the aminopropenal residues with ε-amino groups, reaction of polylysine with IIIa was performed. Polylysine was treated with a 10 molar excess of IIIa at pH 7.5 and 37°C for 14 h, and the reaction mixture was dialyzed against water. No ultraviolet and TBA-reactive polymeric substances were produced in the incubation of IIIa alone under the same conditions. The IIIa-modified polylysine showed an absorption maximum at 289 nm (ε: 28000), but exhibited little fluorescence. The IIIa-modified polylysine was positive in the TBA-reaction, showing an absorption maximum at 532 nm (Fig. 4), and the relative molar TBA-coloration with respect to IIa was 42%. The results suggest that the aminopropenal residues can react with unmodified ε-amino groups to produce new residues. Such a reaction may provide cross-links between molecules of polylysine in the reaction with malonaldehyde.

Polylysine was incubated with 13-hydroperoxylinoleic acid [A], acetaldehyde [B], *n*-hexylaldehyde [C], malonaldehyde [D], a mixture of malonaldehyde and acetaldehyde [E], and a mixture of malonaldehyde and *n*-hexylaldehyde [F] at 37°C for 24 h. The amounts of 13-hydroperoxylinoleic acid and malonaldehyde were one-fourth and one-fifth of polylysine, respectively, while those of acetaldehyde and *n*-hexylaldehyde were in five-fold excess. Fluorescence spectra of the modified polylysines are listed in Table I. It is interesting to note that the monofunctional aldehydes such as acetaldehyde and *n*-hexylaldehyde produced fluorescence, as did the hydroperoxide and malonaldehyde. With respect to the excitation and emission maxima, the modified polylysines were classified into two groups. One group [A, B, C] exhibited spectra with excitation maxima at 340—360 nm and emission maxima at 410—430 nm, and the other group [D, E, F] exhibited spectra with excitation maxima at around 400 nm and emission maxima at 460—470 nm. The combination of malonaldehyde and the monofunctional aldehydes [E, F] might produce the same type of fluorophores as malonaldehyde alone [D], which can be interpreted by assuming that a mixture of malonaldehyde, a monofunctional aldehyde and a primary amine produce 1,4-dihydropyridine-3,5-dicarbaldehyde II with an alkyl substituent at the 4-position.[11]

The fluorescence intensity of the hydroperoxylinoleic acid-modified [A], the acetaldehyde-modified [B] and the *n*-hexylaldehyde-modified [C] polylysines was much lower

Table I. Fluorescence Spectra of the Modified Polylysines

Modified with	Fluorescence spectra			Fluorescence spectra after treatment with 10 mM $NaBH_4$		
	Excitation maximum (nm)	Emission maximum (nm)	Relative molar intensity[a]	% decrease in intensity at the original maximum	New maxima	
					Excitation (nm)	Emission (nm)
A 13-Hydroperoxylinoleic acid	347	425	6.5×10^{-5}	74	329	410
B Acetaldehyde	357	430	9.5×10^{-5}	55	340	419
C *n*-Hexylaldehyde	340	417	3.2×10^{-5}	22	333	407
D Malonaldehyde	398	467	56×10^{-5}	93	—	—
E Malonaldehyde and acetaldehyde	401	470	290×10^{-5}	96	—	—
F Malonaldehyde and *n*-hexylaldehyde	401	470	243×10^{-5}	93	—	—

a) Concentrations of ε-amino groups were 2.25 mM. Relative molar intensity of fluorescence at each maximum of the ε-amino group against that of quinine sulfate in 0.1 N sulfuric acid at 347 nm (excitation) and 452 nm (emission).

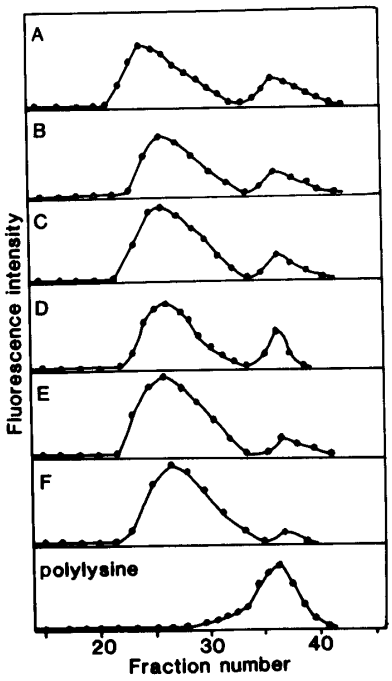

Fig. 5. Gel Filtration of the Modified Polylysines

An aliquot of a solution of polylysine modified with 13-hydroperoxylinoleic acid [A], acetaldehyde [B], *n*-hexylaldehyde [C], malonaldehyde [D], a mixture of malonaldehyde and acetaldehyde [E], or a mixture of malonaldehyde and *n*-hexylaldehyde [F] was applied to a column of Ultrogel AcA 44. One fraction contained 1.40 ml of the effluent. Peaks were detected by fluorescence measurement at the excitation and emission maxima of each modified polylysine. Unmodified polylysine was monitored by fluorescence measurement excitation at 400 nm and emission at 464 nm after all the fractions had been treated with 0.1 ml of 400 mM malonaldehyde at 37 °C overnight.

than that of the polylysines modified with malonaldehyde [D, E, F]. While the fluorescence intensity of the modified polylysines [D, E, F] was almost completely quenched by treatment with a large amount of sodium borohydride, that of the modified polylysines [A, B, C] was considerably but not completely quenched. The modified polylysine [A] lost fluorescence to the extent of 74%, accompanied by the formation of new fluorophores having excitation and emission maxima with shorter wavelength. The decrease in the intensity of the modified polylysines [B, C] produced by the same treatment was much smaller. It is apparent that the fluorophores produced by hydroperoxylinoleic acid are different from those produced by malonaldehyde.

The modified polylysines were fractionated by gel filtration. All the modified polylysines showed two fluorescent peaks (Fig. 5). The molecular weight of the second peaks was the same as that of unmodified polylysine, and was estimated to be about 20000 daltons from the calibration curve. The first peaks showed higher molecular weights of 45000 to 55000 daltons. The results indicate that every modification resulted in the formation of cross-links between two or three molecules of polylysine. The fluorescence spectrum of the first peak of each modified polylysine was similar to that of the second peak. However, the excitation maxima shifted to longer wavelength after fractionation in the case of the modified polylysines [A, B, C], which indicated that the fluorophores were unstable during gel filtration. Cross-links of the malonaldehyde-modified polylysine [D] may be formed by reaction of the aminopropenal residues with the free ε-amino groups, as described above. It is likely that cross-links in the modified polylysines [A, B, C] were formed by different mechanisms from those involved in the case of malonaldehyde. Cross-links in E and F may be formed by malonaldehyde or the monofunctional aldehydes.

Discussion

Lipid oxidation has been implicated in many important biological events, including aging of tissues.[1,2] Malonaldehyde has been suggested to be a major component responsible for these processes.[7] The bifunctionality of the aldehyde permits its crosslinking with free amino groups of proteins. Modification of proteins with malonaldehyde produces fluorescent,

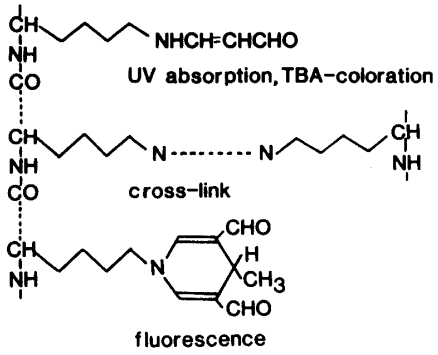

Chart 2

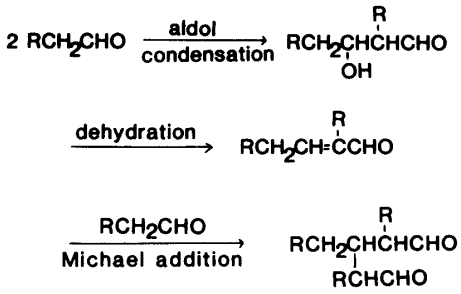

Chart 3

cross-linked and TBA-reactive proteins, and all these properties of the modified proteins have been attributed to the formation of intra- and intermolecular conjugated Schiff bases.[2,6,7] Furthermore, formation of fluorophores and cross-links in the reaction of peroxidized lipids and proteins has been attributed to malonaldehyde, and the fluorescent components in lipofuscin have been considered to be the conjugated Schiff bases.[2,7]

We investigated the reaction of malonaldehyde with polylysine, a protein model exhibiting no ultraviolet absorption and containing no functional groups other than the ε-amino groups and C- and N-terminal groups. All the modified ε-amino groups can be recovered in the polymer fraction. It was found that the ε-amino groups of polylysine were modified in three ways (Chart 2). Most of the malonaldehyde was incorporated into polylysine as unstable aminopropenal residues which were non-fluorescent, strongly ultraviolet absorbing and TBA-reactive. The residues might subsequently react with unmodified ε-amino groups to form cross-links. The third reaction was the formation of stable 1,4-dihydropyridine-3,5-dicarbaldehyde residues with high fluorescence (excitation at 398 nm and emission at 470 nm), but the extent of formation was very much lower than that of the aminopropenal residues. Previously we pointed out that the structure of the fluorophore of ribonuclease[6] and hemoglobin A[22] modified with malonaldehyde should be 1,4-dihydropyridine-3,5-dicarbaldehyde, and the cross-links in the modified proteins should be produced by a different mechanism.[22] The present results support the above proposal.

Reaction of polylysine with hydroperoxylinoleic acid produced fluorophores with an excitation maximum at 347 nm and an emission maximum at 425 nm; the intensity was much lower than that in the case of malonaldehyde-modified polylysine. The fluorescence spectrum was similar to those of the reaction products of primary amines and bovine serum albumin with the hydroperoxide,[23,24] and very different from those of malonaldehyde-modified polylysine and proteins.[6,25] It is unlikely that the fluorophores produced in the interaction of polylysine with the hydroperoxide were derived from malonaldehyde. Hence cross-links in the hydroperoxide-modified polylysine could not be produced by mechanisms involving malonaldehyde.

It is interesting that monofunctional aldehydes such as acetaldehyde and *n*-hexylaldehyde produced fluorophores and cross-links in polylysine. Formation of cross-links by these aldehydes might be explained as follows. Self-condensation of three molecules of the aldehyde by aldol condensation and subsequent Michael reaction might produce a bifunctional aldehyde as shown in Chart 3, which would cross-link ε-amino groups of polylysine. Formation of the fluorophores might involve such self-condensation reactions, as suggested by Yoden *et al.*[26] The fluorescence characteristics of these monofunctional aldehyde-modified polylysines were similar to those of the hydroperoxide-modified polylysine. It is possible that formation of fluorophores and cross-links by the hydroperoxide was caused by secondary products such as those in the case of monofunctional aldehydes.

It is suggested that the reaction of polylysine with malonaldehyde produces fluorescent 1,4-dihydropyridine-3,5-dicarbaldehyde residues and cross-links by formation of aminopropenal residues and subsequent derivatization. It is not likely that formation of fluorophores and cross-links by hydroperoxylinoleic acid was due to malonaldehyde, a possible secondary product of the hydroperoxide. The fluorophores derived from the hydroperoxide might involve the reaction of monofunctional aldehydes, alternative secondary products of the hydroperoxide.

There are several reports demonstrating the formation of fluorescent substances in reactions of hydroperoxy fatty acids with primary amines, proteins or nucleic acids.[23-25,27] The present results are consistent with those obtained by Fujimoto *et al.*[27] who showed that deoxyribonucleic acid formed fluorescent products as a result of the breakdown of lipid oxidation products in the presence of metals and ascorbic acid into reactive materials other than malonaldehyde.

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