Biochimica et Biophysica Acta, 981 (1989) 235–242
Elsevier

BBA 74433

Changes in the fluorescence parameters of bound *N*-(1-pyrene)maleimide by lipid peroxidation of intestinal brush-border membranes

Takao Ohyashiki [1], Taketo Yamamoto [1] and Tetsuro Mohri [2]

[1] *Department of Biochemistry and* [2] *2nd Division, Research Laboratory for Development of Medicine, School of Pharmacy, Hokuriku University, Kanazawa, Ishikawa (Japan)*

(Received 22 November 1988)

Key words: Lipid peroxidation; Protein conformation; Fluorometry; *N*-(1-Pyrene)maleimide; Brush-border membrane; (Porcine small intestine)

Using a fluorogenic thiol reagent, *N*-(1-pyrene)maleimide (NPM), we have examined the effect of lipid peroxidation on the microenvironment around SH groups of the membrane proteins in porcine intestinal brush-border membrane vesicles. The lipid peroxidation of the membranes was performed with various concentrations of *t*-butylhydroperoxide (*t*-BuOOH) in the presence of 100 μM ascorbic acid and 10 μM Fe^{2+}. Treatment of NPM-labeled membranes with these oxidizing agents resulted in a decrease of the fluorescence lifetime, suggesting modification of the environmental properties around the bound dye. Measurement of the steady-state fluorescence anisotropy of the labeled membranes indicated restriction of the motion of the bound dye by the lipid peroxidation of the membranes. This interpretation was further supported by an elevation of the transition temperature of the anisotropy, a decrease in the quenching rate constant of the fluorescence with acrylamide and a decrease in the SH reactivity of the membrane proteins for NPM by lipid peroxidation. Based on these results, the possibility of conformation changes in the vicinity of SH groups in the membrane proteins associated with lipid peroxidation has been discussed.

Introduction

Lipid peroxidation has been widely studied in relation to oxygen toxicity and is known to be a primary process in a variety of pathological events in cells [1–5].

It seems that biological membranes are readily susceptible to lipid peroxidation because the membrane lipids are rich in highly unsaturated fatty acids. Therefore analysis of the lipid structure and lipid–protein interaction in membrane damage resulting from lipid peroxidation will give an important clue to elucidation of the mechanism of oxidative injury of cells.

Several investigators have demonstrated that lipid peroxidation of artificial and biological membranes induces decrease in their lipid fluidity [6–9] and, in consequence, modification of the activities of membrane-bound enzymes [10–12]. Recently, we have reported [13] that the reactivity of SH groups in the porcine intestinal brush-border membranes with a fluorogenic thiol reagent, *N*-(7-dimethylamino-4-methylcoumarinyl)maleimide, decreases upon lipid peroxidation of the membranes, and we have suggested that the membrane protein conformation is modified by lipid peroxidation. However, there have been few lines of research concerning the effect of lipid peroxidation on the protein conformation in biological membranes.

In the present study we have examined the possibility of conformational change affecting the physical property of the microenvironment of protein SH groups in the porcine intestinal brush-border membranes in association with lipid peroxidation.

Materials and Methods

Chemicals. NPM was purchased from Molecular Probes and dissolved in acetone to make a stock solution (1 mM). 2-Thiobarbituric acid, *t*-BuOOH and BHA were obtained from Wako Pure Chemical. Superoxide dismutase (3000 U/mg protein) and catalase (3100 U/mg protein) were obtained from Sigma.

Abbreviations: NPM, *N*-(1-pyrene)maleimide; BHA, 3(2)-*t*-butyl-4-hydroxyanisole; *t*-BuOOH, *t*-butylhydroperoxide; TBAR, thiobarbituric acid-reactive substances; AcCys, *N*-acetylcysteine; MDA, malondialdehyde.

Correspondence: T. Ohyashiki, Department of Biochemistry, School of Pharmacy, Hokuriku University, Kanagawa-machi, Kanazawa, Ishikawa 920-11, Japan.

Preparation of membrane vesicles. The brush-border membrane vesicles were prepared from the porcine small intestine by the calcium-precipitation method described in our previous paper [14] and suspended in 10 mM Tris-maleate buffer (pH 6.85). Protein concentration was assayed by the procedure of Lowry et al. [15] using bovine serum albumin as standard.

Labeling of AcCys or membranes with NPM. Labeling of AcCys with NPM was carried out by incubating 0.5 mM AcCys with 10 μM NPM in 10 mM phosphate buffer (pH 6.5) at 0°C for 10 min. On the other hand, labeling of the membranes with NPM was performed as follows. The membrane vesicles (2 mg protein/ml) were incubated with 10 μM Tris-maleate buffer (pH 6.85) for 30 min at 0°C. The concentration of acetone in the reaction mixture was 1% or less. The reaction was terminated by dilution with a large volume of 10 mM Tris-maleate buffer (pH 6.85) and centrifugation at 25000 × *g* for 20 min. The pellets were washed twice with the same buffer. After centrifugation at 25000 × *g* for 20 min, the final pellets obtained were suspended in 10 mM Tris-maleate buffer (pH 6.85).

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was conducted in a 7.5% separating and a 5% stacking gel system in the presence of 0.1% sodium dodecyl sulfate. Proteins employed as molecular weight standards were obtained from Sigma: triosephosphate isomerase (26.6 kDa), lactate dehydrogenase (36.5 kDa), fumarase (48.5 kDa), pyruvate kinase (58 kDa), fructose-6-phosphate kinase (84 kDa), β-galactosidase (116 kDa) and α_2-macroglobulin (180 kDa). Staining of the protein fractions in a gel was carried out using Coomassie brilliant blue. The fluorescence bands in a gel were detected by using a Shimadzu double-beam flying-spot scanner CS-9000 with the excitation wavelength of 340 nm.

The labeling pattern of the membranes with NPM is shown in Fig. 1. Examination of the electrophoretograms revealed that NPM was mainly incorporated into 36 and 80 kDa protein fractions in the membrane proteins under the conditions of NPM-labeling employed in the present study.

Lipid peroxidation of the membrane. Lipid peroxidation of NPM-labeled membranes was performed as follows. The NPM-labeled membranes (2 mg protein/ml) were incubated with 100 μM ascorbic acid, 10 μM $FeSO_4$ and 0.6 mM *t*-BuOOH in 10 mM Tris-maleate buffer (pH 6.85) for 30 min at 37°C, unless otherwise specified. The reaction was terminated by dilution with a large volume of 10 mM Tris-maleate buffer (pH 6.85) and centrifugation at 25000 × *g* for 20 min. The pellets obtained were washed twice with and resuspended in the same buffer. In the measurement of thiobarbituric acid-reactive substance (TBAR) formation, the reaction of the membranes with oxidizing agents was terminated by the addition of 5 mM BHA

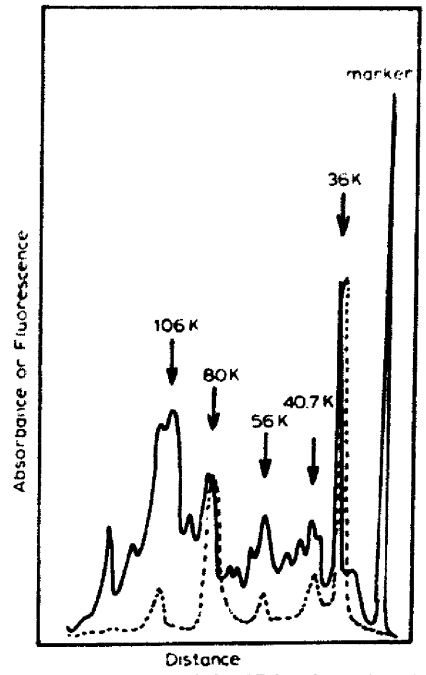

Fig. 1. Densitometric pattern of the SDS-polyacrylamide gel electrophoresis of NPM-labeled membranes. The experimental conditions and procedure of gel electrophoresis are described in Materials and Methods. ———, Coomassie brilliant blue staining; - - - - - -, fluorescence image.

(as a final concentration). The amount of TBAR formed during the reaction was determined by measuring the absorbance at 530 nm ($\varepsilon_{530} = 1.56 \cdot 10^5\ M^{-1} \cdot cm^{-1}$, Ref. 10) after mixing the reaction mixture with 2-thiobarbituric acid as described in our previous paper [16]. Conjugated diene formation was measured by monitoring the absorbance at 233 nm of the detergent-dispersed membranes (0.02 mg protein/ml of 10 mM phosphate buffer (pH 7.1) containing 1% Lubrol) as described in Ref. 17.

Fluorescence measurements. Fluorescence measurements were carried out using a Hitachi spectrofluorometer 850 at 25°C by circulating water through the cell holder unless otherwise specified. The excitation and emission wavelengths used were 340 and 392 nm, respectively. The error due to light scattering of the sample emission could be entirely prevented using a 350 nm cutoff filter. The steady-state fluorescence anisotropy is defined as the value of $(I_V - I_H)/(I_V + 2I_H)$, where the fluorescence intensity upon excitation with vertically polarized light is measured with the emission polarizer in the vertical (I_V) and horizontal (I_H) directions. Measurement of the fluorescence lifetime was performed using an Ortec PRA-3000 nanosecond fluorometer (Photochemical Research Associates, Ontario, Canada) at 25°C. The data were analyzed by a Digital computer system (Digital Equipment, Maynard, U.S.A.).

Quenching studies. Quenching experiments were carried out by adding small amounts of 5 M acrylamide

solution in 10 mM Tris-maleate buffer (pH 6.85) at 25°C as described in our previous paper [18]. The stock solution of the quencher was used within a few days after preparation. Analysis of the quenching data was performed by using the following equations proposed by Stern and Volmer [19] and Lehrer [20].

$$I_0/I = K_Q[Q] + 1$$

$$I_0/(I_0 - I) = \frac{1}{f_a K_Q[Q]} + \frac{1}{f_a}$$

where I_0, I, K_Q, [Q] and f_a denote the fluorescence intensities in the absence and presence of quencher, the quenching constant, the quencher's concentration and the effective fraction of the fluorescence that is quenchable, respectively. The quenching rate constant, k_q, was estimated by the equation, $k_q = K_Q/\tau_0$, where τ_0 is the fluorescence lifetime in the absence of quencher.

Results

Effect of t-BuOOH on the membrane lipid peroxidation

Fig. 2 shows the *t*-BuOOH concentration dependence of TBAR formation in the membranes in the presence of 100 μM ascorbic acid and 10 μM Fe^{2+}.

In the absence of *t*-BuOOH, the amount of TBAR formed by incubation of the membranes at 37°C for 30 min was 0.4 ± 0.03 nmol/mg protein as malondialdehyde. On the other hand, TBAR formation was markedly stimulated by treatment of the membranes with these reagents in the presence of *t*-BuOOH. The stimulation of TBAR formation was strongly dependent on the concentration of *t*-BuOOH, showing the maximum at about 0.6 mM *t*-BuOOH. In addition, the absorbance at 233 nm of detergent-dispensed membranes after treatment with 100 μM ascorbic acid, 10 μM Fe^{2+} and various concentrations of *t*-BuOOH also increased depending on the hydroperoxide concentrations (inset of Fig. 2).

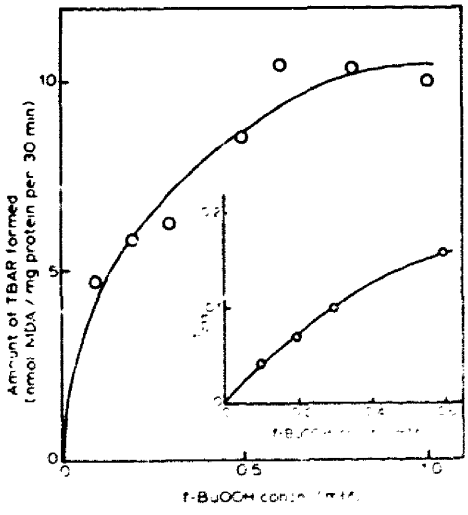

Fig. 2. *t*-BuOOH concentration-dependence of TBAR formation in the membranes at 25°C. Membrane protein concentration was 0.5 mg/ml. The *t*-BuOOH concentration was varied from 0.05 to 1.0 mM. Other experimental conditions of lipid peroxidation are described in Materials and Methods. The amounts of TBAR formed are represented as differences in the values in the presence and absence of *t*-BuOOH and expressed as the mean of triplicate determinations. MDA, malondialdehyde. The inset shows conjugated diene formation in the membranes. After lipid peroxidation, the membranes (0.02 mg protein/ml) were dissolved in 10 mM phosphate buffer (pH 7.1) containing 1% Lubrol and the absorbance was measured at 233 nm. Values are expressed as the means of triplicate determinations.

TABLE I

Effect of radical scavengers on TBAR formation of the membranes

The membranes (0.3 mg protein/ml) were incubated with 100 μM ascorbic acid, 10 μM Fe^{2+} and 0.6 mM *t*-BuOOH at 37°C for 30 min. After termination of the reaction by addition of 5 mM BHA, the amount of TBAR formed was measured. Values are expressed as means ± S.D. for triplicate determinations.

Scavengers	Concn	TBAR formation (%)
No addition	–	100
Superoxide dismutase	100 μg/ml	94.9 ± 7.48
Catalase	100 μg/ml	64.5 ± 1.63
Thiourea	30 mM	45.6 ± 7.07

Next we have examined effect of radical scavengers on TBAR formation by treatment of the membranes with 100 μM ascorbic acid, 10 μM Fe^{2+} and 0.6 mM *t*-BuOOH.

As can be seen in Table I, TBAR formation was markedly and moderately inhibited by addition of thiourea and catalase, respectively, but superoxide dismutase did not affect TBAR formation. From these results, it could be considered that an increased TBAR formation by treatment of the membranes with ascorbic acid/Fe^{2+}/*t*-BuOOH is due to lipid peroxidation of the membrane lipids and hydroxy radical mainly involved in the reaction.

Changes in the fluorescence lifetime of NPM-labeled membranes

The fluorescence decay curve of the membranes labeled with NPM, a SH-directed fluorogenic dye [21], could not be analyzed by a single exponential function (Fig. 3). This fact suggests that NPM bound to SH groups in the membrane proteins is in a multiplicity of physical states.

The fluorescence of NPM-labeled AcCys followed a single exponential decay with a single lifetime (τ = 4.03 ± 0.51 ns). By computer fitting of the data for the membranes, assuming two components in the decay process, one of the decay times was estimated to be 14.8 ± 0.80 ns (τ_1), accounting for 45% of the total fluorescence, and the other to be 56.0 ± 1.82 ns (τ_2), accounting for the remaining 55%.

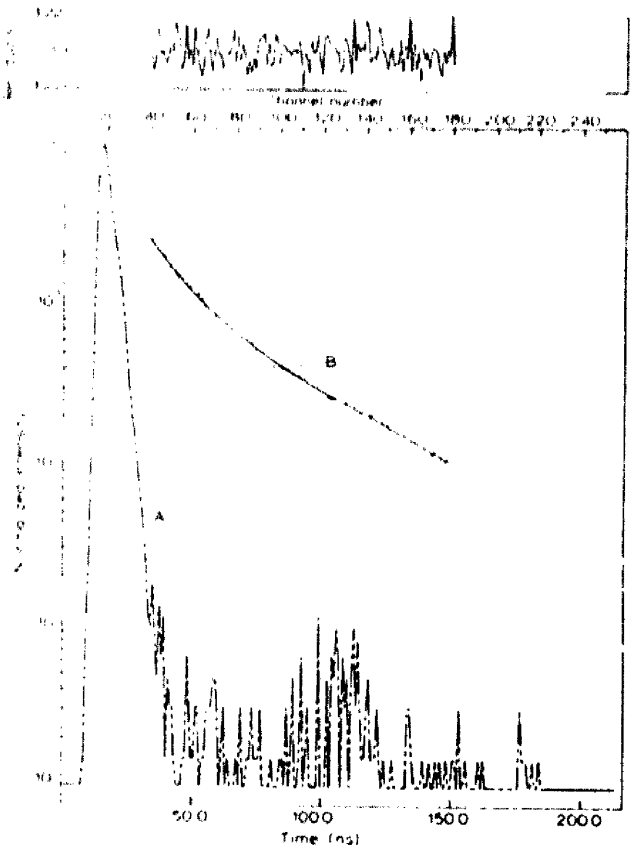

Fig. 3. Fluorescence decay curve of NPM-labeled control membranes in 10 mM Tris-maleate buffer (pH 6.85). Membrane protein concentration was 0.1 mg/ml. Excitation wavelength used was 340 nm. Curves A and B represent excitation pulse and fluorescence of NPM-labeled control membranes, respectively. The solid line in the curve B shows the fitted curve by the non-linear least-squares method. The residual values are shown in the top part of the figure.

Fig. 4 shows the change in the fluorescence lifetime of NPM-labeled membranes as a function of the amount of TBAR formed by treatment of the membranes with ascorbic acid/Fe^{2+} together with various concentrations of *t*-BuOOH.

The fluorescence lifetime of the complex decreased up to about 6 nmol malondialdehyde per mg protein formed and then reached a constant level. On the other hand, the τ_1 and τ_2 values at 25°C of NPM-labeled membranes after treatment with 100 μM ascorbic acid, 10 μM Fe^{2+} and 0.6 mM *t*-BuOOH alone were 14.4 ± 0.80 and 55.3 ± 1.51 ns, 15.0 ± 1.02 and 54.0 ± 0.93 ns, and 15.1 ± 0.82 and 56.5 ± 1.80 ns, respectively. From these results, it is concluded that a decreased fluorescence lifetime of the NPM-labeled membrane reflects the changes in the environmental properties around NPM-labeled SH groups in the membrane proteins by lipid peroxidation.

To obtain further information about the influence of lipid peroxidation on the environmental properties around the fluorescence-labeled SH groups, we have examined the steady-state fluorescence anisotropy and

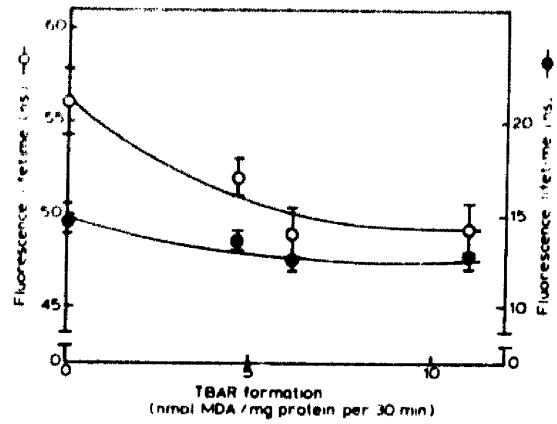

Fig. 4. Change in the fluorescence lifetime of NPM-labeled membranes by lipid peroxidation. The membranes with different levels of lipid peroxidation were prepared by incubating the NPM-labeled membranes (2 mg protein/ml) with 100 μM ascorbic acid and 10 μM Fe^{2+} in the presence of various concentrations of *t*-BuOOH (0.1–0.6 mM) at 37°C for 30 min. The protein concentrations of the labeled membranes employed in measurements of the fluorescence lifetime and TBAR formation were 0.1 and 0.5 mg/ml, respectively. Other experimental conditions were the same as described in the legend to Figs. 3 and 1, respectively. Values are expressed as the means ± S.D. for triplicate determinations. ●, τ_1 value; ○, τ_2 value. MDA, malondialdehyde.

fluorescence quenching efficiency for acrylamide of NPM-labeled membranes.

Fig. 5 shows the temperature-dependence profiles of the fluorescence anisotropy of the control and peroxidized membranes.

The degree of the anisotropy of the control membranes decreased with increase in temperature, showing a transition point at about 32.0 ± 0.2°C. In the peroxidized membranes, the degree of the anisotropy was larger in all temperatures tested as compared to that of the control ones and the transition point shifted to about 37.0 ± 0.1°C.

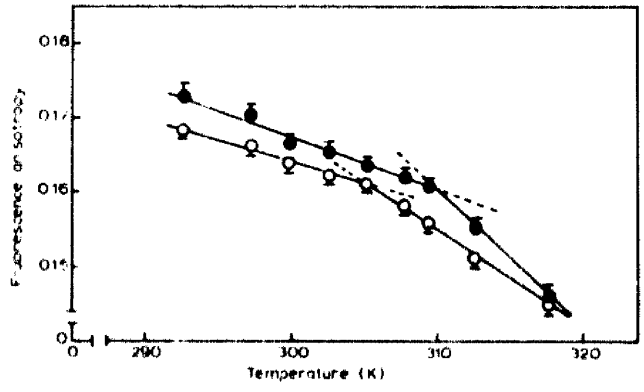

Fig. 5. Temperature-dependence of the fluorescence anisotropy of NPM-labeled membranes. The membrane concentration was 0.1 mg protein/ml. The temperature was varied from 20 to 45°C. ○, control membranes; ●, peroxidized membranes (100 μM ascorbic acid/10 μM Fe^{2+}/0.6 mM *t*-BuOOH). Values are expressed as the means ± S.D. for triplicate determinations.

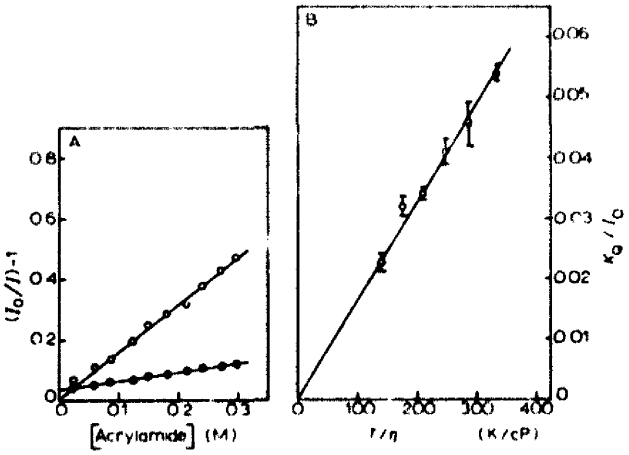

Fig. 6. Quenching studies of NPM-labeled AcCys and membranes by acrylamide at 25°C. (A) Stern-Volmer plots of NPM adducts. The concentrations of NPM-labeled AcCys and membrane protein were 10 μM and 0.1 mg/ml, respectively. The acrylamide concentration was varied from 33.1 to 300 mM. ○, NPM-AcCys adduct; ●, NPM-labeled membranes. Values are expressed as the means of 3 (○) and 5 (●) determinations, respectively. (B) Effect of viscosity (η) on the quenching of fluorescence intensity of NPM-AcCys adduct by acrylamide. NPM-AcCys adduct concentration was 1 μM. Changes in viscosity were produced by the addition of sucrose (5–25%, w/v). Acrylamide concentration was varied from 19.8 to 187.5 mM. The I_0 values represent the fluorescence intensity in the absence of acrylamide in each system. Values are expressed as the means ± S.D. for triplicate determinations.

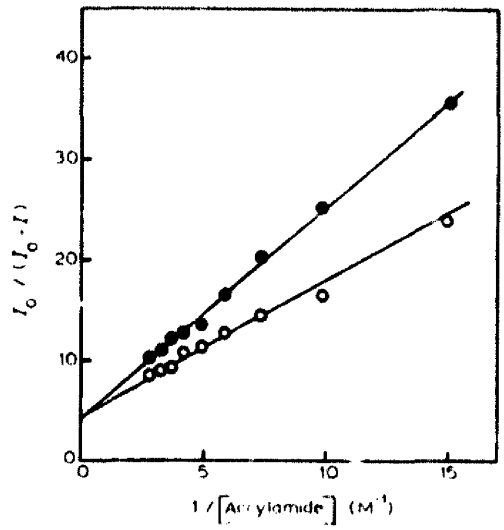

Fig. 7. Modified Stern-Volmer plots of NPM-labeled control and peroxidized membranes. The conditions and procedure of the quenching experiment were the same as those described in the legend to Fig. 6A. ○, control membranes; ●, peroxidized membranes (100 μM ascorbic acid/10 μM Fe^{2+}/0.6 mM *t*-BuOOH). Values are expressed as the means of triplicate determinations.

Quenching studies of NPM-labeled membranes

Figs. 6 and 7 show the results of the quenching experiments using acrylamide of NPM adducts with the membranes or AcCys.

The fluorescence intensity of NPM-AcCys adduct decreased with increasing concentrations of acrylamide and the plot of $(I_0/I)-1$ versus [Q] showed a linear relation up to 0.2 M acrylamide (Fig. 6A).

Since quenching phenomena can be predominantly of either the static or the dynamic type, we have examined the effect of viscosity on the quenching efficiency of NPM-AcCys adduct by acrylamide.

As shown in Fig. 6B, in the presence of increasing concentrations of sucrose, the K_Q/I_0 value of NPM-AcCys adduct fluorescence decreased linearly. This indicates that acrylamide causes a dynamic type of quenching of NPM fluorescence and that the mechanism of quenching involves predominantly a collisional process.

On the other hand, quenching of the fluorescence of NPM-labeled membranes by acrylamide did not follow a simple Stern-Volmer law; the intercept on the ordinate in the plot of $(I_0/I)-1$ versus [Q] derived from zero (Fig. 6A). Therefore, determination of the fluorescence quenching parameters of NPM-labeled membranes was performed using a modified Stern-Volmer equation proposed by Lehrer [20].

As shown in Fig. 7, the plots of $I_0/(I_0-I)$ versus 1/[Q] of the control and peroxidized membranes were both linear over the concentration range of the quencher tested, suggesting that the dye molecules in both the membranes are subjected to a similar degree of fluorescence quenching. The quenching parameters determined from these plots are presented in Table II.

Fig. 8 shows the relationship between the quenching rate constants and the amounts of TBAR formed by treatment of the membranes with 100 μM ascorbic acid and 10 μM Fe^{2+} in the presence of various concentrations of *t*-BuOOH.

TABLE II

Quenching parameters for acrylamide of NPM-labeled membranes

Data were obtained from Fig. 6. Values are expressed as means ± S.D. for triplicate determinations.

System	K_Q (M^{-1})	k_{q_1} (10^7 $M^{-1}\cdot s^{-1}$)	k_{q_2} (10^7 $M^{-1}\cdot s^{-1}$)	f_a
Control membranes	2.85 ± 0.10	19.5 ± 0.10 [a]	5.15 ± 0.15 [b]	0.25 ± 0.02
Peroxidized membranes	1.86 ± 0.08	14.4 ± 0.11 [a]	3.76 ± 0.10 [b]	0.25 ± 0.01

[a] Calculated using the τ_1 value
[b] Calculated using the τ_2 value

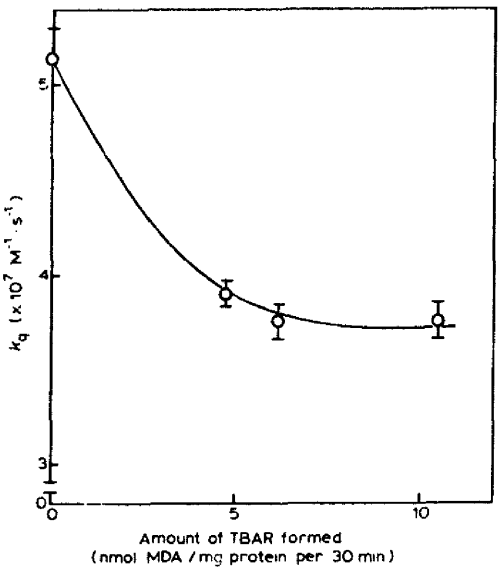

Fig. 8. Relationship between the quenching rate constant (k_q) and the amount of TBAR formed. The data of fluorescence lifetimes and TBAR formation were obtained from Figs. 4 and 2, respectively. The conditions and procedure of quenching experiment were the same as those described in the legend to Fig. 6A. Values are expressed as the means ± S.D. for triplicate determinations. MDA, malondialdehyde.

The quenching rate constant for acrylamide of the peroxidized membranes was smaller than that of the control ones (Table II) and the degree of decrease in the quenching rate constant by treatment of the membranes with ascorbic acid/Fe^{2+}/*t*-BuOOH was dependent on TBAR formation.

Reaction of the membranes with NPM

The incorporation of NPM into the SH groups in the control and peroxidized membranes was examined.

As can be seen in Fig. 9A, incubation of the control membranes with the dye causes a progressive development of NPM fluorescence at 392 nm with two phases, fast and slow reactivities. From the slope of the semilogarithmic plots of the fluorescence change versus the time of reaction with NPM, the pseudo-first-order rate constants, k', of the reaction of the SH groups belonging to the fast and slow phases in the control membranes were estimated to be 5.50 ± 0.27 and $1.90 \pm 0.14 \cdot 10^{-4}$ s^{-1}, respectively. On the other hand, in the peroxidized membranes (100 μM ascorbic acid/10 μM Fe^{2+}/0.6 mM *t*-BuOOH), the degree of NPM fluorescence development was markedly reduced and the k' values of the fast and slow phases were 3.68 ± 0.19 and $1.01 \pm 0.15 \cdot 10^{-4}$ s^{-1}, respectively. A good correlation between the degree of decrease in the k' values and *t*-BuOOH concentration is presented in Fig. 9B.

On the other hand, the time-course of the NPM fluorescence development of the membranes treated with ascorbic acid, Fe^{2+} and *t*-BuOOH alone were almost the same to that of the control membranes (data not shown).

Discussion

The effect of lipid peroxidation on the membrane structure was examined by monitoring the changes in the fluorescence characteristics of covalently bound NPM with SH groups in intestinal brush-border membrane proteins.

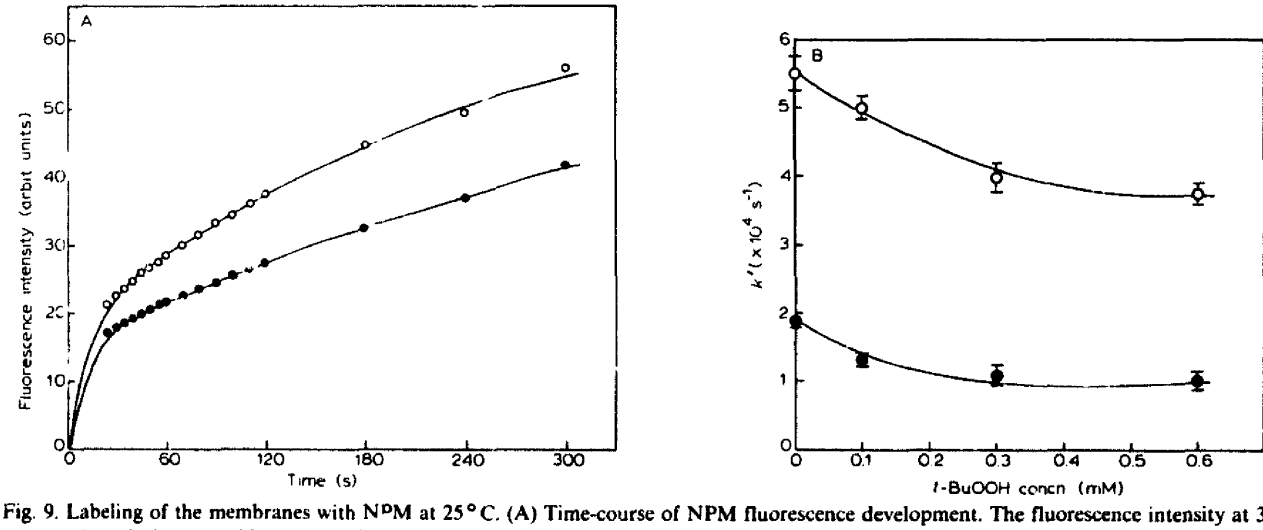

Fig. 9. Labeling of the membranes with NPM at 25 °C. (A) Time-course of NPM fluorescence development. The fluorescence intensity at 392 nm was monitored after the addition of 5 μM NPM (a- a final concentration) to the membrane suspension (0.1 mg protein/ml) in 10 mM phosphate buffer (pH 6.5). The excitation wavelength used was 340 nm. ○, control membranes; ●, peroxidized membranes (100 μM ascorbic acid/10 μM Fe^{2+}/0.6 mM *t*-BuOOH). (B) Relationship between the rate constants of NPM-labeling and *t*-BuOOH concentration. The membranes with different levels of lipid peroxidation were prepared as described in the legend to Fig. 4. The conditions employed in NPM-labeling measurement were the same as described in the legend to (A). ○, k' for the fast phase; ●, k' for the slow phase. Values are expressed as the means ± S.D. for triplicate determinations.

The fluorescence decay of NPM-labeled membranes (Fig. 3) indicated a multiplicity of physical states of the bound dye molecules in the membrane proteins, because the fluorescence decay of NPM-AcCys adduct follows a monoexponential function. The fluorescence lifetime of NPM-labeled membranes decreased depending on the amount of TBAR formed after treatment of the membranes with ascorbic acid/Fe^{2+}/*t*-BuOOH (Fig. 4). Since NPM specifically binds to SH groups of macromolecules [21], this change in the lifetime may reflect modification of environmental properties around NPM-labeled SH groups in the membrane proteins by lipid peroxidation.

Perturbation of the membrane structure in association with lipid peroxidation was also observed in the temperature-dependence of the steady-state fluorescence anisotropy of NPM-labeled membranes (Fig. 5). The degree of the fluorescence anisotropy gives us information about the mobility of fluorescence molecules [22]. Therefore an increase of the fluorescence anisotropy of bound NPM and a shift of the transition temperature of the anisotropy from about 32.0 to 37.0 °C (Fig. 5) by treatment of the membranes with oxidizing agents suggest a restricted motion of the bound NPM molecules by lipid peroxidation. This possibility was also suggested by a decrease in the acrylamide quenching rate for NPM fluorescence in the membranes.

The results of quenching experiments using NPM-AcCys adduct revealed that quenching of NPM fluorescence by acrylamide follows a simple Stern-Volmer law (Fig. 6). However, in the case of NPM-labeled membranes, the intercept on the ordinate in the plot of $(I_0/I) - 1$ versus the quencher concentration deviated from zero, indicating that several classes of fluorophore having different Stern-Volmer constants are present [20,23]. From the intercept on the ordinate in the modified Stern-Volmer plot, a value of $f_a = 0.25$ was obtained (Fig. 7 and Table II), indicating that about 25% of NPM molecules bound to the membrane proteins is accessible for quenching by acrylamide and the other 75% is not affected by acrylamide in the concentration range of acrylamide tested. By lipid peroxidation, the quenching rate constant for acrylamide of NPM fluorescence in the membranes decreased sensitively as shown in Table II. On the other hand, the f_a value did not change by lipid peroxidation of the membranes. This suggests that the amount of quenchable dye molecules in the membranes is not affected by lipid peroxidation. Therefore, it seems that a decrease in the quenching rate constant of NPM-labeled membranes after lipid peroxidation is mainly due to a decrease of the penetration of acrylamide in the vicinity of NPM molecules bound to SH groups in the membrane proteins.

The relationship between the quenching rate constant and TBAR formation (Fig. 8) suggests that the change in the accessibility of the quencher to the bound dye molecules in the membrane proteins is completed after about 6 nmol malondialdehyde per mg protein was formed. A similar phenomenon was observed in the relation between the fluorescence lifetime of the complex and TBAR formation (Fig. 4). The concentration-dependence of *t*-BuOOH on TBAR formation of the membranes in the presence of 100 μM ascorbic acid and 10 μM Fe^{2+} revealed that about 10.5 nmol malondialdehyde per mg protein is formed in the presence of 0.6 mM *t*-BuOOH (Fig. 2). From these results, it is suggested that the change in the environmental properties around the bound dye molecules is saturated at a certain degree of lipid peroxidation.

We have previously reported [9] that lipid fluidity of the membranes decreases depending on TBAR formation. This may reflect an overall increase in lipid–lipid interaction and packing density by lipid peroxidation. Such a modulation of the lipid fluidity may influence the topology and conformation of protein molecules in biological membranes [24,25]. However, the exact explanation for how a decrease in the lipid fluidity influences the protein conformation in the membranes is unclear at present. Recently, several investigators have reported [26–28] that membrane lipid composition is more important a factor in the regulation of certain protein-mediated activities than membrane lipid fluidity per se. Therefore, further detailed experiments, such as reconstitution study using liposomes containing various types of lipid, are necessary to obtain exact information about the contribution of physical state of membrane lipids to the protein conformation in the intestinal brush-border membranes.

Recently, Fodor and Marx [29] have reported that lipid peroxidation of rabbit intestinal microvillus membranes is induced even at a physiological iron concentration. This finding suggests that an overload of iron is capable of inducing lipid peroxidation of the microvillus membranes of enterocytes in vivo. Although the observations of in vitro experiments cannot be directly related to in vivo conditions, it seems that the results obtained in the present study may help us in the analysis of mechanism of cellular injury development in enterocytes by lipid peroxidation.

Acknowledgement

This study was supported by a Grant-in-Aid (No. 61571071, 63571065) from the Ministry of Education, Science and Culture of Japan.

References

1 Ames, B.N. (1983) Science 221, 1256–1264.
2 Halliwell, B. (1984) Med Biol. 62, 71–77.
3 Cerutti, P. (1985) Science 227, 357–381.
4 Till, G.O., Hatherill, J.R., Tourtellote, W.W., Lutz, M.J. and Ward, P.A. (1985) Am. J. Pathol. 119, 376–384.

5 Nakamoto, S., Yamanoi, Y., Kawabata, T., Sadahira, Y., Mori, M. and Awai, M. (1986) Biochim. Biophys. Acta 889, 15–22.
6 Evans, C.R. and Hochstein, P. (1981) Biochem. Biophys. Res. Commun. 100, 1537–1542.
7 Eichenberger, K., Bohni, P., Winterhalter, K.H., Kawato, S. and Richter, C. (1982) FEBS Lett. 142, 59–62.
8 Yamaguchi, T., Fujita, Y., Kuroki, O., Ohtsuka, K. and Kimoto, E. (1983) J. Biochem. 94, 370–386.
9 Ohyashiki, T., Ohtsuka, T. and Mohri, T. (1986) Biochim. Biophys. Acta 861, 311–318.
10 Sun, A.Y. (1972) Biochim. Biophys. Acta 266, 350–360.
11 Baba, A., Lee, E., Ohta, A., Tatsuno, T. and Iwata, H. (1981) J. Biol. Chem. 256, 3679–3684.
12 Lee, E., Baba, A., Ohta, A. and Iwata, H. (1982) Biochim. Biophys. Acta 689, 370–374.
13 Ohyashiki, T., Ohtsuka, T. and Mohri, T. (1988) Biochim. Biophys. Acta 939, 383–392.
14 Ohyashiki, T., Takeuchi, M., Kodera, M. and Mohri, T. (1982) Biochim. Biophys. Acta 688, 16–22.
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
16 Ohyashiki, T., Koshino, M., Ohta, A. and Mohri, T. (1985) Biochim. Biophys. Acta 812, 84–90.
17 Braughler, J.M., Duncan, L.A. and Chase, R.L. (1986) J. Biol. Chem. 261, 10282–10289.
18 Ohyashiki, T., Taka, M. and Mohri, T. (1985) J. Biol. Chem. 260, 6857–6861.
19 Stern, O. and Volmer, M. (1919) Z. Phys. 20, 183–188.
20 Lehrer, S.S. (1971) Biochemistry 10, 3254–3263.
21 Weltman, J.K., Szaro, R.P., Frackelton, Jr., A.R., Dowben, R.M., Bunting, J.R. and Cathou, R.E. (1973) J. Biol. Chem. 248, 3173–3177.
22 Shinitzky, M. and Barenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394.
23 Eftink, M.R. and Ghiron, C.A. (1976) Biochemistry 15, 672–680.
24 Borochov, H. and Shinitzky, M. (1976) Proc. Natl. Acad. Sci. USA 73, 4526–4530.
25 Gut, J., Kawato, S. Cherry, R.J., Winterhalter, K.H. and Richter, C. (1985) Biochim. Biophys. Acta 817, 217–228.
26 East, J.M., Jones, O.T., Simmonds, A.C. and Lee, A.G. (1984) J. Biol. Chem. 259, 8070–8071.
27 Connolly, T.J., Carruthers, A. and Melchior, D.L. (1986) J. Biol. Chem. 260, 2617–2620.
28 Brasitus, T.A., Dehiya, R., Dudeja, P.K.and Bissonnette, B.M. (1988) J. Biol. Chem. 263, 8592–8597.
29 Fodor, I. and Marx, J.J.M. (1988) Biochim. Biophys. Acta 961, 96–102.