Changes in the Fluorescence Characteristics of *N*-(1-Pyrene) Maleimide Bound to the Intestinal Brush-Border Membranes by Neuraminidase Treatment

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The effects of neuraminidase treatment on the dynamic properties of the porcine intestinal brush-border membranes have been examined by using a fluorogenic thiol reagent, *N*-(1-pyrene)maleimide (NPM). Desialylation of the membranes by treatment with neuraminidase resulted in changes in the fluorescence parameters of NPM-labeled membranes, *i.e.* a decrease of the fluorescence lifetime and a suppression of the temperature-dependent decrease of the fluorescence intensity. These results suggest that the environmental properties around NPM-labeled SH groups in the membrane proteins were modified by neuraminidase treatment. Perturbation of the microenvironment around NPM-labeled SH groups associated with desialylation by the enzyme treatment was also determined by measuring the increase of fluorescence anisotropy and decrease of quenching efficiency with acrylamide or CH_3COOTl of the complex. Based on the results, it is suggested that the dynamic properties of the conformation around NPM-labeled SH groups in the membrane proteins are sensitively influenced by neuraminidase treatment.

Keywords neuraminidase; sialic cid; fluorometry; fluorescent thiol reagent; brush-border membrane

It has been recognized[1-3] that the carbohydrate associated with the plasma membrane of animal cells as a constituent of glycoprotein or glycolipid components is involved in many of the physiological functions of the cells, *e.g.* cell adhesion, recognition, growth regulation and binding of specific antibodies or hormonal peptides. In addition, the surface carbohydrate moiety was recently proposed as a candidate regulatory element of the plasma membrane for cellular activities such as those mentioned above, presumably through influencing the dynamic properties of the lipid layers and proteins in biological membrane systems. The biological importance of sialic acids has been reported in many types of cells such as erythrocytes[4] and endothelial cells.[5]

Uchida *et al.*[6] have reported that the molecular packing of the hydrocarbon region in dipalmitoylphosphapidylcholine-ganglioside mixed dispersion is dependent on the number and linkage position of the sialic acid residues in the gangliosides. In addition, we have also recently demonstrated[7] that desialylation of the porcine intestinal brush-border membranes by treatment with neuraminidase causes an increase of the membrane lipid fluidity.

As is well known,[8,9] the dynamic properties of the lipid and proteins and their interactions in biological membranes are important factors in the regulation of physiological functions. Therefore it is of interest to study the effect of modification of sialic acid residues on the dynamic properties of the protein moieties in the membranes.

N-(1-Pyrene)maleimide (NPM) is itself almost nonfluorescent but fluoresces strongly after reacting with organic compounds or proteins containing sulfhydryl groups.[10,11] In addition, the adduct of NPM with proteins has a long lifetime, *e.g.* $\tau = 94$—101 ns for NPM-labeled bovine serum albumin, myosin and aldolase.[10] Therefore this probe is useful for analysis of the properties of the environment around SH groups in the membrane proteins.

In the present study, we examined using NPM the effect of neuraminidase treatment on the physical properties of the porcine intestinal brush-border membranes.

Experimental

Materials NPM was purchased from Molecular Probe Co. and dissolved in acetone to make a stock solution (1 mM). Neuraminidase (*Clostridium perfringens*, type VI) was obtained from Sigma Co. and dissolved in 0.1 M Tris–acetate buffer (pH 5.5) containing bovine serum albumin (0.3 mg/ml) and stored at −20 °C until use.

Preparation of Membrane Vesicles The brush-border membranes were prepared from the porcine small intestine by the calcium-precipitation method described in our previous paper[12] and the final pellets were suspended in 10 mM Tris–maleate buffer (pH 6.85). Protein concentration was assayed by the procedure of Lowry *et al.*[13] using bovine serum albumin as standard.

Labeling of *N*-Acetylcysteine or Membrane Proteins with NPM Labeling of *N*-acetylcysteine (AcCys) with NPM was carried out by incubating 0.5 mM AcCys and 10 μM NPM in 30 mM Tris–maleate buffer (pH 6.85) at 0 °C for 5 min. Reaction of the membranes with NPM was performed as follows. The membrane suspension (4—5 mg protein/ml) was incubated with 10 μM NPM in 30 mM Tris–maleate buffer (pH 6.85) at 0 °C for 30 min. The final concentration of acetone in the reaction mixture was 0.33%. The reaction was terminated by addition of a 20-fold excess of 2-mercaptoethanol over NPM added. The reaction mixture was then gel-filtered on a Sephadex G-50 column (1.2 × 11 cm) equilibrated with 10 mM Tris–maleate buffer (pH 6.85) to remove NPM-2-mercaptoethanol adduct. The membrane-containing eluate was centrifuged at 25000 × *g* for 20 min. The pellets were washed twice with and resuspended in 10 mM Tris–maleate buffer (pH 6.85). Determination of the amount of NPM bound to the membrane proteins was performed as follows. The NPM-labeled membranes (0.4 mg protein/ml) were incubated in 10 mM Tris–maleate buffer (pH 9.0) containing 1% sodium dodecyl sulfate at 37 °C for 18 h. The amount of NPM bound to the membrane proteins was determined using the molar extinction coefficient of 17400 at 339 nm for the ring-opened type of NPM-2-mercaptoethanol adduct. Under the conditions employed in the present study, about 6.1—6.9 nmol of NPM per mg of protein was incorporated into the membrane proteins.

Treatment of the Membranes with Neuraminidase The NPM-labeled membranes (1 mg protein/ml) were incubated with neuraminidase (10 μg/ml) in 30 mM phosphate buffer (pH 6.5) at 37 °C for 30 min, unless otherwise specified. The reaction was terminated by dilution with a large volume of 10 mM Tris–maleate buffer (pH 6.85), followed by centrifugation at 25000 × *g* for 20 min. The pellets were washed twice and resuspended in the same buffer. The control membranes were treated in a similar manner without neuraminidase. The content of sialic acids in the membrane vesicles was assayed according to the thiobarbituric acid method of Warren.[14]

Fluorescence Measurements Fluorescence measurements were performed using a Hitachi MPF-4 spectrofluorometer equipped with a rhodamine B quantum counter. The sample compartment was maintained at 25 °C by circulating water through the cell holder unless otherwise specified. The excitation and emission wavelengths used were 340 and 375 nm, respectively. The error due to light scattering of the sample emission could be entirely prevented by 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. The fluorescence lifetime measurements were performed by the single photon counting method with an Ortec PRA-3000 nanosecond fluorometer (Photochemical Research Associates Inc., Ontario, Canada). Analysis of data was carried out using a Digital MINC-11 computer system (Digital Equipment Co., Maynard, U.S.A.).

Quenching Studies Quenching studies were carried out at 25 °C by adding a small amount of 0.1 M thallium (I) acetate (CH_3COOTl) or 5 M acrylamide in 10 mM Tris–maleate buffer (pH 6.85). The stock solutions of these quenchers were used within a few days after preparation. The quenching constant (K_Q) was calculated according to the following equations given by Stern and Volmer (1)[15] and Lehrer (2)[16]:

$$(I_o/I) - 1 = K_Q[Q]$$

$$I_o/(I_o - I) = \frac{1}{f_a K_Q[Q]} + \frac{1}{f_a} \quad (2)$$

where I_o, I, [Q] and f_a denote the fluorescence intensities in the absence and presence of quencher, the quencher concentration and the fraction of fluorophores accessible to the quencher, respectively. A plot of $I_o/(I_o - I)$ *versus* $1/[Q]$ intercepts the y axis at $1/f_a$. The quenching rate constant (k_q) was estimated from the relation $k_q = K_Q/\tau_o$, where τ_o is the fluorescence lifetime in the absence of quencher.

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 Co.: triosephosphate isomerase (26.6 kilodaltons (kDa)), lactic 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 fluorescent bands in a gel were detected by using a Shimadzu CS-9000 double-beam flying spot scanner with the excitation wavelength of 340 nm.

Results and Discussion

Labeling of the Membranes with NPM Incubation of the porcine intestinal brush-border membranes with NPM resulted in a marked development of the dye fluorescence. The emission spectrum of the complex exhibits a superposition of the intense fluorescence band from 375 to 410 nm with a shoulder peak at about 420 nm.

On the other hand, incubation of NPM with liposomes prepared from phosphatidylcholine or phosphatidylethanolamine did not induce fluorescence development, indicating that NPM does not react with amino groups in the membrane lipids. In addition, the partition coefficient of NPM to aqueous buffer/*n*-hexane mixture was found to be about 1 : 8. From these results, it is concluded that NPM is able to react with SH groups located in the polar and nonpolar regions in the membrane proteins. As shown in Fig. 1, the labeling pattern of the membrane proteins with NPM revealed that NPM was incorporated into 5 protein fractions corresponding to 36, 40.7, 56, 80 and 106 kDa in molecular weight and that the strongly fluorescent bands were mainly those of the 36 and 80 kDa proteins (Table I).

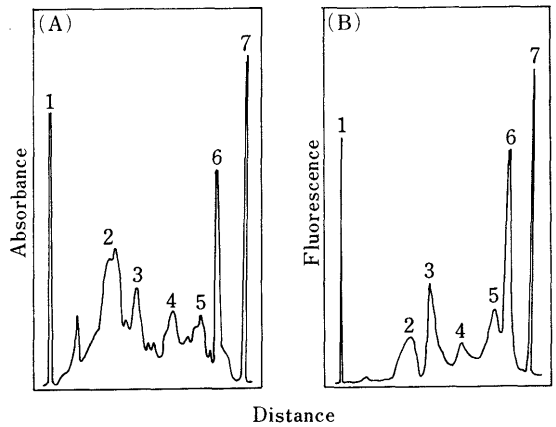

Fig. 1. Densitometric Pattern after SDS-Polyacrylamide Gel Electrophoresis of NPM-Labeled Membranes

(A) Coomassie brilliant blue staining; (B) fluorescence image. Positions: 1, gel top; 2, 106 kDa; 3, 80 kDa; 4, 56 kDa; 5, 40.5 kDa; 6, 36 kDa; 7, marker.

Changes in Fluorescence Parameters of NPM-Labeled Membranes on Neuraminidase Treatment The temperature dependence of the fluorescence intensity of NPM-labeled membranes was examined in the range from 14 to 48 °C. As can be seen in Fig. 2, the fluorescence intensity of the control membranes progressively decreased with elevating temperature, indicating that the environmental properties around the dye molecules bound to the membrane proteins are sensitively modified by temperature variation. On the other hand, in the neuraminidase-treated membranes, the degree of temperature-dependent decrease of the fluorescence intensity was suppressed in all temperatures tested.

The fluorescence lifetime of NPM molecules bound to the membrane proteins was also changed by neuraminidase treatment. The fluorescence decay of NPM-AcCys adduct was well characterized as a single exponential system and the lifetime was determined to be 6.1 ± 0.1 ns. This value is very close to that reported by Weltman *et al.*[10] for the cysteine (6.5 ns) or 2-mercaptoethanol (5.9 ns) adduct.

On the other hand, the fluorescence decay of NPM-labeled membranes did not follow a single exponential function

Table I. Apparent Distribution of Fluorescent Bands in a Gel

Gel peak position	Protein fraction (kDa)	Fluorescence area[a] (%)
2	106.0	15.9
3	80.0	22.6
4	56.0	10.8
5	40.7	13.1
6	36.0	23.3

a) These values are expressed relative to total fluorescence in a gel.

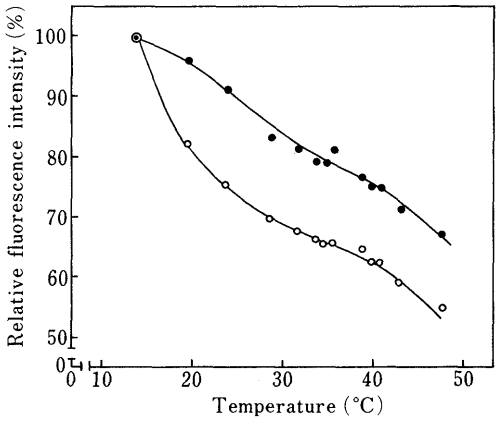

Fig. 2. Temperature Dependence of Fluorescence Intensity of NPM-Labeled Membranes in 10 mM Tris–Maleate Buffer (pH 6.85)

The concentrations of NPM-labeled membrane proteins were 69.1 and 69.6 μg/ml for the control (○) and neuraminidase-treated (●) membranes, respectively. Temperature was varied from 14 to 48 °C. The fluorescence intensity is shown relative to that at 14 °C in each system.

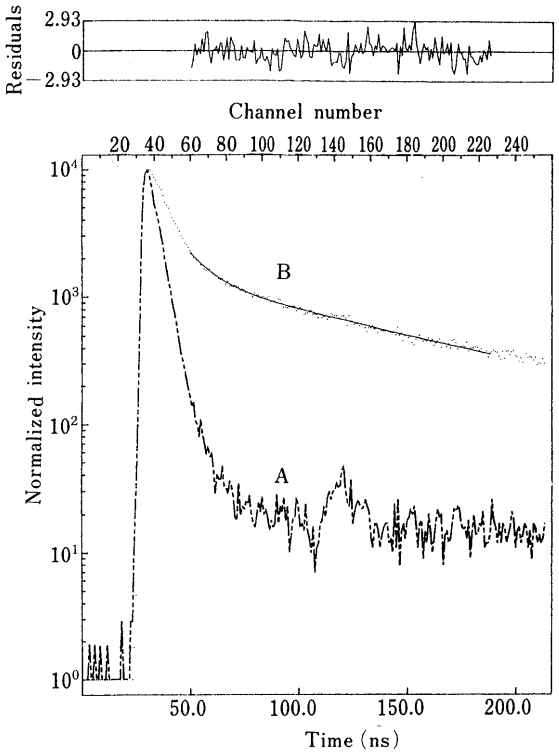

Fig. 3. Fluorescence Decay Curve of NPM-Labeled Control Membranes

Membrane protein concentration was 150 μg/ml. Curves A and B represent excitation pulse and fluorescence of NPM-labeled membranes, respectively. The solid line in curve B shows the result of curve fitting by the non-linear least-squares method. The value of the residuals is shown above the graph. 25 °C.

TABLE II. Fluorescence Lifetime of NPM-Labeled Membranes in 10 mM Tris–Maleate Buffer (pH 6.85) at 25 °C

Membranes	Lifetime (ns)	
	τ_1	τ_2
Control	12.4±0.24	130.1±1.16
Neuraminidase-treated	8.8±0.19	104.1±1.00

The concentration of NPM-labeled membranes was 150 μg protein/ml. The values are expressed as mean (n=4) ±S.D.

(Fig. 3). This indicates the existence of heterogeneity in the emitting population of the dye molecules due to differences in the environmental properties around the labeled SH groups in the membrane proteins. We assumed tentatively that there are two components in the decay process of the fluorescence to analyze its lifetime. By computer fitting, the mean values of the lifetime of the short and long components of NPM-labeled control membranes were estimated to be 12.4 ns (τ_1), accounting for 15% of the total fluorescence, and 130.1 ns (τ_2), accounting for 85%, respectively (Table II). As shown in Table II, the fluorescence lifetime of the complex was markedly decreased by treatment of the membranes with neuraminidase.

Next we examined the temperature dependence of the fluorescence anisotropy of NPM-labeled membranes in order to obtain information about the movement of NPM molecules bound to SH groups in the membrane proteins, because the degree of the fluorescence anisotropy reflects the mobility of the fluorophore attached to macromolecules.[17]

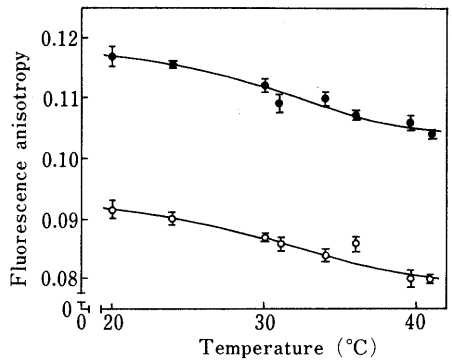

Fig. 4. Temperature Dependence of Fluorescence Anisotropy of NPM-Labeled Membranes in 10 mM Tris–Maleate Buffer (pH 6.85)

The membrane protein concentration was 80 μg/ml. Temperature was varied from 20 to 41 °C. ○, control membranes; ●, neuraminidase-treated membranes. Values are expressed as mean ± S.D. for triplicate determinations.

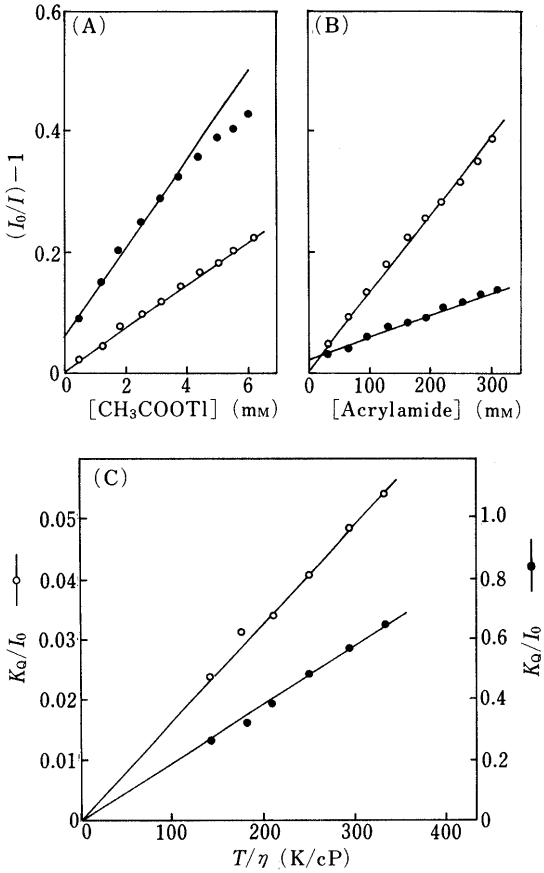

Fig. 5. The Stern–Volmer Plots of Fluorescence Quenching of NPM Adducts by Tl^+ (A) and Acrylamide (B) at 25 °C in 10 mM Tris–Maleate Buffer (pH 6.85) and (C) Viscosity Dependence of Fluorescence Quenching of NPM-AcCys Adduct

The concentrations of NPM-AcCys adduct and NPM-labeled membranes in (A) and (B) were 1 μM and 80 μg protein/ml, respectively. The CH_3COOTl and acrylamide concentrations were varied from 0.66 to 6.25 mM and from 33.1 to 312.5 mM, respectively. ○, NPM-AcCys adduct; ●, NPM-labeled membranes. For (C) the viscosity was varied by the use of sucrose (0—25%). The acrylamide and CH_3COOTl concentrations were varied from 19.8 to 187.5 mM and 0.66 to 6.25 mM, respectively. ○, acrylamide quenching; ●, Tl^+ quenching. The I_0 value shows the fluorescence intensity in the absence of quencher in each system. Other experimental conditions were the same as for (A) and (B).

As shown in Fig. 4, the degree of the fluorescence anisotropy of the neuraminidase-treated membranes was slightly but definitely larger than that of the control ones over the temperature range tested (15—41 °C). This result

TABLE III. Effect of Neuraminidase Treatment on the Quenching Parameters of NPM-Labeled Membranes with Acrylamide or Tl^+

Quencher	Control membranes			Neuraminidase-treated membranes		
	K_Q (M^{-1})	$k_q \times 10^7$ ($M^{-1} \cdot s^{-1}$)	f_a	K_Q (M^{-1})	$k_q \times 10^7$ ($M^{-1} \cdot s^{-1}$)	f_a
Acrylamide	3.09±0.56	2.37±0.20	0.248±0.021	1.94±0.23	1.49±0.27	0.288±0.032
CH_3COOTl	344.1±15.0	330.5±12.8	0.448±0.018	247.1±11.2	237.1±11.5	0.469±0.012

The k_q values were calculated using the τ_2 values from the data shown in Fig. 6 and are expressed as mean ($n=4$—6)±S.D.

suggests that the environmental constraint around the labeled NPM molecules is increased by treatment of the membranes with neuraminidase. In this case, it seems that increased fluorescence anisotropy of the labeled membranes caused by neuraminidase treatment is not due to an increase of energy transfer efficiency in the excited state between the labeled NPM molecules, because the temperature dependence profiles of the fluorescence intensity (Fig. 2) and the anisotropy of the complex are different.

Fluorescence Quenching Studies Prior to measurements of quenching of NPM bound to the membrane proteins, the effects of acrylamide and CH_3COOTI on the fluorescence of a model adduct, NPM-AcCys, were examined.

The fluorescence intensity of the adduct decreased with increasing concentrations of these quenchers and the plots of $(I_0/I)-1$ *versus* [Q] showed good linear relations over the tested concentration ranges of these quenchers (Figs. 5A and B).

In general, quenching phenomena can be predominantly of either the static or dynamic type. In dynamic quenching, the quenching efficiency is controlled by the collisional process between the fluorophore and quencher molecules, and the rate constant for quenching should vary inversely with the bulk viscosity because $k_q \alpha D_Q$, where D_Q is the diffusion coefficient of the quencher.[18] In addition, $k_q = K_Q/\tau_0$ is proportional to K_Q/ϕ_0 or K_Q/I_0 because τ_0 is proportional to the quantum yield ($\phi_0 \approx I_0$) in the absence of quencher. Therefore, in the diffusion-controlled quenching mechanism, K_Q/I_0 is proportional to T/η, where T and η represent the absolute temperature and the viscosity, respectively. On the other hand, in static quenching, the K_Q value is independent of the viscosity.

In order to clarify which mechanism is involved in the NPM fluorescence quenching by acrylamide and CH_3COOTl, we have examined the effects of viscosity on the quenching efficiency of NPM-AcCys adduct by these quenchers. As shown in Fig. 5C, the degrees of the fluorescence quenching of NPM-AcCys adduct by these quenchers were dependent on the viscosity of the medium. From these results, it is clear that the mechanism of quenching of NPM fluorescence with acrylamide or CH_3COOTl involves predominantly a collisional process.

On the other hand, the plots of $(I_0/I)-1$ of NPM-labeled membranes against the concentration of quencher are distinctly different, having a larger ordinate intercept (Figs. 5A and B). This result suggests that NPM molecules bound to the membrane proteins are located in various environments with different accessibility for the quencher molecules.[16,19] This possibility is also supported by the existence of heterogeneity in the fluorescence decay of NPM-labeled membranes (Table I). Therefore the quenching parameters for acrylamide and Tl^+ in the control and neuraminidase-treated membranes were determined by calculation from modified Stern–Volmer plots.

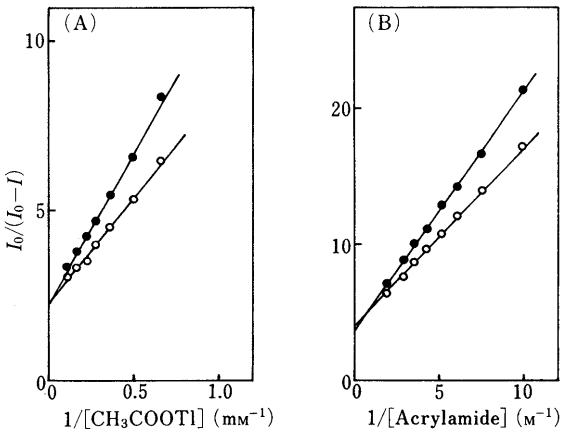

Fig. 6. The Modified Stern–Volmer Plots of Quenching of NPM-Labeled Membranes

The experimental conditions were the same as described in the legend to Figs. 5A and B. ○, control membranes; ●, neuraminidase-treated membranes. (A) CH_3COOTl; (B) acrylamide.

As can be seen in Fig. 6, the plots of $I_0/(I_0-I)$ *versus* 1/[Q] of the control and neuraminidase-treated membranes showed good linear relationships in the ranges of quencher concentration tested. The slopes in these plots for the neuraminidase-treated membranes were larger than those for the control membranes, suggesting that the quenching efficiencies of acrylamide and Tl^+ for NPM fluorescence in the membrane are decreased by neuraminidase treatment. In fact, the values of the quenching rate constant, k_q, for these quenchers were decreased by the treatment (Table III).

On the other hand, the f_a values for these quenchers in the control membranes, 0.25 for acrylamide and 0.45 for Tl^+, allow us to estimate that about 25 and 45% of NPM molecules bound to the membrane proteins are susceptible to acrylamide and Tl^+ quenching, respectively. These f_a values were scarcely changed by treatment of the membranes with neuraminidase (Table III). Therefore it seems that the decrease of the quenching efficiencies of these quenchers after the enzyme treatment is mainly attributed to a decrease of collisional efficiency between the quencher molecules and fluorophores in the membrane proteins.

However, the efficiency of fluorescence quenching by an ionized quencher is also influenced by the dielectric properties of the microenvironment surrounding the fluorophore and its ability to quench should be dependent on the location of neighboring charged groups.[16,20] Therefore the decreased quenching efficiency with Tl^+ of NPM-labeled membranes would imply a decreased accessibility of the quencher molecules to NPM-labeled SH groups and/or a

decreased susceptibility of the conformation around the dye-labeled SH groups in the membrane proteins for Tl^+-binding to negatively charged groups among the membrane components, *i.e.* an increase of rigidity of the conformation around the labeled SH groups.

Judging from all of these results, it is suggested that changes in the dynamic feature of the membranes associated with neuraminidase treatment are sensitively reflected in the fluorescence properties of NPM molecules bound to the membrane proteins. Since NPM specifically binds to SH groups in macromolecules,[10,11] it could be considered that the fluorescence changes observed in the present study may reflect changes in the vicinity of NPM-labeled SH groups in the membrane proteins.

A suppression of the temperature-dependent decrease of the fluorescence intensity (Fig. 2), an increased fluorescence anisotropy (Fig. 4) and a decreased quenching efficiency with acrylamide or Tl^+ (Table III) of NPM-labeled membranes after treatment with neuraminidase suggest that the environmental constraint around NPM-labeled SH groups in the membrane proteins is increased by desialylation.

Previously, we have demonstrated[7] by measurement of the rotational correlation time of diphenylhexatriene embedded in the membrane lipids that the lipid fluidity is increased by neuraminidase treatment of the membranes. Therefore there is the possibility that changes in the fluorescence characteristics of NPM molecules bound to the membrane proteins observed in the present study are partly related to the nature of the lipid organization in the membranes, because the dynamic features of protein molecules in biological membranes are closely related to the nature of lipid organization.[21,22] Further detailed investigations including reconstitution studies could shed additional light on this problem.

Recently Handa and Nakamura have demonstrated[23,24] by the chemical modification technique that the carboxy groups of sialic acids have an important role in the immunogenicity. These findings suggest that sialic acids are closely related to the physiological functions of biological membrane systems. Although it is not clear at present whether or not the results obtained in the present study are directly related to the physiological functions of sialic acids, it is possible that sialic acids play an important role in regulating the dynamic properties of the porcine intestinal brush-border membranes.

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