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Characterization of interaction between Tb^{3+} and porcine intestinal brush-border membranes

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Effects of ionic strength and temperature on the interaction between Tb^{3+} and porcine intestinal brush-border membrane vesicles were studied. When Tb^{3+} was added to the vesicle suspension, Tb^{3+} fluorescence increased with increasing concentration of Tb^{3+}, showing a saturation. The apparent dissociation constant of one of at least two components of this binding reaction was estimated to be about 12.5 μM at 25°C, pH 7.4. But the affinity of Tb^{3+} for the membrane vesicles was variable with changes of ionic strength and temperature. The affinity was lowered by addition of KCl to medium and by increase of temperature above 30°C. In addition, temperature-induced change in the affinity of Tb^{3+} for the membranes was reversible over a temperature range from 13 to 46°C. Temperature-dependence profiles of the excimer formation efficiency of pyrene-labeled membranes and of the harmonic mean of the rotational relaxation times of pyrene molecules in the membranes revealed that the phase transition of the membrane lipids occurs at about 30°C. Based on these results, characteristics of Tb^{3+} binding to the membranes are discussed in relation to the nature of lipid phase and surface charges of the membranes.

Introduction

It has been recognized that Ca^{2+} plays as a trigger ion of many important cellular events [1,2] and that the lipid organization of biological membranes is modified by interaction with Ca^{2+} [3], but the specific membrane structures relevant to the binding of calcium ions are so far not fully characterized. Therefore, it is of interest to study what kinds of factors are concerned with the interaction of Ca^{2+} and the membrane components in biological membrane systems at the molecular level. One experimental approach that has been applied to the problem is the use of the trivalent lanthanide ions.

As is well known, a lanthanide ion, Tb^{3+}, can be substituted for Ca^{2+} in the study of interaction of Ca^{2+} with biomolecules [4], because these two cations share a very similar ionic radius and coordination number [5]. Moreover Tb^{3+} has the advantage of fluorescence emission. Tb^{3+} thus has been widely used in analyses of Ca^{2+}-binding sites of biological membrane systems such as erythrocytes [6], mitochondria [7], chromaffin granules [8] and intestinal mucosa [9] as well as Ca^{2+}-binding proteins [4,5,10].

In the present study, we have utilized Tb^{3+} in characterizing Ca^{2+}-binding sites of porcine intestinal brush-border membranes and explored the effects of ionic strength and temperature on Tb^{3+}-binding capacity of the membranes. Results suggested that the Tb^{3+}-binding capacity is partly related to the nature of lipid layers of the membranes as well as the membrane surface charges.

Materials and Methods

Preparation of membrane vesicles

The brush-border membranes were prepared from porcine small intestine according to the method of calcium precipitation described in our previous paper [11] and suspended in 10 mM Tris-HCl buffer (pH 7.4). Protein concentration was assayed by the procedure of Lowry et al. [12] using bovine serum albumin as standard.

Labeling of membrane vesicles with pyrene

Suspension of the membrane vesicles (1 mg/ml protein) in 10 mM Tris-HCl buffer (pH 7.4) were incubated with 0.5–1.0 μM pyrene at 0°C for 30 min. The reaction was terminated by dilution of the reaction mixture with an excess amount of the same buffer and centrifuged at $25\,000 \times g$ for 20 min. The pellets obtained were washed twice with the same buffer and resuspended in 10 mM Tris-HCl buffer (pH 7.4).

Fluorescence measurements and calculation of fluorescence parameters

Fluorescence measurements were performed using a Hitachi spectro-fluorometer, MPF-4, equipped with a rhodamine B quantum counter. The excitation and emission wavelengths used were 280 and 550 nm for Tb^{3+} fluorescence and 280 and 350 nm for native fluorescnece measurements, respectively, unless otherwise mentioned. The excimer formation efficiency of pyrene was expressed as the value of the excimer-to-monomer fluorescence ratio, I_E/I_M, calculated from the fluorescence intensities at 470 nm (for the excimer) and 392 nm (for the monomer) with excitation at 340 nm as described in our previous paper [13].

The degree of fluorescence polarization, P, of pyrene was expressed as the value of $(I_V - I_H)/(I_V + I_H)$, where I_V and I_H are the intensities of vertically and horizontally polarized fluorescence with vertically polarized light, respectively. The harmonic mean of the rotational relaxation times, ρ_h, of pyrene was determined using the slope of a $1/P$ versus T/η plot, fluorescence lifetime, and the following equation [14] according to Perrin [15] and Weber [16]:

$$\rho_h = \left(\frac{1}{P_0} - \frac{1}{3}\right)\frac{3\tau}{\text{the slope}} \cdot \frac{\eta}{T}$$

where P_0 is the limiting polarization at $T/\eta = 0$, η the viscosity of the solvent, τ the fluorescence lifetime and T the absolute temperature, respectively.

Fluorescence lifetime was measured by the single photon technique with an Ortec PRA-3000 nanosecond spectrofluorometer with a 7100 multichannel analyzer (Phtochemical Research Associates, Ontario, Canada). Analysis of data was carried out using a Digital NINC-11 computer system (Digital Equipment Co., Maynard, MA, U.S.A.).

Reagents

$TbCl_3$ and pyrene were purchased from Mitsuwa Pure Chemical Co. and Wako Pure Chemical Co., respectively. The stock solution (1 mM) of $TbCl_3$ was made by dissolving in water and adjusted to pH 7.4 with 1 M KOH. Pyrene was recrystallized from ethanol once before use and dissolved in ethanol to make a stock solution (1 mM). All other chemicals were of the purest grade commercially obtainable.

Results and Discussion

Fluorescence characterization of Tb^{3+} bound to membrane vesicles

Fig. 1 shows the Tb^{3+} fluorescence spectra in the presence and absence of porcine intestinal

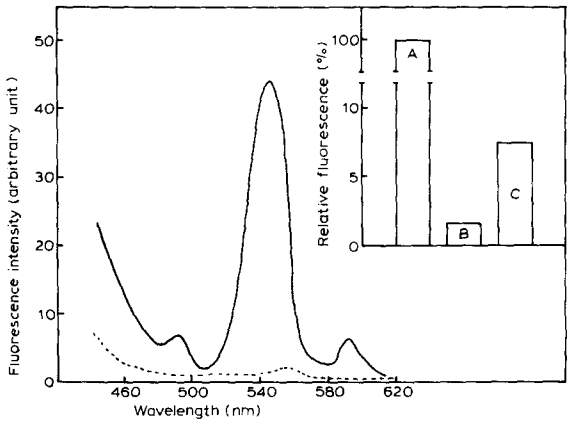

Fig. 1. Emission spectra of Tb^{3+} in the presence and absence of the membranes with excitation of 280 nm at 25°C. 10 mM Tris-HCl buffer (pH 7.4). The membrane protein concentration was 0.100 mg/ml. Tb^{3+} concentration was 6.7 μM. ———, Tb^{3+}-membrane complex; - - - - - -, Tb^{3+} alone. Inset: Excitation and emission wavelengths are 280 and 550 nm (A), 260 and 550 nm (B) and 290 and 550 nm (C), respectively. The fluorescence intensity was expressed relative to that of (A).

brush-border membranes at pH 7.4. When the membrane suspension was mixed with 6.7 μM Tb^{3+}, the fluorescence intensity of Tb^{3+} at 494, 550 and 590 nm was markedly enhanced with excitation at 280 nm. The development of Tb^{3+} fluorescence was the highest with excitation at 280 nm. With the excitation wavelength of either 260 or 290 nm, the magnitude of Tb^{3+} fluorescence at 550 nm was less than ten percent of that with 280 nm (inset in Fig. 1). Appearance of Tb^{3+} fluorescence by addition of the membrane suspension is mainly and probably due to energy transfer from tyrosine residues in the membrane proteins to bound terbium ions [4,5].

As shown in Fig. 2, the development of Tb^{3+} fluorescence with the membranes was dependent on Tb^{3+} concentrations. The double reciprocal plot of the fluorescence intensities against Tb^{3+} concentrations showed a conspicuous bending in the curve (inset in Fig. 2), suggesting more than one types of Tb^{3+}-binding sites with different affinities in the membrane components. One of the apparent dissociation constants (K_d) was estimated at 12.5 μM. This value is comparable with a K_d value of rat intestinal mucosal membranes [9].

Fig. 3 shows the effect of ionic strength on the interaction of Tb^{3+} with the membrane vesicles. The fluorescence intensity of Tb^{3+}-membrane

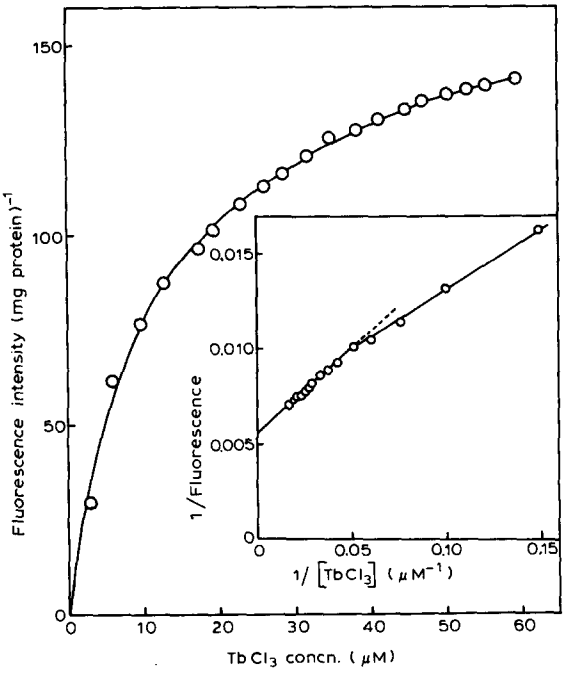

Fig. 2. Titration profile of the membranes with Tb^{3+}. The membrane protein concentration was 0.183 mg/ml. Tb^{3+} concentration was varied from 3.32 to 59.6 μM. The fluorescence intensity was expressed as the value per mg protein. The inset figure represents the double reciprocal plot of Tb^{3+} fluorescence in the membranes against the Tb^{3+} concentration. Excitation and emission wavelengths used were 280 and 550 nm, respectively. Other experimental conditions were the same as described in the legend to Fig. 1.

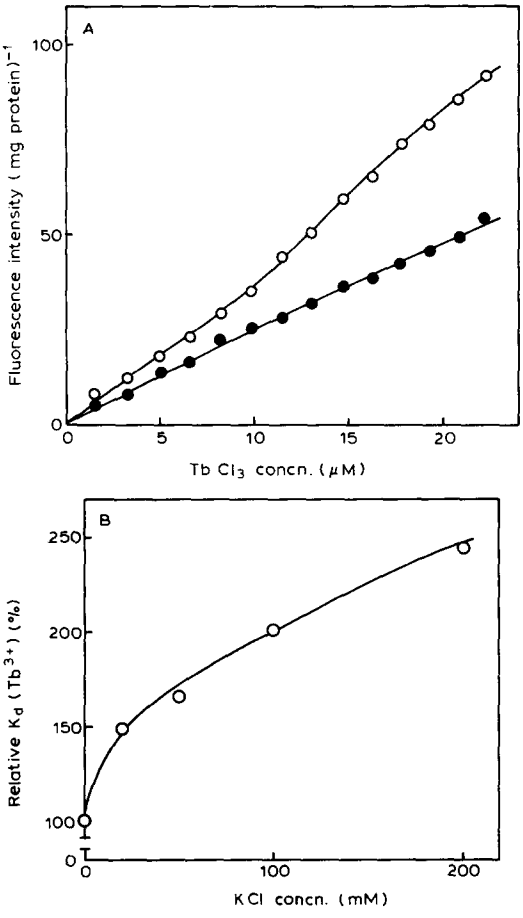

Fig. 3. Effect of KCl on the interaction between Tb^{3+} and the membranes. (A) Titration profiles of the membranes with Tb^{3+} in the presence (●) and absence (○) of 50 mM KCl. The membrane protein concentration was 0.179 mg/ml. The Tb^{3+} concentration was varied from 1.5 to 22.2 μM. Other experimental conditions were the same as described in the legend to Fig. 2. (B) The plot of apparent dissociation constant, $K_d(Tb^{3+})$, of the Tb^{3+}-membrane complex against the KCl concentration in medium. The $K_d(Tb^{3+})$ values were expressed as relative to that in the absence of KCl.

complex markedly reduced by addition of 50 mM KCl (Fig. 3A). It can be deduced from the result shown in Fig. 3B that the decrease of Tb^{3+} fluorescence by the addition of KCl is due to the reduction of the binding affinity of Tb^{3+} for the membrane vesicles. It would be considered that the binding of Tb^{3+} to the membrane components is mainly electrostatic and that negative charges of the membrane surface are neutralized by addition of KCl.

We previously demonstrated that shielding of negatively charged groups of the membrane components of rabbit [17,18] and porcine [19] intestinal brush-border membranes by increasing ionic strength of medium results in an increase of the membrane fluidity. So we attempted then to examine the relationship between Tb^{3+}-binding capacity and lipid rearrangement of the membrane vesicles. Characterization of the thermotropic transition of the lipids and Tb^{3+}-binding of the membrane system would be useful [20] for the purpose.

Effect of temperature on Tb^{3+}-binding to membranes

Fig. 4 shows the change of fluorescence intensity of the complex of Tb^{3+} (6.7 μM) and the membranes at pH 7.4 as a function of temperature. The fluorescence intensity of the complex gradually decreased with increasing temperature, showing the half-maximal at about 28.5°C. In addition, this fluorescence change of the complex induced by elevating temperature was reversible over a temperature range tested.

Fig. 5 shows a temperature dependence profile of the Tb^{3+}-binding parameter for the membranes. The apparent dissociation constant of Tb^{3+}-membrane complex was estimated by the procedure of the double-reciprocal plot of fluorescence intensities at varying temperatures against Tb^{3+} concentrations.

The dissociation constant did not notably change below 30°C, but the value was steeply elevated as the temperature was increased above 30°C up to 47°C. In this case, the temperature-dependence profile of the native fluorescence of the membranes revealed reversibility in the temperatures ranging from 13 to 46°C (Fig. 6), suggesting that the protein denaturation did not occur over a temperature range tested. From these results, therefore, it seems likely that decrease of the fluorescence intensity of the Tb^{3+}-membrane complex with temperatures above about 30°C is attributed to decrease in the binding affinity of Tb^{3+} and/or extension of the effective distance for the energy transfer between the donor (tyrosine residues) and

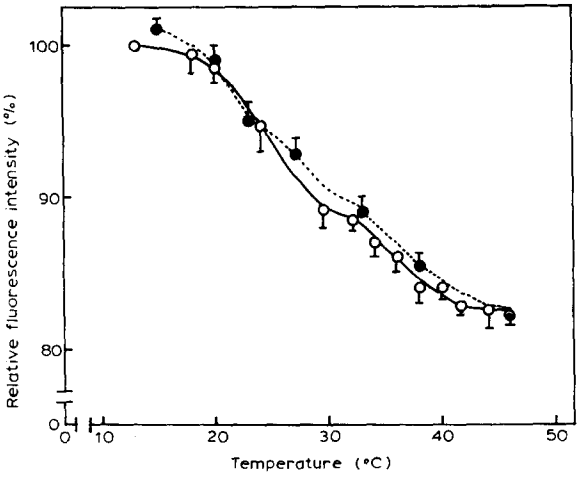

Fig. 4. Temperature dependence of fluorescence intensity of Tb^{3+}-membrane complex. The Tb^{3+} and membrane protein concentrations were 6.7 μM and 0.210 mg/ml, respectively. The temperature was varied from 13 to 46°C. The fluorescence intensity was expressed as relative to that at 13°C. Symbols ○ and ● represent heating and cooling curves, respectively. Other experimental conditions were the same as described in the legend to Fig. 2. Values are expressed as mean ± S.D. of triplet experiments.

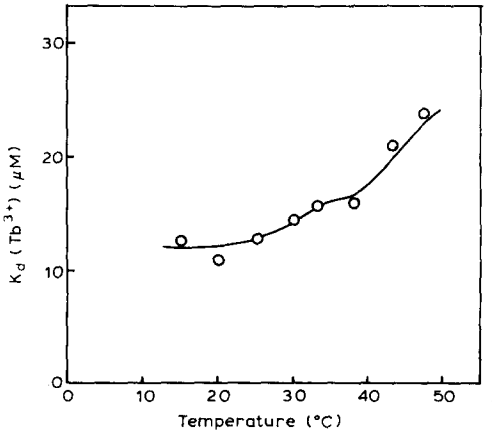

Fig. 5. Temperature dependence of binding affinity of Tb^{3+} for the membranes. The temperature was varied from 15 to 47°C. The Tb^{3+} concentration was varied from 3.32 to 59.6 μM. Other experimental conditions were the same as described in the legend to Fig. 2.

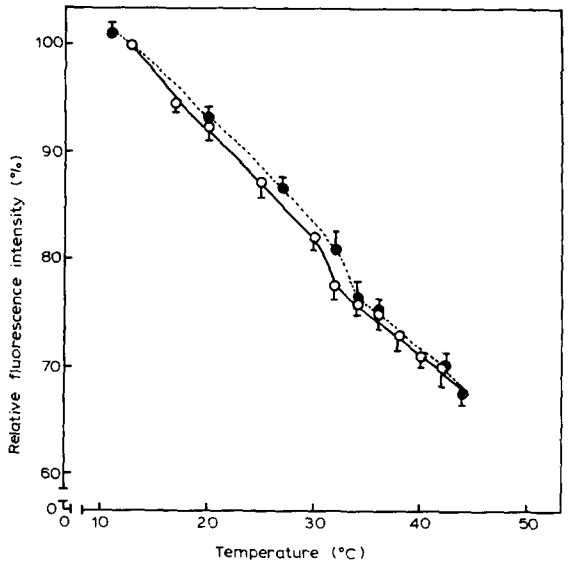

Fig. 6. Temperature dependence of native fluorescence of the membranes. 10 mM Tris-HCl buffer (pH 7.4). The membrane protein concentration was 0.08 mg/ml. Excitation and emission wavelengths used were 280 and 350 nm, respectively. The fluorescence intensity was expressed as relative to that at 13°C. The temperature was varied from 13 to 46°C. Symbols ○ and ● represent heating and cooling curves, respectively. Values were expressed as mean ± S.D. of triplet experiments.

the acceptor (Tb^{3+}) as the result of temperature-induced alteration in the conformation around the ligand-binding sites of the membrane components.

Next we explored the effects of temperature on the excimer formation efficiency and fluorescence polarization of pyrene-labeled membranes to obtain the information about the nature of arrangement of hydrocarbon cores of the membranes.

Temperature dependence of excimer formation efficiency of membrane-bound pyrene

As is well known [21,22], the excimer formation of pyrene is dependent on the solvent viscosity and the dye concentration. An increased excimer fluorescence of the dye is plausible explained to make arrangement of concentrated pyrene molecules favorable to excimer formation due to increase of the membrane fluidity.

As shown in Fig. 7, the ratio I_E/I_M of pyrene-labeled membranes increased with increasing temperature and a distinct bending point appeared at about 30°C, suggesting that the lipids of the membranes become more fluid in higher temperatures

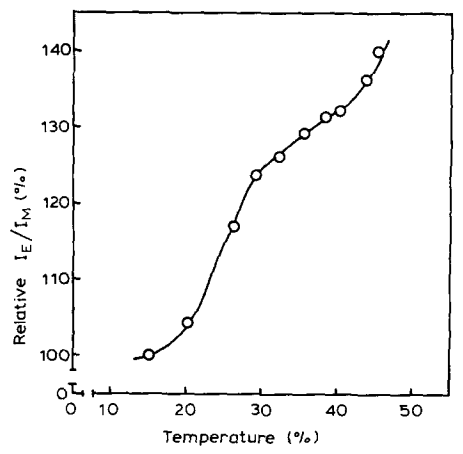

Fig. 7. Temperature dependence of excimer formation efficiency of pyrene-labeled membranes. 10 mM Tris-HCl buffer (pH 7.4). The membrane protein concentration was 0.05 mg/ml. The temperature was varied from 15 to 45°C. The ordinate is expressed as the relative to that at 15°C. The excitation and emission wavelengths used were 340 and, 392 and 470 nm, respectively.

with a phase transition at around 30°C. This result is consistent with our previous findings with 1,6-diphenyl-1,3,5-hexatriene [11]. Perturbation of lipid phase in the membranes associated with temperature was also followed by measuring the fluorescence polarization of pyrene-labeled membranes.

The thermal Perrin plot of fluorescence polarization of membrane-bound pyrene

Fig. 8 shows the temperature dependence of

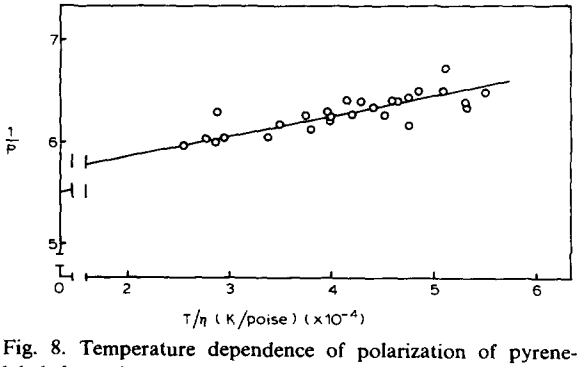

Fig. 8. Temperature dependence of polarization of pyrene-labeled membranes. The membrane protein concentration was 0.100 mg/ml. Temperature was varied from 15 to 47°C. The excitation and emission wavelengths used were 340 and 392 nm, respectively.

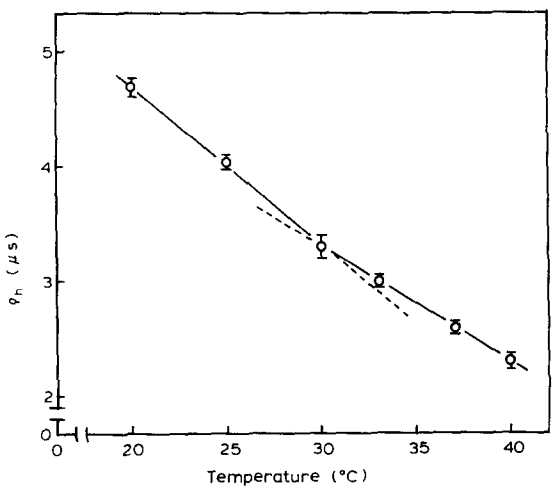

Fig. 9. Temperature dependence of harmonic mean of the rotational relaxation times (ρ_h) of pyrene molecules in the membranes. The ρ_h values were estimated from the slope of the thermal Perrin plot described in Fig. 8 by introducing the values of the fluorescence lifetimes at various temperatures. Values were expressed as mean ± S.D. of triplet experiments.

fluorescence polarization of the bound pyrene over a temperature range from 15 to 47°C. The degree of polarization of the complex slightly, but definitely, decreased with increasing temperature, showing an appreciable slope in the $1/P$ versus T/η plot. This suggests that the constraint of the movement of the dye molecules in the membranes decreased, i.e. increase of membrane fluidity, with increase of temperature.

This interpretation was confirmed from decrease of the harmonic mean of the rotational relaxation times, ρ_h, of pyrene molecules in the membranes with increasing temperature with a transition point at about 30°C (Fig. 9). These results indicate that the Tb^{3+}-binding capacity of the membranes is modified near the thermotropic transition temperature of the membrane lipids.

On the other hand, the temperature-dependence profile of the native fluorescence of the membrane proteins also showed a bend at about 30°C (Fig. 6), suggesting a close relationship between the lipid and protein components of the membranes as described in our previous paper [11]. Therefore, it is difficult to characterize exactly which component of the membranes changes mainly to modify the Tb^{3+}-binding capacity, but it could be considered at least that the nature of the lipid layers in the membranes is one of the important factors affecting the interaction between Tb^{3+} and the membrane components.

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