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TEMPERATURE-INDUCED TRANSITIONS OF PORCINE INTESTINAL BRUSH BORDER MEMBRANES

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Thermotropic transitions of the membrane components in porcine intestinal brush border membranes were studied by means of fluorimetry using a fluorogenic thiol reagent, *N*-[7-dimethylamino-4-methylcoumarinyl]maleimide (DACM), and a lipophilic fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). 1. The reactivity of the sulfhydryl groups of the membrane proteins with DACM was dependent on temperature, with a transition point at about 33°C. A conspicuous transition was also observed in the relation between temperature and the fluorescence intensity of DACM-labeled membranes at 35°C. 2. Temperature dependence profiles of the solubilization of DPH in the membranes and of the fluorescence polarization of DPH-membrane complex suggested that the phase transition of the lipid from gel to liquid-crystalline state occurs over a temperature range of 30 to 35°C. 3. Efficient fluorescence energy transfer was observed from tryptophan residues of the membrane proteins to DPH located in the lipid phase of the membranes, and its efficiency was extremely enhanced, dependent on temperature, above 35°C. The intensity of the tryptophan fluorescence of the membrane proteins decreased with increasing temperature and a discontinuity was observed at about 33°C. Based on these results, it may be concluded that there are co-operative interactions between proteins and lipids in the membranes and that the temperature-induced conformational changes of the membrane proteins are closely related to the dynamics of the hydrocarbon cores of the lipid.

Introduction

It has been recently recognized that structure and dynamics of lipids and proteins in biological membranes are important factors in their function. To investigate the interactions of proteins with lipids, therefore, is of interest to elucidate the mechanism of membrane functions.

It has been found [1] that a dramatic alteration in the organization of membrane lipids is induced as a result of the phase transition of the lipid bilayers from gel to liquid-crystalline state.

Since the fluidity of the lipid layers of biological and synthetic membranes is dependent on temperature, the characterization of lipid thermotropic transition has been studied using a number of membrane preparations [2–6].

Recently, Livingstone and Schachter [2] have demonstrated that the activities of the enzymes 5′-nucleotidase and alkaline phosphatase, of rat hepatocyte plasma membranes, change significantly at the transition temperature of the lipid phase of the membranes.

Similar evidence has been obtained for other

Abbreviations: DACM, *N*-[7-dimethylamino-4-methylcoumarinyl]maleimide; DPH, 1,6-diphenyl-1,3,5-hexatriene.

enzymes such as $(Ca^{2+} + Mg^{2+})$-ATPase in sarcoplasmic reticulum [3,7,8], *p*-nitrophenylphosphatase in rat intestinal microvillus membranes [8], and Mg^{2+}-ATPase in *Mycoplasma mycoides* [9], and for membrane activities such as transmembrane transport mechanisms of β-glucoside and β-galactoside in *Escherichia coli* [10,11] and of D-glucose in rat intestinal microvillus membranes [8].

These results strongly suggest that temperature-dependent changes in the physical state of the lipid phase in biomembranes influence the membrane activities, producing conformational changes of the membrane proteins.

The use of a fluorescence technique is valuable for defining the dynamic properties of macromolecules including biological membranes and proteins [12–14], so that a large number of fluorescent dyes have been developed and employed as probes in studies of membrane structures [12,13].

In the present study, we have demonstrated by fluorimetry that the temperature-dependent alterations of the protein structures in porcine intestinal brush border membranes are closely related to the nature of the lipid phase in the membranes.

Materials and Methods

Preparation of membrane vesicles

Brush border membranes were isolated as vesicles from porcine intestine according to the method of Malathi et al. [15], and stored at 4°C until use in 5 mM Tris-HCl buffer (pH 7.4)/300 mM mannitol. Protein concentration was assayed by the procedure of Lowry et al. [16] using bovine serum albumin as standard.

Reaction of the membranes with DACM and measurement of fluorescence

(1) Time course of reaction of membranes with DACM. The time course of fluorescence development was studied with a reaction mixture (3 ml) consisting of 0.51 mg/ml membrane protein and 10 mM Tris-maleate buffer (pH 6.85), containing 300 mM mannitol. At zero time, 0.1 μM DACM was added to the system.

(2) Effect of temperature on fluorescence efficiency of DACM-labeled membranes. Membranes were first labeled with DACM by incubation of a mixture (3 ml) consisting of 1–2 mg membrane protein and 10 mM Tris-maleate buffer (pH 6.85), containing 300 mM mannitol and 67 μM DACM, at 0°C for 5 min. The reaction was terminated by adding 500-times as much β-mercaptoethanol as DACM (molar proportion). 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)/300 mM mannitol to remove the DACM-β-mercaptoethanol adduct. The membrane-containing eluate was then centrifuged at 27000 × *g* for 30 min at 4°C. The pellets were washed with 10 mM Tris-maleate buffer (pH 6.85). The final pellets suspended in 10 mM Tris-maleate buffer (pH 6.85), in protein concentrations ranging from 0.03 to 0.51 mg/ml were subjected to fluorescence measurements at various temperatures as specified in the results.

Fluorescene measurement was carried out using a Hitachi spectrophotometer MPF-4 equipped with a circulating water bath. Excitation and emission wavelengths used were 397 and 457 nm, respectively.

Determination of amount of bound DACM

Membrane vesicles were labeled with DACM in the same way as described in subjection 2 above, except that various temperatures ranging from 15 to 45°C were applied. The final pellets were washed with 50 mM Tris-HCl buffer (pH 9.0), and then incubated in the same buffer containing 1% sodium dodecyl sulfate for 18 h at 37°C. The amount of DACM bound to the membrane proteins was determined using the molar extinction coefficient of 19800 at 380 nm for the ring-opened type of DACM-β-mercaptoethanol adduct [17] and expressed as nmol per mg protein. Optical absorption was measured at room temperature with a Union Giken High-Sensitivity Spectrophotometer SM-401.

Measurement of DPH fluorescence

Fluorescence development was followed after addition of 10 μl 1 mM DPH (dissolved in tetrahydrofuran) to 3.0 ml 10 mM Tris-HCl buffer (pH 7.4), containing 0.09–0.175 mg/ml of membrane protein at various temperatures as mentioned in the results. Excitation and emission wavelengths used were 350 and 420 nm, respectively. Fluores-

cence polarization was determined at the specified temperatures from 15 to 46°C more than 30 min after addition of DPH (0.67 μM) to 3.0 ml of the same buffer as above containing membrane proteins at 0.12 mg/ml.

Reagents

DACM and DPH were purchased from Wako Pure Chemical Co. and Sigma Chemical Co., respectively. Stock solutions of DACM and DPH were made by dissolving in acetone and tetrahydrofuran, respectively, and stored at 4°C until use.

Results

Reaction of DACM with the membrane proteins

The reaction of DACM with sulfhydryl groups of the membrane proteins can be easily followed by measuring the fluorescence development because DACM is in itself non-fluorescent, but the adduct with sulfhydryl groups is strongly fluorescent [17–19].

The time course of fluorescence development at 20 and 37°C is illustrated in Fig. 1A.

It can be seen that the fluorescence development of DACM at 457 nm is composed of two phases at either temperature, suggesting the existence of at least two kinds of sulfhydryl group with different reaction rates in the membrane proteins.

And it seems that the reaction is completed within 1 h after the addition of DACM at either temperature.

The rate constant of the reaction of DACM with the membranes was determined from the slope of semilogarithmic plots (Fig. 1B) of the values of $I_{max} - I_t$ divided by I_{max} against reaction time for each of the temperatures, where $I_{max} - I_t$ represents the difference between the fluorescence intensities at the end-point (I_{max}) of the reaction and time t (I_t), because the reaction follows pseudo-first-order kinetics.

From these semilogarithmic plots of the fluorescence change vs. time the pseudo-first-order rate constants of sulfhydryl groups belonging to the faster and slower phases in these plots were estimated to be 0.019 and 0.013 s^{-1} at 20°C and, 0.025 and 0.009 s^{-1} at 37°C, respectively.

Temperature-dependent behavior of DACM reaction with the membrane proteins was next studied in detail directly by determination of DACM bound to the sulfhydryl groups in a range of temperatures from 15 to 45°C as shown in Fig. 2.

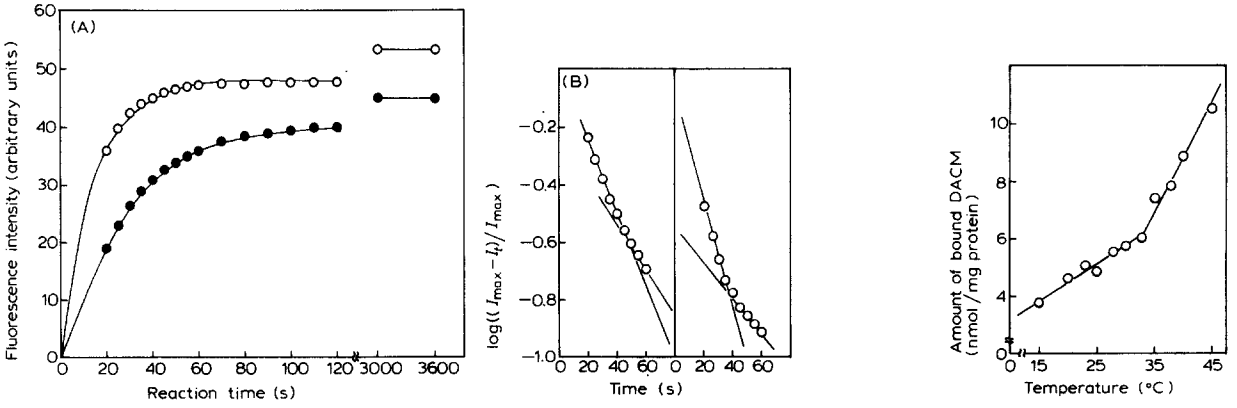

Fig. 1. (Left-hand figures.) (A) Time course of development of the fluorescence of a mixture of membrane vesicles and DACM. Solvent: 10 mM Tris-maleate buffer (pH 6.85). 0.51 mg/ml membrane protein, 0.1 μM DACM. The intensity of the DACM fluorescence was measured after the addition of the dye to the membrane suspensions at 20 (●) or 37°C (○). (B) Semilogarithmic plots of the reactivity of sulfhydryl groups with DACM. Data were obtained from (A). I_{max} and I_t represent the fluorescence intensities at the end point of the reaction (after 1 h of the addition of DACM) and time t, respectively.

Fig. 2. (Right-hand figure.) Temperature dependence of reaction of membrane vesicles with DACM. Details of the assay procedure are described in Materials and Methods.

The number of DACM molecules bound to the membrane proteins progressively increased with elevated temperatures and a discontinuity in the temperature-dependence appeared at about 33°C.

Effect of temperature on fluorescence efficiency of DACM-labeled membranes

We investigated the effect of temperature at the time of measurement on the fluorescence efficiency of DACM-labeled membranes. DACM labeling was carried out at 0°C for 5 min for all the samples in this experiment.

As shown in Fig. 3, the fluorescence intensity of the complex decreased with increasing temperature between 15 and 35°C, and then it turned to increase above 35°C.

This result suggests that the conformation around the DACM-labeled sulfhydryl groups in the membrane proteins changes dramatically with varying temperatures.

Temperature dependence of DPH solubilization

Fig. 4 shows the change in fluorescence signal with time of incubation of the membranes with DPH at 25 and 37°C.

Since DPH lacks any polar group in the molecule, increase in the fluorescence intensity can be ascribed to increased solubilization of the dye in the hydrocarbon cores of the membranes.

At 37°C the rate of development of DPH fluorescence was larger for several minutes at the start and diminished sooner than was the case at 25°C.

These results suggest that the solubilization of DPH molecules in the membranes is promoted with temperature.

The relative intensity of the DPH fluorescence measured 3 min after the addition of the dye to the membrane suspensions was plotted as a function of temperature (Fig. 5).

Increase of the fluorescence intensity with elevated temperature followed a sigmoidal curve. From the midpoint of the curve, it is inferred that the thermotropic transition temperature of the lipid phase of the membranes is about 30°C.

Temperature dependence of fluorescence polarization of DPH-membrane complex

Variation of the fluorescence polarization of DPH located in the membranes was observed at temperatures ranging from 15 to 47°C.

Fluorescence polarization (P) is defined as $P = (I_V - I_H)/(I_V + I_H)$, where I_V and I_H are the intensities of vertically and horizontally polarized emission with vertically polarized light, respectively.

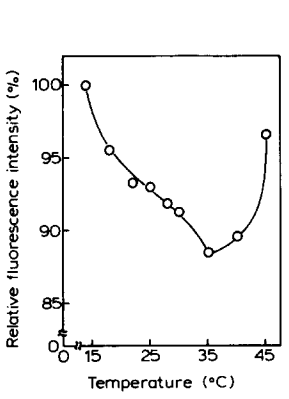

Fig. 3. (Left.) Temperature dependence profile of DACM-labeled membranes. The DACM-labeled membrane protein concentration used was 0.03 mg/ml. Other conditions were the same as described in the legend in Fig. 1A. Fluorescence intensity is relative to that at 15°C.

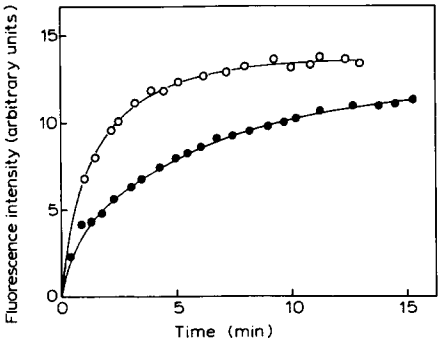

Fig. 4. (Center.) Time course of DPH fluorescence development at 25 (●) and 37°C (○). Membrane protein concentration was 0.09 mg/ml. Details of the procedure and conditions of fluorescence measurement are described in Materials and Methods.

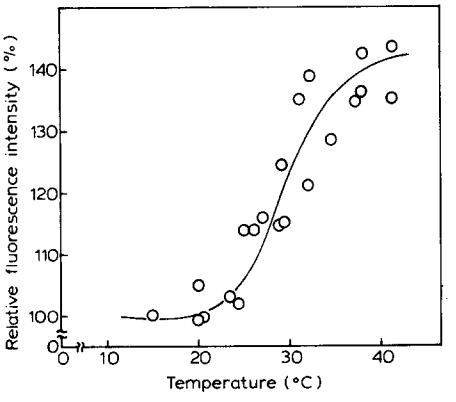

Fig. 5. (Right.) Effect of temperature on DPH fluorescence development. Membrane protein concentration was 0.175 mg/ml. Other conditions were the same as in the legend to Fig. 4. The fluorescence intensity is expressed relative to that at 15°C.

As shown in Fig. 6, plots of the change in fluorescence polarization of the DPH-membrane complex against temperatures lead to two linear regions separated by a breakpoint at around 35°C, which corresponds quite well with the critical temperature mentioned above in the reactivity of sulfhydryl groups of the membrane proteins with DACM.

Energy transfer from tryptophan to DPH

We explored the temperature dependence of energy transfer efficiency from tryptophan residues in the membrane proteins to DPH located in hydrocarbon domains of the membranes in order to obtain further information about the temperature-induced modification of protein-lipid interactions in the membranes.

The emission spectrum of tryptophan residues of the membrane proteins exhibits a maximum at 331 nm, which is very near the excitation maximum of DPH located in the membrane lipid phase (Fig. 7A). Thus, in this system, tryptophan should be an efficient energy donor to DPH.

As can be seen in Fig. 7B, the efficiency of the energy transfer from tryptophans to DPH gradually increased in a linear fashion with increased temperatures up to 35°C and showed a very steep increment above 35°C.

In contrast, tryptophan fluorescence of the membranes decreased linearly with increased temperatures, with a bend in the graph at about 33°C. In addition, a transition phenomenon of the native fluorescence of the membranes around 33°C was also detected as the temperature was lowered from 45 to 25°C, indicating that the thermal transition of the membranes around this temperature is not due to protein denaturation.

Transition temperatures of the membrane components

Thermotropic transition temperatures of the components of porcine intestinal brush border membranes estimated in the present experiment are summarized in Table I.

It is evident from the data that both the lipid phase and the protein molecules of the membranes undergo large structural changes in a narrow range of temperatures from 30 to 35°C, so that the transition temperatures of these components coincide well.

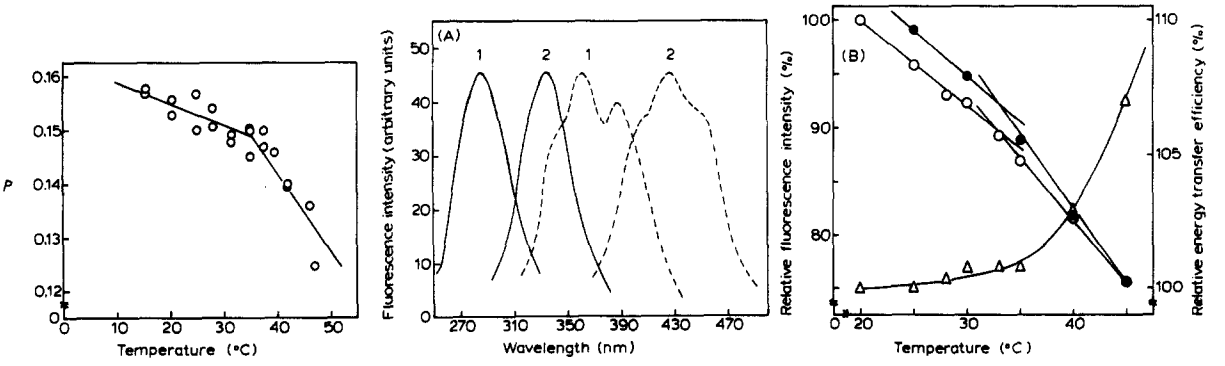

Fig. 6. (Left-hand figure.) Temperature-dependent alteration in the fluorescence polarization (P) of the DPH-membrane complex. Membrane protein concentration was 0.120 mg/ml. Other conditions are described in Materials and Methods.

Fig. 7. (Two right-hand figures.) (A) Fluorescence spectra of native fluorescence (————) and DPH-membrane complex (- - - - -) at 25°C. Membrane protein, 0.047 mg/ml (for native fluorescence), 0.117 mg/ml (for the DPH-membrane complex). The DPH concentration used was 67 μM. The fluorescence spectra were normalized to the same intensities at their maximum peaks. Symbols 1 and 2 represent excitation and emission spectra, respectively. (B) Temperature dependence profiles of energy transfer efficiency and native fluorescence. The DPH labeling of the membranes were carried out by incubation the membrane suspensions (protein concentration, 0.180 mg/ml) with 3.3 μM DPH at 20°C for 30 min. Then the native fluorescence (○, ●) and energy transfer efficiency (△) at various temperatures were measured using 280 and 331 nm (○, ●) and 280 and 420 nm (△) as the excitation and emission wavelengths, respectively, Symbols ○ and ● represent heating and cooling curves, respectively. Solvent: 10 mM Tris-HCl buffer (pH 7.4). Temperature was varied from 20 to 45°C. The native fluorescence intensity and the energy transfer efficiency were represented as the relative to those at 20°C, respectively.

TABLE I

TRANSITION TEMPERATURES OF MEMBRANE COMPONENTS

Data were obtained from Figs. 2, 3, 5, 6 and 7B.

	Parameters	Transition temperature (°C)
I	DACM fluorescence studies	
	SH reactivity	33
	Intensity of labeled membrane	35
II	DPH fluorescence studies	
	DPH incorporation	30
	Polarization	35
III	Energy-transfer studies [a]	35
IV	Tryptophan fluorescence	33

[a] From tryptophans to DPH in the membrane components.

Discussion

In the present study, the relationship of the physical states of proteins with those of lipid phase in porcine intestinal brush border membranes has been investigated as a function of temperature.

Temperature is a convenient factor to induce perturbation of membrane structure such as the gel/liquid-crystalline phase transition of the lipid phase or protein conformational changes [2–6].

DACM becomes fluorescent only after forming an adduct with a sulfhydryl groups [17–19] and is therefore very useful reagent for investigating the environmental changes around sulfhydryl groups of protein moieties in the membranes.

On the other hand, DPH has been utilized by many investigators as a probe for the fluidity measurement of hydrocarbon cores in synthetic [20–23] and biological membrane systems [5,24–27] because of its high lipophilicity.

Thermotropic transition of protein structure of the intestinal brush border membranes was investigated by monitoring the reactivity of sulfhydryl groups of the membrane proteins with DACM (Figs. 1 and 2), fluorescence efficiency of DACM-labeled membranes (Fig. 3) and the native fluorescence of the membranes (Fig. 7B).

A kinetic study of DACM reaction with the membrane proteins showed that the DACM-reactive sulfhydryl groups in the membrane proteins are classified at least into two groups with respect to their reaction rate and that the rate is dependent on temperature in either case. Consequently, the total number of DACM-reactive sulfhydryl groups progressively increased with elevated temperatures with a transition point at 33°C (Fig. 2).

This indicates that marked conformational changes are taking place around the sulfhydryl groups at temperatures in the region of 33°C.

Regional conformation changes around the sulfhydryl groups of the membrane proteins with transition temperature at 35°C were confirmed by measuring the temperature dependence of the fluorescence intensity of DACM-labeled membranes (Fig. 3). Since the fluorescence intensity of the DACM adduct is affected by the viscosity of the medium (unpublished data), the fluorescence change observed in the DACM-membrane system with temperature may be in part interpreted in terms of intramolecular rotatory diffusion of the DACM molecules labeled.

Perturbation of lipid phase in the membranes associated with temperature was followed by measuring the DPH fluorescence development (Figs. 4 and 5) and the fluorescence polarization of DPH-membrane complex (Fig. 6).

Development of DPH fluorescence was also dependent on temperature, with a transition at about 30°C (Fig. 5). The increased fluorescence intensity of DPH is assumed to show enhanced solubilization of the dye in the hydrocarbon regions of the membranes due to rearrangement of the lipid alkyl chains. In fact, the maximum amount of DPH incorporated into the membranes changed from 1.6 to 2.5 nmol/mg protein when the temperature was raised from 24 to 40°C (data not shown).

Polarization of the DPH fluorescence of the membranes decreased with increase in temperature with a conspicuous transition at 35°C (Fig. 6). Since the degree of fluorescence polarization is generally related to the mobility of the fluorophore itself or the moiety carrying the fluorophore [14,28], the above result suggests that the membrane fluidity is increased by the lipid phase transition from gel to liquid-crystalline state with a transition temperature at 35°C. This temperature is close to the

thermotropic transition temperatures of rat intestinal microvillus membranes (31°C) [5] and rat hepatocyte plasma membranes (31–32°C) [2,29].

The coincidence of the transition temperatures of the reactivity of sulfhydryl groups and DPH fluorescence polarization is very interesting in view of protein-lipid interactions in the membranes.

Additional evidence of protein-lipid interactions in the membranes is provided by the energy transfer studies from tryptophan residues to DPH.

As shown in Fig. 7B, the energy transfer efficiency was remarkably enhanced at temperatures above 35°C, well corresponding with more precipitous decrease of tryptophan fluorescence of the membranes at temperatures higher than 33°C. This suggests that the energy transfer change is due partly to changes in the fluorescence yield of the donor associated with conformational changes around tryptophan residues of the membrane proteins. Another possibility for the energy transfer changes is the alteration in location of DPH (acceptor) because the membrane fluidity is markedly increased above 35°C (Figs. 5 and 6). In this case, DPH molecules can be located more closely with tryptophan residues and/or oriented more favorably for energy transfer at higher temperatures in the membranes.

Therefore, in the present system, it can be considered that there are two possible reasons, as described above, for the energy transfer changes.

In addition, the temperature-dependence profile of the tryptophan fluorescence of the membranes revealed reversibility in the temperatures ranging from 25 to 45°C (Fig. 7B), suggesting that the thermotropic transitions observed around 35°C is not due to protein denaturation.

Although it is difficult thus far to characterize the exact changes induced by varying temperature in the structures, the results above showing a very intimate relation between the lipid and protein components of the membranes in response to temperature strongly imply that the structural changes in these two components are capable of affecting each other.

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