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Detection of Protein Conformation under Stress Conditions Using Liposomes As Sensor Materials

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Calcein release from liposomes was analyzed kinetically in the presence of various proteins under the stress condition. In the case of bovine carbonic anhydrase (CAB), a significant release of calcein was observed at pH 4. The conformational change of CAB from the native to the molten-globule (MG) state was confirmed using an aqueous two-phase partitioning method and immobilized liposome chromatography. The CAB-liposome interaction was maximum at a specific pH (4.0), where the CAB conformation was an MG-like state. The results may show that the hydrophobic interaction between CAB and liposomes enhances the perturbation of the liposome membrane, leading to a significant release of calcein from liposomes. These phenomena depended not only on the characteristics of proteins (local hydrophobicity, LH_{pr}) but also on the dynamic properties of liposomes (membrane fluidity). These results obtained with CAB may be extended to other proteins. The manner of protein-induced calcein release was classified into at least two types. Calcein release due to the addition of proteins having either disulfide bonds or being rich in beta-sheet structure was not observed. On the contrary, a significant release of calcein was observed in the case of a reduced protein with a cleaved disulfide bond and in the case of a protein with relatively a low content of beta-sheet structure. These findings suggest that a liposome containing calcein can be used as an effective sensor element for the detection of proteins having large structural fluctuations.

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1. Introduction

Studies of the function of cell membranes have attracted many researchers because environmental signals (stimuli or stress) are normally recognized on the membrane surface and responses of the cell are induced there. A method of evaluating valuable information on the membrane surface, such as signal induction, virus infection, and morphological changes of the cell membrane (endocytosis/exocytosi), has recently been presented using new techniques.[1] Currently, the idea that membrane-protein interactions trigger membrane-related phenomena has become mainstream in research on cell membranes.[2,3] Many researchers have previously investigated the structure and functions of (membrane) proteins related to these phenomena.[2,3] The significance of the role of the cell membrane, including the phospholipid bilayer, has gradually been recognized.[4] It is therefore important and necessary to design and develop an on-line detection system for membrane-protein interactions to quantify the function of the cell membrane.

A liposome is composed of a closed bilayer phospholipid membrane that has previously been used as a model cell membrane. Since an immobilization technique was developed by Lundahl and Yang,[5] liposomes have been used as sensor elements in various monitoring methods, such as surface plasmon resonance (SPR),[6] immobilized liposome chromatography (ILC),[7–9] quartz crystal microbalance analysis (QCM)[10] and dielectric dispersion analysis (DDA).[11,12] It is a common feature of these methods that the liposomes are immobilized as sensor elements on the detector surface or gel support and that the liposome-protein interaction can be directly evaluated. In general, the detection principal is based on the adsorption of protein on the liposomal surface or the retardation of elution behavior resulting from the interaction between liposomes and proteins.[6–12] A liposome entrapping calcein has recently been developed to improve the sensitivity of ILC.[13] It is suggested that calcein release is a useful index for the detection of the protein-liposome interaction because the release of calcein is affected by membrane characteristics such as membrane fluidity and permeability. An electrolyte-entrapped immobilized-liposome electrode (ILE) has recently been developed and applied to the analysis of the protein-lipid membrane interaction using amperometry.[14,15] These methods are based on the release of detectable materials entrapped in an immobilized liposome. Although there have been many reports on this issue, a common mechanism of release has not been presented because of the lack of fundamental knowledge about the relationship between the membrane properties and the release behavior.

The purpose of this study is to obtain fundamental data on the release of detectable materials from immobilized liposomes to design a liposome-based stress sensor effectively. Small fluorescent molecular calcein can be used as a detectable material because calcein becomes entrapped inside liposome during high-concentration quenches. We first investigated calcein release from liposomes in the presence of proteins at various pHs. We also investigated the protein-liposome interaction using immobilized liposomes for comparison with the above results. The effects of lipid composition and protein type on calcein release were also studied. On the basis of these results, a possible model for calcein release due to protein-membrane interaction under the stress condition is presented.

2. Materials and Methods

2.1 *Materials*

The phospholipids used were 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-dimirystoyl-*sn*-glycero-3-phosphocholine (DMPC) (Avanti Polar Lipids (Birmingham, England, UK). Poly(ethylene glycol) (PEG1540, 4000 and 6000, MW=1 k, 3 k, and 6 kD) and dextran (Dex)100 k-200 k (MW=100k-200 kDa) were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan). 1-Anilinonaphthalene-8-sulfonic acid (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Molecular Probes (Junction City, OR, USA). Calcein was purchased from Dojindo (Kumamoto, Japan). The nonionic detergents Triton X-100 and X-405 were purchased from Sigma (New York, NY, USA). Salts and other chemicals of analytical grade were purchased from Wako Pure Chemicals Ltd.

2.2 *Preparation of liposomes*

Phospholipid in chloroform (10 mg/ml) was dried in a round-bottom flask by rotary evaporation. The lipids were redissolved in diethylether twice and then evaporated to form a dry lipid film. The lipid film was kept under vacuum for at least 3 h and then hydrated by being dispersed in buffer T (50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl) or 100 mM calcein solution to form multilamellar liposomes (MLVs). For the preparation of unilamellar liposomes by extrusion, the MLV suspension was frozen in dry ice/ethanol (–80°C) for five cycles, and passed 15 times through two stacked polycarbonate filters with 100-nm pores (Nuclepore, Costar, Cambridge, MA) at room temperature by using an extrusion device (Liposofast; Avestin Inc.). To prepare liposomes entrapping calcein, free calcein was removed by gel permeation chromatography (Sepharose 4B, ϕ 15 mm, height 200 mm). A commercial kit from Wako Pure Chemicals Ltd. was used to determine the lipid concentration.

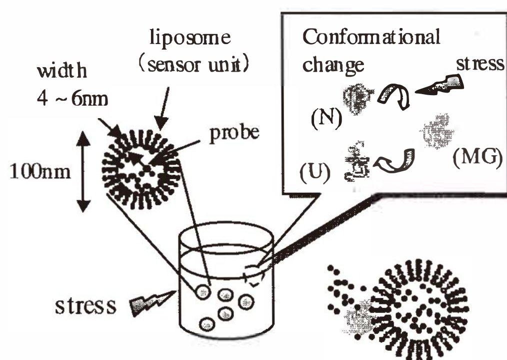
2.3 *Calcein release assay*

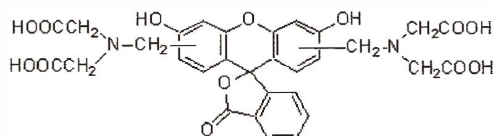
For the calcein release experiments, 10 μl of a solution of liposomes entrapping calcein was added to buffer T with and without CAB (final concentration 10 μM) at various pHs, and 25 μl of a solution of the liposomes was added to a final concentration of 0.1 mM phospholipid (Scheme 1). At pH4 or 5, a citrate buffer containing 150 mM of NaCl was used. The release fraction (RF) of calcein was calculated from its fluorescence intensity (excitation wave length: 490 nm, emission wave length: 520 nm) as

$$RF = 100(I(t) - I_c) / (I_{total} - I_c)\ (\%), \tag{1}$$

where $I(t)$ is the intensity at time t, and I_c and I_{total} are the intensities immediately after the start of the release analysis and immediately after the addition of Triton X-100, respectively. First-order kinetics were employed to analyze calcein release

$$RF = RF_{max}(1 - \exp(-k_{perr}t)), \tag{2}$$





Chemical structure of calcein used as probe.

Scheme 1. Experimental system used in this study and chemical structure of calcein. Symbols are; N: native state; MG: molten-globule state; U: unfolded state.

where RF_{max} and k_{pert} represent the maximum value of RF and the release rate constant, respectively. To determine RF_{max}, all experiments were run until the value of calcein fluorescence reached a constant value.

2.4 *Measurement of local hydrophobicity*

The local hydrophobicity (*LH*) of the proteins was analyzed using an aqueous two-phase partitioning method.[7–9,16] When the pH is set at the pI under low ionic strength conditions, the partition coefficients of the proteins depend mainly on the hydrophobic effect. When Triton X-405 is added to the PEG/Dex system, Triton X-405 partitions preferably to the top (PEG) phase. The protein that binds hydrophobically with Triton X-405 is likely to be partitioned to the top phase. The difference between the partition coefficients of the proteins in two-phase systems with and without 1 mM Triton X-405 gives the *LH* of the proteins. The *LH* of the proteins was defined as

$$LH = \text{In}\ K_{with\ ligand} - \text{In}\ K_{without\ ligand} \quad (3)$$

where $K_{with\ ligand}$ and $K_{without\ ligand}$ are the partition coefficients of the proteins in the aqueous two-phase systems with and without 1 mM Triton X-405, respectively.

2.5 *Membrane properties of liposomes*

The membrane fluidity of the liposomes was determined from fluorescence polarization (P) by measuring the fluorescent intensity of 1,6-diphenyl-1,3,5-hexatriene (DPH) according to the method of Lentz.[17] The fluorescence probes were partitioned into the lipid bilayer in the following way. The solution of DPH (in ethanol) was added to the liposome suspension to maintain the lipid/probe molar ratio at 250($[DPH]_{final}$=2 μM). The mixture was then incubated for at least 1 h at room temperature with gentle stirring. The amount of probe remaining in the outer aqueous phase of the liposomes was negligible because they show little fluorescence in water. The fluorescence intensity of samples was measured with a spectrofluorometer to which excitation and analyzing polarizers (FP-777 JASCO Co.Ltd., Japan) were attached. The sample was excited with vertically polarized light (360 nm) and then the emission intensity at 430 nm both parallel ($I_{//}$) and perpendicular ($I_{\perp}$) to the excited light was recorded. The P of TMA-DPH was calculated using the following equation.

$$P = (I_{//} - I_{\perp}) / (I_{//} + I_{\perp}) \tag{4}$$

The reciprocal value of polarization ($1/P$) was defined as the membrane fluidity. Because DPH is preferably partitioned in the hydrophobic acyl region, $(1/P)_{DPH}$ is considered to be the membrane fluidity of the interior of the liposome membrane.

As for determining the LH of the liposomes, the hydrophobic fluorescence probe ANS was used instead of the hydrophobic probe Triton X-405 because according to a previous study[7] the Triton X-405 probe affects the bilayer structure of liposomes. In brief, 20 μl of 1 mM ANS was added to the liposome suspension ([lipid] = 0.25 mM). The fluorescence of the sample solution (I_{ANS}) was measured at an excitation wavelength of 400 nm and an emission wavelength of 470 nm. The relative I_{ANS} was calculated by selecting the I_{ANS} for a liposome (50 nm in diameter) as the unit LH of a liposome, LH_{lip} =1. The LH_{lip} of liposomes has been confirmed to correspond to the membrane fluidity of the liposome surface as evaluated using a positively charged hydrophobic probe (trimethylammonium-DPH) (data not shown). In this study, only zwitterionic liposomes were used because charge repulsion between negatively charged ANS molecules and charged lipids may occur.

2.6 *Immobilized liposome chromatography*

An immobilized liposome gel matrix was prepared as previously reported.[7–9] In brief, liposomes were immobilized in a gel matrix (TSKG6000PW; Tosoh Corp., Tokyo) using a covalent-binding method. The amount of immobilized liposomes was determined using a phosphate determination kit (Phospholipid Test Wako Kit; Wako). The immobilized liposome gel was packed in a glass column 5.5×0.5 cm (HR5/5; Amersham Biosciences, Sweden), which was connected to an HPLC (AKTA Purifier; Amersham Biosciences, Sweden). The running buffer (pH 2–7.5) was preincubated in a water bath at 25°C. The retardation of proteins on the immobilization column was expressed as the capacity factor, k_s, which is defined as $k_s = (V_s - V_N) / M_{lip}$, as described by Yoshimoto *et al.*[7] The term V_s is the retention volume of proteins (ml) and V_N is the elution volume of proteins (ml), neither

of which was retarded on the column as determined using native proteins in the absence of stress. The term M_{lip} is the apparent amount of immobilized liposomes (mmol).

3. Results and Discussion

3.1 *Calcein release in the presence of proteins at various pHs*

It is well known that calcein can permeate a phospholipid membrane by controlling its membrane fluidity. Calcein release from POPC liposomes, *RF*, was kinetically analyzed in the presence of CAB at various pHs (Fig. 1(a)). At pH4, a significant release of calcein was observed compared with that released at other pHs. The data obtained under various conditions were fitted using eq. (2) and matched a calculated curve well for time intervals of up to 10 min. The parameters obtained were the release rate constant k_{pert} and the maximum release fraction of calcein RF_{max}. Both parameters (k_{pert} and RF_{max}) reached a maximum at around pH4. The RF_{max} values appear to be proportional to the protein concentration and inversely proportional to the lipid concentration (data not shown). When the RF_{max} values were plotted against the protein/lipid ratio (Φ_c) using a double-logarithmic scale, plots identical to Hill plots could be obtained,[18] as shown in Fig. 1(b). The RF_{max} values were found to depend on Φ_c. The Hill coefficients (the slope of the linear part of the curves at low Φ_c) were 1.1 and 1.2 in the cases of pH 5 and 4, respectively. These values are reasonably close to 1, suggesting that the surroundings of the CAB-lipid binding sites correspond with the permeation sites and that there is no cooperativity between CAB and calcein. According to Butko *et al.*,[18] it is not individual molecules of δ-endotoxin CytA, but their aggregates (with Hill coefficient close to 1) that induce the breakdown of the permeable membrane barrier. Their results are not consistent with our results in spite of

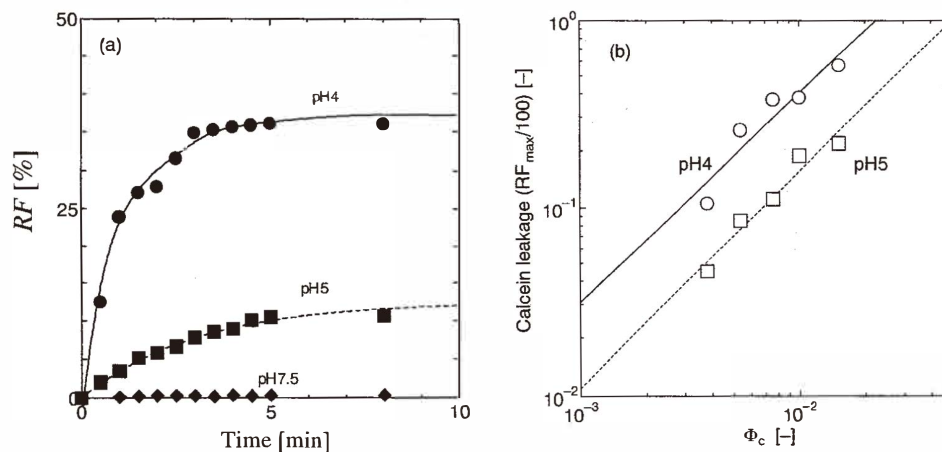


Fig. 1. (a) Time-course of calcein leakage (*RF*) from POPC liposomes in the presence of CAB at various pHs at 25°C. (b) The relationship between the maximum value of RF and the protein-to-lipid molar ratio (G_c).

the similarity in the molecular weight of CytA (27 kDa) and CAB (28.8 kDa). It is therefore suggested that this disagreement arises because CytA exhibits strong cytolytic activity like a detergent[19] although CAB exhibits no such activity and acts via another mode.

3.2 *Variation of CAB-lipid interactions at various pHs*

The variation in calcein release at different pHs (Fig. 1(a)) may be attributed to the conformational changes of proteins. Immobilized liposome chromatography (ILC) is an effective tool for evaluating the extent of the interaction between proteins and lipid membranes by retarding the elution behavior of the target protein.[7–9,13] ILC was used to analyze the interaction between proteins and lipid membranes. Figure 2(a) shows the elution profile of CAB on the ILC column at various pHs. At pH4, CAB was markedly retarded compared with the results obtained at other pHs. To normalize the retardation of CAB due to the interaction between proteins and lipid membranes, the specific capacity factor k_s was determined using eq. (3). Figure 2(b) shows the plots of k_s value as a function of pH. The k_s attained its maximum value at pH 4. It has been reported that the k_s values correspond well with the extent of the hydrophobic interaction between proteins and lipid membranes in the case of electrostatically neutral liposomes.[7–9] The partly-denatured proteins strongly interact with liposome membranes because of the increase in hydrophobic interaction.[7,9,13,20] The conformation of CAB around pH4 is, therefore, considered to be an intermediate state.

The local hydrophobicity of CAB (*LH*) was also evaluated at various pHs using the aqueous two-phase partitioning method. Similar to the case of k_s , *LH* showed a maximum value at around pH4, as shown in Fig. 2(b). The *LH* was evaluated on the basis of the binding of the hydrophobic probe Triton X-405 with the hydrophobic binding sites of

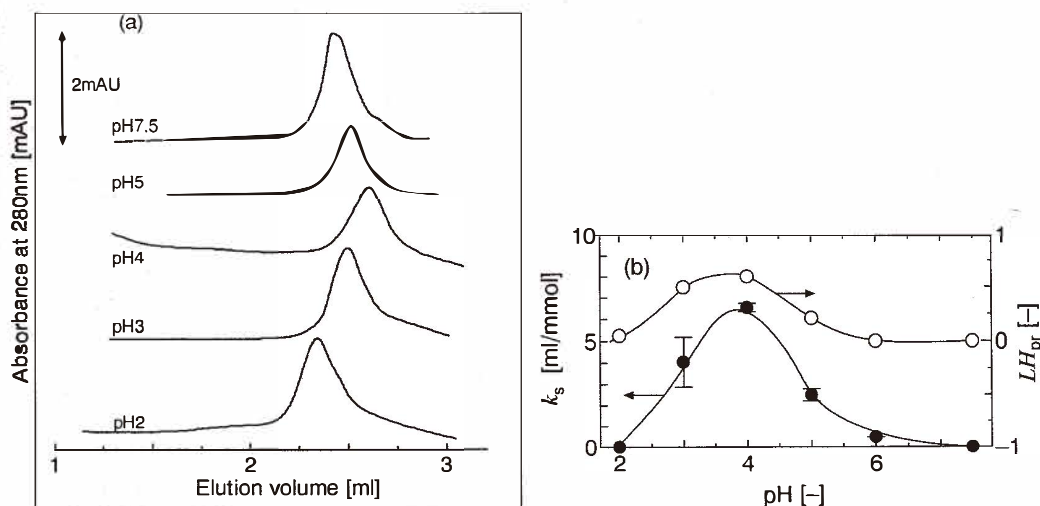


Fig. 2. (a) Elution profile of CAB in the ILC column at various pHs. (b) The pH-dependency of specific capacity factor (k_s) of CAB and its local hydrophobicity (LH_{pr}). k_s and LH_{pr} are defined in the experimental section. All experiments were performed at 25°C.

proteins. A protein in a partly denatured state has a large *LH*.[7–9,16] This is supported by the results of a binding experiment performed with a hydrophobic fluorescent probe, 1-anilinonaphthalene-8-sulfonic acid (ANS), which is an effective probe for proteins in partly denatured states such as the molten-globule (MG) state,[21–23] and the results of circular dichrorism experiments.[22] It is also known that a protein in an MG-like conformation exhibits large fluctuations.[24] As described in our series of reports, the protein conformation with high *LH* may correspond to the partly denatured state exhibiting large fluctuations.

It is concluded that the large membrane perturbation effect of CAB significantly induced the enhancement of calcein release from liposomes through the hydrophobic interaction between CAB and lipid membranes.

3.3 *Effect of lipid composition on calcein release with CAB of intermediate state*

Because calcein release is known to be affected by membrane permeability, the kinetics in our experimental systems may depend on lipid composition. To study the influence of lipid composition on calcein release, the effect of cholesterol on calcein release from liposomes was investigated in the presence of CAB at pH4 (Fig. 3). As the molar ratio of cholesterol was increased, both k_s and RF_{max} decreased. It has been reported that the addition of cholesterol leads to the stabilization of lipid membrane structures. The interaction between CAB and liposomes was considered to decrease due to stabilization of the liposomes by cholesterol.

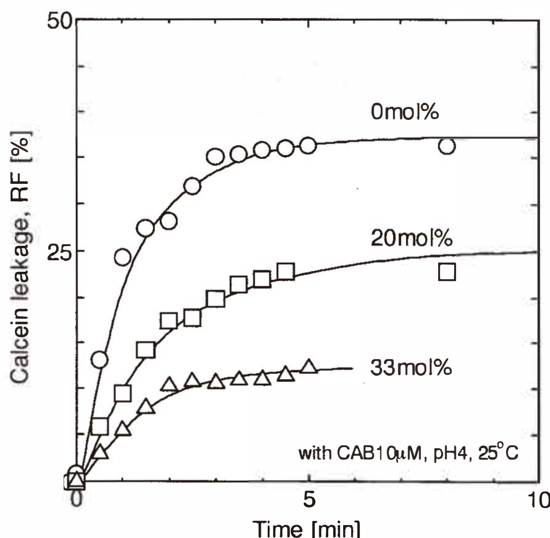


Fig. 3. Time-dependence of calcein leakage (*RF*) from cholesterol-containing POPC liposomes in the presence of CAB at pH4 at 25°C.

The effect of the phospholipid species on kinetic parameters was also studied (Fig. 4). The order of calcein release (its saturation level) was as follows: DOPC > POPC > DMPC > DPPC. POPC and DOPC have one and two unsaturated acyl-chains, respectively, whereas DMPC and DPPC have no such chains. The order of the packing density in lipid membranes is, then, DOPC < POPC < DMPC < DPPC, which is opposite to the order of calcein release. It is known that a low packing density of phospholipid makes lipid membranes unstable.[25] The changes in properties due to the addition of cholesterol and the variation in lipid composition are closely related to the membrane fluidity, which is a characteristic of membrane permeability. The influence of lipid composition on membrane fluidity was then investigated. Figure 5 shows the membrane fluidity of various liposomes evaluated using 1,6-diphenyl-1,3,5-hexatriene (DPH). The addition of cholesterol decreased the membrane fluidity, whereas a liposome composed of phospholipid with a low packing density has a large membrane fluidity. These results are consistent with previous reports.[26] It was thus shown that calcein release may be modulated by controlling membrane fluidity.

3.4 Dependence of calcein release on characteristics of proteins and membranes

On the basis of these results, the kinetics of the CAB-induced release of calcein were shown to depend on (i) the conformational state of CAB and (ii) the dynamic properties of liposomes. It is expected that such phenomena are applicable to cases with other proteins. To quantify the protein-induced release of calcein, a common parameter that can characterize both proteins and liposomes is needed. Judging from Fig. 2(a), proteins at a low pH,

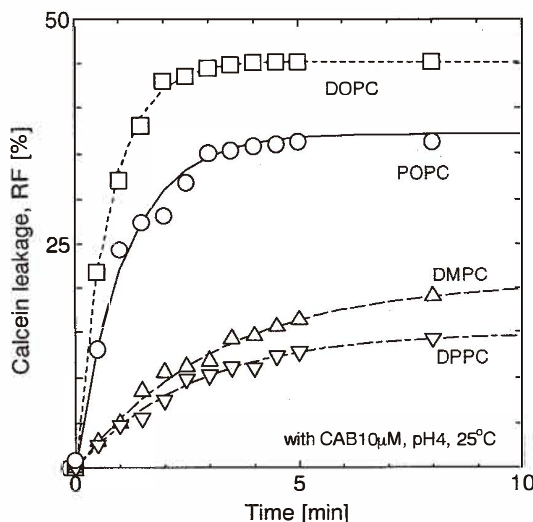


Fig. 4. Time-dependence of calcein leakage (*RF*) from various liposomes in the presence of CAB under pH4 at 25°C.

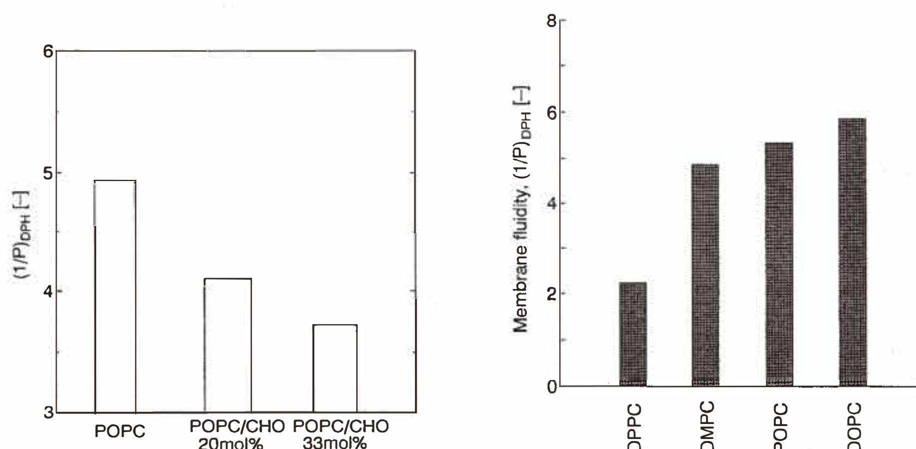


Fig. 5. Membrane fluidity of various of liposomes. Membrane fluidity was calculated from the reciprocal value of the polarization of fluorescent probe DPH. Details are in the experimental section.

where their entire surface is hydrophobic, did not interact significantly with liposomes, indicating that the conventional parameters such as the fluorescent intensity of tryptophan residues cannot be used. The *LH* of proteins is known to reflect the features of the partly denatured proteins that strongly interact with the liposomes. The *LH* was therefore selected as the indicator for the interaction between proteins and liposomes, and the extent of the interaction between proteins and liposomes was then defined as the product of the *LH* of proteins and the *LH* of liposomes, $LH_{lip} \times LH_{pr}$, from the viewpoint of the reaction rate theorem.

Figure 6 shows plots of $RF_{max} / \Phi_c (\Phi_c < 0.3)$ versus $LH_{lip} \times LH_{pr}$. There may be two categories (type A and B) of protein-induced calcein release. In both cases, a linear relationship between the values is observed. These results may reflect the identical features of each protein. Information on the secondary structure of various proteins is summarized in Table 1. The values of RF_{max} / Φ_c of proteins classified as type A, having a structure with relatively low β-sheet content or without disulfide bonds, were larger than those of proteins of type B. On the contrary, the proteins of type B tended to possess a β-sheet-rich structure or disulfide bonds. As for amyloid β protein (1--40), its RF_{max} / Φ_c was small, although neither β-sheet nor disulfide bonds were included in its structure, as shown in Table 1. The orientation of amyloid β protein (1--40) is considered to be different from that of other proteins; the detailed causes of this are still under investigation. These findings indicate that the extent of the protein-liposome interaction depends on the conformation of the proteins, which determines the identical features of the proteins.

3.5 *Model for disturbances due to proteins*

On the basisi of these results, the mechanism of calcein release from liposomes may be discussed. The single-exponential kinetics of calcein release (Figs. 1, 3, 4) indicate that the

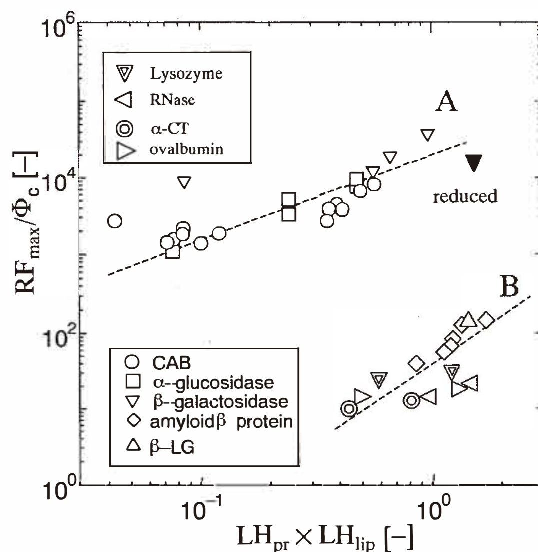


Fig. 6. Relationship between the protein-induced calcein leakage and the hydrophobic protein-liposome interaction. The solid triangle represents the reduced lysozyme.

Table 1
Molecular parameters of the proteins used.

Sample no.	Protein	MW	Fraction(%) of α-helix	Fraction(%) of β-sheet	Fraction(%) of S-S
1	carbonic anhydrae	30000	20	35	0
2	α-glucosidase	65000	—	—	0
3	α-chmotrypsin	25700	8	10	5
4	lysozyme	14300	29	16	4
5	ribonuclease A	13700	18	44	4
6	β-lactogliblin	13400	10	43	2
7	β-galactosidase	540000(4)	11.3	35.4	0
8	ovalbumin	46000	33	30	1
9	amyloid β protein (1--40)	4312	37.5	0	0

membrane association of proteins is a simple one-step process as previously reported.[18] The amount of released calcein approached a constant value within a short time (Figs. 1, 3, 4), resulting in two kinds of dependence of kinetic parameters on *LH* being observed. It has been reported that the capacity factor of proteins and lipid membranes determined by ILC is correlated with *LH* in a single relationship.[7] The difference between the calcein release

and ILC parameters arises because calcein release depends not only on the intensity of protein-liposome interactions but also on the effect of perturbations on the liposome membranes. Many researchers interpreted calcein release as the generation of either transient pores or defects.[27,28] The formation of pores depends on the molecular characteristics of the inducer. According to previous reports, the following two cases can be considered: (i) inducers with membrane-lytic activity;[18,27,28] (ii) inducers without this activity.[15]

Triton X-100 is a good example of the former case. Such a molecule (i.e., surfactant), in general, inserts into the interior of phospholipid membranes because of its hydrophobicity. Above the critical micellar concentration (cmc) in the lipid membranes, the formation of mixed micelles between phospholipids and surfactants occurs in the lipid membranes, and subsequent smaller micelles are formed from the liposomes, leading to the destruction of liposomes.[28] This process depends on the critical micelle concentration of surfactant in the lipid membranes, not on the dynamic properties of the surfactant.

In the latter case, the molecules exhibiting large fluctuations, including those shown in our results, are good examples. Although proteins in native or unfolded states cannot interact with lipid membranes (Figs. 2(a), 2(b)), ordered structures of lipid membranes were destroyed because of their large fluctuations, followed by the breakdown of the permeable barrier of lipid membranes. It is suggested that the fluctuation of proteins, therefore, plays an important role in this process.

The intramolecular force between phospholipid molecules, the so-called *microviscosity*, maintains the ordered structure of lipid membranes and is not markedly influenced by the insertion of surfactants perpendicular to the surface of the lipid membrane. The rapid saturation of calcein release induced by Triton X-100 indicates that the decrease in microviscosity occurs in the extremely limited region around the Triton X-100 molecule. It is just lateral diffusion that, in the broad region of the lipid membrane, provides a force which can overcome the microviscosity. According to reports on membrane-disturbing proteins such as the F-protein of the Sendai virus,[29] proteins that cannot intrude into the lipid membrane may be extended on the liposome membrane, so that the domain of the F-protein inserted into the membrane can move without changing its secondary structure by lateral diffusion on the membrane surface. It is suggested that the α-helix structure is suitable for lateral diffusion rather than the β-sheet structure, judging from Fig. 6. These concepts are supported by the result that the α-helix structure acts as a dynamic domain in the protein molecule.[30] In addition, for the secondary structure of proteins accessing a lipid surface with a hydrophilic surface, proteins may have a hydrophilic surface to some extent. Furthermore, the reduction in the number of disulfide bonds in the lysozyme increased RF_{max} / Φ_c (Fig. 6). Cleavage of disulfide bonds may lead to incremental changes in LH_{pr} accompanying the large fluctuations of protein molecule as a whole. It is assumed that the liposome-protein interaction is dominated by LH.

Specific secondary structures working as dynamic domains may reflect the stability of protein molecules. Their stability is, in general, controlled by (i) hydrophobic interactions, (ii) electrostatic interactions, (iii) hydrogen bonding, and (iv) other interactions including van der Waals interactions. Recently, the findings that suggest hydrogen bonding substantially determines the stability of the protein interior, have accumulated, suggesting that the

strength of hydrogen bonding may affect protein-lipid membrane interactions.[31] Therefore, hydrogen bonding would also be a key factor as well as *LH*. This problem is now under investigation.

On the basis of this discussion, it is suggested that large fluctuations of proteins enable the intense lateral diffusion of their secondary structures, such as the α-helix structure on the liposomal surface, which overcomes the microviscosity of the lipid membranes, which is then followed by the breakdown of the permeable barrier of the liposome membrane against the solutes. The permeability of calcein from the liposomes is controlled by the fluctuation of proteins, characterized by the *LH*. It has thus been shown that liposomes may be used as sensor elements for the detection of conformational changes of proteins under stress. These findings are expected to be applied to the design of liposome-based bioreactors or drug delivery systems.

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