Conformationally Changed Cytochrome *c*-Mediated Fusion of Enzyme- and Substrate-Containing Liposomes

Makoto Yoshimoto,[†] Peter Walde,[‡] Hiroshi Umakoshi,[†] and Ryoichi Kuboi*[,†]

Department of Chemical Science and Engineering, Graduate School of Engineering Science, Osaka University, 1-3 Machikaneyama-cho, Toyonaka, Osaka 560-8531, Japan, and Institut für Polymere, ETH-Zentrum, Universitatstrasse 6, CH-8092 Zürich, Switzerland

The fusion between enzyme-containing liposomes and substrate-containing liposomes was studied, utilizing conformationally altered cytochrome *c* as fusion mediator under stress conditions. The liposomes were composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), and liposome aggregation and subsequent liposome fusion were induced by the addition of cytochrome *c*, which was partially denatured by 0.5 M guanidinium hydrochloride (GuHCl). In the presence of 0.5 M GuHCl, cytochrome *c* was found to have a significantly large local hydrophobicity which was determined with the aqueous two-phase partitioning method. Under these conditions, cytochrome *c* could efficiently bind to POPC bilayer membranes as quantitatively evaluated by immobilized liposome chromatography (ILC). The retardation of cytochrome *c* treated with 0, 0.5, and 1 M GuHCl on ILC could be correlated with the corresponding local hydrophobicity of cytochrome *c*. The enzymatic reaction triggered by liposome fusion involved the proteolytic enzyme α-chymotrypsin and its substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (Suc-AAPF-pNA), which were separately trapped in POPC liposomes. Addition of partially denatured cytochrome *c* (most likely in the molten globule state) to the mixture of enzyme- and substrate-containing liposomes resulted in the release of one of the hydrolysis products, *p*-nitroaniline, to the outer phase of the fused liposomes, indicating that the enzymatic reaction occurred during the liposome fusion process. Such a coupled fusion−reaction system may have specific advantages over the conventional fusion analysis and may find application as drug delivery system.

Introduction

Extensive studies have been made in the past on the interactions between lipid bilayer membranes and water-soluble proteins. In many cases, it has shown that these interactions require a conformational change of the protein which is often induced by "stress conditions", by changes in temperature, osmotic pressure, pH, etc. Furthermore, a number of studies indicate that the molten globule state is a key protein conformation in many processes which involve membrane−protein interactions, e.g., protein refolding/unfolding (*1*−*3*), protein insertion (*4*), and protein translocating across lipid membranes (*5*−*7*), as well as in protein-mediated membrane fusion (*8*−*10*).

The molten globule state of protein has been characterized by using various methods, although its definition has remained obscure. Recently, it has been proposed that protein "local" hydrophobicity, which is determined by using the aqueous two-phase partitioning method, can be used as a measure for conformational changes in proteins, and we have defined that the protein molten globule state is characterized by a large local hydrophobicity compared with other protein conformational states (*11*, *12*). Recently, we have confirmed that stress-induced partially denatured proteins can efficiently interact with liposomal bilayer membranes (*13*, *14*), depending on the local hydrophobicity of proteins. In addition, the interactions between partially denatured protein and membrane have been utilized for liposome-mediated protein refolding (*3*, *15*), protein separation (*16*), and preparation of giant vesicles (*17*).

Stress-induced membrane−protein interactions also play a significant role in the protein-mediated membrane fusion process, which proceeds as follows (*8*, *18*, *19*). In the first step, proteins—such as cytochrome *c*, clathrin, and glyceraldehyde-3-phosphate dehydrogenase—bind to negatively charged membranes at acidic pH and then, in the second step, conformationally altered proteins penetrate into the membranes through hydrophobic interaction, which leads to a membrane disturbance, resulting in membrane fusion. However, several studies indicate that electrostatic interactions at the initial stage of the process are not needed. For instance, it has been reported that peptides with strong hydrophobic and/or amphiphilic regions can bind to neutral (zwitterionic) membranes and cause fusion, most likely through hydrophobic interactions at neutral pH (*20*−*23*).

In this paper, we show that membrane fusion can be triggered by hydrophobic interactions between liposomes and partially denatured cytochrome *c*, which is known as fusion promoting protein (*9*, *10*). The fusion process

[†] Osaka University.
[‡] ETH-Zentrum.
* To whom correspondence should be addressed. Telephone and fax: +81 (0)6 6850 6285. E-mail: kuboi@cheng.es.osaka-u.ac.jp.

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Table 1. Characterization of Enzyme- and Substrate-Containing POPC Liposomes

liposome type	mean liposome diameter[a] (nm)	calcd concn of α-chymotrypsin or S-pNA in a liposome[b] (μM)	[POPC]/[α-chymotrypsin] or [POPC]/[Suc-AAPF-pNA]	calcd no. of α-chymotrypsin or S-pNA molecules per liposome[b]
POPC/α-chymotrypsin	70	168	2920	13
POPC/α-chymotrypsin	115	287	956	113
POPC/α-chymotrypsin	170	273	651	370
POPC/Suc-AAPF-pNA	120	990	265	445

[a] Determined by dynamic light scattering analysis (scattering angle was 90 °C). [b] Calculated on the basis of assumptions: unilamellarity, spherical shape, a bilayer thickness of 3.7 nm, and mean head group area of 0.72 nm^2 (*37*).

was studied by using two types of liposomes as model phospholipid membranes, one type containing an enzyme (α-chymotrypsin) and the other containing substrate molecules for the enzyme. Such a coupled fusion−reaction system may have several advantages over conventional fusion assay systems. In particular, consider that the enzyme trapped inside the liposomes is rather concentrated and stable (*24*, *25*), which allows to detect fusion with high sensitivity and efficiency.

Materials and Methods

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids (U.S.). Stearylamine (SA), dicetyl phosphate (DCP), succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (Suc-AAPF-pNA), and cytochrome *c* were purchased from Sigma (U.S.). α-Chymotrypsin from bovine pancreas (M_r = 24 800) was purchased from Wako (Japan). Calcein was from Dojindo (Japan). All other chemicals were of analytical grade.

Preparation of Enzyme- and Substrate-Containing Liposomes. Enzyme-containing liposomes were prepared essentially according to Walde and Marzetta (*24*). POPC or mixtures of POPC and 10−20 mol % DCP or SA were dissolved in chloroform at a concentration of 10 mg/mL, and then, the solvent was evaporated under reduced pressure in a 100 mL round-bottom flask. The lipids were redissolved in diethyl ether, and the solvent was removed by evaporation again. This procedure was performed twice for the complete removal of chloroform. Then, the lipid film was dried under high vacuum overnight. The dried film containing 20 mg of lipids was dispersed in 2 mL of 50 mM Tris-HCl buffer (pH 8.0) containing 20 mg/mL α-chymotrypsin, followed by eight freezing−thawing cycles. Freezing and thawing treatments were performed in dry ice/ethanol (−80 °C) for 15 min and at 25 °C in a water bath for 15 min, respectively. The concentrations of POPC and α-chymotrypsin in the liposome suspension were 13 and 0.40 mM, respectively. The liposome suspension was extruded 15 times through two stacked polycarbonate membranes with mean pore diameters of 200, 100, and 50 nm (denoted 200, 100, and 50 nm liposomes, respectively) using a small-volume extrusion device (Liposofast; Avestin Inc., Canada) (*26*). Nonentrapped α-chymotrypsin present in the external aqueous phase was removed by gel permeation chromatography with a Sepharose 4B column (1.5 × 30 cm). For this, the liposome suspension (1.5 mL) was loaded on the column and eluted with 50 mM Tris-HCl buffer (pH 8.0) with a flow rate of 0.5 mL/min, collecting 1.5 mL fractions. The liposome-containing fractions (always nos. 8 and 9), which were detected by measuring the turbidity at 400 nm, were used for the following experiments. The concentration of α-chymotrypsin in the pooled liposome fractions was determined enzymatically with 25 μM Suc-AAPF-pNA (see below) after disrupting the liposome membranes with 40 mM sodium cholate. The size and size distribution of the enzyme-containing liposomes were determined by dynamic light scattering, using a DLS-700 Ar system from Otsuka Electric (Japan) equipped with an argon laser. As a measure of membrane fluidity, fluorescence polarization of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) oriented in the lipid bilayer membranes was measured as described before (*3*, *27*).

Suc-AAPF-pNA-containing liposomes were prepared by essentially the same procedure just described for α-chymotrypsin-containing liposomes, except that Suc-AAPF-pNA (2.9 mM) was dissolved in Tris-HCl buffer (pH 9.0), and in the freeze−thawing treatments, the liposomes were thawed at 20 °C to depress hydrolysis of Suc-AAPF-pNA. The characteristics of the enzyme-containing liposomes and substrate-containing liposomes prepared in the present work are summarized in Table 1.

Enzymatic Activity Assay. The enzymatic activity of α-chymotrypsin was determined by measuring the hydrolysis rate of Suc-AAPF-pNA. This substrate, dissolved in distilled water, was added at a concentration of 25 μM to a free enzyme solution or to an enzyme-containing liposome suspension ([POPC] = 38 μM), and the increase in the absorbance at 410 nm, which reflects the release of *p*-nitroaniline (pNA) from the substrate, was continuously monitored with a UV spectrophotometer (Shimadzu, Japan) (*28*, *29*). The molar extinction coefficient of pNA at 410 nm was taken as 8800 $M^{-1}\,cm^{-1}$ (*30*). The entrapped α-chymotrypsin showed constant activity during long-time storage (at least for 1 month), in agreement with previous finding (*24*). The catalytic activity of α-chymotrypsin remained practically unaffected by the presence of 40 mM sodium cholate.

Fusion Assay. In the fusion experiments, pNA, which was formed after mixing the enzyme- and substrate-containing liposomes during 10 min in the absence or presence of 2 μM cytochrome *c*, was separated from the vesicles and from free proteins by gel permeation chromatography using a Superdex 200 column (0.5 × 5 cm) and a AKTA system from Pharmacia Biotech (Sweden). The volume of the sample applied was 10 μL, and elution was performed at a flow rate of 0.25 mL/min with 50 mM Tris-HCl buffer (pH 8.0). The elutions of the liposomes and pNA were monitored by measuring turbidity and absorbance at 600 nm (or 400 nm) and 385 nm, respectively.

In some experiments, the fusion of liposomes was monitored according to the method of Kendall and MacDonald (*31*). Briefly, nonfluorescent Co^{2+}-calcein-containing liposomes and EDTA-containing 100 nm liposomes ([POPC] = 13 mM) were prepared in the presence of either 1 mM $CoCl_3$/0.8 mM calcein or 20 mM EDTA in 50 mM Tris-HCl buffer (pH 8.0) and free solute molecules were removed from liposomes by gel permeation chromatography (Sepharose 4B, 1.5 × 30 cm) (see above). Upon mixing of the two liposome contents (caused by membrane fusion), free fluorescent calcein was formed

due to complexation of Co^{2+} by EDTA. The increase in the relative fluorescence intensity recorded at an emission wavelength of 520 nm was monitored as a function of time after excitation at 490 nm. The maximal fluorescence intensity (100%) was determined by disrupting the liposomes with 2 mM Triton X-100. Liposome aggregation was monitored by turbidity measurements at 600 nm, using a quartz cell with 1 cm path length.

Liposome Chromatography of Proteins. Liposome-immobilized Superdex 200 gel beads were prepared by the covalent coupling method as previously reported (*14*, *32*). Briefly, the POPC liposomes supplemented with 1 mol % of egg phosphatidylethanolamine were mixed with chloroformate-activated Superdex gel beads (*33*) overnight at 4 °C under gentle stirring. The protein−membrane interactions were evaluated on the liposome-immobilized Superdex 200 column (0.5 × 5 cm) connected to a AKTA system. The protein elution behavior was normalized by using the apparent specific capacity factor, k_s (expressed in mL/mmol) (*34*) which is defined as $k_s = (V_r - V_e)/P$, where V_r is the retention volume of the protein under the conditions used (mL), V_e is the elution volume of the same protein in its native state (mL), e.g., in the absence of guanidinium hydrochloride (GuHCl), and P is the apparent amount of immobilized lipids (mmol), which is available for interaction with the proteins, as determined on the basis of the method of Bartlett (*35*).

Evaluation of Hydrophobic Property of Protein. The protein local hydrophobicity (LH (−)) was used as a measure of the conformational changes and was determined by using the aqueous two-phase partitioning method, as previously reported (*11*, *12*). LH is defined as $LH = \Delta \ln K = \ln K_{T,P} - \ln K_P$, where $K_{T,P}$ and K_P are the partition coefficients of a protein (1 mg/mL) in an aqueous two-phase system in the presence of 1 mM Triton X-405 and in an aqueous two-phase system in the absence of Triton, respectively. The aqueous two-phase system used was composed of 9 wt % poly(ethylene glycol, $M_r = 3000$) and 9 wt % dextran ($M_r = 100\,000-200\,000$). The total weight of a aqueous two-phase system was 5 g.

Results and Discussion

Effects of Liposome Physical Properties on Membrane Permeability. Entrapment of α-Chymotrypsin in Liposomes. All the fusion experiments on which we are reporting in this paper require the preparation of substrate-containing liposomes and enzyme-containing liposomes which are not leaky. In the case of α-chymotrypsin-containing liposomes, this is not a problem since α-chymotrypsin is a macromolecule (M_r = 24 800) which—at least in its native state—cannot pass a phospholipid bilayers (*24*). For the substrate, Suc-AAPF-pNA (M_r = 625), however, leakage could be a serious problem. Therefore, the membrane permeability for Suc-AAPF-pNA under various conditions was first examined, in particular because it is well-known that the physical properties of the membrane are important factors in controlling permeability especially for small molecules (*36*). In a series of experiments, the effect of liposome size and membrane fluidity on the membrane permeability for Suc-AAPF-pNA was examined, by using α-chymotrypsin-containing liposomes and following the hydrolysis rate of externally added Suc-AAPF-pNA. As shown in Figure 1, the rate of hydrolysis of Suc-AAPF-pNA was rather low in the case of 100 nm liposomes, while with 50 and 200 nm liposomes, the hydrolysis rate was considerably higher. This observation can be compared with earlier data on the relationship between liposome size and membrane fluidity: small, 50 nm liposomes showed a higher membrane fluidity than 100 nm liposomes (*3*), which explains the observed higher permeability of 50 nm liposomes in comparison with the 100 nm liposomes, in the present work. In the case of the 200 nm liposomes, increased membrane permeability for Suc-AAPF-pNA may be a result of increased multilamellarity, a relatively high polydispersity (*3*, *37*), and surface inhomogeneities, which may cause packing defects as we showed in the previous paper by using freeze−fracture electron microscopy and dynamic light scattering analysis. Although all of this remains to be clarified, it can be concluded that 100 nm liposomes have the most impermeable membranes against Suc-AAPF-pNA among the three types of liposomes examined. Therefore, in all of the following experiments, 100 nm liposomes were used.

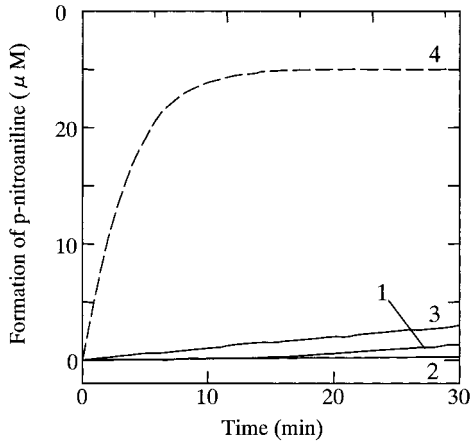

Figure 1. Time course of the hydrolysis rate of Suc-AAPF-pNA added externally to α-chymotrypsin-containing 50 nm (curve 1), 100 nm (curve 2), and 200 nm (curve 3) liposomes. The dotted line (curve 4) shows the hydrolysis of Suc-AAPF-pNA after disrupting α-chymotrypsin-containing 100 nm liposomes with 40 mM sodium cholate. The mean diameters of the 50, 100, and 200 nm liposomes were 70, 115, and 170 nm, respectively, as determined by dynamic light scattering analysis (see Table 1) ([POPC] = 38 μM, $[\alpha\text{-chymotrypsin}]_{overall}$ = 40 nM, $[\text{Suc-AAPF-pNA}]_o$ = 25 μM).

To investigate the effect of membrane fluidity on the permeability properties, α-chymotrypsin-containing 100 nm liposomes were used under different experimental conditions and the rate of hydrolysis of externally added Suc-AAPF-pNA was measured. Either increasing the temperature (up to 40 °C) or adding small amounts of organic solvents (7 vol % ethanol or acetone) led to an increased hydrolysis rate (see Figure 2). This increase correlates with an increase in membrane fluidity, as estimated by fluorescence polarization measurements of TMA-DPH incorporated into the POPC bilayers, see the linear relationship in Figure 2. Since α-chymotrypsin does not leak out of the liposomes and liposomes are stable under the experimental condition (40 °C), it can be concluded that the membrane permeability for Suc-AAPF-pNA can be controlled by the fluidity of the membrane. A general conclusion between membrane fluidity and permeability has been established before (*38*).

Entrapment of S-pNA in Liposomes. Taking into account the series of results reported above, Suc-AAPF-pNA was entrapped at the concentration of 2.9 mM inside 100 nm liposomes and the liposomes were kept below room temperature. The rate of hydrolysis of Suc-AAPF-pNA was then followed after external addition of α-chy-

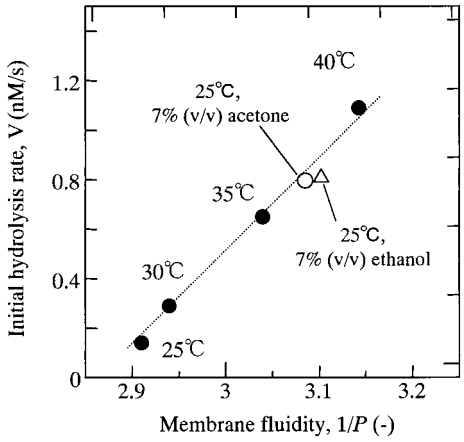

Figure 2. Relationship between membrane fluidity and membrane permeability at different temperatures or at 25 °C in the presence of 7% (v/v) acetone (○) or ethanol (△). The membrane permeability was estimated by measuring the initial rate of hydrolysis (V (nM/s)) of Suc-AAPF-pNA added externally to α-chymotrypsin-containing 100 nm liposomes ([POPC] = 38 μM, $[\text{Suc-AAPF-pNA}]_o$ = 25 μM). The membrane fluidity was estimated by measuring the fluorescence polarization (P (−)) of TMA-DPH in the lipid bilayer membranes ([POPC]/[TMA-DPH] = 250:1).

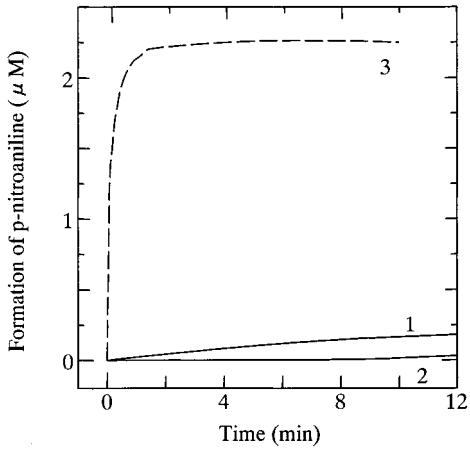

Figure 3. Time course of the hydrolysis of 2.2 μM Suc-AAPF-pNA (overall) entrapped inside 100 nm liposomes upon external addition of 8 μM α-CT (curve 1). Curve 2: Spontaneous hydrolysis of S-pNA in liposomes. Curve 3: Hydrolysis of S-pNA after disrupting the liposomes with 40 mM sodium cholate in the presence of 8 μM α-chymotrypsin. The POPC concentration was 75 μM for all experiments.

motrypsin (8 μM) (see Figure 3). A comparison of curves 1 and 3 in Figure 3 shows that the formation of pNA was drastically increased when liposomes were disrupted by sodium cholate. In the absent of cholate, the liposomes were almost impermeable for Suc-AAPF-pNA, confirming the results reported in Figure 1. The spontaneous hydrolysis of entrapped Suc-AAPF-pNA was negligibly low (see the curve 2 in Figure 3).

Interaction of Cytochrome *c* with Liposomal Bilayer Membranes. Several proteins are known to interact with membranes if the conformation of the protein is changed (*9*, *39*). The degree of protein conformational change and the subsequent protein−membrane interaction may depend on the quality and extent of the stresses applied (pH, heat, denaturant, osmosis, etc.). Under certain conditions, the interactions of (partially denatured) proteins with liposomes can lead to liposome fusion (*8*, *18*, *19*). This means that one can utilize stress conditions for generating protein-mediated membrane fusion. One of the proteins used as a fusion-generating agent is cytochrome *c* (*9*). In the following sections, we will first report on the interaction of cytochrome *c* with POPC liposomes and then discuss experiments on stress-induced, cytochrome *c*-mediated fusion between α-chymotrypsin-containing liposomes and Suc-AAPF-pNA-containing liposomes.

The interaction between liposomal bilayer membranes and cytochrome *c* was examined by using immobilized liposome chromatography (ILC). In this type of chromatography, liposomes are immobilized onto Superdex 200 gel beads, and as previously reported, ILC measurements provide quantitative information on the interaction between membranes (or membrane proteins) and solutes including drugs, peptides, and proteins (*14*, *40*). The stress conditions used in the present work are high concentrations of guanidinium hydrochloride (GuHCl). In Figure 4, the elution behaviors on ILC are shown for cytochrome *c* in the absence or in the presence of 0.5 or 1 M GuHCl in the eluent. The elution profile obtained in the presence of 0.5 M GuHCl was markedly different from the elution profile in the absence of GuHCl or in the presence of 1 M GuHCl. In the case of 0.5 M GuHCl-treated cytochrome *c*, two peaks were observed in the

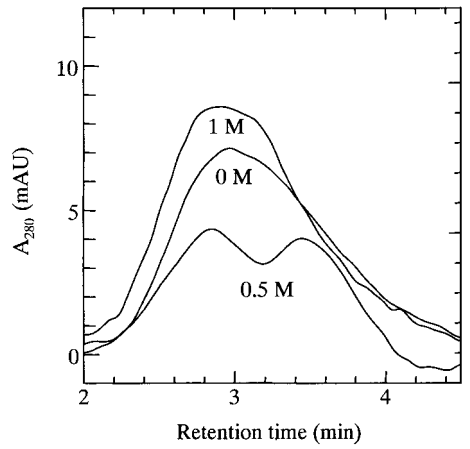

Figure 4. Immobilized liposome chromatography of cytochrome *c* in the absence or in the presence of GuHCl. After pre-equilibration for 2 h in the presence of 0.5 or 1 M GuHCl, elution was performed with 50 mM Tris-HCl buffer (pH 8.0) containing the appropriate amount of GuHCl (column dimension 0.5 × 5 cm, sample volume 10 μL, [cytochrome *c*] = 5 μM, flow rate 0.25 mL/min).

chromatogram: the first peak is at almost the same retention time as in the case of native cytochrome *c*, and the second peak is significantly retarded probably due to interactions between the conformationally altered cytochrome *c* with immobilized liposomal membranes, indicating that two clearly different protein states exist in the presence of 0.5 M GuHCl. One of the two protein states corresponds to the conformationally changed cytochrome *c*, which shows strong binding ability to the POPC membranes of the liposomes. For the 1 M GuHCl-treated cytochrome *c*, no significant retardation was observed, indicating that the cytochrome *c* does not interact with membranes in the presence of 1 M GuHCl. Previously, we have reported that the liposome size is not changed in the presence of GuHCl (up to 1 M) (*3*). We have also confirmed that liposomes immobilized in gel beads are stable in the presence of 1 M GuHCl after at least 200 times chromatographic running (*14*). Furthermore, the membrane fluidity of POPC liposomes remained practically constant in the presence of 0−1 M GuHCl (data not shown). Therefore, we conclude that the change in the elution behavior of cytochrome *c* on ILC

Table 2. Characteristics of the GuHCl-Induced Cytochrome *c*−Liposome Interactions and the Cytochrome *c*-Mediated Fusion of Liposomes

GuHCl (M)	local hydrophobicity, LH[a] (−)	specific capacity factor, k_S[b] (mmol/min)	fusion[c] (%)
0	0		0
0.5	0.61	0.8, 11.8	5.9
1	0.24	1.04	1.0

[a] Determined with the aqueous two-phase partitioning method (*11*, *12*). [b] k_s was defined as $k_s = (V - V_N)/P$, where V (mL) and V_N (mL) represent elution volume of the stressed protein and that of the native protein, respectively. P (mmol) means the amount of immobilized lipid in the column. [c] Determined by fluorescence measurements using the calcein-Co^{2+}-EDTA system (see the Materials and Methods). The fusion process was monitored for 10 min after addition of 2 μM cytochrome *c*. 100% corresponds to the fluorescence intensity after addition of 2 mM Triton X-100.

in the presence of GuHCl is due not to a change in the membrane property but due to a change of the conformation of cytochrome *c*.

The conformational change of cytochrome *c* induced by GuHCl was evaluated in terms of the local hydrophobicity (LH (−)), which can be determined with the aqueous two-phase partitioning method as described before (*11*, *12*). The effectiveness of LH measurement is demonstrated in the previous literature by compared with circular dichroism (CD) measurement (*41*). In Table 2, the effect of GuHCl on the LH value of cytochrome *c* is given, together with the specific capacity factor (k_s (mmol/mL)) for cytochrome *c*, as obtained from ILC. Please note that the higher the k_s, the more the protein interacts with the liposomes. It can be seen that there is a similarity in the trend of the LH value and k_s. In other words, an increase in k_s is paralleled by an increase in the LH value, which means that cytochrome *c* interacts with the POPC membrane depending on its local hydrophobicity. It has been reported before that proteins at the molten globule (MG) state are involved in protein−membrane interactions (*5*). Furthermore, it has also been demonstrated that proteins with reduced disulfide bridges can strongly bind to membranes (*1*). Recently, we have shown in the case of lysozyme and α-lactalbumin that the common characteristics of the MG and the reduced state is the large local hydrophobicity (*15*). Taking all of this together, it means that the local hydrophobicity is one of the dominant factors determining protein−membrane binding. This seems also to be true in the case of cytochrome *c*.

In the next series of experiments, the change in turbidity (measured at 450 nm) of a POPC-liposome suspension ([POPC] = 530 μM) was measured upon addition of cytochrome *c* (2 μM) and GuHCl (Figure 5). In the absence of GuHCl, there was no measurable turbidity change under the conditions used, during the time of observation. On the other hand, the presence of cytochrome *c* and 0.5 or 1 M GuHCl led to an increase in turbidity, indicating the formation of large aggregates which scattered the light more than the 100 nm liposomes. No significant change in the turbidity at 450 nm was observed in the presence of 0.5 or 1 M GuHCl but in the absence of cytochrome *c* (data not shown). Therefore, it can be concluded that cytochrome *c* mediated liposome−liposome interactions (aggregate formation), which occur in the presence of GuHCl. Since, however, liposome aggregation does not necessarily result in liposome fusion—which involves mixing of bilayer lipids as well as mixing of internal aqueous contents (*18*)—a conventional liposome fusion assay was applied.

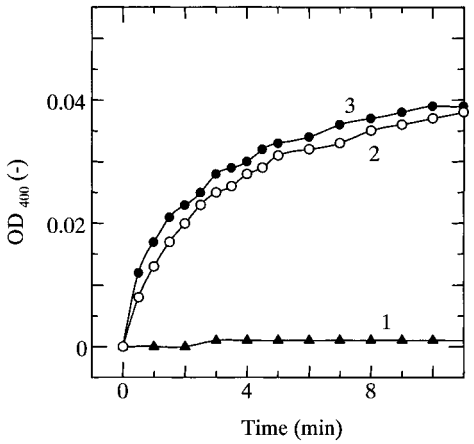

Figure 5. Time course of the change in turbidity of POPC liposomes, induced by cytochrome *c* in the presence of 0 (1, ▲), 0.5 (2, ○), and 1 M (3, ●) GuHCl ([POPC] = 530 μM, [cytochrome *c*] = 2 μM).

Fusion between Enzyme-Containing Liposomes and Substrate-Containing Liposomes. Before using α-chymotrypsin-containing liposomes and Suc-AAPF-pNA-containing liposomes to monitor liposome fusion, one of the conventional methods to detect liposome fusion was used (see the Materials and Methods (*31*)). In these experiments, one type of liposomes contained a quenched fluorescence probe (calcein-Co^{2+}) and the other type of liposomes contained a ligand for the quencher ions (EDTA). The extent of fusion is then monitored by following the increase in fluorescence intensity of the fluorescent probe (calcein) due to the formation of a Co^{2+}-EDTA complex. The results of these fusion measurements are reported in Table 2 for 100 nm liposomes ([POPC] = 50 μM), in the presence of 2 μM cytochrome *c* and 0, 0.5, or 1.0 M GuHCl, respectively. While in the absence of GuHCl no fusion could be detected, a significant fusion was observed in the presence of 0.5 M GuHCl and to a much lesser extent in the presence of 1.0 M GuHCl. These results in combination with the turbidity data presented in Figure 5 and the ILC analysis indicate that, in the presence of cytochrome *c* and 0.5 M GuHCl, the liposomes aggregate and subsequently fuse, while in the presence of cytochrome *c* and 1 M GuHCl, liposome aggregation occurs with a much lesser extent of fusion.

On the basis of the above results, membrane fusion between α-chymotrypsin-containing liposomes and Suc-AAPF-pNA-containing liposomes was examined in the presence of 2 μM cytochrome *c* and 0.5 M GuHCl (see Figure 6). In the absence of cytochrome *c* but in the presence of 0.5 M GuHCl (curve 2), only a large liposome peak was observed, indicating that no mixing of the aqueous contents occurred. However, in the presence of cytochrome *c* and 0.5 M GuHCl (curve 1), two additional peaks were observed, one corresponding to cytochrome *c* and one to pNA, respectively. The appearance of a pNA peak indicates that α-chymotrypsin and Suc-AAPF-pNA came in close contact and that the enzymatic reaction occurred during the cytochrome *c*-mediated fusion process. In the presence of cytochrome *c* but in the absence of GuHCl (curve 3), the appearance of only a small peak of pNA was observed, indicating that native cytochrome *c* at slightly alkaline pH only weakly interacts with POPC membranes (low fusion efficiency). This observation is in agreement with literature (*10*).

It should be clarified that conformationally changed cytochrome *c* does not cause lysis of liposomes, which leads to extensive leakage of entrapped molecules. In the

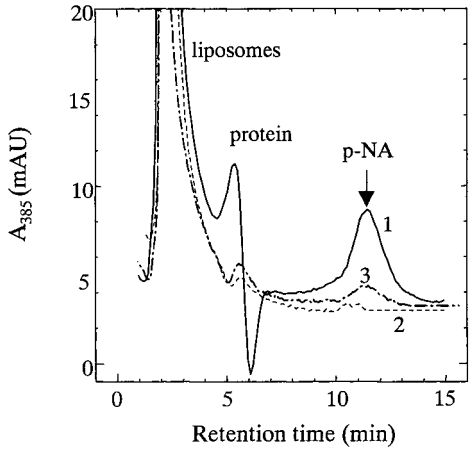

Figure 6. Gel permeation chromatography of α-chymotrypsin-containing liposomes ([POPC] = 1.5 mM) which have been fused with Suc-AAPF-pNA-containing liposomes ([POPC] = 4.5 mM) in the presence of cytochrome *c* (2 μM) and 0.5 M GuHCl (curve 1). After incubation at room temperature for 10 min, the liposome mixture (10 μL) was applied to a Superdex 200 column (0.5 × 5 cm) and eluted with 50 mM Tris-HCl buffer at a flow rate of 0.25 mL/min. The dotted curves represent control measurements in which α-chymotrypsin-containing liposomes and Suc-AAPF-pNA-containing liposomes were mixed in the absence of cytochrome *c* and in the presence of 0.5 M GuHCl (curve 2), in the presence of 2 μM cytochrome *c*, and in the absence of GuHCl (curve 3).

present work, we induced the conformational change of cytochrome *c* by the addition of GuHCl up to 1 M at 25 °C. We had confirmed that 1 M GuHCl had little effect on liposome size (*3*). In addition, in our earlier report, in which liposome-assisted refolding of GuHCl-denatured protein was examined, we showed that lysis of liposomes does not occur in the presence of conformationally changed proteins as evaluated by gel permeation chromatography and dynamic light scattering analysis (*3*). Therefore, under the present experimental condition, liposome itself should be stable. Macromolecules such as proteins essentially do not pass through lipid membranes under physiological conditions. For passing of proteins across lipid membranes, lipid membranes should be extensively perturbed and conformation of proteins should be changed to show high local hydrophobicity under stress conditions. For example, we showed that translocation of β-galactosidase is induced at a temperature of around 45 °C, where lipid membranes are extensively perturbed and, at the same time, local hydrophobicity of protein is pronounced (*13*). Similar phenomena is also observed for other proteins (*16*). The conformational change of α-chymotrypsin is not observed under the experimental condition (25 °C); therefore, the entrapped α-chymotrypsin is stable without leaking out from liposome interior as reported earlier (*24, 25*). On the other hand, it is possible that relatively small molecules such as substrate for α-chymotrypsin leaked out from liposomes upon the interaction with cytochrome *c*. However, leaked substrate is extensively diluted in the outer aqueous phase of liposomes into negligible low concentration. Considering the above results, we considered that enzymatic reaction occurred mostly in the fused liposome interiors.

From earlier investigations, it is known that cytochrome *c* and its derivatives interact with membranes through electrostatic interactions, leading to a penetration of the hydrophobic regions into the membrane, particularly at acidic pH (*10, 42*). For instance, apocytochrome *c* (the heme-free precursor of cytochrome *c*), which is characterized by a high degree of conformational flexibility, is known to mediate membrane fusion of 0−50 mol % phosphatidylserine-containing phosphatydylcholine liposomes at acidic pH (*10*). These findings imply that electrostatic interactions are needed as a trigger of protein−membrane interactions while the subsequent penetration process is dominated by hydrophobic interactions between membranes and the conformationally changed protein with its exposed hydrophobic domains. Shin et al. (*1*), on the other hand, reported that hydrophobic interactions are important in membrane binding of the protein at the molten-globule state with no net charge at neutral pH. Our results suggest that conformationally altered cytochrome *c* with its highly fluctuating structure (see also *43*) and thus with its large local hydrophobicity can bind to and disturb lipid membranes which have no net charge (phosphatidylcholine) under physiological pH conditions.

Comparison of the Cytochrome *c*-Mediated Liposome Fusion with Conventional Liposome Fusion Systems. A general problem of stress-mediated liposome fusion as reported above is the relatively low fusion efficiency (only about 6% of the liposomes fuse). This fusion efficiency (as estimated by the formation of pNA) was also low when 50 mM Ca^{2+} was added to mixed POPC/DCP liposomes (molar ratio 8:2) which separately contained α-chymotrypsin and Suc-AAPF-pNA (fusion efficiency 5%). At a first glance, this observation was surprising, since Ca^{2+} ions are well-known to induce fusion of liposomes containing negatively charged lipids, such as DCP (*44*), and one may have expected a higher fusion efficiency. It might, however, be that the observed low degree of fusion is due to a low fusion selectivity, that is, Ca^{2+} (or cytochrome *c*) randomly cause the fusion of both types of liposomes. Therefore, the extent of the desired fusion between enzyme-containing liposomes and substrate-containing liposomes is low. In other words, the fusion of a substrate-containing liposome with another substrate-containing liposome cannot be monitored (please note that, for practical reason, in the case of the experiment reported in Figure 6, the molar ratio of substrate-containing liposomes to enzyme containing liposomes was 3, indicating the proceeding of nonproductive fusion between substrate-containing liposomes). The fusion event may not be one round process. However, during fusion process, small unilamellar liposomes are transformed into larger and more stable liposomes. Therefore, fusion efficiency may decrease with proceeding fusion process because large liposomes are more stable than small liposomes. For this reason, we considered that an early round of fusion may dominant the reactions in liposomes triggered by the fusion of liposomes. This may be also the case for all other fusion assays which are based on a mixing of the aqueous liposome contents. Indeed, quite a large amount of pNA was formed (with a fusion efficiency of 60%) when α-chymotrypsin-containing positively charged POPC/SA (9:1) liposomes were mixed with Suc-AAPF-pNA-containing negatively charged POPC/DCP (8:2) liposomes. In this case, liposome fusion occurred with a high selectivity between enzyme-containing liposomes and substrate-containing liposomes, based on electrostatic interactions.

Although it is generally desired that the fusion efficiency of our liposome systems should be improved, membrane fusion induced by conformationally altered proteins under stress conditions may have specific advantages over the conventional methods. In drug delivery systems, for instance, membrane fusion may be arbitrarily generated on a target cell if the membrane fusion

can be controlled through the stress conditions. By combining enzymatic reactions with membrane fusion processes, it may be possible to design an effective drug carrier for the synthesis of a particular drug from a prodrug on a target cell surface during a stress-mediated fusion process and the control release of the drug at the target cell. Furthermore, the results obtained in this work suggest that one can utilize enzymatic reactions for fusion assay if enzyme molecules and substrate molecule for the enzyme can be separately and efficiently entrapped in liposomes.

Conclusion

Fusion of POPC liposomes which separately contained enzyme and its substrate was induced by the interaction between POPC membranes and conformationally changed cytochrome *c*. Following conclusions were obtained.

(i) Liposomes which contained the small substrate of α-chymotrypsin, Suc-AAPF-pNA, were prepared for the fusion experiment in which the fusion between α-chymotrypsin- and substrate-containing liposomes was examined. Membrane permeability of liposomes for small molecule Suc-AAPF-pNA could be controlled by membrane fluidity. Suc-AAPF-pNA molecules were efficiently trapped in 100 nm unilamellar liposomes which have lower membrane fluidity than 50 nm liposomes and homogeneous surface.

(ii) By employing the aqueous two-phase partitioning method and the ILC, it was demonstrated that cytochrome *c* in 0.5 M GuHCl had quite large local hydrophobicity and strongly interacted with POPC liposomes with no net charge through hydrophobic interaction. Locally hydrophobic sites of cytochrome *c* strongly perturb hydrocarbon region of lipid membranes by penetrating into membranes, which resulted in aggregation and subsequent fusion of neutral liposomes.

(iii) The enzymatic reaction triggered by the fusion process was examined. Cytochrome *c*-mediated fusion of enzyme-containing liposomes and substrate-containing liposomes resulted in the release of the hydrolysis product, pNA, to outer phase of fused liposomes. The amount of the released pNA was well-correlated with the strength of the liposome−cytochrome *c* interactions as determined by the ILC measurements. Enzymatic reaction triggered by fusion of liposomes can be utilized in designing a functional drug carrier and as a novel fusion assay.

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