Effect of Freeze-Thawing on Phospholipid/Surfactant Mixed Bilayers

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The effect of freeze-thawing of egg phosphatidylcholine (PC) and octyl glucoside (OG) on vesicle growth and on liposomal formation by the subsequent removal of the OG was investigated. When increasing concentrations of OG were mixed with PC vesicles of 10 or 20 mM PC, vesicle growth was observed until the ratio of OG/PC reached about 3.0, and then micellization occurred at a higher concentration of OG, as determined by turbidity change and microscopic observation. On the other hand, a marked increase in turbidity was observed after freeze-thawing of the samples when the OG/PC ratio was less than 3.0. The frozen and thawed mixed bilayer composed of OG/PC = 2 formed closed vesicles having solute-trapping ability as determined by fluorescence microscopy. Interestingly, the trapping volume of liposomes generated after removal of OG from freeze-thawed samples was higher for those from mixed micelles than for those from mixed vesicles composed of OG and PC. When increasing concentrations of OG were mixed with PC vesicles of 0.8 or 2 mM PC, micellization started when the OG was at about its critical micelle concentration (cmc), although marked turbidity was observed when the OG/PC ratio was 2.0 after freeze-thawing. These data suggest that freeze-thawing affects the bilayer-micelle transition and liposomal formation after removal of OG even at concentrations of detergent lower than its cmc.

Key words phospholipid; octyl glucoside; mixed bilayer; liposome

Liposomes have been widely used as a model membrane for investigating the properties of biomembranes and for studying the functions of membrane proteins. The detergent removal method is common for preparing liposomes, especially for the reconstitution of membrane proteins which are also solubilized by some detergents. The non-ionic detergent octyl glucoside (OG) has been commonly used for reconstitution,[1-4] as well as for homogeneous liposome formation,[5] since this detergent has a relatively high critical micelle concentration (cmc) and is believed to be mild without high denaturing activity against membrane proteins. Therefore, many in-depth studies have been done to elucidate the interaction of the detergent with phospholipids.[6-11]

The scheme of the solubilization of phospholipid vesicles by a surfactant is generally thought to be as follows: As a detergent is gradually added to an aqueous suspension of phospholipid vesicles, the detergent partitions into the lipid bilayer until saturation is achieved. The partition coefficient is reported to be about 60 in the case of OG.[6,12] This step is accompanied by a permeability increase of the bilayer.[8,11] Then, the detergent-phospholipid mixed-vesicles are converted into detergent-phospholipid mixed-micelles. Finally, the mole fraction of lipid in the micelles is decreased by further addition of detergent. The detergent-lipid ratio (R_e) for saturation and solubilization is suggested to depend on the detergent cmc and on the bilayer/aqueous phase distribution coefficient (k) regardless of the type of detergent.[12]

Ollivon and co-workers observed an increase in turbidity and release of large molecules like inulin near the transition from mixed-vesicles to mixed-micelles which suggested the opening of vesicles.[6] We postulate that when the concentration of vesicles is rather high, for example, more than 10 mM as phospholipids, micellization should occur after saturation of OG in the vesicles, since the saturation concentration of OG is well above the cmc. On the contrary, when the concentration of vesicles is low, for example, 2 mM as phospholipids, OG partitioning should increase with the detergent's increasing concentrations until near its cmc without micellization since the concentration of OG is below the cmc. If so, freeze-thawing would affect the bilayer-micelle transition of phospholipid and OG, since the freezing step essentially concentrates the detergent to a level higher than its cmc even when the initial concentration is below this. Thus we investigated the vesicle-micelle transition with or without freeze-thawing, and observed almost constant detergent–lipid ratio for making turbid aggregation and/or fusion after the freeze-thawing process in spite of liposomal concentration differences.

Materials and Methods

Materials High-purity egg yolk phosphatidylcholine (PC) was a gift from Nippon Fine Chemical Co. (Takasago, Hyogo). The non-ionic detergent *n*-octyl-β-D-glucoside, 3-(*N*-morpholino)propanesulfonic acid (Mops), and EDTA were purchased from Dojindo Laboratories (Kumamoto). Calcein was obtained from Sigma Chemical Co. (St. Louis, MO). Reduced Triton X-100 was from Aldrich Chemical Co. (Milwaukee, WI). All other reagents were of reagent grade from Wako Pure Chemical Industries (Osaka).

Preparation of Liposomes Sonicated phospholipid vesicles were prepared as follows: PC dissolved in chloroform was dried under reduced pressure and stored *in vacuo* for at least 1 h. Liposomes were produced by hydration of the thin lipid film with 10 mM Mops-buffer, pH 7.2, containing or without 0.1 mM calcein and 0.01 mM EDTA. After three cycles of freeze-thawing with liquid nitrogen, liposomes were sonicated for 10 min at the 50% duty cycle of a Branson Sonifier Model 250. After preparation of the liposomal solution, the liposome concentration was determined by phosphorus assay by a modification of the Bartlett procedure.[13]

Turbidity Measurement To study the interaction of liposomes with OG, turbidity at 450 nm was monitored. Liposomes prepared in 10 mM Mops-buffer, pH 7.2, were mixed with various concentrations of OG. The turbidity was monitored before and after a cycle of freeze-thawing with liquid nitrogen.

Assay of Liposomal Formation after Removal of the Detergent Liposomes prepared in 10 mM Mops-buffer, pH 7.2, containing 0.1 mM calcein and 0.01 mM EDTA were mixed with various concentrations of OG. After a cycle of freeze-thawing or without this, 500 μl of the mixture was dialyzed against 10 mM Mops-buffer, pH 7.2, containing 0.1 mM

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calcein and 0.01 mM EDTA for 48 h with 3 changes of the dialysis solution.

The volume inside the resulting liposomes, or trapping volume, was determined according to a method described previously with a slight modification.[14] In brief, an appropriate aliquot of the reaction mixture was diluted to 2 ml with 10 mM Mops-buffer, pH 7.2, containing 0.01 mM EDTA, and fluorescence intensity was measured before and after addition of 0.1 ml of 10 mM $CoCl_2$. Fluorescence was measured by a Hitachi fluorescence spectrophotometer F-4010 with excitation and emission wavelengths of 488 nm and 520 nm, respectively. The cobalt ion quenches the fluorescence of the calcein that is accessible to it, *i.e.*, that which is not trapped by the liposomes, so that the fraction of the fluorescence remaining after cobalt addition corresponds to the encapsulated calcein. Background fluorescence due to incomplete quenching is almost negligible, a few tenths of a percent of the total fluorescence, and was determined for each sample by lysing the vesicles with 0.1 ml of 10% reduced Triton X-100 after the cobalt addition. Exact trapping volume was calculated from the calcein trapping and the phosphorus content of each sample.

Fluorescence Microscopy To determine the barrier function of phospholipid–OG mixed bilayer, we mixed 1 ml of sonicated liposomes prepared in 10 mM Mops-buffer, pH 7.2, containing 0.1 mM calcein and 0.01 mM EDTA with various concentrations of OG. After three cycles of freeze-thawing, 10 μl of 100 mM $CoCl_2$ was added, and the samples were observed by fluorescence microscopy (Olympus IMT2 equipped with IMT2-RFC).

Results and Discussion

Turbidity of Phospholipid Vesicles in the Presence of OG When OG is added to the PC vesicles, OG partitions into lipid bilayers of the vesicles until solubilization occurs, *i.e.*, the OG and PC make mixed-micelles. Figure 1 shows the interaction of OG and PC vesicles when the PC concentrations were varied.

Figure 1a shows the interaction of OG and PC vesicles when low PC concentrations were used. Figure 1a (solid symbols) shows the turbidity change of PC vesicles by the addition of increasing concentrations of OG without freeze-thawing. The turbidity was increased only slightly by the addition of OG, which occurred at approximately 20 mM OG. This pattern is consistent with previous observations.[6,8] This mild increase in turbidity occurred near the cmc, which is slightly decreased in the presence of phospholipids.[6] A similar turbidity increase was observed with another detergent.[15]

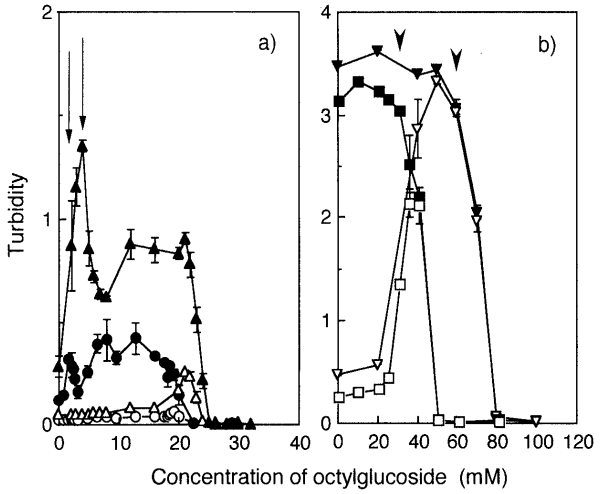

Fig. 1. Effect of OG on Liposomal Turbidity

Turbidity of sonicated PC liposomes was determined after incubation with OG (open symbols) or after freeze-thawing in the presence of OG (closed symbols). a, arrow indicates the molar ratio of OG/PC = 2.0; b, arrowhead in indicates that of 3.0. Concentration of PC: ○, ●, 0.8 mM; △, ▲, 2 mM; □, ■, 10 mM; and ▽, ▼, 20 mM.

When the PC concentration was low and the samples were frozen and thawed (Fig. 1a, open symbols), a marked turbidity increase was observed at the OG/PC ratio of 2.0. de la Maza and Parra reported that the initial steps of bilayer saturation were attained with a free surfactant at a concentration (S_w) lower than its cmc and that when S_w reached the cmc, the solubilization started to occur.[11] If so, freeze-thawing accelerates the saturation of OG in the lipid bilayer, since OG is concentrated above its cmc during the freezing step. A moderate turbidity increase was observed after this sharp increment of turbidity, where PC vesicles were not completely solubilized until the concentration of OG reached its cmc, since the concentration of OG in this phase which increased to above the cmc during the freezing process, dropped below it after thawing. This thawing process is similar to the liposomal formation by detergent removal from or dilution of OG/phospholipid vesicles. Such a phenomenon was observed when phospholipid vesicles were frozen and thawed with strong chaotropes, *i.e.*, sodium trichloroacetate (NaTCA), that solubilize phospholipid vesicles.[16] When sonicated vesicles were frozen and thawed with a low concentration of NaTCA, the resulting liposomes were quite large due to solubilization of phospholipid vesicles during their freezing and reformation during thawing.[17]

When the PC concentration was rather high without the freeze-thawing procedure (Fig. 1b, solid symbols), a gradual and then a marked increase in turbidity was observed as the concentration of OG was raised, followed by a drastic decrease in turbidity due to solubilization of the vesicles. This pattern is consistent with previous observations.[10] The marked increase in turbidity occurred at an OG/PC ratio of about 3.0.

On the other hand, when the samples were frozen and thawed (Fig. 1b, open symbols), high turbidity was observed from the beginning until the solubilization occurred. This might be due to fusion of PC vesicles and PC/OG mixed-vesicles. In fact, large vesicles were produced by freeze-thawing of sonicated PC vesicles.[18]

Barrier Function of OG/PC Mixed Vesicles Next, we determined the barrier function of OG/PC mixed vesicles, since de la Maza and Parra reported that mixed vesicles with an OG/PC ratio of more than 0.72 were quite permeable but still unilamellar vesicles, as determined by transmission electron microscopy.[11] Even if these mixed vesicles are unstable, allowing lipid transfer and/or fusion, they might maintain a barrier function in the absence of collision between them, since collision might be a prerequisite for lipid transfer and/or fusion. Figure 2 shows OG/PC mixed vesicles with an OG/PC ratio of 2.0. These mixed vesicles were formed by freeze-thawing in the presence of calcein, and $CoCl_2$ was added just prior to the fluorescence microscopic observation. As shown in the figure, most of the mixed vesicles retained calcein, indicating an intact barrier function of their membrane composed of PC and OG.

Liposomal Formation from OG/PC Mixed Vesicles and Mixed Micelles We then determined the liposomal formation by removing OG from mixed vesicles as well

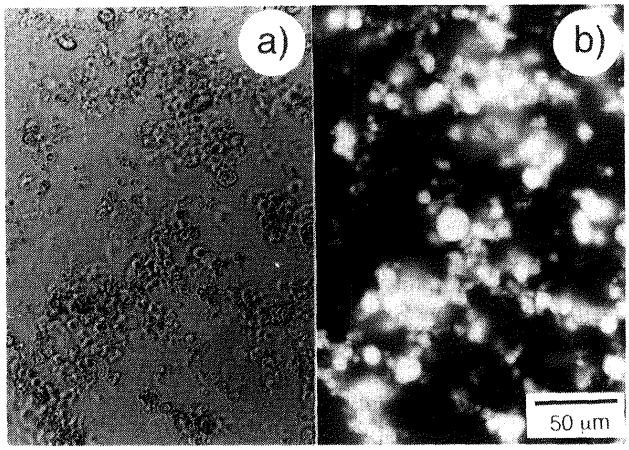

Fig. 2. Mixed Vesicles Composed of PC and OG

Sonicated liposomes (10 mM as PC) were prepared in the presence of 0.1 mM calcein, mixed with 20 mM OG, and freeze-thawed for three cycles. Then, $CoCl_2$ (final concentration of 1 mM) was added, and the preparations were observed by phase-contrast (a) and fluorescence microscopy (b).

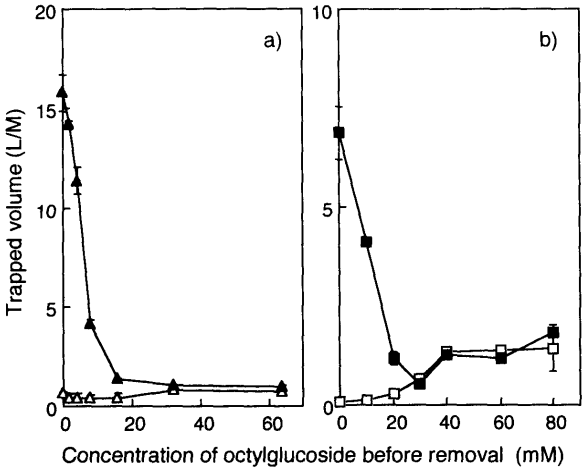

Fig. 3. Liposomal Formation after Removal of OG from OG/PC Mixture

Liposomes (2 mM as PC (a), 10 mM as PC (b)) containing 0.1 mM calcein were mixed with various concentrations of OG. After three cycles of freeze-thawing (open symbols) or none (closed symbols), the mixture was dialyzed against 10 mM Mops-buffer, pH 7.2, containing 0.1 mM calcein and 0.01 mM EDTA for 48 h. The trapping volume (liters per mol PC) was determined as described in Materials and Methods.

as from mixed micelles composed of OG and PC. Figure 3a shows the liposomal formation when 2 mM PC was used. At low concentration of OG, a marked increase in trapping volume was observed with frozen and thawed samples. Figure 3b shows the liposomal formation when 10 mM PC was used. At a low concentration of OG, marked increase in trapping volume was also observed with frozen and thawed samples, although the volume for these generated liposomes was smaller than that generated using 2 mM PC. This evidence is consistent with our previous study that the trapping volume of liposomes after freeze-thawing was higher when a lower concentration of PC was used.[14]

Interestingly, the trapping volume of liposomes generated from mixed vesicles with OG/PC ratios of about 2 to 3, the turbidity of which was rather high, was low compared with that of liposomes generated from mixed micelles. Furthermore, the trapping volume from mixed micelles was dependent on the PC concentration: liposomes generated from 10 mM PC and a high concentration of OG show higher trapping volume than those generated from 2 mM PC with a high concentration of OG. This might be due to the high collision frequency of mixed micelles during vesicle formation by the removal of OG when the concentration of mixed micelles is high.

The present data may be helpful for studies involving reconstitution and vesicle formation from detergent-solubilized phospholipids.

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References

1) Levy D., Gulik A., Bluzat A., Rigaud J. L., *Biochim. Biophys. Acta*, **1107**, 283—298 (1992).
2) Fini C., Thuong V. D., Aliante M., Floridi A., Cannistraro S., *J. Membr. Biol.*, **142**, 137—144 (1994).
3) Hammond J. R., *J. Pharmacol. Exp. Ther.*, **271**, 906—917 (1994).
4) Shapiro A. B., Ling, V., *J. Biol. Chem.*, **270**, 16167—16175 (1995).
5) Choquet C. G., Patel G. B., Beveridge T. J., Sprott, G. D., *Appl. Environ. Microbiol.*, **58**, 2894—2900 (1992).
6) Ollivon M., Eidelman O., Blumenthal R., Walter A., *Biochemistry*, **27**, 1695—1703 (1988).
7) Paternostre M. T., Roux M., Rigaud J. L., *Biochemstry*, **27**, 2668—2677 (1988).
8) Ueno M., *Biochemistry*, **28**, 5631—5634 (1989).
9) Miguel M. G., Eidelman O., Ollivon M., Walter A., *Biochemistry*, **28**, 8921—8928 (1989).
10) Almog S., Litman B. J., Wimley W., Cohen J., Wachtel E. J., Barenholz Y., Ben-Shaul A., Lichtenberg D., *Biochemistry*, **29**, 4582—4592 (1990).
11) de la Maza A., Parra, J. L., *Eur. J. Biochem.*, **226**, 1029—1038 (1994).
12) Lichtenberg D., *Biochim. Biophys. Acta*, **821**, 470—478 (1985).
13) Bartlett G. R., *J. Biol. Chem.*, **234**, 466—468 (1959).
14) Oku N., Kendall D. A., MacDonald R. C., *Biochim. Biophys. Acta*, **691**, 332—340 (1982).
15) Goni F. M., Urbaneja M. A., Arrondo J. L. R., Alonso A., Durrani A. A., *Eur. J. Biochem.*, **160**, 659—665 (1986).
16) Oku N., MacDonald R. C., *J. Biol. Chem.*, **258**, 8733—8738 (1983).
17) Oku N., MacDonald R. C., *Biochim. Biophys. Acta*, **734**, 54—61 (1983).
18) Oku N., MacDonald R. C., *Biochemistry*, **22**, 855—863 (1983).