CHEMISTRY LETTERS, pp. 1249-1252, 1990.

Effective Transfer of Membrane Proteins from Human Erythrocytes to Artificial Boundary Lipid - Containing Liposomes

Junzo SUNAMOTO,* Mitsuaki GOTO,† and Kazunari AKIYOSHI
Department of Polymer Chemistry, Faculty of Engineering, Kyoto University, Sakyo-ku, Yoshida Hommachi, Kyoto 606
† Department of Industrial Chemistry, Faculty of Engineering, Nagasaki University, Bunkyo-machi, Nagasaki 852

Several proteins were directly and effectively transferred from human erythrocyte membranes to dimyristoylphosphatidylcholine (DMPC) liposomes which contain an artificial boundary lipid (1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine, DDPC). Of the membrane proteins, the activity of acetylcholine esterase (AchE) was monitored. The activity of AchE and the amount of total protein transferred were highest when 40 mol% of DDPC was added to DMPC.

We have recently developed a novel artificial boundary lipid, 1,2-dimyristoylamido-1,2-deoxyphosphatidylcholine (DDPC) [1] to make liposomes more stable and cell recognizable by reconstituting glycophorin into egg PC liposomes.[1-4] DDPC has two amide bonds instead of ester bonds of regular lecithins such as 1,2-dimyristoylphosphatidylcholine (DMPC). As expected, DDPC was better to keep glycophorin in eggPC liposomal membranes compared with a naturally occurring sphingomyelin.[1,2] This better reconstituting efficiency of glycophorin into egg PC/DDPC liposomes is due to the hydrogen bonding belt provided by DDPC in lipid bilayers[4] and consequent membrane stabilization.[2] Judging from results of deuterium NMR investigation,[5] it was considered that, in the DDPC/DMPC mixed bilayer which contains glycophorin, DMPC simply behaves as the matrix lipid, while DDPC surrounds glycophorin and certainly plays a role of the boundary lipid. In this paper, we would like to show an excellent effect of DDPC in the transfer of membrane proteins from erythrocytes to liposomes.

Perfect extraction of intrinsic membrane proteins or enzymes from intact cells without any denaturation and deactivation is basic requirement in investigation of the function of membrane proteins and/or in membrane protein engineering. Huestis and his coworkers have developed an elegant technique for transferring intrinsic membrane proteins from red cells to DMPC liposomes.[6-11] When the specifically modified liposomes, which contain an artificial boundary lipid, DDPC, are coincubated with intact cells, several membrane proteins and/or enzymes are able to be effectively transferred from the intact cells to the liposomes.

Protein transfers were carried out according to almost the same procedures as those performed by Huestis and his coworkers,[7] in order to directly compare the present results with those of them. Human erythrocytes were obtained from healthy adult volunteers, separated from serums, and washed as described.[6,7] A mixture of DMPC and DDPC at a given ratio was dissolved in dry chloroform. Lipid thin film was prepared in a round bottom flask by evaporating the solvent under reduced pressure using a rotary evaporator. The lipid thin film so

obtained was dispersed in an aqueous buffered solution by vortexing, and then submitted to ultrasonic irradiation at 25 W for 15 min at 30 s intervals above their phase transition temperatures using a Tomy UR-200P probe type sonifier. All the experiments were conducted in 310 mOsm phosphate buffered saline (pH 7.38) containing 140 mM NaCl, 5 mM KH_2PO_4, 2.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 5 mM glucose, and 50 mM sucrose.[6] A liposome suspension was mixed with a given volume of packed erythrocyte suspension (1.5 ml) and to stand for 2 h at 37.0 °C. This incubation time (2 h) was decided by referring results of Huestis and his coworkers.[7] Cell-liposome suspensions were incubated under agitation at 37.0 °C on a shaking water bath. After the incubation, cells and liposomes were separated by centrifugation at $300 \times g$. Liposome suspensions in the supernatant (2 ml) were further submitted to gel filtration through Sepharose 4B column ($\phi 1.8 \times 45$ cm) in order to eliminate a small amount of soluble proteins as contaminated. Thin layer chromatographic analysis of liposomes after exposure to cells was performed on HPTLC (Merck) as developed by chloroform-methanol-water (65 : 25 : 4 by vol). To a 0.9 ml of the liposome containing eluate of gel filtration using Sepharose 4B was added 2 ml of methanol-chloroform mixture (1 : 1, by vol) in a test tube. The resulting mixture was shaken until solution became cloudy. The organic layer was replaced to a glass tube and evaporated to dryness. One milliliter of water was added to disperse the lipids. Amount of inorganic phosphates was determined using a Phospholipid Test Kit (Wako Pure Chemical Ind., Tokyo).[1]

In thin layer chromatogram no significant difference in the lipid composition of liposomes was observed before and after exposure of liposomes to cells under the present conditions except that very small amounts of sphingomyelin and phosphatidylserine were detected at liposome site after exposure to cells. Judging from a fact that total lipid concentration in the liposome fractions slightly decreased, either some lipids might be transferred from liposomes to cells or several liposomes might fuse with cells.[6,7,11]

Which proteins were transferred from cells to liposomes was investigated by SDS-polyacrylamide gel electrophoresis (PAGE) according to the method described elsewhere.[6,7] Major five proteins were bands of 34, 52, 56, 62, and 68.5 kDa, and, in addition, many other minor proteins were also observed in the SDS-PAGE. Although the amount of proteins transferred was, of course, differed by lipid composition of liposomes employed, the number and sort of major bands on SDS-PAGE were essentially almost the same. Unfortunately, difference between peripheral and integral proteins of total membrane proteins could not be distinguished in this work. Most important and interesting finding is that no hemolysis occurred at all under the conditions employed in this work except the case of DMPC (50 mol%)/DDPC (50 mol%) liposome (run 8 in Fig. 1). This means that any intracellular proteins such as cytoskeleton and/or other soluble proteins most probably do not interact directly with liposomes during the membrane protein transfer.

The lectin-induced aggregation of protein-containing liposomes was investigated by employing TTA (Japan horseshoe crab lectin, Seikagaku Kogyo) which is a lectin specific to sialic acid.[1] After DMPC(60 mol%)/DDPC(40 mol%) liposome was exposed to cells, it was gel filtrated on a Sepharose 4B column. A 2.8 ml of liposome containing eluate was preincubated in a cuvette for 10 min at 25 °C, and an aqueous TTA solution (200 μg / 50 μl of saline) was added. Lectin-induced aggregation of liposome was followed by monitoring an increase in the turbidity at 360 nm. When the turbidity increase was leveled off at 20 min after addition of lectin, 50 μl of 1 M sialic acid solution was added and the recovery of turbidity of the suspension was examined. The liposome exposed to cells was specifically aggregated by sialic acid specific lectin (TTA). This means that some of proteins transferred from cells to liposomes are certainly sialoproteins including glycophorins and AchE. This lectin-induced aggregation of protein reconstituted liposomes is reversible, and the turbidity of liposomal suspen-

sion is almost completely recovered by adding better substrate to the lectin, free sialic acid, into the system (though data is not shown).

Amount of proteins transferred from cells to liposomes was determined by fluorescamine method[12] which was more sensitive and better than that using a kit of Bio Rad. To a liposome suspension (200 μl) diluted by adding saline (1.3 ml) was added 2 %(v/v) aqueous deoxycholate solution (25 μl) to completely destruct liposomes. A 0.5 ml of dioxane solution of fluorescamine (2 mg / 10 ml) was added to the resulting mixture and it was rapidly and vigorously shaken. The product between the protein and fluorescamine emits fluorescence at 475 nm by excitation at 390 nm. The protein content was determined from a calibration curve obtained in advance using bovine serum albumin. The fluorescence intensity was measured at 25 °C on a Hitachi 650-S fluorometer. When egg PC was used as the matrix lipid, protein transfer was hardly observed. Addition of cholesterol to egg PC rather caused a decrease in the transfer efficiency of protein. When 40 mol% of DMPC was added to egg PC, however, the protein transfer efficiency increased. DMPC behaved as the better matrix lipid than egg PC. Egg PC(60 mol%)/DDPC(40 mol%) mixed liposome (run 4 in Fig. 1-A) showed an increased efficiency of protein transfer than egg PC(60 mol%)/DMPC(40 mol%) mixed liposome (run 3 in Fig. 1-A). The highest protein transfer efficiency was observed in the case of DMPC(60 mol%)/DDPC(40 mol%) mixed liposome (run 7 in Fig. 1-A), and addition of more DDPC to DMPC rather decreased the efficiency.

The protein employed as a transfer marker in this work is AchE (acetylcholine esterase), because this is the most easily assayed one of the transferred proteins.[7] Activity of AchE was assayed for both before and after transfer.[13] A typical run used: 3.0 ml of saline (pH 7.38), 20 μl of 0.079 M acetylthiocholine iodide, 100 μl of 0.01 M 5-dithio-bis-2-nitrobenzoic acid (DTNB), 50.0 μl of a protein-containing liposome or cell suspension.

This is accomplished by continuous reaction of thiol with 5,5'-dithiobis-2-nitrobenzoic acid to produce

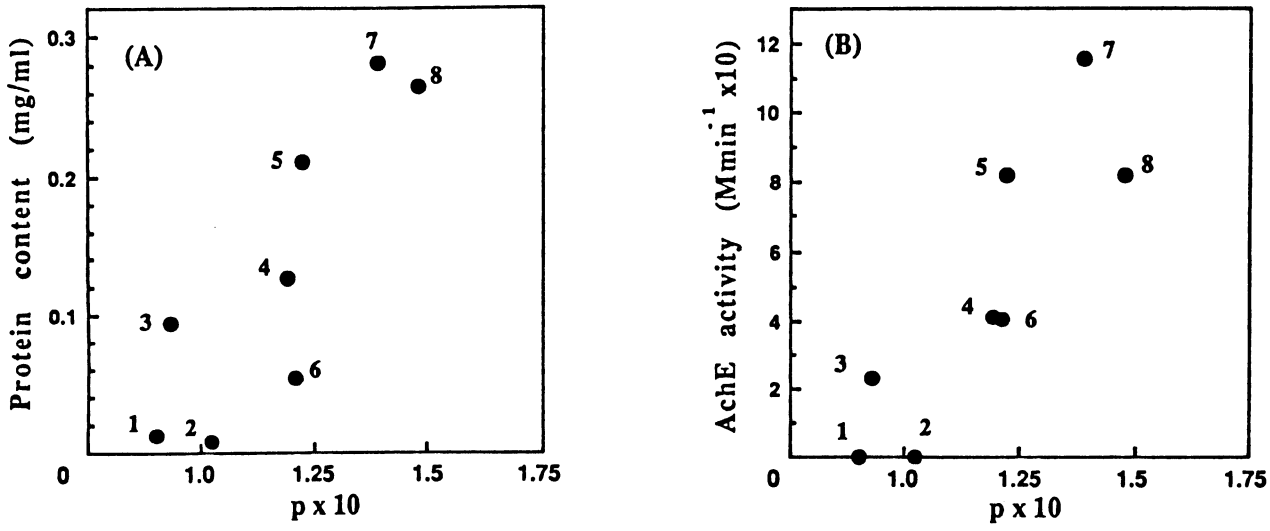

Fig. 1. Protein contents (A) and acetylcholine esterase (AchE) activity (B) in various liposomes after exposure to human erythrocytes in PBS at 37.0°C for 2 h as a function of fluidity of membrane surface (p-value) determined by fluorescence depolarization measured using DSHA as the probe. ①, egg PC; ②, egg PC(60 mol%)/cholesterol(40 mol%); ③, egg PC(60 mol%)/DMPC(40 mol%); ④, egg PC(60 mol%)/ DDPC(40 mol%); ⑤, DMPC; ⑥, DMPC(60 mol%)/cholesterol(40 mol%); ⑦, DMPC(60 mol%)/DDPC(40 mol%); and ⑧, DMPC(50 mol%)/DDPC(50 mol%).

yellow anion of 5-thio-2-nitrobenzoate. An increase in the absorbance was followed as a function of time. The linear portion of the curve describing the hydrolysis was observed during the first 15 - 20 min of the reaction; the slope is the rate in absorbance units/min. Since the extinction coefficient of yellow anion is known ($\varepsilon_{412} = 1.36 \times 10^4$), the rates can be converted to absolute units, *viz.*: rate ($M \cdot min^{-1}$) = ($\Delta Abs \cdot min^{-1}$) / 1.36×10^4.When DMPC(60 mol%)/DDPC(40 mol%) mixed liposome was employed, more than 95% of the enzyme activity remained even after transfer (run 7 in Fig. 1-B). This effect was much better than the case of simple DMPC liposome as reported by Cook, et al.[7] The enzyme activity on liposomal surfaces was exactly parallel to the amount of proteins transferred. Considering that the substrate, acetylthiocholine, is membrane impermeable, the AchE appears to transfer from erythrocytes to liposomes in native orientation.[7]

Addition of DDPC to egg PC or DPPC bilayer membrane makes the membrane less fluid.[1,3] Considering that the protein transfer efficiency is significantly governed by the fluidity of participant membranes,[7] membrane fluidity of liposomes was estimated by fluorescence depolarization technique as described elsewhere.[1,3] The molar ratio of the fluorescent probe employed, dansylhexadecylamine (DSHA), to lipids was kept at 1 : 100 through all runs. Figure 1 shows the amount of protein transferred from cells to liposomes (Fig. 1-A) and AchE activity in liposomes (Fig. 1-B) as a function of the fluidity of liposomal membranes. Both the transfer efficiency of membrane proteins from erythrocytes to liposomes and the enzyme activity on the liposomes are closely correlated with fluidity of surface of liposomal membrane as determined by the use of DSHA as a relatively hydrophilic probe. Although the fluidity of very hydrophobic domain of liposomal membrane was also investigated using 1,6-diphenyl-hexa-1,3,5-triene (DPH) as a lipophilic fluorescent probe (though data are not shown), the fluidity of very hydrophobic part of liposomal membrane was not correlated with behaviors of protein transfer at all. In any event, an artificial boundary lipid, DDPC, was excellent in order for membrane proteins to be effectively transferred from cells to liposomes and be nicely retained in liposomal membranes.

This work was supported by Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (No. 63604581).

References

1) J. Sunamoto, M. Goto, K. Iwamoto, H. Kondo, and T. Sato, Biochim. Biophys. Acta, in press.
2) M. Goto, M. Arakawa, T. Sato, H. Kondo, and J. Sunamoto, Chem. Lett., 1987, 1935.
3) J. Sunamoto, M. Goto, M. Arakawa, T. Sato, H. Kondo, and D. Tsuru, Nippon Kagaku Kaishi, 1987, 569.
4) J. Sunamoto, K. Nagai, M. Goto, and B. Lindman, Biochim. Biophys. Acta, in press.
5) T. Kawai, J. Umemura, T. Takenaka, M. Goto, and J. Sunamoto, Langmuir, 4, 449 (1988).
6) S. R. Bouma, F. W. Drislane, and W. H. Huestis, J. Biol. Chem., 252, 6759 (1977).
7) S. L. Cook, S. R. Bouma, and W. H. Huestis, Biochemistry, 19, 4601 (1980).
8) W. H. Huestis and A. C. Newton, J. Biol. Chem., 261, 16274 (1986).
9) D. L. Daleke and W. H. Huestis, Biochemistry, 24, 5406 (1985).
10) A. C. Newton and W. H. Huestis Biochemistry, 27, 4655 (1988).
11) J. E. Ferrell, K.-J. Lee, and W. H. Huestis, Biochemistry, 24, 2857 (1985).
12) P. Böhlen, S. Stein, W. Dairman, and S. Udenfrien, Arch. Biochem. Biophys., 155, 213 (1973).
13) J. L. Ellman, K. D. Courtney, Jr., V. Andres, and R. M. Fetherstone, Biochem. Pharmacol., 7, 88 (1961).

(Received April 20, 1990)