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Immobilized liposome chromatography for refolding and purification of protein

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Abstract

Small unilamellar liposomes were utilized as a kind of aqueous two-phase system and artificial chaperone which specifically recognize protein conformation with fluctuated structure. Liposomes showed highly selective binding ability to conformationally changed proteins treated with various concentrations of guanidinium hydrochloride, as evaluated by immobilized liposome chromatography (ILC). In refolding of proteins, liposomes bound to refolding intermediate of proteins and prevented them from forming intermolecular aggregates. Refolding of bovine carbonic anhydrase, lysozyme and ribonuclease A was significantly improved in the presence of liposomes. Furthermore, by utilizing ILC, refolding of proteins was also successfully and simply carried out with considerable high reactivation yield.

Keywords: Immobilized liposome chromatography; Protein refolding; Lipid bilayer membranes

1. Introduction

Liposomes are closed phospholipid bilayer membranes and considered as a kind of aqueous two-phase system (ATPS) which is composed of a bulk aqueous phase, a liposome aqueous interior and lipid bilayer membranes. The lipid membranes act as a functional boundary which shows highly selective permeability for small molecules such as ions and amino acids [1]. Therefore, it is possible to concentrate particular materials inside liposomes, as observed in biological cell systems as well as minimal cell models [2]. Compared to the static properties of liposomes, dynamic aspects of liposomes are relatively unknown. Generally, macromolecules such as proteins cannot pass through lipid membranes [3]. However, proteins can bind to and pass through lipid membranes if their conformations are changed under stress conditions which also causes structural fluctuation of lipid membranes [4,5]. If one can control such a stress-mediated function of lipid membranes, the liposome system is very attractive as a novel ATPS with stable and stress-responsive functional boundaries. Typical examples are the bioseparation system [5] and the bioreactor system using liposomes as novel stress-mediated ATPSs [3,6,7]. In the latter system, ploteolytic enzyme was immobilized in the liposome aqueous interior without inactivation, and substrates for the enzyme selectively passed through the lipid membranes, leading to enzymatic reactions in the liposomes.

Recently, we reported that water-soluble proteins interact with liposomes at their partially denatured states, which was detected by using immobilized liposome chromatography (ILC) in a quantitative

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way [8,9]. The membrane-bound proteins fold into native states or translocate across lipid membranes, depending on the surface properties both of proteins and liposomes [4,5,10]. In the present work, the functions of lipid membranes, which appear through the interactions with conformationally changed proteins, were examined especially for the purpose of utilizing liposomes and ILC in refolding and purification of proteins.

2. Experimental

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG), and egg yolk phosphatidylethanolamine (EPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Superdex 200 prep. grade and a glass column (HR 5/5) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Bovine carbonic anhydrase (CAB) and bovine pancreatic ribonuclease A were obtained from Sigma (St. Louis, MO, USA). Hen egg white lysozyme and cholesterol were from Wako (Osaka, Japan). All other chemicals were of analytical grade.

2.2. Preparation of liposomes

Lipids dissolved in chloroform were dried in a round-bottom flask by rotary evaporation under reduced pressure. The lipids were redissolved in diethyl ether and then, the solvent was evaporated again. The lipid film was kept under high vacuum for at least 3 h, and then hydrated with 50 or 100 m*M* Tris–HCl buffer (pH 7.5 or 8.0) at room temperature to form multilamellar vesicles (MLVs). The vesicle suspension was frozen in dry ice–ethanol (−80°C) and thawed at room temperature. This freeze–thawing cycle was performed five times. MLVs were sized down to unilamellar vesicles by extruding the MLV suspension 15 times through two stacked polycarbonate filters with mean pore diameters of 200, 100 and 50 nm using a small-volume extruder [11]. In the case of preparation of liposomes for immobilization to gel beads, small unilamellar vesicles were prepared by probe sonication of a MLV suspension. MLVs were prepared in the same manner except that 1 mol% EPE was supplemented [8,12].

2.3. Immobilization of liposomes

Liposomes were covalently immobilized in gel beads by the method of Yang et al. [12]. Briefly, the sonicated liposomes were mixed with chloroformate-activated gel beads overnight at 4°C. The mixture was washed with 50 m*M* Tris–HCl buffer (pH 8.0) on a glass filter to remove nonimmobilized liposomes. Nonreacted ligands were blocked by circulating buffer containing 50 m*M* ethanolamine on the gel bed overnight at 25°C.

2.4. Chromatography of proteins on immobilized liposomes

Retardation of the proteins on the immobilized liposome gel bed was expressed as an apparent specific capacity factor, k_s, which is defined as $k_s = (V_r - V_e)/M'$, derived from the equation described by Beigi et al. [13], where V_r is the elution volume of proteins (ml), V_e is the elution volume of proteins (ml), which are not retarded on the column as determined by using native proteins in the absence of guanidinium hydrochloride (GuHCl), M' is the apparent amount of immobilized lipids (mmol), which is available for interaction with the proteins. The amounts of immobilized liposomal phospholipids were determined according to the method of Bartlett [14].

2.5. Denaturation and refolding of proteins

Denaturation of proteins was performed in solutions containing 5–6 *M* GuHCl in 50 or 100 m*M* Tris–HCl buffer (pH 7.5 or 8.0). Reduction of lysozyme and ribonuclease A was performed in the presence of 120 m*M* dithiothreitol (DTT). This solution was incubated for at least 5 h at 25°C. For refolding, the denatured (and reduced) protein solution was rapidly diluted with 50 or 100 m*M* Tris–HCl buffer (pH 7.5 or 8.0) in the presence or absence of liposomes ([lipid]=0.25 m*M*). In the case of lysozyme and ribonuclease A, glutathione (GSH) and oxidized glutathione (GSSG) were added to form disulfide bonds. Throughout the refolding ope-

ration, the refolding solution was stirred constantly and thermostated at 25°C. The reactivation yield is defined as the activity of the refolding solution relative to that of control containing active proteins. The enzymatic activity of CAB, lysozyme, and ribonuclease A were measured using *p*-nitrophenyl acetate [15], *Micrococcus lysodeikticus* cells [16], and cytidine 2′:3′-cyclic monophosphate [17] as substrates, respectively.

2.6. Refolding chromatography

Denatured (and reduced) proteins (sample volume: 10 μl) were applied to the ILC column and eluted with 50 or 100 m*M* Tris–HCl buffer (pH 7.5 or 8.0) with a flow-rate of 0.25–1 ml/min collecting 350-μl fractions. In the case of lysozyme, 10 m*M* GSH and 1 m*M* GSSG were immediately added to the collected fractions. After 40 min incubation at 25°C, the enzymatic activities of the pooled fractions were measured. The reactivation yield is defined as the absolute activity of the eluted sample relative to that of native sample corresponding to the same amount of the applied denatured and reduced sample.

3. Results and discussion

3.1. Selective recognition of conformationally changed proteins

First of all, the structure of a liposome, which is immobilized in gel matrix, is schematically shown in Fig. 1. The liposome aqueous interior is separated from the bulk aqueous phase by lipid bilayer membranes which show highly selective membrane permeability to solutes including proteins. In this study, small unilamellar liposomes with a diameter of about 30 nm were covalently immobilized in gel matrix [8,12]. ILC can be considered a variation of liquid–liquid partition chromatography with the functional boundary between the bulk aqueous phase and the stationary aqueous phase (liposome aqueous interior). By using ILC, various aqueous two-phase systems can be created. Furthermore, bio/synthetic polymers can be noncovalently immobilized in the liposome interior without conformational change [3,6]. In the present study, the function of lipid bilayer membranes as a boundary of an aqueous two-phase system was examined with the purpose of utilize ILC in refolding and purification of proteins.

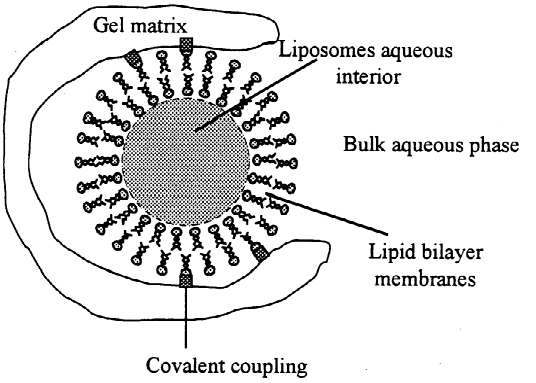

Fig. 1. Schematic illustration of a small unilamellar liposome which is covalently immobilized in gel matrix [12].

The interactions between liposomes and hen egg white lysozyme treated with various concentrations of GuHCl were examined using ILC. The elution behavior of lysozyme at various conformations in ILC is shown in Fig. 2. The elution behavior was significantly different depending on the concentration of GuHCl in eluent. In ILC, lysozyme partitions into

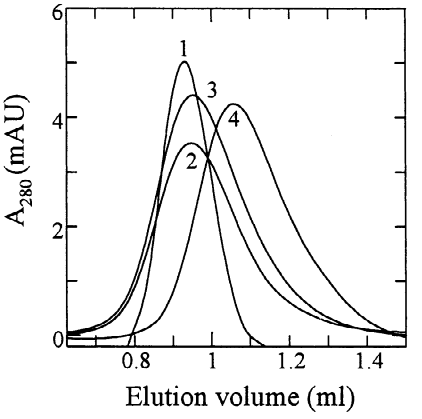

Fig. 2. Immobilized liposome chromatography of lysozyme in the presence of (1) 0 *M* (native lysozyme), (2) 0.25 *M*, (3) 0.5 *M*, and (4) 1 *M* GuHCl in eluent (100 m*M* Tris–HCl, pH 8.0). A 10-μl volume of sample ([lysozyme]=50 μ*M*) was applied to the ILC column, in which SUVs are covalently immobilized in Superdex 200 gel beads (gel volume: about 1 ml), and eluted at a flow-rate of 0.25 ml/min.

lipid bilayer membranes without passing through the membranes, because macromolecules such as proteins essentially do not pass through lipid membranes under physiological conditions [4,5]. 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.

The specific capacity factor of lysozyme in ILC [k_s (ml/mmol)] as well as local hydrophobicity [LH (–)] of lysozyme were plotted as a function of GuHCl concentration, see Fig. 3. The LH value means the degree of the binding affinity between proteins and hydrophobic probe. In the present study, Triton X-405 was used as a hydrophobic probe and the protein–Triton binding was quantified by using the aqueous two-phase partitioning method [18]. It was reported that the conformations of proteins can be evaluated by LH measurement [19], in which the LH measurement was compared with other conventional techniques such as circular dichroism (CD). Both parameters (k_s and LH values) monotonically increased up to 1 *M* GuHCl. The similar increase in these two parameters means that lysozyme interacts with liposomes depending on its local hydrophobicity. We have reported that partially denatured proteins (α-lactalbumin, CAB and cytochrome *c*) also bind to liposomes [6,8,9]. We also demonstrated that similar characteristic for the partially denatured proteins is their large local hydrophobicity [8].

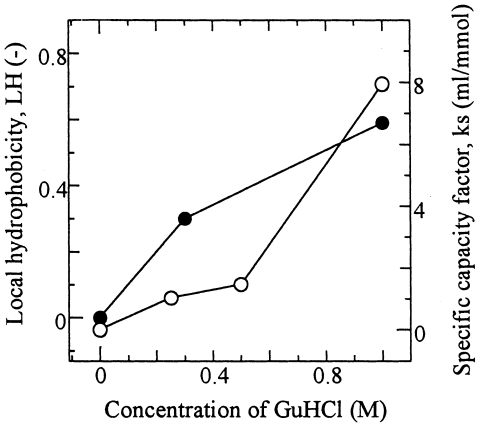

Fig. 3. Specific capacity factor of lysozyme [k_s (mmol/ml)] in ILC (○) and local hydrophobicity [LH (–)] of lysozyme (●) as a function of GuHCl concentration in eluent.

Locally hydrophobic sites of conformationally changed proteins may be derived from their structural fluctuation. Actually, among possible protein conformations, molten globule proteins with highly fluctuated structure usually show quite large local hydrophobicity in agreement with previous literature [20].

The electrostatic interactions between charged liposomes and proteins have been extensively studied in terms of protein-induced fusion of liposomes [21], liposome-induced conformational change of proteins [22], and interactions between blood protein and liposomes [23]. In contrast, the interactions between net-neutral liposomes and proteins are relatively unknown. For binding of net-neutral liposomes to hydrophobic sites of proteins, it is necessary for liposomes to have hydrophobic sites on their surface, although surface of liposomes is considered as hydrophilic as a whole. From the microscopic point of view, however, liposomes at fluid-analogue state have rather fluctuated surface due to dynamic motions of lipid molecules. The inhomogeneity of liposome surface has been observed in lipid membranes at gel state (below phase transition temperature), which induces packing defects in lipid membranes [24]. The packing defects may result in the increased accessibility of hydrophobic region of lipid membranes to aqueous phase. It was reported that such packing defects of the membranes, which are affected by lipid composition, play significant roles in the interactions with hydrophobic region of proteins [25]. For lipid membranes at liquid-crystal phase (above phase transition temperature), fluctuation of lipid molecules may also produce locally hydrophobic sites on liposome surface which can bind to exposed hydrophobic sites of partially denatured proteins. Such dynamic aspects of liposomes have not been well studied, which should be indispensable for the dynamic interaction with conformationally changed proteins. The LH-dependencies of the protein–liposome and protein–biological membrane interactions were also observed for various proteins [4,8,9]. Thus our series of results including Figs. 2 and 3 indicate that lipid bilayer membranes can selectively bind to conformationally changed proteins recognizing local hydrophobicity of proteins.

The stability of immobilized liposomes should be

mentioned. It was reported that no practical difference in the lipid amounts in gel beads was observed before and after a number of chromatographic runs in the presence of high concentrations of denaturant (at least up to 5 *M* GuHCl) and conformationally changed proteins. In addition, recently, dye-containing liposomes were immobilized in gel beads and dye leakage was monitored after a number of chromatographic running and the long-term storage (at least 1 year) [26]. Little leakage of dye molecules from liposomes interior was observed, indicating that spherical shape of immobilized liposomes was kept after the chromatographic running or long-term storage. Therefore, it can be concluded that the immobilized liposomes are quite stable.

The above-described function of liposomes can be applied to various purposes. We have already reported that liposomes have a potential to be utilized in the separation of proteins in which conformational change of proteins was induced by heat conditions for controlling protein–liposome interactions [4,5]. Recently, we also reported that conformationally changed protein has an ability to induce fusion of liposomes with no net charge, which can be utilized as nano-scale bioreactor [6]. In addition, liposomes can be utilized as an artificial chaperone which promote refolding of proteins [9,10]. In this report, we focus on the chaperone-like function of liposomes, in which the recognition of protein conformations by liposomes is utilized.

3.2. Refolding of proteins at lipid–water interface

Refolding of proteins is a series of conformational changes from a denatured state to a native state. During the refolding process, thermodynamically stable intermediate states of proteins are often observed, the so-called molten globule state, with quite large local hydrophobicity [9,10,18]. The effect of liposomes on refolding of proteins were examined. For three proteins, reactivation yields are shown in the presence and absence of liposomes, see Fig. 4. Lysozyme and ribonuclease A have four intramolecular disulfide bonds which should be reformed during the refolding process. In the presence of liposomes, the reactivation yields for three proteins were all significantly improved. Considering the results in the above section, liposomes may

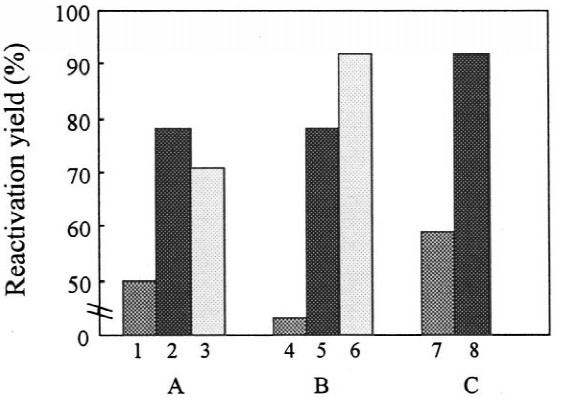

Fig. 4. Reactivation yield of (A) CAB, (B) lysozyme, and (C) ribonuclease A in the presence and absence of liposomes. Bars 1, 4 and 7 represent the yields in the absence of liposomes. Bars 2, 5 and 8 represent the yields in the presence of POPC liposomes extruded through filters of 50 nm pore size. Bars 3 and 6 indicate the yields in the presence of POPC–POPG (molar ratio 95:5) liposomes and POPC–cholesterol (molar ratio 2:1) liposomes, respectively. The final concentrations of proteins were 3.3 μ*M* for CAB, 7.1 μ*M* for lysozyme, and 62 μ*M* for ribonuclease A, respectively. The final concentration of GuHCl was 0.1 *M* for all experiments. All experiments were carried out at 25°C.

selectively recognize the intermediate states and prevent them from forming intermolecular inactive aggregates, as previously observed by ILC analysis [8,9]. It is notable that liposomes do not prevent refolding of these proteins even though liposomes bind to their refolding intermediate. It may be because lipid membranes have fluidity, which may allow dynamic folding of membrane-bound proteins into a native state. In addition, CAB (M_r=28 800) has about a two-times larger molecular mass than ribonuclease A (M_r=13 000) and lysozyme (M_r= 14 000). However, liposomes assisted refolding of proteins regardless of their molecular mass. This means that liposomes have an ability to control their surface structure recognizing the size and conformation of substrates (intermediate state of proteins). Therefore, Fig. 4 also means that liposomes have a potential to be utilized as widely applicable artificial chaperones.

The advantage of liposomes as a kind of artificial chaperone is that liposomes are easily modified with various lipids which have different charge and hydrophobicity. As shown in Fig. 4, if adequate negatively charged lipids (POPG) were added to

lipid membranes, the reactivation yield of lysozyme was further improved, while the chaperone-like activity of liposomes on refolding of CAB was decreased upon the modification with cholesterol. The addition of small amount of charged lipid may improve the binding ability of positively charged refolding intermediate of lysozyme to negatively charged liposomes, which is effective for depressing the formation of intermolecular aggregate. If the lipid membranes are modified with cholesterol, membrane fluidity of liposomes decrease [10], which results in decreased protein–liposome interactions [27]. Therefore, the binding sites for proteins, which is produced by membrane fluidity [10], decrease at the same time, leading to decreased chaperone-like function of liposomes. These results suggest that the adequate charge and hydrophobicity is necessary for liposomes to act as artificial chaperones.

3.3. ILC for refolding of proteins

It is quite useful if refolding of proteins can be carried out in ILC, as the method does not require the separation of liposomes from refolded proteins. In addition, liposomes are rather stable against stress conditions such as high concentrations of denaturant [8,9] and temperature [10,28]. Therefore, liposomes can keep the chaperone-like function for long time compared to natural chaperones such as Gro EL/ES which has been also immobilized in gel matrix for protein refolding [29]. Refolding of proteins in ILC was examined for refolding of CAB and oxidative refolding of lysozyme. The result is shown in Fig. 5. The reactivation yield of these proteins by dilution method is also shown for comparison. By using ILC, the reactivation yields of the two proteins were significantly improved compared to the dilution method. ILC refolding is very attractive because we can obtain biologically active enzyme only by applying denatured protein solution to the ILC column. Not only refolding of proteins but simultaneous purification of target proteins, which have been partly reported as novel aqueous two-phase bioseparation systems [5], is essentially possible by utilizing liposomes which can recognize particular conformations of proteins.

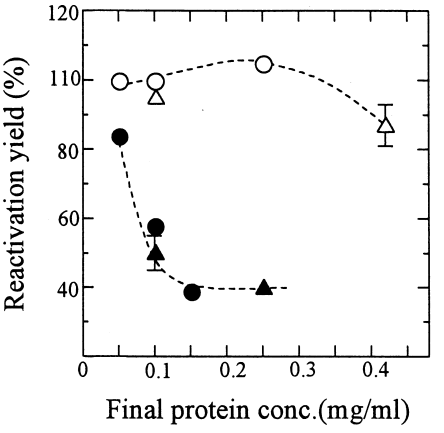

Fig. 5. Reactivation yield of CAB (△, ▲) and lysozyme (○, ●) obtained by ILC (open keys) and dilution method (closed keys) as a function of final protein concentration. In the ILC method, 10 μl of denatured (and reduced) protein solution was applied to ILC column with a flow-rate of 0.035 ml/min for CAB and 1 ml/min for lysozyme [8,9]. Eluted lysozyme was immediately oxidized in the presence of 1 m*M* GSSG and 10 m*M* GSH and incubated for 40 min before enzymatic activity assay [9].

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