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Immobilized liposome chromatography for studies of protein–membrane interactions and refolding of denatured bovine carbonic anhydrase

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Abstract

Small unilamellar vesicles (SUVs) composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 1 mol% phosphatidylethanolamine were covalently coupled to chromatographic gel beads. Interactions of liposomal lipid bilayers with several water-soluble proteins, which had been denatured or partially denatured by 0.1–5 *M* guanidinium hydrochloride (GuHCl), were studied on gel beads containing the immobilized SUVs. The partially-denatured proteins treated with 0.5–1.0 *M* GuHCl were significantly retarded on the immobilized liposome column, whereas little retardation of native or unfolded proteins treated by >2 *M* GuHCl was observed on the same liposome columns. The retardation on the immobilized liposome column was found to be well correlated with local hydrophobicity, which was determined by the aqueous two-phase partitioning method using 1 m*M* Triton X-405 as a hydrophobic probe. It implies that the partially-denatured proteins are likely in a molten-globule state and associated with liposomal lipid bilayers. Chromatographic refolding of denatured bovine carbonic anhydrase (CAB) was achieved on the immobilized liposome column. The enzymatic activity of an unfolded CAB treated by 5 *M* GuHCl was recovered up to 83% after passing it through immobilized liposome column, whereas only 58% of the enzymatic activity was recovered when the denatured CAB was run on a liposome-free column. The refolding process is probably involved in the interaction of molten-globule state of CAB with the liposomal lipid bilayers.

Keywords: Protein–membrane interactions; Enzymes; Bovine carbonic anhydrase

1. Introduction

In biological systems, denatured proteins interact with lipid bilayers and with molecular chaperones, such as heat shock proteins, during folding. It was reported that the interactions between proteins and molecular chaperones were similar to protein–membrane interactions [1]. Recent studies using liposomes as a biological model membrane have clarified the roles of physical properties both of membranes

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and proteins in membrane–protein interactions [2–4]. Partially denatured proteins as in a molten-globule state have been reported to be a key conformation in interacting with membranes [5–7]. It has been demonstrated that the molten globule state has similar secondary structure to the native state but its tertiary structure is not closely packed, resulting in a relatively high hydrophobicity compared to the native state [7–10].

Protein–membrane interactions have been studied by various methods using liposomes as a model membrane [11,12]. Since the interactions between the membranes and the proteins at the molten-globule state are usually weak and highly dynamic, systematic studies on the interactions are, to our knowledge, largely unknown. Chromatographic approaches allow accurate measurements of weak interactions between mobile interactants and immobilized ligands. Liposomes or proteoliposomes have been immobilized in gel beads as a chromatographic stationary phase for analysis of solute–membrane or solute–membrane protein interactions [13–16]. Refolding of protein has been done by chromatography on a gel bead with immobilized mini chaperones [17] and by high-performance hydrophobic interaction chromatography [18].

We have previously reported [2] that refolding of denatured CAB was assisted by SUVs, probably by weak interactions between the proteins and the liposomal lipid bilayers, similar to the protein refolding mediated by molecular chaperones. We have also reported that translocation of β-galactosidase across lipid bilayers was remarkably enhanced at the particular temperature range at which the hydrophobic properties of β-galactosidase significantly change [19,20]. The purpose of the present work is to examine the weak interactions of the water-soluble proteins, especially in their partially denatured state, with lipid bilayers by use of immobilized liposome chromatographic method [15,21]. The chromatographic retention volumes were related to the 'local' hydrophobicity of the proteins determined by the aqueous two-phase partitioning method using 1 m*M* Triton X-405 as a hydrophobic probe [22]. Refolding of the denatured protein upon chromatography on immobilized liposomes was also examined. A covalently coupling method (Yang et al., unpublished) was introduced as a new method for liposome immobilization to gel beads with high stability.

2. Experimental

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and egg yolk phosphatidylethanolamine (EPE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Superdex 200 prep. grade and the glass column (HR 5/5) were purchased from Pharmacia Biotech (Uppsala, Sweden). TSK G6000PW gel was purchased from Tosoh (Tokyo, Japan). Bovine carbonic anhydrase (CAB) was obtained from Sigma (St. Louis, MO, USA). *p*-Nitrophenyl acetate (*p*-NPA) was from Wako (Osaka, Japan). Nitrophenylchloroformate and 4-dimethylaminopyridine were from Aldrich (Milwaukee, WI, USA). 1-Anilinonaphthalene-8-sulfonic acid (ANS) was from Molecular Probes (Junction City, OR, USA). All other chemicals were of analytical grade.

2.2. Liposome preparation

POPC (50–200 μmol) and 1 mol% EPE in chloroform were dried in a round-bottomed flask by rotary evaporation. The lipids were redissolved in diethylether twice and then evaporated to a dry lipid film. The lipid film was flushed with nitrogen, kept under high vacuum for at least 3 h, and then hydrated by dispersing in 0.1 *M* Tris–HCl buffer, pH 7.5 (buffer T) to form multilamellar vesicles (MLVs). Small unilamellar vesicles (SUVs) were prepared by probe sonication of a MLV suspension in a 10-ml plastic tube on an ice-bath at 40 W for 20 min with 1 min intervals for each 1 min. The sonicated vesicle suspension was centrifuged at 100 000 *g* for 60 min at 4°C to sediment titanium particles released from the probe during sonication and residual MLVs. For preparation of unilamellar liposomes by extrusion [23], the MLV suspension was frozen in dry ice–ethanol (−80°C) for five cycles, and passed fifteen times through two stacked polycarbonate filters of 50-, 100- or 200-nm pore sizes (Nuclepore, Costar, Cambridge, MA, USA) at 23°C by using an extrusion device (Liposofast; Avestin) [23].

2.3. Activation of gels with chloroformate

Superdex 200 (denoted Superdex) or TSK G6000PW (TSK) gel was activated by nitrophenyl

chloroformate as described by Wilchek and Miron [24]. A gel slurry was washed in a glass filtering funnel (type 17G3) with distilled water with increasing concentrations of acetone (25, 50, 75 and 99.5%) and finally absolute acetone. The washed gel was mixed with 4-dimethylaminopyridine (0.52 g) in absolute acetone and nitrophenylchloroformate (0.5 g) in absolute acetone was then added dropwise within 2 min at 4°C under gentle stirring. This mixture was kept at 23°C for 1 h, and washed with acetone, acetone–2-propanol (1:1, v/v), 2-propanol–water (1:1, v/v), distilled water, and was finally suspended in coupling buffer (0.1 *M* sodium bicarbonate, pH 8.5). The ligand density of chloroformate was about 43±2 μmol/g gel as determined according to Wilchek and Miron [24].

2.4. Immobilization of liposomes in gels

The liposomes supplemented with 1 mol% of EPE were mixed with chloroformate-activated Superdex or TSK gel overnight at 4°C under gentle stirring. The mixture was washed with buffer T on a glass filter to remove nonimmobilized liposomes and gel beads containing covalently-bound liposomes were packed in a column. The nonreacted ligands were further blocked by circulating buffer T containing 20 m*M* ethanolamine on the gel beads overnight at 23°C.

2.5. Chromatography of proteins on immobilized liposomes

The immobilized-liposome-gel packed in a 50–55×5 mm glass column (HR5/5, Pharmacia Biotech) was connected to a HPLC system (AKTA, Pharmacia Biotech) with a pump, a detector set at 280 nm, a fraction collector interfaced with a PC computer.

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. [25], where V_r is the retention 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, and can be estimated as $M'=V_eC_T$, where C_T is the phospholipid concentration immobilized in a gel bed (mmol/ml). The amounts of immobilized liposomal phospholipids were determined according to Brekkan et al. [26] essentially based on the method of Bartlett [27].

2.6. Refolding of CAB assisted by immobilized liposomes

CAB (0.5–1 m*M*) was denatured by incubation with 5 *M* GuHCl at least for 1 h at room temperature, which reduced the enzymatic activity of CAB to less than 1%. For refolding of CAB by immobilized liposome chromatography (ILC) an aliquot of the denatured protein (10 μl) was applied to a liposome-immobilized column at the flow-rate of 0.035–0.25 ml/min, eluted with buffer T, and fractionated (350 μl in a test tube containing 20 μl of 100 m*M* Na_2–EDTA). EDTA is known to stop the refolding of CAB by forming a chelate complex with Zn^{2+} in CAB, but does not affect enzymatic activity of CAB [28].

2.7. Determination of enzymatic activity of CAB

CAB fractionated from immobilized liposome column or diluted in buffer T was quickly mixed with *p*-nitrophenyl acetate (concentration 1 m*M*) in 0.05 *M* Tris–HCl buffer (pH 7.5) containing 5 m*M* EDTA. The hydrolysis of *p*-nitrophenyl acetate to *p*-nitrophenol was continuously monitored on a spectrophotometer as an increase in the absorbance at 348 nm at 25°C. The initial hydrolysis rate in the first 25 s after a 5-s lag time was taken as a measure of the activity of CAB, essentially as described by Kuboi et al. [29]. The reactivation yield of CAB was estimated from the enzymatic activity of the refolded CAB relative to that of native CAB and expressed as the percentage of the native CAB activity.

2.8. Determination of hydrophobic property of proteins

The binding sites of a protein for a nonionic detergent, Triton X-405, determined by use of an aqueous two-phase system, is called ‘local’ hydrophobicity (LH (-)) and defined as LH=Δln *K*=ln

$K_{T,P}-\ln K_P$, where $K_{T,P}$ and K_P are the partition coefficients of a protein in an aqueous two-phase system in the presence of 1 m*M* Triton X-405 and in another aqueous two-phase system in the absence of Triton, respectively. These two aqueous two-phase systems were composed of 9% poly(ethylene glycol) (w/w) with a molecular mass of 3000 (denoted PEG 4000) and 9% dextran (w/w) with a molecular mass of 100 000–200 000 (denoted Dex 100 000–200 000), respectively, and prepared by mixing PEG 4000 (30%, w/w) and Dex 100 000–200 000 (30%, w/w) stock solutions with protein solution to a total mass of 5 g ([protein]=1 mg/ml) in a plastic tube. The tube was then placed vertically in a thermostat, and after equilibration (for 1 h after mixing), the top PEG phase, the interphase and the bottom Dex phase were carefully separated from each other. The *K* values were determined by measuring the protein concentrations in the top and bottom phases according to Bradford [30].

2.9. Partitioning of ANS into liposomal lipid bilayers

ANS is a hydrophobic fluorescence probe and can partition into lipid bilayers and bind to the hydrophobic core of the bilayers. Immobilized liposomes were mixed with ANS in buffer T to concentrations of 0.25 m*M* and 20 μ*M* for lipid and ANS, respectively, and a final volume of 1 ml. The fluorescence intensity of ANS associated with liposomal membranes was measured on a fluorescence spectrophotometer (FP-777 Jasco, Japan) at excitation and emission wavelengths of 400 nm and 470 nm, respectively, at 25°C. As a control, fluorescence intensity of ANS, which was mixed with the liposome-free gel beads, was measured in the same way as described for the immobilized liposomes.

2.10. Surface hydrophobicity measurement

The surface net hydrophobicity (HFS, (kJ/mol)) of liposomes and liposome-immobilized gel beads was determined with the aqueous two-phase partitioning method as for proteins [29] and liposomes [2,31]. In aqueous PEG–Dex two-phase systems (10.8%, w/w, PEG 1540 (M_r=1540), 9%, w/w, PEG 4000 (M_r=3000) and 9%, w/w, PEG 6000 (M_r=7500)/9% w/w, Dex 100 000–200 000 (M_r= 100 000–200 000), the partitioning behaviors of biomolecules and larger particles are dependent on several effects which are considered to act independently. Partition coefficient [$K=C_{\text{top phase}}/C_{\text{bottom phase}}$(-)] of biomolecules and particles can be expressed as $\ln K=\ln K_{\text{hydrophobic}}+\ln K_{\text{electrostatic}}+\ln K_{\text{salt}}+\ln K_{\text{ligand}}$, where $K_{\text{hydrophobic}}$, $K_{\text{electrostatic}}$, K_{salt} and K_{ligand} are the contributions to the partitioning of biomolecles from hydrophobic, electrostatic, salt and affinity ligand effects, respectively. In the case of POPC liposomes, the membrane surface has no net charge under the experimental conditions used in this study (pH 7.5). Therefore, the partitioning behavior of liposomes in aqueous two-phase systems at low salt concentration and without ligand is mainly dependent on the hydrophobicity ($\ln K_{\text{liposomes}}=\ln K_{\text{hydrophobic}}$). The *K* value of liposomes can be correlated with the known hydrophobic factor of the two phases (HF, (mol/kJ)) which has been previously determined by measuring partition coefficients of amino acids [22] of known hydrophobicities, RH (kJ/mol) [32]. Knowing HF and *K*, HFS can be calculated as $\text{HFS}=\ln K/\text{HF}=\ln K_{\text{hydrophobic}}/\text{HF}$ [22]. The concentration of POPC in aqueous two-phase system was 0.25 m*M* and the *K* values of liposomes in aqueous two-phase systems were determined as described above.

2.11. Dynamic light scattering analysis

The sonicated liposome samples were diluted with filtered buffer T and the average diameter and size distribution of the liposomes were analyzed with a DLS-700 Ar system (Otsuka Electric, Osaka, Japan) equipped with an argon laser (scattering angle was 90°). All measurements were done at 25°C.

3. Results and discussion

3.1. Characterization of liposome-immobilized gel beads

3.1.1. Size of immobilized liposomes

The size distribution of immobilized liposomes, which is difficult to characterize directly, was estimated by the dynamic light scattering (DLS) analy-

sis essentiality according to Yang et al. [15]. The size distribution of immobilized liposomes can be estimated by comparison of the mean sizes and the apparent size distribution of the originally prepared liposomes (Fig. 1a) and those remaining unbound after mixing with the gel beads for liposome immobilization (Fig. 1b). For unbound liposomes (Fig. 1b), its size distribution was sifted to larger particle diameters than that of the liposomes before immobilization (Fig. 1a), suggesting that small liposomes in originally prepared liposomes were selectively immobilized inside the pores of the gel beads.

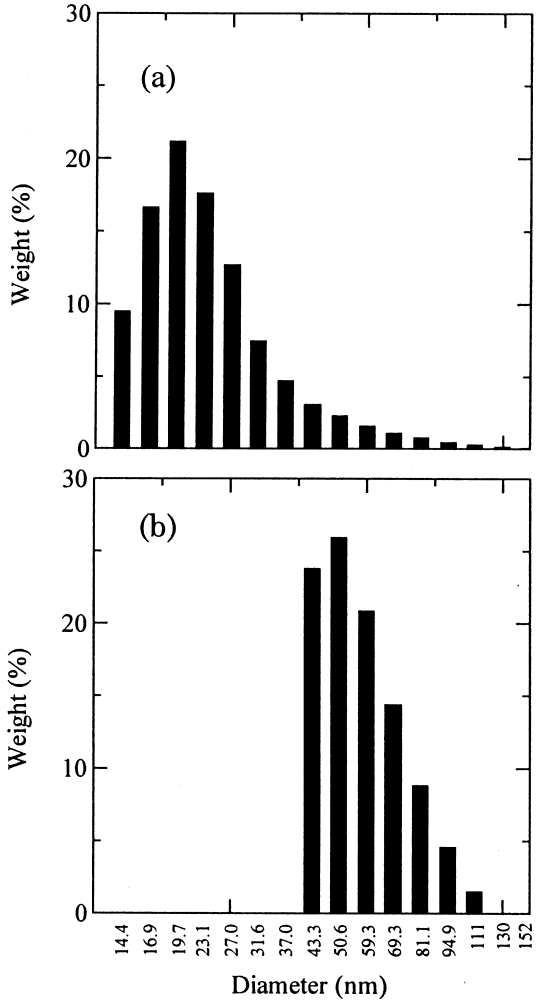

Fig. 1. Dynamic light scattering analysis of apparent size distribution in relative weight for SUVs composed of POPC suspended in buffer T (a) before and (b) after mixing activated gel beads overnight at 4°C.

In the next series of experiments, three sizes of liposomes, which were prepared by extrusion using filters of 200-, 100- and 50-nm pore sizes, respectively, were immobilized in gel beads in order to examine the size dependency on the amount of immobilized lipids (data not shown). The amount of immobilized lipids increased with decreasing liposome size, moreover, the amount of 50-nm liposomes was still lower than that of sonicated liposomes with mean diameter of 26 ± 1 nm. This result is also consistent with size-exclusion properties of Superdex 200 (denoted Superdex) [33]. Therefore, it can be concluded that in the case of Superdex gel beads for liposome immobilization, small liposomes such as sonicated liposomes are the best choice in order to achieve large amounts of immobilized lipids (liposomes).

In this study, TSK G6000PW (TSK) gel beads were partly used for protein refolding experiments. The amount of immobilized lipids was larger for TSK gel beads than for Superdex gel beads (Table 1) when sonicated liposomes were immobilized. Moreover, a significant difference in size the distribution of liposomes between before and after immobilization to TSK was not observed (data not shown), suggesting that sonicated liposomes were immobilized regardless of their size distribution, in agreement with an earlier finding [15]. This is probably because TSK gel beads have a larger pore size than Superdex gel beads.

3.1.2. Surface properties of liposome-immobilized gel beads

The change in the surface properties of gel beads before and after immobilization of liposomes was examined in terms of hydrophobicity. In the hydrophobicity measurements, we distinguish between surface net (HFS (kJ/mol)) and local hydrophobicity (LH (-)) of gel beads. Essentially, both the surface net and the local hydrophobicity can be determined by using the aqueous two-phase partitioning method [22]. Surface net hydrophobicity, which is mainly dependent on the partitioning behavior of particles in three different aqueous two-phase systems, reflects macroscopic (integral) surface properties, see Sec-

Table 1
Characterization of liposome immobilized gel beads[a]

Gel type	Amount of immobilized liposomes (μmol lipid/g moist gel)	Amount of immobilized liposomes after HPLC[b] (μmol lipid/ml gel)	Surface net hydrophobicity[c] (kJ/mol)	ANS fluorescence intensity[d]
Superdex 200	17	12	−53 (−104)[e]	186 (39)[e]
TSK G6000PW	26	26	−41 (−53)[e]	156 (42)[e]

[a] Liposomes were immobilized in gel beads by covalent coupling method, see text.
[b] Chromatographic runs were performed about 200 times and about 50 times for Superdex 200 and TSK G6000PW gel beads, respectively, using buffer T containing 0–2 *M* GuHCl as eluent.
[c] Determined with aqueous poly(ethylene glycol)–dextran two-phase systems [2].
[d] Initial fluorescence intensity just after mixing gel beads (suspended in buffer T adjusting to 0.25 m*M* of immobilized liposomes) and 20 μ*M* ANS to the total volume of 1 ml at 25°C [2].
[e] Values in parentheses; for gel beads before immobilization of liposomes.

tion 2.Local hydrophobicity, on the other hand, is determined as the difference in partitioning coefficients of particles (or proteins) in aqueous two-phase systems in the presence and absence of hydrophobic probe Triton X-405. However, in the presence of Triton X-405, liposomes are affected in their bilayer structure so, we determined the local hydrophobicity of liposomes and liposome-immobilized gel beads by using ANS as another hydrophobic probe which has been used before for proteins [9,34] and liposomes [2]. It has been reported that ANS intensity is compatible with the local hydrophobicity determined by using the aqueous two-phase partitioning method [35]. ANS is a small hydrophobic molecule, and in the case of liposomes, it partitions into the lipid–water interface region of liposome membranes which results in a strong increase in its fluorescence [2]. ANS intensity reflects microscopic surface hydrophobicity of particles, which cannot be detected by the surface net hydrophobicity measurement (HFS value).

Intact liposomes have an essentially hydrophilic surface (low HFS value) and at the same time, the surface has an abundance of locally hydrophobic sites (large LH value) which is not reflected in the HFS value. The local hydrophobicity of liposomes is affected by the order (degree of lipid fluctuation) of lipid molecules especially at the lipid–water interface region. For instance, the local hydrophobicity and the membrane fluidity of SUVs with large surface curvature (disordered membranes) is higher than that of large unilamellar vesicles (LUVs) [2,36] and therefore, SUVs can more effectively interact with proteins or peptides than LUVs [2,37]. Thus, local hydrophobicity measurements of liposomes provide us with important information on liposome–protein interactions.

The surface properties of gel beads were characterized. Generally, nonmodified gel beads for size-exclusion chromatography (SEC) should have a hydrophilic surface in order to minimize the hydrophobic interactions between solutes and the stationary phase in chromatographic analysis. A small HFS value was obtained for gel beads before liposome immobilization (Table 1), that is, the gel beads themselves have a hydrophilic surface. The local hydrophobicity of the gel beads before liposome immobilization was significantly low, see the LH values in Table 1. This means that intact gel beads have few locally hydrophobic sites (binding sites for hydrophobic ANS) on the surface, therefore hydrophobic portions of proteins cannot successfully interact with them [2]. Although after immobilization of liposomes, degree of hydrophilicity was a little reduced (the HFS value was a little increased), the gel beads still keep their hydrophilic nature. On the other hand, the local hydrophobicity of gel beads markedly increased by the immobilization of liposomes. In conclusion, by immobilization of liposomes, gel beads obtained locally hydrophobic sites (binding sites for proteins) without losing their hydrophilic surface. This means that locally hydrophobic sites of immobilized liposomes were not covered by gel beads, and therefore, liposomes can successfully interact with hydrophobic species even in the pore of the gel beads.

3.1.3. Stability of immobilized liposomes

The stability of immobilized liposomes was examined. In earlier studies, liposome chromatography has been mainly used under ordinary (stress-free) conditions and under these conditions, immobilized liposomes have been reported to be stable after running HPLC for a long time [15]. In this study, however, a much higher stability of immobilized liposomes was required because the protein refolding process includes stressful conditions, particularly high osmotic pressures deriving from denaturant (GuHCl) in eluent. The high osmotic pressure affects the liposome stability, and generally, results in shrinkage of liposomes [38]. Therefore, the effect of osmotic pressure on the stability of immobilized liposomes was checked against the number of chromatographic runs. We used some immobilized methods to examine whether immobilized liposomes are stable or not against 0–2 *M* GuHCl in eluent. Recently, a novel method for liposome immobilization in gel beads by utilizing biospecific avidin–biotin binding has been reported. In this method, biotinylated phosphatidylethanolamine in the liposome membrane is bound to avidin which is covalently coupled to the gel beads [15,16]. When the avidin–biotin binding was used for liposome immobilization [15], denaturation of the coupling avidin and subsequent dissociation of biotin from avidin occurred due to the presence of high concentrations of denaturant (GuHCl). A covalent coupling method [39] was found to be more stable than the avidin–biotin binding method. The amounts of immobilized lipids are summarized in Table 1 before and after 50 HPLC runs when eluent containing 2 *M* GuHCl was used. Sonicated SUVs, which were covalently immobilized in both Superdex and TSK gel beads were stable in the presence of 2 *M* GuHCl. These results suggest that the effectiveness of the new covalent coupling method in liposome chromatography especially under osmotic stress conditions.

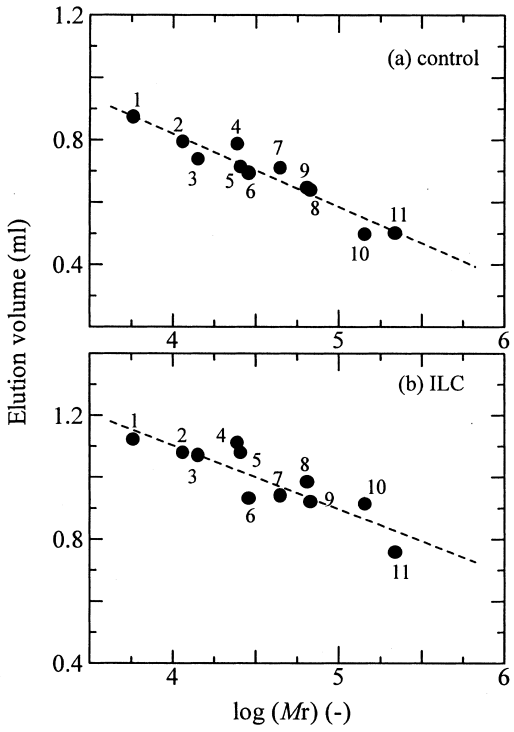

Fig. 2. Relationship between log M_r and elution volume on (a) control column and (b) ILC. 1=insulin, 2=cytochrome *c*, 3=α-lactalbumin, 4=α-chymotrypsin, 5=chymotrypsinogen, 6=CAB, 7=α-amylase, 8=hemoglobin, 9=α-glucosidase, 10=catalase, 11=alcohol dehydrogenase. Molecular masses and local hydrophobicity (LH) of these proteins are summarized in Table 2. A 10 μl volume of solution containing 10 μ*M* each native protein was eluted with buffer T (flow-rate: 0.25 ml/min); detection by absorbance at 280 nm.

3.2. Interactions of partially denatured proteins with immobilized liposomes on chromatography

3.2.1. Elution of native proteins on immobilized liposome chromatography

As seen in Fig. 2a, an almost linear relationship was observed between the protein elution volume on the control (liposome-free) column and the logarithm of the protein molecular mass (log M_r) [40,41]. Fig. 2a shows the size-exclusion properties of Superdex and Fig. 2b shows that of ILC. The linear relationship between log M_r and the elution volume was a little reduced for ILC. This suggests that ILC is so sensitive that weak interactions between native proteins and lipid bilayer membranes could be detected. In Table 2, the properties of the proteins used including its local hydrophobicity (LH (−)) which was determined with the aqueous two-phase partitioning method, are summarized. From Table 2 and Fig. 2b, it can be seen that proteins with large LH values resulted in large elution volumes, suggesting

Table 2
Properties of native proteins used

No.	Protein	M_r[a]	Elution volume[b] (ml)		LH[c] (−)
			Control	ILC	
1	Insulin	5780	0.873	1.12	N.d.
2	Cytochrome *c*	11 700	0.793	1.08	0.072
3	α-Lactalbumin	14 200	0.738	1.07	0.62
4	α-Chymotrypsin	24 800	0.787	1.11	0.15
5	Chymotrpsinogen	25 700	0.713	1.08	0.33
6	CAB	28 800	0.693	0.930	0
7	α-Amylase	45 000	0.710	0.940	0.15
8	Hemoglobin	64 500	0.645	0.983	1.2
9	α-Glucosidase	68 000	0.638	0.920	0.10
10	Alcohol dehydrogenase	144 000	0.498	0.913	1.2
11	Catalase	220 000	0.500	0.758	N.d.

[a] M_r=molecular mass.
[b] Native proteins ([protein]=10 μ*M*) were eluted with buffer T at a flow-rate of 0.25 ml/min (detection wavelength for proteins, 280 nm).
[c] LH (local hydrophobicity) was determined with aqueous two-phase system [22].
N.d: not determined.

that locally hydrophobic parts of native proteins weakly interact with liposomal bilayer membranes.

3.2.2. Elution properties of partially denatured proteins on ILC

Interactions between liposomal bilayer membranes and CAB at different conformations were examined on ILC. Conformations of CAB were controlled by 0–2 *M* GuHCl in the eluent. CAB was preequilibrated with GuHCl solution before applying to the columns. The GuHCl dependence on the elution profiles of CAB is shown in Fig. 3 both for ILC and for the control (liposome-free) column. For the control column (Fig. 3a), no significant CAB retardation was observed in the presence of 1 and 2 *M* GuHCl. In ILC, the extent of the CAB retardation increased as the GuHCl concentration in the eluent was increased to 1 *M* but decreased when the GuHCl concentration was raised to 2 *M* (Fig. 3b).

The retention time of proteins on normal SEC

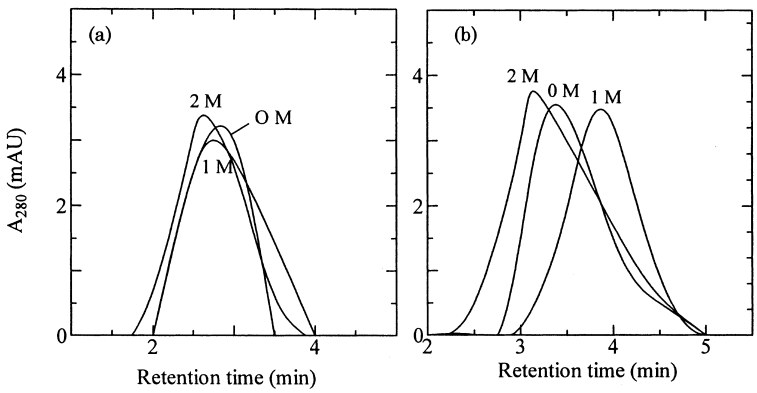

Fig. 3. Elution profiles of native and partially denatured CAB in the presence of GuHCl on (a) Superdex 200 (liposome-free) and (b) immobilized liposome chromatography. CAB samples (10 μl, [CAB]=2 μ*M*) were eluted with buffer T containing 0–2 *M* GuHCl adjusting to CAB denaturation condition (flow-rate: 0.25 ml/min). CAB was preequilibrated with 0–2 *M* GuHCl solution for 2 h before injection.

depends mainly on hydrodynamic volume and shape of solute [42], and for proteins, a change in its retention time indicates a conformational change. The denaturation behavior of proteins [9,10,41,43], has been extensively studied by using SEC because SEC analysis does not shift the equilibrium between the three protein conformations — the native (N), molten-globule (MG) and completely unfolded (U) states [41]. CAB and other globular proteins such as α-lactalbumin exist in the three discrete states (N, MG and U), and the transition between N and MG and between MG and U is an 'all or none' mechanism [44]. This means that proteins in the three different states appear as three different peaks in SEC analysis.

The elution behavior of CAB on normal SEC (Fig. 3a) indicates that CAB in the presence of 1 *M* GuHCl exists in a compact state indistinguishable from the native state. CAB denaturation begins to occur around 1 *M* GuHCl, when a sudden decrease in the biological activity and in the tertiary structure occur simultaneously [9]. We also detected this conformational change in terms of the local hydrophobicity (LH) determined with the aqueous two-phase partitioning method (see Section 3.2.3). Allthough CAB at 1 *M* GuHCl has a compact structure, which cannot be distinguished from the native CAB by normal SEC analysis, its microscopic structure is not completely the same as the native one, especially, in its local hydrophobicity detected with high sensitivity by the aqueous two-phase partitioning method. Fig. 3b indicates that this small conformational change of CAB between 0 and 1 *M* GuHCl can be detected by ILC as a different retention time derived from the different interactions of immobilized liposomes with CAB at 0 and 1 *M* GuHCl. A single peak of CAB at 1 *M* GuHCl on ILC means that CAB is in a compact state almost the same as the native state. The intermediate states of other proteins, for example, heat shock protein, DnaK, appeared as a single peak in SEC analysis at the particular GuHCl concentration range [45]. Liposomal bilayer membranes, therefore, can recognize the only partially denatured CAB in the presence of 1 *M* GuHCl and weakly interact with it as in the case of the interactions between liposomes and native proteins with large local hydrophobicity (see Section 3.2.1). In the following sections, the relationship between the CAB conformational changes and the retention time of CAB on ILC is quantitatively examined.

3.2.3. Relationship between specific capacity factor and local hydrophobicity of denatured proteins

Depending on the concentration of GuHCl used, proteins can be denatured from a native to an unfolded state [9,29,40]. These protein conformational changes have been monitored by measuring enzymatic activity, ANS fluorescence intensity [8,9,34,46], circular dichroism spectra [1], NMR [47] and SEC [9,41,48]. The interactions between the neutral liposomal bilayer membranes and the partially-denatured proteins are presumably dominated by weak hydrophobic bindings. Thus, we studied the local hydrophobicity (LH) of CAB and correlated LH with its chromatographic retention on ILC. Protein hydrophobicity can be determined by various methods, for example, by using aqueous two-phase systems containing hydrophobic ligands such as palmitate [49] or Triton [22,35], by using hydrophobic affinity chromatography [50] and by using the hydrophobic fluorescence probe, ANS. Since Triton is a mild detergent [51], the structure of native proteins is maintained in Triton–protein complexes. We used PEG–Dex aqueous two-phase system containing 1 m*M* hydrophobic probe, Triton X-405. Triton preferably partitions to the top PEG phase, therefore, partition coefficients of proteins with hydrophobic sites increase after binding to Triton. In Fig. 4a, the LH of CAB was plotted as a function of GuHCl concentration. The LH of CAB reached a maximum value in the presence of 1 *M* GuHCl. This change in the LH with GuHCl concentration is similar to the GuHCl dependency of ANS fluorescence intensity reported by Uversky and Ptitsyn [9]. CAB molecules are likely to be in the molten globule-like state in the presence of about 1 *M* GuHCl. CAB in the native and unfolded state, which corresponds to LH values at 0 *M* GuHCl and at 2 *M* GuHCl, respectively, has relatively low LH values.

The specific capacity factor, k_s (ml/mmol) of CAB calculated from the elution behavior on ILC is also plotted as a function of GuHCl concentration (Fig. 4b). Only a single peak was observed both for k_s and LH around 1 *M* GuHCl and the change in the k_s value is compatible with that in the LH value,

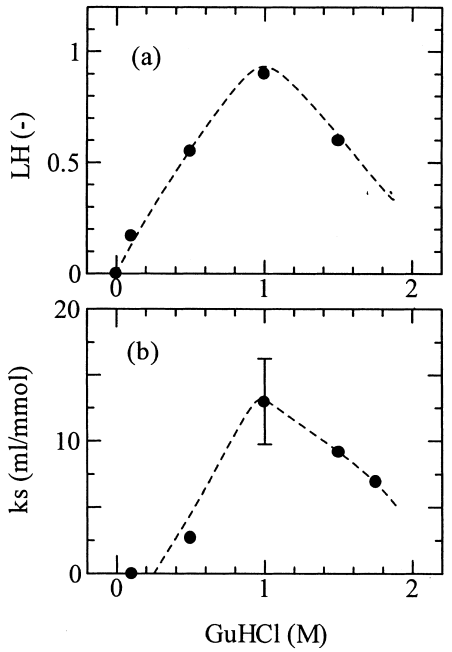

Fig. 4. (a) Local hydrophobicity (LH (−)) of CAB determined with the aqueous two-phase partitioning method and (b) specific capacity factor (k_s (ml/mmol)) as a function of GuHCl concentration; k_s was determined by elution profiles of CAB on liposome-immobilized gel beads (details are in the text).

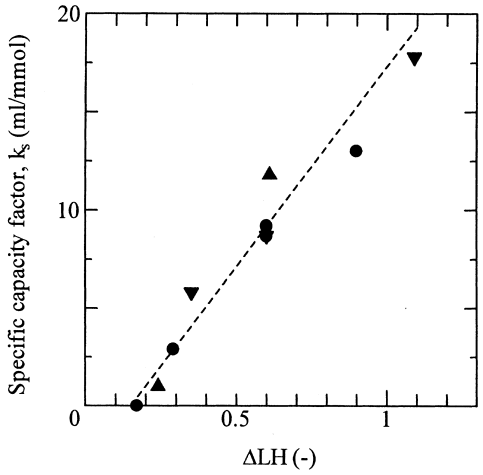

Fig. 5. Relationship between ΔLH and the specific capacity factor, k_s, for CAB (●), cytochrome *c* (▲) and α-lactalbumin (▼). ΔLH is defined as $\Delta LH = LH - LH_{native}$, where LH_{native} means local hydrophobicity of native protein.

strongly suggesting that locally hydrophobic sites of CAB specifically interact with liposomal bilayer membranes. Only proteins in the molten globule-state can interact with lipid membranes as well as molecular chaperones [7,52]. Fig. 4 also suggests that partially denatured CAB molecules in 1 *M* GuHCl are in a molten globule-like state so that the protein can most effectively interact with liposomal bilayer membranes through hydrophobic interaction.

Including other proteins, cytochrome *c* (cyt. *c*) and α-lactalbumin (α-LA), the k_s values obtained by ILC at the different GuHCl concentration were plotted as a function of ΔLH ($\Delta LH = LH - LH_{native}$), see Fig. 5. ΔLH is the difference in LH of proteins, which is determined based on its native state. An approximately linear relationship was observed between the two parameters, k_s and ΔLH. It can be concluded that the k_s value reflects degree of the protein local hydrophobicity relative to its native state, that is, protein conformational change. One can estimate and utilize the protein local hydrophobicity by knowing the k_s value on ILC.

3.3. Refolding of CAB facilitated by ILC

3.3.1. Refolding chromatography

Liposomes composed of POPC have a function similar to molecular chaperones which mediate protein folding [2]. In this study, chromatographic protein refolding of unfolded CAB was attempted using ILC. CAB denatured with 5 *M* GuHCl was applied to the columns and reactivation yield of CAB was examined. In Fig. 6, the elution profiles as well as the enzymatic activity with 1 m*M* *p*-NPA of collected fraction are shown both for the control column and for ILC (control and liposome columns had similar dimensions). CAB was retarded significantly on ILC compared to the control. Moreover, CAB eluted from ILC had higher enzymatic activity than CAB eluted from the control column. SEC itself is not effective for protein renaturation [18]. The CAB retardation and the CAB high reactivation yield on ILC, therefore, are thought to be due to liposome–protein interactions during refolding process. The CAB reactivation yield was affected by flow-rate and protein concentration, (see Table 3). A relatively low flow-rate (0.035 ml/min), which cor-

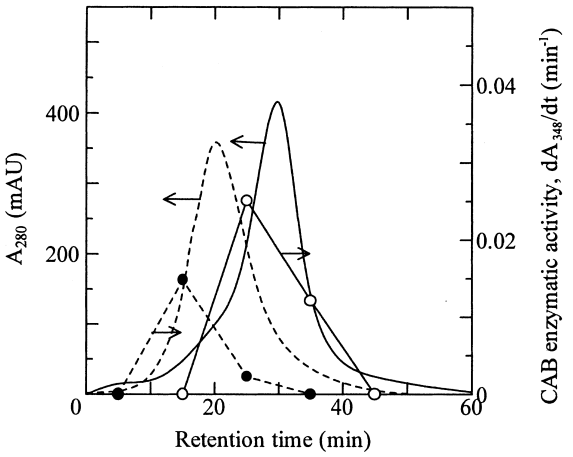

Fig. 6. Liposome chromatography of 5 *M* GuHCl-denatured CAB solution ([CAB]=5 mg/ml). Elution of CAB was determined by measuring absorbance at 280 nm (λ_{280}) and elution of refolded native CAB was determined based on enzymatic activity with 1 m*M* *p*-nitrophenyl acetate (initial increase in absorbance at 348 nm, $d\lambda_{348}/dt$, 5 m*M* EDTA was added to eluted solution in order to stop CAB refolding). Sonicated SUVs were immobilized in Superdex 200 gel beads and packed in 0.5×5 cm column. A 10 μl volume of denatured CAB solution was eluted with buffer T at a flow-rate of 0.035 ml/min, collecting 350 μl fractions for enzymatic activity assay. The dotted lines indicate elution profiles for control (liposome-free) Superdex 200 column.

Table 3
Reactivation yield of 5 *M* GuHCl-denatured CAB under various conditions

Gel type	Flow-rate (ml/min)	Reactivation yield (%)
Superdex 200 (ILC)[a]	0.25	38
Superdex 200 (ILC)	0.1	74
Superdex 200 (ILC)	0.035	83
Superdex 200	0.18	26
Superdex 200	0.035	58
TSK G6000PW (ILC)	0.25	42
TSK G6000PW (ILC)	0.1	85
TSK G6000PW (ILC)	0.035	91 (75)[b]
Batch[c]	–	52

[a] ILC=immobilized liposome chromatography (sonicated SUVs were immobilized in gel beads by covalent coupling method). A 10-μl volume of 5 *M* GuHCl-denatured CAB solution ([CAB]= 0.5 m*M*) was applied to columns with various flow-rates, collecting 350-μl fractions at room temperature. Enzymatic activity of collected samples was determined by measuring CAB enzymatic activity with 1 m*M* *p*-nitrophenyl acetate. The reactivation yield is defined as the total activity of the collected samples relative to that of the control solution containing native CAB.

[b] Value in parentheses: for [CAB]=1 m*M*.

[c] Batch refolding was performed by diluting 40 μl of 5 *M* GuHCl denatured CAB ([CAB]=15 mg/ml) into 1960 μl of 0.1 *M* Tris–HCl buffer (50-fold dilution), pH 7.5, at 25°C.

responds to about 30 min retention time before native CAB was eluted, was required. This refolding time corresponds to the time required for liposome-assisted CAB refolding in solution [2]. At higher flow-rates, denatured CAB cannot effectively interact with immobilized liposomes and as a result, only low reactivation yields can be obtained.

3.3.2. Possible mechanism of immobilized liposomes-assisted protein refolding process

Based on the results of the interactions between liposomal bilayer membranes and CAB at various conformations (see Section 3.2.2 Section 3.2.3), a possible mechanism was proposed for CAB refolding on ILC. Immobilized liposome–CAB interactions during passage through the ILC column are schematically illustrated in Fig. 7. The liposome–CAB interactions are probably dependent on a series of changes in the local hydrophobicity (conformational change) of CAB between the top and the bottom of the column. The change of CAB from the completely unfolded state to the molten globule state is spontaneous and rapid [10], therefore, 5 *M* GuHCl-unfolded CAB transforms into the molten-globule-like intermediate state fast when denaturant concentration is diluted at the early stage of CAB refolding by ILC. This CAB refolding intermediate, which has strong local hydrophobicity can effectively interact with immobilized liposomes. Liposomes assist the conformational change not from unfolded state to the molten globule state but from the molten-globule state to native state by reducing CAB intermolecular interactions which results in forming inactive CAB aggregates. Liposome–CAB interaction can be confirmed as the retardation of CAB peak on the ILC column compared to normal SEC as shown in Fig. 6. It is thought that the interaction between liposomes and partially denatured CAB is a reversible and highly dynamic interaction, i.e., partially denatured CAB molecules undergo a number of steps of binding to and dissociated from immobilized liposomes until it refolds into the native state. If the normal SEC column was used for CAB refold-

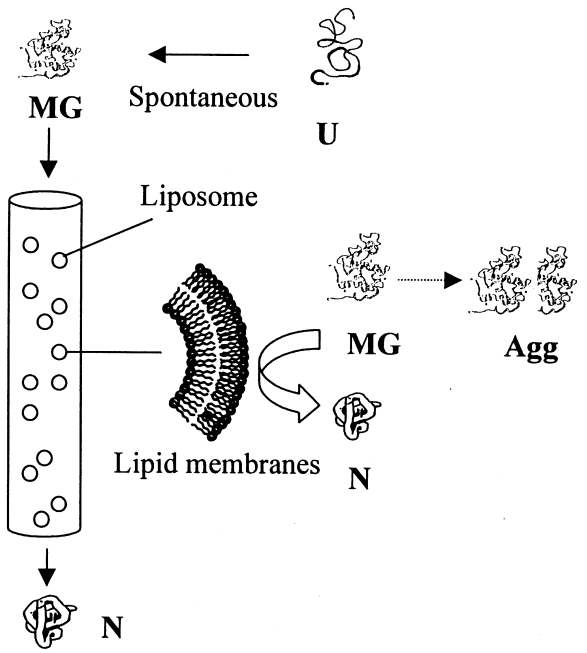

Fig. 7. Schematic illustration of CAB refolding facilitated by ILC. N, MG, and U indicate protein in the native, molten-globule and unfolded states, respectively. Agg indicates protein inactive aggregates.

ing, the formation of inactive aggregates between hydrophobic proteins at the molten-globule state occur and thus the reactivation yield was decreased. Although further studies are needed, our results indicate that refolding of various kinds of protein as well as CAB can also be effectively carried out if the refolding intermediate of the target protein has sufficient local hydrophobicity for binding to immobilized liposomes. The refolding yield can be improved by modulating membrane properties of immobilized liposomes, especially, in terms of membrane fluidity [2].

4. Conclusion

Liposomes could be immobilized on Superdex and TSK gel beads by the covalent coupling method with high stability against osmotic stress. By use of liposome immobilized gel beads, lipid membrane–protein interactions can be quantitatively evaluated. The retardation of CAB at the molten-globule-like state was observed due to membrane–protein interactions on immobilized liposome chromatography. There was a close relationship between the specific capacity factor, k_s, of CAB on ILC and the local hydrophobicity, LH, values of proteins which were determined with the aqueous two-phase partitioning method. In addition, ILC could be applied to the protein refolding process for CAB. CAB, unfolded with 5 *M* GuHCl, was efficiently refolded into the native state after ILC. In this process, the CAB refolding intermediate (at their molten-globule-like state) with strong local hydrophobicity interacts with the lipid membranes and this membrane–protein interaction facilitates CAB refolding.

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