Oxidative Refolding of Denatured/Reduced Lysozyme Utilizing the Chaperone-like Function of Liposomes and Immobilized Liposome Chromatography

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Oxidative refolding of the denatured/reduced lysozyme was examined in the presence of small unilamellar vesicles (SUVs) essentially composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). SUVs facilitated the recovery of the enzymatic activity of lysozyme like molecular chaperones through the interaction with the refolding intermediate of lysozyme. The highest reactivation yield (87%) was obtained by delaying oxidation time (15−30 s) after dilution of the denaturant concentration was initiated in the presence of SUVs. SUVs supplemented with 1 mol % phosphatidylethanolamine were covalently coupled to gel beads, and then the interactions of SUVs with lysozyme at the various conformations were quantitatively examined with immobilized liposome chromatography (ILC). The reduced lysozyme lacking disulfide bonds was found to have a property similar to that of the molten globule state in terms of its local hydrophobicity, which was determined with the aqueous two-phase partitioning method. The reduced lysozyme was more clearly retarded on the ILC column than the native and 1 M guanidinium hydrochloride (GuHCl)-treated lysozymes with intact disulfide bonds. Similar results were obtained for α-lactalbumin, which has three-dimensional structure closely similar to that of lysozyme, regarding the membrane−protein interaction. These results can be interpreted as follows. In the early stage of the oxidative refolding, liposomes bound to the refolding intermediate of lysozyme lacking disulfide bonds. Then, liposomes assisted the formation of the tertiary structure of lysozyme by reducing protein intermolecular interactions, which usually cause the formation of the inactive aggregates. Consequently, the correct formation of the disulfide bonds was promoted. On the basis of the above results, the chromatographic oxidative refolding was also examined with ILC. The denatured/reduced lysozyme (9 mg/mL, 10 μL) was passed through the ILC column with a flow rate of 1 mL/min, which corresponds to the retention of lysozyme about 1 min in the ILC column. Subsequent oxidation of the eluted lysozyme resulted in the almost complete recovery (100%) of the original enzymatic activity of lysozyme.

Introduction

Various recombinant proteins can be obtained as inactive and insoluble protein aggregates called inclusion bodies. To obtain biologically active proteins, inclusion bodies have to be dissolved and reduced followed by an oxidative refolding process. However, it is difficult to obtain native protein with high yield because the refolding intermediate of protein, which is partially denatured and in a so-called molten globule (MG) state, has strong hydrophobicity and thus tends to form intermolecular inactive aggregates (*1*, *2*). To the best of our knowledge, there are few widely applicable methods for recovery of active proteins with high yield from inclusion bodies.

Recently, we proposed a novel protein refolding utilizing the chaperone-like function of liposomes focusing on the effects of the physical properties of liposomes on the hydrophobic interaction between liposomes and the MG state of proteins (*3*). The model protein was bovine carbonic anhydrase, which has strong hydrophobicity in its MG state. Because the interactions between liposomes with no net charge and the partially denatured proteins are nonspecific hydrophobic ones (*4*), liposomes have a potential to be applied to the refolding of various proteins as well as bovine carbonic anhydrase. We have also reported that liposomes can be covalently immobilized in gel beads with high stability (*5*) and that protein refolding can be successfully carried out on the liposome-immobilized column (*4*). Immobilized liposome chromatography (ILC), which we call refolding chromatography, has a possibility to be applied to the simultaneous (oxidative) refolding and purification of a target protein from inclusion bodies containing various contaminants.

Quite generally, proteins with disulfide bonds are difficult to be successfully refolded into their native states from their denatured/reduced states (*6*, *7*). This is due to the simultaneous and competitive formations of the tertiary structure and of the intermolecular disulfide bonds, which usually result in inactive protein aggregates and/or in misfolded proteins (*8*). To control this competitive reaction, it is necessary to understand the features

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of the protein intermediate states which appear in the early stage of protein refolding and often cause the formation of aggregates.

Lysozyme, which has four disulfide bonds, is one of the best characterized proteins in its unfolding and refolding mechanisms in detail (*9*). It has been reported that disulfide-intact lysozyme has no thermodynamically stable intermediate state during its unfolding process (*10*). However, the intermediate state has been observed in the early stage of the oxidative refolding of the denatured/reduced lysozyme (*2*). This intermediate state possesses a conformation like the MG state and does not have the complete disulfide bonds as the native structure (*2*). α-Lactalbumin, which is known to have a three-dimensional structure closely similar to that of lysozyme except that it is a Ca^{2+}-binding protein, has been reported that it has strong binding ability to a heat shock protein GroEL and to phospholipid membranes in its reduced state (*11*, *12*). These previous observations imply that chaperones and phospholipid membranes can mediate the oxidative refolding of the denatured/reduced lysozyme by binding to its refolding intermediate state with few disulfide bonds, that is, to the reduced lysozyme.

In the present study, we show that the chaperone-like function of liposomes can be utilized in the oxidative refolding of the denatured/reduced lysozyme. The mechanism of the liposome-assisted oxidative refolding was proposed on the basis of the interaction of liposomal bilayer membranes with lysozyme at various conformations, which was quantitatively evaluated by using ILC (*4*). We used local hydrophobicity of proteins evaluated with the aqueous two-phase partitioning method as a key factor in protein−liposome interactions (*4*). The effective design of high-yield protein refolding can be carried out by utilizing such a simple and quantitative parameter. As an effective protein refolding, we examined the ILC method, which we call refolding chromatography, as a primary step for utilizing ILC in the practical oxidative refolding and purification of a target protein from inclusion bodies.

Materials and Methods

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and egg phosphatidylethanolamine (EPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Egg white lysozyme, reduced glutathione (GSH), oxidized glutathione (GSSG), dithiothreitol (DTT), and guanidinium hydrochloride (GuHCl) were purchased from Wako Pure Chemicals (Osaka, Japan). Bovine milk α-lactalbumin, bovine pancreatic ribonuclease A, and *Micrococcus lysodeikticus* dried cell were obtained from Sigma (St. Louis, MO). Superdex 200 preparative grade (denoted Superdex) and the glass column (HR 5/5) were obtained from Pharmacia Biotech (Uppsala, Sweden). TSK G6000PW (denoted TSK) gel beads were purchased from Tosoh (Tokyo, Japan). All other chemicals were of analytical grade.

Liposome Preparation. POPC dissolved in chloroform (10 mg/mL) was dried in a 100 mL round-bottom flask by rotary evaporation under reduced pressure. The lipids were redissolved in diethyl ether twice, 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 mM Tris−HCl buffer (pH 8.0) at room temperature to form multilamellar vesicles (MLVs). The vesicle suspension was frozen in dry ice/ethanol (−80 °C) and thawed at 30 °C to enhance the transformation of small vesicles into larger MLVs (*13*, *14*). This freezing−thawing cycle was performed five times. MLVs were sized down to small unilamellar vesicles (SUVs) by extruding the MLV suspension 15 times through two stacked polycarbonate membranes with mean pore diameters of 200, 100, and 50 nm (Nuclepore, Costar, Cambridge, MA) using an extruding devise (Liposofast; Avestin Inc.) (*15*).

In the case of the preparation of liposomes for immobilization to gel beads, SUVs were prepared by probe sonication of a MLV suspension (*4*). MLVs were prepared in the same manner except that 1 mol % EPE was supplemented. The MLV suspension was transferred into a 10 mL plastic tube on an ice bath and sonicated at 40 W for 20 min, followed by centrifugation at 100000*g* for 60 min at 4 °C to sediment titanium particles released from the probe during sonication and residual MLVs.

Liposome Immobilization. SUVs were immobilized in gel beads by the covalent coupling method (*4*, *5*). Briefly, prior to liposome immobilization, gel beads were activated by nitrophenyl chloroformate (*16*). The sonicated liposomes were mixed with the activated gel beads overnight at 4 °C. The mixture was washed with 50 mM Tris−HCl buffer (pH 8.0) on a glass filter to remove nonimmobilized liposomes. The gel beads containing covalently bound liposomes were packed into a glass column (HR 5/5, Pharmacia Biotech), and nonreacted ligands were blocked by circulating buffer containing 20 mM ethanolamine on the gel bed overnight at 25 °C. The mean size of the immobilized liposomes was 26 ± 1 nm, which can be estimated by dynamic light scattering analysis (*4*). The obtained liposome-immobilized gel column was connected to a HPLC system (AKTA, Pharmacia Biotech).

Evaluation of Membrane−Protein Interactions. The elution behaviors of proteins in immobilized liposome chromatography (ILC) were normalized by an apparent specific capacity factor, k_s', which is derived from the equation described by Beigi et al. (*17*) and defined as eq 1. V_r is the retention volume of proteins (mL), V_e is the

$$k_s' = (V_r - V_e)/M' \tag{1}$$

elution volume of proteins (mL), which are not retarded on the liposome-immobilized column as determined by using native proteins in 50 mM Tris−HCl buffer (pH 8.0), and M' is the amount of immobilized liposomes (mmol), which is available for interaction with the proteins. The amount of phospholipid was determined according to the method of Bartlett (*18*).

Determination of the Hydrophobic Property of Proteins. Local hydrophobicity (LH(−)) of proteins was determined by using the aqueous two-phase partitioning method and Triton X-405 as a hydrophobic probe (*4*, *19*−*21*). The binding sites of proteins for nonionic detergent, Triton X-405, were determined using two different aqueous two-phase systems. One was composed of 9 wt % poly(ethylene glycol) (PEG) with a molecular mass of 3000 and 9 wt % dextran (Dex) with a molecular mass of 100000−200000 (total weight of a two-phase system: 5 g). The other had the same composition except that 1 mM Triton X-405 was added. LH is defined as eq 2. $K_{T,P}$ and

$$\mathrm{LH} = \Delta \ln K = \ln K_{T,P} - \ln K_P \tag{2}$$

K_P are the partition coefficients of protein in an aqueous two-phase system in the presence of 1 mM Triton X-405 and in another aqueous two-phase system in the absence of Triton, respectively. Protein (1 mg/mL) was partitioned in the aqueous two-phase systems, and after 1 h, the top PEG phase, interphase, and bottom Dex phase were

separated carefully. The K values were determined by measuring protein concentrations in the top and bottom phases according to Bradford (*22*).

Preparation of Denatured/Reduced Lysozyme. Denaturation and reduction of lysozyme were performed in a solution containing 6 M GuHCl and 120 mM DTT in 50 mM Tris−HCl buffer (pH 8.0). This solution was incubated for at least 5 h at 25 °C. Denaturation and reduction of α-lactalbumin and ribonuclease A were also carried out under the same conditions as described above.

Oxidative Refolding of Denatured/Reduced Lysozyme. For oxidative refolding, the denatured/reduced lysozyme solution was rapidly diluted with 50 mM Tris−HCl buffer (pH 8.0) containing various amounts of GSH and GSSG in the presence or absence of POPC liposomes ([POPC] = 0.25 mM). Unless otherwise indicated, the final concentrations in the refolding solution were 0.1 mg/mL lysozyme, 0.1 M GuHCl, 10 mM GSH, 1 mM GSSG, and 2 mM DTT. Throughout the refolding operation, 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 the control containing native lysozyme.

Refolding Chromatography. Denatured/reduced lysozyme (10 μL) was applied to the ILC column and eluted with 50 mM Tris−HCl buffer (pH 8.0) with a flow rate of 1 mL/min collecting 350 μL fractions. Then, the 10 mM GSH and 1 mM GSSG were immediately added to the fractions. After 40 min of 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 the native sample corresponding to the same amount of the applied denatured and reduced sample.

Enzymatic Activity Measurement. As a measure of lysozyme activity, the rate of enzymatic lysis of *M. lysodeikticus* cells (0.15 mg/mL), which were suspended in 50 mM phosphate buffer (pH 6.2), was taken (*23*). To obtain an adequate refolding yield, the refolding reaction (formation of the disulfide bonds) of lysozyme was quenched (*24*) by the addition of 20 μL of 0.1 M acetic acid solution to each 20 μL lysozyme sample. Then, 960 μL of cell suspension was added to the 40 μL lysozyme solution, and the decrease in the absorbance at 450 nm (A_{450}) was continuously monitored spectrophotomerically (Shimadzu UV-1600) at 25 °C for 60 s after 10 s of lag time. Enzymatic activity was determined from the slope of the straight line obtained by plotting A_{450} vs time.

Results and Discussion

Liposome-Assisted Oxidative Refolding of Denatured/Reduced Lysozyme. Denatured (disulfide-intact) lysozyme was refolded into its native state with high reactivation yield (almost 100%) even at high protein concentration (1 mg/mL, data not shown). However, the oxidative refolding of the denatured/reduced lysozyme did not successfully proceed, and a large amount of protein was led to aggregates. Ramman et al. have shown that the intermediate state of lysozyme and the molten globule (MG) state are similar in their hydrophobic properties (*2*). This means that the refolding intermediate of lysozyme which arises from the denatured/reduced state has strong hydrophobicity and thus has strong intermolecular interactions. Therefore, it is necessary to stabilize the intermediate state in order to promote the formation of the intramolecular disulfide bonds, which is reported as the rate-limiting step (*25*). Various methods have been reported to improve the oxidative refolding of the denatured/reduced lysozyme. Recently, Hevehan and Clark have shown that about 1 M guanidinium hydrochloride (GuHCl) in renaturation buffer minimizes the aggregate formation of lysozyme and improves the reactivation yield although it takes a relatively longer time to reach the maximum yield than at the lower GuHCl concentrations (*6*). Maeda et al. have also reported that residual denaturant in refolding solution positively affects oxidative refolding of lysozyme (*26*, *27*). Moreover, the refolding of proteins with the aid of additives, so-called artificial chaperones, has been extensively studied; for example, protein disulfide isomerase (*8*, *28*), detergents/cyclodextrin (*29*), particular amino acid (*6*), α-crystallin (*30*, *31*), and poly(ethylene glycol) (PEG) (*32*, *33*) were used as a kind of artificial chaperone. Particularly, Rozema and Gellman showed that the addition of cyclodextrin to a detergent−lysozyme complex results in the recovery of active lysozyme with high yield (*29*). In addition, Cleland et al. reported that PEG inhibits aggregate formation and enhances refolding of bovine carbonic anhydrase B (*32*, *33*). These chaperone systems, in principle, utilize the binding between artificial chaperones and proteins in their intermediate state. For instance, Cleland and Randolph reported that PEG binds to the MG-like conformation of bovine carbonic anhydrase B (*33*).

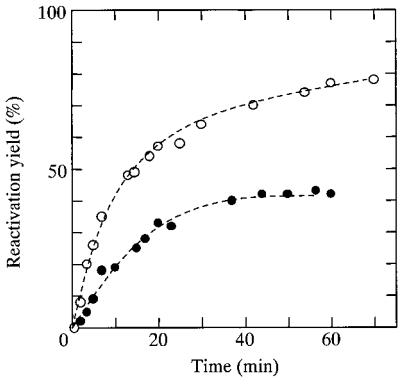

Figure 1. Time course of the reactivation yield of the denatured/reduced lysozyme in the presence (○) and absence (●) of liposomes ([POPC] = 0.25 mM). The denatured/reduced lysozyme was diluted into renaturation buffer to final concentrations of 0.1 mg/mL lysozyme and 0.1 M GuHCl in the presence of 10 mM GSH and 1 mM GSSG at 25 °C.

The denatured/reduced lysozyme was refolded and oxidized in the presence or absence of small unilamellar liposomes (Figure 1). The ratio of GSH/GSSG was fixed at 10 ([GSH] = 10 mM), which has been recommended in the literature as one of the optimal ratios (*2*, *8*). It should be noted that liposomes have no effect on the enzymatic activity measurement of the native lysozyme. From Figure 1, it can be seen that liposomes assist the oxidative refolding of lysozyme. At low final GuHCl concentrations as employed in Figure 1, lysozyme tends to form extensive insoluble aggregates, which results in low reactivation yield in the absence of additives (*29*). This may be because hydrophobic interaction between the intermediate state of lysozyme is pronounced upon the rapid dilution of denaturant concentration. Figure 1 implies that liposomes effectively bind to the refolding intermediate of lysozyme. The effect of liposomes on the reactivation yield of lysozyme was saturated around the lipid concentration of 0.25 mM (data not shown). In addition, liposomes may not slow the refolding of lysozyme because, in the case of oxidative refolding of lysozyme, the rate-limiting step is the disulfide bond formation essentially both in the presence and in the absence of liposomes.

Table 1. Local Hydrophobicity and Relative Activity of Lysozyme at Various Conformations

conformational state of lysozyme	GuHCl (M)/ DTT (mM)	LH[a] (−)	relative activity[b] (%)
native	0/0	0	100
partially denatured	1/0	0.3	96 (38[c])
reduced	0/120	1.6	0
denatured/reduced	1/120	1.0	0

[a] Determined with the aqueous two-phase partitioning method (see the Materials and Methods). [b] The enzymatic activity of lysozyme remained after 15 h in the presence of GuHCl and/or DTT. The enzymatic activity was determined with 0.15 mg/mL *M. lysodeikticus*. The concentration of lysozyme was 0.1 mg/mL. [c] Relative activity in the presence of 6 M GuHCl without DTT.

In the following sections, the mechanism for the liposome-assisted oxidative refolding of lysozyme was examined through the clarification of the hydrophobic properties of lysozyme at the possible conformations.

Variation of Surface Properties of Lysozyme at Various Conformations. The hydrophobic property of lysozyme at the various conformations was examined. As a measure of protein conformational change, the local hydrophobicity (LH(−)) was employed. Protein local hydrophobicity means the degree of the binding affinity between proteins and hydrophobic probes such as nonionic detergent, Triton (*4*, *19*−*21*), or fluorescent probe, 1-anilinonaphthalene-8-sulfonic acid (ANS) (*2*, *34*, *35*). In this study, Triton X-405 was used, and protein−Triton binding was quantified by using the aqueous two-phase partitioning method (see the Materials and Methods). The LH values as well as enzymatic activity are summarized in Table 1 for lysozyme (i) in the partially denatured state, (ii) in the reduced state, and (iii) in the denatured/reduced state. The largest LH value was obtained for lysozyme in the reduced state treated with 120 mM DTT in the absence of denaturant (GuHCl), whereas the partially denatured (disulfide-intact) lysozyme showed a significantly low LH value. The LH value of lysozyme treated with 1 M GuHCl and 120 mM DTT had a larger LH value than the partially denatured lysozyme treated with 1 M GuHCl.

Protein in the MG state possesses a secondary structure similar to that of the native state, but its tertiary structure is partly destroyed. In general, the MG state is a nativelike compact but fluctuated conformation with considerable secondary structure. Its tertiary structure is partly destroyed, but the side chains remain in loose association. Therefore, the hydrophobic parts of protein are partly exposed to its surface. As we have shown previously, the MG state can be detected as having the largest LH value among the possible conformations of protein (*36*, *37*). The MG state detected by LH measurement corresponds well to that detected by other conventional techniques such as circular dichroism (*36*). In the case of the fully unfolded state, its tertiary structure is completely destroyed and the hydrophobic side chains do not associate with each other, which leads to low binding affinity for hydrophobic probes. Our results in Table 1, therefore, indicate that the lysozyme lacking disulfide bonds is likely in the MG state.

From the enzymatic activity measurements, the above results could be confirmed. In the presence of 1 M GuHCl, the enzymatic activity of lysozyme with intact disulfide bonds still remained (96% of original activity), whereas no enzymatic activity was detected in the presence of 120 mM DTT (Table 1). Disulfide-intact lysozyme in the presence of 6 M GuHCl also had 38% of the native activity. Although the refolding of the partially denatured lysozyme should be considered during enzymatic activity measurement (within 60 s), these results imply that the tertiary structure of lysozyme is not strongly destroyed only in the presence of GuHCl (∼6 M) if the disulfide bonds are intact (in the absence of DTT). In the case of breaking disulfide bonds by 120 mM DTT, lysozyme easily loses part of its tertiary structure, which leads lysozyme to the MG-like state with no enzymatic activity and with large local hydrophobicity as indicated by the LH measurement.

Table 2. Local Hydrophobicity of α-Lactalbumin and Ribonuclease A at Various Conformations

conformational state	GuHCl (M)/ DTT (mM)	LH[a] (−)	
		α-lactalbumin	ribonuclease A
native	0/0	0	0.16
partially denatured	1/0	0.6	0
reduced	0/120	2.2	0.59
denatured/reduced	1/120	0.52	0
apo (without Ca^{2+})[b]	0/0	1.8	

[a] Determined with the aqueous two-phase partitioning method (see the Materials and methods). [b] Treated with 1 mM EDTA.

Denaturation of disulfide-intact lysozyme is known to follow the "all-or-none" and two-state transition mechanism (*10*). This indicates that no thermodynamically stable intermediate state, which has usually strong local hydrophobicity, exists except for the native and the unfolded states. In agreement with this mechanism, the LH value of the lysozyme in the presence of 1 M GuHCl without DTT was relatively low, suggesting that the disulfide-intact lysozyme is not in the MG-like intermediate state. For the reduced lysozyme lacking disulfide bonds, however, it has been reported that it strongly binds to ANS, which emits fluorescence upon binding to protein especially in the MG state (*34*). ANS is also reported to bind to the reduced lysozyme and to lysozyme at the initial stage of oxidative refolding (*2*, *35*). Our LH measurements also indicate that the reduced lysozyme is in the MG-like state, as judged by its quite large local hydrophobicity. The reason the reduced lysozyme has such strong local hydrophobicity is probably that, in the absence of disulfide bonds, fluctuation of the tertiary structure of lysozyme is pronounced, which results in the appearance of hydrophobic parts of lysozyme. The reduced lysozyme in the presence of 1 M GuHCl showed a lower LH value than that in the presence of DTT alone. This indicates that tertiary association within lysozyme is further weakened by combined effects of DTT and GuHCl, and as a result, binding sites for the hydrophobic probe are reduced.

In the actual oxidative refolding process, the tertiary structure of lysozyme is formed followed by the slow formation of the disulfide bonds (*25*). That is, the early refolding intermediate is presumed to be in the reduced-like state as reported for other proteins (*38*). Our LH measurements indicate that the reduced lysozyme has strong local hydrophobicity, which usually results in protein aggregates (*37*). Therefore, it is required that the intermolecular interaction of the reduced lysozyme is controlled on the basis of the LH value as a key parameter.

The surface properties of other proteins with disulfide bonds such as α-lactalbumin and ribonuclease A were also examined to clarify the role of the disulfide bonds in the surface properties of proteins. The results are summarized in Table 2. For two examined proteins, their reduced states had large LH values in analogy to the case of lysozyme, although for ribonuclease A, its partially denatured and denatured/reduced states showed an LH

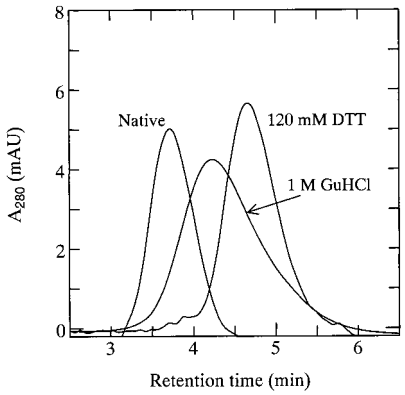

Figure 2. Immobilized liposome chromatography of lysozyme in the native, in the partially denatured, and in the reduced states (flow rate 0.25 mL/min, [lysozyme] = 50 μM).

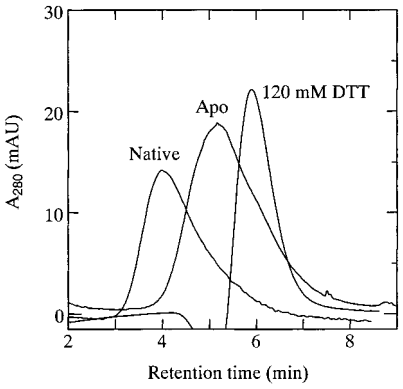

Figure 3. Immobilized liposome chromatography of α-lactalbumin in the native, in the apo, and in the reduced states (flow rate 0.25 mL/min, [α-lactalbumin] = 50 μM).

value of 0. For ribonuclease A, our LH measurements indicate that disulfide bonds keep tight tertiary structure in the native conformation even in the presence of GuHCl, but if they are reduced, the protein loses its tertiary conformation easily by GuHCl in the presence of DTT. For α-lactalbumin, local hydrophobicity similar to that of lysozyme was observed except that its apo (without Ca^{2+}) state also showed a large LH value. The different behaviors between the three proteins are attributed to their different stabilities against chemical stress (DTT and GuHCl) as reported earlier (*36*). However, the common characteristic between the three proteins is that the proteins lacking disulfide bonds have quite large LH values among the possible conformations of proteins.

Evaluation of Liposome−Protein Interactions Using Immobilized Liposome Chromatography (ILC). ILC was previously used as a tool for the evaluation of the weak interactions of liposomal bilayer membranes with partially denatured proteins (*4*). Liposome−protein interactions were quantitatively detected as a retardation of protein in ILC. The elution behaviors of lysozyme in the reduced state and in the partially denatured state in ILC were examined. As seen in Figure 2, the reduced lysozyme was markedly retarded in ILC. Partially denatured lysozyme was also retarded in ILC, but the extent of the retardation was smaller than that of the reduced lysozyme. We have previously reported that the binding sites of proteins for liposomal bilayer membranes are their locally hydrophobic sites (*3*, *4*, *21*). In the case of lysozyme, the retardation was also pronounced with an increase in its local hydrophobicity; i.e., the reduced lysozyme, which has larger local hydrophobicity than the partially denatured lysozyme, was more retarded in ILC than the partially denatured lysozyme. The reduced lysozyme has a lot of locally hydrophobic sites, and thus, it can efficiently interact with liposomal bilayer membranes. In the early stage of the oxidative refolding, lysozyme is presumed to have few disulfide bonds and has large local hydrophobicity as described in the previous section. Lysozyme at the early stage of the oxidative refolding, therefore, has an ability to efficiently bind to liposomal bilayer membranes as quantitatively evaluated by using ILC.

We also examined the interaction of liposomal bilayer membranes with α-lactalbumin at various conformations because α-lactalbumin has a tertiary structure similar to that of lysozyme. The interactions of α-lactalbumin with a molecular chaperone and lipid membranes have been extensively studied (*11, 12*). It has been reported that α-lactalbumin lacking Ca^{2+} (apo state) has fluctuated structure but does not effectively bind to a molecular chaperone, GroEL (*11*). Katsumata et al. have shown that the conformation of α-lactalbumin for binding to a molecular chaperone is the reduced form, which has a more flexible tertiary structure than the apo-α-lactalbumin (*39*). ILC analysis shown in this study also supports these previous observations. As seen in Figure 3, the largest retardation was observed for the reduced α-lactalbumin. This result can also be explained on the basis of the difference in the LH value of α-lactalbumin between the apo and the reduced states. That is, the reduced α-lactalbumin has a larger LH value than the α-lactalbumin at the other conformations including the apo state (see Table 2).

The quantitative relationship between protein local hydrophobicity and the elution behaviors of proteins was examined. LH values and the retention time of proteins in ILC can be normalized by ΔLH (ΔLH = LH − LH_{native}, where LH_{native} is the LH value of native protein) and the specific capacity factor, k_s' (see the Materials and Methods), respectively. We have previously reported that there is a linear relationship between these two parameters (*4*). For the three proteins examined in this study, lysozyme, α-lactalbumin, and ribonuclease A, including their reduced and partially denatured states, the linear relationship was also observed; see Figure 4. Figure 4 shows that membrane−protein interactions can be controlled on the basis of protein local hydrophobicity.

Improvement of Liposome-Assisted Oxidative Refolding of Lysozyme. On the basis of the results of the variation of the local hydrophobicity of lysozyme and of the liposome−lysozyme interactions, we attempted to improve the oxidative refolding of lysozyme. For the successful oxidative refolding, the key point is how to depress the formation of both the protein aggregates and the intermolecular inappropriate disulfide bonds. The latter is easy to occur if the formations of the tertiary structure and of disulfide bonds start at the same time. If the intermediate states, which have nativelike tertiary structure, are separately trapped from each other, the following formation of the disulfide bonds may proceed successfully. From the above point of view, Matsubara et al. have demonstrated that the "delayed oxidation" method is effective for the oxidative refolding of the urea-denatured/reduced lysozyme (*40*). We also examined the effect of the oxidation time (the time when GSH/GSSG was added to the refolding solution) after dilution of the denaturant concentration was initiated in the presence of liposomes. The time course of the reactivation yield is shown in Figure 5 for 30 s of delayed oxidation. The yield was significantly improved by delaying oxidation time.

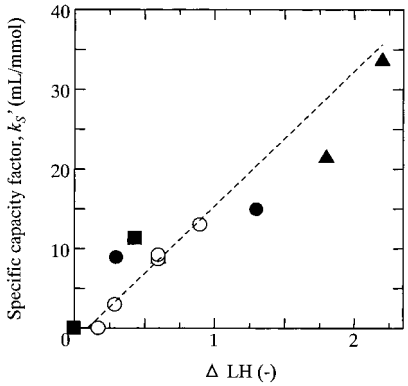

Figure 4. Relationship between ΔLH and the specific capacity factor, k_s', for lysozyme (●), α-lactalbumin (▲), ribonuclease A (■), and bovine carbonic anhydrase (○). The multiple points for one protein include partially denatured proteins. The data for bovine carbonic anhydrase are from Yoshimoto et al. (*4*).

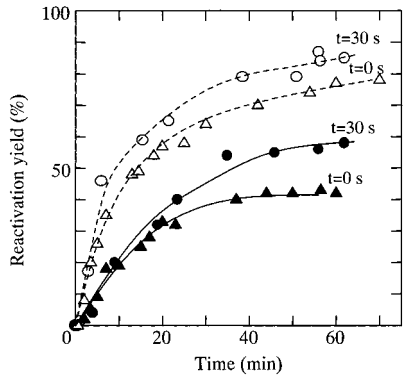

Figure 5. Time course of refolding of the denatured/reduced lysozyme in the presence (○, △) and absence (●, ▲) of liposomes ([POPC] = 0.25 mM). GSH (10 mM) and GSSG (1 mM) were added at 0 s (△, ▲) and at 30 s (○, ●) after refolding was initiated.

The maximum reactivation yield was plotted as a function of the oxidation time; see Figure 6. An effective oxidation time was found to be around 15−30 s after dilution both in the absence and in the presence of liposomes was initiated. The reason delaying the oxidation time was effective can be discussed on the basis of the LH values of lysozyme at various conformations occurring in the oxidative refolding and of membrane−lysozyme interactions. In Figure 7, a possible mechanism of the liposome-assisted oxidative refolding of lysozyme is shown. The denatured/reduce lysozyme is rapidly transformed into the MG-like state with few disulfide bonds and with a large LH value in the absence of GSH/GSSG. This tertiary structure formation occurs within about 15−30 s as indicated by Figure 6. The intermediate state of lysozyme tends to form inactive intermolecular aggregates in the absence of liposomes, and thus the following formation of the correct intramolecular disulfide bonds does not proceed with high efficiency. In the presence of liposomes, however, these intermediate states of lysozyme prefer to bind to liposomal bilayer membranes as quantitatively shown in the ILC measurement, and consequently, they are weakly and separately trapped on the liposome surface. Delayed oxidation of lysozyme during the lysozyme−membrane interaction, which is pronounced just after forming the tertiary structure of lysozyme, is effective as indicated by Figure 6. If the oxidation time was delayed above 30 s, the irreversible formation of aggregates between the intermediate states

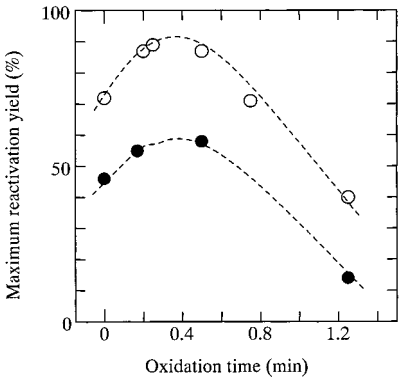

Figure 6. Effect of oxidation time on the reactivation yield of the denatured/reduced lysozyme in the presence (○) and absence (●) of liposomes ([POPC] = 0.25 mM). GSH (10 mM) and GSSG (1 mM) were added at various times after refolding was intitiated ([lysozyme] = 0.1 mg/mL).

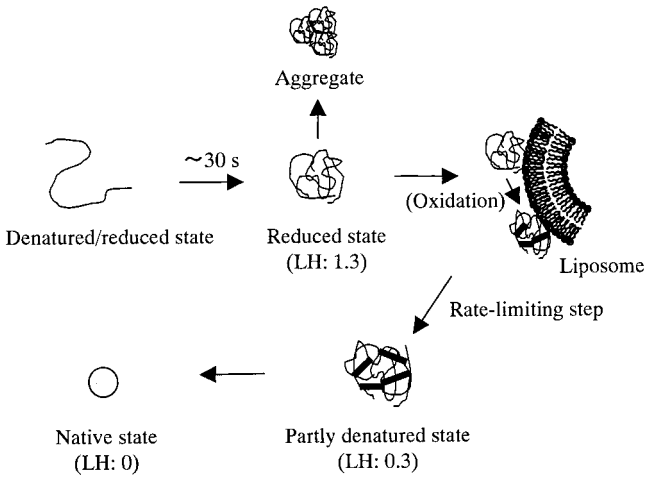

Figure 7. Schematic illustration of the oxidative refolding of the denatured/reduced lysozyme assisted by liposomes. LH values of the intermediate states are also shown, assuming that the conformations listed in Table 1 correspond to the above intermediate states.

was pronounced; thus, the reactivation yield was decreased.

Oxidative Refolding Using ILC (Refolding Chromatography). Chromatographic refolding of proteins has been reported previously (*41*−*43*). To obtain the native proteins from inclusion bodies, an efficient method for oxidative refolding is desired because the reduction process is essential to effectively solubilize inclusion bodies containing many contaminants (*44*). Batas and Chaudhuri have reported that the oxidative refolding of the denatured/reduced lysozyme at high concentration can be successfully carried out by using the normal size-exclusion chromatography although it takes a relatively long time to obtain active lysozyme (*43*).

Chromatographic oxidative refolding was examined using ILC. The results of the oxidative refolding of lysozyme by the simple dilution indicate that it is of significant importance to control the intermolecular interaction especially at the early stage of refolding by utilizing the membrane−lysozyme interactions. On the basis of the results, the denatured/reduced lysozyme (10 μL) was eluted out with Tris−HCl buffer (pH 8.0) containing no GSH/GSSG and with a high flow rate (1 mL/min). Then, the eluted lysozyme was immediately oxidized in solution by the addition of GSH/GSSG. The flow rate of 1 mL/min corresponds to about only 1 min retention of protein in the ILC column. The reactivation

Table 3. Oxidative Refolding of the Denatured/Reduced Lysozyme in ILC

refolding method (gel type)	protein concn of applied sample (mg/mL)	flow rate (mL/min)	reactivation yield[a] (%)
ILC (Superdex)	9	1	105
ILC (TSK)	9	1	100
batch (−)	9	-	54

[a] Lysozyme was oxidized after being eluted from the column.

yields of the denatured/reduced lysozyme in ILC are summarized in Table 3. By utilizing the ILC method, very high reactivation yields could be obtained at 9 mg/mL lysozyme. The following mechanism can be proposed. The denatured/reduced lysozyme is diluted in the ILC column followed by the formation of the intermediate state of lysozyme, which has nativelike tertiary structure but has no disulfide bonds. This reduced lysozyme can bind to immobilized liposomes because it has large local hydrophobicity. Immobilized liposomes are presumed to prevent the reduced lysozyme from forming intermolecular aggregates and promote folding into nativelike conformation as in the case of CAB refolding in ILC (*4*). In the case of using the ILC method, the high reactivation yields were obtained even though the oxidation time was delayed more than 30 s, which is the critical time for the effective oxidative refolding as shown in the previous section. The reason is that, in the chromatographic operation, the dilution of the denaturant concentration proceeds not rapidly but gradually so that the formation of protein aggregates is not pronounced compared with the bath-type operation.

Ultimately, simultaneous protein refolding and separation from the protein mixture using ILC may be possible by combining this chromatographic refolding with the heat-induced separation process utilizing protein translocation across phospholipid membranes (*21*, *45*−*47*).

Conclusion

By employing the aqueous two-phase partitioning method and immobilized liposome chromatography (ILC), the following conclusions were obtained regarding the membrane−protein interactions and the liposome-assisted oxidative refolding of the denatured/reduced lysozyme.

(1) The reduced lysozyme had the largest local hydrophobicity among the possible conformations of lysozyme like the protein at the molten globule state, and it strongly interacted with liposomal bilayer membranes. Similar results were obtained for α-lactalbumin.

(2) Liposomes assisted the oxidative refolding of lysozyme through the interaction with the reduced-like lysozyme, which is formed in the early stage of refolding (within 30 s after refolding was initiated). The oxidation being delayed 15−30 s after refolding was initiated was found to be the most effective for forming the correct tertiary structure of lysozyme followed by the formation of disulfide bonds.

(3) The denatured/reduced lysozyme was successfully refolded into the native state by using ILC. Passing through the ILC column with a high flow rate (1 mL/min) and subsequent oxidation in solution resulted in the high reactivation yield.

Notation

ILC immobilized liposome chromatography
K_P partition coefficient of protein (−)
$K_{P,T}$ partition coefficient of protein in the presence of Triton X-405 (−)
k_s' specific capacity factor of proteins in ILC (mL/mmol)
LH local hydrophobicity of proteins (−)
M' amount of immobilized lipids in gel beads (mmol)
V_e retention volume of native protein in ILC (mL)
V_r retention volume of protein in ILC (mL)

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