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Polymerization of Amino Acids in Liposomal Membrane under Reduced Pressure and Normal Pressure

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Incorporation of polypeptides with hydrophobic side groups into lipid bilayers was achieved by the polymerization of *N*-carboxy anhydrides of γ-dodecyl L-glutamate and of γ-benzyl D-glutamate in DMPC liposomal membrane under reduced pressure and normal pressure. The effects of polypeptide conformations on the barrier properties of liposomal membrane incorporating polypeptide were examined. There was little difference in the trapping volume when liposomes prepared under reduced pressure and normal pressure were compared. The incorporation of polypeptides into the membrane bilayer had a tendency to decrease the volume trapping efficiencies. It was found that in the membrane-free aqueous phase, polymerization of *N*-carboxy amino acid anhydrides promoted formation of β-structure and in the membrane bilayer formation of α-helix structure was enhanced. The content of α-helical polypeptide was maximum for liposomes prepared under reduced pressure. The activation energies for water permeation across the liposomal membrane were lowered by the incorporation of polypeptdes into the membrane bilayer. The incorporation of α-helical polypeptide was more ceffective than that of β-structured one for the lowering of activation energies. The permeability barrier properties of liposomal membrane were larger for liposome incorporating poly(γ-dodecyl L-glutamate) (PDOLG) than for liposome incorporating poly(γ-benzyl D-glutamate)(PBDG). This seems to be due to a difference in miscibilities with lipid in the membrane bilayer.

The interactions of liposomes with a variety of polypeptides have been studied as attractive models for the molecular interactions occuring in biological membranes. The interactions cause major changes in the permeability of the membrane bilayer,[1-3] the polypeptide conformation,[4,5] and the morphology of the liposomes.[6] Poly(L-glutamic acid) interacts on the surface of the phosphatidylcholine liposomes and remains in random coil configuration upon complexation with the liposome.[7] Poly(L-lysine) goes from a random coil to an α-helix when it interacts with phosphatidylserine.[5] The copolypeptide lysine–tyrosine has only a small effect on the membrane conductance and it gives a β-structure upon interaction with phosphatidylserine or phosphatidylcholine liposome.[4] Most results so far reported have been obtained by the use of a variety of water-soluble polypeptides that were adsorbed by electrostatic interaction to the outer surface of liposomal membrane. It is now well-recognized that nonpolar interactions play a major role in the determination of protein tertiary structure, catalytic activity, and ligand binding activities and it is to be anticipated that such interactions would also play an important role in lipid-proton interactions of the cell membrane. Our attention has been focused on the incorporation of hydrophobic polypeptide into the membrane bilayer and the effects of their conformations.[8]

In the present study, polypeptides with nonpolar side groups were used as a lipophilic protein model which was embedded into the hydrophobic domain of the membrane. Incorporation of polypeptides with hydrophobic side groups into the membrane bilayer was achieved by polymerization of *N*-carboxy anhydrides of γ-dodecyl L-glutamate and of γ-benzyl D-glutamate in DMPC liposomal environment under reduced pressure and normal pressure. The effects of conformation and hydrophobic side group of polypeptides on the water permeability of liposomal membrane incorporating polypeptide were studied by the osmotic method.

Experimental

Materials. Dimyristoylphosphatidylcholine (DMPC) and dihexadecyl hydrogenphosphate (DHP) were obtained from Sigma. *N*-Carboxy anhydrides of γ-dodecyl L-glutamate (DOLG-NCA) and γ-benzyl D-glutamate (BDG-NCA) were prepared by the method of Blout and Karlson.[9] Diethyl ether and dichloromethane were distilled under nitrogen atmosphere. Water was distilled, the second time from alkaline potassium permanganate solution.

Preparation of Liposome. Large unilamellar liposomes were prepared according to the ether injection method of Deamer and Bangham[10] with some modifications. Injecting lipid solution was prepared by solubilizing DMPC, DHP, and *N*-carboxy amino acid anhydride in mixture of diethyl ether and dichloromethane (3:2 (v/v)) at an appropriate molar ratio (1:0.1:1). This solution was gradually injected with the aid of a mechanical drive through a needle into an aqueous phase, located within the internal chamber of the modified Liebig condenser. 45 ml of the lipid solution (2.5 μmol ml^{-1}) was injected into 6 ml distilled water at 60 °C under normal pressure. For the preparation of liposomes under reduced pressure the apparatus was placed under water vacuum. The temperature of the aqueous phase inside the water vacuum line was maintained at 35 °C for 460 mmHg (1 mmHg≈133.322 Pa) and 45 °C for 510 mmHg. *N*-Carboxy amino acid anhydride represents a class of high-energy amino acid derivatives which polymerize to form

polypeptides. Since the negative-charged portion on the liposomal surface was expected to be favorable for the spontaneous polymerization of *N*-carboxy amino acid anhydrides,[11] the preparation of liposome was evolved for DMPC liposome containing 10 mol% of DHP.

Isolation of Polypeptides from Liposomes. The liposome suspension prepared was filtered through gel column (Cellulofine-GC-700) to remove residual organic solvent, non-incorporated monomer and polymer. To isolate polypeptide from liposomes incorporating polypeptide (150 μmol DMPC), the liposome suspension was first lyophilized and the residue was added to 10 ml diethyl ether or methanol. After shaking, a dispersed precipitant appeared. The precipitated polypeptide was filtered off, washed with methanol, and dried in vacuo.

Osmotic Shrinkage Measurements. For the osmotic shrinkage measurements the liposome suspension was mixed quickly by using a rapid mixing device (Otsuka Electronics MX-7) with hypertonic glucose solution (100 mM) and a 1:1 mixing ratio. The osmotic shrinking of liposomes was followed by the time-course of turbidity change at 450 nm. For osmometers with membrane completely impermeable to the solute a proportionality exists betweem the volume change and the difference in osmotic pressure of the outer and inner volume. Since the initial volume change is directly proportional to the relative initial shrinkage rate (initial absorbance change), r_0, the following relation holds:[12,13]

$$r_0 = 1/A_0(\mathrm{d}A/\mathrm{d}t) = k(\mathrm{d}V/\mathrm{d}t) = kP_wSRT\Delta C \qquad (1)$$

where $1/A_0(\mathrm{d}A/\mathrm{d}t)$ is the initial shrinkage rate, $\mathrm{d}V/\mathrm{d}t$ is the volume change with time, P_w is the permeability coefficient for water, S is the area occupied by the membrane, R is the gas constant, T is the absolute temperature, and ΔC is the concentration difference in the permeable solutes between the inside and outside the liposome, respectively. On the assumption that permeability coefficients could be expressed in the form of Arrhenius's equation

$$P_w = P_w^0 \exp(-\Delta E_a/RT) \qquad (2)$$

one can obtain from Eqs. 1 and 2:

$$r_0/T = 1/A_0\,(\mathrm{d}A/\mathrm{d}t)/T = KP_w^\circ \exp(-\Delta E_a/RT) \qquad (3)$$

where K ($=kSR\Delta C$) is a constant at fixed ΔC. An activation energy, ΔE_a, for water permeation across the liposomal membrane could be determined by plotting the logarithms of r_0/T as a function of the reciprocal of absolute temperature.

Results and Discussion

Effects of Conditions for Preparing Liposome on Trapping Volume. Polypeptides isolated from the liposomes prepared under reduced pressure and normal pressure were in yields of 20% and 14% for PDOLG and of 12% and 9% for PBDG. The incorporation of these polypeptides into the liposomal membrane tends to be somewhat larger for reduced pressure (460 mmHg) than for normal pressure.

A parameter we have used to characterize ether injection liposomes is the trapping volume, calçulated by the solute diffusion method.[14] Table 1 summarizes the typical values of volume trapping efficiencies of the liposomes with the without polypeptide prepared under reduced pressure and normal pressure. We observed little difference between the trapping volume of the liposomes prepared under reduced pressure polypeptides into the bilayer tended to decrease volume trapping efficiencies. The values of the trapping volume ranged from 8 to 11 l mol^{-1} and were larger than the values for the typical sonicated liposome by a factor of about 10.

Conformation of Polypeptides Prepared in Membrane Bilayer. Figures 1 and 2 show the infrared spectra for PDOLG and PBDG isolated from liposomes prepared under reduced pressure (460 mmHg) and normal pressure, together with that for the same polypeptides obtained by polymerizing *N*-carboxy amino acid anhydrides in membrane-free aqueous phase. The spectra which are characteristic of poly-

Table 1. Effects of Conditions for Preparing Liposome on Volume Trapping Efficiencies

Composition	*P*	*T*	Trapping volume
	mmHg	°C	l mol^{-1}
DMPC:DHP	760	60	11.2
	460	35	10.8
DMPC:DHP: DOLG-NCA	760	60	8.7
	460	35	8.3
DMPC:DHP: BDG-NCA	760	60	8.8
	460	35	9.3

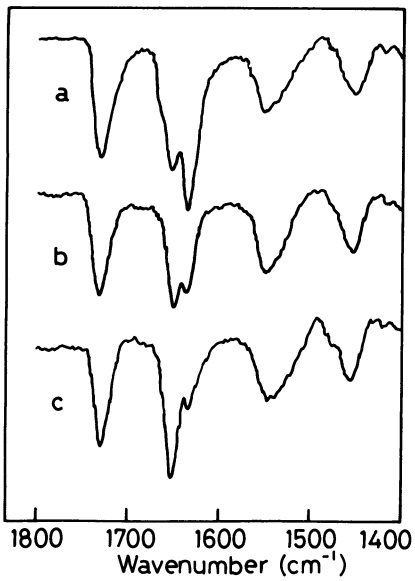

Fig. 1. Infrared spectra of poly(γ-dodecyl L-glutamate) polymerized in membrane free aqueous phase(a), isolated from liposomes prepared under normal pressure(b) and reduce pressure(c).

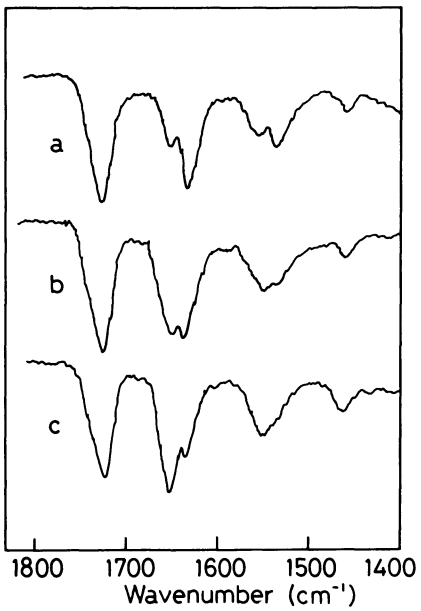

Fig. 2. Infrared spectra of poly(γ-benzyl D-glutamate). Symbols, a, b, and c, are the same as in Fig. 1.

peptide having α-helix structure show major diagonostic at about 1650 cm^{-1} for the amide I band and 1550 cm^{-1} for the amide II band. The β-structure gives rise to a strong band at lower frequecy (about 1635 cm^{-1} for the amide I band and 1520 cm^{-1} for the amide II band) than the α-helix structure. The contents of α-helix structure and β-structure are qualitatively estimated by the comparison of the peak height between 1650 and 1635 cm^{-1} for the amide I band. The conformations of PDOLG and PBDG polymerized in the membrane-free aqueous phase were enhanced in the β-structure (Figs. 1-a and 2-a). Taking into account the fact that both PDOLG and PBDG polymerized in organic solvent (dioxane or anisole) take the α-helix structure,[8] the hydrophilic environments for the polymerization of *N*-carboxy amino acid anhydrides may promote the formation of the β-structure. Yaron et al.[15] have reported that the helical structure of poly[N^5-(3-hydroxypropyl)-L-glutamine] was stabilized under hydrophobic environments such as methanol or *N*,*N*-dimethylformamide and that the addition of water into this solution resulted in a lowering of the helix content. Apparently the presence of water as a protein solvent is not necessarily the most favorable environment for helical structures of polypeptide. On the other hand, polymerization of DOLG-NCA and of BDG-NCA in the membrane bilayer under normal pressure increased the α-helical content (Figs. 1-b and 2-b) and under reduced pressure led to further increase of the α-helix structure (Figs. 1-c and 2-c). Although the α-helicities of PDOLG and PBDG have a tendency to change with the reaction environments, PDOLG differs from PBDG in the α-helix content of polypeptides prepared under different conditions. The polymerization of DOLG-NCA in the membrane-free aqueous phase gave spectra of the β-configuration. PDOLG prepared in the bilayer under normal pressure was enhanced in α-helix, and under reduced pressure the α-helix structure was present in much larger quantities. For the polymerization of BDG-NCA in the membrane-free aqueous phase, on the other hand, the spectra showed mostly β-structure, similar to that of DOLG-NCA. The content of α-helix for PBDG prepared in the bilayer under normal pressure was nearly equal to that of the β-structure, and under reduced pressure the helical content again became much larger than that of the β-structure.

From the results mentioned above, it is found that in the membrane-free aqueous phase the polymerization of *N*-carboxy anhydrides of γ-dodecyl L-glutamate and of γ-benzyl D-glutamate promotes the formation of the β-structure, while in the lipid bilayer membrane the α-helix structure predominates. The content of α-helical polypeptide was maximum for liposomes prepared under reduced pressure. Considering these results, we suggest that the hydrophobic anchor side groups of these *N*-carboxy anhydrides are embedded locally with some degree of translational or rotational freedom into the lipid bilayer matrix of liposomes, and functional groups of *N*-carboxy anhydrides may be brought into positions favorable to the formation of spiral arrangements of the molecules. Consequently, this will induce the formation of the α-helical structure. It is important to delineate the factors affecting the conformation of biological macromolecules. The force dictating the conformation of polypeptide can arise from intramolecular forces, from intermolecular forces, from the solvent, or from surrounding dissimilar medium (environments), which represents a special type of intermolecular interaction. The liposomal environments found to be favorable for the preparation of α-helical polypeptide.

Water Permeation through Liposomal Membrane. The water permeability of liposomal membrane incorporating polypeptide is a sensitive function of the extent of interaction between polypeptides and membrane lipids, and was studied by the measurements of initial osmotic shrinkage rates.

Figures 3, 4, and 5 show the experimental data for the polypeptide-free liposome, and the polypeptide incorporating liposomes prepared under reduced pressure (460 and 500 mmHg) and normal pressure. A linear relationship between the logarithms of r_0/T and the reciprocal of absolute temperature as in Eq. 3 was obtained in all cases investigated. The activation energies estimated from the gradient in Figs. 3—5 are given in Table 2. The values listed in columns 2 and 3 are the pressure and the temperature of the aqueous phase, respectively.

The activation energies for water permeation which

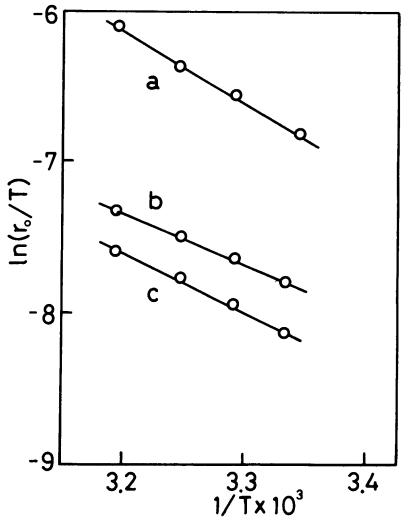

Fig. 3. Arrhenius plots for water permeability across lipid bilayers of liposomes prepared under normal pressure and at 60 °C.
a) Polypeptide free liposome, b) liposome incorporating poly(γ-dodecyl L-glutamate), c) liposome incorporating poly(γ-benzyl D-glutamate).

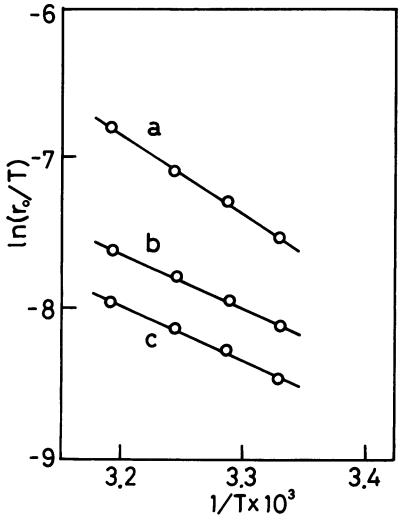

Fig. 4. Arrhenius plots for water permeability across lipid bilayers of liposomes prepared under reduced pressure of 510 mmHg and at 45 °C. Symbols, a, b, and c, are the same in Fig. 3.

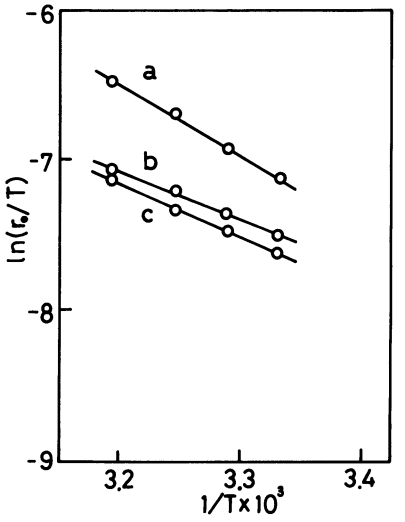

Fig. 5. Arrhenius plots for water permeability across lipid bilayers of liposomes prepared under reduced pressure of 460 mmHg and at 35 °C. Symbols, a, b, and c, are the same in Fig. 3.

Table 2. Activation Energies for Permeation of Water across Liposomal Membranes under Different Conditions

Composition	P	T	ΔE_a
	mmHg	°C	kcal mol^{-1}
DMPC:DHP	760	60	9.6
	510	45	10.3
	460	35	9.5
DMPC:DHP:	760	60	8.6
DOLG-NCA	510	45	8.3
	460	35	7.2
DMPC:DHP:	760	60	7.6
BDG-NCA	510	45	7.1
	460	35	6.3

DMPC:DHP=1:0.1(mol/mol). DMPC:DHP:NCA=1:0.1:1(mol/mol).

laid in the range 6.3—10.3 kcal mol^{-1} were dependent of the conditions for preparing liposomes. The preparation of polypeptide incorporating liposomes under reduced pressure tended to increase α-helical polypeptide in membrane bilayer as shown in Figs. 1 and 2. This lowers the activation energies for water permeation through polypeptide incorporating liposomal membrane. That is to say, the incorporation of α-helical polypeptide was more effective than that of β-structured one for the lowering of activation energies. Another reason why the activation energies are lowered in the presence of polypeptide is in miscibility between polypeptide and DMPC. Much information has been gained by the recognition of analogies between monomolecular films and biological membranes.[16] PDOLG and PBDG form a monomolecular film at the air-water interface, and the surface-pressure-area (π-A) curves are shown in Fig. 6. The curves of PDOLG with alkyl side groups and PBDG with benzyl side groups have differences related to hydrophobic side chain interactions and side chain flexibility.[17,18] Therefore, the difference in properties of monomolecular films must be reflected in the orientation and the packing of DMPC in polypeptide incorporating membrane bilayer which is associated in permeability barrier properties. Actually, we obtained the results that the permeability barrier properties were larger for liposome incorporating PDOLG than for that incorporating PBDG (Table 2).

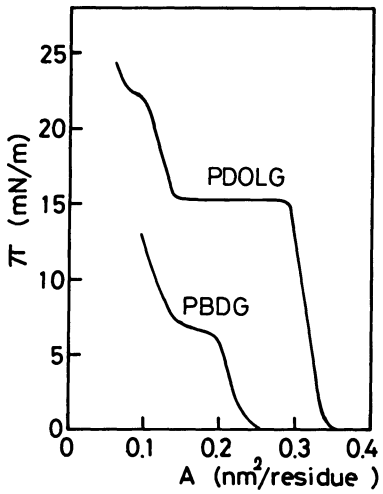

Fig. 6. Surface pressure-area (π-A) curves of poly-(γ-dodecyl L-glutamate) and poly(γ-benzyl D-glutamate).

Conclusion

Incorporation of polypeptides with hydrophobic side groups into lipid bilayers was achieved by the polymerization of DOLG-NCA and BDG-NCA in DMPC liposomal membrane under reduced pressure and normal pressure. Polymerization of *N*-carboxy amino acid anhydrides in the liposomal membrane promoted formation of the α-helix structure. The presence of polypeptides in membrane bilayer resulted in a lowering of activation enegries for permeation of water across the liposomal membrane, and the lowering was maximum for incorporation of α-helical polypeptide.

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