Interaction of α-Helical Glycopeptides with Lipid Bilayer Membrane

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α-Helical polypeptides having a glyco moiety at one end and a fluorescent probe at the other were prepared and investigated on the interaction with a lipid bilayer membrane. A glycopeptide composed of poly(alanine) and maltose was incorporated into the lipid membrane with the glyco moiety exposed to the aqueous phase and the peptide chain buried in membrane above the phase-transition temperature of the lipid membrane. However, below the phase-transition temperature, the glycopeptide induced the aggregation of dimyristoylphosphatidylcholine (DMPC) small unilamellar vesicles (SUV). Another glycopeptide composed of poly(γ-benzyl glutamate) and glucose also induced the aggregation of DMPC SUV, indicating that such the hydrophobic peptide tends to disturb the membrane structure significantly upon binding. The arrangement of the glycopeptide in a membrane was investigated by using a fluorescent probe connected to the end of the peptide chain. The terminal region of the peptide chain was located at the hydrophobic core of lipid membrane. However, the glycopeptide composed of 25 Ala residues had a smaller fraction taking perpendicular orientation to the membrane than that composed of 15 Ala residues. It was therefore considered that a part of the former glycopeptide molecules exits on the membrane surface, forming aggregates.

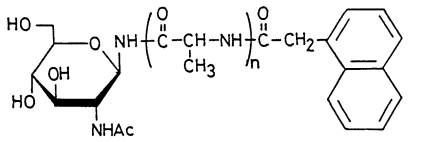
The transmembrane region of intrinsic membrane proteins is considered to take an α-helical structure, because their primary structure consists of helix-forming amino acid residues and the length of successive hydrophobic amino acid residues in the α-helical conformation corresponds to the thickness of a lipid bilayer membrane.[1,2] In addition, the α-helical conformation in the lipid membrane is energetically stable due to shielding from the hydrophobic environment of the polar amide groups by intrachain hydrogen bondings.[3,4]

However, the backbone conformation of membrane proteins has not been well investigated by spectroscopy. It is still unknown whether the α-helical structure is formed spontaneously in the lipid membrane, or what determines the orientation of the α-helical segment in the lipid membrane. Therefore, it is of interest and significance to investigate the interaction with a lipid bilayer membrane of model peptides taking an α-helical conformation. In the present investigation, α-helical oligopeptides having a glyco moiety at one end of the chain and an 9-anthracenecarbonyl group at the other end were prepared. In these glycopeptides, the hydrophilic glyco moiety will reside on the surface of the lipid membrane upon binding of the hydrophobic peptide segment to the lipid membrane.[5] Therefore, the arrangement and orientation of the peptide chain in the lipid membrane can be deduced from the location of the other terminal of the peptide chain by fluorescence spectroscopy.

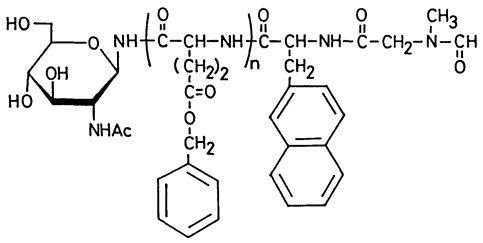
Results and Discussion

Polymerization of NCA. The molecular structure of glycopeptides used in the present investigation and the synthetic routes are shown in Figs. 1 and 2, respectively.

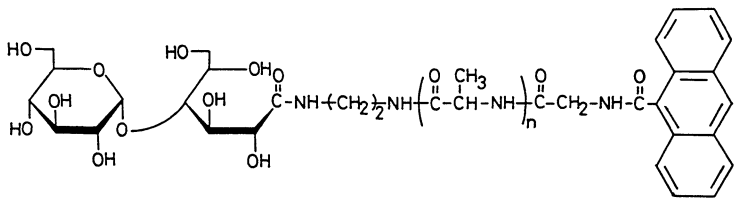
The elution profile of GlcNAc-20-Glu(OBzl) through gel chromatography was bimodal. It has



n=15 ; GlcNAc-15-Ala



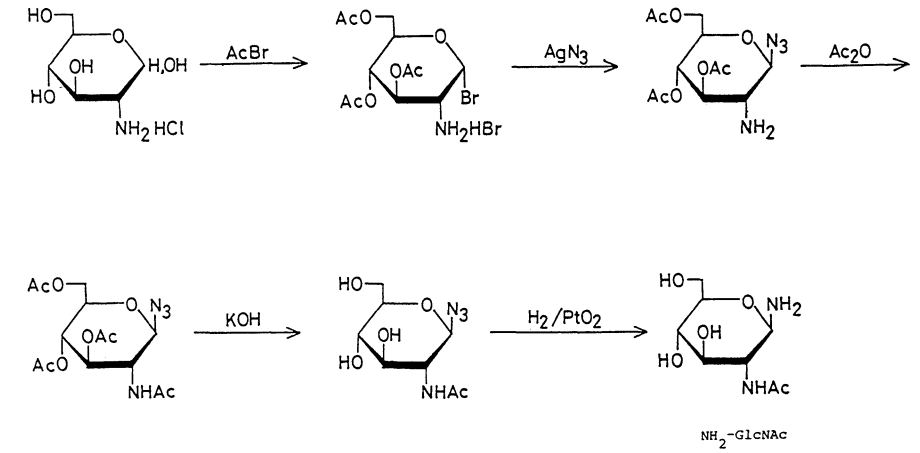
n=20 ; GlcNAc-20-Glu(OBzl)

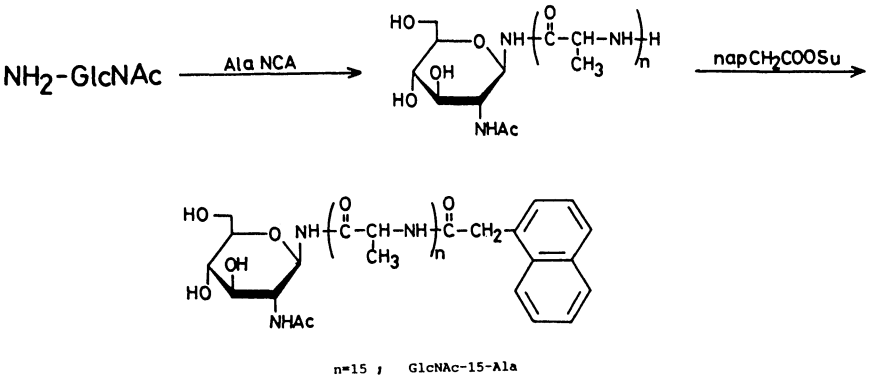


n=15 ; Mal-15-Ala
n=25 ; Mal-25-Ala

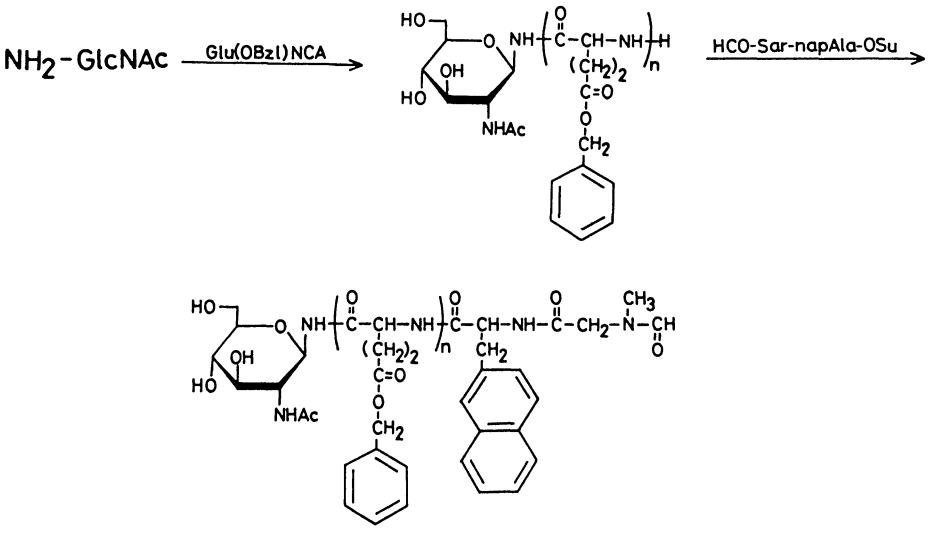
Fig. 1. Molecular structure of glycopeptides synthesized.

been reported that poly(γ-benzyl glutamate) obtained by polymerization in *N,N*-dimethylformamide showed a narrow molecular weight distribution,[9] although that in dioxane had a very large inhomogeneity of the molecular weights.[10] Unfavorable side reactions such as reaction of NCA with hydroxyl groups of the initiator or deamination of α-amino group of the initiator might occur slightly.[11] On the other hand, polymerization using Ac-Mal-EDA as initiator is considered to have proceeded according to the "nucleophilic addition mechanism," because the average degree of polymerization coincides with the ratio of [NCA]/[Initiator] (Table 1).[12] The average degree of polymerization was determined by NMR measurements of glycopeptides by comparing the area of C^{α}-CH_3 signal of Ala with that of acetyl signal of the





n=15 ; GlcNAc-15-Ala



n=20 ; GlcNAc-20-Glu(OBzl)

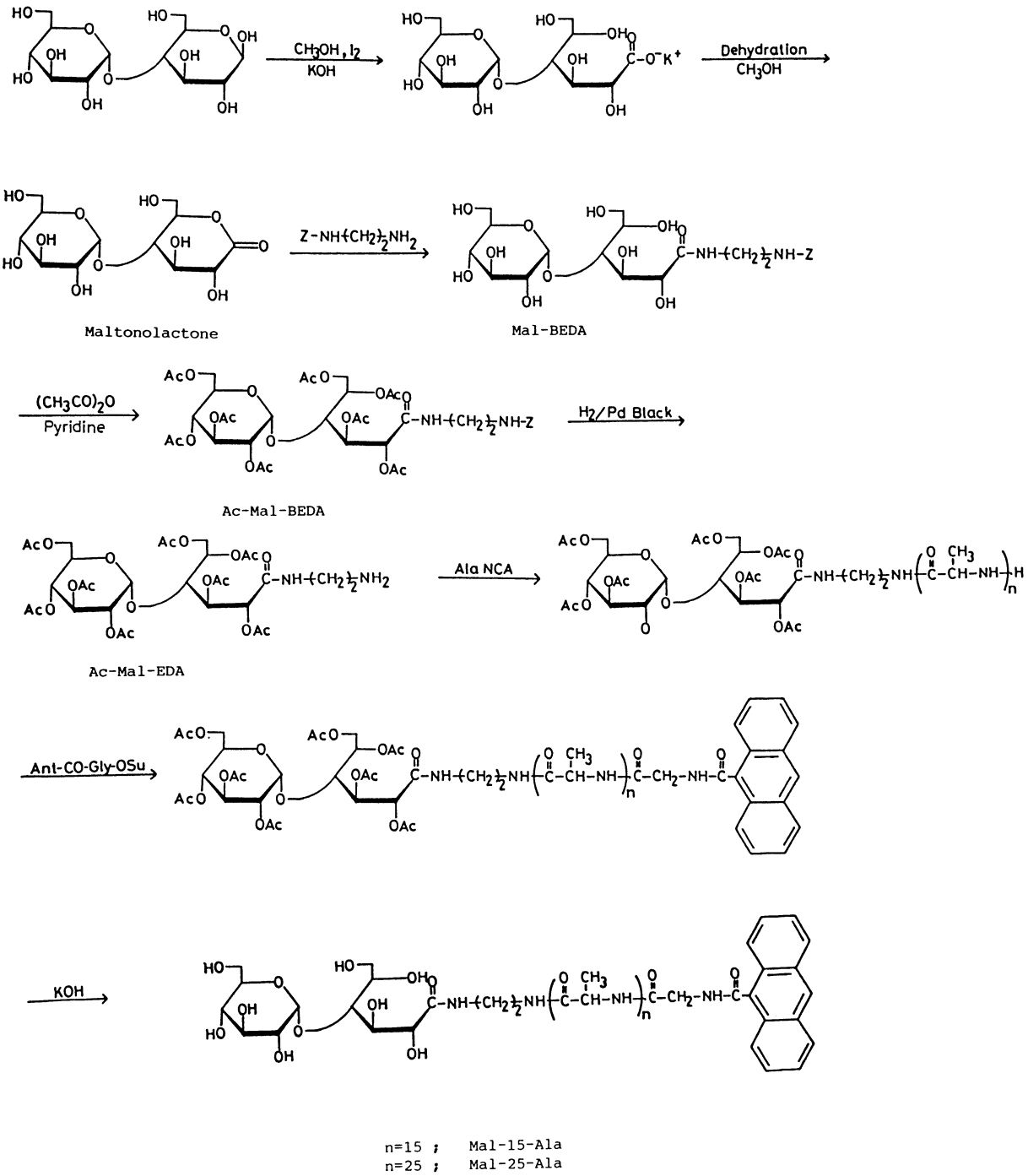


Fig. 2. Synthetic route to glycopeptides.

glyco moiety.

The amount of the fluorescent probe introduced into the N-terminal of polypeptides was estimated by UV spectroscopy. In the case of GlcNAc-20-Glu(OBzl), the content of naphthylalanine moiety was as low as 10%, while it was around 20 % for Mal-15 or 25-Ala (Table 1). It has been reported that the amino group at the terminal of a polymer chain easily lost the nucleophilicity.[12]

Conformation. IR spectra of Mal-15-Ala and Mal-25-Ala showed the absorptions at 1645 cm^{-1} and 1540 cm^{-1}, which are assigned to amide I and II absorptions of the α-helical polypeptide, respectively (data not shown).[13] In addition, CD spectra of GlcNAc-15-Ala, Mal-15-Ala, and Mal-25-Ala showed the double minima of negative Cotton effects in hexafluoro-2-propanol (data not shown). These results indicate that the peptide chain of glycopeptides takes an α-helical conformation both in a solid state and in solution.

Distribution to Lipid Bilayer Membrane. The distribution of glycopeptides to the lipid bilayer mem-

Table 1. Average Degree of Polymerization and Introduction Rate of Fluorescent Probe of Mal-15-Ala and Mal-25-Ala

Glycopeptide	[NCA]/[Initiator]	Average degree of polymerization	Content of 9-anthracene carbonyl group/%
Mal-15-Ala	15	16.3	22.1
Mal-25-Ala	25	23.7	23.8

brane was investigated by fluorescence spectroscopy. The emission of the glycopeptides synthesized in a buffer solution was not stable, because these glycopeptides were not well dispersed in a buffer solution and formed aggregates due to the high hydrophobicity. However, in the presence of DMPC SUV, GlcNAc-15-Ala and Mal-15 or 25-Ala emitted stable fluorescence above the phase-transition temperature of the lipid membrane, indicating the distribution of the glycopeptides to the lipid bilayer membrane. On the other hand, GlcNAc-20-Glu(OBzl) induced the aggregation of DMPC SUV above the phase-transition temperature. The significant destabilization of the membrane structure of DMPC SUV induced by the distribution of GlcNAc-20-Glu(OBzl) should be related with tight binding of GlcNAc-20-Glu(OBzl) with the lipid membrane due to the hydrophobic peptide chain. Hereafter, the interaction of glycopeptides with the lipid membrane was examined by using Mal-15 and 25-Ala, because glycopeptides obtained by using GlcNAc as initiator might contain a low-molecular-weight fraction and poly(γ-benzyl glutamate) induced the aggregation of the lipid membrane.

DPPC SUV aggregated on adding Mal-15-Ala below the phase-transition temperature of the membrane, while it did not above the phase-transition temperature. The addition of Mal-25-Ala to DPPC SUV below the phase-transition temperature did not induce the aggregation. However, the vesicles containing Mal-25-Ala aggregated, when the temperature was raised above the phase-transition temperature and then decreased below the phase-transition temperature again. It was considered that Mal-25-Ala, when added below the phase-transition temperature, was loosely bound to the lipid membrane, but that upon raising temperature above the phase-transition temperature Mal-25-Ala was incorporated into the lipid membrane. It was shown that the fluorescence depolarization (1/P) of Mal-25-Ala in the presence of DPPC SUV decreased upon raising temperature above the phase-transition temperature, indicating the incorporation of N-terminal of the peptide chain into the hydrophobic core of the membrane (Fig. 3). These experimental observations indicate that the glycopeptides are bound by the lipid bilayer membrane in a liquid-crystalline state so tightly that the membrane structure is destabilized to cause the aggregation of vesicles at lower temperatures than the phase-transition temperature. This observation is consistent with the report that the defect formation in the

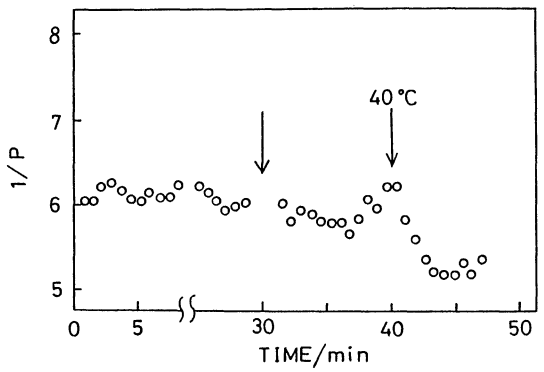


Fig. 3. The temperature dependence of fluorescence depolarization of 9-anthracenecarbonyl group of Mal-25-Ala in the presence of DPPC SUV. Temperature was raised up gradually from the point indicated by the left-side arrow and reached 40 °C at the point indicated by the right-side arrow. [DPPC]=1 mM. [Mal-25-Ala]=6.7×10^{-5} M.

lipid membrane triggered by the distribution of additives are more significant at lower temperatures than the phase-transition temperature.[14]

Interaction with Lectin. Con A is a well-known agglutinin which specifically binds to glucose unit.[15] The addition of Con A to DPPC SUV containing Mal-15 or 25-Ala induced agglutination of vesicles as shown by the increased turbidity of the suspension (Fig. 4). In the absence of glycopeptides the turbidity change was not significant. Therefore, it was suggested that the glyco moiety of the glycopeptides is exposed to the aqueous phase, and the peptide chain is buried in the lipid membrane.

However, the aggregated DPPC SUVs were not segregated by the addition of maltose. It was supposed that a tight aggregation occurred through the interaction of dehydrated surface of the lipid membrane after Con A-induced preliminary association of vesicles. The dehydration of membrane surface should be related with the presence of defects formed in the lipid membrane upon distribution of the glycopeptides.[14]

Orientation of Glycopeptides in Lipid Membrane. Fluorescence quenching of Mal-25-Ala by acrylamide, which is a water-soluble quencher, in the presence of DMPC SUV was less significant than in a buffer solution (data not shown). This means that the 9-anthracenecarbonyl group of glycopeptides is buried in the hydrophobic core of the lipid bilayer membrane.

A quenching group can be located at a certain depth of the lipid bilayer membrane by distributing 5- or 16-DS to the membrane.[16] Figure 5 shows that the emission of each glycopeptide in the presence of DPPC SUV was quenched more efficiently by 16-DS than by 5-DS, implying that the N-terminal region of the glycopeptides is located near the middle of the membrane.

CF Leakage. The addition of glycopeptides to the suspension of CF-trapped vesicles enhanced the CF leakge from the vesicles (Fig. 6). However, a simultaneous addition of Con A and glycopeptide to CF-entrapped vesicles did not change the rate of CF

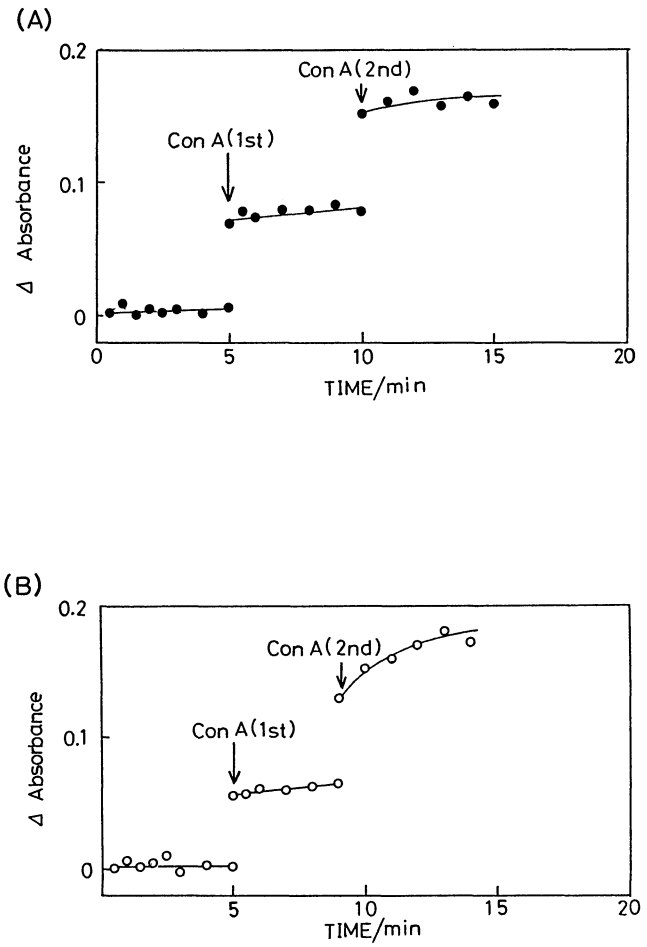


Fig. 4. Con A-induced aggregation of DPPC SUV containing Mal-15-Ala (A) or Mal-25-Ala (B) at 50 °C. Aggregation was monitored by the change of absorbance at 360 nm.
[DPPC]=1 mM. [glycopeptides]=6.7×10^{-7} M. [Con A]=1.7×10^{-6} M (at the 1st addition), 3.4×10^{-6} M (at the 2nd addition).

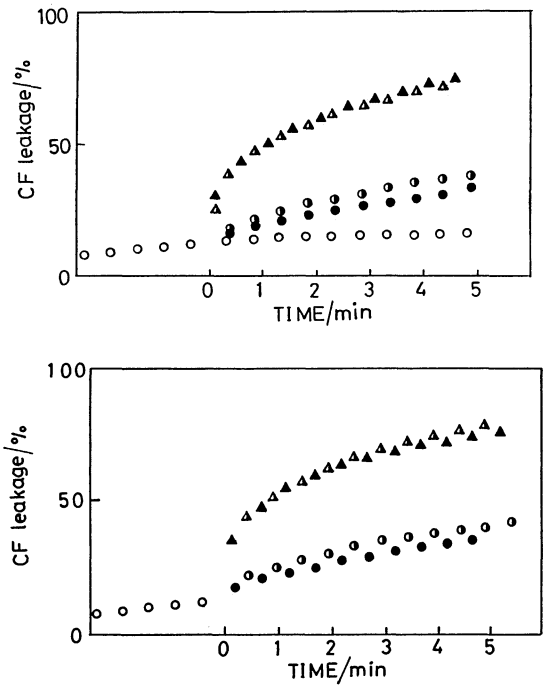


Fig. 6. CF leakage from DPPC SUV with the addition of Mal-15-Ala (A) and Mal-25-Ala (B), and the effect of Con A at 50 °C.
[DPPC]=3.1×10^{-4} M. ○; without additives. ●; [glycopeptide]=1.6×10^{-6} M. ◑; [glycopeptide]=1.6×10^{-6} M, and [Con A]=4.0×10^{-7} M. ▲; [glycopeptide]=8.0×10^{-6} M. ◭; [glycopeptide]=8.0×10^{-6} M, and [Con A]=4.0×10^{-7} M.

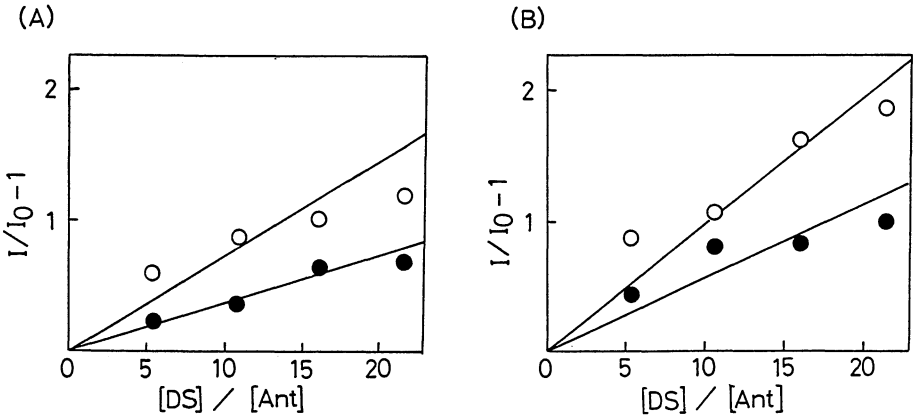


Fig. 5. Quenching of Mal-15-Ala (A) and Mal-25-Ala (B) by 5- (●) and 16-DS (○) in the presence of DPPC SUV according to the Stern-Volmer's plot at 50 °C.
[DPPC]=1 mM. [glycopeptides]=1.7×10^{-5} M.

leakage. Therefore, the binding of Con A to glyco moiety of glycopeptides, accompanying their cross-linking should not influence the CF-leakage. It seems that these glycopeptides are not molecularly dispersed in the membrane, but are distributed by forming aggregates.

The Effect of Membrane Potential on the Orientation of Glycopeptides. A bundle of α-helical rods in the lipid membrane has been proposed to form a voltage-dependent channel.[17] It has been considered that the orientation of the α-helical rod in the membrane can be changed in response to membrane potential due to its macrodipole moment.[18]

Membrane potential was generated according to the concentration difference of K^+ across the SUV membrane by the addition of K^+ ionophore, valinomycin.[19] It was confirmed that the membrane potential was maintained for more than 3 min under the present conditions, which was detected by using the fluorescence indicator of membrane potential, diS-C(3)-5.[20] The location of N-terminal portion of some glycopeptides in the lipid membrane can be evaluated by measuring the excitation spectra of 9-anthracenecarbonyl group connected to the N-terminal in the presence of energy donor, *N*-stearoyltryptophan.[21]

The excitation energy transfer from *N*-stearoyltryptophan to 9-anthracenecarbonyl group of the present glycopeptides in the presence of DMPC SUV was measured and is summarized in Table 2. Notably, the energy-transfer efficiencies of Mal-15-Ala and Mal-25-Ala were lower than that of Ant-CO-Gly-OEt. Since Ant-CO-Gly-OEt is supposed to be located near the membrane surface because of its low hydrophobicity,[21] the low energy-transfer efficiencies of glycopeptides indicate that the *N*-terminal of the glycopeptides is deeply incorporated into the hydrophobic core of the lipid membrane. This result is consistent with the results of the fluorescence quenching experiments described before. However, the energy-transfer efficiency of Mal-25-Ala was higher than that of Mal-15-Ala, indicating that the proportion of Mal-25-Ala residing near the membrane surface is higher than that of Mal-15-Ala.

Table 2. The Excitation Energy Transfer from *N*-Stearoyltryptophan to the Glycopeptides Carrying 9-Anthracenecarbonyl Group in the Presence of Egg Yolk Lecithin SUV

Peptide	Energy-transfer efficiency/%	
	Without membrane potential	Under membrane potential
Mal-15-Ala	4.63	5.12
Mal-25-Ala	8.80	10.03
Ant-CO-Gly-OEt	21.51	22.02

[egg yolk lecithin]=6.0×10^{-4} M. [glycopeptides]=1.3×10^{-5} M. [Ant-CO-Gly-OEt]=2.4×10^{-6} M. [*N*-stearoyltryptophan]=6.0×10^{-6} M. Egg yolk lecithin SUV containing 0.1 M KCl was suspended in buffer solution containing 0.1 M NaCl. Membrane potential was generated by the addition of valinomycin (1.6×10^{-5} M). The energy-transfer efficiency was evaluated from the excitation spectra on the basis of the molecular extinction coefficients; ε_{291} of tryptophan=4870 and ε_{365} of anthracene=5780.

Upon application of membrane potential, the energy-transfer efficiency was slightly increased. Since the K^+ concentration inside the vesicle was higher than that outside the vesicle, the Nernst equation predicts that the membrane potential was generated with higher positive potential in the external aqueous phase than in the internal aqueous phase. If the macrodipole of glycopeptides interacts with the electric field generated across the membrane, the fraction of glycopeptides pointing perpendicularly to the membrane surface should increase, resulting in decreased energy-transfer efficiency. However, this was not the case. It is considerd that the lipid bilayer membrane became thinner due to the membrane potential generated, leading to reduced distance between the 3-indolyl group and the 9-anthracenecarbonyl group in the membrane. This is likely explanation for the increased energy-transfer efficiency under the membrane potential.

In conclusion, the glycopeptides consisting of a maltose unit and 15 or 25 Ala residues were incorporated into the SUV membrane, taking a transmembrane orientation. When the peptide chain consists of 25 Ala residues, however, the glycopeptide tended to stay at the membrane surface. Presumably, the glycopeptides should form aggregates at the membrane surface due to intermolecular interactions. It has been shown that polypeptides such as poly(leucine) and poly(alanine) tend to take β-sheet structure and to form aggregates.[22] In addition, it has been reported that a naturally occurring transmembrane glycoprotein was reconstructed in the SUV membrane with a hairpin configuration having the N- and C-terminal regions exposed to external aqueous phase.[23] Taking these facts into consideration, the glycopeptides Mal-15-Ala and Mal-25-Ala should not necessarily take the transmembrane orientation spontaneously though the peptide chain is long enough to span across the membrane. The interaction of the peptide chain with lipid molecules should be influential in the orientation of the glycopeptide in the lipid membrane.

Experimental

Materials. The synthesis of glycopeptides used in the present investigation are summarized below briefly.

GlcNAc-15-Ala: L-Alanine *N*-carboxyanhydride (Ala NCA) was polymerized in *N*,*N*-dimethylformamide by using 2-*N*-acetyl-α-D-glucosylamine (NH_2-GlcNAc) as initiator, which was synthesized according to the method reported by Makino et al.[6] ([Ala NCA]/[Initiator]=15).

After disappearance of IR absorptions characteristic to the NCA, 1-naphthylacetic acid succinimide ester ($napCH_2COOSu$, 3-equivalent amount of the initiator) was added. After standing for two days at room temperature, the solution was poured into methanol, and the precipitate obtained was washed with methanol.

GlcNAc-20-Glu(OBzl): γ-Benzyl-L-glutamate *N*-carboxyanhydride (Glu(OBzl) NCA) was polymerized by using NH_2-GlcNAc as initiator ([Glu(oBzl)NCA]/[Initiator]=20) in *N,N*-dimethylformamide/dichloromethane (2/8) mixture. After standing for three days at room temperature, *N*-formylsarcosyl-2-naphthylalanine succinimide ester (HCO-Sar-napAla-OSu, 6-equivalent amount of the initiator) was added. This dipeptide was prepared by the condensation of *N*-formylsarcosine and 2-naphthylalanine benzyl ester using dicyclohexylcarbodiimide as a coupling reagent followed by hydrogenation, and esterification with *N*-hydroxysuccinimide. The solution was added to acetone/ether (1/1, v/v) mixture. The precipitate was fractionated by gel chromatography on a Sephadex LH-60 column using *N,N*-dimethylformamide as eluant.

Mal-15 or 25-Ala: Maltonolactone (0.655 g) was prepared according to the method reported by Williams et al.,[7] and reacted with *N*-benzyloxycarbonylethylenediamine (0.36 g) in *N,N*-dimethylformamide (2 ml) overnight. The product was purified on a silica-gel column using methanol/chloroform (3/7, v/v) mixture as eluant. The compound obtained (Mal-BEDA, 0.35 g) was acetylated with acetic anhydride (1.86 ml) and pyridine (2.66 ml) at 0 °C. White solid (Ac-Mal-BEDA) was obtained by precipitation upon addition of water. Calcd for $C_{38}H_{50}N_2O_{21}$: C, 51.35; H, 5.89; N, 3.15%. Found: C, 51.30; H, 5.64; N, 2.77%. Ac-Mal-BEDA (0.051 g) was subjected to the catalytic hydrogenation using Pd/C in *t*-butyl alcohol/ethyl acetate (1/1, v/v) mixture. After completion of hydrogenation, the solution was concentrated and precipitated by the addition of hexane. The product obtaind (Ac-Mal-EDA) was used as an initiator for the polymerization of Ala NCA in *N,N*-dimethylformamide. [Ala NCA]/[Initiator] was adjusted to 15 or 25. After disappearance of IR absorptions characteristic to the NCA, *N*-(9-anthrylcarbonyl)glycine succinimide ester (Ant-CO-Gly-OSu, 3 equivalent of the initiator) was added. Standing for two days at room temperature, the solution was poured into ether, and the precipitate was washed with methanol, acetone, and *N,N*-dimethylformamide/acetic acid (1/1, v/v) mixture.

The protected glycopeptides (20 mg) were dissolved in hexafluoro-2-propanol (500 μl) and hydrolyzed in the presence of 2 M KOH (76 μl). Standing for several hours at 27 °C, the solution was poured into an excess amount of water. The white solid obtained was washed with water, methanol, and ether. The removal of acetyl groups from the glyco moiety was confirmed by IR spectroscopy. The products are represented as Mal-15-Ala and Mal-25-Ala for n=15 and 25, respectively.

Dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), egg yolk lecithin, concanavalin A (Con A), 5- or 16-doxylstearic acid (5- or 16-DS, doxyl=4,4-dimethyl-3-oxazolidinyloxy), 5/6-carboxyfluorescein (CF), and valinomycin were purchased from Sigma (USA).

Preparation of Small Unilamellar Vesicle. Small unilamellar vesicle (SUV) composed of DPPC, DMPC or egg yolk lecithin was prepared by sonication of the lipid dispersion in a buffer solution (10 mM 2-[4-(2-hydroxymethyl)-1-piperazyl]ethanesulfonic acid (HEPES), 0.1 M NaCl or KCl, 0.1 mM EDTA, pH 7.4) and ultracentrifugation at 100000 g. Lipid content was assayed by colorimetric method using phospholipase D (Diacolor, Toyobo, Japan).

Measurement. NMR, fluorescence, UV, and IR spectroscopy was carried out on a JEOL-FX90Q (JEOL, Japan), a MPF-4 fluorescence spectrophotometer (Hitachi, Japan), a UVIDEC-1 UV/VIS spectrophotometer (JASCO, Japan), and an A-202 infrared spectrophotometer (JASCO, Japan), respectively.

CF leakage from DPPC vesicles was measured according to the method reported by Barbet et al.[8] Fluorescence excitation and monitoring wavelengths of CF were 470 and 515 nm, respectively. Aggregation of vesicles induced by the addition of Con A was monitored by the absorption at 360 nm. Fluorescence depolarization of 9-anthracenecarbonyl group connected to the peptides was measured on a MPF-4 fluorophotometer installed with an equipment as previously reported.[5] Fluorescence excitation and monitoring wavelengths of 9-anthracenecarbonyl group of the glycopeptides were 365 and 425 nm, respectively.

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