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Polypeptides as Models for Membrane-Active Proteins

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ABSTRACT: A cyclic oligopeptide, linear sequential polypeptides, and glycopeptides were synthesized and their activities in lipid assemblies were investigated in relation to conformational properties. First, a cyclic octapeptide, cyclo(D-Leu-L-Pro)$_4$, transported efficiently Ba^{2+} across $CHCl_3$ membrane. It transported calcium picrate across a liposomal membrane, which was evidenced by the fluorescence of chlorotetracyclin dissolved in the interior of the liposome. Secondly, sequential peptides, Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl ($n=3$ and 4), and a sequential polymer, poly(Leu-Lue-D-Phe-Pro) took 3_{10} helix conformation in nonpolar organic solvent, and the polymer yielded an electric current under an electric field, when it was added to bilayer lipid membrane (BLM) composed of oxidized cholesterol. This result indicates the formation of pores on the BLM through which ions permeate. The pores were possibly formed by folding and intramolecular association of the sequential polymer with 3_{10} helix conformation in BLM. Thirdly, a linear dipeptide Boc-Asp-ε-Lys-OMe was prepared, in which the α-amino group of the Lys unit was protected with pyrene butyryl group as a fluorecent probe and the β-carboxyl group of Asp unit was linked by an amide bond to 1-β-amino-acetylglucosamine which interacts specifically with a lectin, wheat germ agglutinin (WGA). Liposomes containing the glycodipeptide were associated by the addition of WGA. The association did not occur by the addition of unspecific lectin, phytohemagglutinin. The fluidity of liposome decreased on adding the glycodipeptide, but was restored by the further addition of WGA. Similar phenomena were observed when Ca^{2+} was added to liposomes containing a dipeptide without the sugar group.

KEY WORDS cyclo(D-Leu-L-Pro)$_4$ / Ion Transport / Liposome / Poly(Leu-Leu-D-Phe-Pro) / 3_{10} Helix / Ion Channel / Bilayer Lipid Membrane / Glycodipeptide / Wheat Germ Agglutinin / Patching /

It has been widely recognized that cell is an epitome of life. Almost all life phenomena take place in cell membrane, and proteins or glycoproteins in cell membrane play an essential role in the life phenomena. Therefore, it may be helpful to use synthetic peptides as models for membrane-active proteins in understanding the mechanism of life phenomena.

Muscle contraction, transmittance of neuro impulse, some of antibiotic actions, and generation of membrane potential take place as a result of ion permeation across cell membrane, which is mediated by ionophore. There are two types of ionophores, one of them being carrier ionophores and the other channel-forming ionophores.

Naturally occurring carrier ionophores such as valinomycin and enniatin B transport metal ions and ionic molecules across biomembrane.[1] In my laboratory, cyclo(L-Leu-L-Pro)$_4$ has been synthesized previously as a carrier ionophore model.[2] It bound alkali and alkaline-earth metal ions[3] and transported these ions efficiently across $CHCl_3$ membrane.[4] However, it did not transport metal ions across lipid membrane, which was ascribed to a slow complex formation with metal ions in the lipid membrane system.[4] Cyclo(D-Leu-L-Pro)$_4$ was expected to be an efficient ion carrier across liquid and lipid membranes, because it is highly symmetric and lipophilic. Therefore, we investigated conformational properties of and metal-ion transport by synthetic cyclic octapeptide, cyclo(D-Leu-L-Pro)$_4$.

The mechanism of ion transport through biological membranes mediated by channel-forming peptides such as gramicidine A[5] and alamethicin has

not been well understood. In the present study, sequential oligopeptides Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl ($n=1$, 2, 3, and 4) and a sequential polypeptide, poly(Leu-Leu-D-Phe-Pro) were synthesized, which contain a part of β-turn sequence in gramicidine S and are expected to have successive β-turns with a large internal diameter as models for ion channel. We investigated conformation of sequential polypeptide poly(Leu-Leu-D-Phe-Pro) and formation of ion channel across bilayer lipid membrane (BLM).

Transmembrane glycoproteins[6] are important components of cell membranes, acting as receptors for numerous external agents such as plant lectins,[7] viruses and hormones. A glycodipeptide (CC2), in which *N*-acetyl-D-glucosamine is substituted at C_1 site with a chromophoric dipeptide, was synthesized. This sugar group is specific for wheat germ agglutinin (WGA),[8] which is a lectin from tritcum vulgaris, but not specific for phytohemagglutinin (PHA), which is a lectin from phaseolus vulgaris. The response of CC2 in vesicles to lectins was investigated by spectroscopic methods.

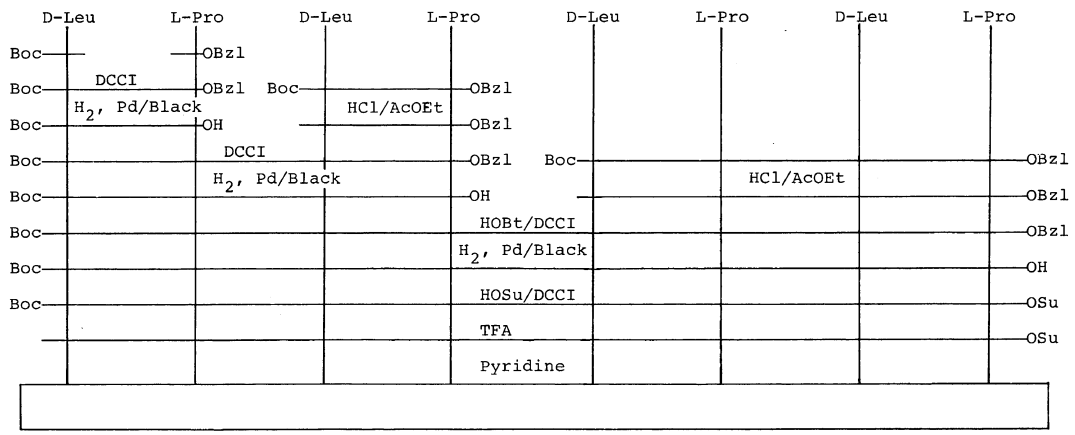
EXPERIMENTAL

Cyclo(D-Leu-L-Pro)$_4$ was synthesized, as shown in Figure 1, by cyclization of the linear oligopeptide under high dilution, which was prepared by the fragment condensation.

Conformation of cyclo(D-Leu-L-Pro)$_4$ in solution was investigated by nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy. The state of hydrogen bonding of cyclo(D-Leu-L-Pro)$_4$ was investigated by H–D exchange reaction. The metal ion transport by cyclo(D-Leu-L-Pro)$_4$ (140 μmol dm^{-3}) through CH_2Cl_2 or $CHCl_3$ membrane (20 cm^3) was measured using U-shaped tube.[9] Metal chloride (10 mmol dm^{-3}) and picric acid (25 mmol dm^{-3}) were dissolved in aqueous phase I (15 cm^3, pH 7.2), and the ion transport was monitored by the absorption of picrate anion in aqueous phase II (15 cm^3). Calcium ion transport across the liposomal membrane by cyclo(D-Leu-L-Pro)$_4$ (5—10 μmol dm^{-3}) was judged by fluorescence of chlorotetracyclin, which fluoresces upon coordination with a divalent metal cation.[10] A dispersion of liposomes of dipalmitoyl phosphatidyl choline (DPPC) (1 mmol dm^{-3}) was prepared by sonication. In the interior aqueous phase, chlorotetracyclin (2.5×10^{-5} mol dm^{-3}) was dissolved. In the aqueous phase outside the liposomes, calcium picrate (1 mmol dm^{-3}) was present.

Sequential oligopeptides Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl ($n=1$, 2, 3, and 4) and a sequential polypeptide poly(Leu-Leu-D-Phe-Pro) were synthesized by the scheme shown in Figure 2.

Conformation of the sequential oligopeptides and the sequential polypeptide in solution was investigated by NMR and CD spectroscopy. The state of hydrogen bonding was investigated by infrared (IR) and NMR spectroscopy and the space-filling molecular model. Ion gating in BLM by the sequential polypeptide was investigated as follows. An electric cell was filled with aqueous solution (pH 6.8, 0.5 mol dm^{-3} KCl, tris buffer) and divided into two



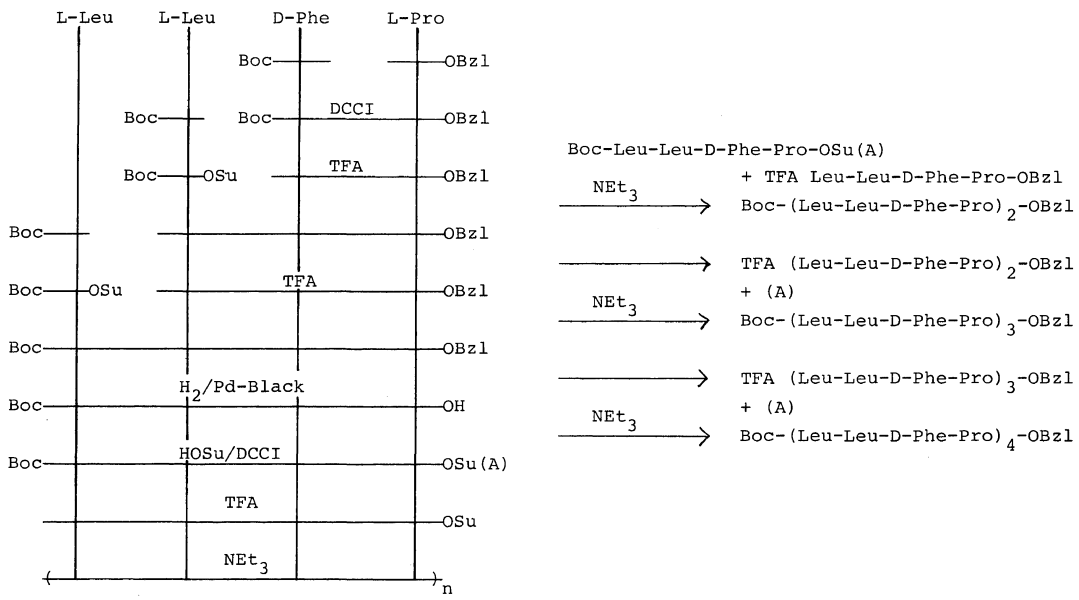


Figure 2. Synthetic scheme of poly(Leu-Leu-D-Phe-Pro) and Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl ($n=1$, 2, 3, and 4).

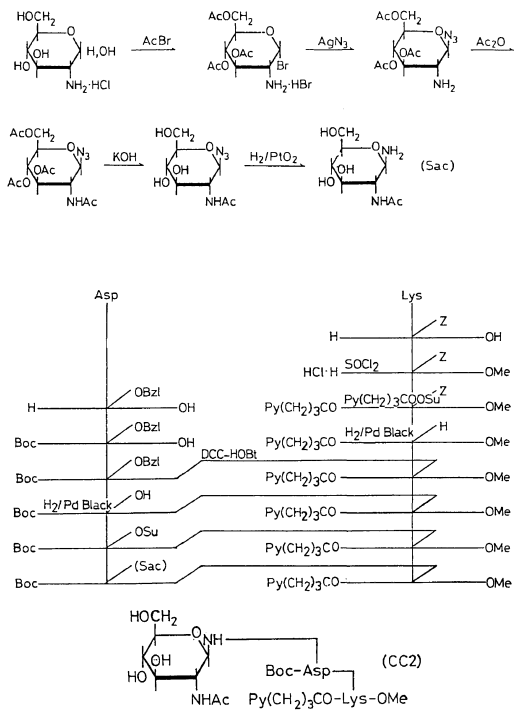


Figure 3. Synthetic scheme of glycodipeptide CC2.

halves with an oxidized cholesterol membrane. A trifluoroethanol (TFE) solution of the sequential polypeptide was added in the positive chamber under an application of electric field, and the current-voltage response was recorded.

A linear glycodipeptide (CC2), Boc-Asp(β-COX)-ε-Lys(α-NHY)-OMe in which X represents 1-β-amino-acetylglucosamine group and Y represents pyrene butyryl group, was synthesized according to the scheme of Figure 3.

Vesicles of dimyristoyl, dipalmitoyl, and distearoyl phosphatidyl choline (DMPC, DPPC, and DSPC) were prepared as follows. Lipids were dissolved in chloroform and a thin film of lipid was obtained by evaporation. To the film buffered aqueous solution was added and the suspension was sonicated at temperatures higher by 10°C than phase-transition temperatures (T_c). Ultracentrifugation (10^5 g, 0.5 h) gave a small unilamellar vesicle (SUV). Large unilamellar vesicles (LUV) with 0.1 μm diameter were prepared according to the method reported by Szoka, Jr. *et al.*[11]

Aggregation of CC2-containing vesicles induced by lectins and segregation induced by inhibitors were monitored by the change of turbidity. Intravesicular association of CC2 was monitored by the increase of emission intensity due to pyrene

excimer (I_D) over that due to pyrene monomer (I_M). The change of membrane fluidity at the surface of vesicle induced by the addition of CC2 and lectin was investigated by the fuorescence polarizability of 8-anilinonaphthalene sulfonic acid (ANS), and that in the bulk of vesicle by the fluorescence polarizability of 1,6-diphenylhexatriene (DPH).[12]

RESULTS AND DISCUSSION

Conformational Properties of and Metal Ion Transport by Synthetic Carrier Ionophore, Cyclic Octapeptide, Cyclo(D-Leu-L-Pro)$_4$

Conformation of cyclo(D-Leu-L-Pro)$_4$ in solution was investigated by spectroscopy. The NH region of ^{1}H NMR spectrum of cyclo(D-Leu-L-Pro)$_4$ in CD_2Cl_2 is shown in Figure 4. The spectrum at 30°C is characteristic of all-*trans* C_4- symmetric conformation, as evidenced by the single NH signal. Lowering the temperature, the NH signal disappeared at −30°C and reappeared at δ 5.5 and 7.5 at lower temperatures. In the C=O region of ^{13}C NMR spectrum, as shown also in Figure 4, one and four signals were observed at 30 and −80°C, respectively. These observations can be explained in terms of a rapid conformational averaging of cyclo(D-Leu-L-Pro)$_4$ taking place at room temperature.

CD spectral change of cyclo(D-Leu-L-Pro)$_4$ took place in alcoholic solution by the addition of Ca^{2+} and Ba^{2+}, which indicates metal ion binding by the cyclic octapeptide accompanying a conformational change. CD spectral change was not induced by the addition of K^+, Na^+, Mg^{2+}, Ag^+, and Cd^{2+} indicating an ion-selective binding. It is contrasting that CD spectrum of cyclo(L-Leu-L-Pro)$_4$ in alcoholic solution changed by the addition of K^+.[3] The binding constant was calculated from the variation of the molar ellipticity of CD spectrum. Thus the binding constant of cyclo(D-Leu-L-Pro)$_4$ with Ba^{2+} was determined to be 400 dm^{-3} mol^{-1}, which is nearly the same as 360 dm^3 mol^{-1} reported for cyclo(L-Leu-L-Pro)$_4$/Ba^{2+} complex.[3] The rate constant of complex formation of cyclo(D-Leu-L-Pro)$_4$ with Ba^{2+} in MeOH–H_2O (95:5, v/v) was determined from time-resolved ellipticity of CD spectrum to be larger than 2×10^3 dm^3 mol^{-1} min^{-1}. This is much larger than 0.7 dm^3 mol^{-1} min^{-1} reported for cyclo(L-Leu-L-Pro)$_4$ and Ba^{2+}.[3] It was found by NMR spectroscopy that cyclo(D-Leu-L-Pro)$_4$/Ba^{2+} complex took an all-*trans* C_4-symmetric conformation.

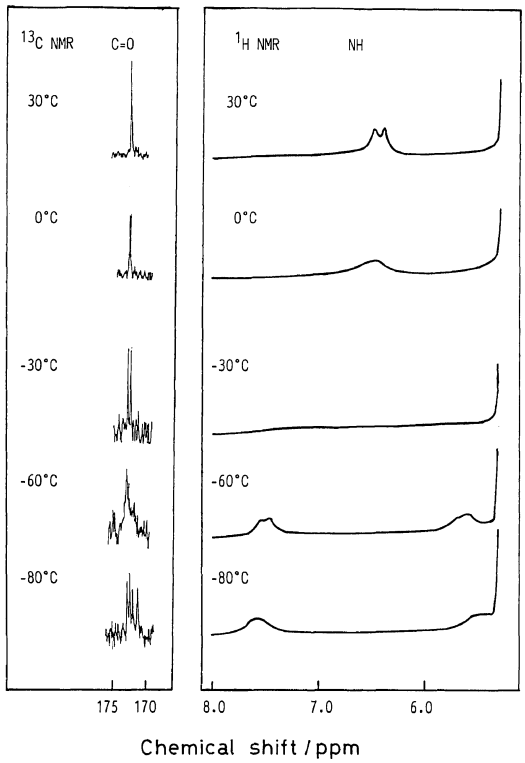


Figure 4. ^{1}H and ^{13}C NMR spectra of cyclo(D-Leu-L-Pro)$_4$ in CD_2Cl_2 at different temperatures.

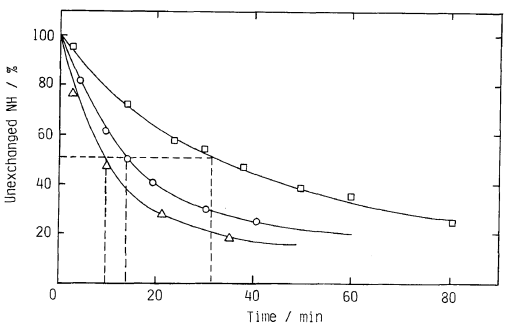


Figure 5. Time course of H–D exchange of amide NH: □, cyclo(D-Leu-L-Pro)$_4$; ○, cyclo(D-Leu-L-Pro)$_4$ + $Ba(ClO_4)_2$; △, *N*-methylacetamide.

Figure 5 shows the time course of H–D exchange of cyclo(D-Leu-L-Pro)$_4$ in $CDCl_3$ containing CD_3OD (40 eq/NH).[13] The half life of unexchanged NH is 31 and 15 min for the free peptide and the

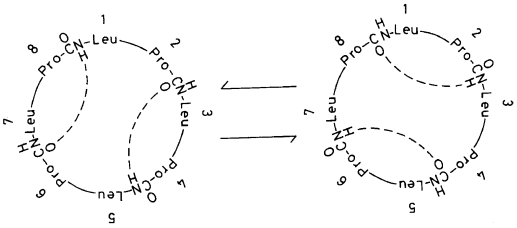


Figure 6. Conformational equilibrium between two C_2-symmetric conformers of cyclo(D-Leu-L-Pro)$_4$ in CH_2Cl_2.

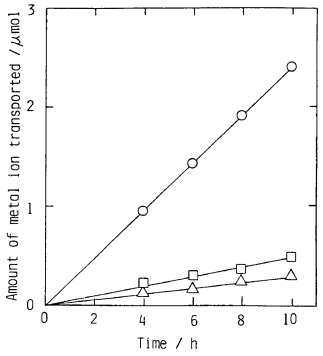


Figure 7. Ion transport across $CHCl_3$ membrane by cyclo(D-Leu-L-Pro)$_4$: ○, Ba^{2+}; □, Ca^{2+}; △, K^+.

Ba^{2+} complex, respectively. The latter value is nearly the same as the half life of *N*-methylacetamide, indicating that the probability of peptide hydrogens involved in the free peptide being exposed to solvent is half as large as that for the Ba^{2+} complex.

It was concluded on the basis of these experimental results that free cyclo(D-Leu-L-Pro)$_4$ assumes two C_2-symmetric conformations and a rapid interchange between them takes place as shown in Figure 6 at room temperature. Free energy of the conformational change was determined to be *ca.* 3 kcal mol^{-1} by ^{1}H NMR spectroscopy, which indicates that the conformational change does not involve the isomerization of peptide bonds.

The efficient and fast binding of metal ion accompanying the scission of internal hydrogen bonding and the internal rotation of single bonds as well as the high lipophilicity of the metal ion complex represents promising properties of cyclo(D-Leu-L-Pro)$_4$as a synthetic ionophore.

The metal ion transport through a liquid membrane was investigated. The amounts of metal ions transported across $CHCl_3$ membrane by cyclo(D-Leu-L-Pro)$_4$ are shown in Figure 7. Ba^{2+} was transported most efficiently. The same selectivity has been observed for cyclo(D-Leu-L-Pro)$_4$.[4] The K^+ transport by cyclo(D-Leu-L-Pro)$_4$ was about 1/3 times as effective as that by dicyclohexyl 18-crown-6 under similar conditions.[14] Although Ca^{2+} was a

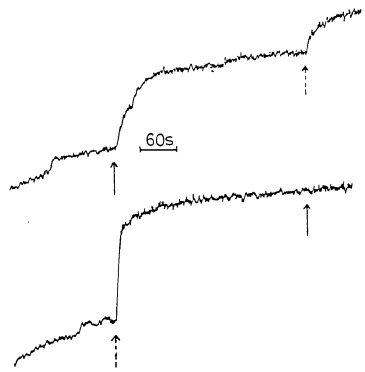


Figure 8. Time-dependent increases in chlorotetracyclin-Ca^{2+} fluorescence on adding ionophore. Ionophore addition marked with an arrow, cyclo(D-Leu-L-Pro)$_4$, 6 μmol dm^{-3} and with a broken arrow, X537A, 10 μmol dm^{-3}.

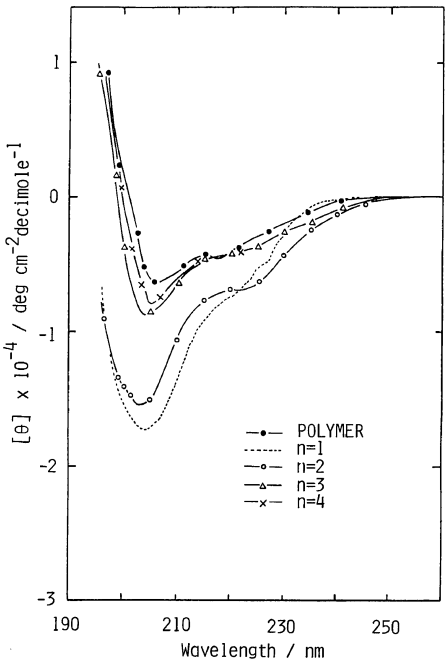


Figure 9. CD spectra of Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl (n=1, 2, 3, and 4) and poly(Leu-Leu-D-phe-Pro)$_n$ in CF_3CH_2OH: conc., 0.3 mg cm^{-3}; room temp.

specific guest ion for cyclo(D-Leu-L-Pro)$_4$, Ca^{2+} transport across $CHCl_3$ membrane by cyclo(D-Leu-L-Pro)$_4$ was not efficient. The extraction coefficient from aqueous phase to organic phase and the rate of complexation should be more closely related to the efficiency of ion transport than the equilibrium binding constant in the organic phase.

Ca^{2+} transport across DPPC membrane by cyclo(D-Leu-L-Pro)$_4$ was investigated by fluorescence of chlorotetracyclin. When cyclo(D-Leu-L-Pro)$_4$ was added to the dispersion of DPPC liposomes in aqueous solution containing calcium picrate, chlorotetracyclin contained in the internal aqueous phase of liposome fluoresced as shown in Figure 8, indicating ion transport across the liposomal membrane.

Conformation of Sequential Polypeptides Poly(Leu-Leu-D-Phe-Pro) and Formation of Ion Channel across BLM

CD spectra of Boc(Leu-Leu-D-Phe-Pro)$_n$-OBzl ($n=1$, 2, 3, and 4) and poly(Leu-Leu-D-Phe-Pro) in TFE are shown in Figure 9. $[\theta]_{207}$ decreased and the shoulder at 220 nm became more marked with increasing chain length of the peptide. This change could be interpreted in terms of the stabilization of a regular structure. The pattern of CD spectrum of poly(Leu-Leu-D-Phe-Pro) is similar to the oligopeptides with $n=3$ and 4, suggesting a similar conformation in solution. Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl changed the conformation in THF depending on temperature, and Boc-(Leu-D-Phe-Pro)$_4$-OBzl took different conformations in polar and nonpolar solvents. In the amide NH and $C^{\alpha}H$ regions of ^1H NMR spectrum of poly(Leu-Leu-D-Phe-Pro) in $CDCl_3$, a drastic change was observed on adding trifluoroacetic acid (TFA). These findings point to a certain secondary conformation of the sequential peptides in nonpolar solvents.

As shown in ^{13}C NMR spectra of Figure 10, the oligopeptides Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl ($n=$ 1 and 2) took multiple conformations in $CDCl_3$

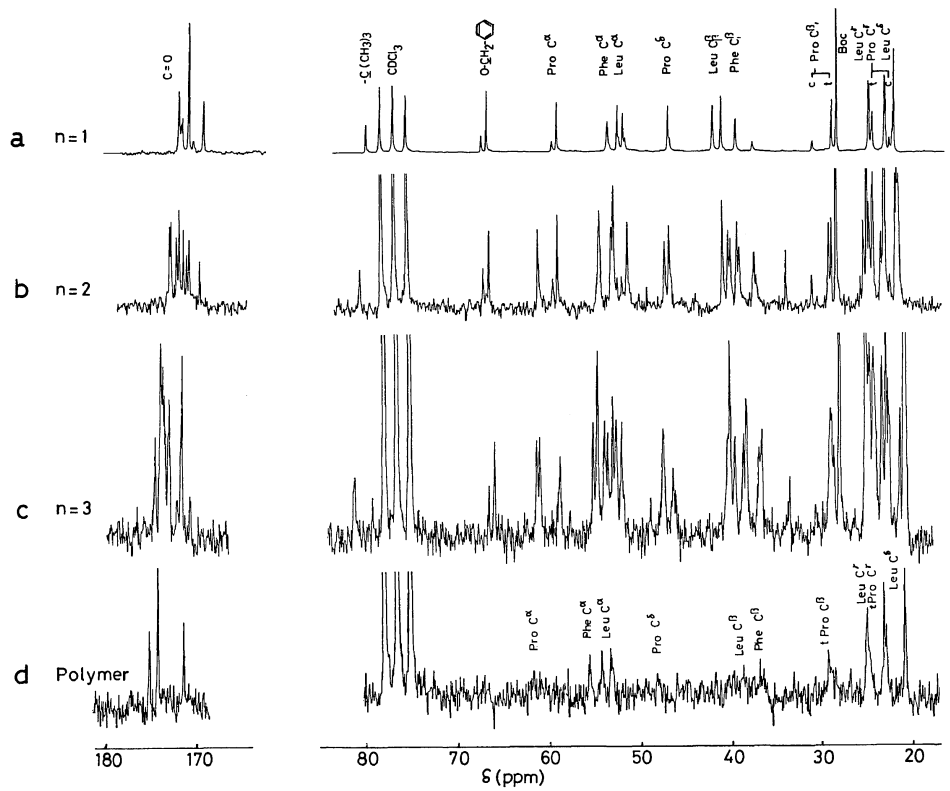


Figure 10. ^{13}C NMR spectrum of Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl. (a) $n=1$, 170 mg cm^{-3}, (b) $n=2$, 70 mg cm^{-3}, (c) $n=3$, 70 mg cm^{-3}, and (d) poly(Leu-Leu-D-Phe-Pro), 33 mg cm^{-3} in $CDCl_3$ at 30°C.

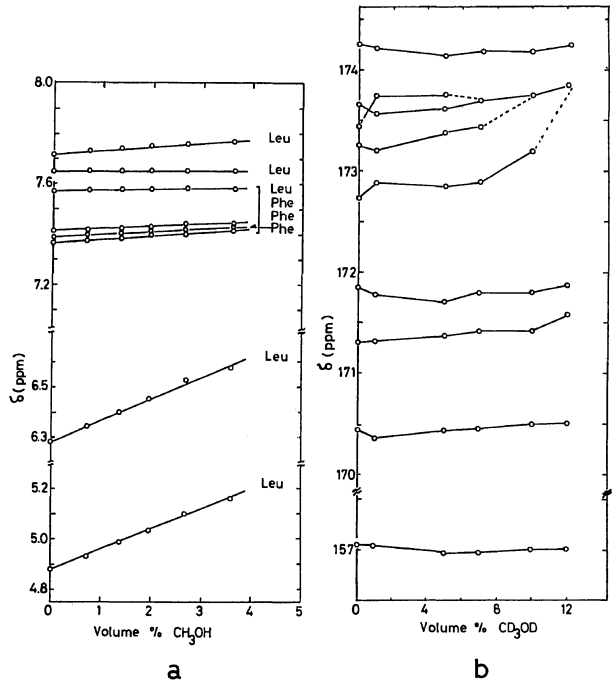


Figure 11. Change of chemical shift in ^{1}H and ^{13}C NMR of Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl in $CDCl_3$ (10 mg cm^{-3}) by the addition of methanol (1—5 vol%) at 25°C. (a) 400 MHz ^{1}H NMR; (b) ^{13}C NMR.

according to the *cis/trans* isomerization of Pro-peptide bonds and that Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl and poly(Leu-Leu-D-Phe-Pro) took similar conformations in which Pro-peptide bonds were confined to take *trans* configuration. The above experimental results indicate that the conformation in solution of poly(Leu-Leu-D-Phe-Pro) can be identified by the investigation on the conformation of Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl.

In order to investigate the exposure to solvent of amide and urethane linkages of Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl, shifts of ^{1}H NMR signals (Figure 11a) and ^{13}C NMR signals (Figure 11b) induced by the addition of methanol to $CDCl_3$ solution of Boc(Leu-Leu-D-Phe-Pro)$_3$-OBzl were measured. These experimental results showed that two Leu-NHs are exposed to solvent and the urethane carbonyl group is shielded from solvent.

IR spectroscopy on $CDCl_3$ solutions of Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl ($n=1$, 2, 3, and 4) and poly(Leu-Leu-D-Phe-Pro) (see Figure 12) revealed that NH protons of the oligopeptide with $n=1$ are free from hydrogen bonding, those with $n=2$, 3, and 4 are partly hydrogen bonded, and those of

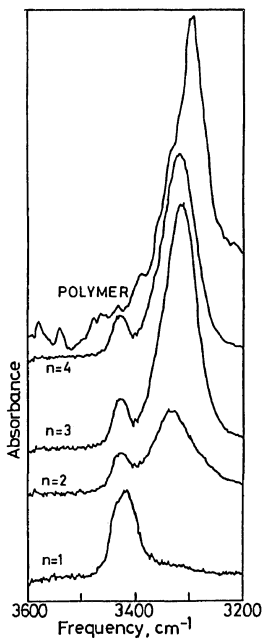


Figure 12. Amide A absorption regions of IR spectra of Boc-(Leu-Leu-D-Phe-Pro)$_n$-OBzl and poly(Leu-Leu-D-Phe-Pro) in $CDCl_3$ (*ca.* 1×10^{-3} mol dm^{-3}).

Table I. Hydrogen-bonding characteristics for various conformations of Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl

Conformation	H-bond-free NH	Urethane NH	Urethane C=O
α-Helix	Leu[1], Leu[2], D-Phe[3]	Not H-bonded	Not H-bonded
3_{10}-Helix	Leu[1], Leu[2]	Not H-bonded	H-bonded
$\beta^{4.4}$-Helix[a]	Leu[1], Leu[2], D-Phe[3]	Not H-bonded	H-bonded
$\beta^{6.3}$-Helix[b]	Leu[1], Leu[2], D-Phe[3], Leu[5], Leu[6]	Not H-bonded Not H-bonded	H-bonded
$\uparrow\downarrow\beta^{5.6}$-Helix (left hand)	Leu[2]	H-bonded	Not H-bonded
$\uparrow\downarrow\beta^{5.6}$-Helix (right hand)	Leu[1], D-Phe[3]	Not H-bonded	Not H-bonded

[a] 6→1 hydrogen-bonding.
[b] 8→1 hydrogen bonding.

polypeptide are completely hydrogen bonded. From the measurements at different concentrations of Boc-(Leu-Leu-D-Phe-Pro)$_4$-OBzl, the hydrogen bonding was concluded to be intramolecular.

The states of hydrogen bonding in possible conformations of Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl were considered and are listed in Table I. The agreement of Table I with the information from ^{1}H NMR spectroscopy is obtained only with 3_{10}-helix, leading to the conclusion that Boc-(Leu-Leu-D-Phe-Pro)$_3$-OBzl takes 3_{10}-helix conformation in $CDCl_3$. The 3_{10}-helix has been often found in Aib-containing peptides,[15,16] and is formed by the continuation of the 4→1 hydrogen bonds of type III β-turn.[17] When a Pro residue is involved in 3_{10}-helix, ^{13}C NMR chemical shift of C^α shifts to lower magnetic field than 60 ppm,[17] which was the present case. The present conclusion is that the sequential polypeptide, poly(Leu-Leu-D-Phe-Pro), takes 3_{10}-helix conformation in $CDCl_3$.

The current trace recording of poly(Leu-Leu-D-Phe-Pro) in the oxidized cholesterol membrane is shown in Figure 13.

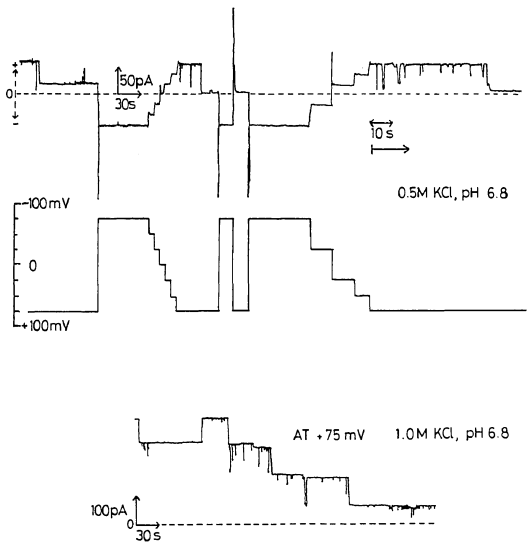


Figure 13. Current trace recording of poly(Leu-Leu-D-Phe-Pro) in oxidized cholesterol membrane.

The current-voltage response across the membrane was observed. At 75 mV, an electric current of 55 pA was observed after 20—30 min. However, fluctuation in current was observed and the current disappeared by the prolonged application of 75 mV. On the other hand, by the application of −75 mV a stationary current of about −55 pA was observed. In this case fluctuation of current was not observed. In the stationary state the current–voltage relation showed the Ohmic behavior as shown in Figure 14.

With increasing KCl concentration from 0.5 mol dm^{-3} to 1.0 mol dm^{-3} the magnitude of the discrete current jump became about 100 pA. This observation suggests a formation of channels having a definite pore size.

In a 3_{10}-helix, the pitch per a residue is 2 Å. Since the thickness of oxidized cholesterol membrane is 40—60 Å, a 3_{10}-helix consisting of 20—30 residues is necessary to penetrate the membrane. The molecular weight of the present poly(Leu-Leu-D-Phe-Pro) lies between 15,000 and 25,000 indicating the numbers of residue to be 130—200. It is considered that by the application of electric field the polypep-

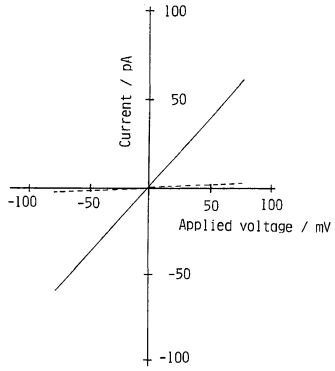


Figure 14. Current-voltage relationship for an oxidized cholesterol membrane treated with poly(Leu-Leu-D-Phe-Pro). —, pore open; ---, pore closed.

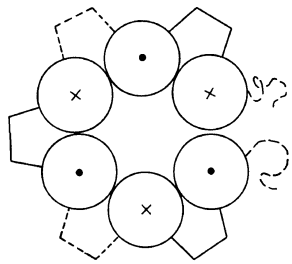


Figure 15. Proposed model for a channel formed by poly(Leu-Leu-D-Phe-Pro). Cross section in the plane of the membrane.

tide folds into a bundle of at least six 3_{10}-helices, as depicted in Figure 15. We see the bundle of 3_{10}-helices from the top to the bottom or *vice versa*. In this model, adjacent 3_{10}-helices run in opposite directions. Ions permeate through the central cavity with coordination to peptide bonds. Incorporation of the sequential polypeptide into BLM should be vectorial, so that a discrete current jump was observed by an electric field having a minus sign. Application of electric field having a positive sign caused a fluctuation and disappearance of current. This type of channel should lead to a constant pore size, which is different from that formed by intermolecular association of helical chains as in the case of alamethicin.[18]

Interactions of Synthetic Glycopeptides with Lectins in Lipid Bilayer Membranes

Using fluorescent quenching with acrylamide, the incorporation of CC2 into DMPC, DPPC, and

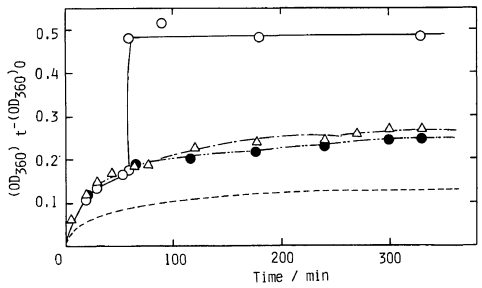


Figure 16. Aggregation of DPPC LUV (1 mmol dm^{-3}) containing CC2 (1.65×10^{-5} mol dm^{-3}) by lectin at room temperature. ---, liposome only; $-\cdot\cdot-\triangle-\cdot\cdot-$, liposome + CC2; —○—, liposome + CC2 + WGA (3.3×10^{-7} mol dm^{-3}), $-\cdot\cdot-\bullet-\cdot\cdot-$, liposome + CC2 + PHA ($8.7\times10^{-8}$ mol dm^{-3}).

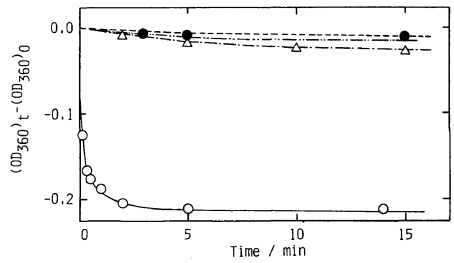


Figure 17. Segregation of associated DPPC LUV by inhibitor for lectin at room temperature. ---, liposome only; $-\cdot\cdot-\triangle-\cdot\cdot-$, liposome + CC2 + GlcNAc; —○—, liposome + CC2 + WGA + GlcNAc; $-\cdot\cdot-\bullet-\cdot\cdot-$, liposome + CC2 + PHA + GlcNAc.

DSPC vesicles was confirmed. When WGA was added to the dispersion of vesicles containing CC2, the turbidity increased drastically, as shown in Figure 16, which indicates intervesicular aggregation taking place. This turbidity change represents a specific interaction of the sugar group of CC2 with WGA, because the change of turbidity was not observed when PHA was added to the dispersion of vesicles containing CC2.

As evidenced by the sudden decrease of turbidity shown in Figure 17, the vesicular aggregation was segregated by the addition of *N*-acetyl-D-glucosamine (GlcNAc), which is an inhibitor of WGA, but not by the addition of *N*-acetyl-D-galactosamine (GalNAc), which is an inhibitor of PHA. The segregation by GlcNAc proves that the turbidity increase by WGA (Figure 16) was caused by the aggregation of vesicles and not by the fusion

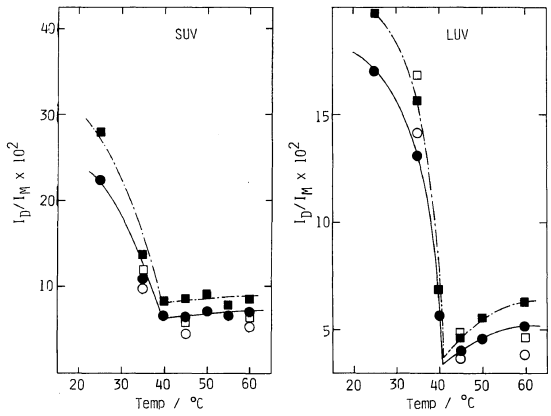


Figure 18. Phase separation of DPPC liposomes (1 mmol dm^{-3}) induced by CC2 (1.9×10^{-5} mol dm^{-3}). I_D, 480 nm; I_M, ● and ○ 376 nm; ■ and □ 397 nm; ○ and □, temperature rising; ● and ■, temperature lowering.

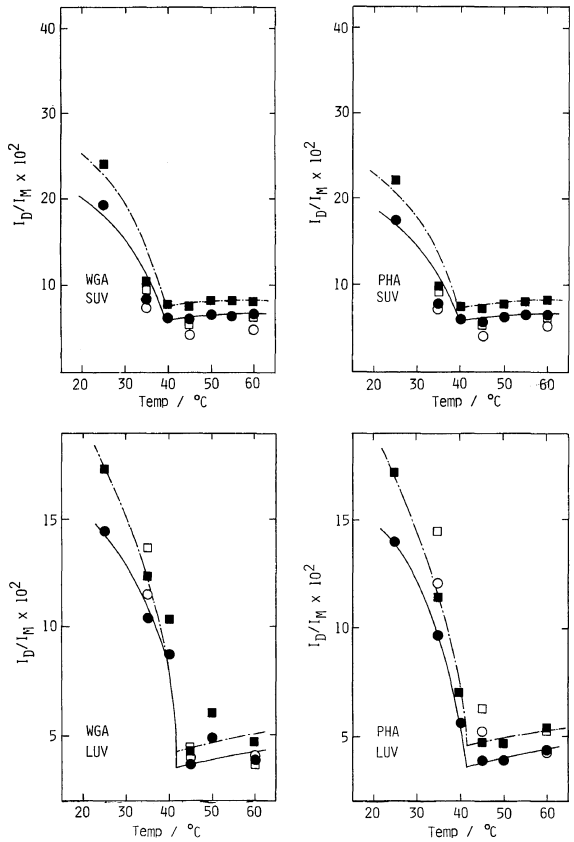


Figure 19. Lectin-induced intravesicular aggregation of CC2 (1.65×10^{-5} mol dm^{-3}) in DPPC liposomes. [WGA] = 1×10^{-6} mol dm^{-3}. [PHA] = 1.74×10^{-7} mol dm^{-3}. Notations are the same as those in Figure 18.

of vesicles. These experimental results also confirm the specific interaction of CC2 embeded in vesicles with WGA.

By fluorescent spectroscopy the ratio of I_D over I_M of CC2 was determined in the temperature range from 25 to 60°C. In the HEPES-buffered saline solution at pH 7.4, I_D/I_M did not change with the temperature change. However, as shown in Figure 18, in the DPPC vesicular system I_D/I_M changed sharply at T_c. From the effect of temperature on the quantum yield of monomer and excimer fluorescence both in solution and vesicular systems, it was concluded that below T_c, CC2 formed an excimer

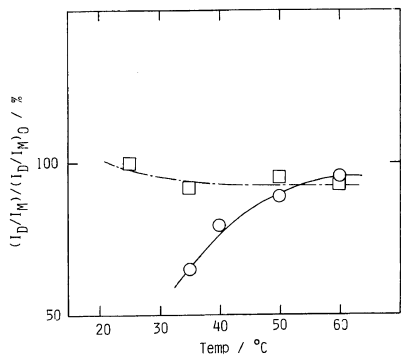


Figure 20. Lectin-induced release of CC2 from DPPC SUV (1 mmol dm^{-3}). —○—, liposome + CC2 + WGA; -·-□-·-, liposome + CC2 + PHA; $(I_D/I_M)_0$, liposome + CC2; I_M, 367 nm.

by patching phenomenon[19] due to phase separation of lipid membrane.

It was expected that WGA might induce an intravesicular interaction of CC2 to result in the increment of I_D/I_M. However, as shown in Figure 19, a little decrease of I_D/I_M was observed on adding WGA.

To eliminate complexities arising from the disordering of the lipid bilayer structure at T_c, I_D/I_M in the presence of lectin was determined at different temperatures. As shown in Figure 20, the relative intensity ratio was nearly unity at any temperatures when PHA was used, whereas it decreased from unity below T_c when WGA was used. These experimental results are explained in terms of the partial abstraction of CC2 by WGA from the BLM. This explanation was supported by the quenching of the monomer fluorescence of CC2-containing liposomal system with acrylamide on adding WGA.

The change of membrane fluidity induced by the addition of CC2 and lectin was investigated and the results are shown in Figure 21.

The addition of CC2 was found to decrease the membrane fluidity, which might be due to the formation of gel-like domain. The addition of WGA to the vesicles containing CC2 increased the membrane fluidity, whereas PHA had little effect. Therefore, the fluidity increase is due to a specific interaction between CC2 and WGA, such as an intravesicular crosslinking of CC2 with lectin lead-

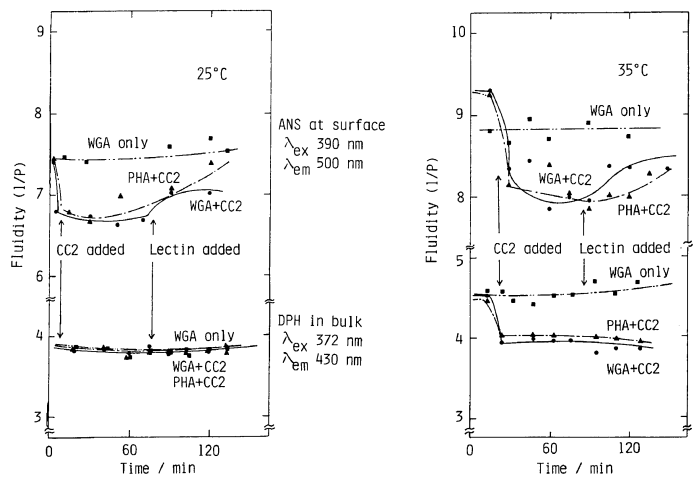


Figure 21. Change of membrane fluidity of DPPC SUV (1 mmol dm^{-3}) by CC2 (1.9×10^{-5} mol dm^{-3}) and lectins below T_c. [WGA] = 3.3×10^{-7} mol dm^{-3}, [PHA] = 8.7×10^{-8} mol dm^{-3}, [ANS] = 1.6×10^{-4} mol dm^{-3}, [DPH] = 1.6×10^{-5} mol dm^{-3}.

ing to an increase of fluid regions of membrane. This idea received a support from the experimental result that the fluidity increase was also observed with a chromophoric dipeptide with carboxyl group but without sugar group, $Py(CH_2)_3CO$-Lys-(ε-Boc-Asp)-OCH_3, interacting with divalent cations in liposome. The absence of the increase of excimer formation between CC2 molecules induced by WGA in liposome in spite of the crosslinking might be due to chromophoric groups being kept too distantly to form an excimer. On the other hand, the addition of WGA to the vesicles containing CC2 was found not to affect the bulk fluidity. The fluidity of vesicles, which do not contain CC2, was not affected by WGA either at the surface or in the bulk. It is concluded that the present system reproduces well some cell phenomena such as the mitogen-induced phase separation of cell membrane[20] and constitutes a basis for the development of biocompatible synthetic materials.

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