Effects of Cation Binding to Hydrophobic Helical Peptides on Orientation, Aggregation, and Ion-channel Activity in Phospholipid Bilayer Membranes

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Effects of cation binding to the C-terminal of α-helical peptides on conformation, orientation, aggregation, and ion-channel activity in a phospholipid bilayer membrane have been studied. The hydrophobic helical peptides having a crown ether unit at the C-terminal region, Boc-[Ala-Aib]$_n$-Ala-Cr (Cr represents a benzo-18-crown-6 unit, n = 4,8), and an anthryl group at the N-terminal region as well, Boc-Ser(Ant)-[Ala-Aib]$_n$-Ala-Cr (Ant represents an anthrylmethyl group, n = 4,8), have been synthesized. The helix content of the peptides increased upon complexation with K^+ because of interaction of the negative pole of the helic macrodipole with the cation. The peptides were incorporated into a phospholipid bilayer membrane, and aggregated taking a transmembrane orientation. Boc-[Ala-Aib]$_4$-Ala-Cr showed an ion-channel-like activity in a bilayer membrane. The activity was higher than that of Boc-[Ala-Aib]$_4$-OMe, since the crown ether unit functions as a cation-binding site of the channel. The aggregation of the crown-peptides was promoted especially in the presence of Rb^+ and Cs^+ due to formation of a sandwich-type crown/cation complex. For this reason, Boc-[Ala-Aib]$_4$-Ala-Cr showed a higher channel-like activity in the presence of Cs^+ than K^+. On the other hand, Boc-[Ala-Aib]$_8$-Ala-Cr aggregated upon incorporation into a bilayer membrane, and showed an ion-channel-like activity. In any one of the peptides, the connection of a crown ether unit to the hydrophobic helical peptide augmented its channel-forming ability by facilitating aggregation with a transmembrane orientation.

Alamethicin, suzukacillin, and trichotoxin are known to form a voltage-dependent ion channel in bilayer membranes.[1-5] These peptides it is suggested form aggregates of helices spanning across the membrane. The aggregate should hold a pore at the centre of the helix bundle, through which ions may permeate. Although the gate mechanism of the voltage-dependent ion channel is still controversial,[6] several model peptides have been designed on the basis of this idea, and shown to form an ion channel.[7-9]

We have shown that a hydrophobic α-helical peptide composed of 21 amino acid residues assembles into an α helix bundle with a transmembrane orientation in bilayer membranes.[10] The peptide showed a channel-like activity in a bilayer lipid membrane.† However, the peptide was less potent in exhibiting the ion-channel-like activity than alamethicin. One of the reasons is difficult access of a cation to the terminal portion of the hydrophobic peptide. In order to obtain a more potent channel-forming peptide, an ion-binding site was connected to the end of hydrophobic helical peptide in the present investigation. A crown ether was chosen for this purpose and connected to the C-terminal of Boc-[Ala-Aib]$_n$-OMe (n = 4,8) (Fig. 1). The crown ether unit of the peptides is expected to play several important roles in ion-channel formation as follows: (i) to facilitate the ion binding to the terminal portion of a hydrophobic helix bundle at a water/lipid interface; (ii) to promote a transmembrane orientation of the peptide due to the primary amphiphilicity;[11] and (iii) to stabilize the aggregate in the membrane by forming a sandwich-type complex with large cations.

A few examples have been reported to develop an ion channel in a lipid membrane by using a crown ether unit.[12,13] However, the molecular structure of the synthetic compounds in the membrane was usually unclear. In the present study, an anthryl group was introduced into the *N*-terminal part of the peptides as a fluorescent probe to interpret the channel-forming property of the peptide on the basis of peptide–lipid bilayer interactions. Fluorescence spectroscopy is advantageous for low concentrations of samples to be required for measurement. This point is essential for the analysis of membrane-active peptides, which generally function below micromolar concentrations. By using this method, effects of cation binding at the crown ether unit on the peptide orientation and aggregation in the lipid membrane were analyzed and correlated with the channel-forming ability of the peptides.

Results and Discussion

Binding Constant of K^+.—The electric conductivity of a KCl solution in a mixture of methanol and water (1 : 1, v/v) decreased with the addition of the peptides at various concentrations due to complexation of K^+ with the crown ether part of the peptides. The binding constants were determined from the changes of the conductivity consistently by assuming the formation of 1 : 1 complex as follows: Boc-Ala-Cr, 4.9×10^4; Boc-[Ala-Aib]$_4$-Ala-Cr, 4.8×10^4; and Boc-[Ala-Aib]$_8$-Ala-Cr, 1.1×10^5 dm^3 mol^{-1}. The binding constant of the heptadecapeptide is clearly higher than others. Since the helix content of Boc-[Ala-Aib]$_n$-OMe increased with the chain length,[14] the higher binding constant of Boc-[Ala-Aib]$_8$-Ala-Cr should be ascribed to the macrodipole produced by the long helix.[15,16] The negative pole of the helix macrodipole at the C-terminal might stabilize the complex with K^+ at this moiety. However, the stabilization energy was 0.49 kcal mol^{-1} ‡ calculated from the difference of the binding constants between Boc-[Ala-Aib]$_8$-Ala-Cr and Boc-Ala-Cr, which is relatively small compared

† *Abbreviations.* A8-Cr = Boc-Ser(CH$_2$Ant)-[Ala-Aib]$_4$-Ala-Cr; A16-Cr = Boc-Ser(CH$_2$Ant)-[Ala-Aib]$_8$-Ala-Cr; BLM = bilayer lipid membrane; CD = circular dichroism; CDI = carbonyldiimidazole; CF = 5/6-carboxyfluorescein; CH$_2$Ant = anthrylmethyl; Cr = benzo-18-crown-6 unit; DCCI = dicyclohexylcarbodiimide; DMF = dimethylformamide; DPPC = dipalmitoylphosphatidylcholine; HOBt = *N*-hydroxybenzotriazole; TFA = trifluoroacetic acid; TLC = thin layer chromatography.

‡ 1 cal = 4.18 J.

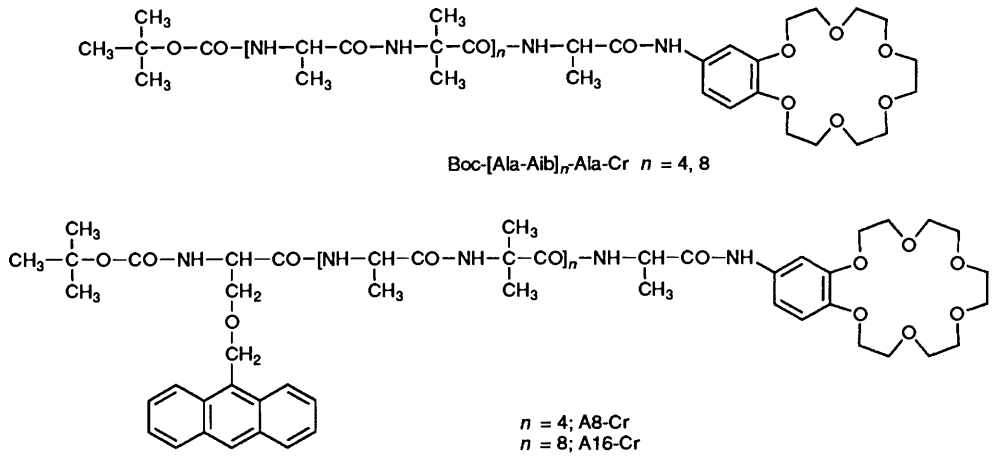

Fig. 1 Molecular structure of hydrophobic helical peptide/crown ether conjugates

with the theory.[16] Two possible reasons are considered as follows: (i) the helix content of Boc-[Ala-Aib]$_8$-Ala-Cr is about 45% in ethanol and water (1:1, v/v) [Fig. 2(*c*)], and the macrodipole should not be so large; and (ii) the high ionic concentration shields partially the electrostatic interaction.[17]

Complexation and Helix Content.—CD spectroscopy revealed that the helix content of Boc-[Ala-Aib]$_4$-Ala-Cr in an ethanol–water (1:4, v/v) mixture increased upon addition of K^+ [Fig. 2(*a*)]. Since Boc-[Ala-Aib]$_4$-OMe did not show a change in its CD spectrum upon K^+ addition, the increase of the helix content may be ascribed to complexation of Boc-[Ala-Aib]$_4$-Ala-Cr with K^+, which stabilizes the helical conformation due to interactions of the macrodipole of the helix with the cation.

The CD spectrum of Boc-[Ala-Aib]$_8$-Ala-Cr in an ethanol–water (1:4, v/v) mixture also changed upon K^+ addition [Fig. 2(*b*)]. However, CD spectra showed a strong negative Cotton effect around 226 nm, which is ascribed to an aggregated helix, so called α_{II} helix, which has been reported for rhodopsin.[18] The negative Cotton effect decreased upon K^+ addition, indicating increasing aggregation of helices. Such a change was not detected in the case of Boc-[Ala-Aib]$_8$-OMe. Complexation of the peptide with K^+ may increase the helix content, and thereby the peptide bears a primary amphiphilicity. The amphiphilic property facilitates aggregation of helix. The hydrophobic peptide chain of Boc-[Ala-Aib]$_8$-Ala-Cr is longer than that of Boc-[Ala-Aib]$_4$-Ala-Cr, and intermolecular hydrophobic interactions of the former peptide may be stronger, leading to aggregation. When K^+ was added to Boc-[Ala-Aib]$_8$-Ala-Cr in an ethanol–water (1:1, v/v) mixture, the strong negative Cotton effect was not seen around 226 nm, but a double minimum typical for α-helical conformation was seen [Fig. 2(*c*)]. The difference in the CD spectra of Boc-[Ala-Aib]$_8$-Ala-Cr in 20 and 50% aq. ethanol can be explained by dissociation of the aggregate in the latter solution due to weakened hydrophobic interactions.

Distribution to Phospholipid Membrane.—Interactions of crown ether/peptide conjugates with phospholipid bilayers were studied by fluroescence spectroscopy. Fluorescence spectra of A8-Cr [Fig. 3(*a*)] and A16-Cr [Fig. 3(*b*)] in a buffer solution changed with the addition of DPPC liposomes. Since the monomer emission at 415 nm increased in the presence of DPPC liposome, the N-terminal region of the peptides should be incorporated into the hydrophobic core of the membrane. The peptide should take a α-helical structure with intramolecular hydrogen bondings, because free amide protons and carbonyl groups are energetically unfavourable in the hydrophobic environment. On the other hand, the hydrophilic C-terminal region is considered to stay at the membrane surface.

The intensity ratio of monomer and excimer emission (I_{415}/I_{470}) was plotted against the lipid concentration in Figs. 4(*a*) and (*b*). In the case of A8-Cr, the relative intensity of the excimer increased in the presence of a small amount of lipids. Under these conditions, the peptides aggregate, because the peptides are concentrated into a small region of the membrane. With an increasing amount of the lipid, the intensity of the monomer emission increased due to dilution of the peptides in the membrane. On the other hand, excimer emission of A16-Cr prevailed in a buffer solution, and decreased with the addition of DPPC lipsome, because of a decrease in the size of the peptide aggregate upon transfer from aqueous solution to lipid membrane.

It should be noted that the relative intensity of the excimer of A16-Cr is stronger than that of A8-Cr under these conditions. I_{415}/I_{470} Reached a plateau above 0.2 mmol dm^{-3} of DPPC concentration, where the excimer emission was still significant in the fluorescence spectra, indicating aggregation of A16-Cr even in the presence of excess amounts of lipids.

Complexation with K^+ in a Lipid Membrane.—Complexation of the peptide with K^+ influenced distribution of the peptides in the lipid membrane. The distribution of the peptides in a lipid membrane was assessed by fluorescence depolarization and excimer formation of an anthryl group connected to the peptides [Figs. 5(*a*) and (*b*)]. In the case of A8-Cr, fluorescence depolarization increased and I_{415}/I_{470} decreased with the addition of K^+. The changes are ascribed to complexation of the crown ether group with K^+, because the change was not observed with the addition of Li^+. 18-Crown-6 does not form a stable complex with Li^+. The increase in excimer intensity implies promotion of aggregation upon K^+ complexation. The amphiphilic property of A8-Cr is strengthened by cation binding at the C-terminal region. It has been shown by theory as well as experiment that peptides with a primary amphiphilicity are incorporated into a lipid membrane by taking a perpendicular orientation to the lipid membrane.[19,20] It was also shown that peptides with a perpendicular orientation to a lipid membrane tend to aggregate in the lipid membrane.[10] The aggregation of A^8-Cr/K^+ complexes in lipid membranes conform with these expectations. In the aggregate of A8-Cr/K^+ complexes, anthryl groups may be aligned to allow energy migration, resulting in fluorescence depolarization as reported previously.[10]

Rb^+ and Cs^+ are known to form a sandwich-type complex with 18-crown-6.[21–23] Upon addition of these cations to lipid membranes containing A8-Cr, I_{415}/I_{470} decreased, representing

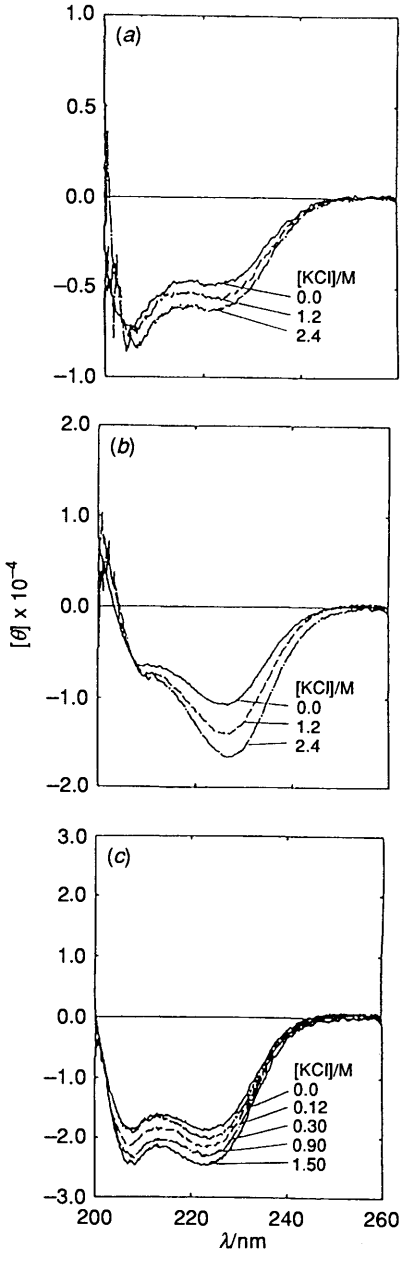

Fig. 2 Change of CD spectra with the addition of KCl of Boc-[Ala-Aib]$_4$-Ala-Cr (*a*, 1.6 × 10^{-4} mol dm^{-3}, in 20% ethanol–aq. solution) and Boc-[Ala-Aib]$_8$-Ala-Cr (*b*, 7.7 × 10^{-5} mol dm^{-3} in 20% ethanol–aq. solution; *c*, 3.8 × 10^{-5} mol dm^{-3} in 50% ethanol–aq. solution)

association of peptides in the lipid membrane due to formation of sandwich-type complexes [Fig. 5(*b*)]. However, in these cases, the fluorescence depolarization values decreased [Fig. 5(*a*)]. Aggregation of liposomes upon cation addition was not detected by UV spectroscopy. The mobility of the anthryl group may be restricted upon formation of a large sandwich-type complex. The steric effect should overwhelm the effect of energy migration on fluorescence depolarization.

On the other hand, fluorescence depolarization and the I_{415}/I_{470} of A16-Cr did not change by addition of these cations [Fig. 5(*b*)]. The distribution of A16-Cr in a lipid membrane may not be affected upon complex formation with cations, because A16-Cr aggregates in the lipid membrane in the absence of cations as shown above.

Ion-channel Formation.—Hydrophobic helical peptides, Boc-[Ala-Aib]$_n$-OMe (n = 4,8) were shown to form a voltage-

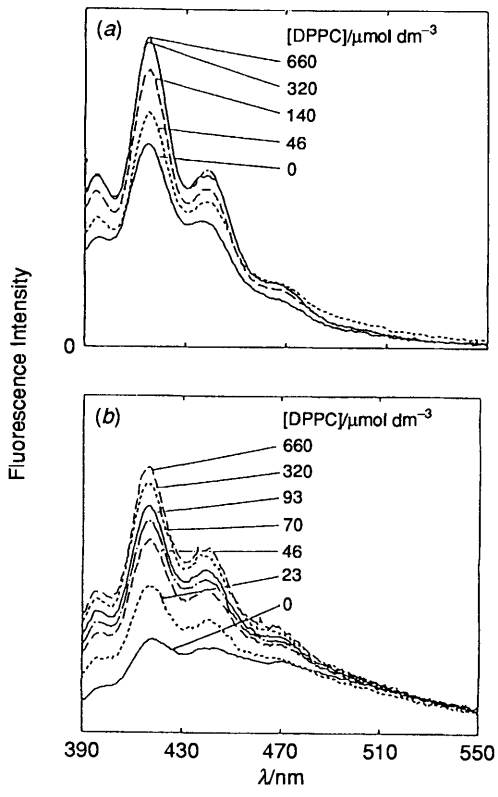

Fig. 3 Change of fluorescence spectra with the addition of DPPC liposome of (*a*) A8-Cr (1.6 × 10^{-6} mol dm^{-3}); and (*b*) A16-Cr (1.1 × 10^{-6} mol dm^{-3}); numbers in the figure represent DPPC concentration

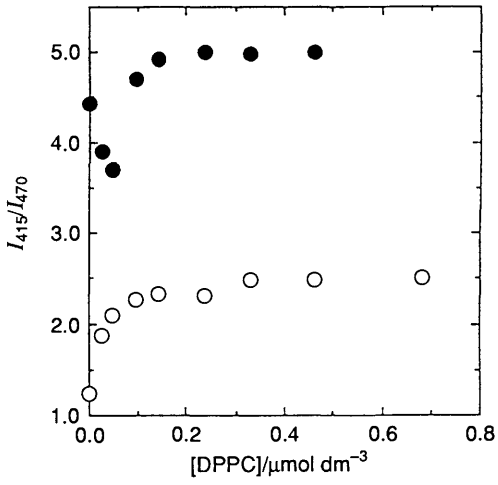

Fig. 4 Change of fluorescence intensity ratio of monomer and excimer emission (I_{415}/I_{470}) of: ●, A8-Cr; and ○, A16-Cr with the addition of DPPC liposome

dependent ion channel across a lipid membrane by forming a bundle structure.[8] The current/voltage (I/V) response across BLM in the presence of Boc-[Ala-Aib]$_4$-Ala-Cr was investigated and compared with Boc-[Ala-Aib]$_4$-OMe (Fig. 6). Boc-[Ala-Aib]$_4$-Ala-Cr was more effective in increasing the conductance than Boc-[Ala-Aib]$_4$-OMe.

When the voltage applied to the lipid membrane containing Boc-[Ala-Aib]$_4$-Ala-Cr was slightly higher than the critical voltage, above which current increases drastically, stepwise conductance changes were observed (Fig. 7). These phenomena represent channel opening and closing, and are characteristic of single channel formation.[7] Since the current changed over a relatively wide range, the peptide formed numerous ion channels, which have highly correlated openings. When the

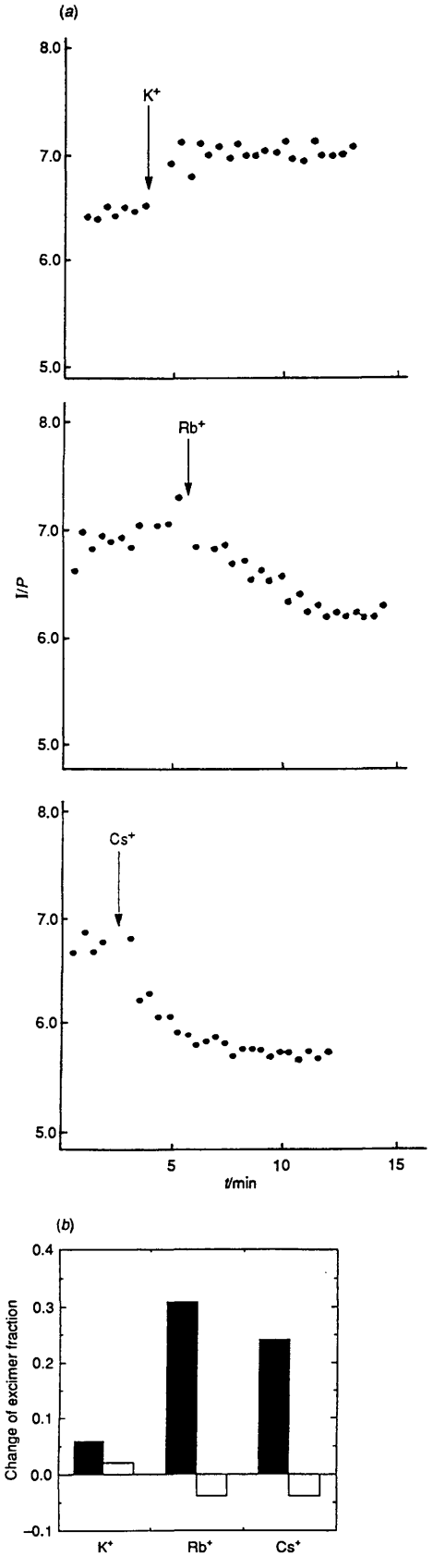

Fig. 5 Effects of addition of K^+, Rb^+ or Cs^+ on: (*a*) fluorescence depolarization ($1/P$, cation addition is indicated by an arrow in the figure) of A8-Cr; and (*b*); I_{415}/I_{470} of ■, A8-Cr; and □, A16-Cr. The ordinate in (*b*) represents $[(I_{415}/I_{470})_0 - (I_{415}/I_{470})_c]/(I_{415}/I_{470})_0$, where $(I_{415}/I_{470})_0$ and $(I_{415}/I_{470})_c$ mean I_{415}/I_{470} in the absence and presence of cation, respectively, [A8-Cr] = 2.9×10^{-6}; [DPCC] = 3.6×10^{-4}; [cation added] = 7.1×10^{-2} mol dm^{-3}.

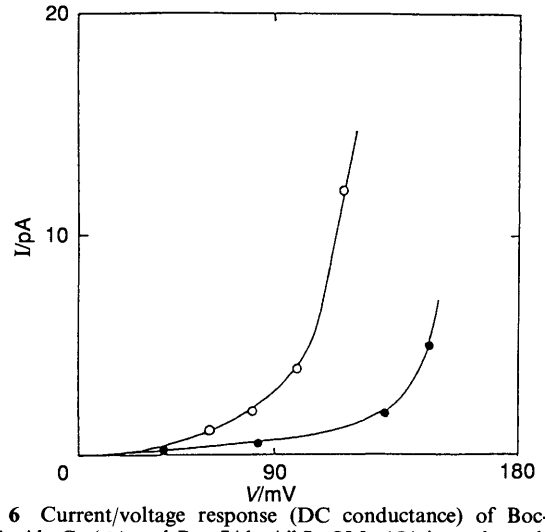

Fig. 6 Current/voltage response (DC conductance) of Boc-[Ala-Aib]$_4$-Ala-Cr (○) and Boc-[Ala-Aib]$_4$-OMe (●) in a planar bilayer membrane. The diameter of the hole in the Teflon sheet was 0.15 mm. The peptide concentration was 3×10^{-6} g cm^{-1}, [KCl] = 1 mol dm^{-3}.

added salt was changed from KCl to LiCl, the current fluctuations were not observed. These results support the fact that the crown ether group of Boc-[Ala-Aib]$_4$-Ala-Cr facilitates the formation of voltage-dependent ion channels and acts as an ion-binding site of the ion channel. It is notable that there are current fluctuations due to the formation of a single ion channel at an applied voltage as low as 20 mV in the case of BLM containing Boc-[Ala-Aib]$_8$-Ala-Cr (Fig. 8).

The effect of cation species on the channel formation of Boc-[Ala-Aib]$_4$-Ala-Cr was investigated. The peptide showed current fluctuations due to the formation of a single ion channel in the presence of Cs^+ at 140 mV, but did not in the presence of K^+ under the same conditions. Since the peptide tends to aggregate upon formation of sandwich-type complexes with Cs^+, a bundle structure of helices is formed in the lipid membrane, acting as an ion channel. On the other hand, Boc-[Ala-Aib]$_8$-Ala-Cr showed a higher potential for channel formation on complexation with K^+ than Cs^+. Since the aggregation of the peptide is not influenced by the nature of the cation, the higher the binding constant with the cation ($K^+ > Cs^+$), the higher the channel conductance.

The channel-forming potency of hydrophobic helical peptides was increased by connection of a crown ether unit to the C-terminal region of the peptide. The crown ether unit should have a variety of important roles to play in the formation and activity of an ion channel: (i) easy access of cations to the channel by ion binding; (ii) increasing primary amphiphilicity of the peptide by cation binding at the C-terminal region, resulting in transmembrane orientation of the peptide; and (iii) promotion of peptide aggregation for the ion channel by forming a sandwich-type complex with Cs^+. However, the precise control of the aggregate size in a lipid membrane remains to be solved for construction of an efficient and ion-specific ion channel.

Experimental

Materials.—DPPC (Sigma) and CF (Sigma) were used without further purification. Azolectin (Associated Concentrates) was purified by the method reported by Kagawa *et al.*[24]

Peptide Synthesis.—General procedure. The peptides were synthesized by a conventional liquid-phase method. The final products as well as intermediate peptides were identified by ^{1}H NMR, and the purity was checked by TLC. Analytical TLC

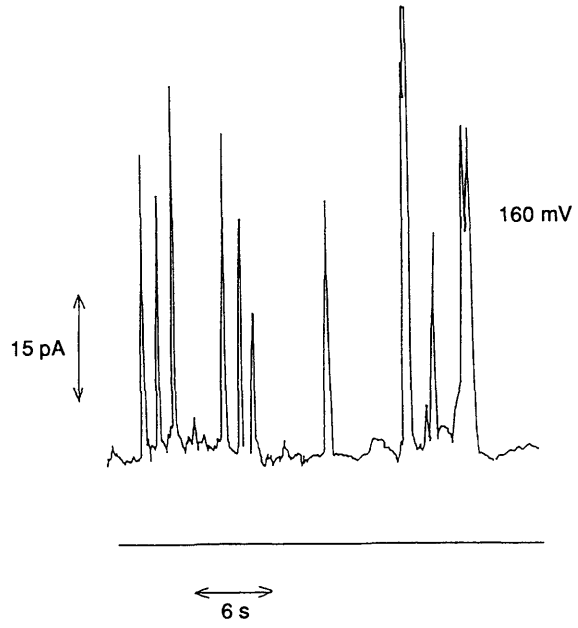

Fig. 7 Current fluctuations at constant applied voltage, 160 mV, in the presence of Boc-[Ala-Aib]$_4$-Ala-Cr (2.0×10^{-6} g cm^{-3}). The diameter of the hole in the Teflon sheet was 0.3 mm. [KCl] = 1 mol dm^{-3}. The fluctuations overlapped on a large base current. The data cannot be directly compared with those of Fig. 6, because of different properties of the membrane.

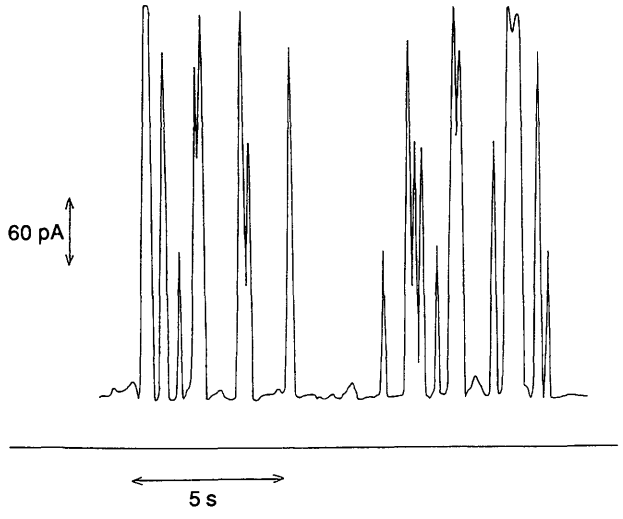

Fig. 8 Current fluctuations at constant applied voltage, 20 mV, in the presence of Boc-[Ala-Aib]$_8$-Ala-Cr (0.5×10^{-6} g cm^{-3}). The diameter of the hole in Teflon sheet was 0.3 mm. [KCl] = 1 mol dm^{-3}. The fluctuations overlapped on a large base current. The data cannot be directly compared with those of Fig. 6, because of different properties of the membrane.

was performed on Merck silica gel 60 F^{254} aluminium plates, with detection by UV light and/or the ninhydrin test. The solvent systems of the TLC were (I): butanol–acetic acid–water–pyridine 15:3:12:9 (v/v/v/v); and (II): ethyl acetate–pyridine–formic acid–water 63:21:10:6 (v/v/v/v). Boc-amino acid derivatives and coupling reagents, CDI, DCCI and HOBt, were purchased from the Kokusan Chemical Works, and Peptide Institute, respectively. Extracts were evaporated under reduced pressure below 40 °C. Purification by chromatography was performed on a LH-20 column (Pharmacia) using methanol as eluent and/or a Jasco-880 HPLC system with a Cosmosil 5C18P column using 5% water–95% methanol as eluent. Analytical HPLC was performed with a Cosmosil 10C18P using water–methanol 5:95 (v/v) as eluent unless specified. The flow rate was 1 cm^3 min^{-1}. Boc-[Ala-Aib]$_4$-OMe and Boc-Ser(CH_2Ant) were synthesized by the method reported previously.[10]

Boc-Ala-Cr. Boc-Ala-Cr. Boc-Ala (318 mg) was activated by CDI (312 mg) in a DMF solution at 0 °C for 1 h, and aminobenzo-18-crown-6 (422 mg, prepared by the method reported previously[25]) was added. The solution was stirred for 8 h at 0 °C and 48 h at room temp., and evaporated. The residue was dissolved in chloroform, and washed successively with 10% citric acid, 4% aq. $NaHCO_3$, and water. The product was purified on an LH-20 column and a reverse-phase column (5C18P). Yield, 150 mg. Analytical HPLC, 3.00 cm^3.

Boc-[Ala-Aib]$_4$-Ala-Cr. Boc-[Ala-Aib]$_4$-OH (150 mg, pre-prepared by alkaline hydrolysis of Boc-[Ala-Aib]$_4$-OMe) was coupled with H-Ala-Cr HCl (90 mg, prepared by HCl–dioxane treatment of Boc-Ala-Cr) in the presence of triethylamine (34 mm^3)* by using DCCI (54 mg) and HOBt (41 mg) as coupling reagents. The product was purified on an LH-20 column using methanol as eluent. Yield, 90 mg, R_f(I) 0.78, R_f(II) 0.90.

A8-Cr. To a DMF solution of Boc-Ser(CH_2Ant) (20 mg) and H-[Ala-Aib]-Ala-Cr HCl (35 mg, prepared by $CHCl_3$–dioxane treatment of Boc-[Ala-Aib]$_4$-Ala-Cr) were added HOBt (11 mg), triethylamine (7.1 mm^3), and DCCI (12 mg) at 0 °C. The solution was stirred for 3 h at 0 °C and 24 h at room temp. The product was purified on an LH-20 column using methanol as eluent and a reverse-phase HPLC column using methanol–water–acetic acid (95:5:1, v/v/v) as eluent. Yield, 32 mg. R_f(I) 0.81, R_f(II) 0.92. Analytical HPLC, 3.20 cm^3.

Boc-[Ala-Aib]$_8$-Ala-Cr. Boc-[Ala-Aib]$_4$-Ala-Cr (40 mg) was dissolved in DMF (20 mm^3), and HCl (4 mol dm^{-3}) dioxane (0.2 cm^3) was added. After storage for 2 h, the solution was concentrated and dried under reduced pressure. The peptide was dissolved in a DMF solution of Boc-[Ala-Aib]$_4$-OH (30 mg), HOBt (11 mg), and DCCI (12 mg), and triethylamine (6.5 mm^3) was added. After being stirred for 2 days, the product was purified on a LH-20 column. Yield, 43 mg. R_f(I) 0.81, R_f(II) 0.94.

A16-Cr. Boc-[Ala-Aib]$_8$-Ala-Cr (20 mg) was dissolved in TFA. After 1 h at room temp., the solution was evaporated and dried under reduced pressure. TFA-H-(Ala-Aib)-Ala-Cr, Boc-Ser(CH_2Ant) (9 mg), HOBt (3 mg), and DCCI (3.5 mg) were dissolved in a DMF solution, and triethylamine (2.4 mm^3) was added. After being stirred for 2 days, the product was purified on a LH-20 column by semi-preparative HPLC using methanol–water–acetic acid, 95:5:1 (v/v/v). Yield, 12 mg. R_f(I) 0.84, R_f(II) 0.96. Analytical HPLC, 4.73 cm^3.

Preparation of Liposome.—Small unilamellar vesicles were prepared by sonication of DMPC or DPPC dispersion in a buffer solution (5 mmol dm^{-3} Hepes, 50 mmol dm^{-3} NaCl, 0.05 mmol dm^3 EDTA, pH 7.4) and ultracentrifugation at 100 000 g. Lipid concentration was determined by a colorimetric method using phospholipase D (Diacolor, Toyobo).

Measurements.—CD and fluorescence spectra were measured on a JASCO J-600 and a Hitachi MPF-4-fluorophotometer, respectively. Steady-state fluorescence depolarization was measured by equipment installed on the fluorophotometer reported previously.[26]

Binding Constant.—Binding constants of Boc-Ala-Cr and Boc-[Ala-Aib]$_n$-Ala-Cr (n = 4,8) with K^+ were determined by the conductometric method. The change of conductivity of a methanol–water (1:1, v/v) solution of KCl (3×10^{-5} mol dm^{-3}) on addition of a methanol–water (1:1, v/v) solution of the peptide with various concentrations was

* 1 mm^3 = 1 μl

determined by using a Wayne Kerr B-224 universal bridge at 25 °C.

CF Leakage.—CF-encapsulated DPPC vesicles were prepared by the method reported by Barbet *et al.*[27] The excitation and monitor wavelengths of CF were 470 and 515 nm, respectively. A complete release of CF from DPPC vesicles was determined by disruption of vesicles with Triton X-100.

Bilayer Experiments.—A thin Teflon film (0.25 mm thickness) with an aperture of 0.15 or 0.3 mm diameter was clamped between two halves of a Teflon trough. The hole was precoated with hexadecane–hexane (6:4, v/v). The azolectin membrane was formed by the method reported by Montal *et al.*[28] The electrolyte solution was 1 mol dm^{-3} KCl, CsCl, or LiCl unbuffered. Before measurements, an electric potential of AC 200 mV (peak-to-peak, 1 kHz) was applied for 30 min across the membrane for aging, which ensured virtually solvent-free planar bilayer membranes formed on an aperture of 0.3 mm diameter.[7] However, the membranes were not stable enough to examine the current/voltage dependence for a wide range of applied voltages. Therefore, we prepared membranes on an aperture of 0.15 mm diameter which, although stable against the applied voltage, were not completely solvent-free.[7] The Ag/AgCl electrodes were connected to the troughs by agar-salt bridges. The peptides were added to both aqueous phases after the AC treatment of the membrane. A dry battery was used for applying the voltage across the membrane. Polarization effects at either the electrode/solution or membrane/solution interfaces were not significant.

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