Ion-channels of cyclic template-assembled alamethicins that emulate the pore structure predicted by the barrel-stave model

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Single channel measurements of cyclic template-assembled synthetic peptides (TASP) AL$_n$-cyclo2n [AL = Ac-UPUAUAQUVUGLUPVUUG-, U = α-aminoisobutyric acid; cyclo2n = *cyclo*(-Lys-Abz-)$_n$, Abz = *m*-aminobenzoic acid] in lipid bilayer membranes reveal that the lowest and the next lowest conductance states of the alamethicin ion-channel are made up of three and four parallel helices, respectively, as predicted by the barrel-stave model.

Alamethicin is a 20-mer antibiotic peptide (Ac-UPUAUAQ-UVUGLUPVUUEQ-Pheol) isolated from a fungus, *Trichoderma viride*, which has a phenylalaninol (Pheol) at the C-terminus and eight α-aminoisobutyric acids (U).[1] It has an amphipathic helix structure and forms voltage-dependent ion channels with various conductance states in lipid bilayer membranes.[2–5] For the pore structure of the alamethicin channels, the barrel-stave model has been generally accepted.[4–5] According to this model, parallel amphipathic helices aggregate so as to direct their polar faces towards the central pore, through which ions flow. The pore size would vary depending on the number of helices in the aggregates. However, the channel structure has never been proved and is still controversial.[6] Further, the number of helices in the aggregates has not been directly determined for each conductance state, although a simple macroscopic approximation to channel structure provides rough estimates for the higher conductance states.[5]

In order to confirm the validity of the barrel-stave model, we have synthesized three template-assembled alamethicins in which three to five 18-mer alamethicin fragments (AL = Ac-UPUAUAQUVUGLUPVUUG-) were arranged to mimic the barrel-stave structure (Fig. 1). The synthetic proteins are named AL$_n$-cyclo2n, where n is the number of ALs. The templates used are cyclic pseudopeptides in which three to five units including a lysine and a *m*-aminobenzoic acid[7] are integrated into a cyclic structure. The corresponding linear pseudopeptides with protection at ε-amino group of lysine were successfully cyclized in DMF by the aid of (benzotriazolyloxy)tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) in 53 to 78% yields. The deprotected cyclic peptides gave signals at *m*/*z* 742 [M + H$^+$], 990 [M + H$^+$] and 1237 [M + H$^+$], respectively, on FAB MS analyses. The AL was prepared by solution phase method according to Nagaraj *et al.*[8] and gave *m*/*z* 1653 [M + Na$^+$] by FAB MS. The C-terminal ends of ALs were attached to the ε-amino groups of the lysines in the cyclic templates with BOP reagent. AL$_n$-cyclo2n were purified with Sephadex LH-60 column (DMF). They showed no remaining amino groups upon Kaiser test[9] and reasonable retention times, 10.56, 10.25 and 10.08 min, respectively, upon size exclusion chromatography with TOSOH, TSK-GEL G3000HxL (DMF). The CD spectra of AL$_n$-cyclo2n in methanol were similar to that of alamethicin, indicating that the assembled ALs also have a helix structure like alamethicin (Fig. 2).

Lipid bilayer membranes were formed from diphytanoyl-sn-glycero-3-phosphocholine in 1 mol dm^{-3} KCl by the monolayer folding technique.[10] After alamethicin and AL$_n$-cyclo2n were added to one side of the membrane, current fluctuations were measured by applying various voltages at room temperature. As shown in Fig. 3, significant differences were found between alamethicin and AL$_n$-cyclo2n. Alamethicin induced ion-channels with multiple conductance states such as the first state (level 0) of 0.07 nS, the second state (level 1) of 0.5 nS and the third state (level 2) of 1.6 nS at 250 mV.[3–5] However, AL$_3$-cyclo6 showed ion-channels with a single-conductance state. The conductance state of 0.11 nS at 280 mV corresponds to the level 0 of alamethicin channel. AL$_4$-cyclo8 again showed a single conductance state (0.25 nS at 130 mV) but corresponding to the level 1 of alamethicin channel. AL$_5$-cyclo10 formed ion-channels with more variable conductance states.

Another difference can be observed in the lifetime of channels in Fig. 3(*a*–*d*). The opening of the AL$_n$-cyclo2n channels occasionally lasted more than one second whereas the

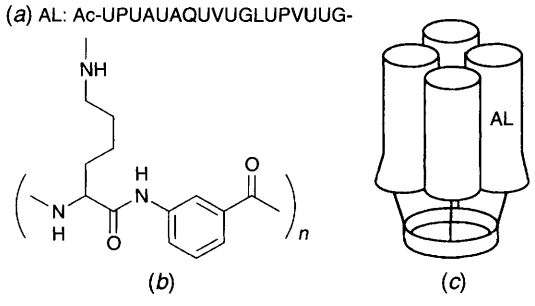

Fig. 1 (*a*) Modified alamethicin fragment (AL) in which U denotes α-aminoisobutyric acid; (*b*) a repeating unit forming cyclic pseudopeptides (cyclo2n); (*c*) illustration of supposed structure of AL$_4$-cyclo8 in which four ALs are C-terminally attached to lysine residues of a cyclic pseudooctapeptide

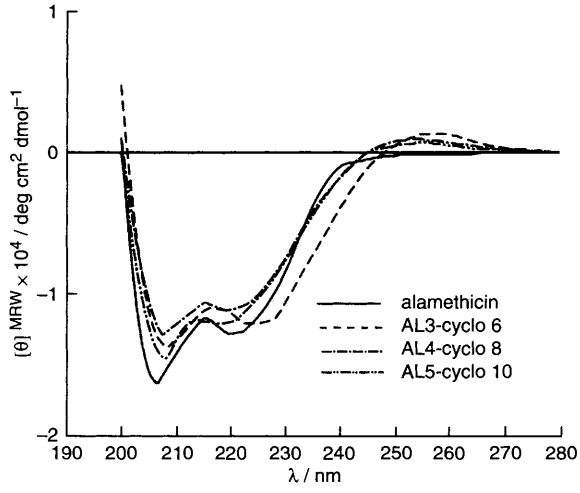

Fig. 2 CD spectra of alamethicin and AL$_n$-cyclo2n in methanol at 30 mmol dm^{-3}

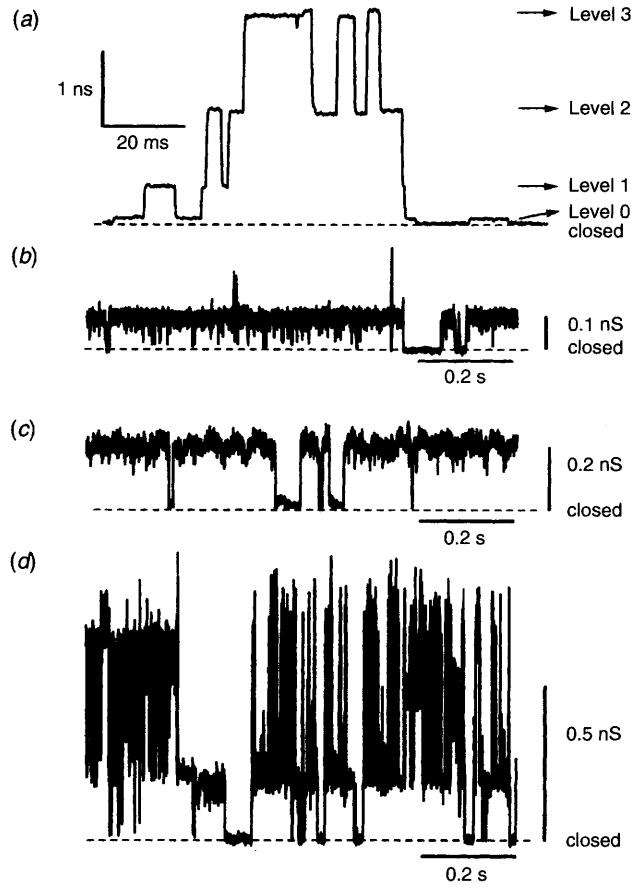

Fig. 3 Single-channel recordings for (*a*) 0.25 mg dm^{-3} alamethicin at 250 mV; (*b*) 2.5 ng dm^{-3} AL_3-cyclo6 at 280 mV; (*c*) 7.5 ng dm^{-3} AL_4-cyclo8 at 130 mV and (*d*) 20 ng dm^{-3} AL_5-cyclo10 at 150 mV

open lifetime of the alamethicin channels was in the millisecond range. The prolonged open lifetime of the AL_n-cyclo2*n* channels indicates that the attachment of peptides to the templates stabilizes the channel structure as pointed out for some template-assembled synthetic proteins.[11] Recently, You *et al.* reported that alamethicin covalent dimers form six-alamethicin molecule channels with prolonged lifetime.[12]

According to the single-channel recordings for AL_n-cyclo2*n* as mentioned before, we plotted the channel conductances for AL_n-cyclo2*n* against the applied voltages (Fig. 4). The plots for AL_3-cyclo6 and AL_4-cyclo8 were in fair agreement with those of the levels 0 and 1 for the alamethicin, respectively (Fig. 3). This finding reversely suggests that the alamethicin channels of the levels 0 and 1 are made of up three and four helical molecules, respectively. In the case of AL_5-cyclo10, however, the conductance values scattered between those of levels 0 and 2 for alamethicin. This result implies that the AL_5-cyclo10

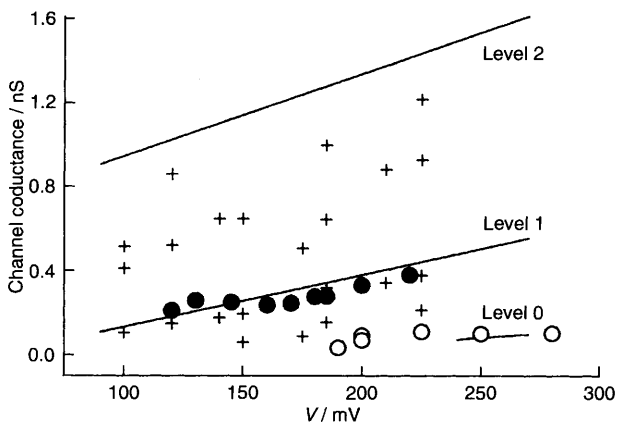

Fig. 4 Single channel conductances of AL_3-cyclo6 (○), AL_4-cyclo8 (●) and AL_5-cyclo10 (+) as a function of applied voltage. The channel conductances of the levels 0, 1 and 2 of alamethicin, which increased linearly with applied voltage, were represented by the three solid lines.

channels have various conductance states that could be attributed to the conformational flexibility of AL_5-cyclo10 whose large cyclic template is less rigid than the templates of AL_3-cyclo8 and AL_4-cyclo10.

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