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Biochimica et Biophysica Acta 1234 (1995) 133–138

BBA Biochimica et Biophysica Acta

Rapid Report

C-terminal amino groups facilitate membrane incorporation of gramicidin derivatives

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Received 15 November 1994; accepted 6 December 1994

Abstract

Gramicidin derivatives with (positively-charged) C-terminal amino groups are found to incorporate readily and refold quickly when added to dioleoylphosphatidylcholine lipid vesicles from concentrated methanol solutions. Neutral and negatively-charged derivatives do not.

Keywords: Gramicidin; Peptide; Lipid; Membrane; Protein folding

The interaction of gramicidin with lipids has been extensively studied as a model for understanding peptide–membrane interactions [1–3]. A central remaining question in this area is the mechanism whereby a membrane environment can induce refolding of gramicidin and stabilization of a particular three-dimensional form of the peptide [2,4,5]. The process has direct analogies to membrane protein folding in vivo [6], and may help to elucidate molecular determinants of the in vivo process.

In organic solvents, gramicidin dimerizes in a variety of intertwined double-helical conformations [7]. These have been characterized by a range of physical techniques (e.g. [4,5,8,9]). In membranes, gramicidin adopts a single predominant structure: the $\beta^{6.3}$ single helix (which can dimerize end to end). This structure has also been well characterized by a variety of physical techniques [5,10]. What remains less clear is the mechanism by which a double-helix becomes two single helices when the peptide is added to membranes from a concentrated organic solution.

Under most conditions, the rate at which a gramicidin double-helix becomes a single helix is slow (> 6 h, 60°C) [11,12]. Often, elevated temperatures and sonication are required to drive the transition to completion [2,12–14]. The rate of the transition depends also on the 'solvent history' of the gramicidin sample [2,15]. It is well known that the nature of the solvent influences the relative proportions of dimers and monomers [7,16–19]. Polar solvents (particularly trifluoroethanol), in which gramicidin is predominantly monomeric, lead to a more rapid adoption of the $\beta^{6.3}$ form than more non-polar solvents (chloroform or tetrahydrofuran). When gramicidin is added to membranes from very dilute solutions (as is done for single channel measurements of gramicidin channels), no solvent history dependence is observed [20], again consistent with a more rapid folding of monomeric peptide to the $\beta^{6.3}$ form.

A favourable interaction of tryptophan residues with the membrane interfacial region appears to provide some of the driving force for the double-helix to single-helix transition [4]. To what extent this interaction plays a role in the transition itself is unclear. Cross and colleagues [4] have proposed a mechanism whereby an antiparallel left-handed double-helical species (species 3, in the nomenclature of Veatch and Blout [7,8]) could unwind to form two single $\beta^{6.3}$ helices in a membrane. The mechanism involves a zipper-like unwinding of the helix (as had been proposed previously [21–23]) with concomitant flipping of peptide planes so that the helix-sense changes from left-handed to right-handed as each peptide unit unwinds. Refolding of this sort might be expected to be slow since many H-bonds must be broken in the process.

In the course of studies aimed at designing gramicidin channels with novel properties, we have synthesized a series of gramicidin derivatives with C-terminal amino groups. We noticed that when these peptides were added to preformed membrane vesicles, even from very concen-

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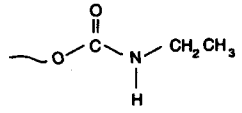
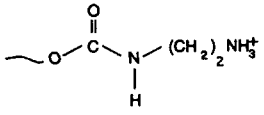
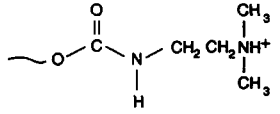
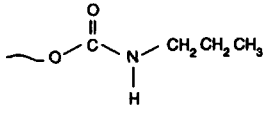
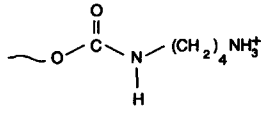
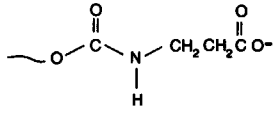
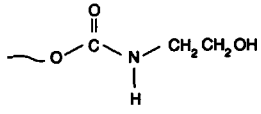
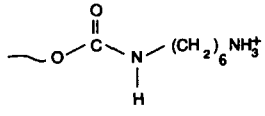
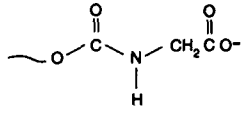
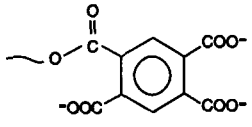
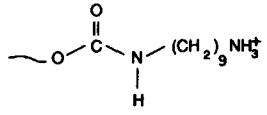
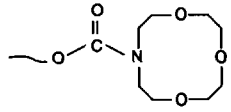
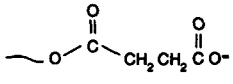
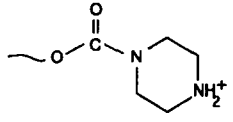
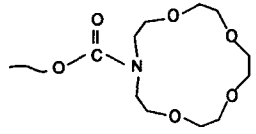
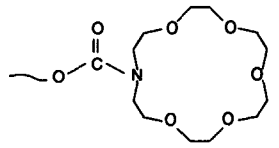
SSDI 0005-2736(94)00307-6

Table 1
Structures of gramicidin and C-terminal derivatives

'g' = HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-
L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-$CONHCH_2CH_2$ ---(OH)

g-ethylamine		g-AC-2		g-NN-dimethyl	
g-propylamine		g-AC-4		g-β-alanine	
g-ethanolamine		g-AC-6		g-glycine	
g-PM		g-AC-9		g-12-crown-4	
g-succinate		g-piperazine		g-15-crown-5	
				g-18-crown-6	

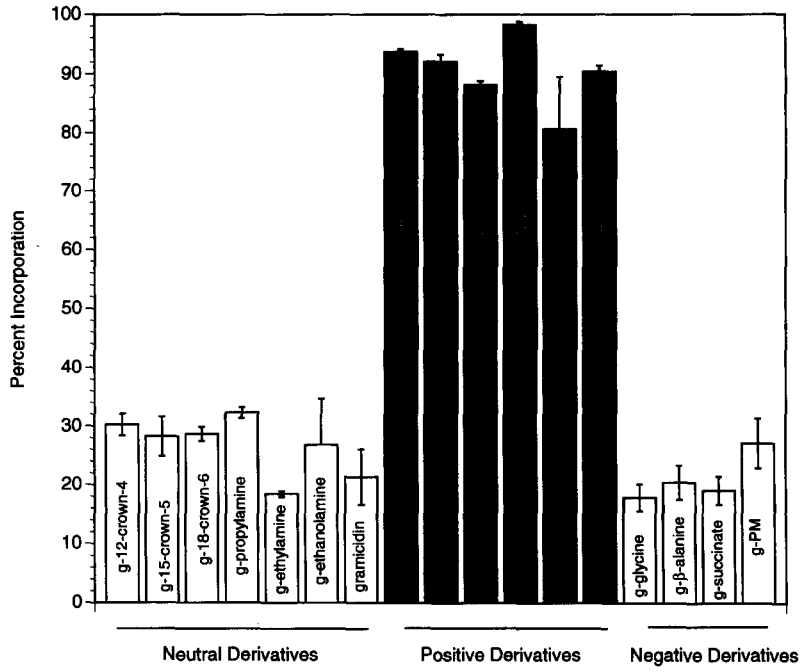


Fig. 1. Membrane incorporation of gramicidin and derivatives. Solutions (5 mM) of gramicidin and derivatives in methanol were added to sonicated DOPC vesicles as described in the text. Bars represent the mean (with standard deviation) of three separate assays. If lipid was omitted, all the positive derivatives then gave incorporation values less than 10% (not shown).

trated solutions in methanol, incorporation appeared to be instantaneous. In stark contrast with gramicidin itself under these conditions, no sonication or heating was required. We report here a systematic study which quantifies this effect. The finding may provide clues to general mechanisms of peptide folding in membranes.

Materials and methods. Gramicidin D (85% gramicidin A), *p*-nitrophenyl chloroformate and Sephadex LH-20 were obtained from Sigma (St. Louis, MO). All other reagents and solvents were obtained from Aldrich (Milwaukee, WI) and were of the highest grade available. Dioleoylphosphatidylcholine (DOPC) was obtained from Avanti Polar Lipids (Alabaster, AL).

Synthesis of gramicidin derivatives. Structures of the gramicidin ('g') derivatives studied are shown in Table 1. The wavy line connected to an oxygen atom represents the peptide connected to the oxygen of ethanolamine. The derivatives are of two types: carbamate-linked and ester-linked extensions to the C-terminal end of the peptide. A general procedure for the synthesis of each type is described below.

(i) Carbamate-linked derivatives. Gramicidin-ethylenediamine (g-AC-2) (for example) was synthesized as follows: Commercial gramicidin D (20 μmol) was esterified (1 h, 4°C) with *p*-nitrophenyl chloroformate (200 μmol) in dry tetrahydrofuran (2 ml) containing triethylamine (TEA) (100 μl). The resulting carbonate ester was either separated by gel filtration (LH-20 in methanol) or filtered through celite into a 100-fold excess of ethylenediamine in 2 ml dimethylformamide. The product was then separated by gel-filtration using LH-20 in methanol and then, if necessary, by passage through a short silica gel column (Merck 60 Å, 230–400 mesh, (C/M/W, 65:25:4)). Other derivatives were made by replacing ethylenediamine with the appropriate amine, diamine or amino acid. All derivatives were characterized by UV, TLC, and FAB-mass spectrometry. Besides the predominant component, Val^1-gramicidin A [10], signals for Ile^1-gA were also observed by FAB-MS. TLC (C/M/W, 65:25:4): gramicidin: R_f = 0.73; g-AC-2: R_f = 0.47, FAB-MS (MH^+ 1969); g-AC-4: R_f = 0.42, FAB-MS (MH^+ 1998); g-AC-6: R_f = 0.44, FAB-MS (MH^+ 2025); g-AC-9: R_f = 0.48, FAB-MS (MH^+ 2068); g-piperazine: R_f = 0.63, FAB-MS (MH^+ 1995); g-*N*,*N*-dimethyl: R_f = 0.52, FAB-MS (MH^+ 1996); g-propylamine: R_f = 0.77, FAB-MS (M^+ 1968, MNa^+ 1991); g-ethylamine: R_f = 0.76, FAB-MS (MNa^+ 1976); g-ethanolamine: R_f = 0.72, FAB-MS (MH^+ 1970, MNa^+ 1992); g-12-crown-4: R_f = 0.78, FAB-MS (MNa^+ 2106); g-15-crown-5: R_f = 0.78, FAB-MS (MNa^+ 2150); g-18-crown-6: R_f = 0.81, FAB-MS (MNa^+ 2195); g-glycine: R_f = 0.32, FAB-MS (MNa^+ 2006); g-β-alanine: R_f = 0.43, FAB-MS (MH^+ 1998, MNa^+ 2020). All derivatives with free amino groups gave a positive reaction with ninhydrin on TLC plates.

(ii) Ester-linked derivatives. These were prepared by reaction of gramicidin D with appropriate anhydrides as described previously [24]. Gramicidin D (20 μmol), dissolved in 1 ml dimethylformamide containing 200 μl TEA and 4-dimethylaminopyridine (100 μmol) as catalyst, was treated with a 10-fold excess (200 μmol) of pyromellitic anhydride or succinic anhydride and allowed to react at room temperature overnight. After hydrolysis of the excess

anhydride, purification was carried out as with the carbamate-linked derivatives. All derivatives were characterized by UV, TLC, and FAB-mass spectrometry. TLC (C/M/W, 65:25:4): gramicidin: $R_f = 0.73$, g-succinate: $R_f = 0.54$, FAB-MS (MH^+ 1983); g-pyromellitate (g-PM): $R_f = 0.23$, FAB-MS: (MNa^+ 2141).

Incorporation assay. A suspension of dioleoylphosphatidylcholine (DOPC) sonicated unilamellar vesicles (SUV's) (prepared as described [25]) (0.5 ml, 10 mg/ml DOPC, 5 mM phosphate buffer, pH 6.8) was placed in a 3.5 ml Pyrex centrifuge tube. Solutions of peptide derivatives in methanol (5 mM (calculated using $\epsilon_m = 22\,600$) were prepared and stored at room temperature for > 2 days to allow conformational equilibration [7]. A 50 μl volume of each solution was pipetted carefully to the centre of the lipid sample. The tube was covered and allowed to stand for 5 min before centrifuging for 10 min at top speed on a Model CL International Clinical Centrifuge. After centrifugation, 100 μl of supernatant was removed and added to 6 ml MeOH. Absorbance at 280 nm was then measured in a 1 cm cell. All assays were done in triplicate.

Circular dichroism (CD) spectroscopy. CD spectra of the peptides in methanol and incorporated into vesicles (the supernatants of the incorporation assays) were recorded using a Jasco J-700A spectropolarimeter using quartz cells of either 0.01 cm or 0.001 cm pathlength. Concentrations of the peptides were based on the absorbance measured at 280 nm ($\epsilon_m = 22\,600$ assumed). Reported spectra are smoothed averages of 2–7 separate scans. The spectropolarimeter was calibrated using (+)-10-camphorsulfonic acid and all measurements were made at room temperature (22 ± 2°C).

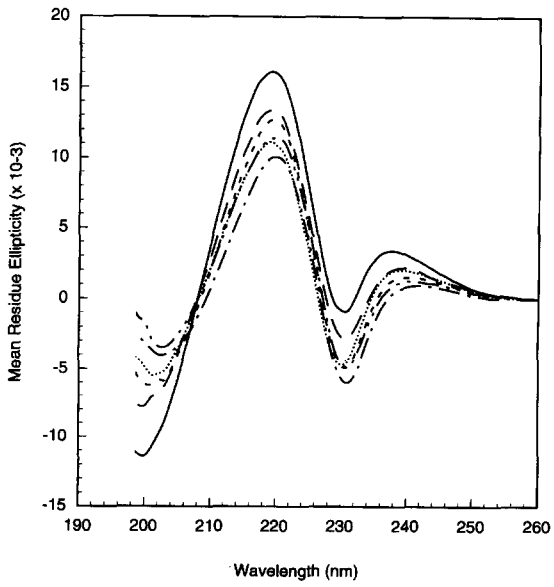


Fig. 2. CD spectra of C-terminal amino derivatives incorporated into DOPC vesicles. g-AC-2 (— · · —); g-AC-4 (— —); g-AC-6 (- - -); g-AC-9 (— · —); g-piperazine (· · · · · · ·); g-*N*,*N*-dimethyl (—).

Results and discussion. When gramicidin derivatives were added to vesicles from a concentrated solution (5 mM) in methanol one of two outcomes was observed. Either a white precipitate formed immediately or the solution remained clear. This effect was quantified by measuring the absorbance at 280 nm (where the Trp residues of gramicidin absorb) of the supernatant obtained after centrifugation of the samples (Fig. 1). The peptides fell into two distinct groups. All those peptides bearing C-terminal amino groups (which would be positively-charged in aqueous phosphate buffer, pH 6.8) incorporated readily and completely. Gramicidin itself, neutral derivatives and negatively-charged (carboxyl-bearing) derivatives all precipitated. Many of the latter could subsequently be incorporated by sonicating and heating the samples. Variations in the precise structure of the C-terminal extension (Table 1) did not seem to play a role in the observed effect. Derivatives with primary, secondary and tertiary amino groups, and from two to nine methylene groups all incorporated readily.

The observed difference was not simply due to a greater aqueous solubility of positively-charged derivatives; if no lipid was present, all the positively-charged derivatives precipitated immediately.

We then tested whether those peptides which had incorporated had adopted the normal $\beta^{6.3}$ channel form of gramicidin in membranes. This form displays a circular dichroism (CD) spectrum characterized by a maximum near 220 nm and a minimum near 230 nm [5]. All the incorporated peptides displayed this spectrum with minor differences in absolute ellipticity values (Fig. 2). A timed experiment with gramicidin-ethylenediamine (g-AC-2) (Table 1) demonstrated that the $\beta^{6.3}$ CD spectrum was obtained as quickly as a scan could be completed after addition of the peptide to vesicles (~ 3 min).

We then wished to determine if some systematic difference were present in the conformational properties of these classes of derivatives in methanol. If the C-terminal amino derivatives were uniformly monomeric in methanol, for example, this might account for their relative ease of incorporation [14]. CD spectroscopy is again informative in this regard. The CD spectrum is sensitive to the structures of all the conformations present and their relative proportions. At peptide concentrations of 5 mM (the concentration added to vesicles), two types of spectra are seen (Fig. 3). The spectra with stronger ellipticity are typical of the family of gramicidin dimeric helices [5,7,8]. The spectra with weaker ellipticity correspond to a mixture of double-helices and gramicidin monomers. Both types of spectra are distinct from those observed for gramicidin in trifluoroethanol, where the peptide is almost completely monomeric [5]. Furthermore, there is no correlation between the spectrum type and the presence or absence of a C-terminal amino group (Fig. 3). Derivatives with C-terminal amino groups thus appear to form a mixture of double-helical dimers in solution, as does gramicidin itself

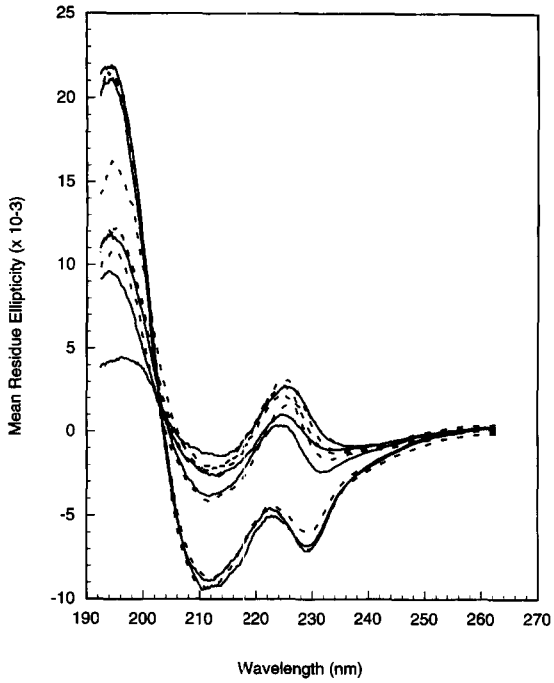


Fig. 3. CD spectra of gramicidin derivatives at 5 mM in methanol. Both C-terminal amino derivatives (—) and neutral and carboxyl derivatives (- - - -), show evidence of dimer formation. The nature of the C-terminal extension does not correlate with the shape of the spectrum. Derivatives giving pronounced negative ellipticity at 212 nm: g-propylamine, g-15-crown-5, g-AC-6, g-AC-9. Derivatives with less pronounced negative ellipticity: g-12-crown-4, g-18-crown-6, g-PM, g-ethanolamine, g-AC-4, g-ethylamine, g-piperazine, g-propylamine, g-AC-2.

and neutral and negatively-charged derivatives. Therefore, unless there are differences in the nature of the conformational ensemble of amino-derivatives which are not evident from the CD analysis, we are forced to conclude that the influence of the C-terminal amino group is on the refolding process itself rather than on the conformation of the derivatives in organic solution.

The molecular mechanism of the observed effect is, at present, unknown. The polarity of the amino groups, alone, cannot be responsible since even triply-charged negative derivatives do not incorporate easily. Although the vesicles have no net charge, it is possible that the amino groups interact with lipid phosphate groups (more strongly than carboxylates interact with choline groups) and provide some sort of anchor for the C-terminal end of the peptides during the refolding process. Alternatively, the amino groups may facilitate the flipping of peptide planes proposed by Zhang et al. [4] or the making and/or breaking of inter-peptide hydrogen bonds during helix unwinding.

An intrinsic charge-anisotropy of membranes is in the orientation of the membrane dipole potential. This potential is oriented such that the interior of a membrane is positive with respect to the surface [26]. An interaction with the membrane dipole potential may be a factor in the refolding of these derivatives since positively-charged species will interact differently from negatively-charged species.

Importantly, positive charges on membrane proteins are known to play a decisive role in determining membrane protein topology in vivo [6]. The finding that short, positively-charged loops are rarely translocated has given rise to the 'positive inside rule' for predicting membrane protein topology. As in the present case, the exact structure of the positively-charged group does not appear to be important (Lys, Arg and even His residues are effective). Negative charges play much less of a role [6]. The molecular basis for these effects has, likewise, not been established. The gramicidin model system may thus be useful for further investigations on the molecular basis for these effects. Finally, many of these gramicidin amino-derivatives have now been analyzed using single channel techniques and form active transmembrane channels. Thus, from a practical point of view, addition of a C-terminal amino group provides a way of incorporating large amounts of $\beta^{6.3}$ gramicidin species into membranes without difficulty.

This work has been supported by NSERC and the CCFF.

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