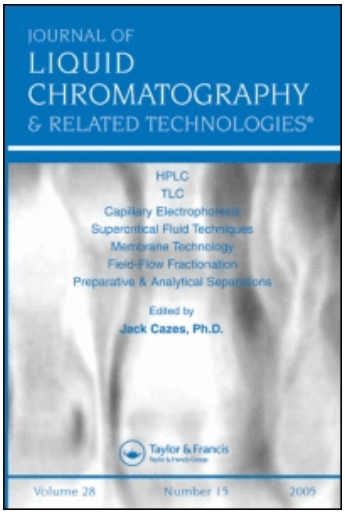

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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Lorenzo Braco[a]; María Del Carmen Baño[a]; Francisco Chillaron[a]; ConcepciÓN Abad[a]
[a] Departamento de Bioquímicay Biología Molecular, Universidad de Valencia, Burjassot, Spain

To cite this Article Braco, Lorenzo , Baño, María Del Carmen , Chillaron, Francisco and Abad, ConcepciÓN(1987) 'HPLC Characterization of the Gramicidin A Dimer-Monomer Conformational Equilibrium in Ethanol and Study of the Effect of Calcium Ion', Journal of Liquid Chromatography & Related Technologies, 10: 15, 3463 — 3480
To link to this Article: DOI: 10.1080/01483918708081884
URL: http://dx.doi.org/10.1080/01483918708081884

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(15), 3463-3480 (1987)

HPLC CHARACTERIZATION OF THE GRAMICIDIN A DIMER-MONOMER CONFORMATIONAL EQUILIBRIUM IN ETHANOL AND STUDY OF THE EFFECT OF CALCIUM ION

Lorenzo Braco*, María Del Carmen Baño, Francisco Chillaron, and Concepción Abad

Departamento de Bioquímica y Biología Molecular
Facultades de Ciencias
Universidad de Valencia
Dr. Moliner 50
46100 Burjassot, Spain

ABSTRACT

The usefulness of size-exclusion high-performance liquid chromatography for the study of gramicidin A (GA) dimer-monomer conformational equilibrium in polar organic solvents is demonstrated for the first time. The monomerization process of GA in ethanol has been analyzed using an Ultrastyragel 1000 Å column isocratically equilibrated with tetrahydrofuran, which has allowed the determination of kinetic and thermodynamic constants. Furthermore, the kinetics of interaction of Ca^{2+} with GA in ethanol has been followed using this methodology, and the binding mechanism has been investigated in terms of the polypeptide dimer-monomer conformational equilibrium.

Present address: Dept. of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139

INTRODUCTION

Gramicidin A is a linear pentadecapeptide which consists almost exclusively of strongly hydrophobic amino acids in an alternating L-D sequence (see ref. 1 for a review). A well-known property of GA is its ability to form cation-selective transmembrane channels in model and biological membranes (see ref. 2 for a recent review). It is currently believed, on the basis of ^{13}C-NMR (3-5), infrared (6,7) and circular dichroism measurements (8,9), that this channel consists of either a dimer formed by the amino end-to-amino end juxtaposition of two monomers in a left-handed $\beta^{6.3}$ helical conformation (3-5,8,9) or an antiparallel double-stranded β-helix (6,7). However, it has been recently pointed out from temperature-jump and voltage-jump experiments that a tetramer could be the smallest conductance unit of an active GA channel (10).

In the last years, experimental (11,13) and theoretical (14,16) studies have focused on the selectivity and detailed mechanism of ion passage through the GA channel. In this connection, the interaction of gramicidin with monovalent and divalent cations both in vesicles (11,12,17) and in organic solvents (17,18) has been examined by spectroscopic and conductance measurements. The effect of channel blocking by divalent cations such as Ca^{2+} or Ba^{2+} has been particularly a matter of considerable interest. Transport studies using artificial lipid bilayers have revealed that Ca^{2+} is not transported through the channel, but it rather binds to a specific site at the channel entrance (17). On the contrary, monovalent cations such as Na^{+} and K^{+} are transported at rates of about 10^{7} ions/s (19). As concerning the studies in organic solvent, it has been shown that in trifluoroethanol Ca^{2+} is strongly bound to GA whereas Na^{+} and K^{+}

exhibit little or no interaction (18). It has been also described from spectroscopic data (infrared, CD and ^{13}C-NMR) that Ca^{2+} interacts with GA in ethanol and methanol and it has been suggested that one gramicidin molecule has two calcium-binding sites located near the COOH end (17).

In spite of all the literature data available on the interaction of Ca^{2+} with GA, which have been obtained basically from spectroscopic techniques, we have not found any reference analyzing the mechanism of this interaction in terms of the conformational equilibrium of the polypeptide. In fact, it has been widely described that gramicidin in organic solvents exhibits a dimer-monomer conformational equilibrium, the dimer:monomer ratio being dependent on temperature, solvent polarity and total polypeptide concentration (20,22). Although Heitz and Gavach (17) have recently carried out a comprehensive study of the binding of calcium to GA in ethanol and methanol, the conformational equilibrium of the peptide in the alcoholic solution was not taken into account and the changes observed upon addition of calcium were explained in terms of a rather ambiguous calcium-induced GA "transconformation". However, factors such as polypeptide concentration and the time at which measurements were performed (at equilibrium conditions or not) should have been considered, because the actual dimer:monomer ratio strongly depends on them.

The most direct evidence for the existence of a dimer-monomer conformational equilibrium of GA in non-polar solvent has been recently obtained in our laboratory by using HPSEC (22,23). The advantages of HPLC techniques in the evaluation of equilibrium and rate constants have been demonstrated in a set of experimental conditions (23,24).

contained (0.5 micron) filter; and conversion of the internal loop valve (200-10,000 nanoliter) to an external loop valve (10,000 nanoliter and higher) (14). For low k' samples, where column efficiency is least, and using a 75 mm X 3 mm i.d. column, up to 50% decrease in column efficiency was found with the direct coupling compared to using a 50 X 0.007 inch connector tube with this valve.

The 7410 valve injector from Rheodyne (Cotati, CA) uses a loop disc with a fixed loop (either 500, 1,000 or 5,000 nanoliter) but the 7413 valve can be ordered with a triple loop disc of various combinations of the volumes (500, 1,000, 2,000 and 5,000 nanoliter) (14). Rheodyne also offers a fixed volume injector (Model 7520) "designed for microbore LC" with volumes of 200, 500, or 1,000 nanoliters. This valve is similar to the earlier 100 nanoliter JASCO valve (ML-422) that was reduced to 20 nanoliter by Takeuchi and Ishii (15). Rheodyne also offers three 6-port valves that can use loops from 5,000 nL up, some with smaller internal channels, for sharper peaks, (but requiring more pressure to load large samples). They note that in addition to the factors sample volume, or pressure to load, other factors may be more important in a particular application, such as volume to properly load (flush) the valve, ease of setting tension to prevent leaks, port spacing for inserting fittings, peak spreading (especially at k' below ca. 5, with non-gradient elution), and wastage

and emission filters wavelengths were 254 and 338 nm respectively) and a Varian Varichrom variable wavelength ultraviolet-visible detector set at 294 nm. The system was equipped with a 100-nm nominal pore size Ultrastyragel column (Ultrastyragel 1000 Å, 30 x 0.78 cm I.D.) from Waters Assoc. The chromatograms were monitored using a Yokogawa Electric Works dual-channel recorder.

Procedures

All chromatographic experiments were conducted at room temperature. The column was always eluted isocratically with THF at a flow rate of 1.0 ml/min.

Monomerization kinetics of gramicidin A in ethanol

GA samples were dissolved in ethanol and aliquots were taken at different times and injected. It was previously verified that the injection of moderate volumes of ethanol (5-10 µl) resulted in temporary damage of the resolution power of the column. Therefore, a GA concentration of 0.5 mg/ml was used in all the experiments which allowed an adequate detection of the peaks with an injection volume of only 2 µl of alcoholic solution.

Kinetics of Ca^{2+}-gramicidin A interaction in ethanol

Prior to the study of the kinetics of Ca^{2+}-GA interaction in ethanol, three procedures for the preparation and the injection of the sample containing the reaction mixture were tested and compared: No. 1) direct injection of 2 µl of the ethanolic solution; No. 2) dilution 1:25 (v/v) in THF of the reaction mixture, vortexing for 45 s and further injection of 50 µl (containing 2 µl of alcohol); and No. 3) the same procedure as in No. 2 but including an additional step of filtration of the THF-diluted sample through a Millipore 0.45-µm Millex HV filter previously washed with THF. It was verified that in both latter cases the dilution in THF

prior to injection practically stops the reaction. Methods No. 1 and 2 yielded identical results (error < 2%) whereas No. 3 altered resolution, probably due to some component of the filter partially dissolved by THF. Consequently, method No. 2 was selected since the higher absolute injected volume provides a higher accuracy.

For the kinetic analysis, a 0.5 mg/ml solution of GA in ethanol was prepared and allowed to equilibrated overnight, prior to the addition of calcium. Zero time was taken in all cases upon addition of an aliquot (13.5 µl) of a 0.1 M solution of Ca^{2+} in ethanol to 10 ml of previously equilibrated polypeptide solution. The reaction mixture was immediately stirred for 1 min. As indicated above, the injection volume was always 50 µl of the THF-diluted sample containing 2 µl of ethanol.

In all the experiments, tightly stoppered glass tubes were completely filled with the corresponding solutions to minimize hydration and, when stored overnight, they were maintained at 25 °C . Other details of the experimental conditions used can be found in the corresponding legends to Figures.

RESULTS AND DISCUSSION

Monomerization kinetics of gramicidin A in ethanol

Fig. 1A depicts, as an example, some elution profiles corresponding to the monomerization of GA in ethanol, for a concentration of injected polypeptide of 0.5 mg/ml. Two peaks are eluted (which absorb at 294 nm and also exhibit fluorescence) at the same elution volumes as those previously reported for the dimeric (V_e = 7.9 ml) and monomeric (V_e = 8.4 ml) conformational species of GA in THF using an Ultrastyragel 1000 Å column (23).

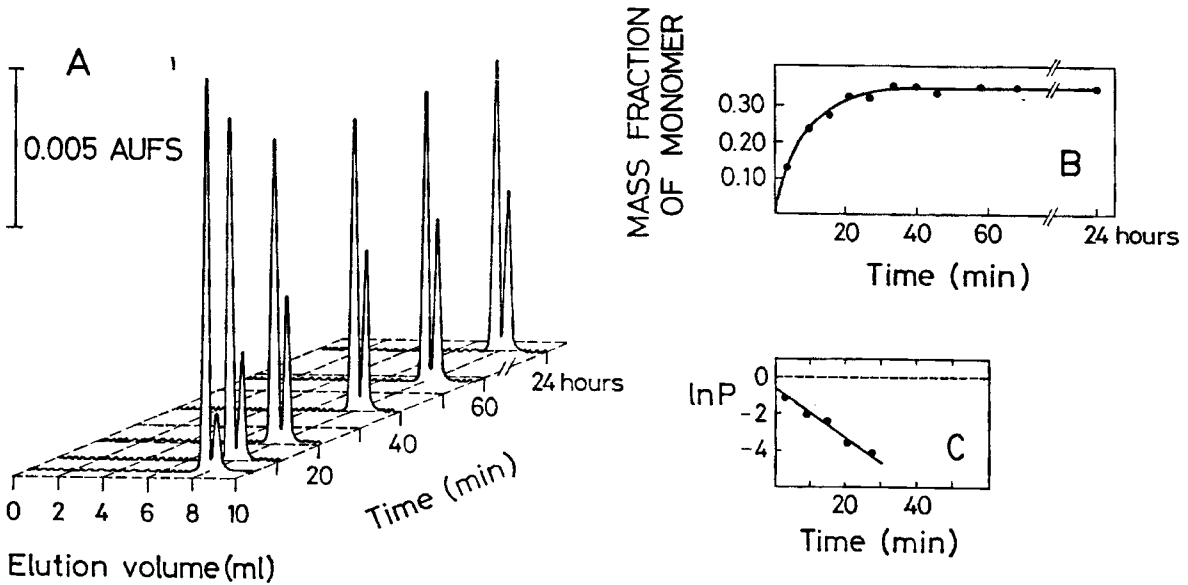

FIGURE 1. A) Elution profiles of a 0.5 mg/ml GA sample in ethanol as a function of incubation time. The column was isocratically eluted with THF at a flow rate of 1.0 ml/min. Samples were monitored by absorbance at 294 nm. The injection volume was 2 µl in all cases. The peak corresponding to ethanol (not shown) appears in all the chromatograms at 10.5 ml. B) Kinetic profiles of GA monomerization in ethanol; the mass fraction of monomer is plotted against the time at which each aliquot was taken. C) Plot of ln P vs. time according to eqn. 2 using the data in Fig. 1B.

Based on this fact and on literature spectroscopic data of GA conformational equilibrium in ethanol (21), which have pointed out that the monomerization rate of the peptide is much higher in this polar solvent than in non-polar ones, some considerations can be made on the mechanisms involved in the elution process. After injection, the 2 µl of ethanol are immediately removed from the polypeptide conformational species due to a size-exclusion effect. On the other hand, it is not likely that the rate of dimeric and monomeric species is altered through the column since the conformational equilibrium of GA in THF (eluent) is extremely slow (several days (23,24)) and the time required for the analysis is only 9 min. Therefore, the chromatograms in Fig. 1A can be considered as reproducing the actual situation of the conformational equilibrium existing in alcoholic solution before injection, i.e., the actual dimer:monomer ratio in ethanol at a given time, t.

We have recently demonstrated that, when the monomerization kinetics of GA is followed by absorbance measurements at 294 nm using THF as eluent, it is possible to evaluate the mass fraction of each species directly from the peak heigths in the chromatograms (24). Fig. 1B shows the mass fraction of monomer as a function of time for a 0.5 mg/ml solution of GA in ethanol. It is evident that the elapsed time results in a displacement of equilibrium from the GA dimer (zero time) towards monomeric forms (Figs. 1A and B). At this polypeptide concentration, equilibrium is reached in about one hour, in contrast to the several days required in THF (23,24).

Using the results in Fig. 1B, a detailed kinetic analysis has been carried out both to verify our above assumption of a simple dimer-monomer equilibrium of GA

in ethanol and to determine the rate constant for the dissociation process.

Let us assume the following equilibrium for GA:

$$M_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 2\,M \qquad (1)$$

The integrated form of the velocity equation can be written as (24):

$$\ln \frac{[M_2] - [M_2]_e}{\frac{[M_2]_o}{[M_2]_e}([M_2]_o - [M_2]_e) + ([M_2]_o - [M_2])} = \ln \frac{[M_2]_e}{[M_2]_o} - \frac{[M_2]_o + [M_2]_e}{[M_2]_o - [M_2]_e} k_1 t \qquad (2)$$

where $[M_2]_o$, $[M_2]_e$ and $[M_2]$ refer to dimer concentration at zero time, at equilibrium and at a given time, t, respectively. k_1 (s^{-1}) denotes the rate constant of the direct process. For simplicity, the whole fraction of the first member will be referred to as P. k_1 value can be determined from the slope of the straight line in eqn. 2.

Fig. 1C plots the chromatographic results in Fig. 1B according to eqn. 2. A good fitting is observed which demonstrates, on the one hand, the validity of the model in eqn. 1, and on the other, the accuracy in the chromatographic determination of dimer and monomer concentrations. From three independent sets of measurements of the GA monomerization kinetics, an average k_1 value of $(5.0 \pm 0.4) \times 10^{-4}$ s^{-1} has been obtained. On the other hand, an average value for the dissociation equilibrium constant, K_d, has been obtained from the data at equilibrium (see Fig. 1B as an example), this value being $K_d = (1.0 \pm 0.3) \times 10^{-4}$ M. The k_1 and K_d values determi-

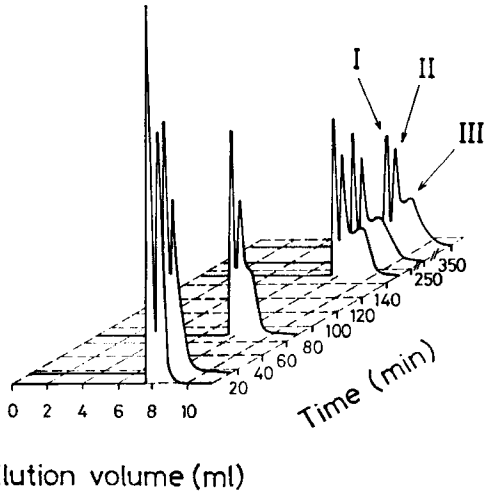

FIGURE 2. Fluorescence detection-monitored elution profiles of Ca^{2+}-GA interaction kinetics in ethanol, at a 0.5 mg/ml peptide concentration, for a Ca^{2+}:GA molar ratio R = 0.5. Eluent:THF; flow rate: 1.0 ml/min. The chromatogram at zero time corresponds to the injection of the GA sample equilibrated before the addition of calcium.

ned from the analysis of chromatographic results are of the same order of magnitude as those reported by Veatch and Blout (21) from spectroscopic measurements of GA in ethanol.

Kinetics of Ca^{2+}-gramicidin A interaction in ethanol

Fig. 2 depicts, as an example, some elution profiles at different incubation times of the reaction of calcium with GA previously equilibrated in ethanol, at a 0.5 mg/ml peptide concentration, for a Ca^{2+}:GA molar ratio, R = 0.5. Samples were monitored simultaneously with fluorescence and absorbance detectors. However, in order not to overcrowd the Figure, only the fluorescence

response is shown. The chromatogram at zero time corresponds to the injection of the GA sample equilibrated in ethanol before the addition of calcium. Two peaks are observed in the chromatograms which are eluted at exactly the same elution volumes as GA free dimeric (peak 1) and monomeric (peak II) species. A broad tailed peak becomes apparent with elapsed time at a higher elution volume, which could be attributed to a calcium-polypeptide complex (peak III).

Two possible non-exclusive alternative explanations can be given for the rather large elution volume of this species: i) a Ca^{2+}- induced GA conformational change resulting in a lower hydrodynamic volume. It should be noted that GA exhibits in non-polar solvent a β-helical conformation (20,21) and in THF a Ca^{2+}-polypeptide complex would be expected to adopt a tighter, more compact structure. ii) a retention of the Ca^{2+}- GA complex on the support hydrophobic matrix. In this connection, it has been recently pointed out, when studying the elution behavior of Ca^{2+}- binding proteins by hydrophobic interaction chromatography (HIC-HPLC) using a phenyl group-derivatized support, that the elution volume strongly depends on the presence or absence of calcium in the eluent (25). The retention observed for the calcium-bound protein relative to the calcium-free one has been ascribed to a Ca^{2+}- dependent exposure of some protein hydrophobic sites (26), so that it would interact more strongly with the hydrophobic gel matrix. Fig. 2 also shows that the addition of calcium causes two effects: a decrease in the absolute heights of free dimer and monomer peaks and a parallel increase in the area of the peak of complex.

Fig. 3 plots (solid line) the results corresponding to the kinetics of interaction of Ca^{2+} with GA for

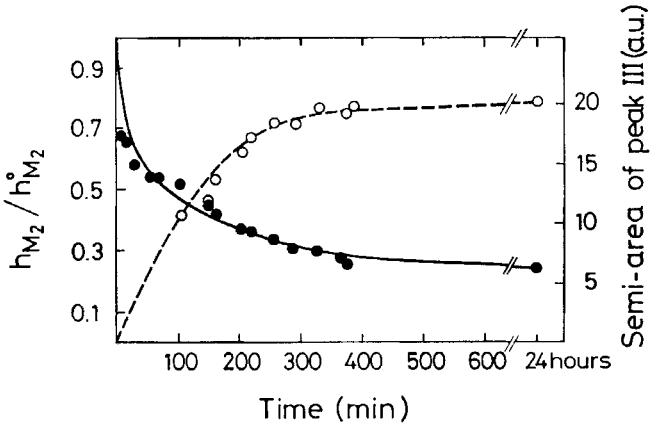

FIGURE 3. Solid line: Variation of the relative height of the free dimer peak, $h_{M_2}/h^{\circ}_{M_2}$, with incubation time for the Ca^{2+}- GA interaction kinetics in ethanol at a 0.5 mg/ml peptide concentration, for R = 0.5. $h^{\circ}_{M_2}$ denotes the height of the free dimer peak (in arbitrary units) at zero time. Dashed line: Time-dependent Ca^{2+}- GA complex formation expressed as the variation of the semi-area of peak III in the chromatograms.

a 0.5 mg/ml peptide concentration and for R = 0.5, expressed as the variation of the relative height of the free dimer peak (peak I), $h_{M_2}/h^{\circ}_{M_2}$, as a function of incubation time. $h^{\circ}_{M_2}$ denotes the height of the free dimer peak (in arbitrary units) at zero time, i.e., that corresponding to the injection of the equilibrated GA sample before the addition of Ca^{2+}. We have considered it more adequate to use the heights of the dimer peak in the kinetic plots, because the free monomer peak is partially overlapped with the complex one.

On the other hand, as an estimation of the appearance of the Ca^{2+}-GA complex, the semi-area of peak III (in arbitrary units, dashed line) is also plotted in Fig. 3 as a function of incubation time. A good time

correlation between the disappearance of free dimer and the formation of the complex can be observed, the sample reaching practically equilibrium at about 9-10 hours.

Since the free dimer:monomer ratio seems to be maintained with elapsed time (see Fig. 2), the apparent dissociation constant, K_d^{app}, for the $M_2 \rightleftharpoons 2M$ equilibrium in the presence of Ca^{2+} was calculated as a function of incubation time. We have previously verified that, when monitoring a GA sample with both UV and fluorescence detectors, for the same height of the dimer peak, the height of the monomer one was always higher with fluorescence than with UV detection, the factor being 1.20 ± 0.05 as determined from a set of 10 independent injections. This behavior is due to the fact that GA dimer and monomer have different quantum yields in organic solvent (21), and particularly in THF the monomer quantum yield is higher than the dimer one ((23), and manuscript in preparation). So, in order to determine K_d^{app} directly from the heights of the peaks, a correction was made in all cases for the height of the monomer peak, which was divided by the factor 1.20.

Table 1 summarizes the values of K_d^{app} as a function of incubation time. It can be seen that, until ~ 2.5 hours of incubation, K_d^{app} values remain practically constant and similar to the value in the absence of Ca^{2+}, whereas al longer times, there is a non-significant slight increase in K_d^{app} probably due to an overestimation of the monomer concentration, as a consequence of the overlapping between free monomer and complex peaks. Therefore, it seems reasonable to assume that equilibrium between free polypeptide species ($M_2 \rightleftharpoons 2M$) is maintained during the interaction kinetics. If so, Ca^{2+} could bind either to both conformational species with the same affinity, or only to one of them shifting the conformational equilibrium between free species.

TABLE 1

Values of the Apparent Dissociation Constant, K_d^{app}, as a Function of Incubation Time

t(min)	$10^4 \times K_d^{app}$(mol/l)	t(min)	$10^4 \times K_d^{app}$(mol/l)
0*	1.0	204	1.3
4	1.1	219	1.4
14	1.4	257	1.5
29	1.0	288	1.5
53	1.1	331	1.5
68	1.2	367	1.9
104	1.0	376	1.8
148	1.0	24 hours	1.9
164	1.3		

* Before the addition of calcium.

In this connection, we have obtained additional evidence supporting that Ca^{2+} binds preferentially to the polypeptide monomeric form (not shown). For this purpose, a Ca^{2+}-containing aliquot was added to freshly dissolved GA dimeric (commercial GA) and monomeric (GA lyophilized from acetic acid, (23)) ethanolic solutions and the samples were chromatographed at very short incubation times (< 5 min at a Ca^{2+}:GA molar ratio R=2). Upon addition of Ca^{2+}, both a rapid decrease and a significant deformation of the peak corresponding to monomeric GA was observed, whereas the peak corresponding to dimeric GA did not appreciably change.

The results presented above allow to propose the following qualitative mechanism of Ca^{2+}-gramicidin A interaction in ethanol in terms of the dimer-monomer conformational equilibrium:

$$M_2 \rightleftharpoons 2M \quad + n\ Ca^{2+} \downarrow \text{complex}$$

Such a mechanism evidences a preferential binding of the divalent cation to the monomeric form relative to the dimeric one. On the other hand, this fact seems to be in line with Urry's model of GA channel, which essentially consists of the N-terminal-to-N-terminal juxtaposition of two monomers (3,4). Furthermore, based on spectroscopic and theoretical data (15,17) supporting that the calcium binding site is close to the ethanolamine terminal of gramicidin A, it could be suggested from our results that the carboxyl-end is more accesible in a monomeric conformation than in a double helical dimeric one.

In summary, HPSEC has proved to be a valuable tool for the elucidation of the mechanism of interaction of GA with Ca^{2+} in terms of the polypeptide conformational equilibrium. Experiments are at present being carried out in our laboratory to confirm the mechanism proposed and to characterize the stoichiometry of the binding process as well as to determine the kinetic and thermodynamic constants. This methodology can also be extended to the study of the interaction of GA with monovalent cations (Na^+, K^+...) which are transported through the gramicidin A transmembrane channel (8,11,12).

ACKNOWLEDGEMENT

This work was supported by grant No. 598/84 from CAICYT.

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