Volume 250, number 1, 67–71 FEB 07235 June 1989

HPLC study on the 'history' dependence of gramicidin A conformation in phospholipid model membranes

M.C. Bañó, L. Braco and C. Abad

Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, E-46100 Burjassot, Valencia, Spain

Received 11 April 1989

A novel HPLC methodology for the study of gramicidin A reconstituted in model membranes has been tested in comparison with circular dichroism data. It is shown that this chromatographic technique not only corroborates most of the recent spectroscopic results but allows one to explain them in terms of mass fractions of different actual conformational species of GA in the phospholipid assemblies. In particular, the dependence of the inserted peptide configuration on the organic solvent and other parameters involved in the 'history' of the sample preparation and handling has been analyzed by HPLC in two phospholipid model systems: small unilamellar vesicles and micelles. Moreover, a slow conformational transition of GA towards a $\beta^{6,3}$-helical configuration, accelerated by heat incubation, has been also chromatographically visualized and quantitatively interpreted.

Gramicidin A conformation; Phospholipid vesicle; HPLC; CD

1. INTRODUCTION

It is well known that the conformation of the 15-amino-acid linear hydrophobic polypeptide, gramicidin A (GA), can vary greatly in organic solvents as well as when incorporated into phospholipid model membranes, depending on a number of factors [1]. Interestingly, it has been recently reported by several researchers, and now appears to be quite established, that the conformational state of the peptide in both artificial lipid bilayers and micelles can be dictated to some extent by the 'history' of the sample preparation [2–6]. In particular, a solvent dependence of the spectral characteristics of GA samples has been shown.

Most of this information has been inferred essentially from circular dichroism (CD) and NMR measurements. However, although these techniques are undoubtedly valuable and very useful in monitoring differences in conformation, interpretation of the data is often qualitative and quite ambiguous so that the spectra cannot always be unequivocally ascribed to either a single species or (more commonly) to a mixture of known composition of different conformational species. All this results in the fact that the exact nature of the observed spectroscopic changes remains in some instances rather unclear.

In this regard, we have demonstrated in recent years the usefulness of high-performance size-exclusion chromatography (HPSEC) for the accurate characterization in organic solvents of self-association equilibria of GA [7–12] and other synthetic hydrophobic peptides such as DL-oligophenylalanines [13]. In particular, we have recently proposed an HPSEC-based novel strategy which permits the simple, rapid, accurate and direct monitoring of different conformational species of GA incorporated into phospholipid model membranes [14].

This paper provides the first account of a direct comparison between CD and HPSEC results in relation to the history-dependent conformation of inserted GA and shows that its spectral characteristics can be reliably correlated to and explained by chromatographic data. Preliminary

Correspondence address: C. Abad, Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, C/Doctor Moliner 50, 46100 Burjassot, Valencia, Spain

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

tests demonstrate that this completely new HPLC methodology can provide valuable, quantitative information on the conformational state of GA in liposomes and micelles. Furthermore, it offers a great potential in the investigation of conformational transitions of this and other self-associating peptides incorporated into model membrane systems, with all the obvious advantages inherent in high-performance chromatographic techniques, particularly the direct visualization of the different species.

2. EXPERIMENTAL

2.1. *Materials and chemicals*

GA (natural mixture) was supplied by Koch Light Labs and was used without further purification. Egg yolk phosphatidylcholine (PC) was purchased from Merck (Darmstadt) and purified according to Singleton et al. [15]. Egg yolk L-α-lysolecithin (LPC) was from Sigma (St. Louis, MO). Tetrahydrofuran (THF) and all other organic solvents were spectroscopic grade (Merck).

2.2. *Chromatographic measurements*

The liquid chromatograph (from Waters Chromatography Division, Millipore, Milford, MA) and the general experimental conditions for elution were as described [14]. All measurements were made in quadruplicate and the standard deviation was always $<3\%$.

2.3. *CD measurements*

CD measurements were performed at 25°C with a Jobin Yvon mark III spectropolarimeter. Blank runs of vesicles in H_2O were subtracted from the measured spectra of GA molecules. CD results were expressed as mean residue weight ellipticities, in units of degree $\cdot$ cm^2 $\cdot$ dmol^{-1}. The reported spectra are the average of three scans from two independent preparations for each sample.

2.4. *Preparation of PC vesicles*

Unless stated otherwise, dispersions of small unilamellar vesicles (SUV) were prepared as in [14]: PC and GA were codissolved in a given organic solvent by mixing identical volumes (each 100 μl) of stock solutions. The solvent was rapidly evaporated under a stream of N_2 and later under high vacuum overnight to remove any traces. The samples were then hydrated, sonicated for 10 min on ice and centrifuged for 15 min at 35000 $\times$ g to remove probe particles and the remaining multilamellar liposomes. The final GA concentration was 0.074 mg/ml and the lipid/polypeptide mole ratio was 50:1.

2.5. *Preparation of LPC micelles*

GA-containing micelles were prepared according to Masotti et al. [16].

3. RESULTS AND DISCUSSION

Fig.1 shows, as an example, a comparison between the elution profiles corresponding to samples of GA dissolved in a given organic solvent (left) and incorporated into SUV by the cosolubilization method from the same individual solvents (right). As described in [7], peak I corresponds to eluted double-stranded (DS) dimers whereas peak II refers to monomers ($\beta^{6,3}$-helical conformation). When GA-containing liposomes are injected peak I reliably and accurately reflects the actual proportion of DS dimeric species in the vesicles, since such species are stable during elution [7,8]. As reported [14], the monomers observed in the chromatograms (peak II) are supposed to arise from actual Urry's head-to-head (HH) dimers present in the initial liposomes before injection and being dissociated during elution. This point is also strongly supported by further evidence in this paper deduced from direct comparison of CD and HPLC data. At any rate, a small percentage of actual monomers in the bilayer cannot be completely discarded, as it has been also indicated for CD measurements [3]. The mass fraction of each species was directly evaluated from the peak heights as reported [8].

As expected, the DS dimer/monomer ratio in pure organic solvents dramatically decreases with solvent polarity. Similar behavior is observed when the GA-containing SUV are comparatively analyzed, which shows a clear correlation between the conformational state of the peptide in freshly prepared liposomes and in the corresponding pure organic solvent before evaporation. In general, a slightly lower proportion of DS dimers is observed in the vesicles as compared to the starting solvent. This is due to the fact that during the PC + GA cosolubilization step, even if the incubation period is very short, some lipid-induced time-dependent GA monomerization may occur [7], especially in this case where the lipid-to-peptide mole ratio is relatively high. Besides, we have verified that sonication also causes some dissociation of DS dimers (not shown).

Since the organic solvent used in the preparation of GA-containing model membranes can be crucial to the molecular organization of the freshly incorporated peptide, we also investigated by HPLC the influence of a number of solvents (and mixtures) typically reported in the literature protocols, but used in some cases in a rather arbitrary way. The results are summarized in table 1 in terms of the

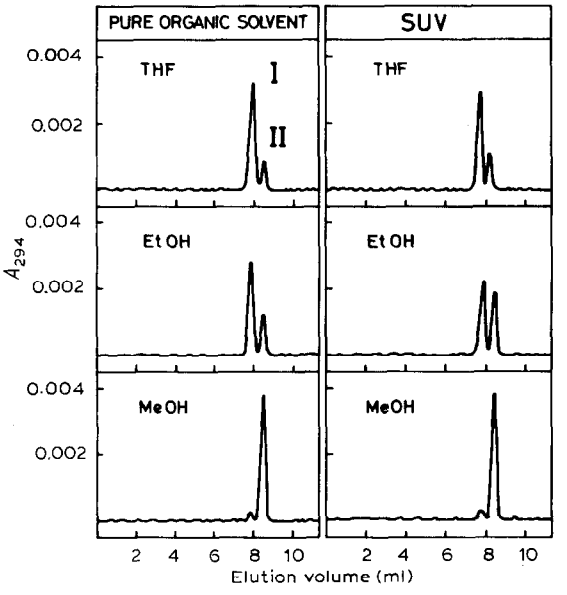

Fig.1. Elution profiles of samples of GA equilibrated in different organic solvents (left) and incorporated into freshly prepared SUV by cosolubilization with PC in the same solvents (right). [GA] = 0.074 mg/ml; column, Ultrastyragel, 1000 Å; mobile phase, TFH; flow rate, 1.0 ml/min; injection volume, 2 μl.

DS dimer composition. The trend is clear: for the nonpolar solvents the predominant conformational species in the liposomes are the DS dimers, whereas for more polar solvents the proportion of these dimers is drastically reduced at the expense of the $\beta^{6.3}$-helical forms.

Next, a direct comparison between CD and HPLC data was made for GA-containing SUV obtained and treated in different ways. This allowed us to interpret more quantitatively the spectral changes in terms of mass fraction values chromatographically derived for the peptide conformational species. Fig.2 depicts the CD spectra of GA incorporated into freshly prepared vesicles from THF (curve a) and ethanol (curve b) as starting solvents in the cosolubilization step, the same vesicles after 7 days of incubation at 25°C (curve c, from ethanol; curve d, from THF), and vesicles from THF after extensive incubation at 60°C for 20 h (curve e). This latter treatment has been reported to lead to a channel configuration [17,18]. The CD spectrum of GA incorporated from trifluoroethanol (TFE) is similar to that of curve e (not shown). Curve e is characteristic of a

Table 1

Mass fraction of GA DS dimers determined by HPLC in PC SUV freshly prepared by cosolubilization from different organic solvents (or mixtures)

Solvent	Mass fraction of GA DS dimers
Benzene	0.82
THF	0.75
Chloroform	0.70
Ethanol	0.55
Benzene/methanol (95:5)	0.73
Chloroform/methanol (2:1)	0.18
Methanol	0.08
Trifluoroethanol (TFE)	<0.02

Chromatographic conditions and peptide concentration were the same as given in fig.1

$\beta^{6.3}$-helix, which is involved in the HH dimer channel conformation [1,19], and displays positive extrema at 218 and 235 nm with a weak negative inflexion in between (at 229 nm) and a negative ellipticity below 208 nm. In contrast, the nonchannel conformation reported when using, for example, ethanol as original solvent (curve b in fig.2) is characterized by a large negative peak at 229 nm, a weaker positive peak at 218 nm and a positive ellipticity below 208 nm [3]. Simultaneous HPLC measurements of these samples provided the following values in terms of the mass fraction of DS dimers: curve a, 0.75; curve b, 0.55; curve c, 0.16; curve d, 0.14; and curve e, <0.03. It is evident that there is a correlation between the data from both techniques: as the proportion of DS dimers decreases, the spectra approach that reported by Urry for the channel ($\beta^{6.3}$-helix) configuration (curve e). In fact, when the percentage of monomers observed on chromatography is >97% CD shows a clear $\beta^{6.3}$-helix spectrum. Contrastingly, for the SUV freshly prepared from the least polar solvent assayed (THF), HPLC shows the highest percentage of DS dimers (75%) and the corresponding spectrum (curve a) displays the most pronounced nonchannel characteristics (even more than for ethanol, curve b).

It must be emphasized at this point that these HPLC results clearly demonstrate the actual configuration of GA in CD spectra similar to curves a or b, reported as corresponding to nonchannel conformation: there is indeed a mixture of DS

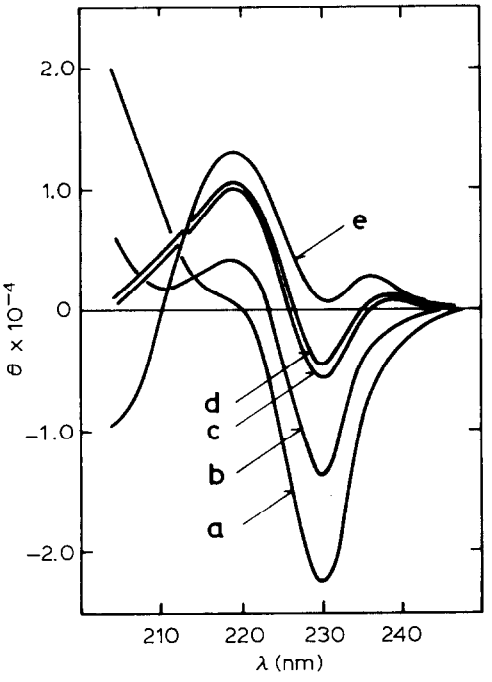

Fig.2. CD spectra of GA-containing SUV prepared and treated in different ways (see text).

dimers and monomeric $\beta^{6.3}$ -helices (perhaps juxtaposed as HH dimers), where the first ones are the predominant species. In this regard, studies are in progress in our laboratory in order to fit empirical data from both techniques, which ultimately would allow theoretical reproduction or simulation of a given CD spectrum, even if it cannot be experimentally obtained. As for curves b–d, these intermediate situations correspond in order on HPLC to intermediate proportions of DS dimers and monomers. The decrease in DS dimers as a function of incubation time at 25°C (curves c,d) is not surprising, since it has been reported that the 'aging' of GA-containing liposomes where the peptide was incorporated in a nonchannel state results in a slow conformational transition of the peptide towards the thermodynamically stable configuration in the bilayer, a $\beta^{6.3}$-helix [3]. Recently, we have identified via chromatograph this transition as a dissociation of DS dimers [14]. Fig.2 and the corresponding HPLC data prove that this transition occurs regardless of the starting organic solvent, in agreement with previous observations [3].

Finally, this chromatographic methodology was tested for a different model system. A direct comparison with CD results was established for GA incorporated into LPC micelles using the experimental conditions described by Masotti et al. [16]. Fig.3 shows the results obtained from simultaneous CD (A) and HPLC (B) measurements for freshly prepared micelles at 25°C and after overnight heating of the sample at 68°C. The CD spectrum at 25°C (nonchannel conformation) corresponds to >97% of DS dimers by HPLC. Note that the peptide was externally added to the preformed micelles as a dry powder, where 100% of DS dimers are present [7]. In contrast, a characteristic channel conformation spectrum appeared after extensive incubation at 68°C, in agreement with previous results [16]. HPLC showed in this case >97% of monomeric forms. Thus, it can be concluded that the temperature-dependent conformational transitions observed by CD upon heating of vesicles (fig.2) and micelles (fig.3) are due to the conversion of DS dimers into a $\beta^{6.3}$-helical configuration organized as HH dimers in the functionally active model membrane.

We believe that this promising chromatographic methodology introduces a new, rapid, sensitive and reliable approach for the direct monitoring of conformational transitions of self-associating hydrophobic peptides (GA and others) incorporated in model membranes, and consequently, will permit a better, quantitative understanding of the mechanisms involved in these processes. A broad range of experimental conditions and parameters

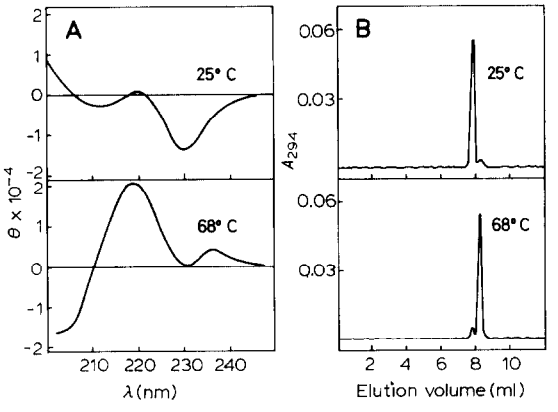

Fig.3. Comparison of CD (A) and HPLC (B) data for GA-containing LPC micelles, freshly prepared at 25°C and after overnight heating at 68°C.

can be investigated, and some qualitative but still ambiguous spectroscopic information can be clarified. Studies in this direction are now in progress.

Acknowledgements: The authors are grateful to Professor J.G. Gavilanes for the use of the circular dichrograph in his laboratory. This work was supported by grant PB87-0996 from the Dirección General de Investigación Científica y Técnica (DGICYT), Spain.

REFERENCES

[1] Wallace, B.A. (1986) Biophys. J. 49, 295–306.
[2] Tournois, H., Killian, J.A., Urry, D.W., Bokking, O.R., De Gier, J. and De Kruijff, B. (1987) Biochim. Biophys. Acta 905, 222–226.
[3] Killian, J.A., Prassard, K.U., Hains, D. and Urry, D.W. (1988) Biochemistry 27, 4848–4855.
[4] Killian, J.A. and Urry, D.W. (1988) Biochemistry 27, 7925–7301.
[5] Killian, J.A., Nicholson, L.K. and Cross, T.A. (1988) Biochim. Biophys. Acta 943, 535–540.
[6] LoGrasso, P.V., Moll, F., iii and Cross, T.A. (1988) Biophys. J. 54, 259–267.
[7] Braco, L., Abad, C., Campos, A. and Figueruelo, J.E. (1986) J. Chromatogr. 353, 181–192.
[8] Braco, L., Bañó, M.C., Chillarón, F. and Abad, C. (1986) Biophys. Chem. 25, 297–305.
[9] Braco, L., Chillarón, F., Bañó, M.C., De la Guardia, M. and Abad, C. (1987) Spectrochim. Acta 43A, 1365–1370.
[10] Braco, L., Bañó, M.C., Chillarón, F. and Abad, C. (1987) J. Liq. Chromatogr. 10, 3463–3480.
[11] Braco, L., Bañó, M.C., Campos, A. and Abad, C. (1988) Biophys. Chem. 30, 93–103.
[12] Braco, L., Bañó, M.C., Chillarón, F. and Abad, C. (1988) Int. J. Biol. Macromol. 10, 343–348.
[13] Bañó, M.C., Braco, L., Celda, B. and Abad, C. (1988) Biophys. Chem. 31, 3–9.
[14] Bañó, M.C., Braco, L. and Abad, C. (1988) J. Chromatogr. 458, 105–116.
[15] Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.C. (1965) J. Am. Oil Chem. Soc. 42, 53–56.
[16] Masotti, L., Spisni, A. and Urry, D.W. (1980) Cell Biophys. 2, 241–251.
[17] Urry, D.W., Spisni, A. and Khaled, M.A. (1979) Biochem. Biophys. Res. Commun. 88, 940–949.
[18] Shungu, D.C., Hinton, J.F., Koeppe, R.E., ii and Millett, F.S. (1986) Biochemistry 25, 6103–6108.
[19] Urry, D.W., Goodall, M.C., Glickson, J.D. and Mayers, D.F. (1971) Proc. Natl. Acad. Sci. USA 68, 1907–1911.