A semi-empirical approach for the simulation of circular dichroism spectra of gramicidin A in a model membrane

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ABSTRACT In an extension of our previous work (Bañó, M. C., Braco, L., and Abad, C. 1991. *Biochemistry.* 30:886–94), the kinetics of dissociation of gramicidin A double-stranded dimers into $\beta^{6.3}$-helical monomers in small unilamellar vesicles prepared following different protocols, were investigated using in combination circular dichroism (CD) and high-performance liquid chromatography (HPLC). The analysis of the data from both techniques according to a two-component model strongly supports that any given CD pattern of gramicidin incorporated in the phospholipid bilayer can be deconvoluted essentially as a linear combination of the reference subspectra calculated for the double-stranded dimer and the helical monomer. An HPLC-based, semi-empirical approach is proposed for the simulation of gramicidin CD curves in the model membrane used, and it is shown that the congruence between theoretical and experimental spectra is very satisfactory.

INTRODUCTION

Circular dichroism (CD) spectroscopy, particularly in the far-ultraviolet region, has been extensively used for about two decades as a routine, exquisitely sensitive tool for the analysis of secondary structure of proteins and peptides in solution (for a recent review see Johnson, 1988). At present, in spite of the advent of other concurrent, powerful, spectroscopic techniques also allowing the analysis of biological macromolecules in solution, such as two-dimensional nuclear magnetic resonance and Fourier-transform infrared spectroscopy, CD is resurging to offer interesting perspectives (particularly in combination with other techniques [Kuwajima et al., 1991]), especially in the investigation of the ever-growing number of recombinant and engineered proteins (see e.g., Shire et al., 1991).

Traditionally, the methods for analyzing the CD spectrum of a protein for secondary structure have been based on the fitting of the spectrum to a combination of reference spectra corresponding to pure structural components (α-helix, β-sheets, β-turn, coil, et cetera) derived either from model polypeptides (Greenfield and Fasman, 1969; Brahms and Brahms, 1980) or proteins with known secondary structure (see, e.g., Chang et al., 1978; Siegel et al., 1980). More recent approaches, however, analyze the CD of a protein of unknown structure as a linear combination of the spectra of a set of proteins (reference spectra) whose structure has been determined by x-ray diffraction (Provencher and Glöckner, 1981; Yang et al., 1986; Manavalan and Johnson, 1987). It appears that to date, a reasonable degree of refinement has been achieved in the deconvolution of CD curves and the prediction of protein and peptide secondary structure (see, e.g., Perczel et al., 1991; Pancoska and Keiderling, 1991). Unfortunately, these methods of analysis cannot be applied to biochemical systems where less common elements of secondary structure are present, if the corresponding reference spectra are not available. In this regard, perhaps one of the most outstanding examples of an extraordinary profusion in the use of CD to monitor peptide conformation without paradoxically any successful attempt of a quantitative treatment of the data, is the case of gramicidin A incorporated in phospholipid model membranes (Wallace, 1986, 1990; Killian and Urry, 1988; Killian et al., 1988; LoGrasso et al., 1988).

Gramicidin A is a hydrophobic linear polypeptide antibiotic consisting of 15 amino acids with alternating L- and D-configuration, which forms monovalent cation specific channels in model membranes (for recent reviews see Andersen et al., 1988; Wallace, 1990). The amino acid sequence for gramicidin A is: HCO-L-val^1-gly^2-L-ala^3-D-leu^4-L-ala^5-D-val^6-L-val^7-D-val^8-L-trp^9-D-leu^{10}-L-trp^{11}-D-leu^{12}-L-trp^{13}-D-leu^{14}-L-trp^{15}-NHCH$_2$-CH$_2$OH (Sarges and Witkop, 1965). It is, at present, generally accepted that the functionally active gramicidin A channel consists of a dimer formed by the *N*-terminal-to-*N*-terminal transmembrane juxtaposition of two $\beta^{6.3}$-helical monomers (Szabo and Urry, 1979; Urry et al., 1983; Arseniev et al., 1985; Durkin et al., 1990), as originally proposed by Urry (1971). This latter conformation can be directly observed in reconstituted model systems after incorporation from solvents such as trifluoroethanol (TFE) or dimethylsulfoxide (DMSO) (Masotti et al., 1980; Tournois et al., 1987; LoGrasso et al., 1988; Killian et al., 1988; Killian and Urry, 1988). However, a wide variety of different CD patterns (typically designed as 'nonchannel' conformation) has been also reported depending on the experimental conditions used for reconstitution, or in other words, on the "history" of the sample preparation (Urry et al., 1979; Wallace et al., 1981; LoGrasso et al., 1988; Killian et al., 1988; Bañó et al., 1989, 1991). Interestingly, these "nonchannel" spectra exhibit a variation as a function of time or after heat treatment (Masotti et al., 1980; Lo-

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Grasso et al., 1988; Killian et al., 1988; Bañó et al., 1989, 1991) towards the $\beta^{6.3}$ configuration (channel type) which is considered to be the thermodynamically stable form in the lipid environment (Killian et al., 1988). In any case, the CD descriptions of the peptide in reconstituted systems have been generally analyzed with some ambiguity, and usually in terms of a certain degree of similarity among the observed spectra.

Recently, the combination of high-performance liquid chromatography (HPLC), as a novel technique in the investigation of conformational transitions of gramicidin inserted in model membranes (Bañó et al., 1989, 1991), with CD data has brought about a clarification in the interpretation of the abundant spectral information. By making possible the physical separation and quantitation of membrane-inserted antiparallel double-stranded (APDS) dimers and β-helical monomers (Bañó et al., 1988), HPLC has provided strong supportive evidence that (*a*) a mixture of at least these two conformers in different proportions must contribute to the observed "nonchannel" CD patterns, and (*b*) the so often reported transition undergone by gramicidin from the "nonchannel" state towards the channel configuration can be correlated with a quantitative conversion from APDS dimers to $\beta^{6.3}$-helical monomers (Bañó et al., 1989, 1991).

Whereas for gramicidin in organic solution it was previously reported that the CD spectrum of an equilibrium mixture of conformers is an average of the spectra of all the forms present in solution, weighted by their relative abundance (Veatch et al., 1974; Wallace, 1986), for the membrane-associated peptide the questions still remain whether any CD spectrum can be described as being additively composed of two terms, i.e., APDS dimers and $\beta^{6.3}$-helical monomers, and whether there may be other membrane-incorporated structures different from these ones also contributing to the observed CD spectra. So far, the answer to these questions had been hindered by the practical impossibility of physically isolating the membrane-inserted conformational species and therefore obtaining their corresponding reference CD spectra.

Because HPLC provides a unique opportunity for this purpose, to address this issue we now report the results of a quantitative, combined CD-HPLC study of the conformational transitions of gramicidin in phosphatidylcholine bilayers as a model system. Both spectroscopic and chromatographic data have been successfully fitted using a tentative two-component analysis, which gives us a simple way to estimate the reference spectra for each component or element of secondary structure (APDS dimer and a $\beta^{6.3}$-helical monomer). The results strongly support the previous assumption (Bañó et al., 1991) that any given CD spectrum in this system can be deconvoluted as a linear combination of two well-defined reference subspectra, regardless of how the sample was prepared. Finally, using the calculated reference ellipticity values, different CD spectra have been simulated and compared to those obtained experimentally from samples prepared by cosolubilization from different organic solvents.

MATERIALS AND METHODS

Materials

Gramicidin (natural mixture) was supplied by Koch Light Labs. (Buckinghamshire, UK) and was used without further purification. Egg yolk phosphatidylcholine (EPC) was purchased from Merck (Darmstadt, Germany) and purified according to Singleton et al. (1965). Tetrahydrofuran (THF) and all other organic solvents were either HPLC or spectroscopic grade, from Merck. THF (chromatographic mobile phase) was passed through a 0.45-μm regenerated cellulose filter (Micro Filtration Systems, Dublin, CA) before use. Deionized water was purified through a Millipore Milli-Q system (Millipore, Milford, MA).

Sample preparation

Gramicidin-containing small unilamellar vesicles (SUV) were prepared basically as previously reported (Bañó et al., 1991). Briefly, polypeptide and lipid were codissolved in the appropriate organic solvent (THF or ethanol) by mixing identical volumes (100 μl of each) of stock solutions. The solvent was rapidly evaporated under a nitrogen stream and later under high vacuum overnight to ensure complete removal of traces. The film was then hydrated (1 ml) with vortexing for 10 min at room temperature. The opaque suspension of multilamellar vesicles was then sonicated for 10 min on ice by using an ultrasonic generator with a microtip probe (Vibracell, Sonics and Materials Inc., Danbury, CT) at power setting 4 and 50% duty cycle. After sonication, the samples were centrifuged for 15 min at 35,000 *g*. The lipid content and gramicidin concentration in the resulting SUV were determined as in Bañó et al. (1991). The samples were deliberately incubated for a long time to enable an appropriate, reliable determination of the rate constants.

High-performance liquid chromatography

The liquid chromatograph (from Waters Chromatography Div., Millipore, Milford, MA) as well as the general strategy and experimental conditions for elution were as previously described (Bañó et al., 1991). Briefly, the method consisted of the direct injection of a few microliters (typically 2 μl) of the gramicidin-containing SUV suspension onto an Ultrastyragel 1,000-Å column, which was eluted with a nonpolar solvent such as THF. Upon injection, the liposomes are immediately disrupted on top of the column by the mobile phase, releasing to the eluent stream the peptide conformational species and lipid molecules, which are separated by a size-exclusion mechanism. Interestingly, the percentage of APDS dimers and β-helical monomers eluted from the column is not expected to differ from the values originally in the vesicles, on the basis of the extremely slow dimer-monomer transition undergone by gramicidin in nonpolar solvents (Braco et al., 1986; Bañó et al., 1988). Therefore, the proportion of conformers obtained in a given chromatogram can be considered as a very reliable, accurate measurement of their actual proportion in the original SUV. All chromatographic measurements were made in triplicate and the standard deviation was always <3%.

Circular dichroism

CD measurements were carried out at 25°C with a spectropolarimeter (Mark III, Jobin Ivon Instruments Division, Longjumeau, France) using a 0.5-mm optical pathlength cell. Blank runs of vesicles in water were subtracted from the measured spectra of gramicidin-containing samples. Ellipticity values (θ) were expressed on a mean residue basis,

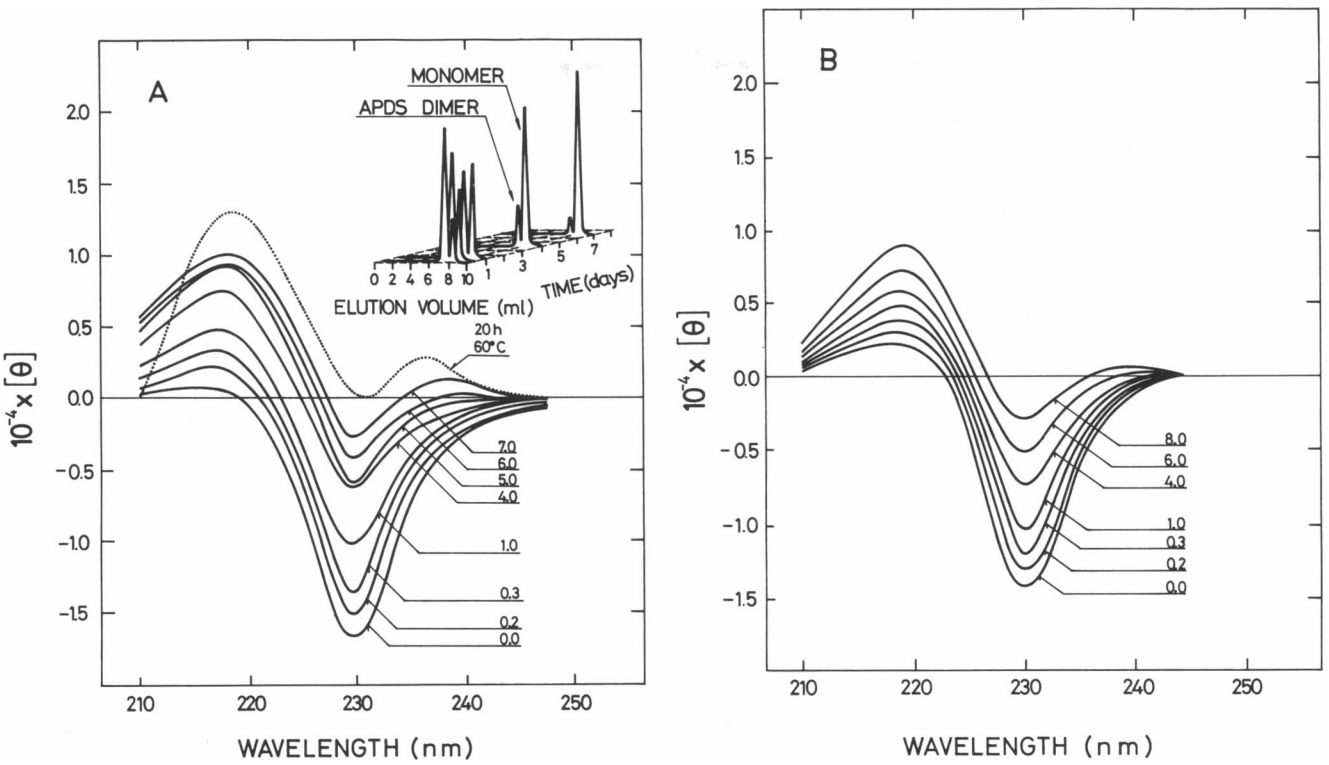

FIGURE 1 Changes in the CD spectra of gramicidin A incorporated in EPC SUV prepared by cosolubilization from THF (*A*) and ethanol (*B*), associated to the dissociation of double-stranded dimers into $\beta^{6.3}$-helical monomers, as simultaneously monitored by HPLC (*inset*). The numbers in the figure indicate the time of vesicle incubation at room temperature, in days. The dotted spectrum corresponds to a sample which was incubated for 20 h at 60°C. The final peptide concentration was 0.074 mg/ml and the lipid/peptide mole ratio was 50. (*Inset*) elution profiles obtained at different incubation times. Chromatographic conditions: eluent, THF; flow-rate, 1.0 ml/min; UV detection at 294 nm; injection volume, 2 μl.

in units of deg cm^2 d mol^{-1}. Each reported spectrum was the average of three independent scans. For a strict comparison of CD and HPLC results, the samples were simultaneously measured by both techniques.

RESULTS AND DISCUSSION

To verify whether the hypothesis of a two-component model can satisfactorily account for the CD spectral patterns of gramicidin A inserted in a phospholipid bilayer, a kinetic study of the peptide conformational transition simultaneously monitored by CD and HPLC becomes critical.

In the effort reported here experimental conditions for protocol preparation were deliberately selected to obtain fresh EPC SUV vesicles containing a high proportion of APDS dimers, which as reported recently would be expected to assume a characteristic 'nonchannel' CD pattern (Bañó et al., 1991). This is reflected in the CD spectra at zero time shown in Fig. 1 (*bottom curves*) for vesicles prepared from THF (*A*) or ethanol (*B*), exhibiting the typical large negative peak at 229 nm. Because it is known (Killian et al., 1988, LoGrasso et al., 1988, Bañó et al., 1991) that the system proceeds with elapsed time towards the thermodynamically stable $\beta^{6.3}$-helical channel conformation (Fig. 1, *A* and *B*), the above strategy permits to maximize the range of CD spectral changes. Note, however, that even after seven days of incubation, the spectra do not correspond exactly to the characteristic well-defined $\beta^{6.3}$-helical configuration which, as expected (Urry et al., 1979; Masotti et al., 1980; Killian et al., 1988; Bañó et al., 1989), is generated much faster after extensive incubation of the sample at high temperature (see *dotted curve*, Fig. 1 *A*).

The inset in Fig. 1 *A* illustrates, as an example, the elution profiles corresponding to the slow time-dependent variation in the proportion of APDS dimers and $\beta^{6.3}$-helical monomers associated to the spectral changes for the THF-derived sample. Similar profiles were obtained for gramicidin incorporated from ethanol (not shown). It is evident that immediately upon peptide insertion in the bilayer from both solvents the observed "nonchannel" conformations (Figs. 1, *A* and *B*, zero time) imply a predominance of APDS conformers. However, neither of the "nonchannel" patterns can be considered as representative of a pure gramicidin APDS component because even at zero time for the vesicles prepared from THF there is already an appreciable proportion of monomeric forms (~25%). On the other hand, after a long incubation period (seven days) a complete monomerization is not either achieved (~12% of APDS dimers remain in the vesicles) which explains the noncoincidence of the corresponding spectra (Figs. 1, *A* and

B, solid top curves) with the channel pattern. HPLC analysis of the sample heated at 60°C for 20 h (channel conformation) yielded >98% of monomeric forms.

Next, the experimental data from both techniques were fitted to a first-order kinetic model. In the case of CD, to avoid inconsistencies derived from the change of sign of the ellipticity values, the kinetic data were analyzed in terms of differences in ellipticity, $\Delta\theta^{\lambda}_{obs}(t)$, at any fixed wavelength, λ, as a function of incubation time, t. $\Delta\theta^{\lambda}_{obs}(t)$ is defined as:

$$\Delta\theta^{\lambda}_{obs}(t) = \theta^{\lambda}_{obs}(\infty) - \theta^{\lambda}_{obs}(t), \quad (1)$$

where $\theta^{\lambda}_{obs}(t)$ denotes the observed ellipticity at a given time, and $\theta^{\lambda}_{obs}(\infty)$ refers to the experimentally determined ellipticity after extensive heating of the sample (see dotted curve in Fig. 1 *A*). Hence, the first-order linearized equation can be expressed as:

$$\ln \Delta\theta^{\lambda}_{obs}(t) = \ln \Delta\theta^{\lambda}_{obs}(0) - k_1^{CD}t, \quad (2)$$

where $\Delta\theta^{\lambda}_{obs}(0)$ corresponds to the ellipticity difference at zero time and k_1^{CD} refers to the rate constant for the gramicidin dissociation process in the membrane bilayer as determined by CD.

In a parallel manner, in the case of HPLC, the kinetic results were expressed as the variation of the mass fraction of APDS-dimers as previously reported (Bañó et al., 1991). The corresponding first-order linearized integrated equation for the irreversible transition:

$$M_2 \xrightarrow{k_1^{HPLC}} 2M \quad (3)$$

is given by:

$$\ln [M_2] = \ln [M_2]_0 - k_1^{HPLC}t, \quad (4)$$

k_1^{HPLC} denoting the rate constant for the APDS dissociation process in the lipid environment as determined chromatographically. Note that if the above working hypothesis is correct both k_1^{CD} and k_1^{HPLC} values are expected to be the same.

Fig. 2, *A* and *B*, depict the kinetic progress curves obtained from both techniques for the samples prepared using THF or ethanol as cosolubilizing solvent. A wavelength of 229 nm was selected to maximize the observed time-dependent ellipticity changes (Fig. 2 *A*). As expected, in agreement with the previously reported solvent memory of the inserted peptide conformation (Bañó et al., 1991) the proportion of double-stranded dimers was higher for the vesicles prepared from the less polar solvent (Fig. 2 *B*). This correlated well with the more negative zero time θ_{229} value for the THF-derived sample (Fig. 1 *A*). Note that regardless of the differences observed for both samples at zero time the curves become congruent after a long incubation period.

In all cases, good fittings were obtained when plotting the kinetic data in Figs. 2, *A* and *B* according to Eqs. 2

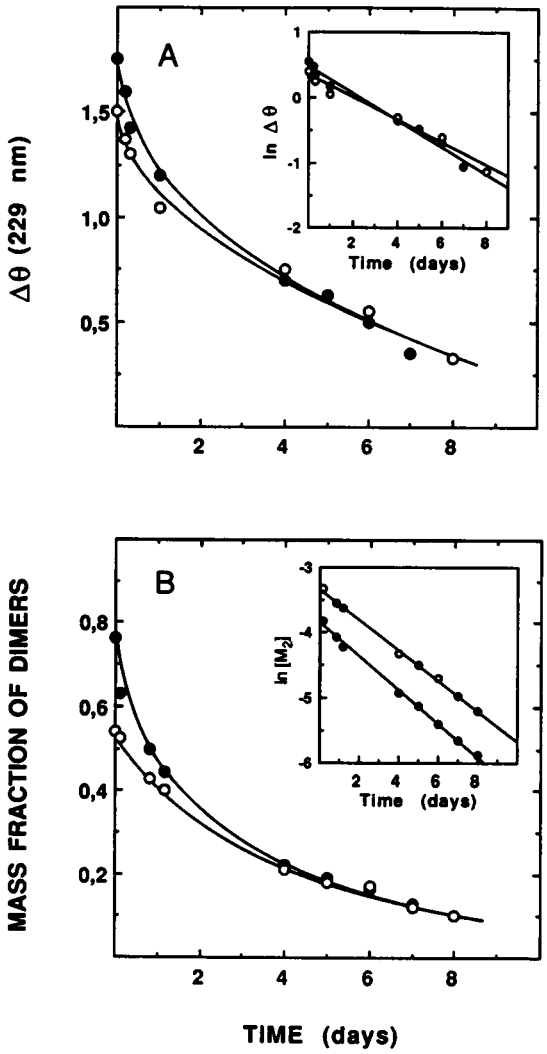

FIGURE 2 Kinetic progress curves of gramicidin double-stranded dimer dissociation in EPC SUV simultaneously monitored by CD (*A*) and HPLC (*B*). The lipid and peptide were cosolubilized in THF (●) or ethanol (○). The insets depict the fitting of the data to Eq. 2 (*A*) and Eq. 4 (*B*). Peptide and lipid concentrations as in Fig. 1.

and 4 (see *insets*). k_1^{CD} values of 2.3×10^{-6} s^{-1} (THF) and 2.0×10^{-6} s^{-1} (ethanol) were determined, in excellent agreement with those obtained for k_1^{HPLC}, 2.4×10^{-6} s^{-1} (THF) and 2.2×10^{-6} s^{-1} (ethanol). Thus, it seems established that once gramicidin is incorporated in the bilayer, the rate of APDS-dimer dissociation is independent of the solvent used in the cosolubilization step. More interestingly, the consistency of these results from both techniques seems to support that only two components are sufficient to interpret the CD data in Fig. 1. This encouraged us to express the observed ellipticity $\theta^{\lambda}_{obs}(t)$, at any fixed wavelength, as a linear combination of the ellipticity values of the pure conformational species inserted in the membrane and isolated by HPLC. Thus, $\theta^{\lambda}_{obs}(t)$ would be represented by the weighted values, f_i, of the appropriate reference spectra:

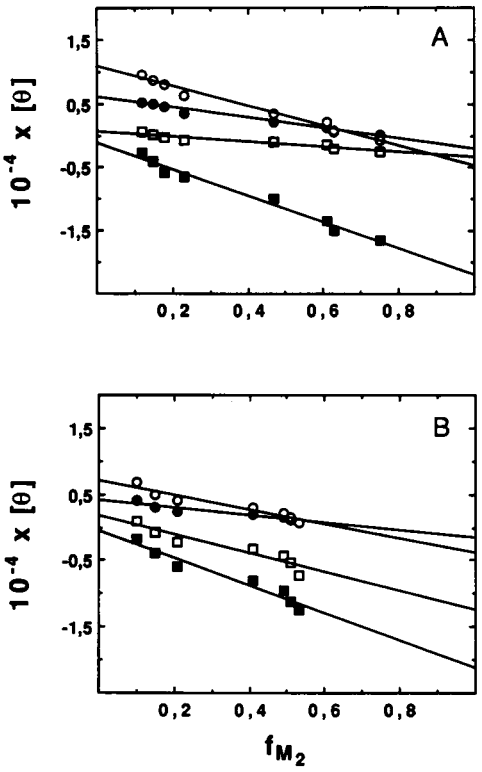

FIGURE 3 Fitting of the kinetic data from CD (Fig. 1) at different wavelengths, and from HPLC (Fig. 2 *B*), according to Eq. 6 for samples cosolubilized from THF (*A*) and ethanol (*B*). The selected wavelengths were: 210 (●), 220 (○), 230 (■), and 240 (□) nm in *A*; 212 (●), 222 (○), 226 (□), and 232 (■) nm in *B*.

$$\theta^{\lambda}_{obs}(t) = f_{M_2}\theta^{\lambda}_{M_2} + f_M\theta^{\lambda}_M, \quad (5)$$

where f_{M_2} and f_M refer to the HPLC-determined mass fractions of APDS-dimers and monomers respectively. $\theta^{\lambda}_{M_2}$ and θ^{λ}_M denote the reference ellipticities at λ wavelength of the APDS-dimer and the $\beta^{6.3}$-helical monomer in the lipid environment, respectively.

As $f_{M_2} + f_M = 1$, it can be written:

$$\theta^{\lambda}_{obs}(t) = \theta^{\lambda}_M + (\theta^{\lambda}_{M_2} - \theta^{\lambda}_M)f_{M_2}. \quad (6)$$

If the proposed statement is correct, a plot of $\theta^{\lambda}_{obs}(t)$ vs f_{M_2} at any given wavelength should yield a straight line, so that θ^{λ}_M and $\theta^{\lambda}_{M_2}$ can be easily determined from the intercept values at $f_{M_2} = 0$ and $f_{M_2} = 1$, respectively.

When the CD (Fig. 1, *A* and *B*) and HPLC (Fig. 2 *B*) kinetic data were plotted according to Eq. 6 in all the range of wavelength values from 210 to 240 nm (at two-nm intervals), for vesicles prepared from THF or ethanol, good fittings were obtained in all cases. As an example, Fig. 3 shows the results for a representative set of wavelengths when the cosolubilizing solvent was THF (Fig. 3 *A*) or ethanol (Fig. 3 *B*). Again, these results provide supportive evidence for the working hypothesis. As expected, the values of $\theta^{\lambda}_{M_2}$ and θ^{λ}_M at each wavelength (see below), estimated independently for the THF- and ethanol-derived samples, did not differ significantly, in agreement with the assumption that once inserted in the membrane the conformation of both APDS dimer and $\beta^{6.3}$-helical monomer must be dictated by the particular lipid environment and not by the solvent used for reconstitution (Bañó et al., 1989; 1991). Hence, for each pure conformer an average of each pair of reference ellipticity values (THF and ethanol) at each wavelength was used in further calculations.

Fig. 4 depicts the virtual reference spectra estimated for both double-stranded dimer and monomer in EPC SUV (*dotted top* and *bottom curves*). Overall, the spectral features of the reference spectrum calculated for the monomer (Fig. 4, *dotted top curve*), broad positive band at 217 nm, less intense band at 237 nm and minimum at 229 nm, are characteristic of the $\beta^{6.3}$-helical conformation (Urry et al., 1983). In fact, this spectrum does not differ appreciably from that experimentally obtained after heating of the vesicles at 60°C (see Fig. 1 *A*) and is very similar to those previously reported for the $\beta^{6.3}$-helical conformation both after extensive heating of the samples (Masotti et al., 1980; Arseniev et al., 1985; Shungu et al., 1986; Killian et al., 1988) or after incorporation

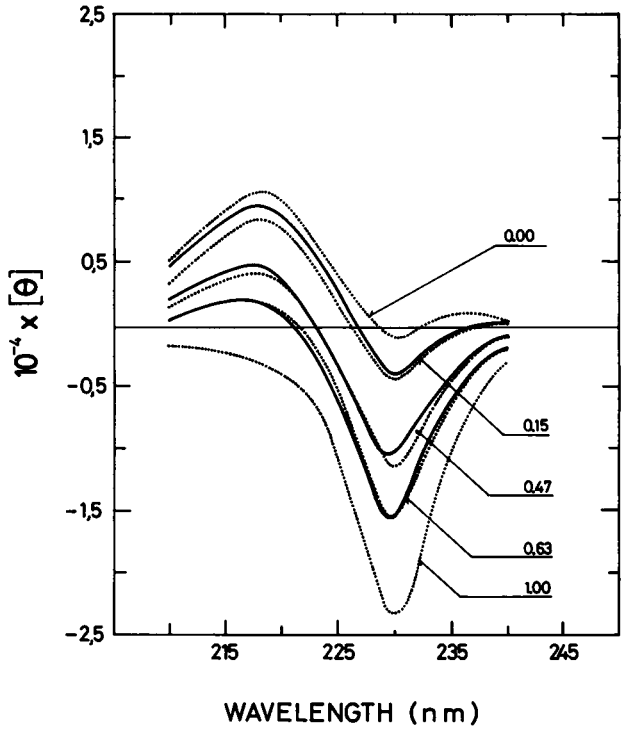

FIGURE 4 Simulation of CD spectra of gramicidin A in EPC SUV (*dotted curves*) by using Eq. 6, for different proportions of double-stranded dimers in the vesicles, indicated in the figure as mass fraction values. Note that the curves denoted by 1.00 and 0.00 correspond to the reference spectra of pure double-stranded dimer and $\beta^{6.3}$-helical monomer, respectively. For comparison are included the experimental spectra (*solid curves*) obtained for mixtures of both conformers in the vesicles (taken from Fig. 1 *A*), at the same dimer mass fractions as for the theoretical ones.

from solvents such as TFE or DMSO (Tournois et al., 1987; LoGrasso et al., 1988; Killian et al., 1988) where monomerization is known to be quantitative (Bañó et al., 1989, 1991). Conversely, the CD reference spectrum estimated for the double-stranded dimer (Fig. 4, *bottom dotted curve*) exhibits a completely distinct pattern with an intense negative ellipticity at 229 nm.

Some considerations are worth being made at this point. Unfortunately, as opposed to the case of the $\beta^{6.3}$-helical monomer, the reference spectrum estimated for the dimer cannot be contrasted with any experimentally measured in this lipid environment, and therefore should be strictly considered as a virtual curve representative of this pure conformer in this model membrane system. This is so because even if conditions for reconstitution are selected to try to preserve this configuration, e.g., use of nonpolar solvents, high peptide concentration, low lipid-to-peptide ratio, short sonication time, etc. (Bañó et al., 1991), it must be realized that the manipulations inherent in the preparation protocol (cosolubilization with the lipid, sonication, heating, et cetera) will cause a partial dissociation of the APDS dimers (Bañó et al., 1991) and a certain proportion of monomeric forms (typically >20%) will be already present in the fresh vesicles. Moreover, the alternative strategy of directly adding the peptide to preformed vesicles either from a nonpolar solvent or as a powder does not seem to result in an adequate incorporation into the bilayer unless the sample is subjected to extensive heating (Masotti et al., 1980) and/or sonication (Tournois et al., 1987).

It is interesting to note, however, that the estimated reference spectrum for the double-stranded dimer in Fig. 4, although not identical, qualitatively resembles that described for the antiparallel double-helical dimer in dioxane (Veatch et al., 1974) or those reported for lysophosphatidylcholine (LPC) micelles at short times after incorporation of the peptide as a powder (Urry et al., 1979; Masotti et al., 1980; Bañó et al., 1989) or from chloroform/methanol (1/1, v/v) and ethanol solution (Killian and Urry, 1988). Curiously, this reference spectrum is also similar to that recently reported for gramicidin B ($Trp^{11} \rightarrow Phe$) incorporated into LPC micelles, which shows a certain inability to relax toward the 'channel' conformation upon extensive heating (Sawyer et al., 1990). In this regard, it should be stressed that although the $\beta^{6.3}$-helical configuration is thermodynamically favored for gramicidin A in a phospholipid environment, it may not always be necessarily the preferred conformation for amino acid-substituted gramicidins (Andersen et al., 1988), particularly with replacement of the tryptophans (Van Mau et al., 1988), especially in the light of our recent HPLC finding that the energetically favored conformation for the optically reversed gramicidin M ($Phe^{9,11,13,15}$) is a double-stranded dimer in both small unilamellar vesicles and LPC micelles (not shown). Interestingly, Andersen and co-workers have recently reported from conductivity studies that the rearrangement of Trp and Leu residues in a gramicidin analog increases the stability of a conducting double-stranded conformer with respect to $\beta^{6.3}$-helical monomers and dimers (Koeppe et al., 1991).

Once the theoretical reference spectra for APDS dimer and $\beta^{6.3}$-helical monomer were estimated in EPC SUV, the possibility was checked of simulating gramicidin CD curves by simply using Eq. 6, for vesicles containing different proportions of conformers. Fig. 4 shows, as an example, the simulated curves for double-stranded dimer mass fraction values of 0.63, 0.47, and 0.15 and the corresponding experimentally determined spectra for the same conformer proportions (*curves,* Fig. 1 *A* corresponding to incubation times of 0.2, 1, and 6 d). A remarkable resemblance can be observed between theoretical and experimental spectra. Also, a good agreement was obtained for the rest of curves in Fig. 1 (not shown). From these results it seems clear that at zero time (fresh vesicles) as well as at any given incubation time after incorporation in the membrane the observed gramicidin CD spectra can be essentially considered as linear combinations in different proportions of double-stranded dimers and $\beta^{6.3}$-helical monomers. Thus, provided that the mass fraction of the inserted conformers is known (at present HPLC seems to be the method ideally suited for this purpose) it is possible to reproduce the CD pattern for a given sample of gramicidin in this model system, regardless of the protocol followed for vesicle reconstitution.

Finally, it should be emphasized that because the reference spectra for the intertwined dimers and helical monomers in this work have been obtained in EPC SUV, care should be taken in any attempt to extrapolate them from a quantitative point of view to other lipid environments. There is no reason to believe that there is a unique CD spectrum for an APDS-dimer (or β-helical monomer) in different phospholipid model membranes. It can be expected that constraints imposed by a type of lipid organization (SUV, LUV, MLV, micelles, hexagonal H_{II} phase, et cetera) or a specific class of phospholipid or lipid composition could affect the reference spectra for either conformer. Thus, for instance, orientational effects have been suggested to be involved in the slight differences observed for the CD spectrum of gramicidin incorporated in dilauryl-phosphatidylcholine aligned multilayers as compared with that obtained in small unilamellar vesicles (Huang and Olah, 1987).

CONCLUSION

We provide in this paper convincing quantitative evidence by using HPLC and CD in combination that a given CD pattern of gramicidin incorporated in a phospholipid bilayer can be deconvoluted essentially as a linear combination of the reference spectra of two components, double-stranded dimers and $\beta^{6.3}$-helical monomers. Also, the strategy employed has made it possible

for the first time to reliably estimate a virtual reference spectrum for the pure (not contamined by other conformations) gramicidin intertwined dimer in EPC small unilamellar vesicles. On the basis of our results, a semiempirical approach is proposed for the simulation of CD curves of gramicidin incorporated in this lipid environment, provided that the proportion of conformers is known. Finally, it should be mentioned that this treatment could be easily extended to the investigation of other self-associating peptides (e.g., amino acid-substituted gramicidin analogs [Becker et al., 1991; Koeppe et al., 1991]) or perhaps more complex systems which are suspected to undergo self-association processes in membranes.

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