Characterization of Gramicidin A in an Inverted Micellar Environment. A Combined High-Performance Liquid Chromatographic and Spectroscopic Study†

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Received March 4, 1992; Revised Manuscript Received May 26, 1992

ABSTRACT: We have investigated the conformational adaptability of gramicidin A incorporated into reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate (AOT)/isooctane/water, a so far unexplored "host" membrane–mimetic model system for this peptide. A high-performance liquid chromatographic strategy previously developed for the study of gramicidin in phospholipid vesicles and normal micelles [Bañó et al. (1989) *FEBS Lett. 250*, 67; Bañó et al. (1991) *Biochemistry 30*, 886] has been successfully extended to this system. The method has permitted the separation of peptide conformational species, namely, double-stranded dimers and monomers, and an accurate quantitation of their proportion in the inverted micellar environment. It has been demonstrated that, once inserted in the micelle, the double-stranded dimers undergo a dissociation process toward a thermodynamically stable monomeric configuration, whose monomerization rate constant (k_1) is dependent in a bell-shaped manner on the water:surfactant mole ratio, w_0. A tight correlation between k_1 and the double-stranded dimer backbone conformation has been found from the comparison of chromatographic and circular dichroism data. In addition, fluorescence experiments indicate that the peptide tryptophans are in a rather nonpolar environment, with a restricted accessibility to water-soluble quenchers such as acrylamide.

Whereas a lot of attention has been paid in past years to the investigation of the structure and functioning of many proteins and enzymes (water-soluble as well as membrane-associated) in surfactant-based reverse micellar systems [for a review, see, e.g., Martinek et al. (1986), Luisi and Steinmann-Hofmann (1987), Luisi et al. (1988)], relatively poor information has been obtained so far on the behavior of bioactive peptides of moderate or high hydrophobicity (Kleinkauf & Von Döhren, 1990) in these organized microassemblies. In particular, it seems surprising that no study has been reported dealing with the conformational behavior in an inverted micellar environment of the hydrophobic polypeptide antibiotic gramicidin A, a prototypic model for membrane-spanning proteins which has been profusely investigated in phospholipid bilayers and aqueous micelles (as well as in organic solution) for about two decades [for a review, see Wallace (1990)].

Gramicidin A is a linear, D,L-alternating pentadecapeptide of sequence formyl-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-ethanolamine, which can act as a transbilayer channel specific for monovalent cations when incorporated into a model membrane (Andersen, 1984; Urry, 1985). Among the diverse folding motifs described for this peptide both in organic solvents (Veatch et al., 1974; Sychev et al., 1980; Braco et al., 1988a; Roux et al., 1990) and in membrane-like environments (Wallace, 1986, 1990; Bañó et al., 1991), there is at present strong supportive evidence that the predominant membrane-associated conformers are (antiparallel) double-stranded (APDS)[1] dimers and $\beta^{6.3}$-helical monomers (Bañó et al., 1989), its actual ratio being determined by the "history" of the protocol used for reconstitution (Bañó et al., 1991, 1992). In this respect, an absence of solvent-history effects on gramicidin single-channel properties has been found in planar lipid bilayers, likely due to the very low peptide/lipid ratios used relative to spectroscopic studies (Sawyer et al., 1990). It has also been recently demonstrated that the APDS dimers slowly interconvert into monomers in phospholipid bilayers as well as in normal micelles (Bañó et al., 1991, 1992). On the other hand, it is generally accepted that the functionally active gramicidin channel consists of a dimer formed by the N-terminal to N-terminal transmembrane juxtaposition of two $\beta^{6.3}$-helical monomers (Urry et al., 1983; Andersen, 1984; Arseniev et al., 1986; Wallace, 1986; Nicholson & Cross, 1989; Teng et al., 1991).

A study of gramicidin in a reverse micellar milieu seems interesting for several reasons. (i) It has been shown to promote in a conformation-dependent manner the formation of inverted nonbilayer structures [for a review, see Killian and De Kruijff (1986)], particularly hexagonal H_{II} phases (Tournois et al., 1987a), from both model and biological membranes (Tournois et al., 1987b). (ii) Reverse micelles have also been used as membrane–water interface mimetic systems to investigate membrane-bound (Binks et al., 1989; Nicot & Waks, 1989) or membrane-associated (Misiorowski & Wells, 1974) proteins which in some cases can self-associate or be part of multimeric systems. In this regard, gramicidin offers a valuable opportunity of analyzing in detail the behavior of a relatively simple, self-associating membrane peptide as a model for more complex, protein oligomeric assemblies. (iii) From a more general point of view, the information inferred from this system could be exploited for eventual biotechnological or therapeutical applications, e.g., the biocompatible lipid-based microemulsion-mediated transdermal delivery of water-insoluble (polypeptide) drugs (Willimann & Luisi, 1991).

In this preliminary work, the sodium bis(2-ethylhexyl)-sulfosuccinate (AOT)/isooctane/water system has been selected as a "host" membrane–mimetic, optically transparent, inverted micellar model system for gramicidin, because it is simple and well characterized (Luisi & Steinmann-Hofmann,

† This work was supported by Grant PB90-0578 from DGICYT (Spain).

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[1] Abbreviations: AOT, sodium bis(2-ethylhexyl)sulfosuccinate; APDS, antiparallel double-stranded; CD, circular dichroism; HPLC, high-performance liquid chromatography; NATA, *N*-acetyl-L-tryptophanamide; TFE, trifluoroethanol; THF, tetrahydrofuran.

0006-2960/92/0431-8072$03.00/0

1987; Luisi et al., 1988). Taking advantage of a recently reported, versatile high-performance liquid chromatography (HPLC) strategy allowing the separation of gramicidin conformational species in diverse model membranes (Bañó et al., 1988, 1989, 1991), we have evidenced and quantitatively characterized a dimer–monomer conformational transition undergone by the peptide in the reverse micelles. To gain insight into the peptide location and backbone conformation in the micellar environment, the chromatographic information has been complemented with circular dichroism (CD), steady-state fluorescence, and fluorescence quenching and anisotropy experiments. The results are discussed in the light of current knowledge on the conformational behavior of gramicidin in model membrane systems.

EXPERIMENTAL PROCEDURES

Materials. Gramicidin (a natural mixture predominantly composed of gramicidin A, ca. 85%) was purchased from Koch Light Laboratories (Buckinghamshire, U.K.) and was used without further purification. AOT was obtained from Serva (Heidelberg, FRG), purified by preparative reversed-phase HPLC according to the method proposed by Luisi and Steinmann-Hofmann (1987), and routinely vacuum-dried overnight prior to use to ensure a minimum water content. *N*-Acetyl-L-tryptophanamide (NATA) was obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide was from Merck (Darmstadt, FRG). Tetrahydrofuran (THF) and all other organic solvents were of 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. Bidistilled water was purified through a Millipore Milli-Q system (Millipore, Milford, MA).

Preparation of Gramicidin-Containing Reverse Micelles. The micellar solutions were prepared by adding measured volumes of isooctane to dried, preweighed amounts of AOT and further injecting the required volume of water and shaking. A small volume (3–20 μL) of equilibrated, concentrated stock solutions of the peptide [in THF or trifluoroethanol (TFE)] was then injected into the preformed microemulsion using a Hamilton syringe, and the sample was vortexed until optically clear. In the case of incorporation of gramicidin as a dimer (from THF), the sample was always measured immediately after addition of the peptide and shaking for 30 s (zero time), to minimize the initial monomerization. To incorporate gramicidin as a monomer, the peptide was added from TFE. Care was taken to ensure that the final volume of the added solvent never exceeded 1% (v/v) in HPLC or 0.1% (v/v) in fluorescence experiments. Whenever possible (depending on how fast the kinetic changes monitored occurred), the samples were centrifuged or filtered to remove any undissolved, non-incorporated material. The water:surfactant mole ratio was expressed as w_0. Gramicidin concentration was determined by HPLC (Bañó et al., 1991) or spectrophotometry by dilution of the sample in methanol using a molar extinction coefficient of 20 700 cm^{-1} M^{-1} (Killian et al., 1988). In a typical experiment, the surfactant concentration was 100 mM and the surfactant:peptide mole ratio of about 1850 (HPLC and CD experiments) or higher (fluorescence experiments). In the study of the influence of the AOT:gramicidin mole ratio, where lower mole ratios were assayed, the procedure for peptide incorporation consisted of AOT and gramicidin cosolubilization in THF, followed by solvent evaporation to form a mixed film prior to the addition of isooctane and the desired amount of water. In all of the experiments where NATA was used, it was added to the micellar solution from a concentrated TFE stock solution.

High-Performance Liquid Chromatography. The liquid chromatograph (from Waters Chromatography Division, Millipore) and 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 reverse micellar solution onto an Ultrastyragel 1000-Å column which was eluted with THF. Since the mobile phase is water-miscible, the surfactant microassemblies are expected to be immediately disrupted on top of the column, releasing to the eluent stream the peptide conformational species and AOT molecules, which will be separated by a size-exclusion mechanism. Interestingly, since the gramicidin dimer–monomer transition in THF is extremely slow as compared to the elution time (Braco et al., 1986a,b; Bañó et al., 1988), the percentage of APDS dimers and β-helical monomers in a given chromatogram can be considered as a very reliable, accurate measurement of their actual proportion in the original reverse micellar medium. It was verified that AOT as well as isooctane/water (all eluted overlapped at >10 mL) did not interfere either peak height or peak resolution of gramicidin conformers.

Circular Dichroism. CD measurements were carried out at 25 °C with a Jobin Yvon Mark III spectropolarimeter using a 1-mm optical path length cell. The spectra of peptide-containing reverse micelles were corrected by subtracting blank runs of peptide-free micellar solutions performed at appropriate w_0 values. Ellipticity (θ) values were expressed on a mean residue basis, in units of deg cm^2 $dmol^{-1}$. Each reported spectrum was the average of three independent scans.

Fluorescence Measurements. Corrected fluorescence emission spectra were recorded from 290 to 450 nm on a Perkin-Elmer LS-5B spectrofluorometer at 25 °C. Excitation and emission slits with a nominal bandpass of 2.5 nm were used. Excitation wavelength was 280 nm. Each reported spectrum was obtained after the corresponding peptide-free reverse micelle blank spectrum under identical conditions at appropriate w_0 value was subtracted. An inner filter correction was applied according to the method of Parker (1968). All fluorescence experiments were carried out at a surfactant:peptide mole ratio of 18 500.

In the quenching experiments, for each w_0 assayed, the titration by acrylamide was performed by preparing the micelles containing the appropriate quencher concentration and then adding the peptide. An inner filter correction for the absorbance by acrylamide was found not to be necessary.

In steady-state anisotropy measurements, emission was measured at 330 nm for an excitation wavelength of 280 nm. The nominal bandpass of the excitation and emission slits was 10 nm. The rest of the experimental conditions were similar to those indicated above. The anisotropy, r, values were obtained as previously reported (Braco et al., 1988b) and represent in each case an average of 20 data acquisitions. In all cases, the relative standard deviation of intensity measurements was less than 1%, resulting in anisotropies precise to within ±0.001.

Other details of experimental conditions are given in the corresponding legends to figures.

RESULTS

High-Performance Liquid Chromatography. It has been previously reported that for a given peptide concentration the proportion of APDS dimers and monomers in organic solution dramatically depends on the polarity of the solvent (Braco et

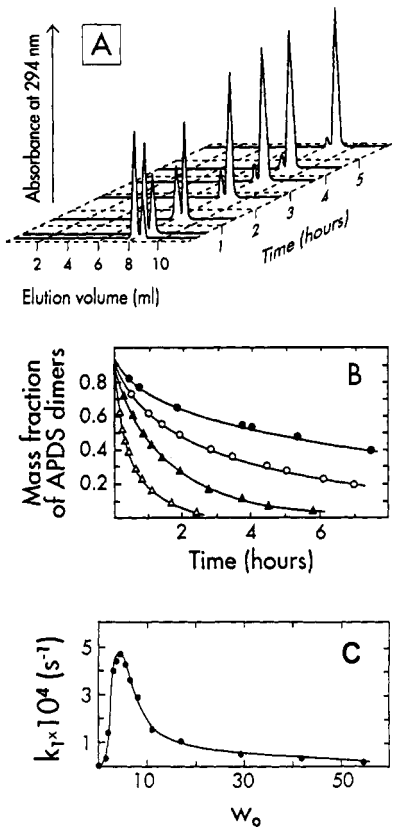

FIGURE 1: (A) Time course of the conformational transition of gramicidin A incorporated in reverse micelles of 100 mM AOT/isooctane, w_0 = 11.2, as monitored by HPLC. The peptide was added to the micellar solution from a THF stock solution, and its final concentration was 0.1 mg/mL, which corresponds to a surfactant:peptide mole ratio of ca. 1850. Chromatographic conditions: mobile phase, THF; flow rate, 1.0 mL/min; injection volume, 2 μL; UV detection at 294 nm. For clarity, the peaks corresponding to AOT and isooctane/water (eluted at >10 mL) are not shown in the chromatograms. (B) Kinetic profiles of the monomerization of gramicidin in reverse micelles at different w_0 values, expressed as the variation with incubation time of the mass fraction of APDS dimers. (●) w_0 = 54.9; (○) w_0 = 29.2; (▲) w_0 = 11.2; (△) w_0 = 6.7. (C) Dependence of the HPLC-determined gramicidin monomerization rate constant, k_1, in 100 mM AOT reverse micelles, on the droplet water content. Peptide concentration: 0.1 mg/mL.

al., 1988a; Bañó et al., 1989). Thus, when dissolved in THF at high concentration, >95% of the peptide exists as APDS dimers, whereas in TFE it exhibits predominantly a monomeric configuration (Braco et al., 1986a; Bañó et al., 1989). In all of the chromatographic experiments reported below, gramicidin was deliberately added to the AOT/isooctane reverse micellar solution from a concentrated stock solution in THF so that the peptide was almost quantitatively inserted in the micelles as APDS dimers. It must be pointed out that solubilization of gramicidin in pure isooctane was not possible, as verified from the analysis of the filtrate from a suspension of the peptide powder. However, when the peptide was added to the AOT microemulsion, it could be easily solubilized in a wide range of w_0 values, yielding in all cases optically transparent solutions. Therefore, gramicidin concentration in the bulk isooctane can be considered negligible, the peptide being actually incorporated into the reverse micelles.

Figure 1A depicts, as an example, the elution profiles obtained after injection onto the HPLC column of aliquots of a gramicidin-containing reverse micelle sample taken at different incubation times. In all cases, two well-resolved peaks were eluted at 7.9 and 8.4 mL (exactly the same elution volumes obtained for an AOT-free system), corresponding to double-stranded dimers and monomers, respectively (Braco et al., 1986a; Bañó et al., 1991), which corroborates the aforementioned hypothesis about the micelle disruption by the mobile phase and the consequent release of the two peptide conformational species, separated on the basis of a size exclusion mechanism. No interference due to the surfactant was detected either compromising resolution or altering peak shape. As the figure shows, a time-dependent transition can be easily monitored by HPLC corresponding to the progressive dissociation of APDS dimers into monomers. The mass fraction of APDS dimers and monomers can be directly determined at any time from the chromatograms, as previously reported (Bañó et al., 1989). Interestingly, when w_0 was varied at constant surfactant concentration, the rate of APDS dimer dissociation also significantly varied, as shown in Figure 1B. The trend was similar in all cases, however, regardless of the droplet water content: the dimers underwent a transition toward a monomeric configuration, which seems to be thermodynamically favored in the micelle environment. This was supported by the observation that when gramicidin was added to the microemulsion from a stock TFE solution, HPLC analysis of the peptide-containing reverse micelles revealed >98% of monomers already at zero time, a situation which did not change upon sample incubation.

To verify that the transition observed actually corresponded to a process of dissociation of APDS dimers (M_2) into monomers (M), the data in Figure 1B were fitted to the integrated equation for an irreversible dissociation kinetic model ($M_2 \rightarrow (k_1)\ 2M$) (Bañó et al., 1991)

$$\ln [M_2] = \ln [M_2]_0 - k_1 t \qquad (1)$$

where $[M_2]_0$ and $[M_2]$ refer to the APDS dimer concentration at zero time and at any given t time, respectively. k_1 denotes the rate constant for APDS dimer dissociation in the reverse micelles. The excellent fittings obtained in all cases (not shown) corroborate the above assumptions and demonstrate the accuracy in the chromatographic determination of k_1.

At this point, it seemed exciting to exploit the HPLC strategy to investigate the effect (if any) on k_1 of different experimental variables, such as w_0, AOT:gramicidin mole ratio, and incubation temperature. Figure 1C shows the variation of the monomerization rate constant with the droplet water content at room temperature and constant AOT concentration. k_1 values were obtained by fitting of the data in Figure 1B (and other data not shown) to eq 1. A bell-shaped curve was obtained with a maximum centered around w_0 = 5. This behavior seems particularly interesting because it offers a novel facet of the often encountered bell-shaped w_0 dependence of a biopolymer property, especially in the case of the enzymatic activity vs w_0 curves [see, e.g., Fletcher et al. (1985), Walde et al. (1988), and Khmelnitsky et al. (1989)]. It is worthwhile noting that when *p*-xylene was used instead of isooctane as an organic solvent, a similar k_1 vs w_0 bell-shaped curve was also obtained, although with lower absolute rate constant values (not shown).

With regard to the influence of incubation temperature, HPLC analysis revealed that, as a general trend in the whole range of temperatures assayed, the higher the temperature the higher the monomerization rate constant. As an example, activation energies for the dimer dissociation of 120 (w_0 = 1.6) and 100 (w_0 = 29.2) kJ/mol were determined, which are higher than that recently reported for the same transition in phosphatidylcholine small unilamellar vesicles (Bañó et al., 1991).

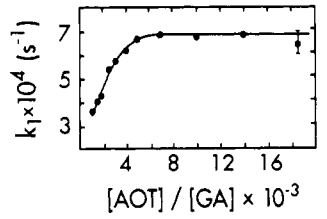

FIGURE 2: Dependence of the HPLC-determined gramicidin monomerization rate constant, k_1, in 100 mM AOT reverse micelles, on the surfactant:peptide mole ratio (the peptide concentration was varied), at w_0 = 5.6.

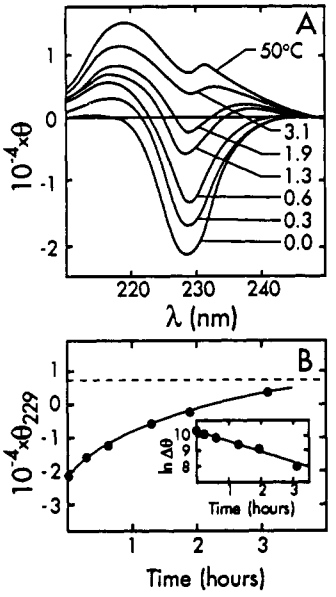

FIGURE 3: (A) CD patterns corresponding to the time course at 25 °C of the gramicidin monomerization in 50 mM AOT/isooctane reverse micelles (w_0 = 11.2). Peptide concentration: 50 μg/mL. The numbers in the figure indicate the time of incubation of the micellar solution, in hours. The CD spectrum denoted by 50 °C corresponds to an aliquot of the gramicidin-containing reverse micellar sample which was heated for several hours at this temperature. (B) Variation of the ellipticity at 229 nm from the curves in part A with the incubation time. The dashed line corresponds to the θ_{229} for the spectrum of the sample heated at 50 °C (quantitative monomerization of gramicidin). (Inset) First-order linearization of the data in part B, where $\Delta\theta$ denotes the difference in ellipticity at 229 nm between the asymptotic value (dashed line) and any given value at a given time.

The chromatographic strategy was also applied to investigate the influence of the surfactant:peptide mole ratio, R, on k_1. As Figure 2 shows, for constant AOT concentration and w_0 values, k_1 increases as R increases, leveling off at an R value of about 6000. Qualitatively, this trend is in agreement with recent data reported in phospholipid vesicles, where it has been suggested that a high peptide-to-lipid ratio can stabilize dimeric (nonchannel) conformations (Killian et al., 1988; Bañó et al., 1991). In addition, the assumption that k_1 decreases as gramicidin concentration increases as due to increasing peptide–peptide interactions is also supported by the fact that for R values clearly lower than 1000 phase separation occurred at room temperature.

Circular Dichroism. Figure 3A depicts the CD spectral changes associated with the gramicidin conformational transition in reverse micelles (w_0 = 11.2) from an initial situation where the mass fraction of APDS dimers was 0.90 (as revealed by HPLC, zero time) toward a final, equilibrium situation consisting predominantly of monomers. To ensure a quantitative monomerization, the sample was further heated at 50 °C for 2 h (top curve), the chromatographic analysis revealing

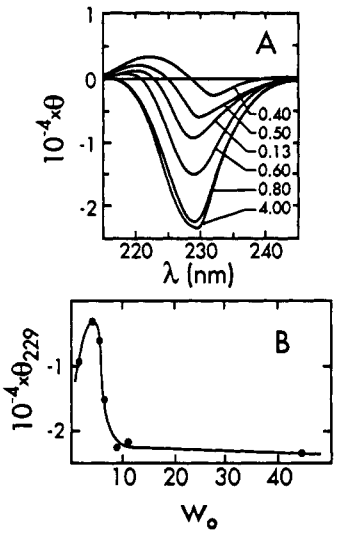

FIGURE 4: (A) CD patterns of 50 mM AOT reverse micelles containing gramicidin predominantly as a double-stranded dimer (see text) as a function of the droplet water content. Peptide concentration: 50 μg/mL. The numbers in the figure refer to the percentage of water in the micellar solution (v/v). (B) Variation of the ellipticity at 229 nm from the curves in part A as a function of w_0.

in this case >98% of monomeric forms. Interestingly, the CD spectra corresponding to the two extreme situations in reverse micelles are very similar to those recently reported for APDS dimers (nonchannel configuration) and $\beta^{6.3}$-helical monomers, in phospholipid model membranes (Killian et al., 1988; LoGrasso et al., 1988; Bañó et al., 1991, 1992) as well as in lysophosphatidylcholine aqueous micelles (Killian & Urry, 1988; Bañó et al., 1989). It seems reasonable to assume that any intermediate spectrum in Figure 3A between the bottom and the top ones is due to the sum of two independent contributions assigned to two well-defined conformational states of the polypeptide, i.e., APDS dimers and monomers (Bañó et al., 1992). In fact, the time dependence variation of the ellipticity at 229 nm (Figure 3B) could be satisfactorily fitted to a first-order model (see inset). Under these conditions, a rate constant of 2.0 × 10^{-4} s^{-1} was obtained. Thus, the changes in ellipticity with elapsed time in Figure 3A reflect the increasing contribution to the total spectrum of the monomeric configuration.

To investigate the dependence of the CD spectra of each conformer on the droplet water content, the following experiment was designed. In the case of the dimer, gramicidin was injected from a THF solution to microemulsion samples differing in w_0, the samples were stirred, and their CD spectra were immediately recorded to ensure that most of the polypeptide was maintained in this configuration during the spectroscopic measurement. This point was verified by simultaneous HPLC determination (>80% dimers in all cases). The results are depicted in Figure 4A, where dramatic changes in the CD spectroscopic pattern of the dimer are apparent as the water content varies. Note also the clear red shift in the position of the minimum which is not observed in Figure 3A. When the ellipticity at 229 nm was plotted against w_0 again, a bell-shaped curve centered around w_0 = 5 was obtained (Figure 4B). This result has an important implication; i.e., there is a clear correlation between dimer backbone conformation and rate of dissociation (Figure 1C) as a function of w_0. Around w_0 = 5 (top curve in Figure 4A) the APDS dimer seems to be somewhat more distorted and/or its two strands are not completely intertwined, which would facilitate dissociation into the monomeric forms. In the case of the

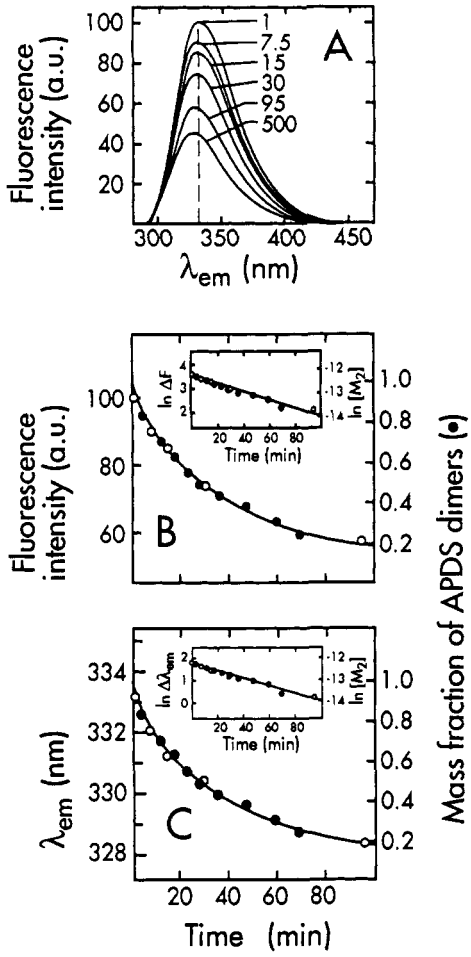

FIGURE 5: (A) Corrected emission spectra corresponding to the time course of the gramicidin monomerization in 100 mM AOT/isooctane reverse micelles at $w_0 = 11.2$. Excitation wavelength: 280 nm. Peptide concentration: 10 μg/mL. The numbers in the figure indicate the time of incubation of the sample at 25 °C, in minutes. The curve at 500 min corresponds to a complete monomerization, as revealed by HPLC. (B, C) (○) Variation of the fluorescence intensity at the maximum emission wavelength, F (B), and of the maximum emission wavelength, λ_{em} (C), from the curves in part A, as a function of the incubation time. (●) Time-dependent variation of the mass fraction of APDS dimers as simultaneously determined by HPLC. (Insets) First-order linearization of the data in parts B and C, where ΔF and $\Delta\lambda_{em}$ correspond to the difference in each magnitude between any value at a given time and the value corresponding to complete monomerization.

monomer, however, no significant dependence of the CD spectral pattern was observed as a function of w_0 (not shown).

Steady-State Fluorescence. Figure 5A depicts, as an example, the changes in the fluorescence emission spectrum of gramicidin in reverse micelles ($w_0 = 11.2$) associated to the transition from an APDS dimeric (>90%, zero time) toward a monomeric configuration. The time dependence of the fluorescence intensity, F, and of the maximum emission wavelength, λ_{em}, are plotted in parts B and C, respectively, of Figure 5. The decrease with elapsed time of the mass fraction of APDS dimers, as simultaneously determined by HPLC, is also included. The excellent congruence of the data from both techniques strongly supports that the spectral changes observed are due to the peptide monomerization. Moreover, when both sets of data were linearized by fitting to a first-order model (see insets), the same rate constant value, 3.0×10^{-4} s^{-1}, was obtained.

As in the case of the CD study, the w_0 dependence of the emission spectral parameters of both gramicidin dimer and

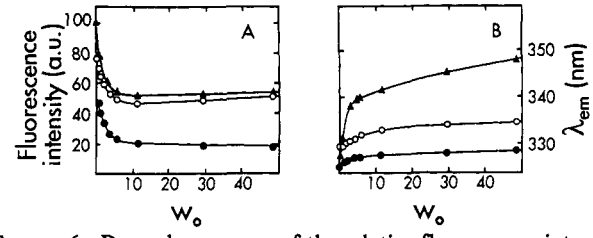

FIGURE 6: Dependence on w_0 of the relative fluorescence intensity (A) and the maximum emission wavelength (B) of NATA (▲), gramicidin APDS dimer (○), and monomer (●) in 100 mM AOT reverse micelles. Peptide concentration: 10 μg/mL. The molar concentration of tryptophanyl residues was kept the same for both gramicidin and the model fluorophore.

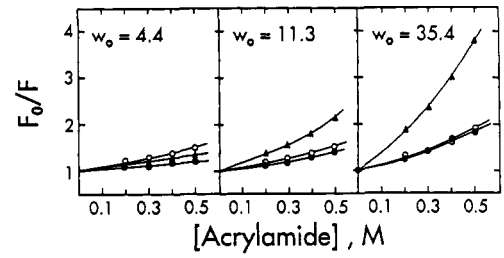

FIGURE 7: Stern–Volmer plots for the quenching by acrylamide of the tryptophan fluorescence of NATA (▲), gramicidin APDS dimer (○), and monomer (●) in 100 mM reverse micelles at different w_0 values. Excitation and emission wavelengths were 280 and 330 nm, respectively. Fluorophore concentrations are the same as in Figure 6. The acrylamide concentrations refer to the water pool.

monomer was also investigated (Figure 6). The data obtained for the model fluorophore NATA under the same experimental conditions are also included for comparison. In general, an increase in the droplet water content results in a decrease in the fluorescence quantum yield concomitant with an increase in λ_{em} of both the model fluorophore and the gramicidin conformational species. In the case of NATA the progressive, remarkable red shift is likely due to a gradual change in the polarity of the environment of the probe, although other factors have been also suggested to be involved, such as changes in the physical properties of entrapped water, particularly dielectric constant and viscosity (Nicot & Waks, 1989). Interestingly, the red shift of gramicidin tryptophans is very small as compared to that of NATA. This is a first indication of the relative insensitivity of both dimer and monomer fluorophores to the increase in micelle water content and that even at high w_0 the tryptophans' environment must be rather nonpolar. Note also the difference of about 4–5 nm in λ_{em} of the monomer relative to the dimer.

Fluorescence Quenching. Fluorescence quenching has been one of the most widely used approaches to determine the membrane penetration depth of a fluorophore (Bolen & Holloway, 1990; Killian et al., 1990; Chattopadhyay & McNamee, 1991). To further investigate the location of gramicidin in the reverse micelles, quenching experiments were performed using the aqueous quencher acrylamide. Figure 7 shows the Stern–Volmer plots for both gramicidin APDS dimer and monomer as well as for NATA, at three different w_0 values. The upward curvature observed has been reported in the past for this quencher on the indole ring fluorescence and has been extensively accounted for using the "sphere of action" model (Lackowicz, 1983). Again, the behavior of the model fluorophore, especially at moderate and high water contents, markedly differed from that of the peptide. Whereas at low w_0 a very slight quenching was observed for all species, at high w_0 the gramicidin tryptophans from both conformers seemed to be much less accessible to acrylamide than that of NATA, although not completely shielded from the quencher.

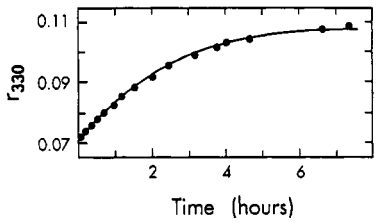

FIGURE 8: Time-dependent variation of the fluorescence anisotropy, r, corresponding to the monomerization of gramicidin in 100 mM AOT/isooctane reverse micelles ($w_0 = 11.2$). Peptide concentration: 10 μg/mL.

Steady-State Fluorescence Anisotropy. In general, upon transfer of a peptide from solution into a reverse micelle, an increase in the anisotropy value is observed, mainly due to a restriction in the peptide internal dynamics caused by an enhanced viscosity of the micellar medium and by the interaction with the surfactant monolayer (Gallay et al., 1987). As expected, in the case of gramicidin the r values for both conformers in the micelles were also higher than those previously obtained in organic solution (Braco et al., 1988a,b). Figure 8 depicts the changes in anisotropy associated to the kinetics of monomerization of a gramicidin sample incorporated mainly as APDS dimers (>90% as revealed by HPLC) in reverse micelles ($w_0 = 11.2$). The time framework of the changes observed with this technique is well correlated with that shown in previous sections (the data fit well to a first-order model with a rate constant of 2.5×10^{-4} s^{-1}). The higher r value for the monomer relative to the dimer, indicative of a more restricted overall rotational motion for the monomeric species, seems consistent with a better fit of this conformer in the surfactant monolayer. On the other hand, when the dependence of r with w_0 was studied for gramicidin incorporated either as a dimer or as a monomer, a gradual decrease in anisotropy with increasing droplet water content was observed (not shown), qualitatively similar to that found for NATA in control experiments.

DISCUSSION

In the present paper, a characterization of the behavior of gramicidin in the AOT/isooctane/water reverse micellar model system has been carried out using a combination of chromatographic and spectroscopic techniques. To the best of our knowledge, this is the first study on gramicidin A incorporated in reverse micelles and, particularly, the first opportunity to show how such an HPLC strategy can be applied to the direct visualization of different association states of a peptide in the micellar milieu. It must also be emphasized that the chromatographic information has greatly facilitated the interpretation of the CD and fluorescence spectral changes associated to the dimer–monomer transition.

It has been shown that gramicidin can be effectively incorporated into the reverse micelles, either as a double-stranded dimer or as a monomer. In this environment, a solvent "memory" dependence of the "freshly" inserted peptide conformation is observed, similar to that recently reported for small unilamellar vesicles and normal micelles (Bañó et al., 1991). Interestingly, HPLC has convincingly demonstrated that once inserted in the micelle the APDS dimers invariably undergo a transition toward the monomeric configuration (see Figure 1). It is possible that some relationship could exist between this observation and the known ability of gramicidin, in a channel configuration ($\beta^{6.3}$-helical monomers) but not in a nonchannel one (which can be ascribed predominantly to double-stranded dimers), to induce hexagonal H_{II} phases (Tournois et al., 1987a). It was proposed in this respect that the pronounced truncated cone shape of (monomeric) gramicidin (Brasseur et al., 1986) originated by the presence of four bulky tryptophan residues in the C-terminal end could explain the H_{II} phase-inducing ability of the molecule (Brasseur et al., 1987), suggesting a good accommodation of the peptide in the curved lipid leaflets of the hexagonal structure. This argument, in the present case, could reasonably apply for the gramicidin monomer in the reverse micellar monolayer.

The chromatographic approach used has permitted the accurate determination of, in a simple manner, the rate constant for the APDS dissociation, as a function of a number of experimental variables. Thus, k_1 has proved to vary quite surprisingly in a bell-shaped manner with w_0 (see Figure 1C). At either very low or very high droplet water content, the very slow monomerization of gramicidin reflects a kinetic barrier for the conversion (the dimeric configuration is as if "frozen" in the micellar environment) rather than the fact that the dimer is a true stable form under these conditions; this is supported by the observation that after a long enough incubation time, the dimers were eventually completely dissociated. An explanation for the k_1 vs w_0 bell-shaped curve may not be immediate. It is evident that the arguments usually invoked to account for this type of behavior when enzymatic activity in reverse micelles is analyzed are not applicable in this case. In principle, the rate of monomerization of gramicidin may be affected by several concurring factors, e.g., the hydration degree of the surfactant polar heads, the collision frequency between micelles, or a dimer vulnerability significantly influenced by structural or geometrical factors related to the AOT monolayer curvature. This latter assumption seems consistent with the CD results in Figure 4 which parallel the bell-shaped behavior of k_1 and show a clear, quite dramatic dependence of the dimer backbone conformation on the droplet water content. In fact, at $w_0 = 5$ where monomerization proceeds fastest, the CD pattern of the dimer apparently reflects the most perturbed backbone conformation.

The results from fluorescence measurements are in overall suggestive that gramicidin is inserted in the surfactant monolayer in a relatively nonpolar environment and that water seems to have a restricted accessibility to the peptide fluorophores. In contrast with the behavior observed for NATA, both gramicidin dimer and monomer tryptophans are rather insensitive to the droplet water content, exhibiting, even at very high w_0, λ_{em} values as low as 334 and 329 nm, respectively. It must be emphasized that the interpretation of the fluorescence results is complicated in this system due to the presence of four tryptophanyl residues close to each other in the peptide (eight in dimeric gramicidin), so that measurements should be rather considered as an average response of all fluorophores in the molecule. Thus, although the time-dependent decrease in fluorescence intensity upon monomerization (Figure 5B) does not seem to have an immediate explanation, it could be suggested that as the peptide folds into the monomeric form some tryptophan–tryptophan interactions (ring stacking) may occur leading to self-quenching of fluorescence (Scarlata, 1988, 1991). On the other hand, an explanation for the slightly higher emission wavelength of the dimer relative to the monomer regardless of the droplet water content (Figure 5C) is not either clear. Taking into account that the axial length of the APDS dimer (ca. 3.2 nm) (Ivanov & Sychev, 1982) is more than twice that of an AOT molecule (ca. 1.2 nm) (Zulauf & Eicke, 1979), the dimer moiety virtually protruding out of the monolayer into the oil phase could be

somewhat "covered" by monomeric surfactant molecules interacting through their polar heads with the peptide and therefore rendering the environment of some of the tryptophans more polar. In this respect, it is well documented that in nonpolar solvents phosphatidylcholine polar heads strongly interact with the indole groups of gramicidin, causing an appreciable red shift in the fluorescence emission (Braco et al., 1986c). Also, it cannot be discarded that gramicidin could transiently act as a micelle-connecting bridge, thereby increasing the average time of contact between micelles sharing the peptide (the micelles would become more "sticky"). This view would be supported by the observation that the addition of the transmembrane channel-forming peptide gramicidin S to an AOT/isooctane/water microemulsion system resulted in a considerable decrease in the percolation threshold temperature (Maitra et al., 1990).

In summary, this work represents a preliminary step in the characterization of conformational transitions of self-associating membrane peptides in inverted micellar systems. AOT reverse micelles offer a stable, manageable, optically transparent model system which can be regarded as an alternative membrane–mimetic environment to investigate the conformational behavior of gramicidin. Obviously, other inverted micellar aggregates can be tested in the future as hosts for the peptide, especially those based on phospholipids (Walde et al., 1990), such as flexible polymerlike lecithin reverse micelles (Schurtenberger et al., 1991) or cubic phases (Portmann et al., 1991), where the picture of the system will be undoubtedly enriched. On the other hand, the implications of the present strategy as a novel experimental approach to gain insight on how the conformational adaptability of self-associating bioactive peptides (or proteins) can be modulated by an inverted micellar environment deserve further consideration. In particular, taking into account that different nonlamellar, inverted lipid structures have been often suggested to be involved in relevant processes (e.g., membrane fusion) occurring in biomembranes [for a review, see Seddon (1990)].

ACKNOWLEDGMENT

We express our thanks to Dr. Peter Walde for his valuable suggestions and critical revision of the manuscript. We are also grateful to Prof. J. G. Gavilanes for the use of the circular dichrograph in his lab. D.S. was a recipient of a long-term postgraduate fellowship from the Generalitat Valenciana (Spain).

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Registry No. AOT, 577-11-7; gramicidin A, 11029-61-1.