Solvent Effects on the Conformation of the Transmembrane Peptide Gramicidin A: Insights from Electrospray Ionization Mass Spectrometry

Mario Bouchard, Dennis R. Benjamin, Paula Tito, Carol V. Robinson, and Christopher M. Dobson
Oxford Centre for Molecular Sciences, New Chemistry Laboratory, University of Oxford, Oxford OX1 3QT, United Kingdom

ABSTRACT   The binding of sodium ions to the transmembrane channel peptide gramicidin A has permitted the use of electrospray ionization mass spectrometry to study its conformation in different solvent environments. The mass spectra of the peptide in the various solvents suggest that different conformations of gramicidin A differ in their ability to bind metal ions. The data are consistent with monomeric behavior of gramicidin A in trifluoroethanol and dimethyl sulfoxide solutions, but reveal the presence of noncovalent intermolecular interactions in ethanol solution through the observation of heterodimers formed between the naturally occurring variants of the peptide. The addition of 50% v/v of water to the ethanolic solution causes changes in the circular dichroism spectrum of the peptide, suggestive of a shift in the equilibrium mixture of conformers present toward monomeric species, a result supported by its mass spectrum. The structure of gramicidin A in trifluoroethanol has also been investigated by hydrogen exchange measurements monitored by mass spectrometry. The observation of significant protection against exchange suggests that the monomeric peptide is highly structured in trifluoroethanol. The results indicate that mass spectrometry has the potential to probe the conformational behavior of neutral hydrophobic peptides in environments that mimic their functional states.

INTRODUCTION

Gramicidin A is a hydrophobic linear pentadecapeptide composed of alternating L- and D-amino acids that can form transmembrane channels that induce selective permeability to monovalent cations in lipid membranes (Urry, 1984). In the channel conformation, in lipid membranes and SDS micelles, gramicidin A is considered to exist as an N-terminal to N-terminal right-handed $\beta^{6.3}$ helix dimer (Arseniev et al., 1985, 1986; Nicholson and Cross, 1989; Prosser et al., 1991; Killian, 1992; Lomize et al., 1992). It has been shown, however, that gramicidin A can adopt other conformational states in hydrated lipid bilayers. A number of factors may influence the conformation that gramicidin A ultimately adopts in a phospholipid dispersion. One of these factors is the solvent initially used to cosolubilize the phospholipid molecules and gramicidin A. Indeed, it has been shown that when gramicidin A is added to diacylphosphatidylcholine model membranes, the peptide initially associates with the lipid in a conformation that is dependent on the nature of the organic solvent from which it has been added (Killian et al., 1988; Killian, 1992; Bouchard and Auger, 1993; Bouchard et al., 1995).

The conformation of gramicidin A in pure solvent systems has been the focus of a variety of studies. As a result, various models have been suggested for its structure in different solvents. The models have largely been inferred from spectroscopic techniques such as CD (Urry et al., 1972; Veatch et al., 1974; Chen and Wallace, 1997), nuclear magnetic resonance (Hawkes et al., 1987; Arseniev et al., 1985; Pascal and Cross, 1992), infrared and Raman spectroscopies (Urry et al., 1972; Veatch et al., 1974; Iqbal and Weidekamm, 1980) and high-performance liquid chromatography (Braco et al., 1986). Urry and colleagues (1972) were the first to demonstrate the conformation to be solvent-dependent. Subsequent studies have given a detailed insight into the complexity of the conformational behavior of gramicidin A (Wallace, 1983). For example, in most pure alcohols and dioxan it has been shown that gramicidin A adopts four different parallel, antiparallel, left-, or right-handed double-stranded helices that intertwine with each other (Veatch et al., 1974; Veatch and Blout, 1974; Bystrov and Arseniev, 1988). The kinetics of interconversion between the different conformers has been found to depend on the solvent type and peptide concentration (Veatch and Blout, 1974; Braco et al., 1988; Chen and Wallace, 1997).

In more polar solvents such as DMSO, gramicidin A is present in a monomeric form (Veatch and Blout, 1974). A number of studies have been performed in this solvent (Fossel et al., 1974; Iqbal and Weidekamm, 1980; Heitz et al., 1986; Hawkes et al., 1987). Although all of these studies are consistent with a monomeric conformation, controversy remains about the secondary structure of this monomeric form. Several authors propose an equilibrium between a disordered structure and a $\beta^{6.3}$ helical conformation (Hawkes et al., 1987), while others proposed an LD-ribbon structure (Heitz et al., 1986). In TFE, which is also highly polar, the conformation of gramicidin A has been proposed

---

to be similar to that in DMSO (Urry et al., 1972). Although the peptide does not behave identically in these two solvents, it is clear that gramicidin A is also present predominantly in a monomeric form in TFE (Abdul-Manan and Hinton, 1994).

ESI-MS has become a powerful method for studying protein structure (Loo et al., 1991; Miranker et al., 1993; Wagner and Anderegg, 1994; Yi and Baker, 1996; Chung et al., 1997). The electrospray process produces multiply charged gas-phase ions from a protein solution. The distribution of charged states observed in the mass spectrum is related to the conformations present in the solution. Moreover, in combination with hydrogen exchange measurements, ESI-MS has proved to be successful in studies of protein structure and dynamics, and particularly of protein folding and unfolding (Zhang et al., 1992; Miranker et al., 1996; Robinson et al., 1996). ESI-MS permits accurate measurements of H/D distributions and hence can provide unique information concerning protein structure, the mechanism of conformational changes, and the nature of any intermediate states (Miranker et al., 1993; Wagner and Anderegg, 1994; Wang and Tang, 1996). Furthermore, mass spectrometry is also well suited to the study of electrostatic interactions between ions and biomolecules, and it has been used to characterize the metal ion-binding properties of a variety of peptides and proteins (Senko et al., 1993; Gross and Williams, 1996).

In the present study we have examined the structure of gramicidin A in different solvents using ESI-MS in combination with CD. We have determined that mass spectrometry can provide information on the state of gramicidin A in different solvent systems including unique information about its association with sodium ions. Moreover, when combined with hydrogen exchange measurements, information on the conformational properties of the peptide in TFE has been gained.

MATERIALS AND METHODS

Materials

Gramicidin A was purchased from Fluka Chemika-Biochemika-Analytica (Buchs, CH) and used without purification (purity ≥90% and free of gramicidin A C). DMSO and TFE were obtained from Sigma Chemical Co (Poole, Dorset, UK). Deuterium oxide was obtained from Fluorochem Ltd. (Old Glossop, UK). Ethanol, deuterated ethanol, and deuterated TFE were purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK).

CD

Samples of the gramicidin A were prepared by dilution of a 5 mM ethanol solution of the peptide into solvent mixtures containing appropriate amounts of ethanol and water (1:1 final v/v ratio) or pure TFE (1:99 final v/v). The final concentration of the peptide was 100 μM. The CD measurements were performed on a Jasco J-720 spectropolarimeter using a cell with a 10 mm optical path-length. Solvent spectra were subtracted from the

measured spectra of gramicidin A. The reported spectra are averages of four scans from each of three independent preparations of each sample.

ESI-MS

Mass spectra were acquired in the positive ion mode on a Micromass Platform II mass spectrometer equipped with a nanoelectrospray probe. Samples were introduced into the ionization source from a gold sputtered borosilicate needle. All the spectra represent the raw data with minimal smoothing. The spectra are the averages of 16 scans for the structural study and 6 scans for the hydrogen exchange measurements. Spectra have been recorded under optimal instrumental conditions as described in the figure legends. Average masses are the intensity-weighted average calculated from the sum of the fractional mass of each isotope as given by the following equation: Σ (mass × intensity of peak)/Σ (intensity of total isotopic peaks). Back-exchange was negligible under the conditions used here and no corrections have been performed on the calculated masses.

Conformational study

Samples of gramicidin A in ethanol (5 mM) were diluted in DMSO, TFE, ethanol, and a mixture of water and ethanol (1:1 v/v). The final peptide concentration in each case was 50 μM. The final ethanol concentration in the TFE and DMSO samples was 1%.

Hydrogen exchange labeling of Gramicidin A and the tripeptide Ala-Ala-Ala

Deuterium-labeled gramicidin A samples were prepared by dilution of a 5 mM peptide solution in TFE into TFE-d_1. The final peptide concentration was 50 μM and the quantity of nondeuterated trifluoroethanol present was 1% v/v. In an additional experiment samples containing the tripeptide Ala-Ala-Ala as a reference molecule were produced by mixing both peptides together in TFE followed by dilution into deuterated TFE. The final concentration was 50 μM for both gramicidin A and the Ala-Ala-Ala tripeptide, and 2% nondeuterated trifluoroethanol was present. The hydrogen exchange experiments were all performed at room temperature (23°C), and the mass spectra were acquired immediately after mixing. A new sample was used for each time point.

RESULTS AND DISCUSSION

ESI-MS of gramicidin A in different solvents

Fig. 1, *A* and *B* show ESI mass spectra of gramicidin A in TFE in two different *m/z* regions. The most abundant ion observed in spectrum 1 *A*, *m/z* 1905.02, where *m/z* is in Daltons per elementary charge, is assigned to the singly charged state of the major form of gramicidin A, having a valine residue at the N-terminus, and denoted [1-Val]-GA. The resolving power is high enough for the individual isotopes of the molecular ion to be observed; the multiplicity of the peak arises mainly from the contribution of the 1% natural abundance of the ^{13}C isotope of carbon with smaller contributions from the natural isotopes of oxygen, nitrogen, and hydrogen. We can also observe that the peaks are separated by 1 *m/z* unit, indicating that they arise from monomeric singly charged ions, a result consistent with the monomeric structure adopted by the peptide in this solvent (Urry et al., 1972; Killian, 1992; Bouchard and Auger,
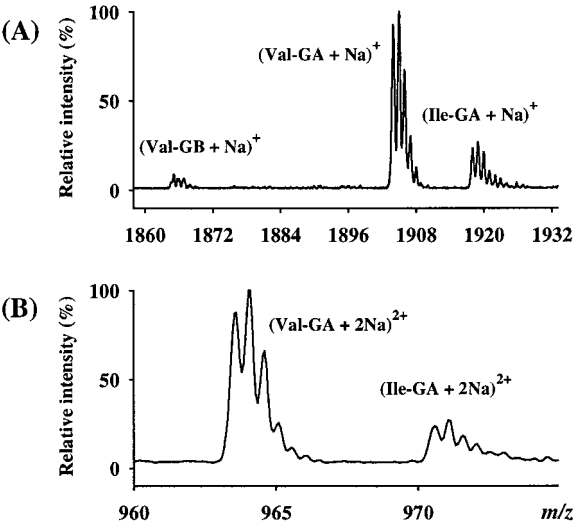
FIGURE 1 ESI mass spectra of monomeric gramicidin A in TFE in two different m/z regions. (*A*) m/z 1860–1932; (*B*) m/z 960–975. The optimum experimental conditions for (*B*) were obtained with a lower cone voltage of 60 V compared to 175 V for (*A*).

TABLE 1   Characteristics of the different species observed in the ESI-MS spectra of gramicidin A in the different solvents studied

Species	Observed m/z	Calculated m/z	Observed Charged State	Number of Na$^+$ Associated
Val-gramicidin A	1883.15	1883.08	+1	0
	1905.02	1905.06		1
	953.07	953.04	+2	1
	964.02	964.03		2
Ile-gramicidin A	1897.11	1897.09	+1	0
	1918.98	1919.08		1
	960.11	960.04	+2	1
	971.07	971.03		2
Val-gramicidin B	1843.95	1844.07	+1	0
	1865.95	1866.05		1
	922.55	922.54	+2	0
	944.54	944.52		2
Ile-Val-gramicidin A heterodimer	1912.19	1912.07	+2	2
Val-gramicidin A-B heterodimer	1885.33	1885.55	+2	2

1993). The observed mass, however, is 23 Da greater than the mass of the free peptide (predicted average mass of the neutral peptide = 1882.08 Da). The molecular ion therefore arises from the complexation of a sodium ion with the free peptide to form a stable monocharged adduct (predicted m/z = 1905.06). This cationization process is interesting because it involves the noncovalent addition of a positively charged ion to a neutral molecule. This results in a charged complex that can be observed by mass spectrometry and therefore allows the analysis of a neutral hydrophobic biopolymer such as gramicidin A. Fig. 1 *B* shows the doubly charged state of the [1-Val]-GA molecular ion at m/z 964.02 with which two sodium ions are associated (predicted m/z = 964.03). The peaks are separated by 0.5 m/z units, in full agreement with a doubly charged monomeric structure.

Gramicidin A occurs naturally as a mixture of variants, and species in addition to that described above can be observed in the spectra presented in Fig. 1. For example, in 5–20% of the gramicidin A molecules, the valine at position 1 is replaced by an isoleucine residue (Gross and Witkop, 1965; Killian, 1992). The peaks arising from this species, denoted [1-Ile]-GA, complexed with a single sodium ion can be observed at m/z 1918.98 (predicted m/z = 1919.08). Furthermore, a peak corresponding to singly charged gramicidin B, denoted [1-Val]-GB, where the tryptophan at position 11 is replaced by a phenylalanine residue (Gross and Witkop, 1965; Killian, 1992), can be observed at m/z 1865.95 (predicted m/z = 1866.05). In Fig. 1 *B* the doubly charged state of [1-Ile]-GA, to which two sodium ions are associated, can be observed at m/z 971.07 (predicted m/z = 971.03). The observed and calculated masses, charge states, and complexion states for the different species are given in Table 1.

Fig. 2, *A* and *B* show mass spectra of gramicidin A in DMSO in two different m/z regions. The conformation of gramicidin A in DMSO is thought to be similar to that in TFE (Killian, 1992) and the peptide also adopts a monomeric state in this solvent (Hawkes et al., 1987; Killian, 1992). As in the spectra of gramicidin A dissolved in TFE, the singly and doubly charged [1-Val]-GA and [1-Ile]-GA and [I-Val]-GB molecules complexed to sodium are ob-
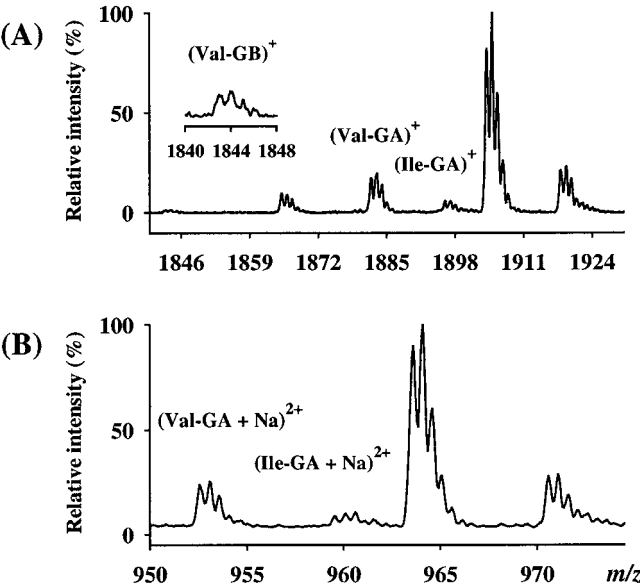

FIGURE 2 ESI mass spectra of gramicidin A in DMSO in two different m/z regions. (*A*) m/z 1840–1930; (*B*) m/z 950–975. The charge state separation shows that the species are monomeric. The optimum experimental conditions for (*B*) were obtained with a lower cone voltage of 60 V compared to 175 V for (*A*).

served. The mass separation between the isotopic peaks is again 1 m/z unit in the case of the singly charged molecule and 0.5 units for the doubly charged peptide, showing that gramicidin A adopts a monomeric structure under these conditions. However, the spectra show the presence of peaks additional to those observed in TFE. In Fig. 2 *A*, three new species corresponding to singly charged protonated states of [1-Val]-GA and [1-Ile]-GA, at m/z of 1883.15 (predicted $m/z = 1883.15$) and 1897.11 (predicted $m/z = 1897.09$) respectively, and [1-Val]-GB at m/z of 1843.95 (predicted $m/z = 1844.07$) can be seen. No sodium ions are complexed to these species. In Fig. 2 *B*, new species corresponding to doubly charged states of the peptide associated with only a single sodium ion are observed. The second charge in both these cases must come from protonation of the peptide. The doubly charged state of the peptide is confirmed by the 0.5 m/z unit separation between the isotopic peaks of the molecular ion.

The observation of singly charged sodium complexes of the linear gramicidin A molecules is similar to the detection of complexes of gramicidin S and alkali metal ions shown in previous work (Gross and Williams, 1996) even though the two types of gramicidin A are functionally and structurally unrelated. In both peptides, the singly charged ions are predominantly attached to Na^+. However, in contrast to gramicidin A, the most abundant doubly charged gramicidin S ions are doubly protonated species rather than complexes with sodium ions.

Fig. 3 shows the ESI-MS of gramicidin A in ethanol in the 1860–1930 m/z region. The most abundant ion observed in the spectrum again corresponds to the singly charged state of the [1-Val]-GA to which a single sodium ion is complexed. Singly charged [1-Ile]-GA and [1-Val]-GB complexed to sodium ions can also be observed in the spectrum. The 1 m/z unit difference between the isotopic peaks of these species shows that gramicidin A in this solvent is monomeric. However, it is well-established that gramicidin A in ethanol adopts four different dimeric double helical conformations (Veatch and Blout, 1974); the observation of monomeric species in the mass spectrum therefore suggests that the peptide undergoes dissociation
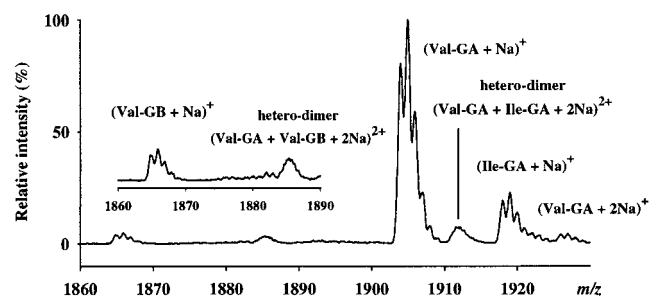
during the ESI process. The peak at m/z 1912.19 does not, however, arise from any of the monomeric species present in the sample. It arises from the formation of a hetero-dimer between [1-Val]- and [1-Ile]-GA complexed to two sodium ions with a charge state of +2 (predicted $m/z = 1912.07$). In addition, the peak at m/z 1885.33 results from the +2 charged heterodimer formed between the [1-Val]-GB species and the [1-Val]-GA ion (predicted $m/z = 1885.55$). No homodimers or singly charged homo and heterodimers are observable under the experimental condition used here; the doubly charged homodimers will have the same m/z as singly charged monomers, and their signals will overlap with the intense signal of the latter. The characteristics of the heterodimer ions observed in ethanol are summarized in Table 1.

The observation of the two heterodimers shows that even though the ESI process produces mainly singly charged monomers by dissociation of the noncovalently associated gramicidin A strands, a proportion of the peptide in the dimeric (double helical) conformation can be detected. These findings, therefore, indicate a partial preservation of the noncovalent interactions between gramicidin A molecules in ethanol, indicating that the peptide retains a history of its ethanol environment in the gas phase. The correlation between the solution and gas-phase structural information observed through the variants of gramicidin A also supports the conclusion that the combination of nanoflow ESI-MS and the use of different protein variants to produce hetero-oligomers represent a powerful means for probing protein complex formation such as protein aggregation (Vis et al., 1999).

ESI-MS of gramicidin A in a 1:1 v/v ethanol/water mixture

Previous studies have characterized the monomeric and dimeric forms of gramicidin A under different conditions using CD spectroscopy (Urry et al., 1972; Veatch and Blout, 1974; Chen and Wallace, 1997). The CD spectrum of any molecule under given conditions is the sum of the spectra of all the individual conformers present in solution weighted by their relative abundance. The spectrum of gramicidin A in ethanol displays two strong negative absorption bands at ~210 nm and 228 nm that can be attributed to the various forms of intertwined double helical species present in this solvent (Veatch and Blout, 1974; Chen and Wallace, 1997). By contrast, the CD spectrum of gramicidin A in TFE, in which the peptide is thought to be monomeric, shows a distinctive positive band at ~223 nm (Urry et al., 1972; Killian et al., 1992; Ogoshi and Mita, 1997). When gramicidin A is dissolved in a mixture of ethanol and water (1:1 v/v) the CD spectrum obtained is almost identical to that of the peptide in TFE (Fig. 4), indicating that the conformational state of the peptide in the mixed solvent system is closely similar to that found in TFE. The addition of 50% of
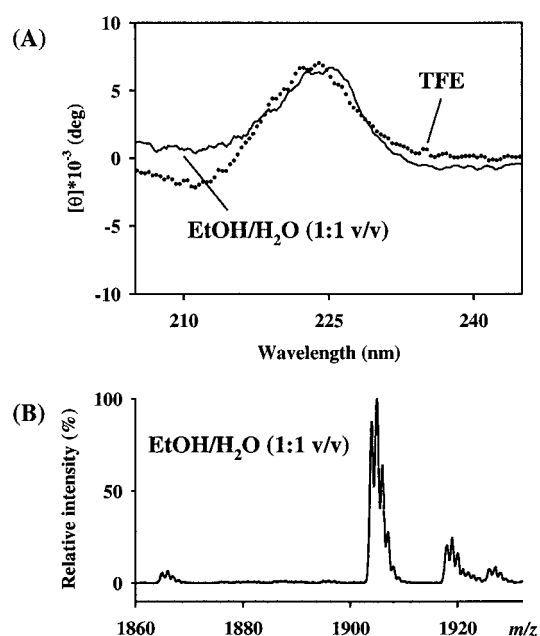

FIGURE 3   ESI mass spectrum of gramicidin A in pure ethanol in the m/z 1860–1930 region. The inset represents an expansion of the spectral region between m/z 1860 and 1890.

FIGURE 4 (*A*) Comparison between the far-UV CD spectrum of gramicidin A dissolved in TFE (*broken line*) and gramicidin A dissolved in a mixture of water and ethanol (1:1 v/v) (*solid line*). (*B*) ESI mass spectrum of gramicidin A in a mixture of water and ethanol (1:1 v/v) in the *m/z* 1860–1930 region.

water to the ethanolic gramicidin A solution has therefore resulted in the peptide converting from its double helical form to a monomeric conformation.

Fig. 4 *B* shows the mass spectrum of gramicidin A dissolved in the ethanol/water mixture. The molecular ions observed are identical to those observed for the peptide in TFE in Fig. 1 *A*, listed in Table 1. The 1 *m/z* unit difference between the isotopic peaks of all the molecular ions again shows that gramicidin A is monomeric under these solution conditions. No peaks associated with any dimeric species were observed in the mass spectrum. In addition, the sodium ion-binding properties of the peptide are similar to those in TFE, in contrast to the situation when the peptide is dissolved in DMSO. The observation of a monomeric state for gramicidin A in the ethanol and water mixture is therefore in full agreement with the monomeric CD pattern observed for the peptide in this mixed solvent system, and again shows the close correlation between the gas phase and the peptide structural characteristics in solution.

Deuterium exchange labeling of gramicidin A in trifluoroethanol monitored by ESI-MS

In order to probe further the characteristics of the monomeric state of gramicidin A in TFE, the hydrogen exchange properties of the peptide have been investigated. Gramicidin A contains 21 labile hydrogens of which 15 are backbone hydrogens. The remaining labile hydrogens consist of

four indole protons and the two exchangeable hydrogens of the ethanolamine terminus. If all 21 hydrogens were to be replaced by deuterons the average mass would increase from 1905.06 Da to 1926.06 Da.

The hydrogen/deuterium exchange experiments were carried out by dilution of a solution of gramicidin A in TFE into pure deuterated TFE to give a 1:99 final v/v ratio of normal to deuterated TFE, at room temperature, and aliquots were collected as a function of time. Fig. 5 *A* shows the time evolution of the mass spectrum of gramicidin A after dilution and hydrogen exchange. The *m/z* region between 1900 and 1950 is shown. The rate of deuterium exchange can also be observed by plotting the molecular mass as a function of time. Fig. 5 *B* shows the time evolution profile of the average mass of the +1 [1-Val]-GA molecular ion complexed to one sodium ion during the hydrogen exchange reaction of the peptide. The data can be fitted to a biexponential function with amplitudes of 5.4 Da and 13.7 Da, and time constants of 37 min and 14 h, respectively. This result is consistent with a model in which the four indole protons and the two labile hydrogens of the
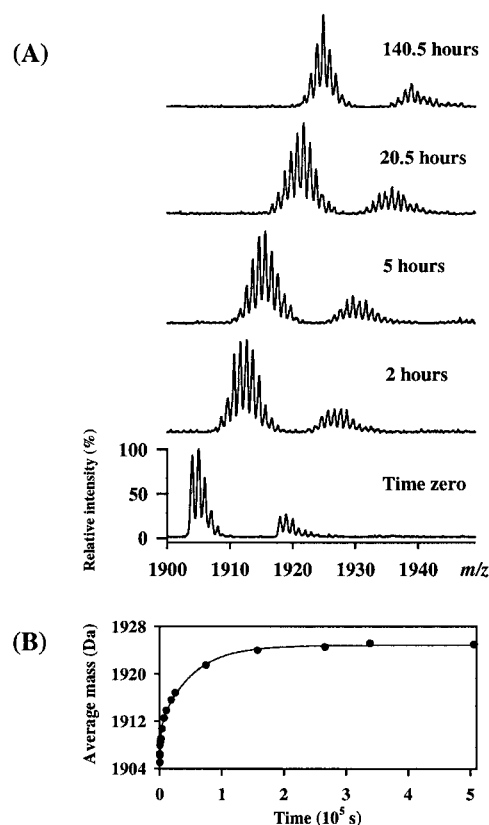

FIGURE 5 (*A*) Time evolution of the ESI mass spectrum of gramicidin A after dilution and hydrogen exchange in deuterated TFE. (*B*) Time evolution profile of the average mass of the +1 [1-Val]-GA molecular ion during the hydrogen exchange of the peptide. The final concentration of nondeuterated TFE is 1% and all spectra are the average of six scans. In (*B*) the average mass has been calculated as described in Materials and Methods.

ethanolamine group of the peptide exchange first, followed by the exchange of the remaining amide hydrogens. Similar results were obtained for the +1 [1-Ile]-GA molecular ion (results not shown).

In the hydrogen/deuterium exchange experiment, protein conformers with reduced hydrogen bonding and/or increased solvent accessibility show more rapid deuterium incorporation than do the more stable conformers. The mechanism for amide exchange in proteins generally involves the unfolding and refolding of the protein conformers. Transient unfolding may also occur via local or sublocal fluctuations of the protein structure. Two limiting processes can be considered to relate the protein structural fluctuations to the observed exchange behavior. These are the EX1 and EX2 exchange mechanism (Miranker et al., 1993; Zhang and Smith, 1993; Chung et al., 1997; Engen et al., 1997, 1999). Exchange occurs in the EX1 regime if the interconversion between open and closed structures is slow compared to the intrinsic amide exchange rate. This mechanism will give rise to two distinct populations, the fully protonated and the fully deuterated forms. In this case the mass spectrum would show two peaks, each associated with individual isotopically labeled populations, and the intensities of the two mass peaks, but not their individual masses, will vary with time. In contrast, an EX2 mechanism is observed if the exchange rate is slow compared to the unfolding/refolding reaction. In this case the structural transition will take place on average many times before exchange occurs and the occupancies of protons and deuterons at different sites on a given molecule need not be correlated. Instead, each molecule in the solution ensemble will have, on average, the same number of exchanged hydrogens and the population will appear in the mass spectrum as a single peak, which shifts to higher masses as the extent of exchange increases.

The time evolution of the mass spectrum observed in Fig. 5 A clearly shows that the hydrogen exchange of gramicidin A in deuterated TFE occurs according to an EX2 mechanism. A single set of peaks is always observed resulting from a random distribution of the deuterons in the peptide. The peak width does not change significantly through the exchange time course sampled, suggesting that a narrow distribution of conformers is present at any time (Chung et al., 1997). The slowness of the exchange observed for gramicidin A in deuterated TFE relative to the intrinsic exchange rate of amides in water suggests protection of the exchangeable hydrogens due to the presence of persistent structure. However, it is well known that intrinsic hydrogen exchange rates are strongly dependent upon the solvent conditions used (Englander and Kallenbach, 1983: Maier et al., 1997) and therefore the kinetic exchange profile observed gives no direct information regarding the structure, or the presence of conformers in rapid equilibrium, of gramicidin A in TFE and a definitive statement to this effect

requires knowledge of the intrinsic exchange rates of amide hydrogens under these conditions.

In order to explore this further, the hydrogen exchange properties of an unstructured tripeptide (Ala-Ala-Ala) were measured in the presence of gramicidin A and compared to those of the latter. MS offers the advantage of being able to follow the exchange properties of different molecular species simultaneously, providing they have different masses. Thus exchange rates of both peptides could be measured under identical solution conditions. Although the rates of exchange vary with sequences, their variations are likely to be less than a factor of 10 for an unstructured peptide (Bai et al., 1993). The structure of the Ala-Ala-Ala tripeptide in TFE has been studied by CD (results not shown) and clearly shows that the peptide possesses no significant secondary structure in TFE.

Fig. 6 shows mass spectra of the mixture of gramicidin A and the alanine tripeptide before (A) and 160s after (B) dilution in deuterated TFE and hydrogen exchange. In Fig. 6 the singly charged Ala-Ala-Ala tripeptide associated with one sodium ion at $m/z = 254.39$ and the previously described singly charged [1-Val]-[1-Ile]-GA ions are shown. After 160 s the mass of the tripeptide has increased from 254.39 to 259.37 Da, showing that exchange of the five labile hydrogens of the tripeptide is virtually complete. However, for gramicidin A, the extent of exchange that has occurred over that time period is small and the peptide remains significantly protonated. Because both of the peptides are studied simultaneously a direct comparison of their exchange rates can be made. This comparison indicates that the average exchange rate of the amide hydrogens of gram-
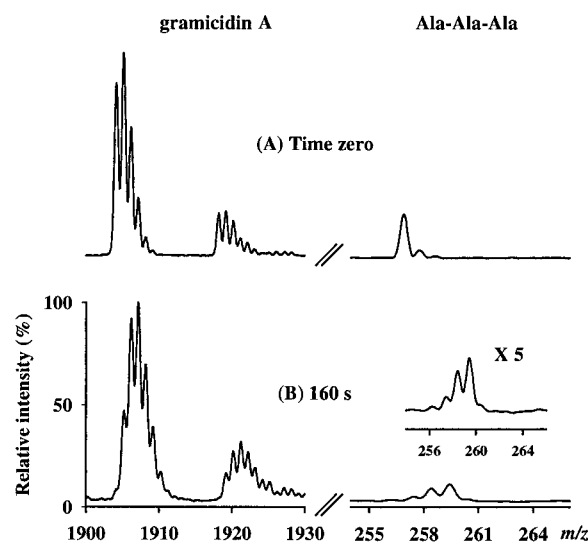

FIGURE 6 ESI mass spectra monitoring and comparing the hydrogen exchange of the Ala-Ala-Ala tripeptide and gramicidin A at room temperature (A) before initiation of the exchange and (B) 160 s after dilution in deuterated TFE. The two peptides were mixed before the exchange, hence the H/D exchange of both species took place under identical conditions.

icidin A is significantly reduced compared to the Ala-Ala-Ala peptide, and therefore suggests that gramicidin A in TFE possesses persistent structure in contrast to the tripeptide. Exchange protection of such magnitude has been observed for other peptides and proteins in TFE (Buck et al., 1993; Bolin et al., 1996). When gramicidin A is incorporated into lipid bilayers from a solution of TFE the peptide directly incorporates in the $\beta^{6.3}$ helical conformation (Bouchard and Auger, 1993; Bouchard et al., 1995) and hence this helical structure is the most probable conformation for gramicidin A in pure TFE. Other studies using CD and nuclear magnetic resonance have found gramicidin A to be monomeric and structured in TFE, possibly existing as species consisting of α or $\beta^{4.4}$ helices (Abdul-Manan and Hinton, 1994). However, the MS results presented here cannot alone verify a particular model for the peptide dissolved in the pure solvent system, and further experiments are necessary to fully determine the structure of gramicidin A in TFE.

CONCLUSIONS

Although originally introduced as a method for molecular weight and primary structure determination of biopolymers, the ability of ESI-MS to provide information on the structure and specific noncovalent interaction of macromolecules in solution is presently adding a new dimension to the technique (Rostom and Robinson, 1999). ESI-MS has been used here to examine the conformational state of the transmembrane channel peptide gramicidin A in different solvents. Four solvents have been studied: ethanol, a 1:1 mixture of ethanol and water, TFE, and DMSO. The interactions of the peptide with sodium ions provided a unique opportunity to observe and investigate the structural properties of gramicidin A by generating a variety of charged species for detection in the mass spectrometer. This may be a general approach to studying strategically neutral hydrophobic peptides of the type frequently associated with membrane environments.

For the peptide in DMSO, TFE, and in the 1:1 mixture of ethanol and water, only monomeric species are observed in the mass spectra. In the case of the peptide in TFE, hydrogen exchange measurements show that the monomeric species present in solution are highly structured. The observation of molecular peaks associated with the formation of heterodimers between different gramicidin A variants in pure ethanol indicates the presence of noncovalent dimers in this solvent, a finding consistent with the double helical structures identified in earlier studies using conventional structural techniques (Veatch et al., 1974; Veatch and Blout, 1974). The ability to observe such species for a relatively small peptide is a testament to the recent development of soft ionization in ESI-MS procedures. These findings and the results of a related study of a repressor proteins (Vis et al., 1999) suggest noncovalent peptide or protein associa-

tion phenomena can in general be probed by using procedures to generate heterodimers in mass spectrometric investigations. The results of this present study also provide information concerning the binding of sodium ions to gramicidin A. The peptide can bind one or two sodium ions in either the monomeric or the dimeric state, depending upon its conformation and the solvent in which it has been solubilized.

REFERENCES

Arseniev, A. S., I. L Barsukov, V. F. Bystrov, A. L. Lomize, and Y. A. Ovchinnikov. 1985. ^{1}H-NMR study of gramicidin A transmembrane ion channel. *FEBS Lett.* 186:168–174.

Arseniev, A. S., A. L. Lomize, I. L. Barsukov, and V. F. Bystrov. 1986. Gramicidin-A transmembrane ion-channel-3-dimensional structure reconstruction based on NMR-spectroscopy and energy refinement. *Biol. Mol.* 3:1077–1104.

Bai, Y. W., J. S. Milne, L. Mayne, and S. W. Englander. 1993. Primary structure effects on peptide group hydrogen exchange. *Proteins: Struct., Funct., Genet.* 17:75–86.

Bolin, K. A., M. Pitkeathly, A. Miranker, L. J. Smith, and C. M. Dobson. 1996. Insight into a random coil conformation and an isolated helix: structural and dynamical characterization of the C-helix peptide from hen lysozyme. *J. Mol. Biol.* 261:443–453.

Bouchard, M., and M. Auger. 1993. Solvent history dependence of gramicidin-lipid interactions: a Raman and infrared spectroscopic study. *Biophys. J.* 65:2484–2492.

Bouchard, M., J. H. Davis, and M. Auger. 1995. High-speed magic angle spinning solid-state ^{1}H nuclear magnetic resonance study of the conformation of gramicidin A A in lipid bilayers. *Biophys. J.* 69:1933–1938.

Braco, L., C. Abad, A. Campos, and J. E. Figueruelo. 1986. Time-dependent monomerization of gramicidin A, enhanced by phosphatidylcholine in non-polar solvents. A high-performance liquid chromatographic and spectrofluorometric study. *J. Chromatogr.* 353:181–192.

Braco, L., C. Bano, F. Chillaron, and C. Abad. 1988. Dimer-monomer conformational equilibrium of gramicidin A in 1-alkanols as studied by HPLC and fluorescence spectroscopy. *Int. J. Biol. Macromol.* 10: 343–348.

Buck, M., S. E. Radford, and C. M. Dobson. 1993. A partially folded state of hen egg white lysozyme in trifluoroethanol: structural characterization and implication for protein folding. *Biochemistry.* 32:669–678.

Bystrov, V. F., and A. S. Arseniev, 1988. Diversity of the gramicidin A spatial structure-two-dimensional H-1-NMR study in solution. *Tetrahedron.* 44:925–940.

Chen, Y., and B. A. Wallace. 1997. Solvent effects on the conformation and far UV CD spectra of gramicidin A. *Biopolymer.* 42:771–781.

Chung, E. W., E. J. Nettleton, C. J. Morgan, M. Gross, A. Miranker, S. E. Radford, C. M. Dobson, and C. V. Robinson. 1997. Hydrogen exchange properties of proteins in native and denatured states monitored by mass spectrometry and NMR. *Protein Sci.* 6:1316–1324.

Engen, J. R., T. E. Smithgall, W. H. Gmeiner, and D. L. Smith. 1997. Identification of and localization of slow, natural, cooperative unfolding in the hematopoietic cell kinase SH3 domain by amide hydrogen exchange and mass spectrometry. *Biochemistry.* 36:14384–14391.

Engen, J. R., T. E. Smithgall, W. H. Gmeiner, and D. L. Smith. 1999. Comparison of SH3 and SH2 domain dynamics when expressed alone or in an SH(3 + 2) construct: the role of protein dynamics in functional regulation. *J. Mol. Biol.* 287:645–656.

Englander, S. W., and N. Kallenbach. 1983. Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q. Rev. Biophys.* 16:521–655.

Fossel, E. T., W. R. Veatch, Y. A. Ovchinnikov, and E. R. Blout. 1974. A ^{13}C nuclear magnetic resonance study of gramicidin A in monomer and dimer forms. *Biochemistry.* 13:5264–5275.

Gross, D. S., and E. R., Williams. 1996. Structure of gramicidin S (M + H + X)$^{2+}$ ions (X = Li, Na, K) probed by proton transfer reactions. *J. Am. Chem. Soc.* 118:202–204.

Gross, E., and B. Witkop. 1965. Gramicidin. IX. Preparation of gramicidin A, B and C. *Biochemistry.* 4:2495–2501.

Hawkes, G. E., L.-Y. Lian, E. W. Randall, K. D. Sales, and E. H. Curzo. 1987. The conformation of gramicidin A in dimethylsulfoxide solution: a full analysis of one and two-dimensional ^{1}H, ^{13}C and ^{15}N nuclear magnetic resonance spectra. *Eur. J. Biochem.* 166:437–445.

Heitz, F., A. Heitz, and Y. Trudelle. 1986. Conformations of gramicidin-A and its 9, 11, 13, 15-phenylalanyl analogue in dimethylsulfoxide and chloroform. *Biophys. Chem.* 24:149–160.

Iqbal, Z., and E. Weidekamm. 1980. Raman and infrared spectroscopic study of gramicidin A conformations. *Arch. Biochem. Biophys.* 202:639–649.

Killian, J. A. 1992. Gramicidin A and gramicidin-lipid interactions. *Biochem. Biophys. Acta* 1113:391–425.

Killian, J. A., K. U. Prasad, D. Hains, and D. W. Urry. 1988. The membrane as an environment of minimal interconversion: a circular dichroism study on the solvent dependence of the conformational behaviour of gramicidin in diacylphosphatidylcholine model membranes. *Biochemistry.* 27:4848–4855.

Lomize, A., V. Yu. Orechov, and A. S. Arseniev. 1992. Refinement of the spatial structure of gramicidin-A transmembrane ion-channel. *Bioorg. Khim.* 18:182–200.

Loo, J. A., R. R. Ogorzalek Loo, H. R. Udseth, C. G. Edmonds, and R. D. Smith. 1991. Solvent-induced conformational changes of polypeptides probed by electrospray-ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 5:101–105.

Maier, C. S., O-H. Kim, and M. L. Deinzer. 1997. Conformational properties of the A-state of cytochrome c studied by hydrogen/deuterium exchange and electrospray mass spectrometry. *Anal. Chem.* 252:127–135.

Miranker, A., C. V. Robinson, S. E. Radford, R. T. Aplin, and C. M. Dobson. 1993. Detection of transient protein folding populations by mass spectrometry. *Science.* 262:896–899.

Miranker, A., C. V. Robinson, S. E. Radford, and C. M. Dobson. 1996. Investigation of protein folding by mass spectrometry. *FASEB J.* 10:93–101.

Nicholson, L. K., and T. A. Cross. 1989. Gramicidin cation channel: an experimental determination of the right-handed helix sense and verification of β-type hydrogen bonding. *Biochemistry.* 28:9379–9385.

Ogoshi, S., and T. Mita. 1997. Conformation of gramicidin in monolayers, organic solvents and phospholipid bilayers. *Bull. Chem. Soc. Jpn.* 70:841–846.

Pascal, S. M., and T. A. Cross. 1992. Structure of an isolated gramicidin A double helical species by high-resolution nuclear magnetic resonance. *J. Mol. Biol.* 226:1101–1109.

Prosser, R. S., J. H. Davis, F. W. Dahlquist, and M. A. Lindorfer. 1991. ^{2}H nuclear magnetic resonance of the gramicidin A backbone in a phospholipid bilayer. *Biochemistry.* 30:4687–4696.

Robinson, C. V., E. W. Chung, B. B. Kragelund, J. Knudsen, R. T. Aplin, F. M. Poulsen, and C. M. Dobson. 1996. Probing the nature of noncovalent interactions by mass spectrometry. A study of protein-CoA ligand binding and assembly. *J. Am. Chem. Soc.* 118:8646–8653.

Rostom, A. A., and C. V., Robinson. 1999. Disassembly of intact multiprotein complexes in the gas phase. *Curr. Opin. Struct. Biol.* 1:135–141.

Senko, M. W., S. C. Beu, and F. W. McLafferty. 1993. Mass and charge assignment for electrospray ions by cation adduction. *J. Am. Soc. Mass Spectrom.* 4:828–830.

Urry, D. W. 1984. Molecular structure of the gramicidin transmembrane channel: Utilization of carbon-13 nuclear magnetic resonance, ultraviolet absorption, circular dichroism and infrared spectroscopies. *In* Spectroscopy of Biological Molecules. C. Sandorfy and T. Theophanides, editors. D. Reidel Publishing Company, Dordrecht, The Netherlands. 487–510.

Urry, D. W., D. F. Mayers, and J. Haider. 1972. Spectroscopic studies on the conformation of gramicidin A. Evidence for a new helical conformation. *Biochemistry.* 11:487–493.

Veatch, W. R., and E. R. Blout. 1974. The aggregation of gramicidin A in solution. *Biochemistry.* 13:5257–5264.

Veatch, W. R., E. T. Fossel, and E. R. Blout. 1974. The conformation of gramicidin A. *Biochemistry.* 13:5249–5256.

Vis, H., C. V., Dobson, and C. V., Robinson. 1999. Selective association of protein molecules followed by mass spectrometry. *Protein Sci.* 8:1368–1370.

Wagner, D. S., and R. J. Anderegg. 1994. Conformation of cytochrome c studied by deuterium exchange-electrospray ionization mass spectrometry. *Anal. Chem.* 66:706–711.

Wallace, B. A. 1983. Gramicidin A adopts distinctly different conformations in membranes and in organic solvents. *Biopolymers*. 22:397–402.

Wang, F., and X-j. Tang. 1996. Conformational heterogeneity and stability of Apomyoglobin studied by hydrogen/deuterium and electrospray ionization mass spectrometry. *Biochemistry.* 35:4069–4078.

Yi, Q., and D. Baker. 1996. Direct evidence for a two-state protein unfolding transition from hydrogen-deuterium exchange, mass spectrometry and NMR. *Protein Sci.* 5:1060–1066.

Zhang, Y.-P., R. N. A. H. Lewis, R. S. Hodges, and R. N. McElhaney. 1992. Interaction of a peptide model of a hydrophobic transmembrane alpha-helical segment of a membrane protein with phosphatidylcholine bilayers: differential calorimetric and FTIR spectroscopic studies. *Biochemistry.* 46:11579–11588.

Zhang, Z., and D. L. Smith. 1993. Determination of amide hydrogen exchange by mass spectrometry: a new tool for protein elucidation. *Protein Sci.* 2:522–531.