Conformational Study of Gramicidin S Using the Phthalimide Group as Nuclear Magnetic Resonance Marker*

Robert Schwyzer and Urs Ludescher

ABSTRACT: Nuclear magnetic resonance spectra of gramicidin S dihydrochloride and of diphthaloylgramicidin S were determined at room temperature in deuterated methanol. Upfield shifts of the protons on carbon atoms 3 and 6 *and* 4 and 5 of the phthaloyl groups on the ornithine side chains were observed (stronger for the second pair of protons). According to model experiments, this can be best explained by the proximity of the phenyl groups of the D-phenylalanine residues. Of all published models for secondary structure of gramicidin S containing *trans*-peptide links, only the antiparallel pleated sheet model proposed independently by Hodgkin and Oughton and by Schwyzer can account for such an interaction. This model is regarded as a definite possibility for the secondary structure of gramicidin S in solution. The distribution of amide proton signals seems to support this view, as four protons at low field could be interpreted as belonging to the four intramolecular hydrogen bonds. Gramicidin S might populate a number of different conformational states in solution. Should this be the case, then those conformers which do not account for the possibility of an interaction between ornithine and phenylalanine ought to be in rapid equilibrium with those that do so.

About 10 years ago, a three-dimensional model for the peptide backbone (secondary structure) of gramicidin S was proposed in which the two tripeptide sequences L-valyl-L-ornithyl-L-leucyl are arranged like two strands of an antiparallel pleated sheet and are connected at each end by the "odd" residues D-phenylalanine and L-proline. Hodgkin and Oughton (1957) suggested this antiparallel pleated sheet structure (as we shall call it) to explain the X-ray diffraction data; one of us chose it independently (Schwyzer, 1958a,b, 1959) in order to explain synthetic anomalies (cyclization of the pentapeptide unit L-valyl-L-ornithyl-L-leucyl-D-phenylalanyl-L-proline tends to produce the cyclodecapeptide gramicidin S (Schwyzer and Sieber, 1958)) and the infrared spectra data (natural and synthetic gramicidin S hydrochloride as well as ditosylgramicidin S in Nujol mull absorbed at 1637 and 1527 cm^{-1} (Schwyzer and Sieber, 1957); another sharp absorption peak at 700 cm^{-1} might be identical with the amide V band found later in antiparallel β structures (Miyazawa, 1960; Miyazawa and Blout, 1961; Miyazawa *et al.*, 1962). For these reasons, it was favored over alternative models built at that time (for example, one containing two short segments of α helix and corresponding to the secondary structure recently proposed by Liquori and coworkers, 1966), and the existing α-ribbon model of Abbott and Ambrose (1953).

Other three-dimensional models have also been put forward (Hodgkin and Oughton, 1957; Warner, 1961; Vanderkooi *et al.*, 1966; Scott *et al.*, 1967). Their advantages and disadvantages have been discussed (Balasubramanian, 1967; Quadrifoglio and Urry, 1967; Liquori and Conti, 1968). The situation is still controversial, because spectral data derived from amino acid polymers in α, β, and coiled conformations are being used as standards of comparison for evaluating spectral properties of a cyclic decapeptide. This contains at the most two times three residues in situations equivalent to α or β structures; at least four residues have other conformations and different surroundings. This, together with the fact that gramicidin S is cyclic and may contain unknown second-order dipole–dipole and other interactions among absorbing or oscillating groups, makes spectral analyses rather difficult (see also Balasubramanian, 1967).

By using charge transfer (Carrion *et al.*, 1967, 1968) or nuclear magnetic resonance markers (Schwyzer and Ludescher, 1968) for detecting intramolecular interactions of certain amino acid side chains, we should be able to specify those side-chain interactions which actually occur and which have to be accounted for by any three-dimensional model that pretends to be realistic. In this piece of work, we have used the phthalimide group as nuclear magnetic resonance marker. The results clearly demonstrate specific interactions between the phthaloyl groups on the ornithyl side chains and the phenyl groups of the D-phenylalanine residues in methanolic solutions of diphthaloylgramicidin S.

The antiparallel pleated sheet model is consistent with these results and may be regarded as a conformational possibility[1] for the secondary structure of diphthaloylgramicidin S in methanol. All those secondary structures seem to be excluded which do not allow for inter-

* From the Laboratory of Molecular Biology, Swiss Federal Institute of Technology, Zürich, Switzerland. *Received March 19, 1968.* Aided by the Swiss National Foundation for Scientific Research (grant no. 3426).

[1] It may be pointed out that there is no reason for assuming the presence of only *one* conformation of gramicidin S in a given solution *unless* an equilibrium between various conformers of similar thermodynamic stability has been ruled out (the popula-

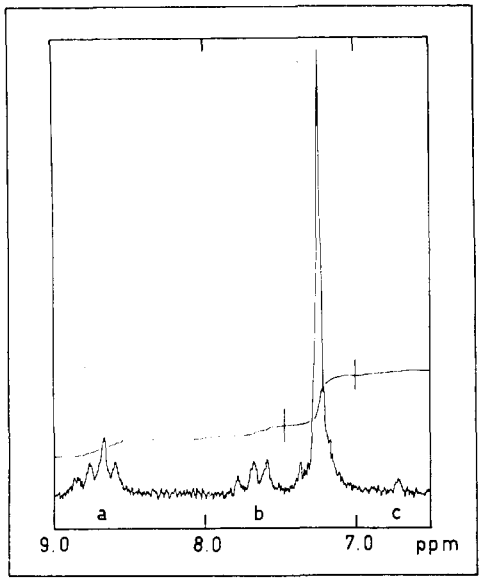

FIGURE 1: Proton nuclear magnetic resonance spectrum (100 Mc) of gramicidin S dihydrochloride in CD_3OD as solvent, tetramethylsilane as reference and at ambient temperature. Region of the 18 amide and phenyl protons (with integration tracing).

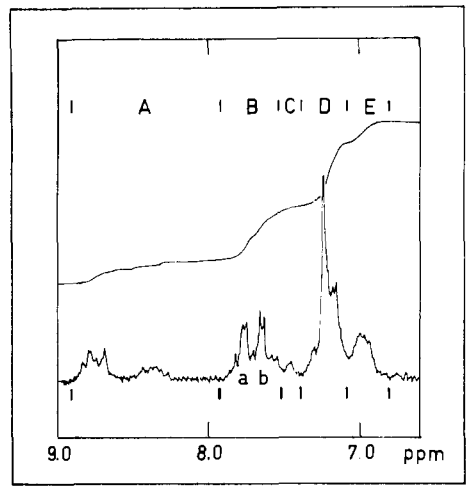

FIGURE 2: Proton nuclear magnetic resonance spectrum (100 Mc) of diphthaloylgramicidin S in CD_3OD as solvent, tetramethylsilane as reference and at ambient temperature. Region of the 26 amide, phenyl, and phthaloyl protons (with integration tracing).

action of phenylalanine and ornithine side chains, unless they are in a state of rapid equilibrium with conformations allowing such interactions.

Experimental Procedures and Materials

Gramicidin S dihydrochloride was synthetic material (Schwyzer and Sieber, 1956, 1957).

Diphthaloylgramicidin S (mp 284–285°) was obtained by reaction of 16 mg of gramicidin S dihydrochloride in 2 ml of ethanol with 2 drops of triethylamine and an excess (~20 mg) of *N*-carbethoxyphthalimide (Nefkens *et al.*, 1960) at room temperature for 3 hr. The product was isolated by evaporation of the solvent at reduced pressure, extraction into ethyl acetate, and washing this solution with dilute HCl and water. After drying with a concentrated solution of NaCl, the organic phase was evaporated at reduced pressure. The residue was recrystallized three times from hot ethanol by slow addition of hot water to a concentration of approximately 35–50% H_2O (see Schwyzer and Sieber, 1957); yield, 14 mg.

Proton nuclear magnetic resonance spectra were obtained from solutions in deuteriomethanol (CD_3OD) with a Varian HA-100 spectrometer at 100 MHz; tetramethylsilane served as reference. The obtained nuclear magnetic resonance spectra revealed the analytical purity of the sample of diphthaloylgramicidin S.

Shifts of phthaloyl protons were measured against *N*-phthaloylglycine methyl ester in deuteriomethanol (two protons at 7.86 and two protons at 7.84 ppm). The signals of 2-bromoethylphthalimide in deuteriochloroform (Bhacca *et al.*, 1962) appear at 7.87 (two protons) and 7.83 ppm (two protons), indicating that these changes in solvent and in structure influence the position of the signals only very slightly. As this was confirmed in other examples, Figure 3 (A, C, D in $CDCl_3$ and B in CD_3OD) seems reasonable for semiquantitative comparison of the results of this investigation with those of the preceding one (Schwyzer and Ludescher, 1968).

Results

Figure 1 shows the proton nuclear magnetic resonance of gramicidin S in deuterated methanol in the region of the amide and phenyl protons. The peak of the phenyl protons of the D-phenylalanyl residues appears at 7.25 ppm; the amide NH protons give rise to three groups of signals centered at 8.67 (a), 7.64 (b), and 6.78 (c) ppm, respectively. Assuming a total of 18 protons in the region between 9 and 6.5 ppm, integration gives 10.3 protons under the phenyl proton peak (limits shown in Figure 1) and 7.7 protons in the remaining area. Taking into account the 0.3 NH proton probably buried under the signal of the 10 phenyl protons, we find 3.9 protons absorbing at the a peak and 4.1 protons in the region of the b and c peaks (2.7 protons at b between 7.5 and 8.1 ppm; 1.4 protons under the phenyl proton and c peaks).

Approximately the same region of the nuclear magnetic resonance spectrum of diphthaloylgramicidin S (in deuterated methanol) is shown in Figure 2. The amide protons (integrated value of 7.9) absorb at 8.74 and 8.37 ppm (the region A of Figure 2 totaling 3.7 protons), and at 7.45 (C) and 6.98 (E) ppm (1.2 and 3.0 protons, respectively). Six of the phenyl protons give rise to a signal at 7.24 ppm, the four remaining protons signalize at 7.18 and 7.15 ppm. The protons of the phthaloyl groups show up very clearly in a composite peak with signals of equal strength (four plus four protons) centered at

tion of four different conformational states was demonstrated by Karle and Karle (1963) even in crystals of cyclohexaglycyl). Furthermore, there are certainly other secondary structures of gramicidin S, besides the antiparallel pleated sheet structure, which allow ornithine–phenylalanine interaction. We therefore chose to use the expression "conformational possibility" (*cf.* Carrión *et al.*, 1968).

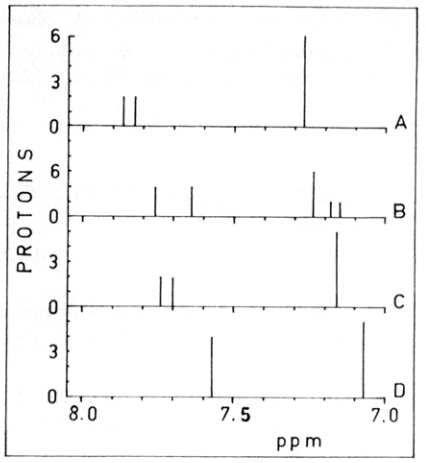

FIGURE 3: Comparison of chemical shifts and number of protons of (A) 2-bromoethylphthalimide and benzene (separate), (B) diphthaloylgramicidin S, (C) phthaloyl-L-phenylalanine methyl ester, and (D) *cis*-2-phenylcyclopentylphthalimide.

7.76 and 7.64 ppm. This represents an upfield shift of 0.10 ppm of the "a" protons, and of 0.20 ppm of the "b" protons against the standard. The separation of the a and b peaks has increased from ~0.04 ppm in *N*-alkylphthalimides to 0.12 ppm in diphthaloylgramicidin S.

All of the signals of gramicidin S assigned to NH disappear in the presence of D_2O–F_3CCOOD (R. Schwyzer, unpublished work).

Discussion

The upfield shift of the phthaloyl protons in diphthaloylgramicidin S against the standard indicates a change in the close surroundings of the phthaloyl groups. The fact that the separation of the a and the b proton peaks is enhanced suggests that the effect of the new surroundings is anisotropic, affecting the b protons more than the a protons (Schwyzer and Ludescher, 1968).

Similarly, the separation of two phenyl protons from the remaining three in each residue of phenlyalanine suggests a difference in the close surroundings of the phenyl rings. These concomitant changes of surroundings are most probably complementary and due to mutual ring-current effects like those observed in model compounds containing phthalimide and phenyl rings (Schwyzer and Ludescher, 1968) (see Figure 3).

If one excludes stacking effects (which are highly improbable in dilute solutions in methanol[2]), this result means that diphthaloylgramicidin S, dissolved in deuterated methanol, must have the possibility of assuming conformations which bring the phthaloyl groups on the ornithine residues into rather close contact with the phenyl groups of the phenylalanine residues. Furthermore, this contact must be such that the b protons (on carbon atoms 4 and 5) of the phthaloyl group lie closer to the center of the phenyl ring and therefore be more subject to shielding by ring-current effects than the a protons (on carbon atoms 3 and 6). As a consequence, certain protons of the phenyl rings of the phenylalanine residues must be closer to the phthaloyl groups than others and, hence, show up at slightly different positions in the nuclear magnetic resonance spectrum (Figure 1 and 2).

[2] This has been confirmed by proton magnetic resonance studies at different temperatures (0–50°).

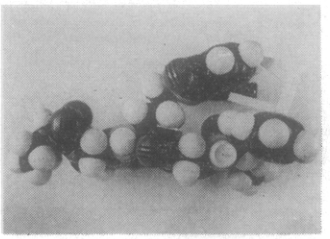

FIGURE 4: Ealing CPK[3] model of the peptide backbone of gramicidin S according to the antiparallel pleated sheet conformation (Hodgkin and Oughton, 1957; Schwyzer, 1958a,b). Side chains of N^{δ}-phthaloyl-L-ornithine (position 2) and D-phenylalanine (position 4) included, to show possibility of interaction between aromatic rings. Side view: the side chains of valine and leucine have been replaced by hydrogen atoms (below the plane of the ring, left and right foreground, and right and left background). Ornithine 7 and phenylalanine 8 are also omitted. A ruler is included between the phenyl and the phthaloyl rings to help visualize the relevant distances of the phthaloyl a and b protons from the center of the phenyl ring. These are for the a protons $z \simeq 2.7$ and $p \simeq 2.7$ ring radii, for the b protons $z \simeq 3.8$ and $p \simeq 1.8$ ring radii. The z axis is the axis normal to the plane of the phenyl ring at its center; the p axis is in the plane of the ring and measures the distance of the protons to the z axis (see Emsley *et al.*, 1965).

Investigations of gramicidin S by spectroscopic methods (ultraviolet and infrared absorption, optical rotatory dispersion, and circular dichroism) (Balasubramanian, 1967; Quadrifoglio and Urry, 1967) indicate that the conformation of the cyclic peptide backbone is rather stable in solution and is hardly affected by changes in solvent and temperature. It is from such a stable conformational platform (or stable equilibrium mixture of conformational platforms), then, that the side chains of N^{δ}-phthaloylornithine and phenylalanine are "free" to interact, as the small energy of interaction of such aromatic systems (Carrión *et al.*, 1968) is most

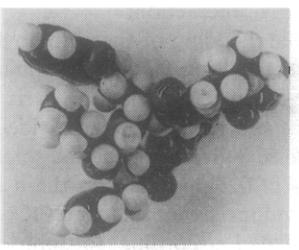

FIGURE 5: Ealing CPK[3] model of the peptide backbone of gramicidin S according to Vanderkooi *et al.* (1966). Side chains of N^{δ}-phthaloyl-L-ornithine (position 2), L-leucine (position 3), and D-phenylalanine (position 4) are included, demonstrating lack of reasonable interaction between aromatic rings even in favorable conformations. The corresponding amino acid side chains at positions 7, 8, and 9 have been omitted. The same is true for side chains of valine 1 and 6; the points of attachment to the α-carbon atoms show clearly as holes at the middle right hand side of the model.

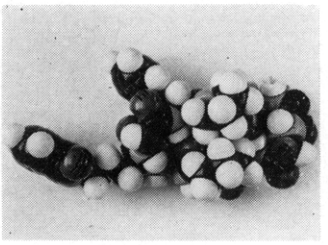

FIGURE 6: Ealing CPK[3] model of the peptide backbone of gramicidin S according to Liquori *et al.* (1966). Side chains of N^{δ}-phthaloyl-L-ornithine (position 2) and D-phenylalanine (position 4) are included, demonstrating lack of reasonable interaction between aromatic rings even in favorable conformations. All other amino acid side chains have been omitted, except those of proline 5 and 10 seen facing us in the model.

probably insufficient to cause gross changes in backbone conformation.

Of the four conformational possibilities with peptide groups in the *trans* configuration that have been put forward for possible conformations of gramicidin S, only the one with six L-amino acid residues in the antiparallel pleated sheet structure (Hodgkin and Oughton, 1957; Schwyzer, 1958a,b, 1959; Schwyzer and Sieber, 1958) satisfies the condition of allowing close interactions between the side chains of N^{δ}-phthaloylornithine and phenylalanine (Figures 4–6).

This conformation would also explain the observation (Figure 1) of four (presumably intramolecularly hydrogen-bonded) NH protons at low field (8.67 ppm) and of four others (nonbonded) at higher field (7.64 and 6.78 ppm). The distortion of this picture in diphthaloylgramicidin S demonstrates the extremely high susceptibility of the NH proton signals to environment which is probably also the reason for the differences between the position of NH signals observed in deuterated methanol (this work) and in deuterated dimethyl sulfoxide (Liquori and Conti, 1968; R. Schwyzer, unpublished observations). In view of these uncertainties, we would not wish to draw any definite conclusions from the signals of the NH protons; suffice it to mention that their positions and integration seem to support the idea of the antiparallel pleated sheet conformation suggested by the phthaloyl–phenyl interaction.

Although the shielding of the phthaloyl protons is an average effect, resulting from the population of many different side-chain conformations, it is interesting to point out that the rather probable side-chain conformation in Figure 3 gives a calculated shielding effect on the b protons of 0.2 ppm and on the a protons of 0.1 ppm (mean values from Ealing CPK[3] and Dreiding models, calculated according to Johnson and Bovey (1958); see Appendix B in Emsley *et al.* (1965)). These values are surprisingly close to the observed values of 0.20 (b) and 0.10 (a).

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