Conformational and Spectral Analysis of the Polypeptide Antibiotic *N*-Methylleucine Gramicidin S Dihydrochloride by Nuclear Magnetic Resonance†

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ABSTRACT: The 220-MHz proton magnetic resonance spectrum of the cyclic decapeptide antibiotic, mono-*N*-methylleucine gramicidin S, is reported and all the resonances have been assigned to specific protons of the constituent amino acids. Three methods—temperature dependence and solvent mixture (methanol–trifluoroethanol and dimethyl sulfoxide–trifluoroethanol) dependence of peptide NH proton chemical shifts and proton deuteron exchange—have been utilized to delineate peptide NH protons. The results of the above methods, coupled with the observed vicinal α-CH–NH coupling constants and chemical shifts, indicate that in trifluoroethanol the peptide NH protons of D-Phe$_4$, D-Phe$_9$, L-Orn$_2$, and L-Val$_6$ are exposed to the solvent, and those of L-Val$_1$, L-Orn$_7$, and L-Leu$_8$ are solvent shielded and intramolecularly hydrogen bonded. In trifluoroethanol, dimethyl sulfoxide, and methanol, the decapeptide has no C_2 symmetry, and there are only minor conformational differences in the different solvents. In the proposed conformation in trifluoroethanol, one-half of the decapeptide retained the hydrogen bonding pattern of gramicidin S, i.e. *cyclo*-(L-Val$_1$ NH···O–C L-Leu$_8$) (a β turn) and *cyclo*-(L-Leu$_8$ NH···O–C L-Val$_1$). The second half of the molecule exhibits a different type of stable β turn involving the ten-atom hydrogen-bonded ring, *cyclo*-(L-Orn$_7$-NH···O–C D-Phe$_4$).

Three possible conformational models for gramicidin S in the solid state have been suggested by Hodgkin and Oughton (1957) on the basis of their X-ray diffraction data. One of the three models was the anti-parallel β-type conformation with four intramolecular hydrogen bonds between the two antiparallel chains involving the two pairs of L-Val and L-Leu residues. A similar β-type conformation was proposed on the basis of ease of cyclization by Schwyzer et al. (1958) and Schwyzer (1959). The same secondary structure was proposed in solution on the basis of nuclear magnetic resonance (NMR) studies and peptide NH exchange rates by Stern et al.[1] (1968), and also the β-type conformation in solution was independently arrived at in this laboratory using ^{1}H NMR temperature delineation of peptide NH moieties (Ohnishi and Urry, 1969). On the basis of proton–deuterium exchange of the peptide protons, temperature dependence of the circular dichroism pattern, and conformational energy calculations, the conformation of gramicidin S is unusually stable (Stern et al., 1968; Ovchinnikov et al., 1970; Urry and Ohnishi, 1970; Quadrifoglio and Urry, 1967; DeSantis and Liquori, 1971).

Mono-*N*-methylleucine gramicidin S dihydrochloride has been synthesized by Sugano et al. (1973a,b) to elucidate structure–biological activity relationships of gramicidin S and its analogs. They found that the antimicrobial activities of mono-*N*-methylleucine gramicidin S dihydrochloride were nearly the same as those of gramicidin S demonstrating that the peptide NH protons of the L-Leu residues are not necessary for exhibiting the biological activity. This presents an opportunity to further consider the conformation and to throw some light on the question of the relationships between specific conformations and biological activity.

It is the purpose of this work to present ^{1}H NMR studies designed to determine the conformational features of mono-*N*-methylleucine gramicidin S dihydrochloride in solution after achieving proton magnetic resonance assignments, which is a prerequisite for extracting structural information contained in the ^{1}H NMR spectrum. The spectral features for distinguishing between solvent-exposed and solvent-shielded peptide NH protons are discussed as they apply to mono-*N*-methylleucine gramicidin S dihydrochloride. A conformational model, in which one-half of the molecule retains its gramicidin S β-type conformation and the second half has a different β-type conformation, and in which the peptide NH proton of the L-Leu residue does not participate in the intramolecular hydrogen bonding, is proposed for mono-*N*-methylleucine gramicidin S dihydrochloride on the basis of the NMR data and steric considerations.

Experimental Section

Proton magnetic resonance spectra were recorded on a Varian Associates HR-220 spectrometer, equipped with an SS-100 computer system. Homonuclear spin decoupling experiments were performed with a field-tracking decoupling accessory; by this accessory a resonance may be irradiated continuously as the remainder of spectrum, upfield or downfield from the decoupling frequency, is either scanned manually or multiscanned on an expanded scale with an SS-100 computer system. This latter technique is extremely useful for resonance assignments of a cyclodecapeptide

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[1] These authors did not indicate the assignment of δ-CH$_2$ protons of L-Orn.

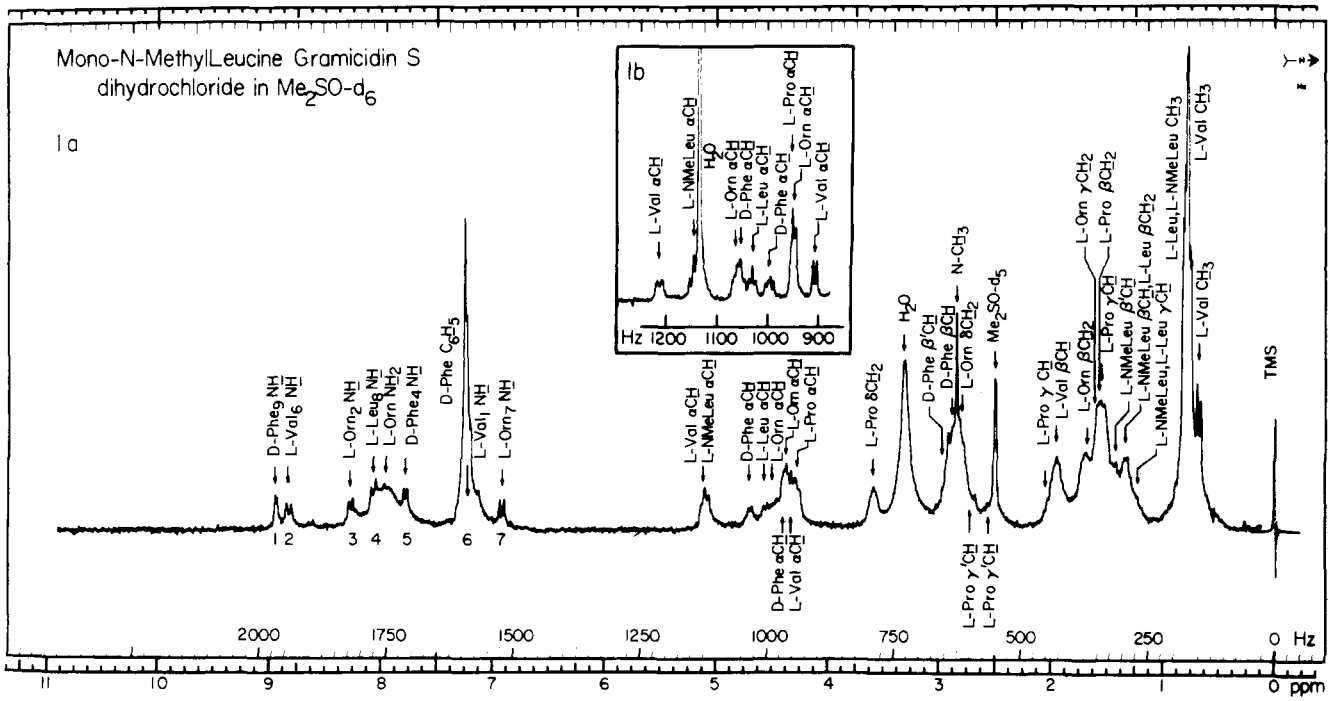

FIGURE 1: Complete 220-MHz proton magnetic resonance spectrum of 3% (w/v) mono-*N*-methylleucine gramicidin S dihydrochloride in (a) dimethyl-d_6 sulfoxide at 30°, and (b) 220-MHz ^{1}H NMR spectrum of the α-CH region in deuteriotrifluoroethanol at 30°.

especially in the absence of symmetry. Chemical shifts were measured relative to tetramethylsilane as an internal reference. The chemical-shift difference between resonances of ethylene glycol or methanol was used to determine the probe temperature to within ±1°. The synthesis of 3-*N*-methylleucine gramicidin S dihydrochloride has been reported elsewhere by Sugano et al. (1973a,b).

Results

Spectral Analysis.[2] The 220-MHz proton magnetic resonance spectrum of mono-*N*-methylleucine gramicidin S dihydrochloride in Me_2SO-d_6 at 30° is presented in Figure 1a together with the complete resonance assignments. The insert in Figure 1b is the α-CH region in deuteriotrifluoroethanol at 30°. The 220-MHz ^{1}H NMR spectra of the peptide proton resonances in Me_2SO-d_6 at 20°, with the decoupling radiofrequency power applied at the resonance frequencies of the α-CH resonances, are presented in Figure 2. The amide resonances are numbered in the order of increasing field as they appear in Me_2SO-d_6. A major complicating spectral feature of mono-*N*-methylleucine gramicidin S dihydrochloride is that there is no C_2 symmetry. Hence, in contrast to gramicidin S, which is spectrally a pentapeptide, assignment of multiplets to specific protons or groups of protons in mono-*N*-methylleucine gramicidin S dihydrochloride involves ten spectrally distinguishable residues. Spectral analysis of a polypeptide with an increasing number of different amino acid residues is often complicated from overlap of α, β, and γ proton resonances of different amino acid residues. Because individual proton transitions in a particular amino acid occur near transitions of other residues the strength of decoupling power (f_2) used for spin decoupling in the α, β, and γ region of the spectrum was less than that utilized for less complicated double resonance experiments.

Assignment of individual resonances to specific protons or groups of protons of the constituent amino acids (Figures 1a and 1b and 2) was achieved in the majority of cases by performing homonuclear spin decoupling experiments not only in different solvents, but also in various solvent mixtures at different temperatures with a field-tracking decoupling accessory. The coupled resonances were then followed as a function of solvent or temperature to determine their positions at the temperature and solvent of interest.

With a completely assigned ^{1}H NMR spectrum of mono-*N*-methylleucine gramicidin S in Me_2SO-d_6, the assignments of the amide protons in CH_3OH and F_3EtOH were confirmed from solvent dependence of the peptide proton chemical shifts in Me_2SO-CH_3OH and Me_2SO-F_3EtOH mixtures as a function of the mole percent of CH_3OH and F_3EtOH, respectively. Assignments of the remaining resonances (α, β, γ, δ, etc.) in F_3EtOH-d_3 were achieved by spin decoupling in deuteriotrifluoroethanol and solvent dependence of the α-CH proton chemical shifts in Me_2SO-d_6-F_3EtOH-d_3 mixtures. The resonance assignments and coupling constants of all the amino acid residues in different solvents are listed in Tables I, II, and III.

It is necessary first to assign a set of resonances to an amino acid and then to place the amino acid in the sequence. For the sequence numbering the method of Sugano et al. (1973a,b) will be used, i.e. *cyclo*-(L-Val_1-L-Orn_2-L-N-$MeLeu_3$-D-Phe_4-L-Pro_5-L-Val_6-L-Orn_7-L-Leu_8-D-Phe_9-L-Pro_{10}). The step from assignment of resonance to the placing of a resonance in a sequence requires the decision, for example, as to which L-Val is residue 1 and which is residue 6. Fortunately, as will be shown below, one-half of the molecule retains the gramicidin S conformation so that chemical shifts, temperature coefficients, and solvent perturbation behavior similar to those of the peptide NH resonances of gramicidin S are exhibited by several peptide NH resonances of the *N*-methyl derivative. Since hydrogen

[2] Supplementary material describing details of spectral analysis will appear following these pages in the microfilm edition of this volume of the journal (see paragraph at end of paper regarding supplementary material).

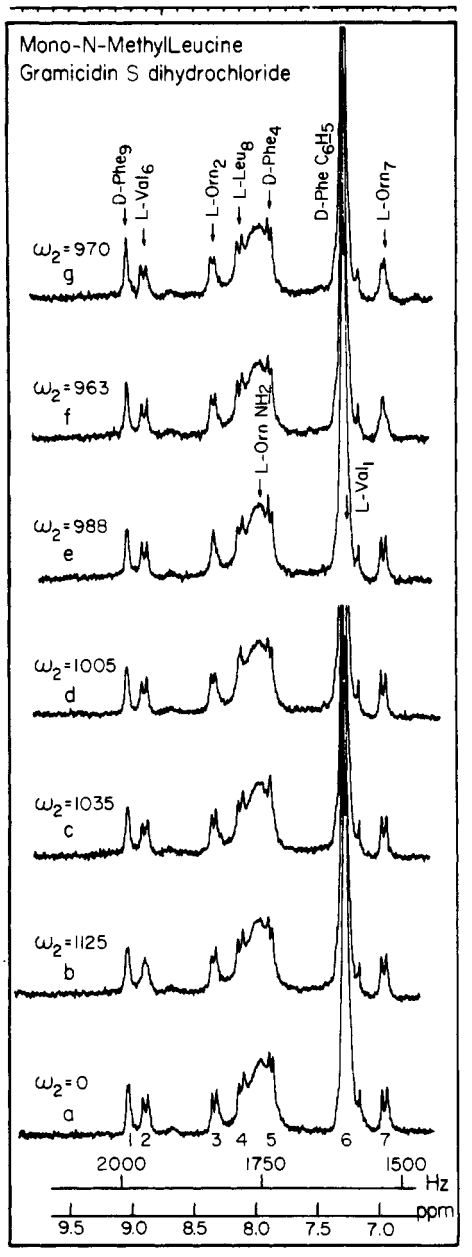

FIGURE 2: The 220-MHz ^1H NMR spectra of the peptide proton resonances in dimethyl-d_6 sulfoxide at 20° with decoupling radiofrequency power applied at f_2, the resonance frequencies of the α-CH resonances: (a) f_2 off, (b) f_2 = 1125, L-Val$_6$; (c) f_2 = 1035, D-Phe$_4$; (d) f_2 = 1005, L-Leu$_8$; (e) f_2 = 988, L-Orn$_2$; (f) f_2 = 963, L-Orn$_7$; (g) f_2 = 970, D-Phe$_9$.

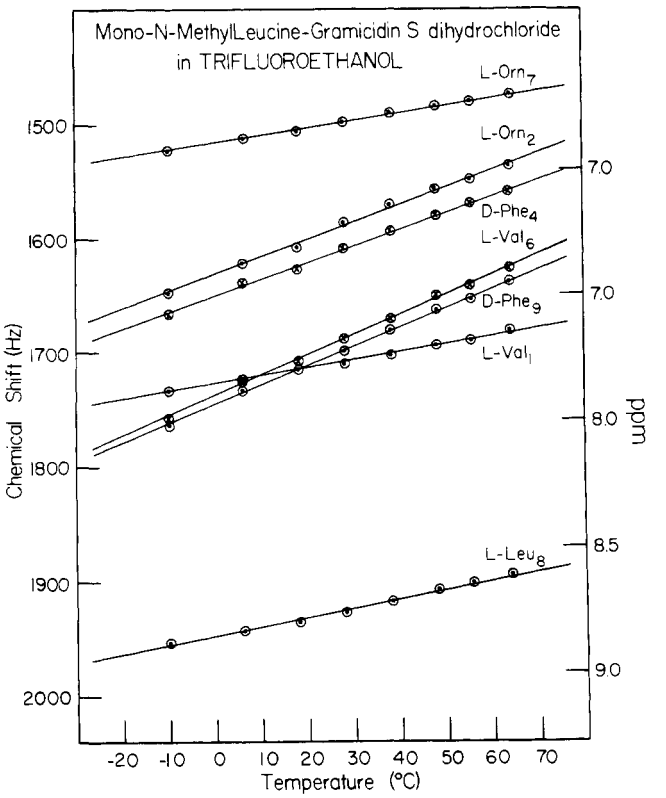

FIGURE 3: Temperature dependence of the peptide proton resonances in deuteriotrifluoroethanol.

bonding in gramicidin S involves the Leu NH, the part of the sequence containing L-N-MeLeu$_3$ cannot be gramicidin S type. Accordingly, the common portion must involve the sequence L-Leu$_8$-D-Phe$_9$-L-Pro$_{10}$-L-Val$_1$. As will be seen below the ornithine residues exhibit very different peptide NH chemical shifts with one peptide NH solvent exposed as in gramicidin S and the other strongly solvent shielded and at high field as in a β turn (Kopple et al., 1969a,b; Urry and Ohnishi, 1970). Since only the L-Orn$_7$ NH can participate in a β turn and retain the gramicidin S conformation for residues 8, 9, 10, and 1, and since the coupling constant data are in accord with a β turn, the solvent shielded L-Orn NH (resonance 7) is assigned to residue 7. A similar β turn has been proposed to occur twice in di-*N*-methylleucine gramicidin S (Kumar et al., 1975). This brief statement of translating assigned resonances to specific residues within the sequence is given at this stage in order that the subsequent listing can be according to sequence.

Temperature Dependence. The temperature dependences of the peptide proton resonances of mono-*N*-methylleucine gramicidin S dihydrochloride in F_3EtOH, Me_2SO-d_6, and CH_3OH are presented in Figure 3 and Table IV. The resonances are numbered in the order of increasing field as they appear in Me_2SO-d_6. Resonances of protons attached to nitrogen broaden out when the rate of proton exchange between the peptide NH moiety, δ-NH_3^+, and residual H_2O becomes comparable to the difference in their respective chemical shifts. In Me_2SO-d_6, all the amide resonances are well resolved between 17 and 84° except resonance 6 (L-Val$_1$) which overlaps with the aromatic protons. At 84°, however, it resonates at 1565 Hz as a well-resolved doublet. Since the peptide protons did not exhibit broadening in the temperature range 17 to 84°, their temperature dependence measurements were performed below their coalescence temperature. In MeOH at and below 6° (6 to −30°), resonance 7 (L-Orn$_7$) overlaps with the aromatic protons, but at 30° all the peptide NH resonances are well resolved. In F_3EtOH, all the peptide resonances are well resolved between 6 and 64° except for resonance 3 (L-Orn$_2$) which becomes broader at 64°. It is possible that the amide proton of the L-Orn$_2$ is influenced by the L-Orn δ-NH_3^+ group. The origin of this differential broadening may be due to quadrapole relaxation of the ^{14}N nuclei or peptide proton exchange. The effect of ^{14}N relaxation on the peptide proton line width could be determined by irradiation at the ^{14}N resonance frequency.

Increasing the temperature of a folded, compact molecule such as mono-*N*-methylleucine gramicidin S dihydrochloride causes a change in the vibrational states of the molecule, so that the average magnetic environment experi-

Table I: Chemical Shifts of the Proton Resonances of Mono-*N*-methylleucine Gramicidin S Dihydrochloride.[a]

Amino Acid[b] Residues	Solvent	ν_α	ν_β	$\nu_{\beta'}$	ν_γ	$\nu_{\gamma'}$	ν_δ	Other ν
L-Val_1	Me_2SO-d_6	952	430		169			
	F_3EtOH-d_3	908	495		225, 210			
	Me_2SO-d_6 + 10% D_2O	~950	430		~175			
L-Orn_2	Me_2SO-d_6	988	370		355		615	NH_3^+ ~1750
	F_3EtOH-d_3	1065	450				~615	
	Me_2SO-d_6 + 10% D_2O	973	~360				~610	
L-N-$MeLeu_3$	Me_2SO-d_6	~1125	295	315	~270		~170	N-CH_3 ~625
	F_3EtOH-d_3	1148	370		~300		~195	~675
	Me_2SO-d_6 + 10% D_2O	1125	300	310	~270		~175	~628
D-Phe_4	Me_2SO-d_6	1035	635	655				Ar ~1600
	F_3EtOH-d_3	1054	650	660				Ar ~1615, ~1590
	Me_2SO-d_6 + 10% D_2O	1035	~640					Ar ~1600
L-Pro_5	Me_2SO-d_6	940	345		450, 340	600, 565	790	
L-Pro_{10}	F_3EtOH-d_3	952	~385		425, 390	595, 550	815	
	Me_2SO-d_6 + 10% D_2O	935	365				~795	
L-Val_6	Me_2SO-d_6	~1125	430		150, 169			
	F_3EtOH-d_3	1215	505		~210			
	Me_2SO-d_6 + 10% D_2O	1110	425		155, ~175			
L-Orn_7	Me_2SO-d_6	963	~370		355		615	NH_3^+ ~1750
	F_3EtOH-d_3[c]	948	450				~615	
	Me_2SO-d_6 + 10% D_2O	~952	~360				~610	
L-Leu_8	Me_2SO-d_6	1005	295		~270		~180, ~175	
	F_3EtOH-d_3	1030	340		300		~200	
	Me_2SO-d_6 + 10% D_2O	1005	295	310			~175	
D-Phe_9	Me_2SO-d_6	970	635	655				Ar ~1600
	F_3EtOH-d_3	997	650	660				~1615, ~1590
	Me_2SO-d_6 + 10% D_2O	960	~640					~1600

[a] At 220 MHz, the chemical shifts in Me_2SO-d_6 and F_3EtOH-d_3 are at 30° and in Me_2SO-d_6 + 10% D_2O at 20°. [b] The amino acid residues are numbered according to the amino acid sequence numbering (Figure 6). [c] In F_3EtOH-d_3, the α-CH proton of the L-Orn residue 7 overlaps with the α-CH protons of the L-Pro residues 5 and 10 but, in Me_2SO-d_6–F_3EtOH-d_3 (7:3, v/v) mixture, it resonates at 986 Hz as a well-resolved resonance.

enced by the peptide protons could change. Hence, it is important to conduct temperature dependence experiments in different solvents in which relative temperature coefficients for solvent-exposed and solvent-shielded peptide protons have been shown to be delineated with linear temperature dependences. The temperature coefficients ($d\delta/dT$, parts per million per degree Celsius) of the peptide protons in Me_2SO-d_6, CH_3OH, and F_3EtOH are listed in Table IV. In Me_2SO-d_6 and CH_3OH, the peptide protons of Leu_8 and Val_1 exhibited almost identical, small temperature coefficients (−0.0027 ppm/°C); their 0° intercepts in Me_2SO-d_6 are 8.16 and 7.33 ppm, and in CH_3OH they are 8.65 and 7.72 ppm, respectively. The coefficient of the upfield Orn peptide proton of residue 7 in Me_2SO-d_6 is small (−0.0016 ppm/°C) with 0° intercept at 6.98 ppm, and in CH_3OH the coefficient is −0.0042 ppm/°C with 0° intercept at 7.24 ppm. The Phe peptide protons of the residues 9 and 4 exhibited relatively large coefficients in Me_2SO-d_6 (−0.0080 and −0.0068 ppm/°C) with 0° intercepts at 9.18 and 8.00 ppm, respectively; they also exhibited almost similar, large coefficients in CH_3OH (−0.0081 and −0.0074 ppm/°C) with 0° intercepts at 9.03 and 8.12 ppm, respectively. The coefficients of Orn_2 and Val_6 amide protons in Me_2SO-d_6 are intermediate (−0.0040 and −0.0041 ppm/°C) with 0° intercepts at 8.40 and 8.96 ppm, respectively, and the coefficients in CH_3OH are relatively large (−0.0061 and −0.0060 ppm/°C) with 0° intercepts at 8.44 and 9.02 ppm, respectively. In F_3EtOH, the peptide protons of Leu_8, Val_1, and Orn_7 exhibited almost identical, relatively small temperature coefficients (−0.0037, −0.0033, and −0.0030 ppm/°C) with 0° intercepts at 8.85, 7.85, and 6.89 ppm, respectively. The coefficients of the remaining peptide protons of $Phe_{9,4}$, Orn_2, and Val_6 are −0.0076, −0.0066, −0.0070, and −0.0082 ppm/°C with 0° intercepts at 7.93, 7.50, 7.41, and 7.90 ppm, respectively. It is important to be aware that traces of acid or base in solution may alter the absolute values of the temperature coefficients of the solvent-exposed and partially solvent-shielded peptide protons by catalyzing the peptide proton exchange, which is of particular concern when broadening is observed with increasing temperature.

Effect of Solvent Variation on the Peptide Proton Chemical Shift and $J_{\alpha\text{-CH-NH}}$ Coupling Constant. Solvent dependences of the peptide proton chemical shifts in Me_2SO–F_3EtOH and CH_3OH–F_3EtOH are presented in Figures 4 and 5. The solvent dependence plots are quite similar. On addition of F_3EtOH to CH_3OH, the amide protons of Val_6, Phe_9, and Orn_2 exhibited dramatic upfield shifts (266, 243, and 223 Hz) greater than 1 ppm in a nearly monotonic fashion, and the peptide proton of Phe_4 also showed a relatively large upfield shift of 160 Hz (~0.73 ppm). The peptide protons of Leu_8 and Val_1 exhibited small downfield shifts of 38 and 28 Hz, and the amide proton of Orn_7 showed a small upfield shift (~70 Hz). Also, on addition of F_3EtOH to Me_2SO-d_6, the peptide protons of residues 6, 9, and 2 shift dramatically upfield by 259, 273, and 224 Hz (>1 ppm), and the Phe_4 amide proton exhibited a relatively large upfield shift of 134 Hz. The peptide protons of the residues 8 and 1 exhibited downfield shifts (~146 and 114 Hz), and the amide proton of Orn_7 exhibited a small upfield shift (~31 Hz). On increasing the mole percent of F_3EtOH in CH_3OH or in Me_2SO, the observed $^3J_{\alpha\text{-CH-NH}}$ coupling constants within experimental error remained constant.

H–D Exchange Rates. Since H–D exchange rates are

Table II: Coupling Constants of the Proton Resonances of Mono-*N*-methylleucine Gramicidin S Dihydrochloride.[a]

Amino Acid[b] Residues	Solvent	$J_{\alpha,\beta}$	$J_{\alpha,\beta'}$	$J_{\beta,\beta'}$	$J_{\beta,\gamma}$	$J_{\gamma,\delta}$	$J_{\delta,\delta'}$
L-Val_1	Me_2SO-d_6				7.0		
	F_3EtOH-d_3	8.0			7.0 ± 0.2		
	Me_2SO-d_6 + 10% D_2O	~7.8					
L-Orn_2	Me_2SO-d_6	~4.0	9.2 ± 0.4			5.9 ± 0.3	
	F_3EtOH-d_3	4.4 ± 0.4	10.0 ± 0.4				
	Me_2SO-d_6 + 10% D_2O						
L-N-$MeLeu_3$	Me_2SO-d_6	~8.0	5.2 ± 0.4			6.8 ± 0.3	
	F_3EtOH-d_3	7.8 ± 0.2	7.8 ± 0.2			7.0 ± 0.2	
	Me_2SO-d_6 + 10% D_2O	~8.4	~6.0				
D-Phe_4	Me_2SO-d_6	~9.0	~6.3	11.5 ± 0.5			
	F_3EtOH-d_3	9.2 ± 0.4	6.0 ± 0.4				
	Me_2SO-d_6 + 10% D_2O	7.5 ± 0.3	7.5 ± 0.3				
L-Pro_5	Me_2SO-d_6	7.5 ± 0.5	~0			5.0 ± 0.5	9.5 ± 0.5
	F_3EtOH-d_3[c]	6.5	~0				
	Me_2SO-d_6 + 10% D_2O	7.2	~0				
L-Val_6	Me_2SO-d_6[d]				7.0		
	F_3EtOH-d_3	10.0 ± 0.4			6.5		
	Me_2SO-d_6 + 10% D_2O	~9.6			~7.5		
L-Orn_7	Me_2SO-d_6					5.9 ± 0.3	
	F_3EtOH-d_3[e]	~5.6 ± 0.4					
	Me_2SO-d_6 + 10% D_2O						
L-Leu_8	Me_2SO-d_6	7.6 ± 0.4				6.8 ± 0.3	
	F_3EtOH-d_3	~7.2	~7.2			~6.5	
	Me_2SO-d_6 + 10% D_2O	5.7 ± 0.3	~8.4				
D-Phe_9	Me_2SO-d_6	~9.0	~6.3	11.5 ± 0.5			
	F_3EtOH-d_3	10.3 ± 0.2	5.8 ± 0.2				
	Me_2SO-d_6 + 10% D_2O						

[a] Coupling constants are in hertz. [b] The amino acid residues are numbered according to the amino acid sequence numbering (Figure 6). [c] The α-CH protons of the L-Pro residues 5 and 10 are well resolved in F_3EtOH-d_3–Me_2SO-d_6 (7:3, v/v) mixture, and resonate at 952 Hz exhibiting 6.5 ± 0.3 Hz vicinal coupling constant. [d] In Me_2SO-d_6, the α-CH proton of the L-Val residue 6 overlaps with the α-CH proton of the L-N-MeLeu residue 3 but, in Me_2SO-d_6–F_3EtOH-d_3 (1:1, v/v) mixture, it resonates at 1172 Hz as a resolved doublet exhibiting 10.0 ± 0.4 Hz $J_{\alpha,\beta}$ coupling constant. [e] In F_3EtOH-d_3, the α-CH proton of the L-Orn residue 7 overlaps with the α-CH protons of the L-Pro residues 5 and 10 but, in Me_2SO-d_6–F_3EtOH-d_3 (7:3, v/v) mixture, it resonates at 986 Hz as a well-resolved resonance exhibiting 5.4 ± 0.2 and 3.6 ± 0.4 Hz $J_{\alpha,\beta}$ coupling constants.

subject to general acid–base catalysis, it is important to note that the Orn δ-NH_2 groups in mono-*N*-methylleucine gramicidin S dihydrochloride are present as salts of a strong acid (hydrochloride, -$NH_3^+Cl^-$) which can catalyze H–D exchange. Hence, the relative exchange rates in nonfluorinated hydroxylic solvents were very fast and delineation between the exchange rates of the peptide protons was not achieved. As anticipated, in Me_2SO-d_6 containing 10% D_2O at 19°, and in CD_3OD at 17°, all the peptide protons exchanged in less than 15 min. In F_3EtOH-d_3, the H–D exchange rates are relatively slow, which is an indication of the poor proton accepting ability of the solvent. The amide resonances, well separated at 51° in F_3EtOH, allow satisfactory delineation between the exchange rates of the Val_1 and Orn_7 peptide protons at 51° in F_3EtOH-d_3. The aromatic protons of D-Phe were used as internal intensity standard for estimating the number of unexchanged protons from the peptide NH integrals. In F_3EtOH-d_3 at 51°, in less than 20 min, the Orn_2, Phe_4, Val_6, and Phe_9 peptide protons had almost completely exchanged. The Leu_8 peptide proton had a slightly slower exchange rate than the peptide protons of residues 2, 4, 6, and 9; it exchanged almost completely in 30 min. The remaining peptide protons of Val_1 and Orn_7 exchanged slowly and had half-lives of 91 min ($k_1 = 7.61 \times 10^{-3}$ min^{-1}) and 64 min ($k_1 = 1.08 \times 10^{-2}$ min^{-1}), respectively. The presence of δ-NH_3^+ groups on the Orn residues appears to catalyze the H–D exchange of the Orn peptide protons relatively faster than the peptide protons of the other amino acid residues (Galardy et al., 1974). At 28°, in F_3EtOH-d_3, delineation between the exchange rates of the peptide protons of residues 1, 7, and 8 was achieved. The peptide proton of Val_1 exchanged very slowly (~20% exchanged after ~18 hr). The peptide protons of Leu_8 and Orn_7 exchanged slowly and had half-lives of ~131 min ($k_1 = 5.30 \times 10^{-3}$ min^{-1}) and 108 min ($k_1 = 6.41 \times 10^{-4}$ min^{-1}), respectively. The remaining peptide protons of residues 2, 4, 6, and 9 exchanged fast.

Discussion

Three methods for identification of the solvent-exposed and solvent-shielded peptide protons—temperature dependence of peptide proton chemical shifts, effect of solvent variation on the peptide proton chemical shift, and proton–deuterium exchange rates taken together with $J_{\alpha\text{-CH-NH}}$ coupling constants—have been shown to be effective in determining solution conformations when applied to small, relatively rigid, cyclic polypeptides (Stern et al., 1968; Schwyzer and Ludescher, 1969; Kopple et al., 1969a,b, 1972; Ohnishi and Urry, 1969; Urry and Ohnishi, 1970; Urry et al., 1970; Ovchinnikov et al., 1970; Bovey et al., 1972; Torchia et al., 1972a,b; Pitner and Urry, 1972; Kumar and Urry, 1973a,b; Kumar et al., 1975). For mono-*N*-methylleucine gramicidin S dihydrochloride, agreement between the three methods for identification of the solvent-exposed and solvent-shielded peptide protons is as good as for gramicidin S.

Once assignments are made, ^1H NMR studies provide three classes of information as constraints for Corey–Paul-

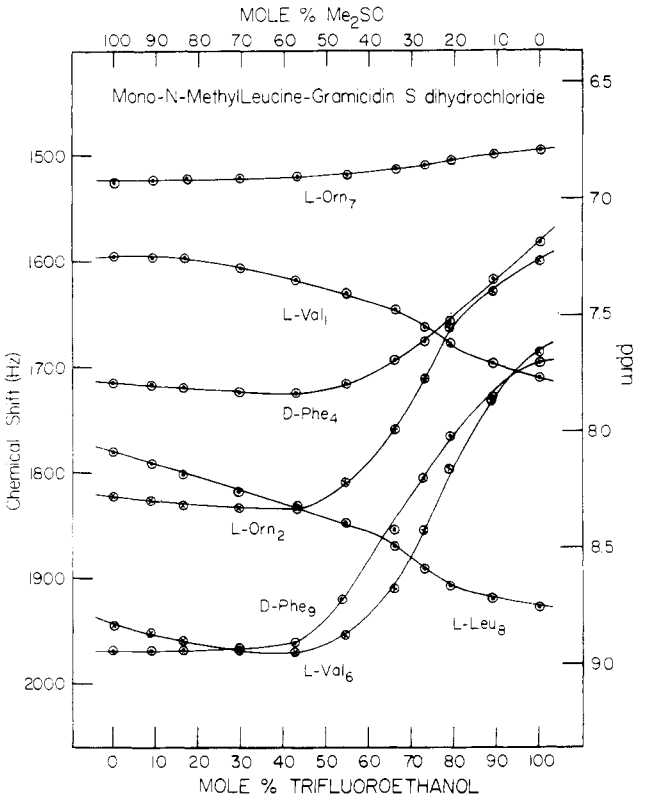

FIGURE 4: Solvent dependence of the peptide proton chemical shifts in dimethyl sulfoxide-trifluoroethanol mixture at 30°.

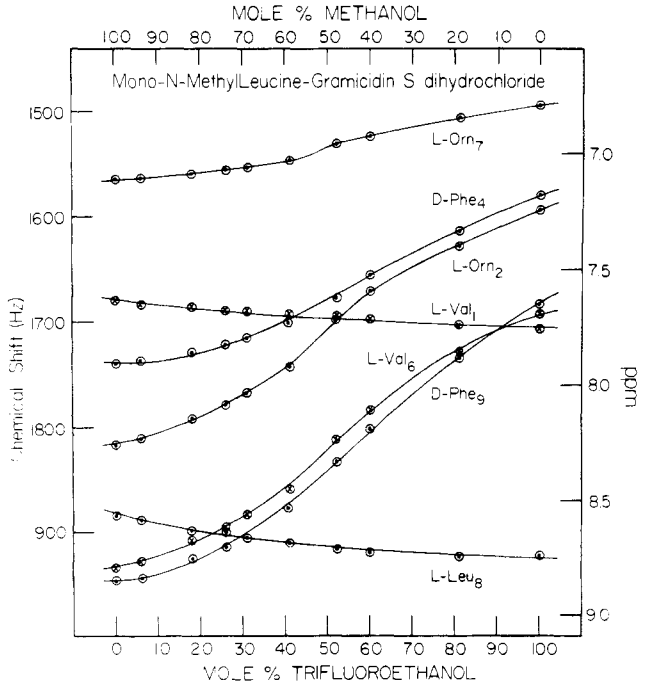

FIGURE 5: Solvent dependence of the peptide proton chemical shifts in methanol-trifluoroethanol mixture at 30°.

ing-Koltun (CPK) space-filling model building. The presence of intramolecular hydrogen bonds is detected by the three methods noted above for delineating peptide protons; dihedral angles are limited by the corresponding $^3J_{\alpha\text{-CH-NH}}$ and $^3J_{\alpha,\beta}$ coupling constants, and the presence of magnetically anisotropic vicinal moieties is implied by the variance of a chemical shift from a characteristic range of values for

Table III: Proton Magnetic Resonance Parameters of the Peptide NH Resonances of Mono-*N*-methylleucine Gramicidin S Dihydrochloride.

Amino Acid[a] Residue (NH Resonance No.)[b]	Solvent	Coupling Constant[c] ($^3J_{\alpha\text{-CH-NH}}$, Hz)	Chemical Shift[d] ν_{NH}, Hz (ppm)
L-Val_1 (6)[b]	Me_2SO-d_6[e]	9.6	~1595 (7.25)
	CH_3OH	9.3 ± 0.3	1680 (7.64)
	F_3EtOH	8.8 ± 0.3	1689 (7.68)
L-Orn_2 (3)[b]	Me_2SO-d_6	8.5 ± 0.5	1824 (8.29)
	CH_3OH	8.2 ± 0.2	1817 (8.26)
	F_3EtOH	8.5	1549 (7.04)
D-Phe_4 (5)[b]	Me_2SO-d_6	6.8 ± 0.3	1718 (7.81)
	CH_3OH	6.5 ± 0.5	1740 (7.91)
	F_3EtOH	6.5	1572 (7.15)
L-Val_6 (2)[b]	Me_2SO-d_6	9.3 ± 0.3	1945 (8.84)
	CH_3OH	9.3 ± 0.3	1947 (8.85)
	F_3EtOH	9.3 ± 0.3	1639 (7.45)
L-Orn_7 (7)[b]	Me_2SO-d_6	8.8 ± 0.3	1526 (6.94)
	CH_3OH	9.3 ± 0.3	1565 (7.11)
	F_3EtOH	8.8 ± 0.3	1480 (6.73)
L-Leu_8 (4)[b]	Me_2SO-d_6	9.3 ± 0.3	1780 (8.09)
	CH_3OH	9.3 ± 0.3	1885 (8.57)
	F_3EtOH	9.3 ± 0.3	1903 (8.65)
D-Phe_9 (1)[b]	Me_2SO-d_6	3.3 ± 0.3	1971 (8.96)
	CH_3OH	3.3 ± 0.3	1936 (8.80)
	F_3EtOH	3.3 ± 0.3	1653 (7.51)

[a] The amino acid residues are numbered according to the amino acid sequence numbering (Figure 6). [b] The peptide proton resonances are numbered in the order of increasing field as they appear in Me_2SO-d_6. [c] Coupling constants within experimental error remained constant between −10 and 64° in F_3EtOH, between −12 and 50° in CH_3OH, and between 18 and 84° in Me_2SO-d_6. [d] At 220 MHz, the chemical shifts in Me_2SO-d_6 and CH_3OH are at 30° and in F_3EtOH at 55°, and at these temperatures the peptide proton resonances are well resolved. [e] In Me_2SO-d_6 at 30°, the L-Val resonance 6 overlaps with the aromatic protons, but at 84° it resonates at 1565 Hz as a well-resolved doublet.

a specific proton. As will be discussed below, NMR data point to a folded conformation for the ring of mono-*N*-methylleucine gramicidin S dihydrochloride. With such a folded conformation, one must be careful in interpreting chemical-shift temperature dependence results; slight rotation about the bonds can alter relative spatial orientation of peptide protons and nearby peptide moieties and can cause significant chemical-shift changes due to magnetic anisotropy of the nearby peptide moiety or due to ring currents from the nearby phenyl rings. However, for *N*-methylleucine gramicidin S dihydrochloride, between the temperature range of −10 to 64° in F_3EtOH, 18 to 84° in Me_2SO-d_6, and −12 to 50° in CH_3OH, the $^3J_{\alpha\text{-CH-NH}}$ coupling constants, within experimental error, remained constant and the peptide proton chemical shifts exhibited linear temperature dependence. This indicates that the average peptide backbone conformation, as reflected in the α-CH-NH dihedral angles, remained essentially unchanged in each of the solvents. The temperature dependence and solvent-mixture titrimetric methods have the ability to detect conformational changes accompanying the solvent transition.

When comparing the methods of delineating peptide protons, it is important to emphasize that H-D exchange results are observations of a kinetically controlled process, dependent on transient activated states, whereas temperature dependence and solvent mixture dependence of the peptide proton chemical shifts are the result of a mole fraction weighting of states in equilibria.

Table IV: Temperature Dependence of the Peptide Proton Chemical Shifts in Different Solvents.

Amino Acid[a] Residue (NH Resonance No.)[b]	Me_2SO-d_6		CH_3OH		F_3EtOH	
	Temp Coeff (ppm/°C)	0° Intercept, Hz (ppm)	Temp Coeff (ppm/°C)	0° Intercept, Hz (ppm)	Temp Coeff (ppm/°C)	0° Intercept, Hz (ppm)
L-Val_1 (6)[b]	−0.0027	1613.0 (7.33)	−0.0027	1698.1 (7.72)	−0.0033	1728.2 (7.85)
L-Orn_2 (3)[b]	−0.0040	1848.1 (8.40)	−0.0061	1856.3 (8.44)	−0.0070	1630.8 (7.41)
D-Phe_4 (5)[b]	−0.0068	1760.0 (8.00)	−0.0074	1787.3 (8.12)	−0.0066	1650.8 (7.50)
L-Val_6 (2)[b]	−0.0044	1972.4 (8.96)	−0.0060	1984.3 (9.02)	−0.0082	1739.0 (7.90)
L-Orn_7 (7)[b]	−0.0016	1535.5 (6.98)	−0.0042	1592.8 (7.24)	−0.0030	1515.2 (6.89)
L-Leu_8 (4)[b]	−0.0028	1796.2 (8.16)	−0.0028	1902.6 (8.65)	−0.0037	1947.5 (8.85)
D-Phe_9 (1)[b]	−0.0080	2020.6 (9.18)	−0.0081	1987.5 (9.03)	−0.0076	1745.1 (7.93)

[a] Amino acid residues are numbered according to the amino acid sequence numbering (Figure 6). [b] The peptide proton resonances are numbered in the order of increasing field as they appear in Me_2SO-d_6.

Delineation of Peptide NH Moieties in Trifluoroethanol. In F_3EtOH, the temperature coefficients of the Val_1 and Leu_8 peptide NH protons are as small or smaller in magnitude ($d\delta/dT$ = −0.0033 and −0.0037 ppm/°C) than the internally hydrogen bonded Val (−0.0037 ppm/°C) and Leu (−0.0041 ppm/°C) peptide NH protons of gramicidin S. On going from CH_3OH to F_3EtOH, the Val_1 NH and Leu_8 NH protons exhibited small downfield shifts of 28 and 38 Hz and from Me_2SO-d_6 to F_3EtOH they exhibited downfield shifts of 114 and 146 Hz. In both cases, the downfield shifts are approximately the same as observed in gramicidin S for the internally hydrogen bonded Val and Leu peptide protons. The peptide protons of Val_1 and Leu_8 exchanged slowly in F_3EtOH-d_3 (see Results section), as did the Val and Leu peptide protons of gramicidin S. In mono-*N*-methylleucine gramicidin S dihydrochloride, the L-Orn δ-NH_2 groups are present as salts of a strong acid (hydrochloride) which could catalyze the H–D exchange, whereas in gramicidin S these groups were present as the free bases (Urry and Ohnishi, 1970) or as salts of a weak acid (acetate salt; Stern et al., 1968). When comparing the H–D exchange results of gramicidin S and mono-*N*-methylleucine gramicidin S dihydrochloride in hydroxylic solvents, it is important to note this feature which contributes to the delineation of the H–D exchange rates of the solvent-exposed and solvent-shielded peptide protons of gramicidin S. Hence, in contrast to gramicidin S, the exchange rates in nonfluorinated hydroxylic solvents (CD_3OD, Me_2SO-d_6, + 10% D_2O) were very fast, and delineation between the exchange rates of the peptide protons was not achieved. By the criteria of temperature coefficients, solvent dependence of the peptide proton chemical shifts, and H–D exchange in F_3EtOH, the Val and Leu peptide NH protons of the residues 1 and 8 are delineated as solvent shielded, most likely through the formation of strong intramolecular hydrogen bonds as in gramicidin S.

In F_3EtOH on the basis of its small temperature coefficient (−0.0030 ppm/°C), small upfield shift on addition of F_3EtOH to MeOH (~70 Hz) and to Me_2SO (~31 Hz), and slow H–D exchange in F_3EtOH-d_3, the Orn peptide proton (residue 7) is delineated as solvent shielded as when intramolecularly hydrogen bonded. At 18° in F_3EtOH, the chemical shifts of the Val_1 and Leu_8 peptide protons are 7.80 and 8.80 ppm, respectively, as compared to 7.85 and 8.90 ppm for the Val NH and Leu NH of gramicidin S. The large temperature coefficients, large MeOH → F_3EtOH, and Me_2SO → F_3EtOH upfield shifts, and fast H–D exchange in F_3EtOH-d_3 (see Results section) delineate the peptide protons of Phe_9, Phe_4, Orn_2, and Val_6 as being solvent exposed in F_3EtOH. The temperature coefficients and solvent upfield shifts for the Phe_9 and Orn_2 peptide protons are similar to the solvent-exposed Phe and Orn peptide protons of gramicidin S. In F_3EtOH, delineation between the temperature coefficients of the solvent-exposed and solvent-shielded peptide protons of mono-*N*-methylleucine gramicidin S dihydrochloride is relatively better than the delineation of peptide protons of gramicidin S.

Delineation of Peptide NH Moieties in Dimethyl Sulfoxide and Methanol. In Me_2SO-d_6 and CH_3OH, the Val_1 and Leu_8 peptide protons exhibited almost identical, relatively small temperature coefficients (−0.0027 ppm/°C), whereas the Val NH and Leu NH of gramicidin S exhibited coefficients (−0.0022 and −0.0034 ppm/°C) in Me_2SO-d_6, but similar coefficients in CH_3OH (−0.0030 ppm/°C). The temperature coefficients and small MeOH → F_3EtOH and Me_2SO-d_6 → F_3EtOH downfield shifts (noted above) delineate the peptide protons of the Val_1 and Leu_8 as being well shielded from the solvent in CH_3OH and Me_2SO-d_6, as when intramolecularly hydrogen bonded. The chemical shifts of the Val_1 and Leu_8 peptide protons are 7.30 and 8.10 ppm in Me_2SO-d_6, 7.66 and 8.59 ppm in CH_3OH, and are not considerably different from their position in a poor proton acceptor solvent (7.80 and 8.80 ppm in F_3EtOH). The chemical shifts of the Val and Leu peptide protons of gramicidin S in Me_2SO-d_6 are 7.20 and 8.35 ppm. The constancy of the chemical shifts in the three solvents in both cases indicates that the Val_1 and Leu_8 peptide protons are in an internal environment unexposed to hydrogen bonding with the solvents.

The Orn_7 peptide proton exhibited a relatively smaller coefficient (−0.0016 ppm/°C) in Me_2SO-d_6 than in CH_3OH (−0.0042 ppm/°C). This spectral feature and the small Me_2SO → F_3EtOH and MeOH → F_3EtOH upfield shifts indicate that the peptide proton is well shielded in Me_2SO and is relatively less shielded in CH_3OH. The constancy of the chemical shifts in Me_2SO (6.95 ppm), in CH_3OH (7.15 ppm), and in F_3EtOH (6.84 ppm) indicates that the Orn peptide proton is in an internal environment unexposed to hydrogen bonding with the solvents. The relatively high-field positions of the Val_1 and Orn_7 peptide protons in the three solvents imply either shielding as by the magnetic anisotropy of an end peptide moiety (Urry and Ohnishi, 1970) or longer intramolecular hydrogen bonds (Kopple et al., 1973) or both.

In Me_2SO-d_6 and CH_3OH, the Phe peptide protons, residues 4 and 9, exhibited large temperature coefficients, and

large MeOH → F_3EtOH and Me_2SO → F_3EtOH upfield shifts (see Results section). These spectral features indicate that the peptide protons are well solvated in Me_2SO and CH_3OH. The temperature coefficient and solvent upfield shifts for the Phe_9 peptide proton are similar to those of the solvent-exposed Phe peptide protons of gramicidin S. In Me_2SO, the Val_6 and Orn_2 peptides exhibited temperature coefficients (−0.0044 and −0.0040 ppm/°C) intermediate between the solvent-exposed (D-$Phe_{4,9}$) and solvent-shielded (Val_1, Orn_2, and Leu_8) peptide protons, whereas in CH_3OH the coefficients are relatively large (−0.0060 and −0.0061 ppm/°C). On going from Me_2SO to F_3EtOH or MeOH to F_3EtOH, the Val_6 and Orn_2 peptide protons exhibited upfield shifts greater than 1 ppm, as in the fully exposed D-Phe_9 peptide proton. This spectral feature delineates the Val_6 and Orn_2 peptide protons as being well exposed to the solvent in Me_2SO and CH_3OH, but the temperature coefficient criteria suggest that the peptide protons are relatively less exposed to the solvent in Me_2SO than in CH_3OH.

Probability of Occurrence of β Turns in Trifluoroethanol, Dimethyl Sulfoxide, and Methanol with Reference to Gramicidin S. It is informative to calculate mole fractions, χ_i, or probabilities of solution molecules present as solvent-shielded peptide protons by using an experimentally observable property such as temperature coefficients or solvent shifts (Urry and Ohnishi, 1974). This does not imply the presence of a locked solution conformation, but rather the frequency of occurrence of such a conformation with solvent-shielded peptide protons in solution. The magnitude of the mole fraction is a relative measure of the solvent-shielded peptide protons with reference to a model system, and in a qualitative sense is a measure of the relative stability of intramolecular hydrogen bonding.

The general mole fraction expression calculated from an experimental observable, a, is:

$$\chi_i = \frac{a_{obsd} - \sum_{j \neq i}\left(1 + \sum_{k \neq i,j} \chi_k\right) a_j}{a_i - \sum_{j \neq i} a_i} \quad (1)$$

Assuming two states, solvent exposed, e, and solvent shielded, s, this reduces to:

$$\chi_s = \frac{a_{obsd} - a_e}{a_s - a_e} \quad (2)$$

For gramicidin S as a reference state, the temperature coefficients in F_3EtOH are $a_s{}^T = -0.0037$ (L-Val) and $a_e{}^T = -0.0083$ ppm/°C (D-Phe). Using the values of $a_{obsd}{}^T = -0.0037$, −0.0033, and −0.0030 ppm/°C for the L-Leu_8, L-Val_1, and L-Orn_7 in eq 2, one obtains values of 1 or greater for χ_s in the three cases. In dimethyl sulfoxide, the temperature coefficients of the reference state (gramicidin S) are $a_s{}^T = -0.0034$ and $a_e{}^T = -0.0081$ ppm/°C. Using the values of $a_{obsd}{}^T = -0.0028$ ppm/°C for L-Val_1 and L-Leu_8 and −0.0016 ppm/°C for the L-Orn_7, the mole fractions of the solvent-shielded peptide protons calculate to be $\chi_s{}^T > 1$ In methanol, for gramicidin S as a reference state, the temperature coefficients are $a_s{}^T = -0.0030$ (L-Val and L-Leu) and $a_e{}^T = -0.0077$ ppm/°C (D-Phe). Using the value of −0.0028 ppm/°C for the L-Val_1 and L-Leu_8 and −0.0042 ppm/°C for the L-Orn_7 in eq 2, one obtains values of >1.0 and 0.75 for χ_s, or >100% of the time L-Val_1 and L-Leu_8 and 75% of the time L-Orn_7 are intramolecularly hydrogen bonded in methanol solution.

From the solvent mixture method, $a_s{}^{SM} = -44$ Hz and $a_e{}^{SM} = 240$ Hz (chemical-shift difference at 220 MHz for solvent-shielded and solvent-exposed peptide protons, in going from CH_3OH to F_3EtOH) for gramicidin S as a reference state. With $a_{obsd}{}^T = -38$, −28, and +70 Hz for the L-Leu_8, L-Val_1, and L-Orn_7 in eq 2, one obtains values of 1.0, 1.0, and 0.86, respectively, for χ_s. Since there is no major conformational change in going from methanol to F_3EtOH, the agreement between the two methods is quite good; the mole fraction (χ_s) for L-Orn_7 calculates to be 0.75 by the temperature coefficient method and 0.86 by the solvent mixture method.

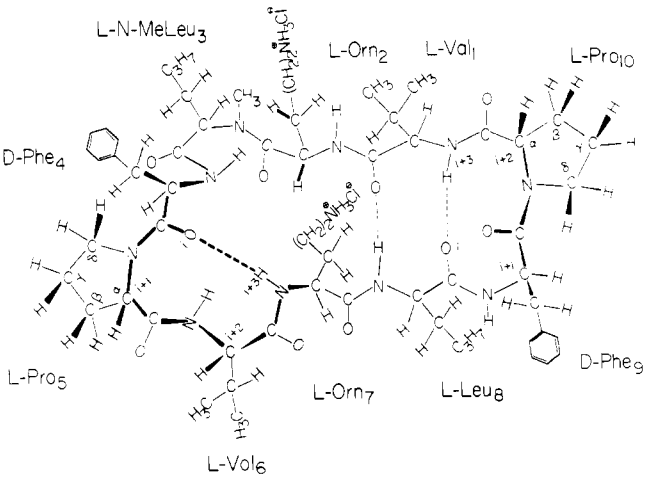

FIGURE 6: One possible conformation of 3-*N*-methylleucine gramicidin S dihydrochloride. The ring is partially unfolded to reveal β-conformational features.

A major limitation of the temperature coefficient method is that $a_{obsd}{}^T$ is itself a function of temperature, i.e.:

$$a_{obsd}{}^T = \sum_i a_i{}^T \chi_i$$

For the two states, solvent exposed and solvent shielded:

$$\chi_s = \chi_e e^{-\Delta F / RT}$$

For rapidly interconverting conformational states ($\Delta F < 12$ kcal/mol), the relative population of the conformational states can change with temperature. This can result in a nonlinear temperature dependence at higher temperatures, because different conformational states are appreciably populated, and it can also give fortuitously large or small temperature coefficients.

Solution Conformation in Trifluoroethanol, Dimethyl Sulfoxide, and Methanol. In proposing conformational models, one must be aware of the fact that there may be more than one conformation which agrees with the experimental data. Usually the term "conformation" stands for an "ensemble average on the NMR time scale", i.e., one is dealing with an ensemble of conformations which may be in rapid equilibrium, so that the resulting ^{1}H NMR spectrum is determined by a weighted time average of spectral parameters of the conformations. With these considerations in mind, a possible conformation will be discussed which satisfies the steric requirements and experimental NMR data. CPK space-filling models were used in constructing the proposed conformational model of mono-*N*-methylleucine gramicidin S dihydrochloride (Figure 6). Being cyclic with two DL sequences, the ring system has the distinct possibility of being folded using DL (as in gramicidin S) and LL sites for the ten-atom hydrogen-bonded rings of β turns (Geddes

et al., 1968; Venkatachalam, 1968; Urry and Ohnishi, 1970; Ramachandran et al., 1970). As reported by Chandrasekaran et al. (1973) for the LL β turn, the conformational angle ϕ_{i+1} at α-C_{i+1} in the minimum energy conformation (ϕ_{i+1} = 50°) closely corresponds to that favorable for the occurrence of a proline residue (ϕ_{i+2} = −60°), so that such a bend could be readily formed by the sequence L-Pro$_{i+1}$-L-Val$_{i+2}$, utilizing L-proline as the first residue in the LL β turn.

In the proposed conformational model (Figure 6) one-half of the decapeptide retained its gramicidin S β-type conformation, and the second half has a different turn. The gramicidin S type β turn utilizes D-Phe$_{i+1}$-L-Pro$_{i+2}$ at the corners of the DL bend with the carbonyl and nitrogen of the end peptide moiety in the most sterically favored orientation (Venkatachalam, 1968; Urry and Ohnishi, 1970; Ramachandran et al., 1970). The L-Val$_1$ peptide proton is intramolecularly hydrogen bonded to the carbonyl oxygen of the L-Leu$_8$ resulting in formation of a ten-atom hydrogen-bonded ring of a β turn. This β conformational feature is supported by the relatively low temperature coefficient, small downfield solvent shifts, and slow H–D exchange in F_3EtOH-d_3. The Leu$_8$ peptide proton is intramolecularly hydrogen bonded to the carbonyl oxygen of L-Val$_1$ as in the case of gramicidin S. This conformational feature is supported by the relatively low temperature coefficient, small downfield solvent shifts, and slow H–D exchange in F_3EtOH-d_3. The $J_{\alpha\text{-CH-NH}}$ coupling constants for the L-Val$_1$ and L-Leu$_8$ are large (9.0 and 9.3 Hz; Table III) corresponding to dihedral angles of near 170°, as expected from the trans orientation of the α-CH and NH protons (Ramachandran et al., 1971; Bystrov et al., 1969).

Another type of β turn has L-Pro and L-Val at the corners of the LL bend with the end peptide moiety in the most sterically favored orientation. This type of β turn with Pro as residue i + 1 was first proposed in solution for the tail of oxytocin (Urry and Walter, 1971), and was subsequently confirmed by the X-ray data of Rudko et al. (1971). Since then, this type of β turn has been proposed for several polypeptides such as telomycin (Kumar and Urry, 1973a,b), elastin peptides (Urry and Ohnishi, 1974; Urry et al., 1974), and di-*N*-methylleucine gramicidin S dihydrochloride (Kumar et al., 1975). The L-Orn$_7$ peptide proton is internally hydrogen bonded to the carbonyl oxygen of D-Phe$_4$, resulting in the formation of a ten-atom hydrogen-bonded β turn with L-Pro$_{i+1}$-L-Val$_{i+2}$ at the corners. The occurrence of this β turn is supported by the low temperature coefficient, small upfield solvent shifts, and slow H–D exchange in F_3EtOH-d_3. The 9.0 $J_{\alpha\text{-CH-NH}}$ coupling constant ($\theta \simeq$ 170°) is consistent with a near trans orientation of the two protons. There is no major conformational change in going from trifluoroethanol to dimethyl sulfoxide or methanol.

Side Chains.[3] The $^3J_{\alpha\text{-CH-}\beta\text{-CH}}$ coupling constants of the D-Phe$_4$ and D-Phe$_9$, in Me_2SO-d_6 and F_3EtOH, are near 10 and 6 Hz (Table II), indicating a major population of a conformer in which the relative orientation of the α,β protons is trans ($\chi_1 \simeq$ 180°) and that of the α,β' protons is gauche. The γ'-CH protons of L-Pro$_{5,10}$ are shifted downfield by 160 Hz relative to the γ-CH protons. The $J_{\alpha\text{-CH-}\beta\text{-CH}}$ vicinal coupling constants in Table II suggest predominantly trans orientation for the Val$_1$ and Val$_6$ α-CH–β-CH bonds. The 4- and 10-Hz $J_{\alpha\text{-CH-}\beta\text{-CH}}$ coupling constants for L-Orn$_2$ suggest a conformer in which the relative orientation of the α,β protons is gauche, and that of α,β' is trans, in contrast to the L-Orn$_7$ in which both the α-CH–β'-CH bonds are predominantly in a gauche orientation.

Conformation of the D-Phe-L-Pro Peptide Bond. According to Patel (1973), for the cis X-Pro bond, the L-Pro α-CH proton resonates at a lower field and exhibits a doublet with one large and one near zero $J_{\alpha,\beta}$ coupling constant, and for the trans X-Pro bond a more complex multiplet is observed at a higher field. In Me_2SO-d_6, the chemical shifts for several polypeptides containing proline residues are within 4.2–4.4 ppm for the trans and 4.26–4.7 ppm for the cis isomer (Patel, 1973). In gramicidin S in CD_3OD at 19°, the L-Pro α-CH resonates at 955 Hz (4.34 ppm) exhibiting 6.5-Hz and near zero coupling constants, and also, in Me_2SO-d_6 at 75°, it appears at 950 Hz (4.32 ppm) exhibiting 6.5-Hz and near zero coupling constants. According to the correlation suggested by Patel (1973), the gramicidin S D-Phe-L-Pro bond would be assigned to the trans configuration on the basis of the chemical shift and to the cis conformation on the basis of the $J_{\alpha,\beta}$ coupling constant. Dorman and Bovey (1973) and other workers (Deslauriers et al., 1972) have reported that the chemical shifts of the γ-carbon resonances could be used to determine the conformation about the X-Pro bond. The reported chemical shift of the γ-carbon resonance for the trans conformer is 168.5 ± 0.5 ppm and for the cis conformer it is 170.3 ± 0.3 ppm, relative to external CS_2. The carbon-13 spectra of gramicidin S in CD_3OD and Me_2SO-d_6 are very similar, and in Me_2SO-d_6, the α-, β-, γ-, and δ-carbon resonances of L-Pro appear at 132.6, 163.4, 168.5, and 146.1 ppm, respectively, with reference to external CS_2 (Dorman and Bovey, 1973; Sogn et al., 1974). Therefore, the proline spectrum of gramicidin S in Me_2SO-d_6 is entirely consistent with that of a typical trans X-Pro bond as suggested by Dorman and Bovey (1973) and as presented by Schwyzer and Ludescher (1968), Stern et al. (1968), Ohnishi and Urry (1969), and Ovchinnikov et al. (1970).

In Me_2SO-d_6 at 22°, we have observed that the L-Pro α-, β-, γ-, and δ-carbon resonances of gramicidin S appear at 59.85, 28.93, 23.05, and 46.0 ppm, respectively, with reference to internal Me_4Si at 25 MHz. For mono-*N*-methylleucine gramicidin S in Me_2SO-d_6, the L-Pro$_{5,10}$ α-CH protons resonate at 940 Hz (4.27 ppm) exhibiting 7.5-Hz and near zero coupling constants, and in F_3EtOH-d_3 they resonate at 952 Hz (4.32 ppm) as a doublet exhibiting 6.5-Hz and near zero coupling constants. The ^{13}C NMR assignments were made in Me_2SO-d_6 at 22° by comparison of the gramicidin S and di-*N*-methylleucine gramicidin S (Kumar et al., 1975) spectra with mono-*N*-methylleucine gramicidin S dihydrochloride spectra. In the proton-decoupled carbon-13 magnetic resonance spectrum of mono-*N*-methylleucine gramicidin S, most α-carbon resonances showed doubling due to the absence of C_2 symmetry, but no doubling is observed for the α-, β-, γ-, and δ-carbon resonances of the L-Pro$_{5,10}$ that would occur if cis–trans isomerism of the D-Phe-L-Pro peptide bond were present. For unhindered amides, the energy barrier for cis–trans peptide bond isomerization is ~20 kcal/mol (Deber et al., 1974). This requires only one of the two isomers. The α-carbon resonances appear at 47.4 (N-MeLeu$_3$), 49.2 (Leu$_8$), 51.85 ± 0.05 (Orn$_{2,7}$), 53.0 and 53.68 (Phe$_{4,9}$), and 55.96 and 56.88

[3] In estimating the C^α–C^β dihedral angles, the observed $^3J_{\alpha\text{-CH-}\beta\text{-CH}}$ coupling constants may correspond to dihedral angles in a locked conformer or may reflect a population weighted average of the values corresponding to the rotational energy minima.

ppm ($Val_{6,1}$) with reference to the internal Me_4Si. The α carbons of L-$Pro_{5,10}$ resonate at 59.9 and 60.4 ppm, and the β, γ, and δ carbons appear at 29.0, 23.1, and 46.0 ppm, respectively. From the ^{13}C NMR spectra in Me_2SO-d_6, the chemical shifts of the L-Pro α-, β-, γ-, and δ-carbon resonances of gramicidin S and mono-*N*-methylleucine gramicidin S dihydrochloride are very similar; therefore, the trans isomer is most reasonable. The intramolecular hydrogen bonding in Figure 6, based on CPK models, requires the trans isomer.

On the Relationship of Conformation to Biological Activity. The primary goal of conformational studies on antibiotics is to achieve an understanding of mechanism of action. The surprising feature in this series of antibiotics—gramicidin S, di-*N*-methylleucine gramicidin S, and mono-*N*-methylleucine gramicidin S—is that they have nearly equivalent biological activities. The result is surprising in that gramicidin S is viewed as having a very stable conformation involving two Leu NH···O-C Val hydrogen bonds. N-Methylation of the Leu residue necessarily disrupts this hydrogen bond, and it was expected that such an obligatory change in conformation would be reflected in a change in biological activity. This did not occur (Sugano et al., 1973a,b).

The conformation of mono-*N*-methylleucine gramicidin S appears to be essentially as stable as that of gramicidin S. If this is the case and if both maintain the proposed solution conformations at the site of action then secondary structure is not important per se and we must look to the backbone as a superstructure on which side chains are arranged in a functional way. In the gramicidin S conformation Stern et al. (1968) and Schwyzer and Ludescher (1972) noted a sidedness in which the charged ornithyl side chains were on one side and the hydrophobic side chains were on the other. This general division is lost in the *N*-methyl derivatives. The relative orientation or spacing between the δ-NH_3^+ and between the phenyl moieties, however, need not be significantly different in the series. Accordingly, these relative orientations may be fundamental to biological activity. The recent demonstration that tyrocidines (the class of antibiotics to which gramicidin S belongs) affect RNA transcription (Schazschneider et al., 1974; Sarkar and Paulus, 1972) would be consistent with involvement of these side chains.

These findings suggest further studies which may clarify the problem. First, it would be of interest to know the biological activity of di-*N*-methylornithine and di-*N*-methylphenylalanine gramicidin S. This would in effect confirm the gramicidin S conformation to be that of the solution conformation. Secondly, the role of the ornithyl and phenylalanine side chains could be considered by formylation or acetylation of the δ-amino moiety and by hydrogenation of the phenyl moiety. The biological activity of these derivatives would be of considerable interest.

Supplementary Material Available

Supplementary material describing details of spectral analysis will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 24× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D.C. 20036. Remit check or money order for $4.50 for photocopy or $2.50 for microfiche, referring to code number BIO-75-2197.

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The Interaction of Heavy Meromyosin and Subfragment 1 with Actin. Physical Measurements in the Presence and Absence of Adenosine Triphosphate†

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ABSTRACT: Viscosity, turbidity, and laser-light fluctuation autocorrelations of acto-heavy meromyosin (HMM) and acto-subfragment 1 (S-1) solutions were measured under conditions where the actin-activated ATPase is close to its maximal value. The results were compared to similar data obtained in the absence of ATP where the actin and myosin fragments were completely complexed, and in the presence of ATP but at 0.1 *M* KCl where the actin and HMM or S-1 were almost completely dissociated. It was found that at maximal actin activation, the viscosity, turbidity, and autocorrelation data were all much closer to the values for the completely dissociated systems than to the values for the completely complexed systems. Assuming that viscosity, turbidity, and autocorrelation measurements approximate a linear measure of binding between actin and HMM or S-1, the results suggest that at maximal actin activation less than 10% of the HMM or S-1 are bound to the actin. Therefore as was suggested previously by ultracentrifuge and kinetic studies, it appears that under conditions of maximal actin activation, most of the HMM and S-1 occur in a refractory state unable to bind to actin.

A key question in the biochemistry of muscle contraction is the nature of the interaction between actin and myosin in the presence of ATP. In vivo X-ray diffraction studies suggest that in the absence of ATP, more than 90% of the myosin bridges are attached to the F-actin filaments (Huxley, 1968), while in the presence of ATP, in activated muscle, less than 50% of the bridges appear to be attached (Miller and Tregear, 1970; Haselgrove and Huxley, 1973). In vitro studies also demonstrate that a marked difference occurs in the binding of actin to myosin in the presence and absence of ATP. In the absence of ATP, the actin–HMM[1] binding is essentially stoichiometric with maximum binding occurring at a molar ratio of two F-actin monomers/HMM molecule (Eisenberg et al., 1972; Margossian and Lowey, 1973). On the other hand, Perry and his coworkers concluded more than 10 years ago from physical measurements that the actin and HMM are almost completely dissociated in the presence of ATP even at very low ionic strength (Leadbeater and Perry, 1963; Perry et al., 1966). Of course these observations could have been due to the occurrence of a weak binding constant between actin and HMM in the presence of ATP, in which case presumably, had the actin concentration been raised to a high enough level all of the HMM would have become complexed with actin. At low ionic strength, however, actin activates the HMM ATPase and, furthermore, this actin activation follows a simple hyperbolic plot so that at very low salt concentration, experiments can be performed at actin concentrations where the actin activated HMM ATPase is nearly at its maximum value (Rizzino et al., 1970; Eisenberg et al., 1972; Eisenberg and Kielley, 1972). At these actin concentrations weak binding between actin and HMM cannot be invoked as an explanation for the dissociation of the actin and HMM. Rather, at saturating actin concentration, the actin and

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[1] Abbreviations used are: HMM, heavy meromyosin; S-1, subfragment 1.