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Spectroscopic Studies on the Conformation of Gramicidin A′. Proton Magnetic Resonance Assignments, Coupling Constants, and H–D Exchange*

J. D. Glickson, D. F. Mayers, J. M. Settine, and D. W. Urry†

ABSTRACT: Analysis of proton magnetic resonances of a commercial preparation (Nutritional Biochemicals) of gramicidin, referred to herein as gramicidin A′ (GA′), shows the preparation to contain gramicidin A (GA), gramicidin B (GB), and gramicidin C (GC) in ratios of 72:9:19, respectively. These gramicidins differ only in the amino acid at position 11. The analysis is based on the relative intensities of tryptophan indole C*H* resonances, the phenylalanine phenyl C*H* resonance (of GB), and the tyrosine ortho C*H* resonance (of GC). Resonances in the 220-MHz proton magnetic resonance spectrum of GA′ in hexadeuteriodimethyl sulfoxide (Me_2SO-d_6) are assigned to specific hydrogens. An ordered conformation is indicated by the following evidence, all of which is consistent with the $\pi_{L,D}$ helices previously proposed for the structure of GA′ in transmembrane channels (Urry, D. W. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 672; Urry, D. W., Goodall, M. C., Glickson, J. D., and Mayers, D. F. (1971a), *Proc. Nat. Acad. Sci. U. S. 68*, 1907). (1) Observation of distinct resonances from side-chain protons that are chemically equivalent except for the sequence positions of their corresponding amino acids indicates that conformational constraints produce magnetically nonequivalent local environments in different segments of the polypeptide chain. In particular, as a result of proximity to the faces of nearby tryptophan indole rings, the methyl resonances of one valine and three leucines are shifted by ring-current interactions to high field from the methyl resonances of the remaining three valines and one leucine. Elimination of these ring-current effects by hydrogenation of the tryptophan indole rings shifts the high-field leucine and valine methyl resonances back to their "normal" position. (2) Rates of deuterium replacement of labile hydrogens of GA′ in 5% D_2O–Me_2SO-d_6 (v/v) indicate that the four tryptophan indole N*H* protons and the ethanolamine O*H* proton are exposed to the solvent, but most of the peptide hydrogens are internally hydrogen bonded. (3) The peptide N*H*–αC*H* coupling constants are significantly larger than values identified with random coil and α-helical polypeptides but are comparable to values anticipated for the $\pi_{L,D}$ helices.

The gramicidins, isolated from *Bacillus brevis*, are bacteriostatic agents active against gram-positive bacteria and are therapeutically administered in combination with tyrocidin as the broad-spectrum antibiotic mixture Tyrothricin (Hunter and Schwartz, 1967; Korzybski *et al.*, 1967). These linear pentadecapeptides should not be confused with the cyclic decapeptide gramicidin S which, in accordance with the suggestion of Goodall (1970), we refer to as tyrocidin S. Commerical preparations of gramicidin,[1] herein referred to as GA′, are composed of at least four structurally related components, gramicidin A (GA), gramicidin B (GB), gramicidin C (GC), and gramicidin D (GD), which have been separated by countercurrent distribution (Gregory and Craig 1948; Craig *et al.*, 1949; Ramachandran, 1963; Gross and Witkop, 1965). We demonstrate here that the composition of GA′ can, for the most part, be determined from its proton magnetic resonance (pmr) spectrum.

Sarges and Witkop (1965a) showed that GA has the following sequence: HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NH-CH_2CH_2OH. GB and GC differ only in that the L-tryptophan in position 11 is replaced by L-phenylalanine and L-tyrosine, respectively (Sarges and Witkop, 1965b,c). GA, GB, and GC each contain 5–20% of a congener in which L-isoleucine replaces the N-terminal valine (Sarges and Witkop 1965a–c). The structure of GD has not yet been determined, but this component is usually present in only trace quantities

* From the Division of Molecular Biophysics, Laboratory of Molecular Biology, University of Alabama Medical Center, Birmingham, Alabama 35233. *Received April 19, 1971.*

† To whom to address correspondence.

[1] Abbreviations used are: GA, GB, GC, and GD, gramicidins A, B, C, and D, respectively.

(Ramachandran, 1963). The basic structural characteristics of the gramicidins are a linear, electrically neutral sequence of hydrophobic amino acids with alternating L and D configurations which begin and terminate with formyl and ethanolamine groups, respectively.

The gramicidins, like a number of other antibiotics which transport cations across natural and synthetic membranes, serve as models for biological transport systems (Pressman, 1965, 1968; Chappell and Crofts, 1965; Tosteson *et al.*, 1968; Goodall, 1970). There is now considerable evidence that, under given circumstances, gramicidin forms channels through lipid bilayer membranes (Hladky and Haydon, 1970; Krasne *et al.*, 1971) and that two molecules of the antibiotic associate to form each channel (Tosteson *et al.*, 1968; Goodall, 1970; Urry *et al.*, 1971). As models for proteins the gramicidins manifest many of the physical properties of their complex analogs, *e.g.*, aggregation and interaction with lipids and metal ions.

It would be highly desirable to explain the pharmacological, transport, and physical properties of the gramicidins in terms of their molecular conformations. On the basis of infrared evidence Sarges and Witkop (1965a) suggested an antiparallel β structure for the gramicidin dimer in which the molecules are cyclically joined head to tail (formyl end to hydroxy end). Steric interactions of bulky side chains and the presence of three cis peptide bonds per molecule are unfavorable features of that conformation. A lipophilic, left-handed helical structure termed the $\pi_{L,D}$ helix, which is both energetically plausible and fulfills the requirements for channel formation, has recently been proposed (Urry, 1971; Urry *et al.*, 1971). Two such helices coaxially joined head to head (formyl end to formyl end) by hydrogen bonds form a channel through their centers which, with the aid of local ion-induced conformational fluctuations, can accommodate the passage of various cations. The dimensions of this channel are consistent with estimates of membrane thickness and with the observed ion specificity of gramicidin: $NH_4^+ > K^+ > Na^+$ (Urry *et al.*, 1971).

With this model as a working hypothesis, we have been pursuing various experimental approaches with the purpose of relating the conformation of GA′ in solution to the mechanism of transmembrane cation transport. High-frequency pmr spectroscopy has been used in elucidating the conformations of a number of peptide antibiotics and their metal complexes, as well as conformations of peptide hormones in solution (Urry and Ohnishi, 1970), *e.g.*, tyrocidin S (Stern *et al.*, 1968; Ohnishi and Urry, 1969), valinomycin (Ivanov *et al.*, 1969; Ohnishi and Urry, 1970; Mayers and Urry, 1972), nonactin (Prestgard and Chan, 1969, 1970), enniatin (Shemyakin *et al.*, 1969), antamanide (Ivanov *et al.*, 1971), oxytocin (Johnson *et al.*, 1969; Urry *et al.*, 1970; Urry and Walter, 1971), actinomycin D (Victor *et al.*, 1969), and lysine vasopressin (von Dreele *et al.*, 1971).

The extent of structural and dynamic information obtainable from pmr spectra depends ultimately on the successful assignment of resonances to specific hydrogens. Here we present the assignment of resonances in the 220-MHz pmr spectrum of GA′ in Me_2SO-d_6 solution. Deuterium-exchange rates are used to monitor the exposure to the solvent of labile hydrogens, and peptide N*H*-αC*H* coupling constants yield estimates of backbone dihedral angles. In subsequent studies pmr spectra are used to monitor chemical modifications of GA′ (Urry *et al.*, 1971) and the effects of solvent variations and chemical denaturants (Urry *et al.*, 1972) on conformation.

Experimental Section

Spectra were recorded using a Varian Associates HR-220 spectrometer. Sample concentrations were 10% (w/v), and the internal reference was tetramethylsilane. The probe temperature was measured to within $\pm 2°$ using the chemical shifts of methanol or ethylene glycol samples. Proton homonuclear spin decoupling was accomplished by the field-sweep method using a side band generated by a Hewlett Packard Model 5103-A frequency synthesizer.

Gramicidin (Nutritional Biochemicals Corp., Cleveland, Ohio) was used without further purification. Me_2SO-d_6 was purchased from Diaprep Corp., Atlanta, Ga. (99.5% D), and from Columbia Organic Chemical Co., Columbia, S. C. (99.8% D). Deuterium oxide (99.7% D) and acetone-d_6 were purchased from Merck Sharpe and Dohme, Montreal, Can. *N*-Acetylethanolamine was prepared according to D'Allelio and Reid (1937). Hydrogenation of gramicidin was accomplished by the procedure of Ruttenberg *et al.* (1966). This reaction was monitored by the 285-nm absorption of aliquots periodically removed from the reaction mixture. L-Alanyl-L-alanine diketopiperazine and the (γ-methyl-L-glutamyl)$_3$tryptophan polymer were prepared by Fox Chemicals, Inc., Los Angeles, Calif.

Results and Discussion

Assignments. The 220-MHz pmr spectrum of GA′ in Me_2SO-d_6 appears in Figure 1a. A trace of trifluoroacetic acid was added to move the water resonance away from overlapping gramicidin resonances. Addition of the acid sufficiently catalyzed the proton-exchange rate of the ethanolamine O*H* hydrogen so that its resonance, which in the absence of trifluoroacetic acid occurred at 1043 Hz (inset of Figure 1a), fused with the water resonance. As will be discussed below, the indicated assignments were accomplished by combined studies of chemical shifts, resonant intensities, deuterium replacement of labile protons, and spin-decoupling experiments.

Replacement of labile hydrogens (*i.e.*, N*H* and O*H* hydrogens) by deuterium yielded the spectrum in Figure 1b. Exchange was accomplished by heating a 10% (w/v) solution of gramicidin in 20% D_2O–Me_2SO-d_6 (v/v) solution for 1 hr at 57° (some samples required 100° for complete exchange). Since spectral changes were observed upon addition of water, it was necessary to minimize the water content of the exchanged sample before meaningful comparisons could be made to spectra of unexchanged GA′ in Me_2SO-d_6. Presumably these spectral changes resulted from aggregation of the highly hydrophobic antibiotic. The solution of GA′ in 20% D_2O–Me_2SO-d_6 was therefore lyophilized and the deuterated sample was redissolved in 2% D_2O–Me_2SO-d_6. A small amount of D_2O was necessary to limit the extent of back-exchange of labile deuterons with inevitable traces of H_2O in the solvent. The resonances eliminated by deuterium replacement were assigned to N*H* and O*H* protons, the latter being readily identified by its characteristic high-field position, single proton intensity, extreme sensitivity to temperature changes, and spin coupling pattern (a triplet coupled to the quadruplet at 735 Hz, $J = 5.4$ Hz). This resonance could only be resolved from neighboring αC*H* resonances in sufficiently wet Me_2SO-d_6 (note the relatively strong intensity of the water resonance in Figure 1a). The chemical shifts of the indole N*H* resonances of a series of tryptophan analogs in Me_2SO-d_6 at 23° are 2358 Hz for skatole, 2452 Hz for β-3-indolacetonitrile, 2388 Hz

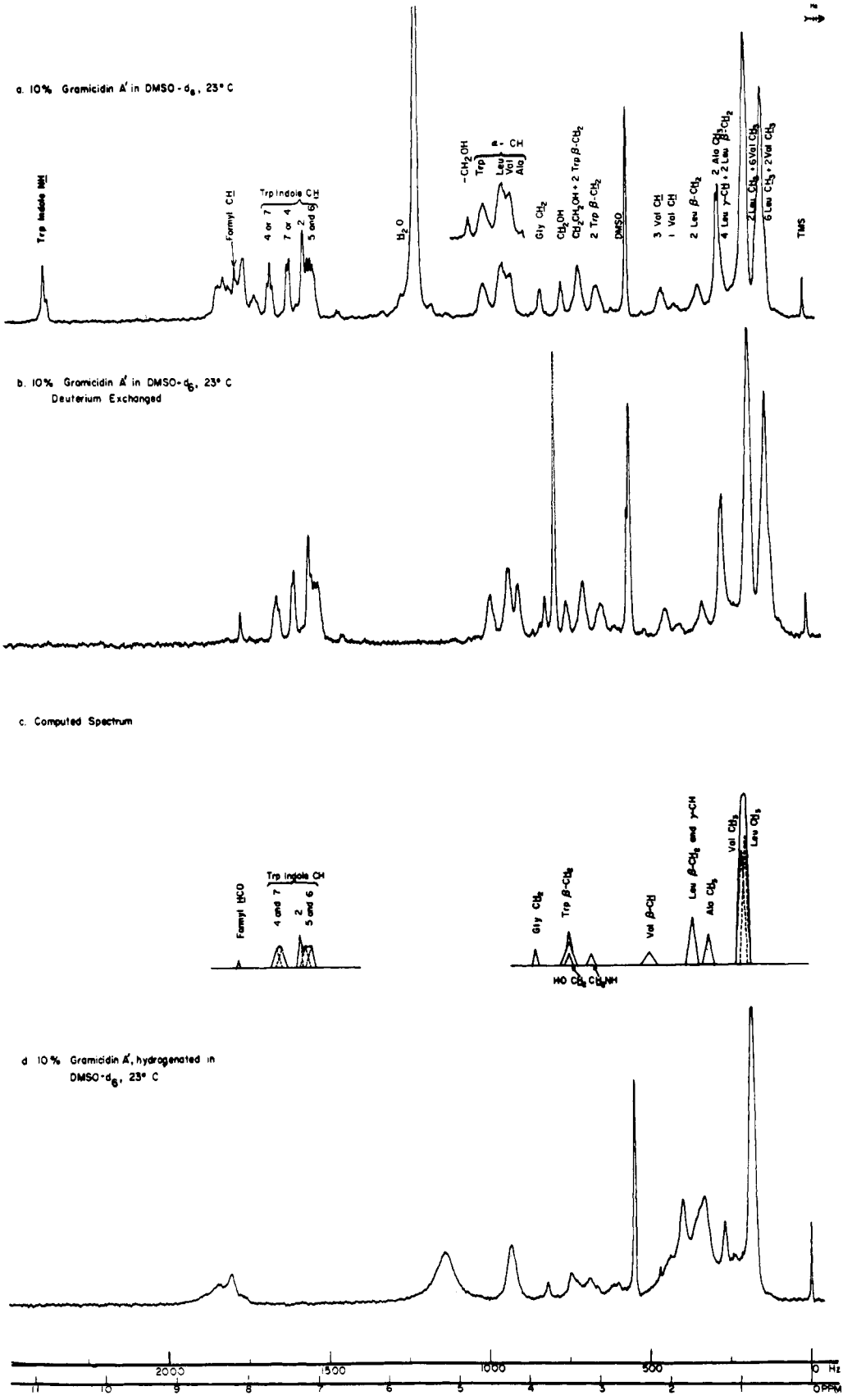

FIGURE 1: Pmr spectra of 10% (w/v) gramicidin A′ in Me_2SO-d_6 at 23°. (a) Spectrum showing assignments of various resonances. A few drops of trifluoroacetic acid were added to shift the water resonance to low field of normally overlapping peaks. The inset shows the αCH and CH_2OH resonances before addition of trifluoroacetic acid. (b) Spectrum of gramicidin A′ after all its labile hydrogens have been replaced by deuterium. The solution contained 2% D_2O. (c) Spectrum, calculated by the algorithm of McDonald and Phillips (1969), of a random coil gramicidin A in neutral D_2O. Resonances of all the labile hydrogens and all the αCH protons except glycine have been omitted. (d) Spectrum of a sample of GA′ whose indoles have been hydrogenated by the procedure of Ruttenberg *et al.* (1966).

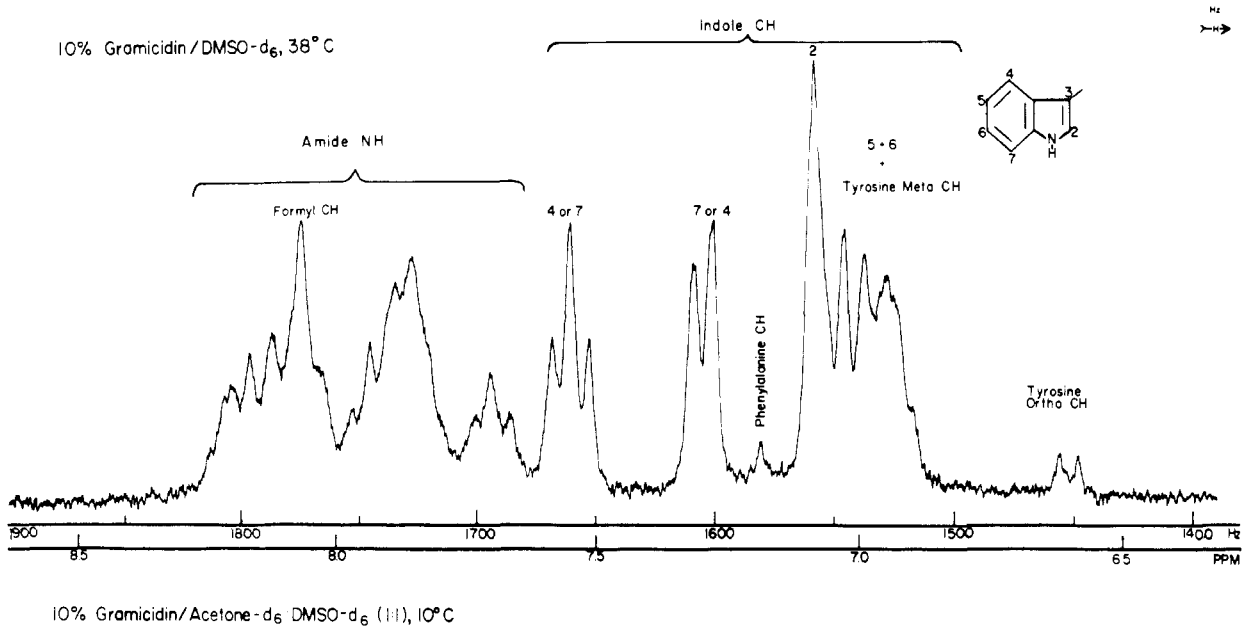

FIGURE 2: Expansion of the 1400- to 1900-Hz portion of the spectrum of 10% gramicidin A′ in Me_2SO-d_6 at 23° (compare to Figure 1a).

for *N*-acetyltryptophan, and 2378 Hz for *N*-acetyltryptophanamide (J. D. Glickson and W. D. Phillips, unpublished data), whereas peptide N*H* resonances generally occur at considerably higher field. The resonance at 2368 Hz in Figure 1a, therefore, originates from the indole N*H* hydrogens of the four tryptophans of GA′. The GA′ indole N*H* absorptions consist of two partially overlapping resonances with relative intensities of 3:1. The remaining labile proton resonances between 1685 and 1860 Hz originate from peptide N*H* hydrogens, the lone formylated N*H*, and the ethanolamine N*H*.

Figure 2 shows the 1400- to 1900-Hz region of the spectrum of GA′ in Me_2SO-d_6 on an expanded scale (section of Figure 1a). Replacement of N*H* hydrogens by deuterium readily distinguished N*H* resonances from C*H* resonances (Figure 1b). The formyl C*H* hydrogen produces the sharp spike at 1775 Hz, which survives deuterium replacement. Formyl C*H* absorptions of various compounds had similar chemical shifts, *e.g.*, dimethylformamide, 1764 Hz (Bhacca *et al.*, 1962). Assignment of the tryptophan indole C*H* resonances is readily accomplished by comparison to the spectrum of the free amino acid (McDonald and Phillips, 1967). Each of the four tryptophans of GA contributes a doublet originating from the C-4 (or C-7) indole C*H* proton to the triplet at 1661 Hz. Two of the doublets coincide as do the remaining two. The resultant two doublets (each of two proton intensity) have one peak in common, and, therefore, appear as an apparent 1:2:1 triplet (J = 8.8 Hz). All four C-7 (or C-4) tryptophan indole C*H* doublets coincide to produce the doublet at 1605 Hz (J = 8.6 Hz). The sharp singlet at 1558 Hz originates from four coincident C-2 indole C*H* singlet absorptions, and the C-5 and C-6 triplets overlap to produce the complex multiplet centered at 1535 Hz.

A number of weak aromatic C*H* absorptions were associated with GB and GC, which contain a phenylalanine and a tyrosine, respectively, in place of Trp-11 of GA (Sarges and Witkop, 1965a). In disordered proteins in D_2O solution the phenylalanine phenyl absorption occurs at 1598 Hz, and the tyrosine aromatic absorptions at 1500 Hz (ortho to OH) and at 1560 Hz (meta to OH) (McDonald and Phillips, 1969). Consequently, it is reasonable to assign the 1681-Hz absorption of GA′ to the GB phenyl hydrogens, and the 1451-Hz doublet to the ortho C*H* hydrogens of the GC tyrosine. The meta C*H* tyrosine doublet overlaps with tryptophan resonances of C-5 and C-6 indole hydrogens (the ortho and meta hydrogens of tyrosine are expected to yield an A_2B_2 pair of doublets). From the relative intensities of the tryptophan, phenylalanine, and tyrosine aromatic C*H* absorptions, we estimate that GA′ is composed of 72% GA, 9% GB, and 19% GC. Other impurities appear to be present in negligible amounts (Ramachandran, 1963; Gross and Witkop, 1965) as may be seen from further assignments of resonances (see below).

Using the algorithm of McDonald and Phillips (1969), we computed the spectrum of the side-chain absorptions of a hypothetical disordered GA in D_2O (Figure 1c). (GA′ is not soluble enough in water to measure its spectrum). Table I compares the chemical shifts of side-chain hydrogens in Me_2SO-d_6 and D_2O. The data in Me_2SO-d_6 were obtained from various compounds and may reflect effects of conformation as well as solvent on chemical shifts. Within about 20 Hz, the leucine, valine, and aromatic tryptophan C*H* chemical shifts in Me_2SO-d_6 agree with the values obtained in D_2O. More pronounced differences are observed for alanyl CH_3, glycyl CH_2, and tryptophan CH_2 resonant frequencies. Despite these differences, the spectrum in Figure 1c serves as a useful starting point for the assignment of aliphatic C*H* resonances. Chemical shifts and line widths of formyl C*H* and ethanolamine CH_2 absorptions were obtained from spectra of gramicidin and *N*-acetylethanolamine, respectively.

Homonuclear proton decoupling was very helpful in the assignment of a number of resonances. Most of the decoupling experiments were performed at 84°, at which temperature more distinct hyperfine structure is observed. A continuous, but small, change in chemical shift and coupling constant was observed as the temperature was raised. These changes may reflect a thermally induced conformational change that is rapid on the pmr time scale, *i.e.*, the rates of transition are much greater than the chemical-shift differences of resonances of a given proton in the various conformations. Con-

sequently, it was possible to perform decoupling experiments at higher temperatures and then to correlate coupled resonances with their positions in the 23° spectrum.

The leucine and valine methyl resonances are split into two absorptions, each of 24 proton intensity (Figure 1a). Spin decoupling from valine βCH resonances indicated that three valines and one leucine contributed resonances to the lower field peak at 180 Hz, which is associated with methyl groups in a predominantly solvated environment because of its coincidence with the "disordered" absorption (Figure 1c). The remaining leucine and valine methyl resonances are high field shifted to 130 Hz by ring-current interactions resulting from the close proximity of methyl groups to the faces of indole rings. Hydrogenation of the gramicidin indoles eliminates these ring currents and shifts the methyl resonances to their "normal" position (Figure 1d).

The presence of similar high-field ring-current-shifted methyl resonances in pmr spectra of proteins has been ascribed to the close proximity of methyl groups and aromatic side chains in the folded native conformation (McDonald and Phillips, 1967; McDonald *et al.*, 1971). Denaturation of the protein exposes both the aromatic and methyl resonances to an essentially equivalent solvated environment, thereby eliminating these ring-current shifts. Because of their extreme dependence on the relative orientation of methyl and aromatic side chains (Johnson and Bovey, 1958), these ring current shifts are valuable parameters for detecting even very subtle conformational changes and for distinguishing between disordered and ordered conformations. Observation of these high-field methyl ring-current shifts in spectra of GA′ indicates that the molecules must be oriented in such a manner as to bring the methyl groups of three leucines and one valine in close proximity to the faces of tryptophan indole rings. It is interesting to note that in the proposed helical transmembrane conformation of GA (Urry, 1971; Urry *et al.*, 1971) the methyl groups of Val-8 and Leu-10, -12, and -14 are indeed in very close proximity to the faces of the indole rings of Trp-9, -11, -13, and -15, respectively (Urry, 1972a,b).

Both alanine residues exhibit magnetically equivalent methyl and αCH hydrogens. Their methyl resonance (Figure 2a) is the distinct doublet at 226 Hz (J = 3.4 Hz) which is coupled to an αCH 1:3:3:1 quartet at 936 ± 2 Hz. Even though the alanine αCH resonance is buried beneath a number of other αCH absorptions, the characteristic spin-coupling pattern of this multiplet could be discerned on an expanded scale once its chemical shifts and coupling constant had been ascertained by spin decoupling.

Since the leucine βCH hydrogens are magnetically nonequivalent, the two βCH hydrogens of a given residue may yield distinct resonances. The four proton resonances at 335 Hz (Figure 1a) originated from four βCH hydrogens of either two or four distinct leucine residues. This assignment was supported by the chemical shift of this resonance (compare to the "disordered" spectrum in Figure 1c) and by its coupling to αCH resonances at 927 ± 5 Hz (spin decoupling was performed at 85°, at which temperature the 335-Hz resonance was a quartet, J = 5.8 Hz—actually a doublet of doublets due to αCH and γCH coupling). The broad background upon which the alanine methyl doublet appears is composed of γCH and βCH absorptions.

The assignment of the high-field ring-current-shifted methyl resonances to one valine and three leucines is based on spin-decoupling experiments performed on the two valine βCH resonances at 449 and 411 Hz (relative intensities three and one, respectively) in Figure 1a. Both resonances appear in the region of the spectrum where valine βCH absorptions are anticipated (compare to Figure 1c). Furthermore, the 449-Hz three proton peak couples to an αCH at 930 ± 5 Hz and to the methyl resonance at 177 ± 5 Hz (the nonring-current-shifted methyl), and the 411-Hz single proton valine βCH couples to an αCH at 911 ± 10 Hz and to the ring-current-shifted methyl absorption at 130 ± 5 Hz. If we associate the 449-H and 177-Hz peaks with unperturbed βCH and methyl resonances of valines, respectively, then the ring-current shifts experienced by one of the four valines are about 38 and 50 Hz for βCH and methyl hydrogens, respectively. This suggests that the valine methyl group falls somewhat closer to the center and plane of the proximal indole ring then does the βCH hydrogen.

The glycine CH_2 resonance at 817 Hz in Figure 1a was readily identified by its characteristically high-field chemical shift for an αCH (Figure 1c) and by its structure (a doublet at 85°, J = 5.1 Hz, but a sharp singlet after deuterium exchange). At lower temperatures the glycine CH_2 resonance is too broad and complex to readily yield a coupling constant. Consequently, the 5.1-Hz coupling constant may not be associated with the most stable conformation. The 758-Hz two-proton resonance, which coupled to the OH at 1043 Hz, emanates from the ethanolamine CH_2OH protons. The remaining ethanolamine $NHCH_2$ methylene resonance contributes two protons to the six-proton resonance centered at 700 Hz. This assignment is supported by the spectrum of *N*-acetylethanolamine in Me_2SO-d_6 in which the *N*-methylene occurred 35 Hz to high field of the *O*-methylene resonance. An alternate assignment of the *N*-methylene to the four proton resonance at 643 Hz was ruled out by failure of this resonance to couple to the *O*-methylene resonance (the 700-Hz resonance was too close to the *O*-methylene resonance

TABLE I: Chemical Shifts of Amino Acid Residue Side-Chain CH Proton Resonances in Me_2SO-d_6 and D_2O.

Amino Acid Residue	Protein	Chemical Shift (Hz) in DMSO-d_6	D_2O[a]
Alanyl[b]	CH_3	272	310
Glycyl	CH_2	760[c]	850
Leucyl	CH_3	205[c], 176[d]	195
	βCH_2	340[c], 330[d]	360
	γCH	375[c], 330[d]	360
Tryptophanyl[e]	CH_2	676	745
Indole	C-2	1569	1584
	C-4 or -7	1663	1658
	C-7 or -4	1603	1638
	C-5 or -6	1542	1566
	C-6 or -5	1533	1549
Valyl	CH_3	176[d], 205[f]	205
	βCH	480[f]	495

[a] Chemical shifts of random coil proteins in neutral D_2O (McDonald and Phillips, 1969). [b] From L-alanyl-L-alanine diketopiperazine (Fox Chemicals, Inc.). [c] From oxytocin (Johnson *et al.*, 1969). [d] From tyrocidin S (Stern *et al.*, 1968). [e] From (γ-methyl-L-glutamyl)$_3$tryptophan polymer (Fox Chemicals, Inc.). [f] From valinomycin (Ohnishi and Urry, 1969).

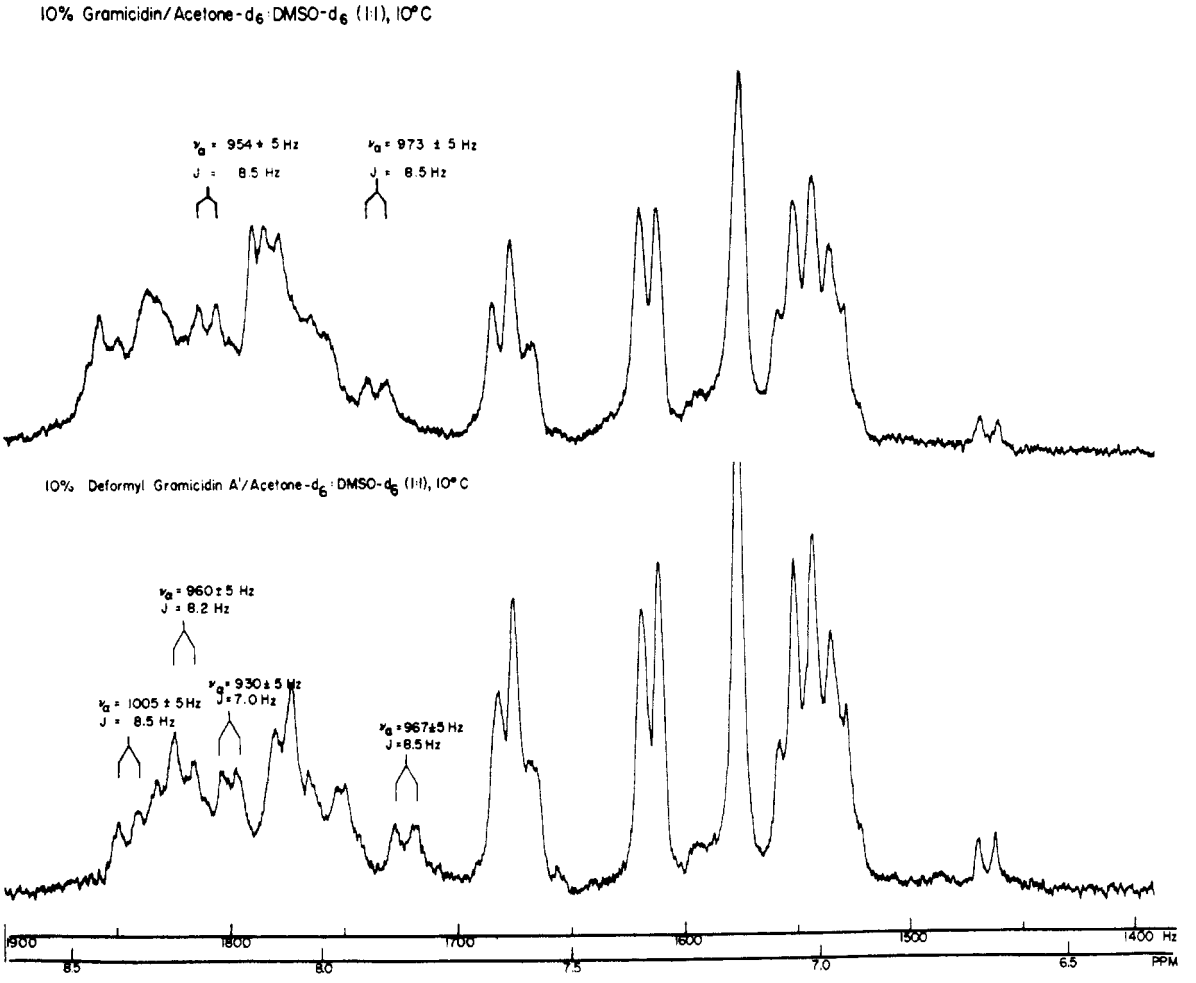

FIGURE 3: The low-field region of 220-MHz pmr spectra of (a) 10% GA′ (w/v) and (b) 10% deformyl-GA′ (w/v) both in Me_2SO-d_6–acetone (1:1, v/v) at 10°.

to permit spin-decoupling experiments). The 643- and 700-Hz resonances must be associated with tryptophan absorptions, either two βCH_2 absorptions per peak or four βCH absorptions per peak (the βCH hydrogens are magnetically nonequivalent). Consistent with this assignment, the four proton resonance at 997 Hz, which is associated with the tryptophan αCH hydrogens, coupled to both tryptophan βCH_2 resonances at 643 and 700 Hz. The remaining absorptions can now be assigned to four leucines, four valines, and two alanine αCH hydrogens.

TABLE II: Chemical Shifts of αCH Resonances of Gramicidin A′ in Me_2SO-d_6.

Residue	αCH Chemical Shift at: 23° (Hz)	85° (Hz)
Glycine	817	823
1 Valine + 3 leucines (methyl resonances ring current shifted)	908	911
2 Alanines	935	930 ± 5
3 Valines + 1 leucine (methyl resonances not ring current shifted)	943	931
4 Tryptophans	997	998

The assignment of αCH resonances is crucial to subsequent spin-decoupling experiments involving peptide NH resonances. Assignment of the 643- and 700-Hz resonances to tryptophan βCH_2 hydrogens indicates that the 997-Hz resonance to which they are coupled originates from the four tryptophan αCH hydrogens. The αCH absorptions of (γ-methyl-L-glutamyl)$_8$tryptophan polymer (Me_2SO-d_6, 23°) glutamic acid and tryptophan residues occurred at 935 and 990 Hz, respectively. An anomalous low-field position may, therefore, be characteristic not only of the βCH_2 resonances but also of the αCH resonances of tryptophan. This is altogether reasonable since both βCH_2 and αCH hydrogens are close to the edges of indole rings, where the ring-current field is expected to produce a low-field shift. Hydrogenation of these indole rings eliminates the ring-current effects and causes all the αCH absorptions, except that of glycine, to collapse into the 937-Hz peak (Figure 1d). This lends further support to the assignment of tryptophan αCH hydrogens and suggests that unless hydrogenation was accompanied by a conformational change—which infrared data suggest did not occur (Urry *et al.*, 1972)—the resonances previously at 911 Hz must have originated from αCH hydrogens that were ring current shifted to high field. Spin-decoupling experiments indicate that one of these αCH resonances originates from a

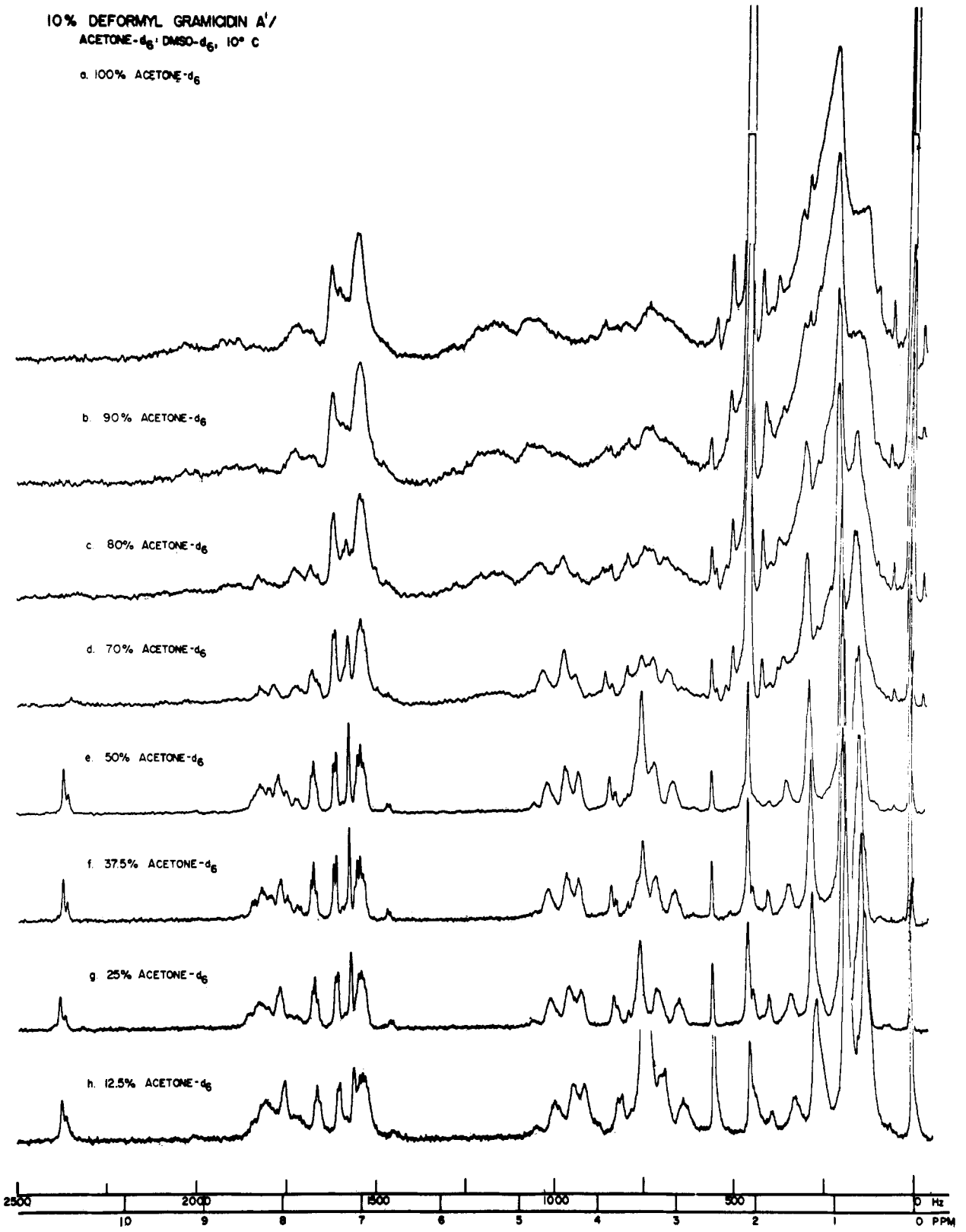

FIGURE 4: 220-MHz pmr spectra of 10% deformyl-GA′ (w/v) at 10° in mixtures of acetone-d_6 and Me_2SO-d_6.

valine whose methyl resonance is ring current shifted to high field. It is, therefore, reasonable to assign the remaining αCH hydrogens contributing to the 911-Hz absorption to three leucines whose methyl resonances are also ring current shifted to high field. The complete assignment of αCH absorptions is summarized in Table II.

Coupling Constants. Theoretically, the vicinal proton–proton-coupling constant J depends in the following manner on the dihedral angle θ, which is defined by these protons and the two atoms to which they are joined

$$J = A \cos^2 \theta + B \cos \theta + C \sin^2 \theta \qquad (1)$$

TABLE III: Peptide N*H*–α*CH* Coupling Constants in Acetone-d_6 Me_2SO-d_6 (1:1, v/v), 10°.

Compound	ν_{NH} (Hz)	$\nu_{\alpha CH}$ (Hz)	J (Hz)	θ (deg)
Gramicidin A′	1735	973 ± 5	8.5	159
	1809	954 ± 5	8.5	159
Deformyl-gramicidin A′	1721	967 ± 5	8.5	159
	1798	930 ± 5	7.0	145
	1818	960 ± 5	8.2	156
	1844	1005 ± 5	8.5	159
$\pi_{L,D}$				
L residues			9.4	175
D residues			7.0	145
$\pi_{L,D}{}^6$				
L residues			9.4	175
D residues			8.9	165
α Helix[a] (L residues)				
Right handed			2.4	108
Left handed			6.0	12
Random coil[b]			6.1	

[a] An α helix of alternating L and D residues would be expected to exhibit about half of its resonances with J = 2.4 Hz and the other half with J = 6.0 Hz. This possibility appears to be readily excluded even with the uncertainties which presently must be associated with an empirical expression for J. [b] From Tonelli and Bovey (1970).

where A, B, and C are constants which depend on electronegativities of adjoining atoms, their states of hybridization, bond lengths, and bond angles (Karplus, 1959, 1963; Barfield and Grant, 1965). While these constants have been calculated for ethane, ethylene, and fluoroethylene (Karplus, 1959), it is best for experimental purposes to empirically determine their values from spectra of molecules similar to the system being studied and having a well defined conformation. Such an approach has proven particularly successful in studies of organic compounds (Bothner-By, 1965) and has recently been applied to the αCHNH fragment of peptides (Stern *et al.*, 1968; Bystrov *et al.*, 1969; Ramachandran *et al.*, 1972).[2] In the present study, we use the most current values—A = 7.9, B = −1.55, and C = 1.35 (Ramachandran *et al.*, 1972)—in applying eq 1 to the N*H*-α*CH* fragment of peptides. Because the values of these constants may require further refinement, dihedral angles determined by eq 1 are recognized to be only approximate.

Overlap of N*H* resonances in the pmr spectrum of GA′ in Me_2SO-d_6 (Figure 2) hampered reliable estimation of coupling constants. Lowering the temperature, which necessitated the addition of acetone to lower the solvent freezing point, permitted resolution of a number of doublets which were decoupled from α*CH* hydrogens by double resonance (Figure 3). Optimum resolution was obtained at 10°; at lower temperatures resonances broadened out, presumably because of aggregation and/or increased viscosity of the sample. Because spectra of deformyl-GA′ were somewhat better resolved in the N*H* absorption region than spectra of GA′, and because spectral similarity of these compounds attests to their conformational resemblance, most of the coupling constants were obtained from the deformyl derivative.

The effect of acetone on the pmr spectrum of deformyl-GA′ is illustrated in Figure 4. The broadness of resonances in the pure acetone spectrum together with the observation that acetone precipitates GA′ suggests that this solvent promotes extensive association of GA′ and deformyl-GA′. A gradual transition complete by about 40% Me_2SO-d_6 occurs as acetone is enriched with Me_2SO-d_6. Consequently, coupling constants measured in acetone-d_6–Me_2SO-d_6 mixtures containing at least 50% Me_2SO-d_6 (v/v) reasonably reflect the Me_2SO-d_6 conformation. Acetone-d_6 promotes the exchange of labile hydrogens. The diminution of tryptophan indole N*H* resonances is particularly apparent, even in spectra obtained immediately after solution of the sample; exchange of peptide hydrogens is considerably slower. This exchange reaction may occur as a result of proton exchange with water impurities in the solvent, *i.e.*

$$2CD_3COCD_3 + H_2O \rightleftharpoons 2CD_3COCHD_2 + D_2O$$

$$D_2O + NH \rightleftharpoons HDO + ND$$

Peptide N*H*–α*CH* coupling constants and frequencies of corresponding α*CH* resonances, as determined by double resonance, and estimated dihedral angles, are summarized in Table III. Rough association of coupling constants with specific amino acids can be accomplished by referring to Table III. The dihedral angles in Table III fall within the range in which eq 1 is single valued (J = >6.4; 140° < $\theta \leq$ 180°). Consequently, there is no ambiguity in θ. Obviously the coupling constants could be associated not with a unique conformation, but could be averaged over a number of rapidly interconverting conformations. Furthermore, only about one-fourth of all the α*CH*–N*H* coupling constants could be determined. Despite these reservations, and despite the recognized uncertainty in the dihedral angle estimates, the coupling constants appear to be well outside the range associated with α-helical and random coil polypeptides. Their large magnitude is, however, consistent with coupling constants anticipated for the $\pi_{L,D}{}^4$ and $\pi_{L,D}{}^6$ helices (Table III).

Deuterium-Exchange Kinetics. Pmr spectroscopy is uniquely capable of monitoring the rates of deuterium replacement of specific labile hydrogens of complex macromolecules when the resonances of these hydrogens are well resolved. In this manner the state of hydrogen bonding of the various peptide N*H* hydrogens of tyrocidin S (Stern *et al.*, 1968) and the tryptophan indole N*H* hydrogens of lysozyme (Glickson *et al.*, 1969; 1971) has been determined. When more poorly resolved spectra are obtained, the envelope of overlapping resonances of labile hydrogens offers a means of nonspecifically monitoring the number of exchanged hydrogens. Wishnia and Saunders (1962) used this method to follow the exchange of peptide N*H* hydrogens of ribonuclease.

We have employed a combination of these techniques to study the accessibility to the solvent of the replaceable protons of GA′ and two of its derivatives—deformyl-GA′ and malonyl-GA′ (Mal-GA′) which consists of two deformyl-GA′ molecules joined amino end to amino end by a malonamide bridge (Urry *et al.*, 1971). The temperature for the exchange reaction was adjusted to produce measurable rates. Replacement of O*H* hydrogens, as indicated by the complete absence

[2] We are indebted to K. D. Kopple for sending us a preprint of this manuscript for use before publication.

of the O*H* resonance, was complete within 16 min, the time required to measure the first spectrum of all three compounds. Consequently, their O*H* protons appear to be exposed to the solvent.

Overlap of peptide N*H* resonances permitted only nonspecific monitoring of the exchange rates of all the peptide hydrogens (Figure 4). Comparison of the intensities of the peptide N*H* and tryptophan indole N*H* resonances to the intensity of the tryptophan indole C*H* doublet (Figure 2) was used to determine the number of nonexchanged hydrogens. In a separate experiment the exchange of GA′ was studied under conditions identical with those used to obtain the data in Figure 4, except that a fivefold molar excess of alanylalanine diketopiperazine was included in the sample. By the time the first spectrum was recorded (20 min after addition of D_2O) all the diketopiperazine peptide hydrogens had exchanged, whereas hardly any of the peptide hydrogens and tryptophan indole N*H* hydrogens of GA′ had been replaced. Consequently, if diketopiperazines are suitable models for solvated peptides, then the much slower rate of exchange of most of the peptide hydrogens of GA′ and its derivatives reflects a conformation which conceals most of the peptide hydrogens from the solvent. Hydrogen bonding, indicated by the chemical shifts of peptide N*H* resonances, appears to be at least partially responsible for this concealment of peptide protons. Chen and Swenson's (1969) observation that cis and trans amide N*H* hydrogens exchange at similar rates suggests that diketopiperazines whose amide hydrogens assume the cis configuration may have similar exchange properties to disordered polypeptides, whose amides are trans. For comparison of exchange rates it was necessary to keep the GA′ and reference compound in the same solution, because exchange rates in Me_2SO were not very reproducible (probably as a result of variable trace amounts of catalytic impurities in the solvent and/or samples).

It is significant that all the hydrogens are replaceable by deuterium. While this is not indicated in Figure 4, it was ascertained that all the reactions there depicted did go to completion. Consequently, GA′ cannot exist in a stable rigid conformation in which a significant number of its labile hydrogens are inaccessible to the solvent. A plausible structural model for this polypeptide must allow for disruptions of ordered structure which expose peptide hydrogens to the solvent. A rapid order $\rightleftarrows$ disorder equilibrium heavily favoring the ordered conformations would be consistent with the pmr data. Alternately, the molecule may exist in a stable ordered conformation, such as one of the $\pi_{L,D}$ helices, but local disruptions of ordered structure associated with small rotations about a few contiguous bonds could occur. In this regard it is interesting to note that the proposed $\pi_{L,D}$ helical conformation allows for considerable flexibility about the backbone bonds (the dihedral angles specifying this structure, $\psi \simeq 270°$, $\phi \simeq 60°$, for the $\pi_{L,D}^4$ helix, occur in a low energy basin of the potential energy–dihedral angle plot (Gibson and Scheraga, 1966)). Tilting of the planes of the amide bonds, such as is required to permit coordination of carbonyl oxygens to the cation in the channel, would tend to expose the peptide hydrogens to the solvent. Consequently, the exchangeability of peptide hydrogens is entirely consistent with the $\pi_{L,D}$ helical conformations.

Exchange of the four tryptophan indole N*H* hydrogens of GA′ followed first-order kinetics ($k = 1.88 \times 10^{-2}$ min^{-1} at 38°) (see Figure 5). Kinetic equivalence of these protons indicates approximately equal accessibility to the solvent. In a separate experiment, the exchange rates of the indole

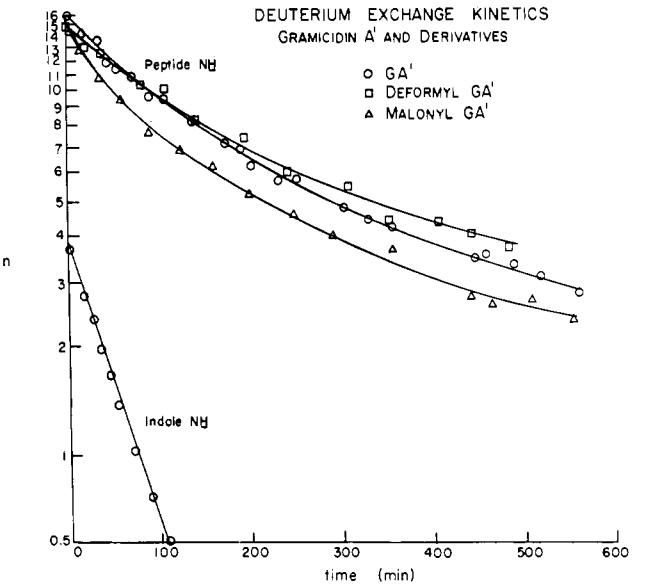

FIGURE 5: Kinetics of peptide proton exchange in 5% D_2O–Me_2SO-d_6 (v/v) of 2.0% (w/v) GA′, Mal-GA′, and deformyl-GA′. The exchange of tryptophan indole N*H* hydrogens of the GA′ sample is also included. The logarithm of the number of unexchanged N*H* protons (n) is plotted as a function of time (t).

N*H* hydrogens of the GA′ tryptophans and of indole, both contained in the same solution, were 1.78×10^{-2} and 3.81×10^{-2} min^{-1}, respectively (2% GA′, 0.8% indole, 5% D_2O–Me_2SO-d_6, 26°). The similarity of the rates of indole N*H* proton exchange of GA′ tryptophans and indole indicates that the four tryptophan indole N*H* hydrogens are exposed to the solvent. This is consistent with both the $\pi_{L,D}^4$ and $\pi_{L,D}^6$ conformations, which have their indole rings on the outside of the helix.

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