Homonuclear Indor Spectroscopy as a Means of Simplifying and Analyzing Proton Magnetic Resonance Spectra of Peptides and as a Basis for Determining Secondary and Tertiary Conformations of Complex Peptides†

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ABSTRACT: Homonuclear indor (internuclear double-resonance) spectroscopy is used (a) to simplify the "conventional" proton magnetic resonance (pmr) spectra of polypeptides and (b) to identify coupled proton transitions even when proton transitions of individual amino acid residues obscure each other by overlap. Indor spectroscopy is shown to be superior to and more generally applicable than "conventional" pmr spectroscopy for studies of polypeptides. (Conventional or normal spectra refer to single resonance spectra or to double-resonance spectra obtained not by *sweeping*, but by *maintaining constant* the decoupling frequency.) Specifically, the following simplifications were achieved. (a) The side-chain proton region of the antibiotic gramicidin S-A (a decapeptide with C_2 symmetry) was reduced from five amino acid residues in the normal pmr spectrum to the single residue valine in the indor spectra; (b) the C_α proton region of the heptapeptide ring fragment of the antibiotic polymyxin B_1 was reduced from seven residues in the normal spectrum to the single residue leucine in the indor spectra; and (c) the amide aromatic proton region of the antibiotic tyrocidin A was reduced from ten residues in the normal spectrum to three residues in the indor spectra. Thus, indor spectra of both backbone and side-chain protons were obtained. The indor spectra of individual residues yielded coupling constants, even with nonfirst-order spin systems. Conformational energy maps, or even better, conformational energy calculations can be used, in conjunction with the coupling constants evaluated from the indor spectra, to calculate the torsional angles that define secondary (backbone) and tertiary (side-chain) structure of the individual amino acid residues. The overall conformation of a polypeptide is simply the sum of the secondary and tertiary structures of the constituent amino acid residues.

There has been considerable interest in recent years in the study of conformation, kinetics of hydrogen exchange, and self-aggregation of peptides by proton magnetic resonance (pmr) spectroscopy. The first step in the determination of the conformation of a peptide by nuclear magnetic resonance (nmr) is an analysis of the pmr spectrum (*e.g.*, Stern *et al.*, 1968). Usually the C_β, C_γ, and C_δ protons of the side chains can be assigned with reasonable certainty by comparison of the spectrum of the peptide to the spectra of the constituent amino acids if there are not too many overlapping resonances. The same procedure, however, cannot be used to assign the C_α and amide protons whose chemical shifts seem much more dependent on secondary and tertiary conformation. Three principal techniques are used to assign the C_α and amide proton chemical shifts: (a) double resonance; (b) temperature, solvent, and pH perturbation of the spectrum; (c) selective deuterium–hydrogen exchange. Spin decoupling (Stern *et al.*, 1968; Hoffman and Forsén, 1966), one form of double resonance, is the most important of these techniques so far in common use, but it suffers from the disadvantage that it produces a perturbation of only a few lines in a polypeptide pmr spectrum containing a large number of lines. This disadvantage can be overcome by employing another double resonance technique, homonuclear indor (internuclear double-resonance) spectroscopy (Baker, 1962; Sciacovelli *et al.*, 1970). An indor spectrum is a much simplified double-resonance spectrum since it contains *only* perturbed transitions and not all the transitions of the whole spectrum. We shall show that indor spectroscopy is not only a useful technique for assignment of backbone, C_α, and amide proton chemical shifts, but is also ideal for obtaining basic stereochemical information about side-chain protons both in free amino acids and in amino acid residues of polypeptides.

Indor spectroscopy was first described by Baker (1962). Kowalewski (1969) has reviewed the application of indor spectroscopy (a) to the study of low sensitivity nuclear species such as natural abundance ^{13}C, (b) to the construction of nuclear spin energy level diagrams, (c) to the determination of relative signs of coupling constants, and (d) to the revelation of the precise location of transition maxima when signals are obscured by quadrupolar broadening. To our knowledge there have been no applications of *homonuclear* indor spectroscopy to amino acids, peptides, or proteins, but Horsley and Sternlicht (1968) used *heteronuclear* indor spectroscopy to study coupling between ^1H and ^{13}C in amino acids and simple peptides.

In an indor experiment a single frequency f_1, which usually corresponds to a single transition, is observed (monitored), while the perturbing (decoupling) frequency f_2 is swept through the selected range of the indor spectrum. Qualitatively, there are only three signals in an indor spectrum: negative, zero, and positive. A zero signal corresponds to the

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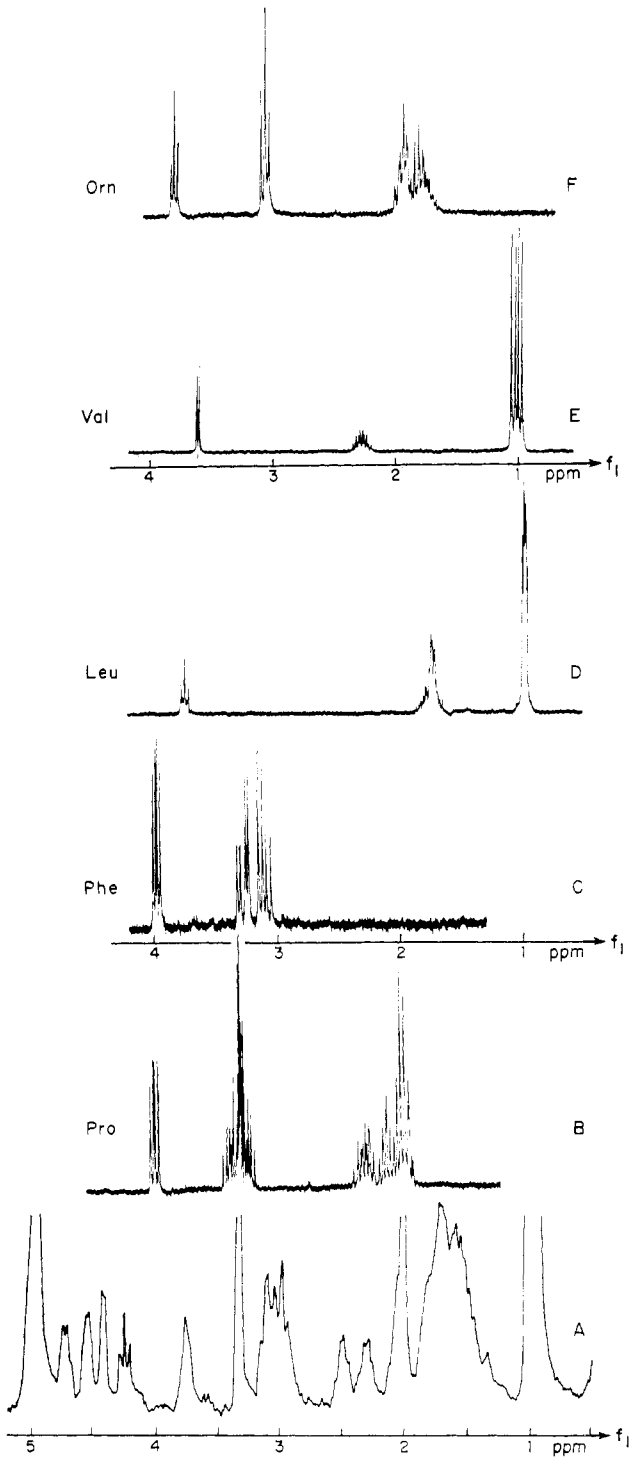

FIGURE 1: Comparison of the normal side-chain spectrum of the polypeptide gramicidin S-A in CD_3OD (A) with the normal side-chain proton spectra of the constituent amino acids in D_2O (B–F). The spectra were taken at 220 MHz at a temperature of 21°. The internal standards were tetramethylsilane (TMS) in CD_3OD and 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) in D_2O. Approximate assignments of side-chain proton transitions in the polypeptide can be made by comparison to the spectra of the free amino acids.

intensity of the monitored transition at f_1 when the second (decoupling) field at f_2 either perturbs no transition at all or perturbs transitions which do not have an energy level in common with the transition at f_1. If the observed (f_1) and perturbed (f_2) transitions have an energy level in common, then a "progressive" connection (Kaiser, 1963; Anderson *et al.*, 1963)[1] leads to a positive indor signal while a "regressive" connection leads to a negative indor signal.

The efficacy of indor spectroscopy is based on the principle that because each amino acid residue in a polypeptide is an isolated spin system, there is no coupling between the protons of one residue and those of another. Therefore, indor spectroscopy can be used to identify mutually coupled protons in a single amino acid residue. *In other words, indor spectroscopy permits great simplification of the entire polypeptide spectrum, the study of single residue spectra, and the revelation of hidden chemical shifts and coupling constants, thereby simplifying the problem of spectral analysis and, hence, of conformational analysis.* Tertiary conformation which has so far resisted analysis by "conventional" nmr methods is amenable to analysis based on indor spectroscopy.

Experimental Section

All indor spectra were taken on a Bruker HX-90 nmr spectrometer utilizing an internal lock. The lock frequency (f_0), monitoring frequency (f_1), and the double irradiation frequency (f_2) are all stable to better than 0.1 Hz, a necessary requirement of indor spectroscopy. The f_1 and f_2 channels can be swept individually. Spin-tickling and total decoupling experiments are performed by setting f_2 on a particular spectral line (transition) and sweeping the monitoring channel f_1. Indor experiments are performed by setting f_1 on a particular line in the pmr spectrum and sweeping the decoupling channel f_2 through the rest of the spectrum. Only those transitions in the spectrum having an energy level in common with the transition(s) being monitored at f_1 appear in the indor spectrum. Great spectral simplifications therefore result. Care is taken to insure that f_1 power is below saturation and that the f_2 power is less than that ulitized for spin-tickling experiments. Experimental methodology and the origins of negative and positive lines of indor spectra have been summarized by others (Kaiser, 1963; Anderson *et al.*, 1963).

Cross-relaxation effects, which could lead to spurious indor signals, are eliminated (a) by utilizing an extremely weak perturbing field (of less strength than that required for spin tickling) and (b) by allowing slow transit of the perturbing field through the spectrum (sweep rate $\lesssim 0.2$ Hz/sec).

All chemical shifts (δ's) are downfield from the standard.

Results and Discussion

We shall make a logical progression from a study of a side-chain proton region in section I to a study of a C_α proton region in section II to a concluding study of an amide proton region in section III.

I. Reducing the Complexity of the Side-Chain Proton Region of a Polypeptide Pmr Spectrum. Figure 1A shows part of the normal 220-MHz pmr spectrum of the cyclic decapeptide antibiotic gramicidin S-A in CD_3OD. Because the molecule has C_2 symmetry, only five different amino acid residues contribute to this spectrum; the normal individual spectra of the five constituent amino acids in D_2O are shown in Figures 1B–F. It is possible from Figure 1 to assign the individual C_β and C_γ side-chain protons of the peptide by

[1] In a progressive connection, the common energy level lies intermediate between the two levels that are not in common, while in a regressive connection, the common energy level lies either above or below both of the other two levels.

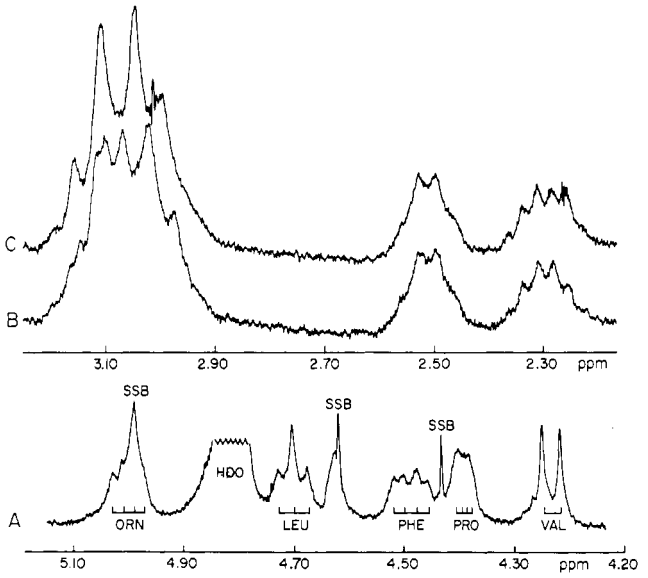

FIGURE 2: The assignment of a C_α proton transition of the phenylalanine residue in gramicidin S-A by conventional spin-decoupling.[2] The concentration was 50 mg/ml, the spectra were taken at 250 MHz, the solvent was CD_3OD, the temperature was 21°, the internal standard was TMS, and the amide protons were preexchanged for deuterium. Spectrum A shows the normal C_α proton region. Spectrum B shows the side-chain proton region between 2.2 and 3.2 ppm *without decoupling*. Spectrum C is the same as spectrum B, but *with decoupling* set as shown in spectrum A at 4.48 ppm, which corresponds to the center of the C_α proton multiplet of the phenylalanine residue.

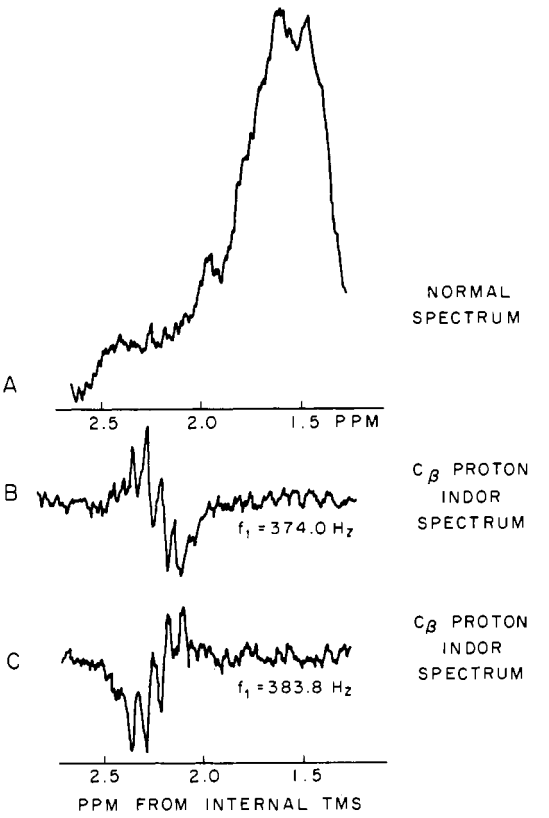

FIGURE 3: A comparison of the normal (A) and indor (B and C) proton spectra of gramicidin S-A in CD_3OD at 90 MHz. The temperature was 27° and the internal standard was TMS. Spectrum A is the normal pmr spectrum of only those protons whose chemical shift is between 1.0 and 2.5 ppm. Indor spectra B and C were obtained by monitoring the intensity of C_α proton transitions with f_1 at 374.0 and 383.8 Hz, respectively, while sweeping the decoupling field (f_2) from 240.0 to 120.0 Hz (2.67–1.33 ppm, respectively) through the C_β proton region.

a comparison to the spectra of the various constituent amino acids. This procedure cannot be used to assign the C_α or amide protons of the spectrum. The chemical shifts of the latter protons, associated with the backbone of the polypeptide, seem much more sensitive to conformation. This point led initially to wrong assignments of the gramicidin S-A spectrum (Liquori and Conti, 1968). The later assignments of the C_α and amide protons (Stern *et al.*, 1968) were made by spin decoupling. Figure 2 shows an example of the assignment of the C_α proton of the phenylalanine residue in gramicidin S-A by decoupling from the C_β protons, which were assigned by comparison of the peptide and phenylalanine pmr spectra; in this experiment, the solvent was CD_3OD and the spectra were taken at 250 MHz. Spectrum B collapsed to spectrum C (N. B. The AB quartet from the two C_β protons centered at 2.97 ppm) when the C_α proton at 4.5 ppm (spectrum A) was irradiated. Thus, it can be discerned that in polypeptides such as gramicidin S-A, spin decoupling permits the assignment of the C_α protons.

Several limitations of spin decoupling become more pronounced as this technique is applied to polypeptides with increasing numbers of amino acid residues. These limitations arise as follows. (a) Individual proton transitions in a particular amino acid residue become progressively more obscured by overlap from other transitions in other residues as molecular size increases. Therefore, it becomes progressively more difficult to assign the C_β protons which are needed as the basis for the assignment of the C_α protons. (b) In order to perturb the pmr spectrum significantly with the decoupling field, it is sometimes necessary to use power of such strength that several different transitions may be perturbed simultaneously. Therefore, as molecular size increases, it becomes more difficult to make correct proton assignments, because those transitions which are coupled cannot be identified with great certainty.

Because spin tickling utilizes less power than spin decoupling, fewer transitions are perturbed simultaneously. Therefore, assignments of lines in a complex spectrum of a polypeptide can be made with more certainty when spin tickling, rather than spin decoupling, is utilized. Nevertheless, in general, spin tickling still suffers the disadvantages outlined above for spin decoupling (Hoffman and Forsén, 1966).

Figure 3A shows the normal 90-MHz side-chain pmr spectrum of gramicidin S-A in the region from 1.0 to 2.5 ppm. The sample was dissolved in CD_3COOD to exchange completely the amide protons for deuterium in order to reduce the complexity of the C_α proton region. Figure 3B,C shows two proton indor spectra in which C_α proton transitions at 374.0 and 383.8 Hz, respectively, are monitored. A comparison of the two indor spectra to the normal spectrum reveals a great reduction in complexity, *viz.*, that although the chemical shift multiplets of the C_β and C_γ protons of leucine, the C_β and C_γ methylene protons of both proline and ornithine, and the C_β proton multiplets of the valine residue occur between 90 and 225 Hz (1.0 and 2.5 ppm, re-

[2] These spectra were recorded at the Carnegie-Mellon University 250 MHz NMR Facility for Biomedical Studies. We are grateful to Dr. J. Dadok, Dr. A. Bothner-By, and Mr. P. Balaram for their kind cooperation.

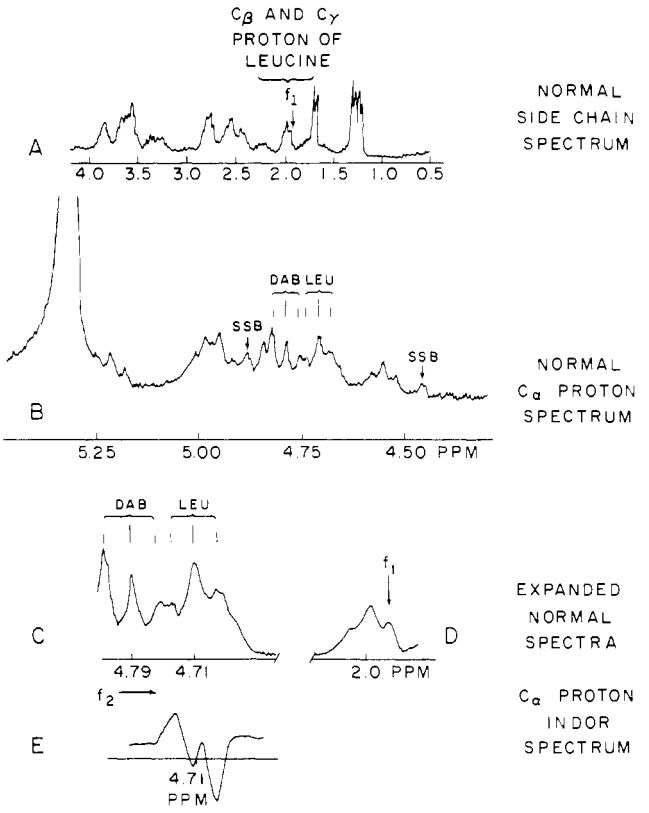

FIGURE 4: Assignment of the C_α proton transitions of leucine in the ring fragment of polymyxin B_1, and reduction of the whole C_α proton region from seven C_α proton residues to the single residue leucine. Spectra A–D were taken at 220 MHz, the solvent was D_2O at pD 2.0, the temperature was 21°, and the external standard was TMS. DAB refers to the residue diaminobutyric acid which appears in the normal, but not in the indor spectra. SSB refers to a spinning side band. Spectra A and B are normal side chain and normal C_α proton spectra, respectively. Spectra C and D are expanded portions of the C_α and side-chain proton regions, respectively. Spectrum E is a C_α proton indor spectrum obtained at 90 MHz by monitoring the intensity of a C_β proton transition with f_1 at 180.0 Hz while sweeping f_2 through the C_α proton region. N.B. The chemical shift scales are not corrected for bulk magnetic susceptibility.

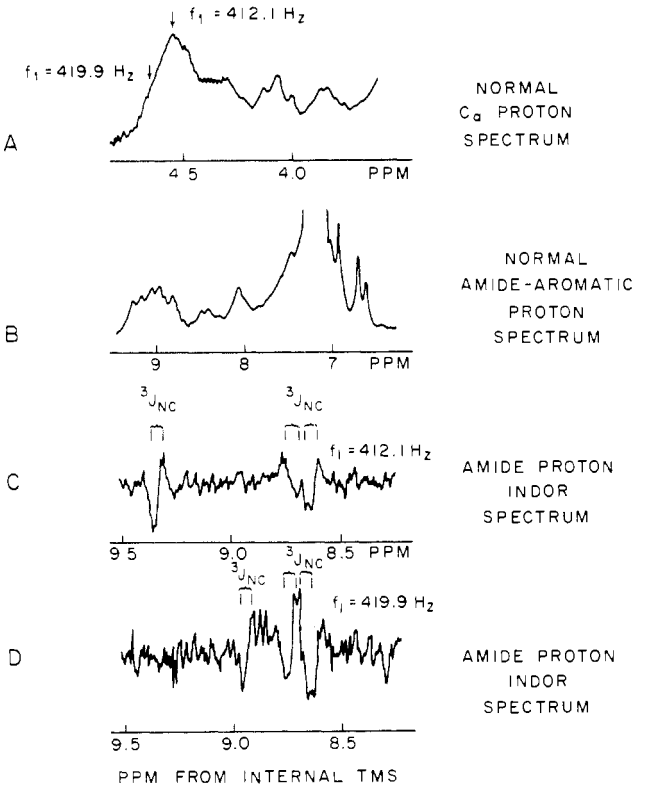

FIGURE 5: A reduction of the complexity of the amide-aromatic proton region of tyrocidin A in dimethyl sulfoxide-d_6 (DMSO-d_6) at 90 MHz. The temperature was 27° and the internal standard was TMS. Spectra A and B are normal C_α and normal amide-aromatic proton spectra, respectively. Indor spectra C and D were obtained by monitoring C_α proton transitions with f_1 at 412.1 and 419.9 Hz, while sweeping f_2 through the amide-aromatic proton region.

spectively) in Figure 3A, *only the latter multiplet appears in Figure 3B,C*. Furthermore, these indor spectra firstly confirm that the two monitored frequencies (f_1's) correspond to the C_α proton doublet of valine and secondly reveal the precise chemical shift of the valine C_β proton which was hidden in Figure 3A. First-order analysis of the indor spectra reveals that $\langle {}^3J_{\alpha\beta} \rangle$ = 8.6 Hz and $\langle {}^3J_{\beta\gamma} \rangle$ = 6.8 Hz for protons in the valine residue.

II. Reducing the Complexity of the C_α Proton Region of a Polypeptide Pmr Spectrum. Figure 4A,B shows the normal 220-MHz pmr spectra of the side chain and C_α proton regions of the pmr spectrum of the cyclic heptapeptide ring fragment of the antibiotic polymyxin B_1 in D_2O at pD 2.0. Expanded portions of these regions are shown in Figure 4C,D. Approximate assignments of the C_β, C_γ, and C_δ side-chain protons of this molecule are again easily made by comparison of the polypeptide spectrum to the spectra of the constituent amino acids. In particular, the unique C_β and C_δ proton multiplet of the leucine side chain centered at 2.00 ppm is identified with reasonable certainty by inspection. By monitoring the intensity of one of the C_β proton transitions of this multiplet with the f_1 channel (on the Bruker HX-90 instrument) set at 180.0 Hz the entire C_α proton region, swept by channel f_2, is reduced to that shown in Figure 4E. This unequivocally identifies the C_α multiplet centered at 4.71 ppm, as belonging to the leucine residue of the polymyxin B_1 ring. Thus, out of the seven C_α proton multiplets of the polymyxin B_1 ring, one obtains only the leucine residue C_α proton indor spectrum (Bockman, 1971). The other assignments in the C_α region of this molecule were made by both indor and conventional spin-decoupling techniques and will be reported in full in a later publication.

The most important point with which to conclude this section is that the role of the C_α and C_β proton transitions in this experiment (Figure 4) are reversed from those in the previous experiment (Figure 3), *i.e.*, in this experiment a C_α proton multiplet is revealed by monitoring a particular C_β proton transition with channel f_1 and sweeping the C_α proton region with the decoupling field f_2, while in the previous experiment a C_β proton multiplet is revealed by monitoring a particular C_α proton transition and sweeping the C_β proton region with the decoupling field.

III. Reducing the Complexity of the Amide Proton Region of a Polypeptide Pmr Spectrum. The normal 90-MHz pmr spectrum of the amide-aromatic proton region of the cyclic decapeptide antibiotic tyrocidin A in dimethyl sulfoxide-d_6 (Me_2SO-d_6) is shown in Figure 5B. It consists of a superposition of resonances from the 9 amide protons, 15 phenyl protons, 4 tyrosine aromatic protons, 3 ornithine amino protons, and the 4 amide protons from the side chains of as-

paragine and glutamine. The 10 overlapping C_α proton multiplets are shown in Figure 5A. By monitoring C_α proton transitions with channel f_1 at 412.1 Hz, the entire amide proton region swept by channel f_2 is reduced to the three amide proton doublets shown in Figure 5C. By setting the monitoring frequency (f_1) at 419.9 Hz, one of these multiplets disappears and is replaced by another multiplet shown in Figure 5D. Thus, by doing only two indor experiments, it was possible to reduce the pmr spectrum from that of 35 protons, as shown in Figure 5B, to that of two indor spectra of three protons each, as shown in Figure 5C,D. These latter indor spectra again reveal the precise chemical shifts of four amide protons and the coupling constant, $^3J_{NC}$, between each amide and C_α proton and demonstrate the power of indor for reducing the complexity of the amide region and making assignments of resonances previously hidden. A more detailed analysis of this spectrum of tyrocidin A will be published soon. The value of $^3J_{NC}$, in conjunction with conformational energy calculations (Gibbons *et al.*, 1970), can be used to ascertain the dihedral angle ϕ, one of the determinants of secondary (backbone) structure.

Conclusion

Homonuclear indor spectroscopy, a double-resonance technique, has been compared to spin decoupling and spin tickling as a means of simplifying and analyzing pmr spectra of polypeptides and shown to be superior to these conventional double-resonance techniques. By monitoring a particular transition in the C_α proton region, one obtains a great reduction in the number of lines in both the side-chain and the amide proton regions of the pmr spectra. Hidden chemical shifts are precisely revealed in indor spectra and the coupling constants between the C_α and the side-chain protons and between the amide and C_α protons are easily evaluated. It is precisely the coupling constant between the amide and C_α protons, in conjunction with energy calculations (Gibbons *et al.*, 1970), that is required in order to determine the secondary (backbone) structure of individual amino acid residues in polypeptides. Additional coupling constants necessary to determine tertiary (side-chain) structure of individual amino acid residues can be found (a) by monitoring the various C_β proton transitions in order to obtain the C_α and C_γ proton indor spectra and (b) by monitoring the various C_γ proton transitions to obtain the C_β and C_δ proton indor spectra. Of course, the overall conformation of a polypeptide is merely the sum of the individual conformations of the constituent amino acid residues.

In an extension (Gibbons *et al.*, 1972) of this work, we have shown that an amino acid can be uniquely classified according to the spin system of the C_α and side-chain protons and that indor spectroscopy can be used (a) to obtain characteristic individual amino acid residue spectra based on the proton spin system classification and (b) to obtain the coupling constants between side-chain protons, even when the spin systems are nonfirst order. In fact, we have shown that homonuclear indor spectroscopy serves as a *better* basis than normal pmr spectroscopy for the spectral analysis of amino acids with non-first-order spin systems. These observations form the basis for the use of both homonuclear and heteronuclear indor spectroscopy in the complete definition of polypeptide conformation.

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