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Homonuclear Internuclear Double Resonance Spectroscopy as a Basis for Determination of Amino Acid Conformation

(free amino acids/amino acid residues/NMR/PMR/INDOR spectroscopy/molecular conformation)

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ABSTRACT **INDOR (Internuclear Double Resonance) spectroscopy is shown to be superior to conventional (spectra obtained not by *sweeping*, but by *maintaining constant* the decoupling frequency) nuclear single- or double-resonance techniques for conformational studies of amino acids and amino acid residues in the following ways: (*a*) INDOR spectra of amino acids are inherently simpler than conventional proton magnetic resonance spectra of amino acids, and INDOR spectra of individual amino acid residues are slightly, if at all, complicated by overlap with either solvent peaks or the transitions of nuclei in other residues. (*b*) For each amino acid, the side-chain and C_α proton belong to a particular class of spin system characterized by unique INDOR spectra, the pattern of which aids in the proper assignment of spectral lines. (*c*) For an amino acid with a first-order spin system, INDOR spectra *directly* reveal hidden chemical shifts and coupling constants. For an amino acid with a spin system other than first-order, INDOR spectra *indirectly* reveal values for chemical shifts and coupling constants as follows: INDOR spectra permit construction of a *topological spin energy level diagram* which, in turn, allows division of the PMR spectrum of the spin system into subspectra that easily yield values for chemical shifts and coupling constants.**

Although we only report INDOR spectra of free amino acids or amino acid derivatives that resemble amino acid residues in polypeptides, *we, in effect, demonstrate a novel method to obtain total polypeptide conformation based on INDOR spectroscopy*, inasmuch as the total conformation is the sum of the individual residue conformations.

The first step in the conformational analysis of a polypeptide by proton magnetic resonance (PMR) spectroscopy is the assignment of the chemical shifts (δs) and the evaluation of all coupling constants (Js) for all protons of each amino acid residue contributing to the PMR spectrum of the entire polypeptide. Double resonance, in the form of either total spin-decoupling or spin-tickling, was the obvious PMR technique for proton assignments in the decapeptide antibiotic gramicidin S-A (1, 2) and other peptides (3–18), but it became apparent that these forms of double resonance have several limitations that become more pronounced with increasing numbers of amino acid residues in a peptide. These limitations arise as follows: (*a*) Individual PMR transitions in a particular amino acid residue become progressively more obscured by overlap from other transitions in other residues as the molecular size increases. Therefore, coupling constants needed for conformational analysis cannot be determined with any certainty. Because there are generally more side-chain protons than backbone protons, overlap is a more severe limitation in the determination of tertiary (side-chain) structure than in that of secondary (backbone) structure. (*b*) In order to perturb the PMR spectrum significantly with the decoupling frequency, it is often necessary to use decoupling 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 that are coupled cannot be identified with great certainty.

Homonuclear INDOR spectroscopy (19–21), a more recent double-resonance technique than spin-decoupling or -tickling, is less subject to the limitations listed above, and has distinct advantages. It has not, to our knowledge, been applied to the simplification and analysis of either amino acid or polypeptide PMR spectra and, consequently, to conformational analysis. Horsley and Sternlicht (22) have elegantly used *heteronuclear* INDOR spectroscopy to obtain carbon-13 spectra of amino acids. In this publication, we show how *homonuclear* INDOR spectroscopy can be used as a basis for the determination of the conformation either of free amino acids or of amino acid derivatives that resemble residues in polypeptides.

EXPERIMENTAL

All PMR spectra were taken on a Bruker HX-90 NMR spectrometer with 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. A conventional decoupled PMR spectrum is generated by sweeping the *monitoring frequency* (f_1), while the *decoupling frequency* (f_2) is maintained constant. On the other hand, an INDOR spectrum is generated by maintaining the monitoring frequency (f_1) constant, while the decoupling frequency (f_2) is swept. The conventionally decoupled spectrum contains nearly all the transitions of the whole spectrum; an INDOR spectrum indicates only those transitions that are coupled to the monitored transition (usually the monitored frequency corresponds to only a single transition).

Qualitatively, there are only three signals in an INDOR spectrum: negative, zero, and positive. A "zero signal"

Abbreviations: INDOR, Internuclear Double Resonance; J, coupling constant (Hz); δ, chemical shift (ppm).

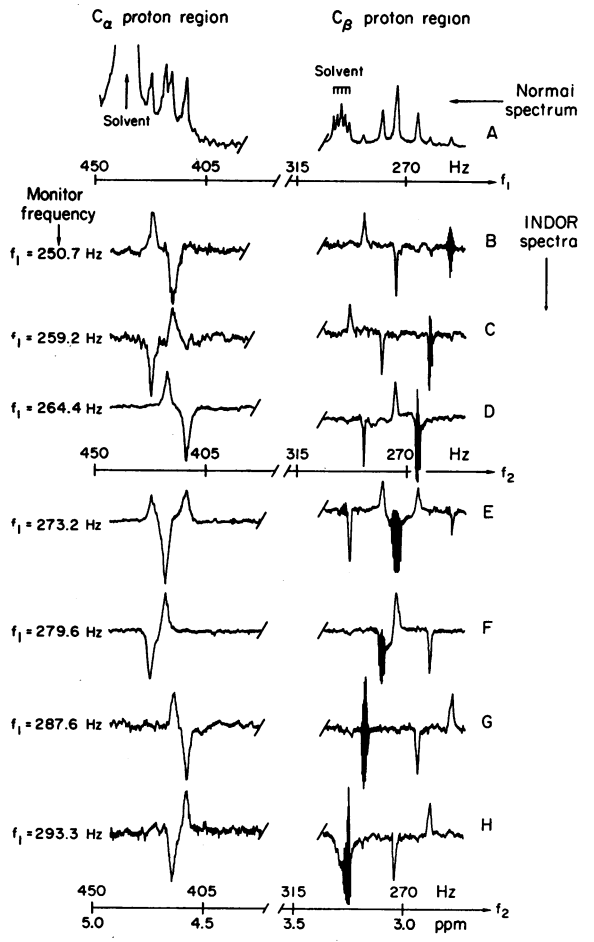

FIG. 1. Normal and INDOR spectra of *N*-acetyl phenylalanine methyl ester in CD_3OD.

corresponds to no change in the intensity of the monitored transition at f_1, and arises when the second (decoupling) field at f_2 either perturbs no transition at all or perturbs transitions that do not have an energy level in common with the transition at f_1. If the monitored (f_1) and perturbed (f_2) transitions have an energy level in common, then a "progressive" connection leads to a positive INDOR signal, while a so-called "regressive" connection leads to a negative INDOR signal (23, 24)‡. In other words, an unperturbed signal serves as the reference level, and only positive and negative perturbations of the monitored signal are detected. Because only transitions coupled to the monitored transition appear in an INDOR spectrum, great spectral simplifications result.

Care is taken to insure that f_1 power is below saturation and that the f_2 power is equivalent to that used for spin-tickling experiments. Experimental methodology and the origins of negative and positive lines of INDOR spectra have been summarized (23, 24).

All chemical shifts (δs) are downfield from tetramethylsilane (Me_4Si) as the internal standard. All spectra were taken at 90 MHz at 27°.

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

RESULTS AND DISCUSSION

INDOR studies of amino acids with an ABX spin system (phenylalanine)

The amino acids asparagine, aspartic acid, cysteine, histidine, serine, phenylalanine, tryptophan and tyrosine have two C_β protons and no C_γ protons. In these amino acids, the two C_β protons are slightly unequivalent chemically, and form an AB spin system. Furthermore, the two C_β protons (AB) are coupled to the C_α proton (X) to form an ABX spin system, in which there is coupling between all pairs of protons. Coupling of the amide proton to the C_α proton leads to an additional complication in the spin system, but this complication can be circumvented by preexchanging the amide proton for deuterium.

The methyl ester of *N*-acetyl phenylalanine (AcMePhe) preexchanged and dissolved in CD_3OD with a tetramethylsilane (Me_4Si) internal standard, was used as an example of an amino acid with an ABX spin system. There are potentially two advantages gained by use of this derivative of phenylalanine rather than the free amino acid. First, the derivative is more soluble than the free amino acid, and second, the derivative more closely resembles a residue in a peptide than does the free amino acid.

Before we discuss the INDOR spectrum of AcMePhe, let us first consider the *normal* 90 MHz PMR spectrum, part of which is shown in Fig. 1*A*. The centers of the characteristic C_α proton X quartet and two C_β proton AB quartets occur at 4.7 and 3.0 ppm, respectively. Because at 90 MHz the geminal coupling constant between the two C_β protons ($^2J_{AB}$) is of comparable magnitude to the difference between chemical shifts of these protons when expressed in the same physical units (Hz), the spectrum is not first-order. Therefore, one cannot evaluate the relevant PMR parameters (namely, coupling constants and chemical shifts) of the three protons by simple inspection of the spectrum. One can use a computer (25), or can follow the methodology described in standard texts (26–29) to evaluate the coupling constants ($^3J_{AX}$, $^3J_{BX}$, and $^2J_{AB}$) and chemical shifts (δ_A, δ_B, and δ_X).

At least five of the eight AB (C_β proton) and two of the four X (C_α proton) transitions must be *resolved* and *identified* in order to evaluate the six parameters that characterize an ABX spin system. In general, in polypeptides in which the

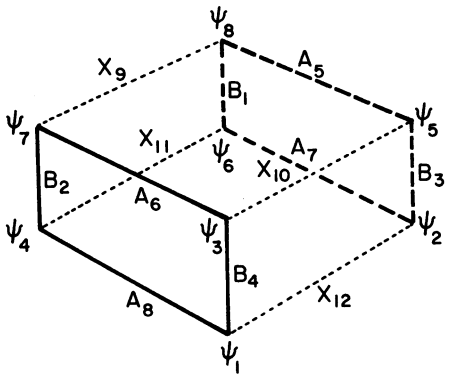

FIG. 2. Topological spin energy level diagram of proton transitions in *N*-acetyl phenylalanine methyl ester in CD_3OD. The *ab* subspectra (—— and - - -) and *x* subspectrum (– – –) are easily separated, since the *ab* subspectra appear as parallelograms separated by four parallel lines, which represent the *x* subspectrum.

proton transitions from individual amino acid residues overlap, it is usually not possible to *resolve*, let alone *identify*, proton transitions of an individual amino acid residue by inspection of a *normal* PMR spectrum. INDOR spectroscopy is ideally suited for the resolution of individual proton transitions that cannot be resolved by inspection of a normal polypeptide PMR spectrum.

In this section, we shall show how INDOR spectroscopy can be used to obtain information necessary for the determination of the tertiary (side chain) structure of amino acid residues with an ABX system, as exemplified by the phenylalanine derivative, AcMePhe. It should be kept in mind that the same technique used to obtain INDOR spectra of AcMePhe can be used to obtain similar spectra of amino acids with ABX spin systems in polypeptides.

Fig. 1*B–H* shows the INDOR spectra obtained by monitoring individually the *eight* transitions of the AB portion of the ABX spin system with the f_1 channel of the PMR spectrometer, while scanning the decoupling frequency, f_2, through the rest of the PMR spectrum. Because two of the transitions are degenerate (i.e., overlap) and, therefore, are monitored simultaneously at the same frequency (273.2 Hz), only *seven* lines in the AB region are monitored. The outstanding features of the INDOR spectra of Fig. 1 are the following: (*a*) They are simpler than the corresponding normal PMR spectra. (*b*) They contain negative, as well as positive, lines. (*c*) The transitions of protons in the *N*-acetyl and *O*-methyl groups, as well as those of the solvent, do not appear in any of these INDOR spectra, because none of these protons are coupled to any of the protons being monitored. (Similarly, because protons in different amino acid residues in a polypeptide are not coupled, spectra of individual residues can be generated by INDOR spectroscopy.)

It is possible to construct only two topological spin energy level diagrams consistent with the INDOR spectral data of Fig. 1 (20, 27). The corners of the cube represent the energy levels corresponding to the spin wave functions (ψs), and the lines are the *allowed* transitions. If we assume that $^2J_{AB}$, the geminal coupling-constant between the two C_β protons, is

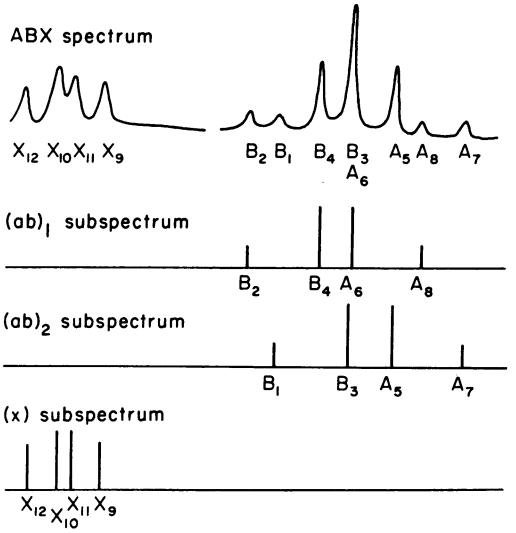

FIG. 3. Diagrammatic normal spectrum and subspectra of the ABX spin system of *N*-acetyl phenylalanine methyl ester.

TABLE 1. *Chemical shifts (δs) and coupling constants (Js) for the C_α proton (X) and two C_β protons (AB) of N-acetyl phenylalanine methyl ester in CD_3OD*

δ_A = 264.4 Hz	$^2J_{AB}$ = −13.95 Hz*
δ_B = 280.4 Hz	$^3J_{AX}$ = +9.0 Hz
δ_X = 419.1 Hz	$^3J_{BX}$ = +5.5 Hz

* We assume that $^2J_{AB}$ is negative (30).

negative (30), the only topological spin energy level diagram consistent with our data is shown in Fig. 2.

Fig. 3*A* shows a diagrammatic representation of the normal spectrum for an ABX spin system with two degenerate transitions (B_3 and A_6); the nomenclature used throughout Fig. 3 is that of Emsley, Feeney, and Sutcliffe (27). Fig. 3*B–D* shows the following three subspectra, which can be identified from Fig. 3*A*: (*i*) subspectrum $(ab)_1$, which corresponds to C_β proton transitions B_2, B_4, A_6, and A_8; (*ii*) subspectrum $(ab)_2$, which corresponds to C_β proton transitions B_1, B_3, A_5, and A_7; and (*iii*) subspectrum x, which corresponds to C_α proton transitions X_{12}, X_{10}, X_{11}, and X_9.

Even when the subspectra cannot be identified from the normal spectrum because individual transitions are obscured by overlap, the subspectra can be readily identified from the topological spin energy level diagram, inasmuch as the two *ab* subspectra appear as two "parallelograms," separated by four "parallel lines" that represent the x subspectrum (see Fig. 2).

Either from the correctly deduced subspectra or directly from the topological spin energy level diagram, it is standard procedure (27, 31) to evaluate the three chemical shifts and three coupling constants (both relative signs and magnitudes) for the single C_α and two C_β protons. Table 1 shows the values of these parameters for AcMePhe, with the assumption that $^2J_{AB}$ is negative.

INDOR studies of amino acids with an A_nMX spin system (threonine and valine)

Threonine, valine, and isoleucine are the only three common amino acids with only one C_β proton. This single C_β proton (M) is coupled both to the C_α proton (X) and to three C_γ protons (A_3) in the case of threonine, or to six C_γ protons (A_6)§ in the case of valine, to form A_3MX and A_6MX spin systems, respectively, with zero coupling between the A and X protons.

Coupling between the C_β hydroxyl proton and the C_β proton in threonine, and coupling between the amide and C_α proton in either amino acid, can be eliminated by preexchange of the hydroxyl and amide protons for deuterium. When these protons are preexchanged, threonine and valine have side chains $(C_\gamma H_3\text{-}C_\beta H(OD)\text{-}$ and $(C_\gamma H_3)_2C_\beta H\text{-}$, respectively, attached to the C_α atom.

In this study, the free amino acid, threonine, and the methyl ester of *N*-acetyl valine (AcMeVal), preexchanged and dissolved in CD_3OD with an internal standard of Me_4Si, were used.

§ Although the chemical shifts of protons in the methyl groups of valine are different, for all practical purposes the protons form an A_6MX spin system.

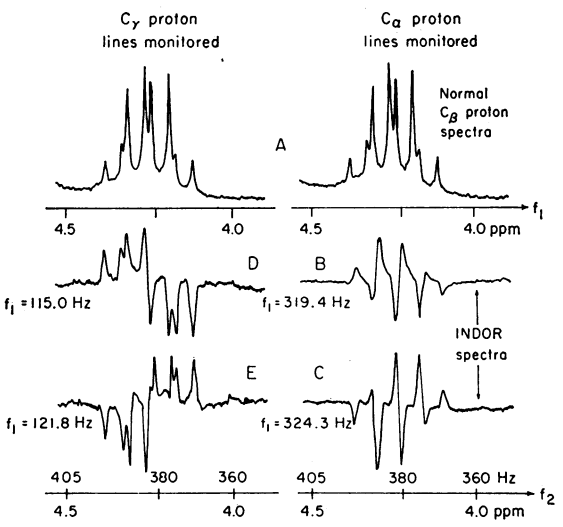

FIG. 4. Normal and INDOR spectra of threonine in CD_3OD.

Figs. 4*A* and 5*A* show part of the normal PMR spectrum of threonine and AcMeVal, respectively. In the INDOR spectra shown in Figs. 4*B–C* and 5*B–C*, the transition of each C_α proton doublet is monitored (f_1), while the spectrum is scanned with the decoupling field (f_2) through the C_β proton region. In the INDOR spectra shown in Fig. 4*D–E*, the transition of each C_γ proton doublet of threonine is monitored, while again the C_β proton region is scanned with the decoupling field. In none of the INDOR spectra are signals obtained from the solvent or the protons of the *N*-acetyl and *O*-methyl groups, since they are not coupled to the observed C_α or C_γ proton transitions. In addition, because C_α and C_γ protons are not coupled, the C_γ proton doublet does not appear in an INDOR spectrum when the C_α proton transitions are monitored and,

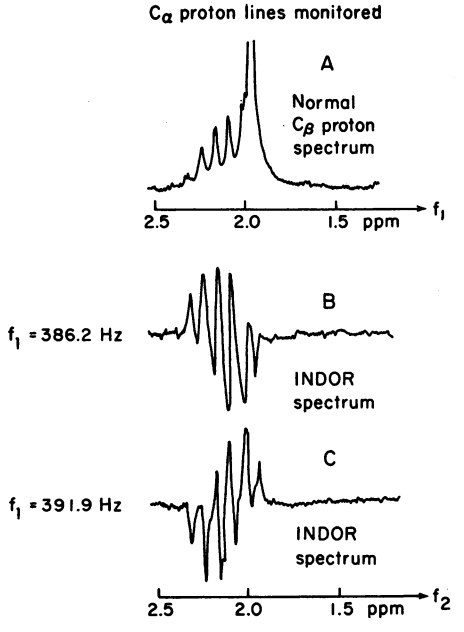

FIG. 5. Normal and INDOR spectra of *N*-acetyl valine methyl ester in CD_3OD.

TABLE 2. *Chemical shifts (δs) and coupling constants (Js) for the C_α (X), C_β (M), and C_γ (A) protons of threonine and N-acetyl valine methyl ester in CD_3OD**

	δ_A†	δ_M	δ_X	$^3J_{AM}$	$^3J_{MX}$
Threonine	118.4 Hz	383.5 Hz	321.8 Hz	+6.8 Hz	+4.9 Hz
AcMeVal	—‡	191.5 Hz	389.0 Hz	—‡	+5.7 Hz

* $^4J_{AX} \approx 0$.
† We assume that all coupling constants are positive.
‡ C_γ (A) proton INDOR spectra are not taken for AcMeVal.

similarly, the C_α proton doublet does not appear when the C_γ proton transitions are monitored.

Because at 90 MHz the magnitudes of the three coupling constants ($^4J_{AX} \approx 0$, $^3J_{AM}$, and $^3J_{MX}$) are much less than the magnitudes of any of the three differences in chemical shifts (between δ_A, δ_M, and δ_X), the A_3MX and A_6MX spin systems are first-order, and all six parameters can be evaluated by inspection of either the normal PMR spectrum or the INDOR spectra. As mentioned previously, however, the normal PMR spectrum is useful only when a sufficient number of individual transitions can be discerned. On the other hand, INDOR spectra are useful to obtain chemical shifts and coupling constants, even when individual lines are obscured by overlap, as in the case in spectra of polypeptides. Table 2 shows the values of these parameters for threonine and AcMeVal, on the assumption that all vicinal coupling constants are positive.

CONCLUSION

We have shown that homonuclear INDOR spectroscopy can be used to simplify the normal PMR spectrum of either free amino acids or amino acid derivatives that resemble residues in polypeptides in order (*a*) to obtain characteristic spectral patterns that can be used to facilitate the assignment of the amino acid residues in polypeptide spectra and (*b*) to obtain easily the coupling constants between side-chain protons. It is precisely these coupling constants that are needed (in conjunction with energy maps or energy calculations) to provide information about the side-chain torsional angles, χs, which define the side-chain conformation of the residue in question.

The method proposed here for total conformational analysis of peptides involves the following sequence of steps: (*a*) Obtain the INDOR spectra of each amino acid residue. (*b*) Construct the topological spin energy level diagram that is consistent with the INDOR spectra. Each amino acid residue will have its own topological spin energy level diagram that is consistent with the spin system class to which its side-chain belongs. For amino acids whose spectra are first-order, a topological spin energy diagram is unnecessary, but such a diagram is essential for amino acids whose spectra are not first-order. (*c*) Division of the NMR spectrum into subspectra, consistent with the topological spin energy level diagram. (*d*) Evaluation of all the chemical shifts and coupling constants for the amino acid by analysis of the subspectra. (*e*) Evaluation of the stereochemistry of the amino acid (or residue) by relating the coupling constants to dihedral angles ϕ, ψ, and χ through Karplus-type relationships. (*f*) Reduction in the number of dihedral angles consistent with a given coupling constant by use of the combined Karplus-type relationship and conformational

energy maps (1) or, even better, by conformational energy calculations.

Usually, the *normal* PMR spectrum can be directly analyzed for the coupling constant between amide and C_α protons in order to provide information about the torsional angle, ϕ, which is one of the three angles needed to define secondary (backbone) structure. There is no reason, however, why homonuclear INDOR spectroscopy cannot be used to obtain the coupling constant between the amide and C_α protons, if either the amide or C_α proton regions are obscured by overlap. Indeed, in another publication (32), we report spectra of the complex amide-proton region of a decapeptide. There is no reason why heteronuclear INDOR spectroscopy cannot also be used to obtain the coupling constant between the C_α proton and the amide nitrogen, specifically labeled with ^{15}N, on the next residue, in order to provide direct information about the backbone torsional angle ψ, in accordance with the suggestion by Gibbons *et al.* (1).

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