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)

W. A. GIBBONS*, H. ALMS*, J. SOGN*, AND H. R. WYSSBROD†

*The Rockefeller University, New York, N.Y. 10021; and †Department of Physiology, Mount Sinai Medical and Graduate Schools, The City University of New York

Communicated by William D. Phillips, January 20, 1978

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 Cα 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 sub-spectra 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, 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 (δα) and the evaluation of all coupling constants (Jαδ) 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 decapptide 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 obscure 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 spin-ticking, 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. Horose 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 look. The lock frequency (fL), monitoring frequency (fD), and the double-irradiation frequency (f2) are all stable to better than 0.1 Hz, a necessary requirement of INDO spectroscopy. The fL and fD channels can be swept individually. A conventional decoupled PMR spectrum is generated by sweeping the monitoring frequency (fD), while the decoupling frequency (f2) is maintained constant. On the other hand, an INDOR spectrum is generated by maintaining the monitoring frequency (fD) constant, while the decoupling frequency (f2) 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"
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 $C_\beta$ protons and no $C_\alpha$ protons. In these amino acids, the two $C_\beta$ protons are slightly unequivalently chemical, and form an AB spin system. Furthermore, the two $C_\beta$ protons (AB) are coupled to the $C_\alpha$ 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 $C_\alpha$ 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$_3$OD with a tetramethylsilane (Me$_4$Si) 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 INDO spectrum of AcMePhe, let us first consider the normal 90 MHz PMR spectrum, part of which is shown in Fig. 1A. The centers of the characteristic $C_\alpha$ proton X quartet and two $C_\beta$ proton AB quartets occur at 4.7 and 3.0 ppm, respectively. Because at 90 MHz the geminal coupling constant between the two $C_\beta$ protons ($J_{AB}$) 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 ($J_{AX}$, $J_{AX}$, and $J_{AB}$) and chemical shifts ($\delta_A$, $\delta_B$, and $\delta_X$).

At least five of the eight AB ($C_\beta$ proton) and two of the four X ($C_\alpha$ 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

---

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.

---

**RESULTS AND DISCUSSION**

![Fig. 2. Topological spin energy level diagram of proton transitions in N-acetyl phenylalanine methyl ester in CD$_3$OD.](image-url)
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. INDO 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 INDO 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 INDO spectra of AcMePhe can be used to obtain similar spectra of amino acids with ABX spin systems in polypeptides.

Fig. 1B-H shows the INDO spectra obtained by monitoring individually the eight transitions of the AB portion of the ABX spin system with the $f_3$ channel of the PMR spectrometer, while scanning the decoupling frequency, $f_D$, 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 INDO 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 INDO 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 INDO spectroscopy.)

It is possible to construct only two topological spin energy level diagrams consistent with the INDO spectral data of Fig. 1 (20, 27). The corners of the cube represent the energy levels corresponding to the spin wave functions $(\psi)$, and the lines are the allowed transitions. If we assume that $\gamma_{AB}$, the geminal coupling constant between the two $C_\beta$ protons, is negative (30), the only topological spin energy level diagram consistent with the data is shown in Fig. 2.

Fig. 3A shows a diagrammatic representation of the normal spectrum for an ABX spin system with two degenerate transitions ($B_3$ and $A_3$); the nomenclature used throughout Fig. 3 is that of Emseley, Feeney, and Sutcliffe (27). Fig. 3B-D shows the following three subspectra, which can be identified from Fig. 3A: (i) subspectrum $(ab)_1$, which corresponds to $C_\beta$ proton transitions $B_3$, $B_4$, $A_6$, and $A_7$; (ii) subspectrum $(ab)_2$, which corresponds to $C_\beta$ proton transitions $B_1$, $B_2$, $B_3$, $A_6$, and $A_7$; and (iii) subspectrum $(x)$, which corresponds to $C_\alpha$ proton transitions $X_{15}$, $X_{16}$, $X_{17}$, and $X_8$.

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 "parallel lines," 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 $C_\alpha$ and two $C_\beta$ protons. Table 1 shows the values of these parameters for AcMePhe, with the assumption that $\gamma_{AB}$ is negative.

**INDOR studies of amino acids with an $A_\alpha MX$ spin system (threonine and valine)**

Threonine, valine, and isoleucine are the only three common amino acids with only one $C_\beta$ proton. This single $C_\beta$ proton (M) is $C_\beta$ proton coupled both to the $C_\alpha$ proton (X) and to three $C_\gamma$ protons $(A_2)$ in the case of threonine, or to six $C_\gamma$ protons $(A_6)$ in the case of valine, to form $A_\alpha MX$ and $A_\alpha MX$ spin systems, respectively, with zero coupling between the $A$ and $X$ protons.

Coupling between the $C_\beta$ hydroxyl proton and the $C_\beta$ proton in threonine, and coupling between the amide and $C_\alpha$ proton in either amino acid, can be eliminated by pre-exchange of the hydroxyl and amide protons for deuterium. When these protons are pre-exchanged, threonine and valine have side chains ($C_\gamma$H2-C9H(OD)-) and ($C_\gamma$H2-C9H(OD)-), respectively, attached to the $C_\gamma$ atom.

In this study, the free amino acid, threonine, and the methyl ester of N-acetyl valine (AcMeVal), pre-exchanged and dissolved in CD2OD with an internal standard of Me3Si, were used.

**Table 1. Chemical shifts (δ) and coupling constants (J) for the $C_\alpha$ proton (X) and two $C_\beta$ protons (AB) of N-acetyl phenylalanine methyl ester in CD2OD**

<table>
<thead>
<tr>
<th>Chemical Shift (δ)</th>
<th>Coupling Constant (J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\delta_A = 264.4$ Hz</td>
<td>$\gamma_{AB} = -13.95$ Hz*</td>
</tr>
<tr>
<td>$\delta_B = 280.4$ Hz</td>
<td>$\gamma_{AX} = +9.0$ Hz</td>
</tr>
<tr>
<td>$\delta_X = 419.1$ Hz</td>
<td>$\gamma_{BX} = +5.5$ Hz</td>
</tr>
</tbody>
</table>

* We assume that $\gamma_{AB}$ is negative (30).

§ Although the chemical shifts of protons in the methyl groups of valine are different, for all practical purposes the protons form an $A_\alpha MX$ spin system.
Figs. 4A and 5A show part of the normal PMR spectrum of threonine and AcMeVal, respectively. In the INDOM spectra shown in Figs. 4B–C and 5B–C, the transition of each \( C_\alpha \) proton doublet is monitored \( (f_1) \), while the spectrum is scanned with the decoupling field \( (f_2) \) through the \( C_\beta \) proton region. In the INDOM spectra shown in Fig. 4D–E, the transition of each \( C_\gamma \) proton doublet of threonine is monitored, while again the \( C_\beta \) proton region is scanned with the decoupling field. In none of the INDOM 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 \( C_\alpha \) or \( C_\gamma \) proton transitions. In addition, because \( C_\alpha \) and \( C_\gamma \) protons are not coupled, the \( C_\alpha \) proton doublet does not appear in an INDOM spectrum when the \( C_\alpha \) proton transitions are monitored and,

\[
\begin{array}{c}
\text{Threonine} \\
\text{AcMeVal} \\
\end{array}
\]

\[
\begin{array}{cccc}
\delta_A^\dagger & \delta_X & \delta_X & \mu_{AM}^\ddagger & \mu_{MX}^\ddagger \\
118.4 \text{ Hz} & 383.5 \text{ Hz} & 321.8 \text{ Hz} & +6.8 \text{ Hz} & +4.9 \text{ Hz} \\
- - - - - - & - - - - - - & - - - - - - & - - - - - - & - - - - - - \\
\end{array}
\]

\* \( \mu_{AX} \approx 0 \)

\| We assume that all coupling constants are positive.

\| \( C_\gamma \) (A) proton INDOM spectra are not taken for AcMeVal.

Similarly, the \( C_\alpha \) proton doublet does not appear when the \( C_\alpha \) proton transitions are monitored.

Because at 90 MHz the magnitudes of the three coupling constants \( (\mu_{AX} \approx 0, \mu_{AM}, \text{and} \mu_{MX}) \) are much less than the magnitudes of any of the three differences in chemical shifts \( (\delta_A, \delta_X, \text{and} \delta_X) \), the \( \Delta_{AX} \) and \( \Delta_{MX} \) spin systems are first-order, and all six parameters can be evaluated by inspection of either the normal PMR spectrum or the INDOM 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, INDOM 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 INDOM 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, \( x_\alpha \), 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 INDOM spectra of each amino acid residue. (b) Construct the topological spin energy level diagram that is consistent with the INDOM 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 \( \phi, \psi, \text{and} \chi \) 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 Cα 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 INDO spectroscopy cannot be used to obtain the coupling constant between the amide and Cα protons, if either the amide or Cα 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 INDO spectroscopy cannot also be used to obtain the coupling constant between the Cα proton and the amide nitrogen, specifically labeled with 15N, 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).

H. R. W. was supported, in part, by The Life Sciences Foundation, Inc. and, in part, by N.I.H. Grant AM-10080. We thank Prof. Lyman C. Craig for his kind encouragement.