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Strong Spin–Spin Coupling in the Two-Dimensional *J*-Resolved 360-MHz ^{1}H NMR Spectra of the Common Amino Acids

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The two-dimensional *J*-resolved ^{1}H NMR spectra at 360 MHz of the 20 common amino acids have been investigated. The characteristic features of strong coupling are described. Advantages and drawbacks of projections, cross sections, and contour plots for the presentation of spectra including strong coupling are illustrated with selected examples. On the basis of the amino acid data practical aspects of the use of two-dimensional *J*-resolved spectroscopy to improve the resolution of high-field ^{1}H NMR spectra of proteins are discussed.

INTRODUCTION

High-resolution nuclear magnetic resonance appears at present to be the only technique capable of truly providing a many-parameter characterization of macromolecular structures in solution (*1*). The practical uses of NMR in this field are, however, often limited by the restricted spectral resolution. Hence the development and the application of new resolution enhancement techniques are of particular interest. Two-dimensional *J*-resolved (2D*J*) spectroscopy is a new concept which promises to enhance NMR spectral resolution (*2–6*) greatly.

Techniques for handling the large data matrices encountered when recording high-field 2D*J* spectra of macromolecules have previously been described (*4*). It was further shown that cross sections and projections are suitable techniques for presentation of the information content of complex 2D*J* spectra containing a large number of weakly coupled spin systems (*5*). The present paper treats questions arising from the occurrence of strongly coupled spin systems of individual amino acid residues (*1*) in the 2D*J* ^{1}H NMR spectra of proteins.

The amino acids histidine, serine, and tyrosine are used to illustrate different features of 2D*J* spectra which may arise from strongly coupled spin systems. In addition to cross-section and projection presentations, contour plots are shown to be an effective means for presentation of strongly coupled 2D*J* spectra. Schematic plots were computed for all the common amino acids which contain strong coupling features in the ^{1}H NMR spectra at 360 MHz. On the basis of these data practical aspects of the use of 2D*J* ^{1}H NMR for structural studies of proteins are discussed.

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MATERIALS AND METHODS

The basic features of two-dimensional J-resolved (2DJ) NMR have been described previously (*2–6*). The 2DJ experiment is initiated by a 90° pulse at the onset of the evolution period of duration t_1, in the middle of which a 180° pulse is applied. Data acquisition starts at the time of the spin echo, which marks the end of the evolution period and the beginning of the detection period, t_2. To obtain the pairs of frequencies giving rise to a peak in the 2DJ spectrum $S(\omega_1,\omega_2)$, a set of experiments with different evolution periods t_1 is made, so that a data set $s(t_1,t_2)$ is obtained. The information from the evolution period enters into the signal observed during the detection period through a phase modulation. For weakly coupled spin systems this modulation comes only from the spin–spin coupling constant. For strong coupling, however, chemical shift effects are not completely refocussed by the 180° pulse. After Fourier transformation of the time domain data in two dimensions the 2DJ spectrum is obtained in the ω_1-ω_2 representation, where the

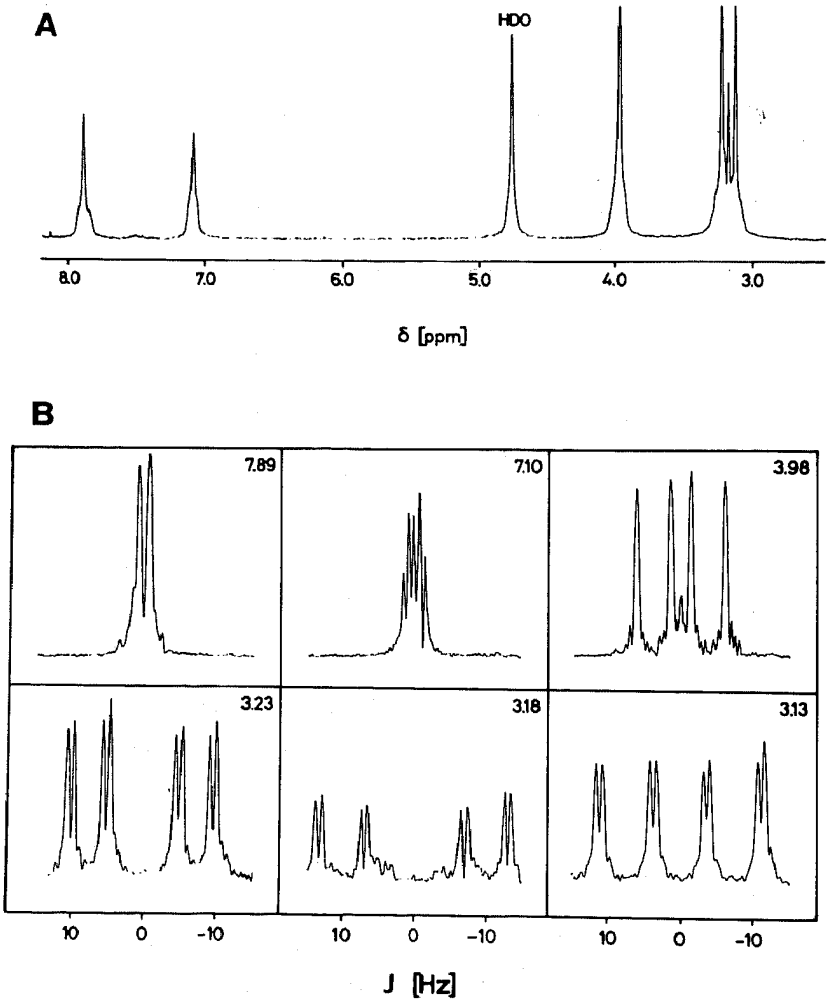


FIG. 1. 2DJ ^{1}H NMR spectrum at 360 MHz of 0.1 M L-histidine in D_2O; pD = 6.8, T = 25°C. (A) Projection of the (δ,J) spectrum along the J axis onto the δ axis. (B) Cross sections parallel to the J axis through the (δ,J) spectrum. The numbers in the upper right corner of each multiplet representation indicate the chemical shift of the peak maxima in the projection, A, where the cross sections were taken.

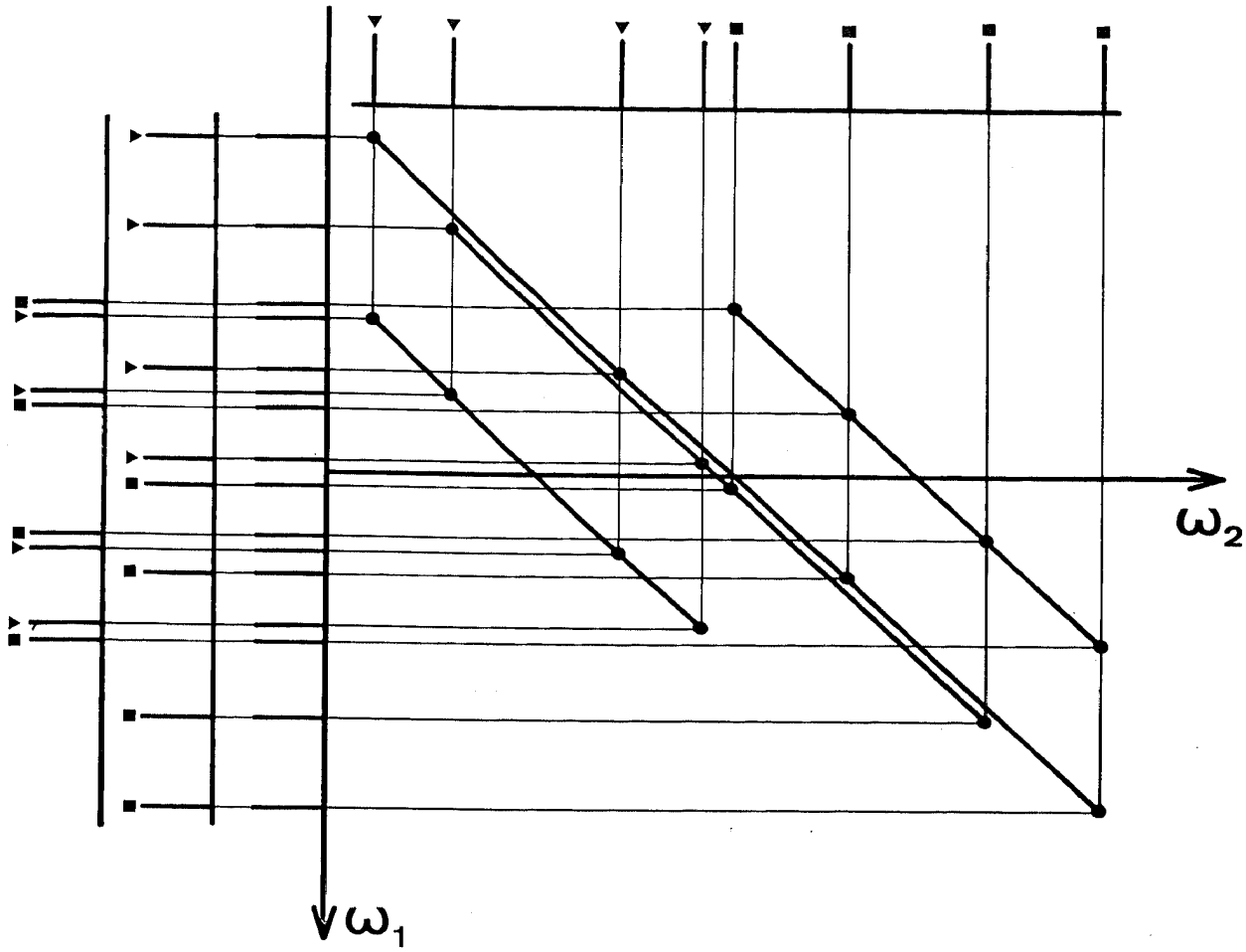


FIG. 2. Scheme for the AB part of the (ω_1,ω_2) representation of the 2DJ spectrum of an ABX spin system. Peaks of the 2DJ spectrum are indicated by solid circles. It is seen that these are aligned on four parallel straight lines at an angle of 45° with respect to the two frequency axes. Projections of the line positions on the ω_1 and the ω_2 axes are also shown, where peaks from nucleus A are identified by ■, and those from nucleus B by ▼. The projections onto the ω_1 axis are shown separately for the central multiplets, the peripheral multiplets, and the sum of the two (see text). The parameters for L-serine, $^{\oplus}D_3N-CH(-CH_2-OD)-COO^{\ominus}$, were used in the drawing.

peaks of each multiplet are aligned on a straight line with 45° slope with respect to both frequency axes. The intersection of the 45° line with the $\omega_1 = 0$ axis defines the chemical shift, δ, for each multiplet. The J-δ representation of the 2DJ spectrum, where the multiplet components are aligned on straight lines perpendicular to the chemical shift axis, is obtained from the ω_1-ω_2 spectrum by a spectrum tilt of 45° (*5*).

^{1}H NMR spectra at 360 MHz were recorded on a Bruker HXS-360 spectrometer equipped with an Aspect 2000 computer. The 20 common amino acids were obtained from Fluka AG. 0.1 *M* amino acid solutions in D_2O were prepared and lyophilized repeatedly to minimize the residual solvent proton concentration. For some amino acids, more dilute solutions had to be used because of the limited solubility. The pD was adjusted to 6.5 by adding DCl or NaOD to the solutions. The pH meter reading was used without corrections for isotope effects (*7*). Sodium-

TABLE 1

COMPARISON OF FUNDAMENTAL FEATURES OF WEAKLY AND STRONGLY COUPLED SPIN SYSTEMS IN 2D*J* SPECTRA AND IN ONE-DIMENSIONAL SPECTRA

Weak coupling	Strong coupling
The (J,δ) representation is symmetric with respect to $J = 0$.	The (J,δ) representation is symmetric with respect to $J = 0$.
The multiplet patterns are identical to those in the corresponding 1D spectrum.	Multiplet patterns with identical line positions but different intensities than the 1D spectrum are seen. Furthermore, unexpected additional peaks show up.
The projection of the (J,δ) representation in the J direction on the δ axis corresponds to a fully decoupled 1D spectrum.	The projection of the (J,δ) representation in the J direction on the δ axis does not correspond to a fully decoupled 1D spectrum, since additional strong coupling peaks appear.
The projection of the (ω_1,ω_2) representation along ω_1 on the ω_2 axis reproduces the corresponding 1D spectrum.	The projection of the (ω_1,ω_2) representation along ω_1 on the ω_2 axis reproduces the corresponding 1D spectrum.[a]

[a] Projection of the absolute value 2D*J* spectrum reproduces only the line positions but not the intensities of the 1D spectrum.

3-trimethylsilyl-[2,2,3,3-$^2H_4$]-propionate (TSP) was added as an internal reference. The solutions were measured at 25°C. In most experiments the time domain data size was 64 × 2048. Depending on the amino acid concentration the time used to record a spectrum was between 1 and 6 hr.

The software used with the Aspect 2000 computer was an improved version of the previously developed 2D program (*4*). The following representations of 2D*J* spectra could be obtained: Stacked plots in the ω_1 or ω_2 direction or, after a spectrum tilt (*5*), in the δ or J direction, which provide a "three-dimensional" image of the 2D spectrum; projection of the (δ,J) spectra on the δ axis; cross sections through individual multiplets; contour plots of (ω_1,ω_2) or (δ,J) spectra. The maximum data size available with full use of the disk storage space was a 64 × 4096 matrix for the time domain data, and a 64 × 4096 matrix each for the positive and negative frequency domain data. During the data processing zero-supplemented Fourier transformation was applied in both dimensions (*8*). To improve the spectral resolution the time-domain data were multiplied in both the t_1 and t_2 directions with a suitable resolution enhancement function (*9*). A phase-shifted sine bell (*10*, *11*) was found proper in the present experiments.

Simulated 2D*J* spectra for the common amino acids were computed with the formalism previously described by Aue *et al.* (*2*), Kumar (*12*), and Bodenhausen *et al.* (*13*). The magnetization component corresponding to a 2D*J* peak was evaluated in the form

$$s_{abcd}(t_1,t_2) = Z_{abcd} \exp(i\omega_{ab}t_2) \exp(i(\omega_{ab} - \omega_{cd})t_1/2), \quad [1]$$

with

$$Z_{abcd} = (I_x)_{ab}(I_x)_{cd}(P)_{bc}(P)_{da} \quad [2]$$

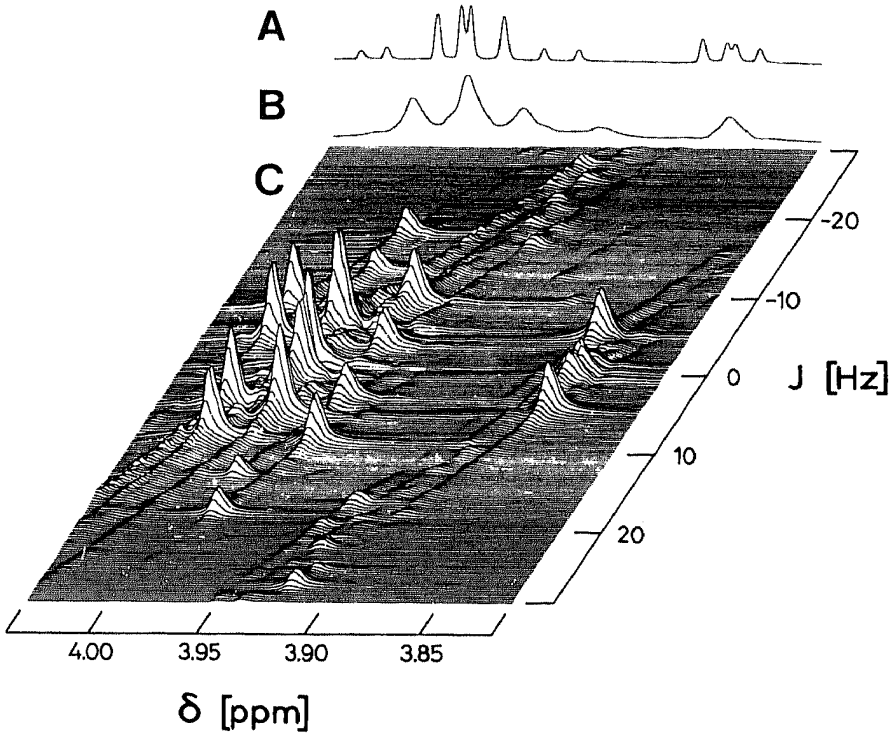


FIG. 3. 360-MHz ^{1}H NMR spectra of 0.1 *M* L-serine in D_2O; pD = 6.5, T = 25°C. (A) 1D spectrum. (B) Projection of the (δ,J) representation of the 2DJ spectrum along the J axis on the δ-axis. (C) Stacked plots in the J direction providing a "three-dimensional" view of the (δ,J) spectrum.

and

$$P = 2^N \prod_{k=1}^{N} (I_x)_k. \qquad [3]$$

The product in [3] is over all the N spins, and a, b, c, and d denote energy levels of the spin system.

A modified LAOCOON III program obtained from R. Freeman was used for the numerical calculations. The frequencies ω_{mn} and the matrix elements of the spin operators $(I_x)_{ij}$ are already available in the LAOCOON III program, so that only the matrix elements of the mixing operator, $(P)_{ij}$, had to be calculated. The numerical output was used to simulate the 2DJ spectra in the (J,δ) and (ω_1,ω_2) representations (*5*). The program is limited to spin systems containing up to six spins 1/2. For the amino acids which contain more than six protons, the simulations were obtained as follows: For phenylalanine and tyrosine the aromatic protons were treated separately from the ABX spin system of the α and β protons. For tryptophan the α and β protons were considered jointly with the C2 proton of the indole ring and a separate calculation was applied for the remaining four indole ring protons. For methionine the ϵ methyl protons were treated separately. For valine, leucine, and isoleucine the methyl groups were replaced by single protons; additional multiplet features from the methyls were then introduced with the assumption that these affected only the vicinal protons. For arginine two separate calculations were made for the α, β, and γ protons, and the β, γ, and δ

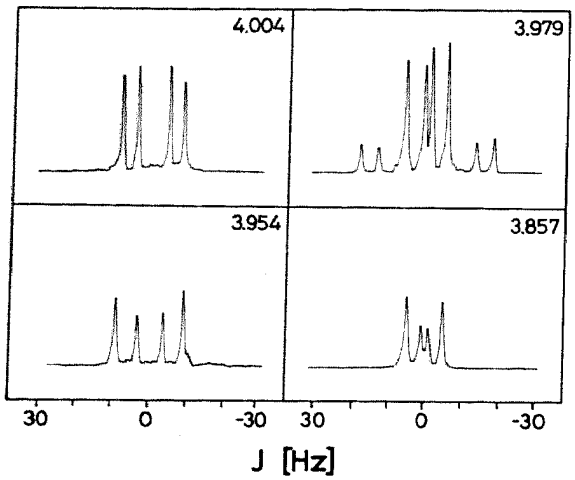


FIG. 4. Cross-section representation of four multiplet patterns in the L-serine ^{1}H NMR spectrum of Fig. 3C. The chemical shifts where the cross sections along the J axis have been taken are indicated by the numbers in the upper right corner of each multiplet representation.

protons, respectively. An approximate simulation of the entire spectrum was then obtained by addition of the α and β resonances from the first calculation and the γ and δ resonances from the second calculation. Similarly the spin systems of lysine and proline were decomposed into overlapping partial systems.

RESULTS

The 2DJ spectrum of L-histidine in Fig. 1 is used to illustrate manifestations of weak and strong spin–spin coupling in the previously described projection and cross-section representations (*5*). The low-field part of the histidine spectrum contains exclusively weakly coupled patterns. In the projection representation the two imidazole ring protons give rise to two somewhat broadened lines. In the cross sections the long-range couplings of the C2 and C4 protons are well resolved and can readily be analyzed. The doublet splitting of 1.6 Hz for C2H at 7.89 ppm is due to coupling with C4H at 7.10 ppm. C4H has additional long-range coupling with the two β-methylene protons. These two coupling constants are coincidentally equal, and at 0.9 Hz they correspond to one-half of the coupling between C2H and C4H. Hence the five-line pattern at 7.10 ppm corresponds to a partially degenerate quartet of doublets. In the high-field part of the spectrum four multiplets could be resolved between 3.0 and 4.0 ppm. The long-range couplings between the imidazole ring protons and the β-methylene protons are seen in the three cross sections between 3.13 and 3.23 ppm. Strong spin–spin coupling is clearly manifested since, in contrast to the weakly coupled case, the projection representation of the β-proton resonances shows three lines instead of the two lines expected for a "broad-band homonuclear decoupled spectrum" (*3*, *5*). Furthermore the appearance of a weak central peak in the α-proton multiplet is due to strong coupling between the two β-methylene protons. As is discussed in the following two sections, much care must therefore be exercised in the analysis of the individual cross sections, since these are affected by strong coupling.

ABX Spin Systems in 2DJ NMR

It is known that ABX subsystems occur quite frequently in protein spectra (*1*). Knowledge of their special features (*12*, *13*) is important for a correct interpretation of 2D*J* spectra of proteins. Figure 2 shows a schematic representation of the AB part of an ABX spin system in the (ω_1,ω_2) representation of the 2D*J* ^{1}H NMR spectrum. As in the weak coupling case all peaks are aligned on straight lines with 45° slope with respect to both frequency axes. The four resonances on each of the four 45° lines represent a symmetrical doublet of doublets. The projection of the 2D*J* spectrum along the ω_1 axis on the ω_2 axis shows the resonance positions of the corresponding 1D spectrum. The projection along ω_2 on the ω_1 axis gives the resonance positions of a *J* spectrum (*3*). For clarity the *J* spectra resulting from projection of the two peripheral and the two central multiplets of the 2D*J* spectrum are also plotted separately on the extreme left side. In the weak coupling case only the peripheral multiplets have nonzero intensities. In the presence of strong coupling the peripheral multiplets decrease and the central multiplets increase in intensity. To determine the NMR parameters the central and the peripheral lines give equivalent information. Some properties of weakly coupled and strongly coupled 2D*J* spectra are compared in Table 1.

The different projections in Fig. 2 may be helpful in correlating corresponding features in 1D and 2D*J* spectra. From the projections of the two peripheral multiplets one can extract the apparent chemical shifts, δ^*, and the apparent coupling constants, J^*, to be used as starting parameters for the 2D*J* simulation program. If only the resonance positions and not the intensities were considered the NMR parameters for the 2D*J* spectrum could also be obtained from a fit of the projections with a LAOCOON program for 1D spectrum simulations.

In Figs. 3 to 6 experimental ^{1}H NMR spectra of L-serine are shown to illustrate advantages and limitations of the presently readily available modes of representation of strongly coupled 2D*J* spectra. The three-dimensional view of the 2D*J* spectrum in Fig. 3C provides a survey of the spectrum. The resonances of the α proton are at 3.857 ppm, and those of the β-methylene protons between 3.95 and 4.01 ppm. Accurate measurements of the chemical shifts and spin–spin coupling constants are obtained from the projections (Fig. 3B) and the cross sections (Fig. 4), respectively. Comparison with the scheme of Fig. 2 shows that the small chemical shift difference between the two groups of four peaks in the central part of the spectrum is not resolved in these presentations. The cross section at 3.979 ppm (Fig. 4) was taken at the position which corresponds to the average chemical shift for these lines. In the contour plot of the (δ,J) representation of the 2D*J* spectrum (Fig. 5) the different chemical shifts in the central part of the β-proton resonances can be distinguished, in particular when comparing the experiment with the simulated spectrum.

Additional strong coupling features, not shown in Fig. 2, appear in the spectral region between the α- and β-proton resonances and in the α-proton multiplet. The weak lines at around 3.92 ppm (Figs. 3C and 5) result from the strong coupling between the α proton and the two β protons. The small intensity of the two center lines in the α-proton multiplet at 3.857 ppm (Fig. 4) and the two extra peaks

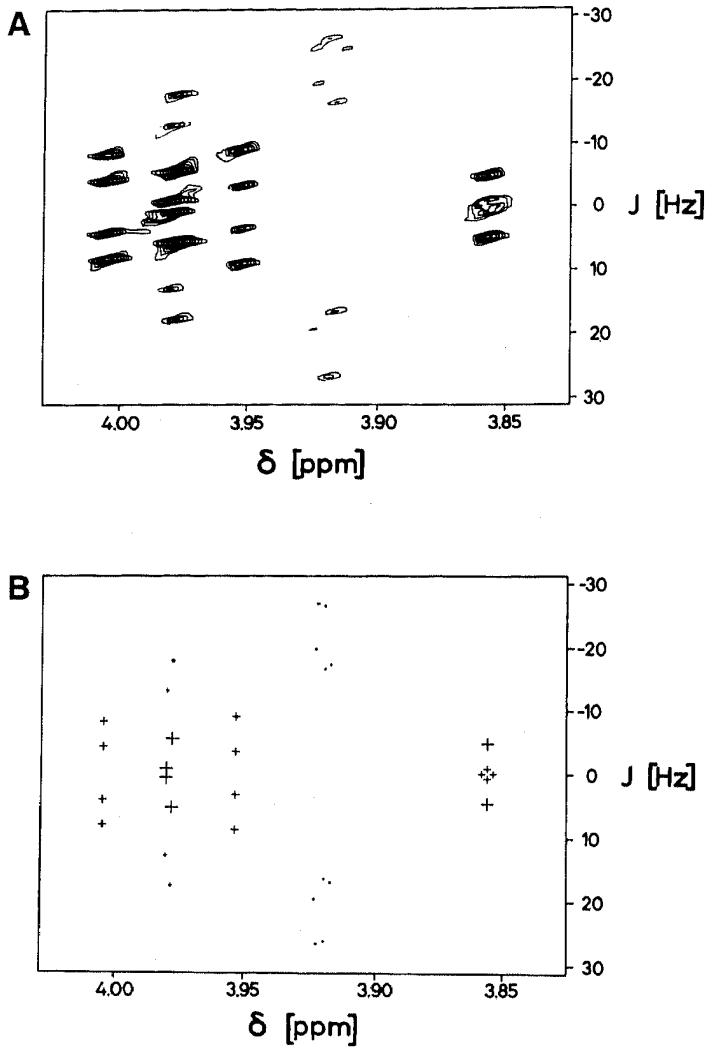


FIG. 5. (A) Contour plot of the 2DJ ^{1}H NMR spectrum of L-serine in Fig. 3C. (B) Simulated 2DJ spectrum of L-serine. Each peak is represented by a cross. The resonance intensity is indicated by the size of the cross.

on the $J = 0$ line (Figs. 5 and 6) are due to the strong coupling between the two β protons. Theoretically the X part of the 2DJ spectrum of an ABX spin system may contain up to 18 resonance lines (*12*, *13*). In our measurements we could never see more than six peaks. This was not unexpected, since the intensities of the additional peaks are at least two orders of magnitude smaller (*12*, *13*).

Strong Coupling Features in the 360-MHz 2DJ ^{1}H NMR Spectra of the Common Amino Acids

To establish a basis for the analysis of protein spectra we measured the 2DJ ^{1}H NMR spectra of the common amino acids. For practical reasons free amino acids were measured. With regard to studies of peptides and proteins the spectra of nonterminal amino acid residues in peptide chains, which may be somewhat different from those of the free amino acids, would, however, be of more direct

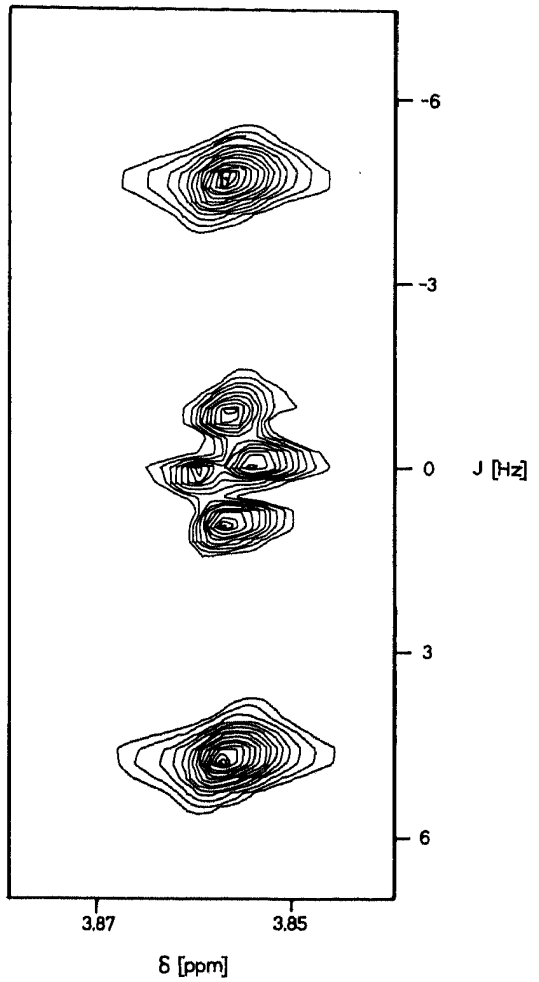
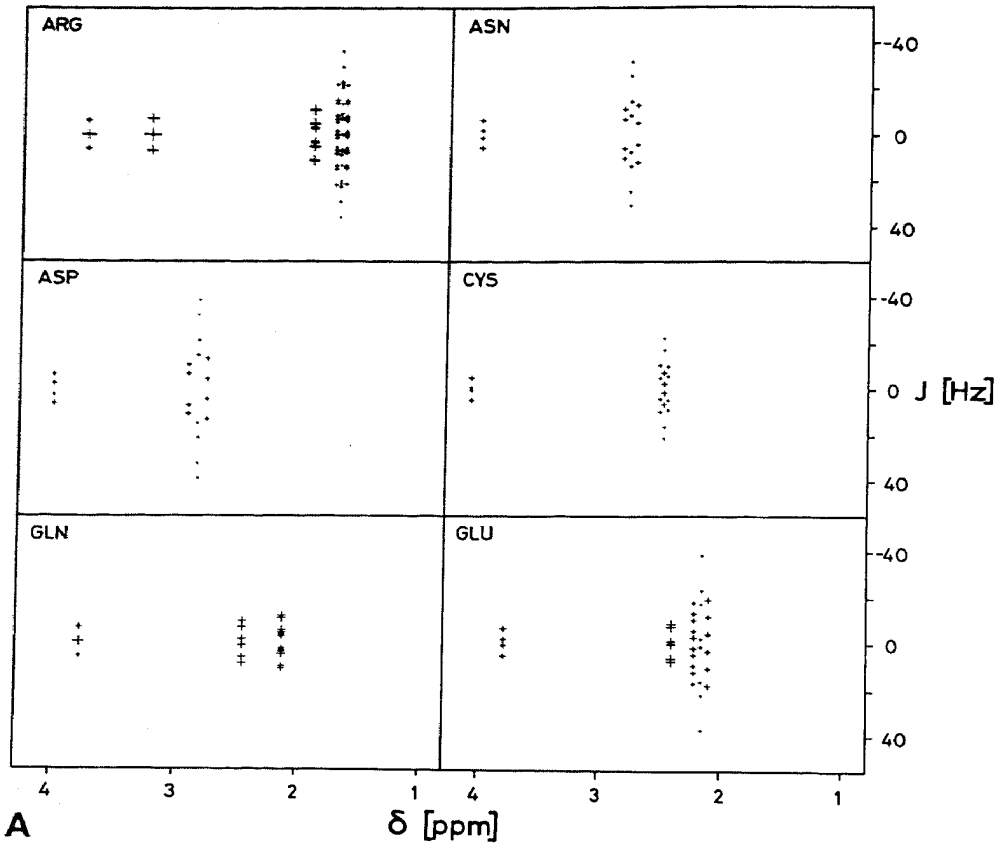


FIG. 6. Contour plot of the α-proton multiplet in the L-serine ^{1}H NMR spectrum of Fig. 3C on an expanded scale.

use (*1*, *14*). We computed schematic 2D*J* spectra with the parameters measured previously for the amino acid residues in linear tetrapeptides (*7*). For proline the parameters published by Pogliani *et al.* (*15*) were used. For all the amino acids the qualitative features of these computed spectra coincided with those measured for the amino acids. Fig. 7 shows the spectra of 16 amino acids; Gly, Ala, Val, and Thr are not shown since they do not show any strong coupling features.

Inspection of Fig. 7 shows that the typical features of an ABX spin system (Figs. 2 to 6) appear also in the spectra of more complex spin systems. Quite generally a 2D*J* pattern similar to that of the AB part of an ABX system is observed for each pair of strongly coupled protons. As in the ABX system there are two peripheral multiplets, yet there may be more than two central multiplets. These were usually not resolved in the experimental spectra. Except for the AA'BB'C spin system of the aromatic protons of phenylalanine, the amino acids contain usually only pairs of strongly coupled protons. The spectra can therefore be analyzed with the ideas developed with the ABX spin system.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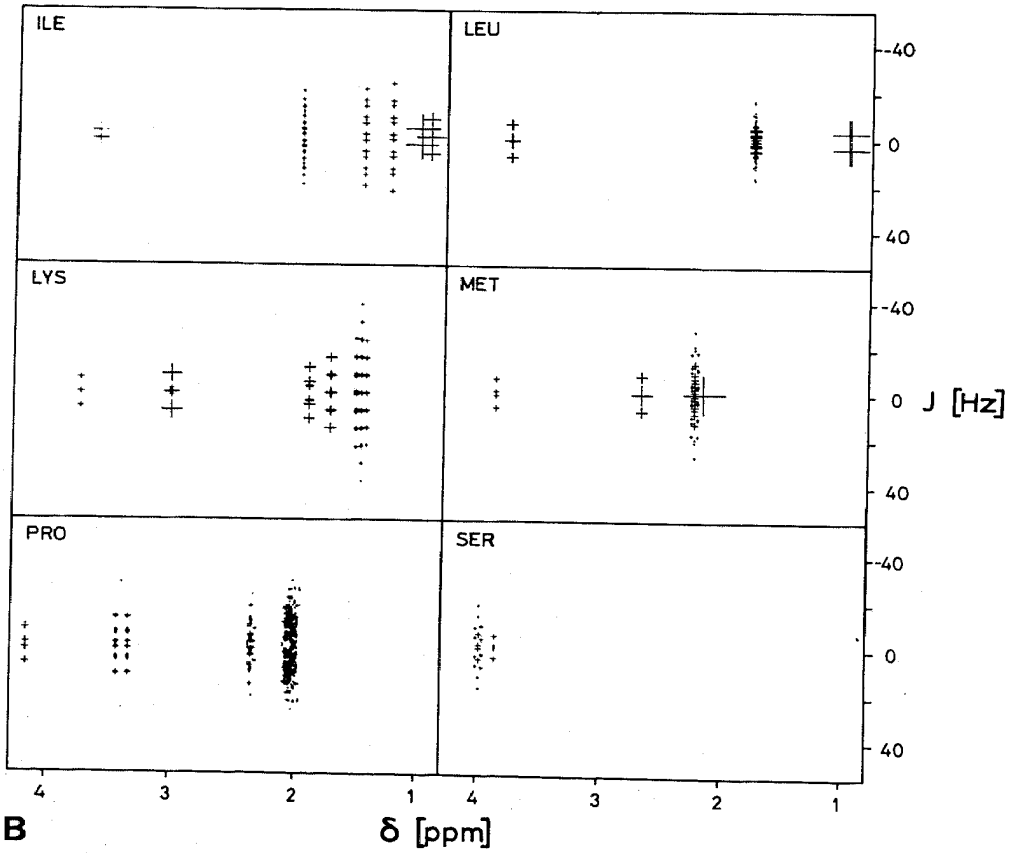
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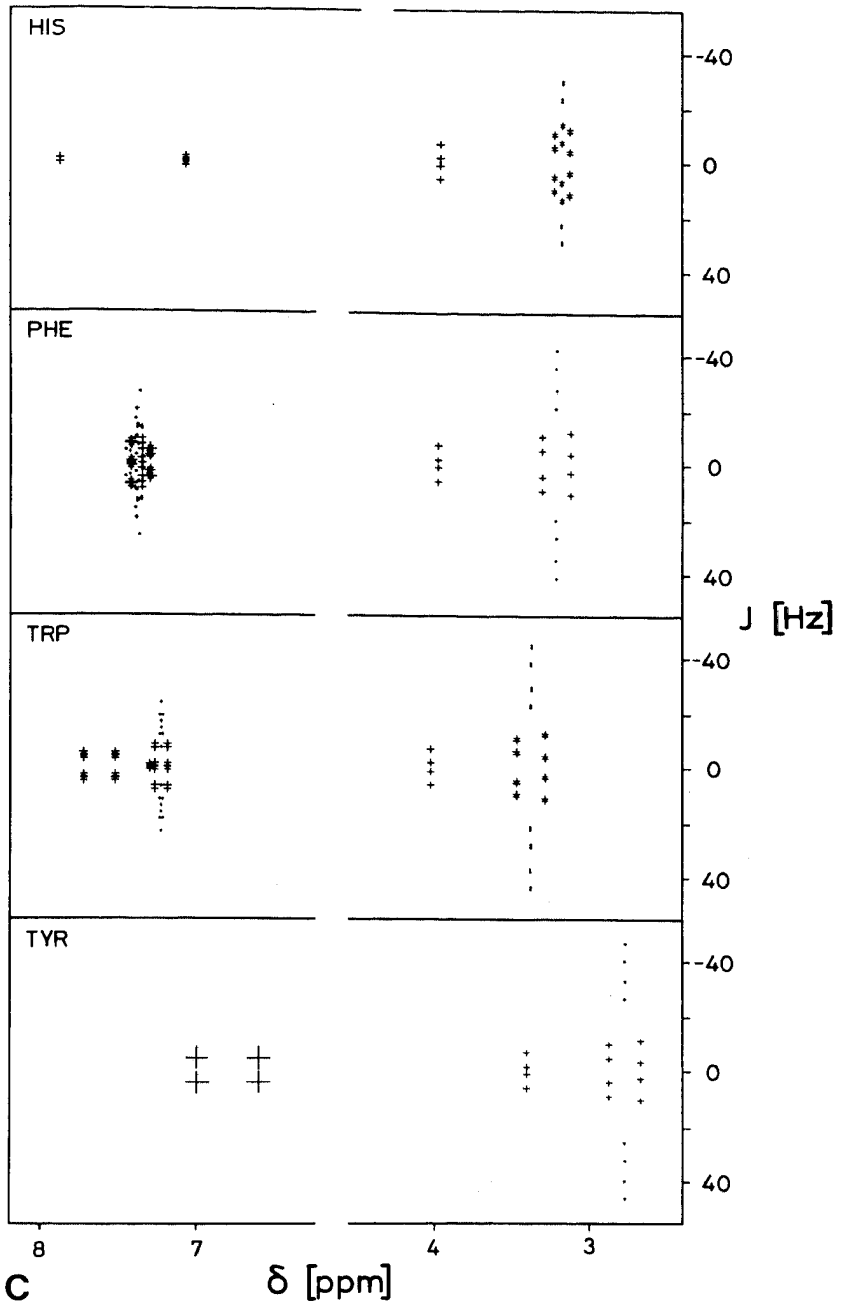

FIG. 7. Schemes of the 360-MHz 2DJ ^{1}H NMR spectra of the 16 common amino acids which give rise to strong coupling features. Not shown are Gly, Ala, Val, and Thr. The plots correspond to that of Fig. 5B; i.e., each resonance peak is represented by a cross and the intensity is indicated by the size of the cross.

Artifacts from Folding Back in 2DJ Spectra

Figure 7 indicates that strong coupling patterns in the 2DJ ^{1}H NMR spectra may cover a much larger frequency range along the J axis than one would expect with the assumption of weak coupling. This is shown in Fig. 8A for the β-methylene protons of L-tyrosine. When the same spectrum was recorded with a smaller spectral range along ω_1 the outermost lines were folded back into the central

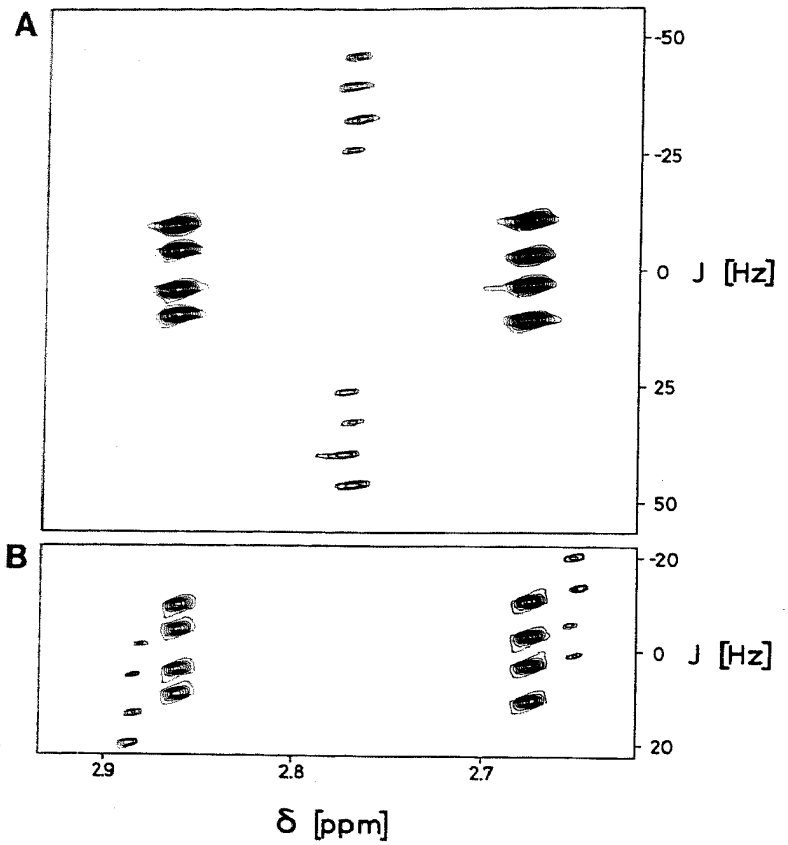

FIG. 8. Contour plot of the (δ,J) representation of the 360-MHz 2DJ ^{1}H NMR spectrum of the β-methylene protons of L-tyrosine. An 0.25 M solution of the amino acid in D_2O was measured at pD 11.6 and T = 35°C. (A) Spectrum recorded with a spectral range of ±56.2 Hz in the ω_1 direction. (B) Spectrum recorded with an ω_1 range of ±22.5 Hz. The strong coupling lines at about 2.77 ppm (A) are folded back into positions at about 2.65 ppm and 2.89 ppm.

spectral region. In the (δ,J) representation of the spectrum this gives rise to artifactual lines at quite unexpected positions (Fig. 8B).

In the practice of 2DJ spectroscopy, easy recognition of backfolding patterns may be of considerable interest (see below). Figure 9 shows schemes of the common backfolding patterns in the (ω_1,ω_2) and (δ,J) representations of homonuclear 2DJ spectra. Details are given in the figure caption.

DISCUSSION

Fundamental aspects of strong coupling in 2DJ spectra were previously discussed (*2*, *12*, *13*). Here we were primarily interested in the practical consequences for the analysis of 2DJ spectra of proteins. When 2DJ ^{1}H NMR spectroscopy is applied to studies of the crowded spectra of proteins, advantages over conventional one-dimensional experiments include the fact that for weakly coupled spin systems well-resolved multiplet patterns, and hence accurate values for the spin–spin coupling constants, can be obtained (*5*, *6*). When the J connectivities have been determined, e.g., with selective spin decoupling in the 2DJ ^{1}H NMR spectra (*16*, *17*) or, more efficiently, with the use of correlated spectroscopy (*18*),

a complete characterization of the spin systems of individual amino acid residues can thus be obtained. The data on individual amino acids presented in this paper show that, in the presence of strong coupling, complex resonance patterns appear in the 2D*J* spectra, which may be difficult to resolve and require particular care to analyze in the spectrum of a protein. This clearly illustrates that the spectral resolution can be optimized only by the *combined* use of high-field and 2D*J* spec-

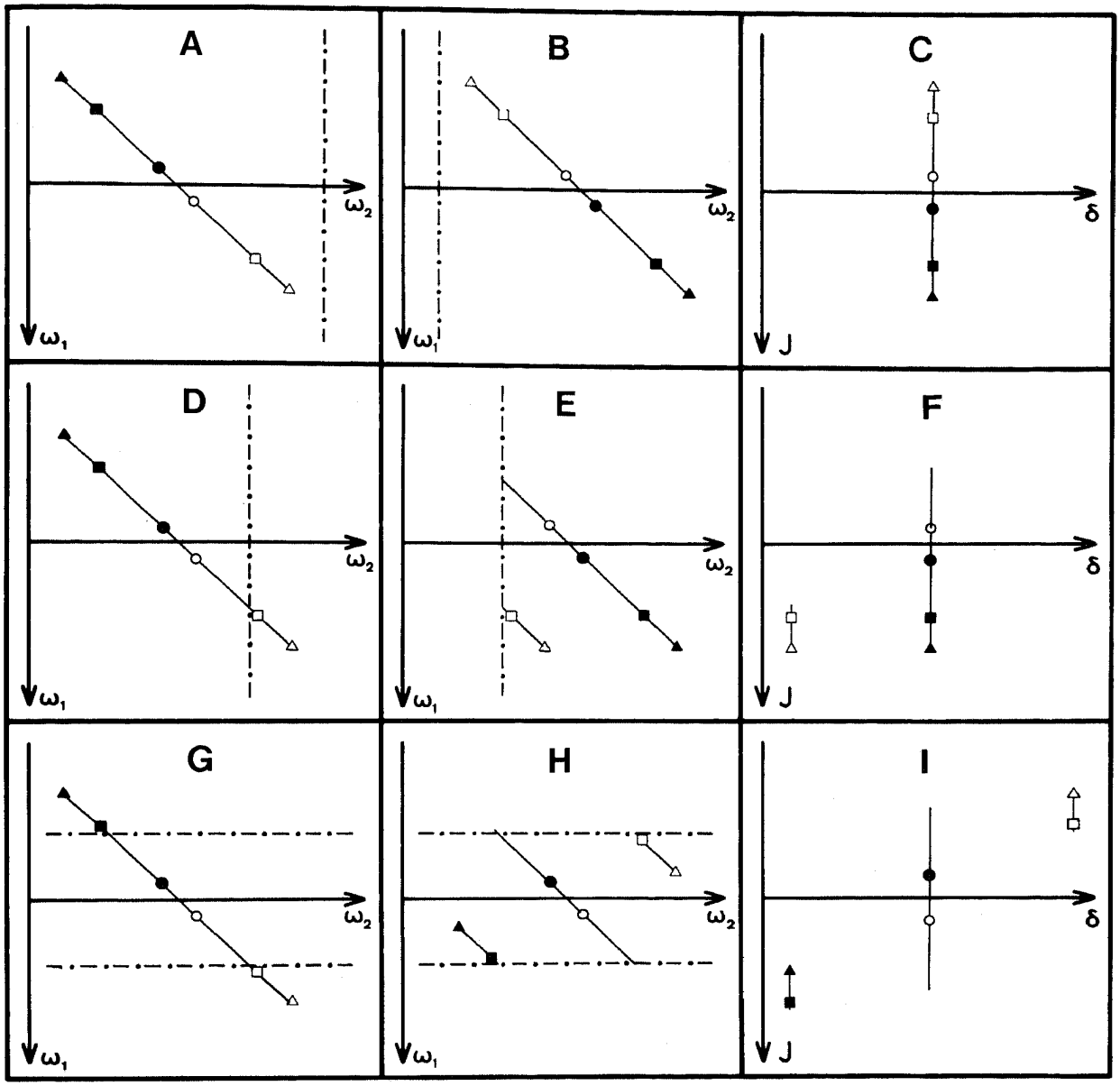

FIG. 9. Schematic illustration of folding-back patterns in 2D*J* spectra. A multiplet pattern with six components, ▲, ■, ●, ○, □, and △ is shown. (A) The top row of figures shows backfolding along ω_2 about a frequency outside the ω_2 range covered by the multiplet (– · – in A) in the (ω_1,ω_2) representation (B) and the (δ,J) representation (C). The center row shows backfolding along ω_2 about a frequency within the ω_2 range of the multiplet (D to F). The bottom row shows folding back with respect to ω_1 about a frequency within the ω_1 range covered by the multiplet (G) in the (ω_1,ω_2) (H) and the (δ,J) representation (I).

troscopy. However, 2DJ spectroscopy cannot replace the use of high fields. The amino acid spectra further indicate that much care must be used when analyzing cross sections through strongly coupled 2DJ ^{1}H NMR spectra in terms of first-order patterns.

For amino acid residues located in interior parts of a globular protein conformation-dependent chemical shifts caused by interactions with the local magnetic fields of neighboring groups (*14*) can greatly affect the ^{1}H NMR. Hence a residue giving rise to a strongly coupled spectrum in a random coil peptide may give a first-order pattern in a protein and vice versa. The spectra of the free amino acids (Fig. 7) thus provide only a rough guideline of the spectral patterns to be expected in proteins. This appears sufficient, however, to distinguish between spectral regions with predominantly weak or predominantly strong coupling. At 360 MHz weak coupling patterns are expected for the methyl resonances (and possibly additional high-field shifted lines) at high field from about 1.6 ppm and for the α-proton resonances between about 3.5 and 6.0 ppm (*1*, *7*, *14*). Both strong and weak coupling are expected for methylene groups between about 1.5 and 3.5 ppm and for the aromatic region (Fig. 7). For work with proteins the combined use of correlated spectroscopy (*18*) to delineate J connectivities and 2DJ spectroscopy to determine the multiplicities and measure the spin–spin coupling constants for the weakly coupled peripheral groups of protons appears to be a successful strategy for characterization of the spin systems of numerous amino acid residues (manuscript in preparation).

The strong coupling patterns cover a much larger ω_1 frequency range than what one would estimate with the assumption of weak coupling (Fig. 7). This makes the selection of an optimal ω_1 frequency range in a 2DJ experiment rather tricky. If the frequency range is chosen sufficiently wide to accomodate the strong coupling multiplets (Fig. 7) one will either have to work with low digital resolution along ω_1 or use a very large data matrix which requires exceedingly long time periods for data acquisition and Fourier transformation. If one chooses to work with a small frequency range adjusted to the multiplets expected in first order the spectral analysis will be complicated by the appearance of backfolded resonance lines (Figs. 8 and 9). As a workable compromise recording of two spectra with wide and narrow ω_1 frequency ranges and without and with backfolding, respectively, is suggested.

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