Torsion Angle Determination in Solid ^{13}C-Labeled Amino Acids and Peptides by Separated-Local-Field Double-Quantum NMR

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Abstract: A novel two-dimensional double-quantum NMR technique for determining the torsion angle ψ in doubly-^{13}C-labeled amino acid residues of solid peptides is presented. The intensity pattern in the two-dimensional NMR spectrum reflects the orientation of the $C_\alpha-H$ bond with respect to the carbonyl moiety, by correlating the $C_\alpha-H$ dipolar coupling with the CO chemical-shift anisotropy. This approach eliminates problems caused by $^{13}C-^{14}N$ dipolar couplings and the relatively small chemical-shift anisotropy of the C_α carbon in two-dimensional double-quantum spectra based only on chemical-shift anisotropies. The double-quantum selection achieves isolated-spin background suppression and increases the spectral resolution by partially removing the inhomogeneous spectral broadening. The experiment is demonstrated on $^{13}C_\alpha-^{13}CO$-labeled leucine. With 50 mg, a useful spectrum was obtained in 3 h. The potential of the technique for distinguishing different secondary structures in peptides is demonstrated by spectral simulations.

Introduction

The determination of segmental conformations in unoriented biological or polymeric solids is a challenging problem of considerable relevance. Nuclear magnetic resonance (NMR) techniques are the most promising and widely used methods for determining the segmental structure in such systems, in terms of internuclear distances,[1−4] relative segmental orientations,[5−10] or chemical shifts.[11,12] A considerable number of such NMR investigations have been aimed at solid peptides and polypeptides[13−15] (including macroscopically ordered systems[16−18]) since these are of biological and medical relevance but often difficult to obtain in the single-crystalline form required for scattering studies. A recently introduced two-dimensional (2D) double-quantum NMR method[19] is particularly promising for torsion-angle determination in solid peptides. Unlike all other NMR techniques in this field, the double-quantum approach does not require isotopic labeling of two different amino acid residues. Instead, it requires only the incorporation of one doubly-^{13}C-labeled amino acid residue into the peptide. This has the crucial advantage that the double label can be introduced biosynthetically, by feeding a suitable auxotroph with the doubly-labeled amino acid. In contrast, the control of two labeled amino acid residues required for REDOR or rotational resonance NMR experiments[1−4] is often impossible in such a biosynthetic process.

However, the double-quantum experiment in its original form is hampered by undesired $^{13}C-^{14}N$ dipolar couplings which are comparable in strength to the chemical-shift anisotropy of the C_α carbon. This paper describes the elimination of the problem by exploiting the large $C_\alpha-H_\alpha$ dipolar coupling instead. The double-quantum selection achieves isolated-spin background suppression and increases the spectral resolution markedly by partially removing inhomogeneous spectral broadening. The potential of the new 2D NMR technique for torsion-angle determination is demonstrated by experimental spectra of an amino acid and by spectral simulations for various peptide conformations.

Experimental Section

NMR Measurements. The experiments were performed on a Bruker MSL 300 NMR spectrometer in a Bruker double-resonance NMR probehead in a 7-T field (^{13}C at 75.74 MHz). The 90° pulse lengths were ca. 3.7 μs, the recycle delay was 1.8 s, and the cross-polarization time was 1 ms. The ^{1}H and ^{13}C radio-frequency field strengths were $\gamma\mathbf{B}_1/2\pi = 68$ kHz. Since the ^{13}C frequency was set in the middle of the spectrum which extends over a range of ±10 kHz,

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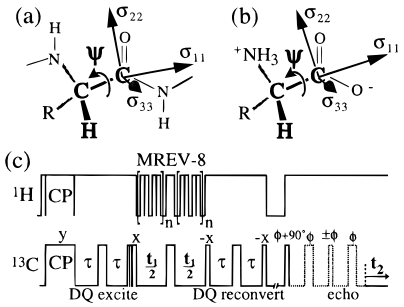

Figure 1. (a) Doubly-^{13}C-labeled amino acid residue in a peptide and (b) doubly-^{13}C-labeled amino acid. The torsion angle ψ determines the orientation of the $C_\alpha-H$ bond relative to the principal-axes system of the carbonyl chemical-shift tensor. Note that the conformation shown in (b) differs by 180° from that found in leucine crystals, where the shorter C═O bond is approximately trans with the C−N bond. (c) NMR pulse sequence for the SELFIDOQ experiment, with double-quantum (DQ) generation (chemical shift refocused by the 180° pulse), $^{13}C-^{13}C$ DQ evolution under $^{13}C-^{1}H$ dipolar couplings, DQ reconversion, and ^{13}C detection under ^{13}C chemical shift and $^{13}C-^{13}C$ dipolar coupling. The two 45° pulses at the end of the DQ generation period, of phases y and $\pm y$, eliminate spectral artifacts at $\omega_1 = 0$.

^{13}C pulse-length effects were quite negligible. In the SELFIDOQ experiment, one double-quantum excitation delay of $2\tau = 140$ μs and eight t_1-increments of full semiwindowless MREV-8 cycles were used. The measuring time was 12 h on 170 mg of sample, and 3 h on 50 mg, in the experimental spectra shown below. The sample was $^{13}C_\alpha-^{13}$CO-labeled L-leucine ($NH_3^+-^{13}CHR-^{13}COO^-$, R = $CH_2CH(CH_3)_2$) purchased from Isotec Inc.

Simulation of 2D SELFIDOQ Spectra. The theoretical 2D spectra were calculated directly in the frequency domain, by scanning the $\mathbf{B}_0$ field direction over all orientations. For each orientation and the given torsion angle, the FORTRAN program calculates the $C_\alpha-H_\alpha$ coupling (ω_1 dimension) and the anisotropic chemical shift of the carbonyl as well as the ^{13} $C_\alpha-^{13}$COO dipolar coupling (ω_2 dimension). The double-quantum generation as well as the dipolar/chemical-shift frequencies and intensities in the detection period were calculated according to the exact formulas,[20] but due to the large (~9 kHz) chemical-shift difference between the two ^{13}C sites, the much simpler weak-coupling limit would have provided a good approximation.[19] Realistic line broadening was generated by convolution with suitable Gaussians (full width at half-maximum of 6 kHz in ω_1 and 1 kHz in ω_2). On a Power Macintosh 7100/66, the spectral simulations, with an angular resolution of 1°, required less than 30 s per spectrum. NMR parameters required as input in the program are the C−C and C−H bond lengths and the intervening bond angle, and the carbonyl chemical-shift tensor orientation. Further details are given in the text and the figure captions below.

Results and Discussion

Figure 1a displays a doubly-^{13}C-labeled amino acid residue in a peptide, Figure 1b an amino acid. The orientation of the principal-axes system (PAS)[21−23] of the chemical shift tensor of the carbonyl site[21,24] is indicated. The figure shows that the torsion angle ψ can be determined from the orientation of the C−H bond relative to the chemical-shift PAS of the carbonyl group. In solid-state NMR, the orientation of the C−H bond with respect to the external magnetic field $\mathbf{B}_0$ can be measured via the $C_\alpha-H_\alpha$ dipolar splitting, while the chemical shift frequency reflects the orientation of its PAS with respect to $\mathbf{B}_0$.[21−23] The relative orientation of C−H and C═O can thus be determined by correlating the $C_\alpha-H_\alpha$ dipolar coupling, along the first frequency dimension ω_1, and the chemical-shift anisotropy of the carbonyl site, along the second dimension ω_2, in a 2D NMR spectrum. Such a correlation is achieved in a separated-local-field[25,26] double-quantum (SELFIDOQ) NMR experiment, using the pulse sequence of Figure 1c. After $^{1}H-^{13}C$ cross-polarization, a $^{13}C_\alpha-^{13}$CO double-quantum state[19,20,27−29] is generated by the $^{13}C-^{13}C$ dipolar coupling during a period 2τ followed by a 90° pulse. The double-quantum coherence evolves during t_1 under MREV-8 multiple-pulse homonuclear proton decoupling[22,23,30] which retains the C−H couplings scaled by 0.5. The 180° pulses in the center of the t_1 period refocus ^{13}C chemical shifts and the undesired $^{13}C-^{14}N$ and $^{1}H-^{14}N$ dipolar couplings. The $^{13}C-^{13}C$ dipolar coupling does not affect the double-quantum state.[19,28] The double-quantum coherence modulated only by the C−H coupling is then reconverted into transverse magnetization in both the carbonyl and C_α sites, which is detected during t_2 as it evolves under the respective chemical shift and the $^{13}C-^{13}C$ dipolar coupling. The carbonyl signal is thus modulated by the evolution of the double-quantum coherence under the $C_\alpha-H_\alpha$ dipolar coupling. After a complex Fourier transformation over t_2 and a real Fourier transformation over t_1, a 2D spectrum correlating the $C_\alpha-H_\alpha$ dipolar splitting with the anisotropic carbonyl chemical shift is obtained.

Since the double-quantum generation and reconversion modulates the signal as $\sin^2(\omega_{C-C}2\tau)$,[29] it enhances and suppresses signals depending on their $^{13}C-^{13}C$ dipole couplings ω_{C-C}. Working with a single value of 2τ thus removes some of the inhomogeneous broadening in the 2D powder spectrum and enhances the spectral resolution. This spectral selection serves the same purpose as would a third spectral dimension that separates the signals by their $^{13}C-^{13}C$ dipolar couplings. In other words, the 2D spectrum with the double-quantum selection for one value of 2τ can be viewed as a slice through a 3D spectrum correlating C−H coupling, the ^{13}CO chemical shift, and $^{13}C-^{13}C$ dipolar coupling, albeit with low resolution in the third dimension.

The experimental spectrum obtained with this SELFIDOQ technique for doubly-^{13}C-labeled L-leucine is shown in Figure 2a. Figure 2b shows the simulation based on the X-ray crystal structure,[31] which shows an asymmetric unit containing two molecules, with $\psi = -26°$ and $\psi = -36°$. For comparison, parts c and d of Figure 2 show the SELFIDOQ patterns for other torsion angles, with significantly changed ridge patterns and spectral splittings along ω_1.

In order to demonstrate the potential of the experiment for distinguishing various peptide conformations, Figure 3 presents the SELFIDOQ peptide spectra for four values of ψ, which correspond to common conformations in proteins. The torsion angle is the most relevant parameter in the simulations, while line-broadening is of lesser importance. Other parameters such as bond lengths, bond angles, and the CO chemical-shift tensor are well known[21,24,31] and can also be determined by auxiliary 2D experiments on the same sample.[32] The orientation of the C−H bond with respect to the three principal axes of the

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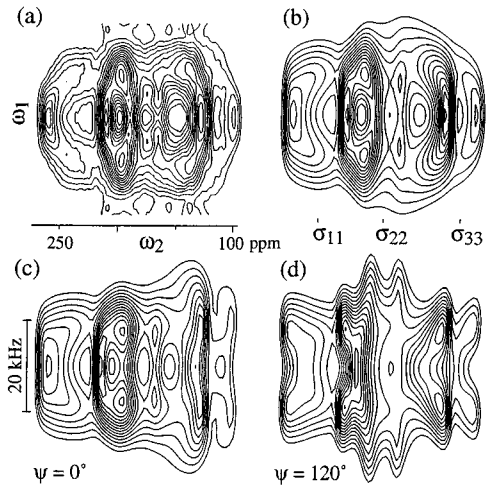

Figure 2. (a) Experimental SELFIDOQ spectrum of doubly-^{13}C-labeled L-leucine. Only the carbonyl chemical-shift region is shown, since the C_α pattern is independent of ψ. (b) Corresponding simulation based on the crystal structure of leucine, which exhibits two molecules with different conformations, $\psi = -26°$ and $\psi = -36°$. Simulations demonstrating some of the possible spectral variations: (c) for a planar conformation with $\psi = 0°$, (d) for $\psi = 120°$. An angle of 9° between the σ_{11} principal axis and the C−C bond was used in the simulations, as determined in auxiliary 2D experiments.

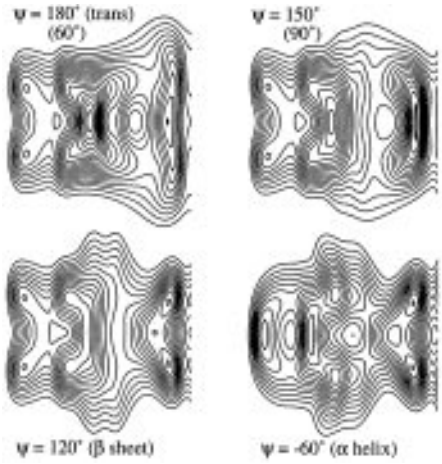

Figure 3. Simulated SELFIDOQ spectra (carbonyl region) for a general amino acid residue (except glycine) in a peptide, for different backbone conformations (trans, β-sheet, α-helix) with torsion angles ψ as indicated. As discussed in the text, $\psi = 180°$ and $\psi = 60°$ produce the same spectral pattern, as do $\psi = 150°$ and $\psi = 90°$. Strong line broadening (6 kHz in ω_1, 1 kHz in ω_2) was applied to produce realistic spectra.

carbonyl chemical-shift tensor, and thus the torsion angle ψ, can be estimated directly by inspection of the splittings in the ω_1 dimension. This is particularly simple for the intensity maxima in the ω_2 dimension, which correspond to $\mathbf{B}_0$ field orientations along the principal axes of the carbonyl chemical-shift tensor. More quantitatively, from a set of simulated spectra covering the ψ range of 180° in steps of 5°, the correct value of ψ can be determined simply by finding the best fit to the experimental spectrum visually or numerically. The accuracy with typical spectral broadenings is estimated to be ±10°, which would compare favorably with the angular resolution achieved in recent rotational-resonance NMR studies of a 10-residue peptide.[13]

For symmetry reasons,[19] the ψ determination from SELFIDOQ 2D patterns has an ambiguity. Pairs of angles $\psi = -60° \pm \psi'$ (or equivalently $\psi = 120° \pm \psi''$), e.g., $\psi = -50°$ and $\psi = -70°$, produce the same spectrum because for the two ψ values the $C_\alpha-H_\alpha$ bond makes the same angle with the CCOO plane and thus with the principal axes of the carbonyl chemical-shift tensor. This ambiguity can be resolved by chemical-shift double-quantum spectroscopy (DOQSY) patterns[19] for the same sample. The symmetry of the C_α chemical-shift tensor which is relevant for the DOQSY spectra results in spectral equivalence for $\pm\psi$. Therefore, the DOQSY 2D patterns for the two angles $\psi = -60° \pm \psi'$ are clearly distinct, even in the presence of the ^{14}N−^{13}C dipolar broadenings. In the case of leucine, where the SELFIDOQ pattern of Figure 2a yields $\psi \approx -60° \pm 30°$, the experimental DOQSY pattern[32] resembles the simulation for $\psi \approx -30°$, but deviates strongly from that for $\psi \approx -90°$.

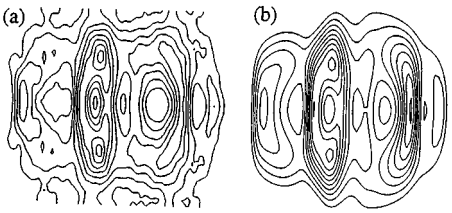

Figure 4. Signal-to-noise demonstration: (a) SELFIDOQ spectrum similar to Figure 2a, but with 50 mg of leucine, an acquisition time of 3 h, and slightly stronger spectral smoothing. (b) Simulation with parameters as in Figure 2b except for smoothing and fewer contour levels.

The size of peptides accessible to the SELFIDOQ NMR is limited only by the sensitivity. Spectral overlap is no problem since the natural-abundance signal is suppressed by the double-quantum selection, and overlap of the carbonyl and C_α powder patterns is minimal. To test the sensitivity, Figure 4 shows the SELFIDOQ spectrum for 50 mg of the labeled leucine sample, acquired in 3 h. Considering that signal averaging over many days is common in biological solid-state NMR, in a 48-h measurement on a 300-mg sample a similar spectrum of 1 residue in 25 can be observed, corresponding to a molecular weight of the peptide, or of the repeat unit in a polypeptide, of ca. 3000. Various interesting linear and cyclic peptides as well as polypeptides fulfilling these size constraints either have been investigated with only relatively low resolution[13,18] or still await detailed structural studies.[33] Applications of the SELFIDOQ technique to biosynthetic polypeptides are currently being pursued in our laboratory.

Conclusions

SELFIDOQ NMR is a promising new tool for structure elucidation in solid peptides and polypeptides. It determines the backbone torsion angle ψ by correlating the $C_\alpha-H_\alpha$ bond direction with the carbonyl tensor orientation in a two-dimensional NMR spectrum. The required $^{13}C_\alpha-^{13}CO$ spin pair can be introduced in a simple way, e.g., biosynthetically, by means of a single doubly-labeled amino acid residue. The analysis of the spectral SELFIDOQ patterns is straightforward, with the torsion angle being the only relevant parameter. Within the size limitation of MW = 3000 for the repeating structural unit, various cyclic and linear peptides as well as biological or biosynthetic polypeptides can be investigated.

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