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Three-dimensional structure determination of a uniformly labeled molecule by frequency-selective dipolar recoupling under magic-angle spinning

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Abstract

The complete three-dimensional (3D) structure of a glycylisoleucine (Gly-Ile) molecule was determined by individually measuring six dihedral angles with a frequency-selective homonuclear dipolar recoupling method, R2TR (rotational resonance in the tilted rotating frame), using a powder sample of diluted uniformly ^{13}C,^{15}N-labeled Gly-Ile. Each dihedral angle was obtained by recoupling a dipolar interaction between three or four bonds distant spins concerned or observing a dipolar correlation 2D powder pattern. The 3D structure of a Gly-Ile molecule was also determined by X-ray crystallography, and a good agreement with the NMR result was obtained. The results demonstrate that the R2TR method in a uniformly labeled powder sample can provide the 3D structure without the need to prepare a lot of selectively labeled samples.

Introduction

Solid state NMR may provide important structural information without requiring long-range order nor solubility. In particular, measurements of dipolar interactions provide direct information on structures. With the aim of applying mainly to biological molecules, a lot of methods for dipolar recoupling under magic angle spinning (MAS) have been developed. While the non-selective measurements of dipolar couplings by broadband recoupling methods do not currently provide any quantitative structural information, selective measurements are practical and useful for obtaining local geometrical structures. In the latter, usually only one specific internuclear distance or dihedral angle is determined using a selectively isotope-labeled powder sample. A large number of techniques for doubly labeled samples have been developed (Raleigh et al., 1988; Gullion and Schaefer, 1989; Tycko and Dabbagh, 1990; Bennett et al., 1992; Gullion and Vega, 1992; Fujiwara et al., 1993; Ishii and Terao, 1995; Lee et al., 1995; Sun et al., 1995; Takegoshi et al., 1996). However, if one tries to determine the three-dimensional (3D) structure composed of sizeable atoms by such approaches, many labeled samples must be prepared (Nishimura et al., 1998). On the other hand, if dipolar recoupling can be frequency-selectively realized, it allows us to recouple a specific dipolar interaction even in a uniformly labeled sample. Therefore, frequency-selective recoupling should have a potential for obtaining all the structural parameters necessary to determine the complete molecular structure one by one using a single sample, which significantly reduces experimental labor for preparing isotope-labeled samples.

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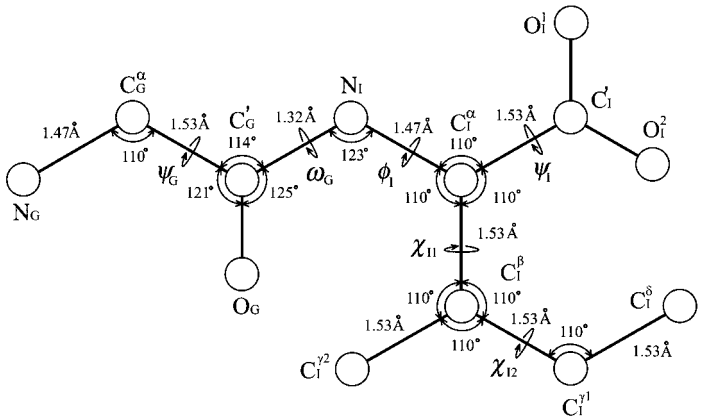

Figure 1. Schematic representation of a glycylisoleucine molecule. Typical values of the bond distances (in Å) and the bond angles (in degrees) are shown, which were used to simulate experimental results and construct the three-dimensional structure of glycylisoleucine. The glycine carbonyl and C_α carbons are labeled as C'_G and C^α_G, and the isoleucine carboxyl, C_α, C_β, $C_{\gamma 1}$, $C_{\gamma 2}$, and C_δ carbons are as C'_I, C^α_I, C^β_I, $C^{\gamma 1}_I$, $C^{\gamma 2}_I$, and C^δ_I, respectively. The glycine amine and the isoleucine amide nitrogens are labeled as N_G and N_I, respectively, and the glycine carbonyl oxygen as O_G, and the two isoleucine carboxyl oxygens as O^1_I and O^2_I. The dihedral angles ϕ_I, ω_G, ψ_G, ψ_I, χ_{I1}, and χ_{I2} are also shown.

Rotational resonance (R^2) (Andrew et al., 1963, 1966; Raleigh et al., 1987, 1988; Levitt et al., 1990) is a very simple and useful technique for selectively recoupling a particular homonuclear dipolar interaction which has been decoupled by MAS. Recoupling occurs when the spinning frequency ω_R is adjusted to one n-th the resonance frequency difference. However, R^2 is not a good means for selective recoupling in a uniformly labeled sample. When the chemical shift difference is small, the spinning speed must be slow. Not only does this yield many crowded spinning sidebands, but the other dipolar couplings cannot be sufficiently decoupled by such slow MAS. Furthermore, R^2 is not frequency selective, but frequency-difference selective; therefore, if more than one carbon pair with the same separation exists, they are simultaneously recoupled. We extended R^2 by applying an rf field with an intensity ω_1 and a frequency ω_0, which we call R2TR (rotational resonance in the tilted rotating frame) (Takegoshi et al., 1995, 1997). Then, rotational resonance occurs in the tilted rotating frame under a zero-quantum (ZQ) condition ($\omega_{eI} - \omega_{eS} = n\omega_R$), a single-quantum (SQ) condition ($\omega_{eI} = n\omega_R$ or $\omega_{eS} = n\omega_R$), or a double-quantum (DQ) condition ($\omega_{eI} + \omega_{eS} = n\omega_R$), where ω_{eX} is the effective field intensity for the X spin, with three experimentally adjustable parameters, ω_1, ω_R, and ω_0 (Takegoshi et al., 1995). This diversity makes it possible, for example, to recouple a pair of spins with a small chemical shift difference while spinning fast enough to decouple all the other dipolar interactions. Furthermore, the R2TR method can selectively recover a particular dipolar coupling by properly choosing the experimental parameters, even when more than two lines have equal chemical-shift differences.

In the present study, we have determined the complete structure of a glycylisoleucine (Gly-Ile) molecule by R2TR using a powder sample of uniformly ^{13}C,^{15}N-labeled Gly-Ile molecules, including all the positions of carbons, nitrogens, and oxygens in both main chains and side chains. This is the first example of the complete structure determination of a molecule by solid state NMR; a preliminary report of this experiment has been presented (Nomura et al., 1999).

Method of structure determination

We determine the 3D structure of a Gly-Ile molecule by obtaining the six dihedral angles shown in Figure 1. In this study, ψ_G, ω_G, ϕ_I, χ_{I1}, and χ_{I2} are defined as dihedral angles N_G–C^α_G–C'_G–N_I, C^α_G–C'_G–N_I–C^α_I, C'_G–N_I–C^α_I–C'_I, C'_I–C^α_I–C^β_I–$C^{\gamma 1}_I$, and C^α_I–C^β_I–$C^{\gamma 1}_I$–C^δ_I, respectively, by taking zero degrees for the *cis* conformation. ψ_I is defined as the angle between the N_I-C^α_I bond and the O^1_I–C'_I–O^2_I plane, being between 0° and 180°.

Except for χ_{I2}, each dihedral angle is determined by performing the R2TR dipolar recoupling experiment for a proper three- or four-bonds distant spin pair, assuming the typical bond lengths and angles shown in

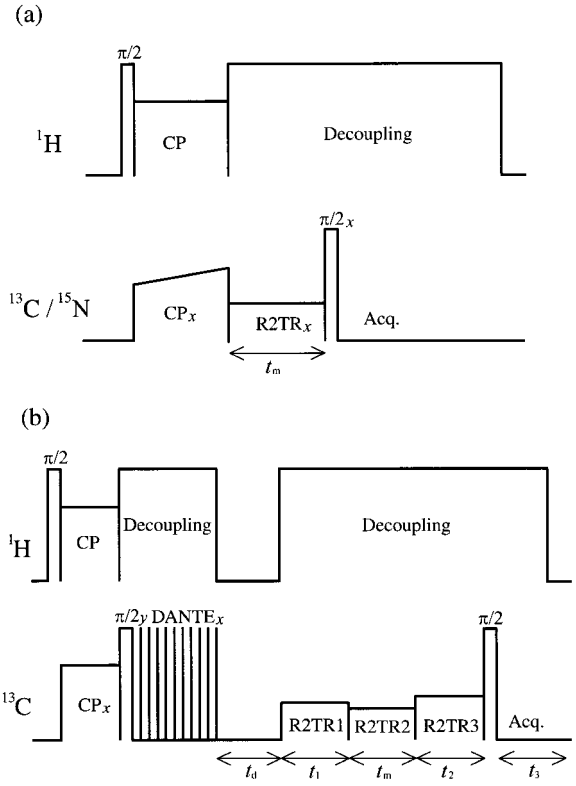

Figure 2. Pulse sequences to determine dihedral angles. (a) Dipolar recoupling experiment. After CP, an rf field satisfying a DQ condition for a particular pair of ^{13}C spins is applied on-resonance to one of the spins for a recoupling time t_m. Then, the transverse magnetization of the spin is observed as a function of t_m. (b) 2D dipolar correlation experiment. After CP, the C magnetization in a four ^{13}C spin (A-B-C-D) system is suppressed by a DANTE pulse train. The A-B and the C-D dipolar interactions are selectively recovered in the t_1 and t_2 periods, respectively. These dipolar powder patterns are correlated by transferring magnetization from A or B to C or D in the mixing period. The AB/CD dipolar correlation spectrum is obtained by observing the C magnetization in the detection period t_3.

Figure 1. We also assumed a planar configuration for the peptide plane.

Figure 2a shows the pulse sequence used in the dipolar recoupling experiments. During the recoupling period, the rf field satisfying a DQ condition is applied on-resonance to one of the pair spins concerned. Then, the recoupling-time (t_m) dependence of the magnetization of the spin is observed, after a 90° pulse is applied to remove the possible small y component of the magnetization. The t_m-dependence of the magnetization is affected by $T_{1\rho}$ relaxation; therefore, we corrected the data, measuring $T_{1\rho}$ as described later.

To determine χ_{I2}, we made a 2D dipolar correlation experiment because of reasons mentioned later. In the R2TR method, we can easily and quickly switch the dipolar interaction to be recoupled from one to another by changing ω_1 and ω_0 under a constant spinning speed. This makes it possible to realize a 2D experiment which correlates the AB and CD dipolar powder patterns in an A-B-C-D ^{13}C spin system. The pulse sequence for this 2D experiment is shown in Figure 2b. After cross polarization (CP), the C magnetization is suppressed by a selective 90° (DANTE) pulse followed by a dephasing period (t_d). In the first evolution period (t_1) the AB dipolar interaction is selectively recoupled, in the mixing period the A or B magnetization is selectively transferred to the C or D spin, and in the second evolution period (t_2) the C magnetization is evolved under the selectively recoupled CD dipolar interaction. The AB/CD dipolar correlated 2D powder pattern is selectively obtained by observing the C spin in the detection period (t_3).

To recouple a specific dipolar interaction, we used a DQ R2TR condition with n=1 ($\omega_{eI} + \omega_{eS} = \omega_R$), except that a ZQ condition is used in the mixing period of the 2D dipolar correlation experiment for efficient polarization transfer. The other conditions of ZQ, SQ, and DQ ($n > 1$) are not suitable for the present experiments for the following reasons. Firstly, to satisfy a ZQ condition, the chemical shift difference between a particular pair of spins must be larger than ω_R. However, chemical shift differences of ^{13}C or ^{15}N spin pairs at a common range of magnetic field intensity are not so large as to satisfy a ZQ condition for ω_R fast enough to completely decouple the other dipolar couplings. Secondly, under an SQ condition, both chemical shift anisotropies (CSAs) of the relevant carbons are dominantly recovered, obscuring the dipolar recoupling effect. Lastly, a DQ condition with $n > 1$ requires a large rf-field intensity for ω_R fast enough to decouple the other dipolar couplings, lowering the selectivity of recoupling.

Numerical simulations were done by a multistep method. Recoupling may take place even in off-R2TR conditions to some extent, and therefore we compensated for the incompleteness of selectivity by including a maximum of three spins into simulations in addition to the relevant two spins. We obtained empirical criteria of spins to be added in simulations by numerous calculations: Let us consider the selective recoupling of A and D in an A-B-C-D ^{13}C system and assume the recoupling-time dependence of the D magnetization to be observed. In this case, not only all the four spins, but also all ^{13}C spins directly bonded to D must be included. Further, a ^{13}C spin within

about 2.5 Å from D should be included if the ω_e difference between the spin and A is less than 1000 Hz. A ^{13}C spin directly bonded to A should be included if the ω_e difference between the spin and D is less than 1000 Hz.

Not only dipolar couplings but also CSA tensors contribute to the results. The ^{13}C and ^{15}N chemical shift parameters used for numerical simulations are collected in Table 1. The CSA tensors for the sp^2 carbon are not very different from peptide to peptide. Hence, we used the mean values of δ and η for the carbonyl carbons in $AcGlyGlyNH_2$, $AcGlyAlaNH_2$, $AcGlyTyrNH_2$, and GlyGly·HCl (Oas et al., 1987a) for C'_G, and those for the carboxyl carbons in glycine (Haberkorn et al., 1981), L-alanine (Naito et al., 1981), L-serine (Naito et al., 1983), L-threonine (Janes et al., 1983), and L-asparagine (Naito et al., 1984) for C'_I. The values for N_G and N_I were determined from spinning sidebands observed at a low MAS speed. The directions of the principal axes of CSA tensors were assumed as follows: For C'_G, the σ_{33} axis is perpendicular to the sp^2 plane (Veeman et al., 1984) and the σ_{22} axis makes an angle of 4.5° with the C'_G-O_G bond toward the C'_G-C^α_G bond (Oas et al., 1987a). For C'_I, the σ_{33} axis is perpendicular to the sp^2 plane and the σ_{11} axis is along the C^α_I-C'_I bond (Veeman et al., 1984). For N_I, the σ_{22} axis is perpendicular to the peptide plane and the σ_{11} axis is along the C'_G-N_I bond (Oas et al., 1987b). For N_G, the σ_{33} direction is along the N_G-C^α_G bond direction and the σ_{11} axis is perpendicular to the N_G-C^α_G-C'_G plane. For the aliphatic carbons, it will be shown that the CSA interactions do not significantly affect simulated results. Hence, we adopted likely values and directions for them.

Experimental

The NMR experiments were performed on Chemagnetics CMX-300 and CMX-400 spectrometers operating at 75.6 MHz and 100.7 MHz, respectively, for ^{13}C with Doty CPMAS probes having a 5 ϕ spinning system. The contact time for cross polarization was 2 ms and the recycling delay time between transients was 4 s. The rf-field strength for TPPM decoupling (Bennett et al., 1995) was 70∼90 kHz and that for CP was 40∼50 kHz. For the dipolar recoupling experiment, a precise adjustment of the rf-field intensity is required so as to accurately fulfill an appropriate R2TR condition for a selected spin pair. We calibrated the rf-field intensity by observing a nutation frequency under MAS using adamantane. The rf-field inhomogeneity was estimated to be 2∼3%, which barely affects the results; we used long caps supplied by Doty Scientific Inc. While the rf-field intensity for recoupling must be very stable in the present experiments, we found that the stability of an rf-field intensity in our spectrometers is insufficient in such low H_1 as used in R2TR experiments. We have solved this problem for a weak pulse by controlling the intensity using a stable attenuater which we added and switching from the final power amplifier to a low power amplifier with high stability.

Uniformly ^{13}C,^{15}N-labeled Gly-Ile was prepared according to the following procedure. Boc-[ul-^{13}C,^{15}N]Gly was prepared from [ul-^{13}C,^{15}N]Gly (130 mg, 1.67 mmol) with di-*tert*-butylcarbonate (Moroder et al., 1976) (250 mg, 84%). [ul-^{13}C,^{15}N]Ile-OMe·HCl was obtained from [ul-^{13}C,^{15}N]Ile (230 mg, 1.67 mmol) with methanol and thionyl chloride (Brenner et al., 1953) (320 mg, quantitative). [ul-^{13}C,^{15}N]Ile-OMe·HCl (320 mg, 1.67 mmol) was neutralized with an equimolar amount of *N*-methylmorpholine and then coupled with Boc-[ul-^{13}C,^{15}N]Gly (250 mg, 1.40 mmol) by the DCC-HOBt method (Könich et al., 1970) (530 mg, quantitative). Boc-[ul-^{13}C,^{15}N]Gly-[ul-^{13}C,^{15}N]Ile-OMe was saponified with methanolic NaOH, followed by deprotection of the Boc group with 4 M HCl in ethyl acetate. The crude [ul-^{13}C,^{15}N]Gly-[ul-^{13}C,^{15}N]Ile was adsorbed on a Dowex 50-X8 column (H^+ form) and eluted with 1.5 M NH_4OH, and finally recrystallized from water (200 mg, 60%).

About 100 mg of a 10% uniformly ^{13}C,^{15}N-labeled sample, obtained by recrystallization of 100% uniformly ^{13}C,^{15}N-labeled Gly-Ile from deionized water with ninefold natural abundance material, was used for NMR measurements. For the 1D-dipolar recoupling experiment (Figure 2a), 16 ^{13}C FIDs or 64 ^{15}N FIDs were accumulated for each t_m. For the 2D correlation experiment (Figure 2b), the 32 t_1 data and the 15 t_2 data were collected after accumulating 16 FIDs for each (t_1, t_2). Total acquisition time was about 10 h.

An X-ray diffraction experiment was done as follows. A colorless prismatic crystal of Gly-Ile having approximate dimensions of 0.70 × 0.40 × 0.20 mm was used. All measurements were made on a Rigaku AFC7R diffractiometer with filtered Cu-K_α radiation. The final conventional *R* factor was 11.8%. We found that Gly-Ile crystallizes in the monoclinic space group $P2_12_12_1$, $Z = 4$, with lattice constants of $a = 6.432$ Å, $b = 30.018$ Å, and $c = 5.522$ Å, and the

Table 1. Chemical shift parameters used for the numerical simulations of the R2TR dipolar recoupling experiments in glycylisoleucine

	C'_G	C'_I	C^{α}_G	C^{α}_I	C^{β}_I	$C^{\gamma 1}_I$	$C^{\gamma 2}_I$	C^{δ}_I	N_I	N_G
δ_{iso}[a] (ppm)	178.9	169.3	64.0	40.3	36.3	26.7	17.0	12.4	120.9	23.1
δ[b] (ppm)	−79.3[c]	−71.0[d]	20.0[e]	−19.7[f]	6.67[g]	−12.3[h]	–	−13.9[i]	103.0[j]	19.5[j]
η[b]	0.82[c]	0.84[d]	0.94[e]	0.44[f]	0.60[g]	0.58[h]	–	0.32[i]	0.31[j]	0.68[j]

[a] ^{13}C and ^{15}N isotropic chemical shifts were obtained with respect to Me_4Si and liquid NH_3, respectively.
[b] The anisotropy δ and the asymmetry parameter η are defined as $\delta = \delta_{zz} - \delta_{iso}$ and $\eta = (\delta_{yy} - \delta_{xx})/\delta$, where δ_{iso} is the isotropic shift and $|\delta_{yy} - \delta_{iso}| \leq |\delta_{xx} - \delta_{iso}| \leq |\delta_{zz} - \delta_{iso}|$.
[c] Average of the values for the carbonyl carbons in some peptides (Oas et al., 1987a).
[d] Average of the values for the carboxyl carbons in glycine (Haberkorn et al., 1981), L-alanine (Naito et al., 1981), L-serine (Naito et al., 1983), L-threonine (Janes et al., 1983), L-asparagine (Naito et al., 1984).
[e] Values for the α carbon in glycine (Haberkorn et al., 1981).
[f] Values for the α carbon in L-alanine (Naito et al., 1981).
[g] Values for the β carbon in isobutane (Facelli et al., 1986).
[h] Values for the α-methylene carbon in *n*-eicosane (VanderHart, 1976).
[i] Values for the methyl carbon in *n*-eicosane (VanderHart, 1976).
[j] Determined by the sideband analysis of a low-speed MAS spectrum.

four molecules in the unit cell are crystallographically equivalent.

Results and discussion

Signal assignment

Figure 3 shows a ^{13}C CPMAS spectrum observed under a spinning speed of 17.5 kHz with TPPM decoupling together with a ^{13}C COSY spectrum obtained with a CPMAS version of the conventional COSY sequence under TPPM decoupling. The observed eight signals (C_1-C_8) show the splitting due to the ^{13}C-^{13}C spin-spin couplings. As indicated in the figure, the C_2 and C_4 signals are connected with each other but isolated from the other carbons. C_2 must obviously be assigned to the glycine carbonyl carbon (C'_G) and C_4 to the glycine C_α carbon (C^{α}_G). The remaining signals C_1, C_3, C_5, C_6, C_7, and C_8, belonging to the isoleucine residue, can be assigned as C_1=C'_I, C_3=C^{α}_I, C_5=C^{β}_I, C_6=$C^{\gamma 1}_I$, C_7=$C^{\gamma 2}_I$, and C_8=C^{δ}_I from the connectivity and common knowledge of ^{13}C chemical shifts. The observed isotropic chemical shifts are collected in Table 1.

A ^{15}N CPMAS spectrum was recorded under a spinning speed of 17.5 kHz with TPPM decoupling (not shown). We assigned the resonance at 120.9 ppm to the amide nitrogen N_I and that at 23.03 ppm to the amine nitrogen N_G, referring to their typical isotropic shift values (Roberts et al., 1987).

Dipolar recoupling experiments

In order to remove the influence of intermolecular dipolar couplings on the dipolar recoupling experiments, we used a sample of uniformly ^{13}C,^{15}N-labeled Gly-Ile 10-fold diluted with natural abundance material. The peaks from the natural abundance material were observed overlapping with those of the fully labeled molecules. To appreciate the contribution of the natural abundance material to the signal intensity observed in the dipolar recoupling experiment, we have done the dipolar recoupling experiment using natural abundance material. Since the dipolar recoupling between naturally abundant nuclei is negligibly small, this experiment corresponds to a $T_{1\rho}$ measurement. As a result, $T_{1\rho}$ was found to be much longer than the longest recoupling time used (10 ms), the magnetization of the natural abundance material being kept almost constant during t_m. Therefore, in the recoupling-time dependence experiments for the 10% uniformly labeled sample, we simply subtract 10% of the intensity at $t_m = 0$ from the intensity observed at each recoupling time t_m.

Further, we must take account of the relaxation effect of the labeled material during the recoupling time t_m. To do so, we did the same experiment except for shifting the spinning frequency by ~ 500 Hz from the R2TR condition. Subtracting the natural abundance contribution from the time dependence of the magnetization, we obtained $T_{1\rho}$ for uniformly ^{13}C,^{15}N-labeled molecules. Then, the relaxation effect can be removed from the experimental data from which the natural abundance contribution has been subtracted, by mul-

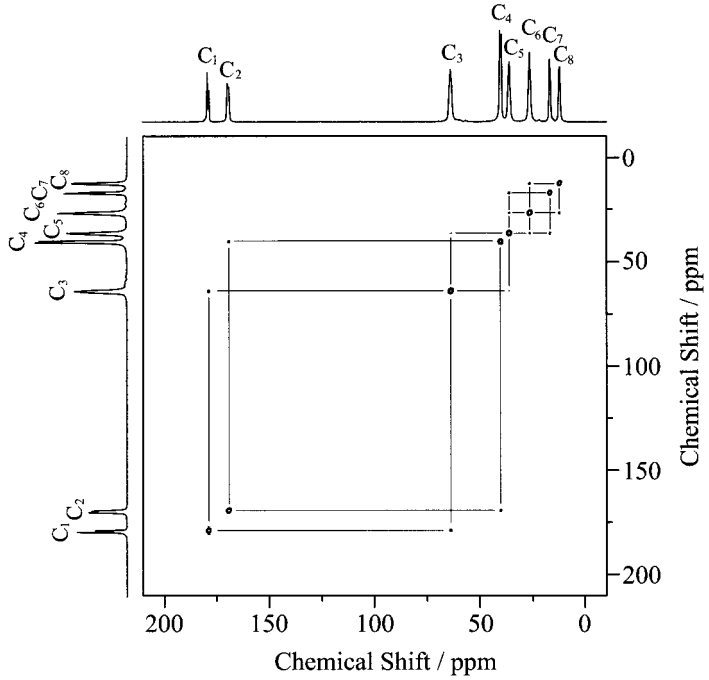

Figure 3. ^{13}C COSY spectrum of uniformly ^{13}C,^{15}N-labeled glycylisoleucine. It was obtained under a spinning speed of 17.5 kHz and TPPM decoupling.

tiplying the data by $\exp(t_m/T_{1\rho})$. In all the dipolar recoupling data shown below, both the subtraction of the natural abundance contribution and the correction for the relaxation effect have been done.

ψ_G *angle*: The ψ_G angle was obtained through a dipolar recoupling experiment for N_G and N_I. The recoupling was achieved by applying an rf field satisfying a DQ condition under $\omega_R = 17.5$ kHz: an rf field with intensity 8300 Hz was applied on-resonance to N_G during the recoupling period t_m. Figure 4a shows the recoupling-time dependence of the spin-locked magnetization of N_G. The root-mean-square deviation (rmsd) between the experimental data and the curves calculated for various ψ_G is shown in Figure 4b, and from the minimum, ψ_G was determined to be 180°. In the present approach, however, recoupling-time dependence is insensitive to the dihedral angle around 0° and 180°, displaying a very broad minimum. Nevertheless, the $\Delta\chi^2$ plot shown in Figure 4b indicates that the 68% confidence interval ($\Delta\chi^2 < 1$) is still ±10°. The recoupling-time dependence calculated for $\psi_G = 180°$ is represented in Figure 4a.

Here, we examine to what extent the assumptions for the chemical shift tensors lead to error in the determination of ψ_G. We assumed that the direction of the σ_{11} axis for the amide nitrogen is parallel to the peptide bond. While this is almost the case in general, in some cases, it deviates from the peptide bond direction to the N-C_α direction by a maximum of about 10° (Oas et al., 1987a). We found that this amount of deviation in the σ_{11} direction of the N_I CSA tensor hardly changes the simulation curve, and that the deviation of 10° in the σ_{33} axis for N_G from the C_G^α-N_G bond direction does not change it either. However, changing the CSA parameters (δ, η)=(103.0 ppm, 0.31) of N_I to those for the amide nitrogens in AcGlyTyrNH$_2$ ((δ, η)=(96.4 ppm, 0.26)), or GlyGly·HCl ((δ, η)=(101.0 ppm, 0.02)) (Oas et al., 1987b) results in the best-fit values of $\psi_G = \pm 154°$ or ±159°, respectively, which are considerably different from the above value of 180°. As shown by this result, the recoupling-time dependence for N_I is sensitive to the CSA parameters δ and η, particularly around $\psi_G = 180°$, because the ^{15}N-^{15}N dipolar interaction is the weakest for $\psi_G = 180°$ and, moreover, the recoupling-time dependence is insensitive to the dihedral angle around 180°. Therefore, it is desirable to measure the CSA parameters of N_I and use them in the simulations for determining ψ_G precisely. Note, however, that the averages of the CSA parameters of the amide nitrogens in AcGlyGlyNH$_2$,

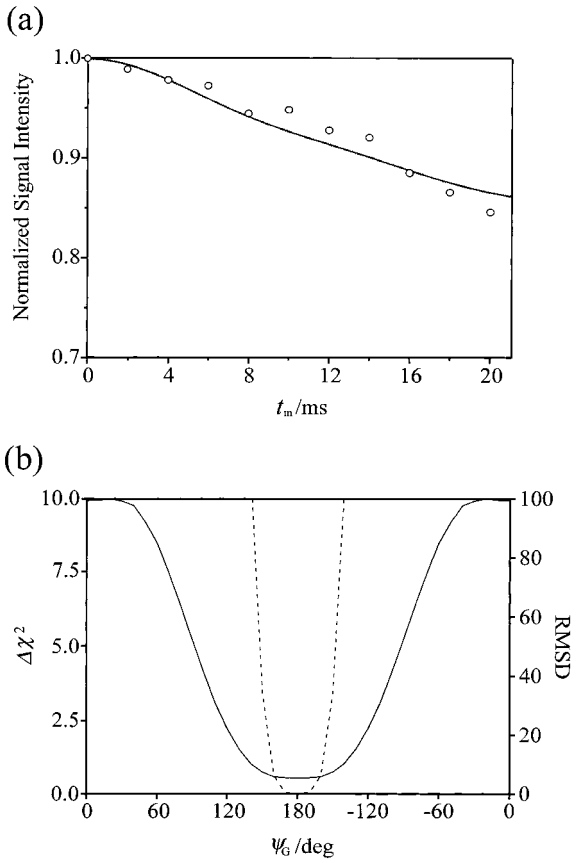

Figure 4. (a) Recoupling-time dependence of the normalized magnetization of N_G in 10% uniformly $^{13}C,^{15}N$-labeled glycylisoleucine. The dipolar recoupling experiment was carried out using the pulse sequence in Figure 2a under an R2TR condition ($\omega_{eI} + \omega_{eS} = \omega_R$) satisfied for N_G and N_I by applying an rf field with intensity 8300 Hz on-resonance to the N_G carbon under MAS with $\omega_R = 17.5$ kHz. Experimental points are shown by circles. The standard deviation for each point is ca. ±0.05. The curves are calculated for the dihedral angle ψ_G of 180°. $T_{1\rho}$ was determined to be 70 ms by an off-R2TR experiment, and used to remove the relaxation effect from the dipolar recoupling data. (b) Dihedral angle (ψ_G) dependence of rmsd (solid line) between the experimental (Figure 4a) and simulated data and of $\Delta\chi^2$ (dashed line).

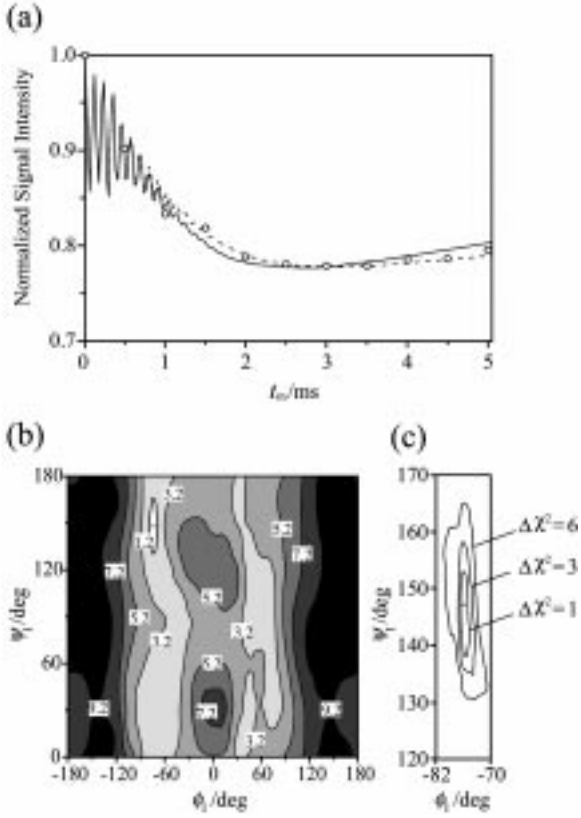

Figure 5. (a) Recoupling-time dependence of the normalized magnetization of C'_I in 10% uniformly $^{13}C,^{15}N$-labeled glycylisoleucine. The dipolar recoupling experiment was carried out using the pulse sequence in Figure 2a under an R2TR condition ($\omega_{eI} + \omega_{eS} = \omega_R$) satisfied for C'_I and C'_G by applying an rf field with intensity 8500 Hz on-resonance to the C'_I carbon under MAS with $\omega_R = 17.054$ kHz. Experimental points are shown by circles. The standard deviation for each point is ca. ±0.03. The solid line is the curve calculated for the dihedral angles (ω_G, ϕ_I, ψ_I) = (180°, −76°, 147°), whereas the dotted line for (ω_G, ϕ_I, ψ_I) = (0°, −86°, 167°). $T_{1\rho}$ was determined to be 500 ms for C'_I in 10% uniformly $^{13}C,^{15}N$-labeled glycylisoleucine, and used to correct the experimental data. (b) The contour plot of rmsd between the experimental spectrum (Figure 5a) and the simulations for various sets of (ϕ_I, ψ_I) and ω_G = 180°. The global minimum is indicated by a dot. (c) Dihedral angles (ϕ_I and ψ_I) dependence of $\Delta\chi^2$ for $\omega_G = 180°$.

$AcGlyAlaNH_2$, $AcGlyTyrNH_2$, and GlyGly·HCl (Oas et al., 1987b) give (δ, η)=(103.2 ppm, 0.22), which are similar to the observed values, and in fact, lead to the same ψ_G value of 180° as above.

$\omega_G, \phi_I, and\ \psi_I$ *angles*: Figure 5a shows the observed recoupling-time dependence of the magnetization of C'_I under a DQ condition satisfied between the resonances of C'_G and C'_I, where an rf field with intensity 8500 Hz was applied on-resonance to the C'_I carbon while spinning with $\omega_R = 17.054$ kHz. Since peptide planes take the *trans* configuration except for cyclic peptides or peptides with proline, we assume ω_G to be 180°. If only the dipolar interaction between C'_G and C'_I contributes to the result, only the absolute value of the dihedral angle ϕ_I can be determined from this experiment. In the present case, however, the large CSAs of both carbons and the relative orientations between the CSA tensors and the dipolar vector would significantly affect the experimental result. Hence, there may be a good chance of

determining not only ϕ_I but also ψ_I. Therefore, we simulated the experimental curve, taking ϕ_I and ψ_I as adjustable parameters. Furthermore, due to the imperfect selectivity of R2TR, three carbons, C_G^α, C_I^α, and C_I^β, do affect the recoupling-time dependence appreciably, so that they were included in addition to C'_G and C'_I in the calculation of the dipolar interactions. Figure 5b shows the contour plot of rmsd between the experimental data and the curves calculated for various ϕ_I and ψ_I. From the global minimum, ϕ_I and ψ_I were determined to be $-76°$ and 147°, respectively. It is worthy to point out here that if the recoupling-time curve depends only on C'_G and C'_I, (ϕ_I, ψ_I) and $(-\phi_I, -\psi_I$ or $180 - \psi_I)$ give identical results. However, the $\Delta\chi^2$ values around $(\phi_I, \psi_I) = (76°, 33°)$ are large (ca. 34). This apparent asymmetry comes from the inclusion of the outer carbons C_G^α, C_I^α, and C_I^β. Hence, we could uniquely determine both ϕ_I and ψ_I. Figure 5c shows $\Delta\chi^2$ around the global minimum; the 68% confidence regions ($\Delta\chi^2 \sim 1$) for ϕ_I and ψ_I are $(-77° \sim -75°)$ and $(138° \sim 153°)$, respectively. The reason why ϕ_I is precisely determined but ψ_I is not is because the dipolar coupling directly contributes to the determination of ϕ_I, but ψ_I is obtained only through the relative orientation of the C'_I CSA tensor to the other tensors. The result of the recoupling experiment for C'_G and C'_I indicates that one dipolar recoupling experiment per amino acid residue is enough to determine dihedral angles (ψ, ϕ) which provide the 3D backbone structure of a peptide. In Figure 5(a) the recoupling-time dependence is shown calculated for the best-fit parameter set $(\phi_I, \psi_I) = (-76°, 147°)$. The rapid oscillation in the initial part of the simulated curve is caused by the presence of CSA and understood as the spinning sidebands in the tilted rotating frame, whose frequencies are given approximately by $|\omega_1 - n\omega_R|$ (n is an integer) (Takegoshi et al., 1997). In experiments, however, we found that this oscillation is damped quickly within a few milliseconds due to rf-field inhomogeneity. Therefore, we damped this oscillation in the simulation to the same extent as the experimental data.

The large CSAs and the tensor orientations for the C'_G and C'_I carbons affect the recoupling behavior. Therefore, we examine the validity of the use of the CSA tensors assumed for C'_G and C'_I in the above simulations. First, as for C'_G, the σ_{22} axis direction was assumed to make an angle of 4.5° with the C'_G=O_G bond toward the C'_G-C_G^α bond. Actually, it is along the C′=O bond direction in $AcGlyGlyNH_2$ and $AcGlyAlaNH_2$ and deviates to the C′-C_α bond direction about 6° and 12° in $AcGlyTyrNH_2$ and GlyGly·HCl, respectively (Oas et al., 1987a). The CSA parameters were assumed to be $(\delta, \eta) = (-79.3$ ppm, 0.82), while they are $(-81.6$ ppm, 0.72), $(-82.3$ ppm, 0.69), (74.7 ppm, 0.94), and $(-80.9$ ppm, 0.82) for $AcGlyGlyNH_2$, $AcGlyAlaNH_2$, $AcGlyTyrNH_2$, and GlyGly·HCl, respectively (Oas et al., 1987a). Simulating the experimental result using the individual CSA parameters and orientations, we obtained the best-fit values of (ϕ_I, ψ_I)=$(-76°, 148°)$, $(-76°, 148°)$, $(-71°, 144°)$, and $(-78°, 139°)$, for $AcGlyGlyNH_2$, $AcGlyAlaNH_2$, $AcGlyTyrNH_2$, and GlyGly·HCl, respectively. As for the tensor orientation of C'_I, we assumed the σ_{11} to be parallel to the C_I^α-C'_I bond, while it may deviate from the bond direction by about 10° (Veeman et al., 1984). We found that this amount of deviation changes the best-fit values to (ϕ_I, ψ_I)=$(-76°, 138°)$. The CSA parameters (δ, η) were assumed to be (δ, η)=$(-68.9$ ppm, 0.93), while they are $(-72.8$ ppm, 0.94), $(-71.0$ ppm, 0.84), $(-68.7$ ppm, 0.84), (70.2 ppm, 0.85), and $(-67.1$ ppm, 0.90) for glycine (Haberkorn et al., 1981), L-alanine (Naito et al., 1981), L-serine (Naito et al., 1983), L-threonine (Janes et al., 1983), and L-asparagine (Naito et al., 1984), respectively. Using these CSA parameters, we obtained (ϕ_I, ψ_I) to be $(-76°, 137°)$, $(-76°, 143°)$, $(-75°, 148°)$, $(-76°, 149°)$, and $(-76°, 150°)$, for glycine, L-alanine, L-serine, L-threonine, and L-asparagine, respectively. These results show that the use of likely CSA tensors for C'_G and C'_I in the simulations causes little error in the determination of ϕ_I, but leads to considerable error in ψ_I. Since the ϕ_I value is directly related to the distance, it is insensitive to the CSA tensors, while the ψ_I value is related mainly to the relative orientations of the CSA tensor of the carboxyl carbon to the other tensor, being more sensitive to them. In fact, Tycko et al. showed that both ϕ and ψ can be determined by correlating the two CSAs of the carbonyl carbons in adjacent amino acid residues (Tycko et al., 1996). To obtain a more precise value for ψ_I, (δ, η) for C'_I should be measured by some method.

In cyclic peptides or peptides with proline, ω_G may be 0°, and therefore we examine the possibility of determining whether $\omega_G = 0°$ or 180°. Obtaining a similar rmsd contour plot for $\omega_G = 0°$, we determined ϕ_I and ψ_I to be $-86°$ and 167°, respectively. In Figure 5a is shown the recoupling-time dependence calculated for both best-fit parameter sets $(\omega_G, \phi_I, \psi_I) = (180°, -76°, 147°)$ and $(0°, -86°, 167°)$. Unfortunately, the simulations for the two sets of dihedral

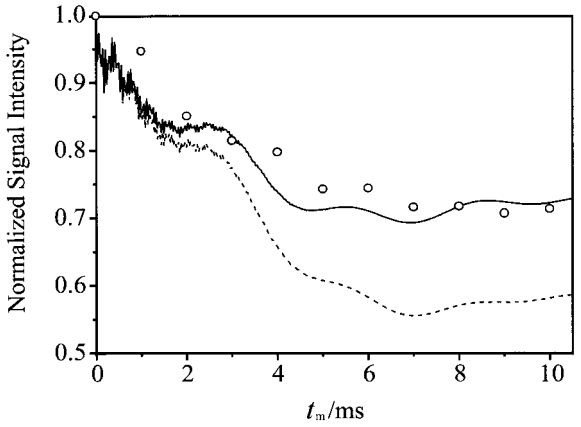

Figure 6. Recoupling-time dependence of the normalized magnetization of C'_I in 10% uniformly $^{13}C,^{15}N$-labeled glycylisoleucine. The dipolar recoupling experiment was carried out using the pulse sequence in Figure 2a under an R2TR condition ($\omega_{eI} + \omega_{eS} = \omega_R$) satisfied for C'_I and C^{α}_G by applying an rf field with intensity 2800 Hz on-resonance to the C'_I carbon under MAS with $\omega_R = 17.03$ kHz. Experimental points are shown by circles. The standard deviation for each point is ca. ± 0.04. The solid line is the curve calculated for the dihedral angles (ω_G, ϕ_I, ψ_I) = (180°, −76°, 147°), whereas the dotted line for (ω_G, ϕ_I, ψ_I) = (0°, −86°, 167°). $T_{1\rho}$ was determined to be 500 ms for C'_I in 10% $^{13}C,^{15}N$-labeled glycylisoleucine, and used to correct the experimental data.

angles are similar, so that we cannot choose the correct set out of the two from this result. To conclude which set of dihedral angles is correct, we did another recoupling experiment for C^{α}_G and C'_I. The recoupling-time dependence can be calculated for given ω_G, ϕ_I, and ψ_I. An rf field with intensity 2800 Hz was applied on-resonance to the C'_I carbon which satisfies a DQ condition under spinning with $\omega_R = 17.03$ kHz. Figure 6 shows the recoupling-time dependence of the magnetization of C'_I together with the simulated curves obtained for the above two sets of dihedral angles. Clearly, the former set reproduces the observation as expected. This indicates that even when we do not know whether $\omega = 0°$ or 180°, we can determine the three dihedral angles (ψ, ω, ϕ) by two dipolar recoupling experiments per amino acid residue.

As shown here, the recoupling-time behavior in a general sp^2-sp^2 ^{13}C spin pair depends not only on the internuclear distance, but also on the relative orientations between the CSA tensors and the dipolar vector as well as the CSA parameters. Fortunately, the orientations and values for sp^2 carbons have been well established and are virtually independent of peptides. Further, owing to the dependence caused by CSA, the magnitudes of not only ϕ but also of ψ can be determined. Moreover, C^{α}_I and C^{β}_I carbons also contribute

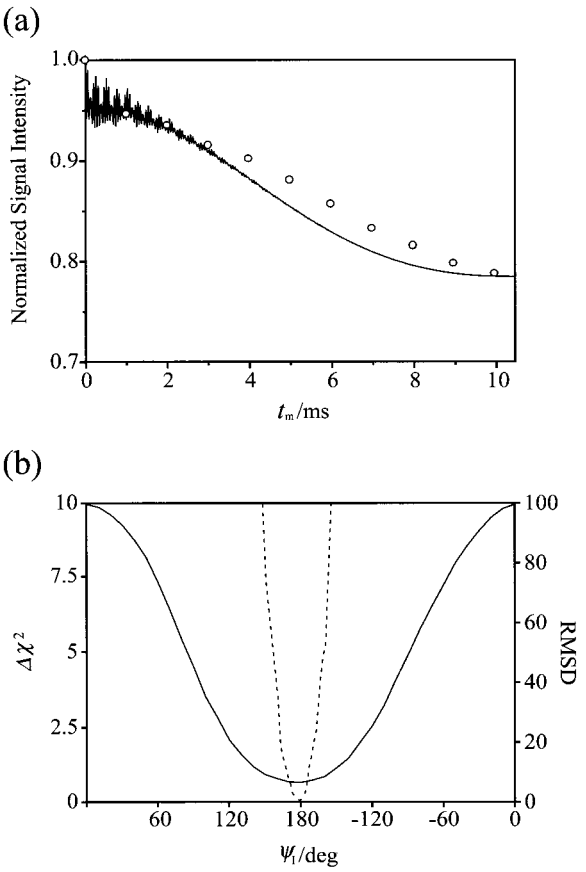

Figure 7. (a) Recoupling-time dependence of the normalized magnetization of C'_I in 10% uniformly $^{13}C,^{15}N$-labeled glycylisoleucine. The dipolar recoupling experiment was carried out using the pulse sequence in Figure 2a under an R2TR condition ($\omega_{eI} + \omega_{eS} = \omega_R$) satisfied for C'_I and $C^{\gamma 1}_I$ by applying an rf field with intensity 2000 Hz on-resonance to the C'_I carbon under MAS with $\omega_R = 17.46$ kHz. Experimental points are shown by circles. The standard deviation for each point is ca. ± 0.03. The curves are calculated for the dihedral angle χ_{I1} of 178°. $T_{1\rho}$ was determined to be 500 ms for C'_I in 10% $^{13}C,^{15}N$-labeled glycylisoleucine, and used to correct the experimental data. (b) Dihedral angle (χ_{I1}) dependence of rmsd (solid line) between the experimental (Figure 7a) and simulated data and of $\Delta\chi^2$ (dashed line).

to the experimental result due to the incomplete selectivity; however, it provides the benefit of being able to determine the signs of ϕ and ψ.

χ_{I1} *angle*: The χ_{I1} angle was obtained by selective dipolar recoupling between C'_I and $C^{\gamma 1}_I$, which was attained by applying an rf field with intensity 2000 Hz on-resonance to the C'_I carbon under MAS with $\omega_R = 17.46$ kHz. Figure 7a shows the observed recoupling-time dependence of the C'_I magnetization. Figure 7b indicates the rmsd between the experimental data and

the curves calculated for various χ_{I1}. We included C_I^{α}, C_I^{β}, and C_I^{δ} besides C_I' and $C_I^{\gamma 1}$ in the simulation, making the rmsd plot slightly asymmetric, as shown in Figure 7b. From the minimum, χ_{I1} was determined to be 178°. The recoupling-time dependence calculated for $\chi_{I1} = 178°$ is shown in Figure 7a. The 68% confidence interval is ±10°, as shown in Figure 7b, being large because $\chi_{I1} \sim 180°$.

Here, we examine the effect of the chemical shift tensors of C_I' and $C_I^{\gamma 1}$. We found that the above deviation of the σ_{11} direction for C_I' does not alter the best-fit value of 178°. Using the above parameters (δ, η) for C_I' in glycine, L-alanine, L-serine, L-threonine, and L-asparagine, we obtained the best-fit values of χ_{I1} to be 178°, 178°, 175°, 178°, and 175°, respectively. As for $C_I^{\gamma 1}$, it was found that changing the CSA orientation hardly affects the calculated recoupling-time dependence, while changing the CSA parameters (δ, η) from (−12.3 ppm, 0.58) for the α-methylene carbon of *n*-eicosane (VanderHart et al., 1976), which was used for the simulation, to (−7.3 ppm, 0.58) results in the best-fit value of $\chi_{I1} = -176°$.

As exemplified by the present case, in a general sp^3-sp^2 ^{13}C spin pair, possible variations of parameters and orientations of their CSA tensors barely affect the recoupling-time dependence. Thus, we may safely use likely CSA parameters (δ, η) and tensor orientations for both carbons.

2D dipolar correlation experiment

χ_{I2} *angle*: To determine the χ_{I2} angle in the same way, it is necessary to selectively recouple C_I^{α} and C_I^{δ}, or $C_I^{\gamma 2}$ and C_I^{δ}. However, since their chemical-shift values are very similar to each other and, moreover, the atomic positions are close to each other, selective recoupling of one of these pairs is difficult. In this sense, the isoleucine residue is one of the most demanding ones for the present approach. Therefore, we adopted another way of observing a C_I^{α}-C_I^{β}/$C_I^{\gamma 1}$-C_I^{δ} dipolar correlation 2D powder pattern using the sequence shown in Figure 2b. The spinning speed ω_R was set to 5110 Hz, satisfying the R^2 condition for C_I^{δ} and C_I^{α}. In the t_1 period, a DQ condition is matched for $C_I^{\gamma 1}$ and C_I^{δ} by applying an rf field with intensity 2540 Hz on-resonance to C_I^{δ}. During the mixing time t_m, the magnetization of C_I^{δ} is selectively transferred to C_I^{α} by R^2. In the t_2 period, a DQ condition is fulfilled for C_I^{α} and C_I^{β} by applying an rf field with intensity 1825 Hz on-resonance to C_I^{α}.

Figure 8a shows the experimental spectrum, which was observed using a 100% uniformly labeled sample for obtaining a high signal-to-noise ratio. The use of the undiluted sample is justified, because in this experiment, the dipolar interactions recoupled in the t_1 and t_2 periods are of directly bonded nuclei, and thus much stronger than the intermolecular dipolar interactions. We simulated 2D spectra for various χ_{I2} angles, including $C_I^{\gamma 2}$ in addition to C_I^{α} C_I^{β} $C_I^{\gamma 1}$ and C_I^{δ}. In the course of our study, we found that the $C_I^{\gamma 1}$-C_I^{δ} bond apparently shows an unusual bond length, which may be caused by a large vibration of C_I^{δ}. With the existence of molecular motion, dihedral angles cannot be correctly determined from distance measurements. However, in a theoretical study of vibrational effects (Ishii et al., 1997), it has been proven that the dipolar 2D powder patterns provide correct dihedral angles even under the presence of molecular vibrations as long as the asymmetry of the vibration is negligible. It is a common problem for various methods of structure determination that we must pay attention to the presence of local motion. Figure 8b shows the χ_{I2} dependence of rmsd between the experimental and calculated 2D spectra. From the minimum, χ_{I2} was determined to be 160°, and the spectrum calculated with $\chi_{I2} = 160°$ is given in Figure 8a. In this simulation, a somewhat long bond length of 1.62 Å was assumed for the $C_I^{\gamma 1}$-C_I^{δ} bond, corresponding to our observation of the unusual vibration of C_I^{δ}, which provides the best agreement with the experimental spectrum. Even though the agreement is not excellent, the $\Delta\chi^2$ plot (Ishii et al., 1998) in Figure 8b shows that the 68% confidence interval is ±5°. The sign of χ_{I2} cannot be determined from this experiment. We calculated the dihedral angle ($C_I^{\gamma 2}$-C_I^{β}-$C_I^{\gamma 1}$-C_I^{δ}) to be 39° from $\chi_{I2} = -160°$, and to be 79° from $\chi_{I2} = 160°$; this suggests that the steric hindrance between the two methyl groups is smaller for the latter case. Therefore, we prefer the positive sign.

In these simulations, chemical shift anisotropies are not included; we found that even if likely chemical shift tensors are included; the spectrum does not change appreciably, under the MAS frequency of ∼5 kHz. This is fortunate, because the CSA tensors of sp^3 carbons depend heavily on circumstances, so that it is very difficult to assume them appropriately for simulations.

For a directly bonded sp^3-sp^3 ^{13}C spin pair, the effect of CSAs can indeed be neglected in comparison with the contribution of the dipolar coupling under MAS with a spinning speed a few times faster than $|\delta|$,

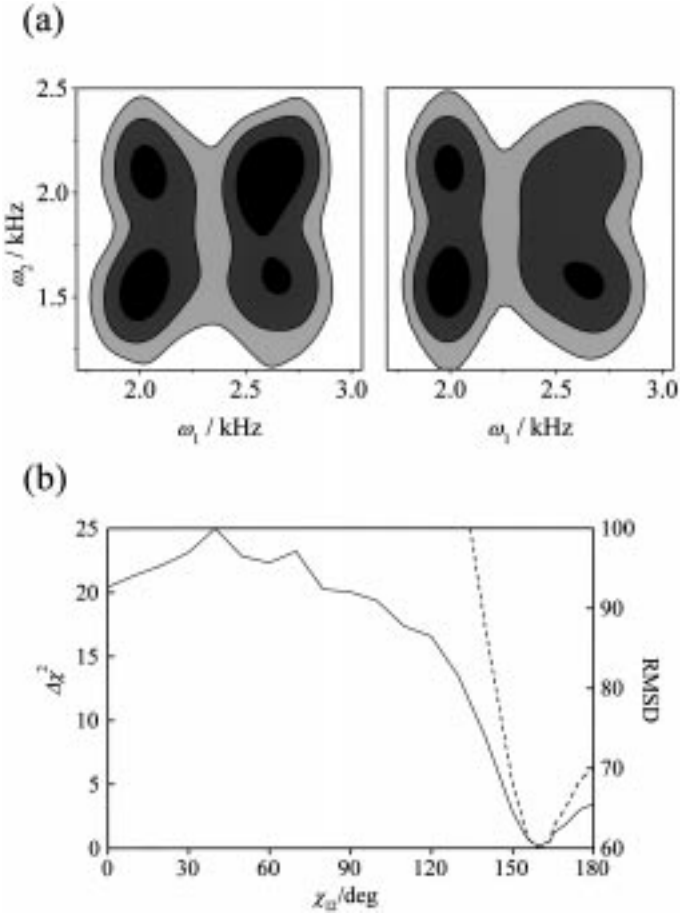

Figure 8. (a) The 2D C_I^{α}–C_I^{δ}/$C_I^{\gamma 2}$–C_I^{δ} dipolar correlation spectrum for uniformly ^{13}C,^{15}N-labeled glycylisoleucine. The experiment was performed using the pulse sequence in Figure 2b. The spinning frequency ω_R is 5110 Hz. The ^{13}C rf-field intensities during the t_1 period, the mixing time, and the t_2 period are $\omega_1/2 = 2540$ Hz (on-resonance to the C_I^{δ} carbon), 0 Hz, and 1825 Hz (on-resonance to the C_I^{α} carbon), respectively. Left: experimental 2D spectrum; right: simulated 2D spectrum calculated for the $C_I^{\gamma 1}$-C_I^{δ} distance of 1.62 Å and the dihedral angle χ_{I2} of 160°. The contour lines are plotted at 65%, 77%, and 88% of the maximum intensity. (b) Dihedral angle (χ_{I2}) dependence of rmsd (solid line) between the experimental (Figure 8a) and simulated 2D dipolar correlation spectra and of $\Delta\chi^2$ (dashed line).

according to our simulations. On the other hand, for a nonbonded, namely weakly dipolar coupled sp^3-sp^3 ^{13}C spin pair, the effect of the CSAs becomes more important than for a directly bonded pair. Therefore, the CSA effect must be reduced for accurate determination of dihedral angles by using a sufficiently fast spinning speed. Then a large ω_1 is needed to satisfy a DQ recoupling condition, so that selectivity is declined. Therefore, when some other sp^3 carbons exist close to the relevant carbons, the dipolar recoupling experiment (Figure 2a) does not work well, and the 2D dipolar correlation experiment (Figure 2b) is recommended.

Three-dimensional structure

Figure 9a shows the 3D structure constructed from the dihedral angles determined by the present R2TR experiments. For comparison, we have also made an X-ray structural analysis; no X-ray structure of glycylisoleucine has been reported. Figure 9b shows the structure by X-ray crystallography. The dihedral angles determined by NMR and X-ray are (ψ_G, ω_G, ϕ_I, ψ_I, χ_{I1}, χ_{I2}) = (180°, 180°, −76°, 147°, 178°, 160°) and (165°, 170°, −70°, 156°, 178°, 170°), respectively. Although of course the result by NMR includes errors, the X-ray structure is not very accurate either: we could not prepare a good sample for the X-ray structural analysis. Nevertheless, both structures are in good agreement, indicating that the present ap-

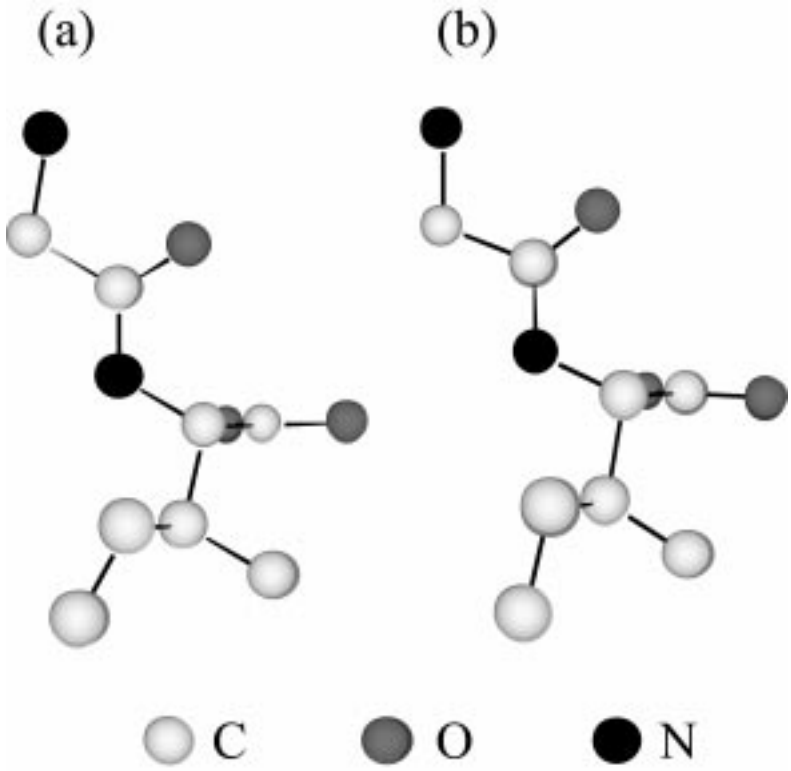

Figure 9. Three-dimensional structure of glycylisoleucine. (a) Present NMR study. (b) X-ray crystallography.

proach is useful for structure determination. Further, the X-ray diffraction showed that the thermal vibration parameters of C_I^{δ} are very large, agreeing with our observation by NMR.

Conclusions

In this work, we have determined the complete 3D structure of a uniformly ^{13}C,^{15}N-labeled Gly-Ile molecule using the R2TR method, by which all the dihedral angles were individually determined by selectively recoupling some dipolar interactions between three- or four-bonds distant spins or observing a dipolar correlation 2D powder pattern. Choosing three adjustable parameters ω_1, ω_0, and ω_R properly, we could find a R2TR condition suitable for selective recoupling of the pair of spins concerned. Furthermore, it is not always necessary that all resonances are resolved; it is possible to monitor the recoupling-time dependence if only one of the pair of spins concerned is resolved well from the others.

The present approach can be applied to determine the 3D structure of a small molecule and also the structure of a restricted region important to the biological function of a large system using a sample in which the region is uniformly or multiply labeled. So far quantitative structural information obtained by solid state NMR has been almost limited to a single internuclear distance or dihedral angle. The present approach increases the obtainable quantity of structural information in a single powder sample.

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