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Determination of the mutual orientation of the ^{15}N and ^{13}C NMR chemical shift tensors of $^{13}C-^{15}N$ double labeled model peptides for silk fibroin from the dipolar-coupled powder patterns

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Abstract

The ^{15}N and ^{13}C chemical shift tensors, and the orientation of the principal axis system relative to the molecular symmetry axes were determined for ^{15}N and ^{13}C carbonyl carbon sites of $^{13}C-^{15}N$ double labeled model peptides for *Bombyx mori* silk fibroin, that is, Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe, Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac, Boc-AlaGly[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac, Boc-[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac, Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac and Boc-[1-^{13}C]Gly[^{15}N]ValGlyAla-OPac, where Boc is t-butoxycarbonyl, OMe is methyl ester, OPac is phenacyl ester, Ala is alanine, Gly is glycine and Val is valine. From the comparisons of the ^{15}N chemical shift tensors and the orientations of the principal axis system relative to the molecular symmetry axes among three compounds having [1-^{13}C]Ala[^{15}N]Gly units, it is concluded that the intermolecular interactions such as hydrogen bonding are different between Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe and two compounds, Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac and Boc-AlaGly[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac although the latter two compounds have similar structures. A similar conclusion has also been obtained from the ^{13}C chemical shift tensors of these compounds. © 1998 Elsevier Science B.V.

Keywords: Solid state ^{15}N and ^{13}C NMR; Chemical shift tensor; Dipolar coupling; Model compounds of *Bombyx mori* silk fibroin

1. Introduction

Recent advances in orientation-dependent solid state NMR such as chemical shift anisotropy and dipolar coupling have proven useful in structural studies of biomolecules [1–3]. The NMR spectra of oriented molecules are very sensitive to the orientation of the molecule with respect to the magnetic field and have been interpreted in terms of molecular orientations. Accurate determination of structure is critically dependent on an accurate knowledge of the principal values and orientation of the chemical shift or dipolar tensors in the molecule of interest.

Fibrous proteins are particularly difficult to study using standard structure determination techniques. X-ray diffraction from fibers in which proteins are aligned along the long axis of the fiber typically yields general features of molecular organization and packing, but lacks atomic resolution details [4]. However, the natural alignment of the protein within the fiber

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provides an important advantage that can be utilized by solid state NMR. On the basis of determination of the relative orientation of peptide planes with solid state NMR, we have shown that the local structure of the Gly site of [^{15}N]Gly labeled *Bombyx mori* silk fibroin protein fiber is similar to the structure reported by X-ray diffraction [3,5–8]. *Bombyx mori* silk fibroin is a fibrous protein whose amino acid composition (in mol%) is 42.9 (glycine), 30.0 (alanine), 12.2 (serine), 4.8 (tyrosine), 2.5 (valine). The whole primary structure consists largely of a repeating sequence of six residues (Gly–Ala–Gly–Ala–Gly–Ser)$_n$ [9]. This work forms part of a program to determine the structure of the silk fibroin fiber, especially ϕ and φ angles for each residue, using solid state NMR spectroscopy [3,6,8,10]. This can be done by determining the Euler angles for isotopically labeled atoms along the peptide backbone, which express the relative orientation of the principal axis system and the molecular symmetry axis frames of reference [6]. For this purpose, we have prepared [1-^{13}C]Ala and [1-^{13}C]Gly labeled silk fibroins and five types of [^{15}N] labeled silk fibroins, labeled Gly,Ala,Ser,Tyr and Val sites, by oral administration of isotope-labeled amino acids into fifth stage silkworm larvae or cultivation of the silkglands in a medium containing isotope-labeled amino acids [3,6–8,10–13].

The standard method for obtaining the values of the Euler angles requires a large single crystal and knowledge of the crystal structure [14]. However, it is usually difficult to prepare such a large single crystal. An alternative method involves the use of powder samples. When the nucleus is dipolar-coupled to a second nearby nucleus, the dipolar tensor is manifested in the powder patterns as well [15]. As first pointed out by Kaplan et al. [16] for a ^{13}C/^{15}N pair in acetonitrile, this dipole-coupled chemical shift powder pattern contains sufficient information to allow the determination of the relative orientation of the chemical shift and dipolar tensors. Because the dipolar tensor is an axially symmetric tensor whose unique axis is known to lie along the internuclear vector, the orientation of the chemical shift tensor relative to this molecular axis can be directly determined from the dipole coupled powder patterns [15–22].

In this paper, we will determine the ^{15}N and ^{13}C chemical shift tensors, and Euler angles, α_D and β_D of 15N and [1-^{13}C] sites for the ^{13}C-^{15}N double labeled model peptides of *B. mori* silk fibroin, that is, Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe, Boc-[1-^{13}C]Ala[^{15}N]Gly-AlaGly-OPac, Boc-AlaGly[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac, Boc-1-^{13}C]Gly[^{15}N]Ala-GlyAla-OPac, Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac and Boc-[1-^{13}C]Gly-[^{15}N]Val GlyAla-OPac, where Boc is t-butoxycarbonyl, OMe is methyl ester, OPac is phenacyl ester, Ala is alanine, Gly is glycine and Val is valine. Here, the Euler angles, α_D and β_D are denoted as α_{DNC} and β_{DNC} for the Euler angles relating to the ^{15}N principal axis system (PAS), and α_{DCN} and α_{DCN} relating to the ^{13}C PAS. These values will be used in determining the structure of such sequences in *B. mori* silk fibroin chain with solid state NMR. We will also discuss the difference in the local structure including intermolecular interactions such as hydrogen bonding among these compounds on the basis of the NMR parameters obtained.

2. Experimental

2.1. Materials

Six ^{13}C–^{15}N double labeled model peptides for *B. mori* silk fibroin, that is, Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe, Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac, Boc-AlaGly[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac, Boc-[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac, Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac and Boc-[1-^{13}C]Gly[^{15}N] ValGlyAla-OPac were synthesized by the liquid phase method. The synthesis was performed using dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) as the coupling reagents. A typical route for the synthesis is summarized in Fig. 1 for Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac. The isotopically labeled amino acids, [^{15}N]Ala (99.8% enrichment, ISOTEC Inc., Miamisburg, Ohio), [^{15}N]Gly (99% enrichment, Masstrace, Inc., USA), [^{15}N]Val (99%, ICON, NY), [1-^{13}C]Ala (99%, ICON) and [1-^{13}C]Gly (99%, ICON,) were used in the preparation of isotope labeled peptides. All of these model sequences have β-sheet structure judging from the ^{13}C chemical shifts of the Cβ carbons of the Ala residue in the ^{13}C CP/MAS NMR spectra of these peptides [23–27].

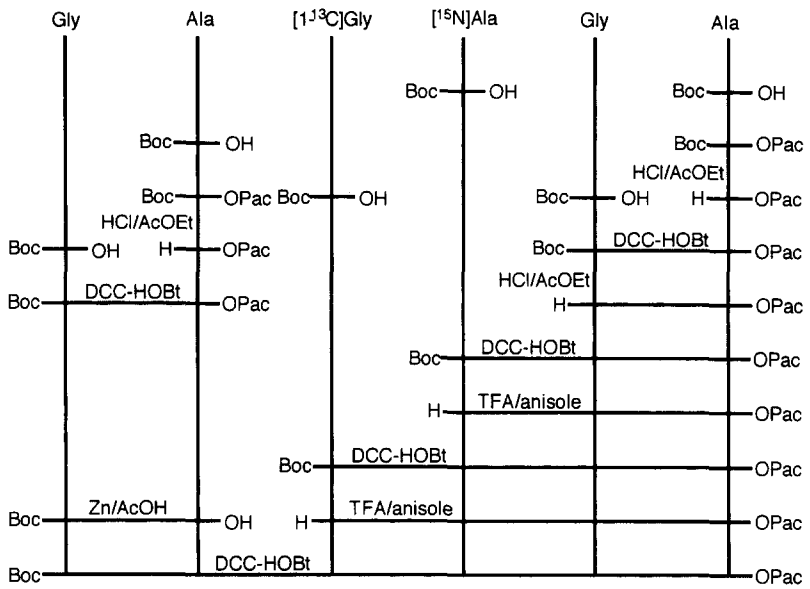

Fig. 1. Typical route for the synthesis of Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac by the liquid phase method. Boc is t-butoxycarbonyl, Gly is glycine, Ala is alanine, OPac is phenacyl ester, DCC is dicyclohexylcarbodiimide, HOBt is 1-hydoxybenzotriazole, Zn is powdered zinc, AcOH is acetic acid, HCl is hydrogen chloride, AcOEt is ethoxyacetic acid, TFA is trifluoroacetic acid.

2.2. NMR experiments

The solid state ^{15}N cross polarization (CP) NMR spectra were obtained on a JEOL GX 400 NMR spectrometer equipped with a solids NMR unit (NM-GSH-40MU/VT). Typical experimental conditions utilized a ^{15}N observation frequency of 40.5 MHz, 6.0 μs 90° pulse, pulse delay time of 7 s, contact time for CP condition of 5 ms. The ^{15}N CP NMR spectrum of Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe was also observed on a Bruker WP200 NMR spectrometer at Florida State University (Prof. T.A. Cross). The ^{15}N chemical shifts were externally referenced to $^{15}NH_4NO_3$ powder. ^{13}C CP and CP/MAS NMR spectra were obtained on a JEOL GX 400 spectrometer operating at 100.4 MHz. The ^{13}C and ^{15}N CP NMR spectra of Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac were obtained on a JEOL EX 270 spectrometer at Toyo Seikan Co., R&D Group. The methyl ^{13}C peak of hexamethylbenzene (17.3 ppm from TMS) was used as a ^{13}C chemical shift reference.

3. Calculation

The powder pattern spectrum of a dipolar-coupled nuclear site can be expressed in the following form [20,28]:

$$S(\nu) = \sum_i S(\nu_i) = \sum \int_{\theta=0}^{180} \int_{\phi=0}^{360} g_i[\nu, \nu_i(\theta, \phi, \alpha_D, \beta_D)] \times \sin\theta \partial\theta\partial\phi$$

where θ and ϕ are polar angles that represent the orientation of the principal axis system with respect to the magnetic field, B_0, fixed in the laboratory frame. The angles α_D and β_D are rotations that transform the dipole interaction into the principal axis system of the chemical shift tensor. Here the orientations of the chemical shift tensor element as well as the Euler angles are shown for ^{15}N and ^{13}C carbonyl carbon nuclei in Fig. 2. The ^{15}N and ^{13}C CSA PAS are frames in which the CSA tensors are diagonal, with principal components σ_{11}, σ_{22} and σ_{33} (taking into account the negative gyromagnetic ratio of ^{15}N). ν is the observed frequency and $\nu_i(\theta,\phi,\alpha_D,\beta_D)$ is the transition frequency for a particular orientation of the chemical shift tensor and dipolar tensor; the superposition of the ^{15}N or ^{13}C chemical shifts and ^{15}N–^{13}C dipolar interactions. The lineshape function for $\nu_i(\theta,\phi,\alpha_D,\beta_D)$ is g_i, and a line broadening function such as the Gaussian function can be used for peak

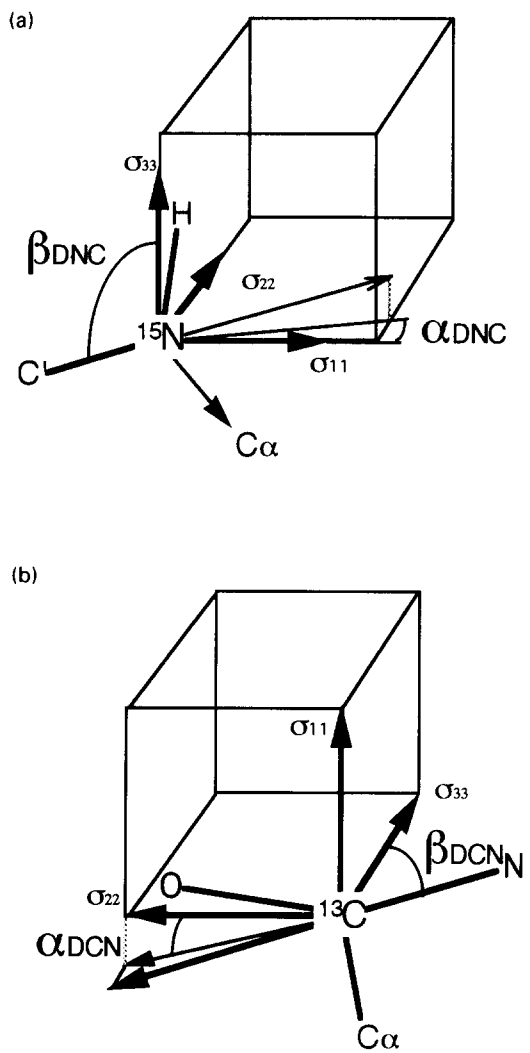

Fig. 2. Transformations from the principal axis system (PAS) to the molecular symmetry axis (MSA) system of (A) ^{15}N and (B) $^{13}C_1$ sites in a peptide plane. Both ^{15}N and $^{13}C_1$ tensors have two tensor elements in the peptide plane as a result of the Euler angles α_{DNC} and α_{DNC} being 0° and 90°. Consequently, in this definition, σ_{22} and σ_{11} are the unique elements for the ^{15}N and $^{13}C_1$ tensors that do not lie in the peptide plane, respectively.

simulation. The dipolar interaction affects the chemical shift spectral lineshape by an orientational dependent splitting of the chemical shift resonances. The powder pattern simulation is accomplished by transforming the dipolar interaction tensor into the chemical shift tensor via rotations α_D and β_D. Then both interaction tensors are transformed into the laboratory frame via the polar angles θ and ϕ. For powder spectra, integrations over θ and ϕ are performed for values of α_D and β_D that are chosen by a trial and error process [17–20]. The values of the chemical shift principal elements are used as variables.

4. Results

4.1. ^{15}N powder pattern spectra

Fig. 3 shows the observed and simulated ^{15}N powder pattern spectra of Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe observed at 40.5 and 20.3 MHz. The shapes of

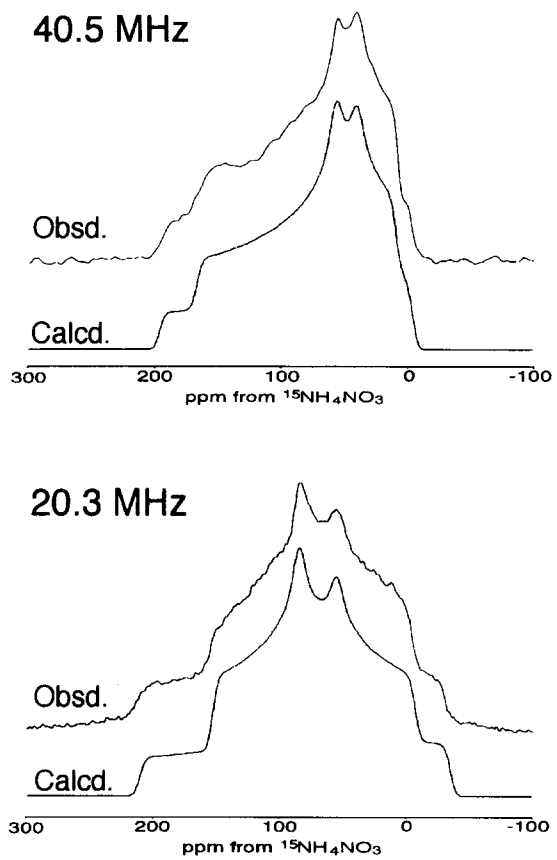

Fig. 3. The ^{15}N CP NMR spectra of Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe powder observed at 40.5 and 20.3 MHz. The simulated spectra are also shown. The chemical shift tensors and the Euler angles, α_{DNC} and β_{DNC}, are described together with the method for the simulation in the text.

Table 1
Chemical shift tensors and Euler angles relating to the molecular symmetry axis system (MSA) for ^{15}N principal axis system (PAS) of various $^{13}C-^{15}N$ double labeled model peptides and ^{15}N single labeled *B. mori* fibroins (silk II) which were determined from spectral simulation with line broadening (lb)

Sample	σ_{11}	σ_{22}	σ_{33}	σ_{iso}	α_{DNC}	β_{DNC}	lb
Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe	27	25	182	78	0	97	8
Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac	12	60	180	84	0	102[a]	8
Boc-AlaGlyl[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac	17	49	188	85	0	104	8
Boc-[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac	33	50	196	93	0	103	8
Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac	37	60	203	100	0	109	8
Boc-[1-^{13}C]Gly[^{15}N]ValGlyAla-OPac	40	58	197	98	0	109	8
[^{15}N]Gly-silk fibroin	22	54	186[a]	87	0	–	
[^{15}N]Ala-silk fibroin	33	56	195	99	–	–	

[a] Ref. [6].

the powder pattern spectra reflect both the ^{15}N chemical shift tensors and $^{13}C-^{15}N$ dipolar interactions present in the peptide. The chemical shift tensor depends on the strength of magnetic field, B_0, while the $^{13}C-^{15}N$ dipolar interaction does not depend on B_0 [29]. The spectra are therefore quite different. Thus, it is possible to analyze the spectra in terms of the principal values of the chemical shift tensor and the orientation of the N–C vector in the chemical shift tensor frame. As defined in Fig. 2(A), β_{DNC} is the angle between the N–C bond and the σ_{33} axis, and α_{DNC} is the angle between the projection of the N–C bond onto the $\sigma_{11}-\sigma_{22}$ plane and the σ_{11} axis. The observed 40.5 and 20.3 MHz spectra can be well reproduced by the simulations as described in Section 3; the parameters are determined as $\sigma_{11} = 27$ ppm, $\sigma_{22} = 25$ ppm, $\sigma_{33} = 182$ ppm, and $\alpha_{DNC} = 0°$, $\beta_{DNC} = 97°$ with high precision (Table 1). The errors in the α_{DNC} and β_{DNC} determinations are within $\pm 1°$. It is interesting that the magnitude of the chemical shift tensor is reversed between σ_{11} and σ_{22}, that is, σ_{22} is a higher field component than σ_{11}. This occurs when α_{DNC} (the angle between the projection of the N–C bond onto the $\sigma_{11}-\sigma_{22}$ plane and the σ_{11} axis) is 0°. When α_{DNC} is assumed to be 90°, σ_{22} is a lower field component than σ_{11}. However, the latter assumption is clearly incorrect [18]. Namely, $\alpha_{DNC} = 0°$ is consistent with the notion that, due to the planar symmetry of the peptide bond, one of the three principal axes of the chemical shift tensor (in this case σ_{22}) is perpendicular to the peptide plane. These problems concerning the definition of the chemical shift tensors have been discussed by Teng et al. [30].

The ^{15}N powder pattern spectra of the other samples were observed at 40.5 MHz except for Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac. Fig. 4 shows the observed (solid line) and simulated (broken line) ^{15}N CP NMR spectra of Boc-[1-^{13}C]Ala[^{15}N] GlyAlaGly-OPac powder at 27.3 MHz. The ^{15}N CP NMR spectra, observed (solid line) and simulated (broken line) ones, of Boc-AlaGly[1-^{13}C]Ala[^{15}N] GlyAlaGly-OPac powder at 40.5 MHz are also shown. The changes in the simulated spectra of Boc-1-^{13}C]Ala-[^{15}N]GlyAlaGly OPac powder at 27.3 MHz are shown in Fig. 5 when the Euler angles, α_{DNC} and β_{DNC}, are changed individually by assuming that the ^{15}N chemical shift tensors are fixed to be $\sigma_{11} = 12$ ppm, $\sigma_{22} = 60$ ppm and $\sigma_{33} = 180$ ppm. The simulated spectra change depends on the change in α_{DNC} (A) and β_{DNC} (B), and thus it is possible to determine the Euler angles with high precision (the error is within $\pm 2°$). The spectral pattern of Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac in Fig. 4 is quite different from that of Boc-[1-^{13}C]Ala[^{15}N]Gly OMe, although the observed NMR frequency is different among these spectra. This mainly comes from the difference in the chemical shift tensors, especially the σ_{22} value, as summarized in Table 1. The ^{15}N chemical shift tensors of Boc-[1-^{13}C]Ala[^{15}N] GlyAlaGly-OPac are close to the ^{15}N chemical shift tensor values of the Gly residue of silk fibroin reported previously [6]. α_{DNC} and β_{DNC} values were determined to be 0° and 102°,

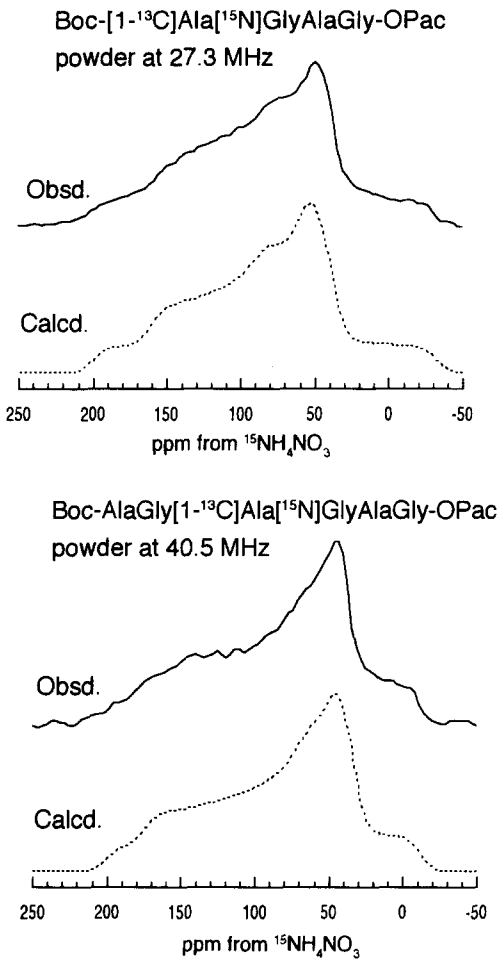

Fig. 4. The observed (solid line) and simulated (broken line) ^{15}N CP NMR spectra of Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac powder at 27.3 MHz (upper). Observed (solid line) and simulated (broken line) ^{15}N CP NMR spectra of Boc-AlaGly[1-^{13}C]Ala[^{15}N] GlyAlaGly-OPac powder at 40.5 MHz are also shown (lower).

respectively. A slightly larger value of β_{DNC} (104°) is obtained by the simulation of the spectrum of Boc-AlaGly[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac observed at 40.5 MHz, although α_{DNC} is still 0° [6,8,10,18,21]. Thus, the β_{DNC} value for the [^{15}N]Gly residue tends to be larger when the length of the sequence containing the Gly residue becomes longer.

^{15}N CP NMR spectra are shown for the ^{15}N Ala residue in Boc-[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac (A) and Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac powders (B) in Fig. 6. The chemical shift tensor values are almost the same as those of the ^{15}N Ala residue of silk fibroin as summarized in Table 1. The β_{DNC} values increase for the longer peptide compound which is in agreement with the case of the ^{15}N Gly residue mentioned above. The ^{15}N NMR powder pattern spectrum of ^{15}N Val residue of Boc-[1-^{13}C]Gly[^{15}N]Val-GlyAla-OPac was also observed as shown in Fig. 6(C). The α_{DNC} and β_{DNC} values were 0° and 109°, respectively (Table 1). The chemical shift tensor values are almost the same as those of the ^{15}N Ala site.

4.2. ^{13}C powder pattern spectra

Fig. 7 shows the observed (solid line) and simulated (broken line) ^{13}C powder pattern spectra of carbonyl carbons in Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe (100.4 MHz), Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac (68 MHz) and Boc-AlaGly[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac (100.4 MHz). The ^{13}C powder pattern spectra are more symmetrical than the ^{15}N powder pattern spectra. A similar simulation to that for the ^{15}N powder pattern spectra was performed in order to determine Euler angles, α_{DCN} and β_{DCN}, where α_{DCN} was 90°[17,29–31]. The definition of the Euler angles, α_{DCN} and β_{DCN} is shown in Fig. 2(B); for example, β_{DCN} is the angle between σ_{33} and the C–N bond where σ_{11} is perpendicular to the peptide plane and σ_{33} and σ_{22} lie in the peptide plane. The magnitude of the chemical shift tensor, σ_{22} of Boc-[1-^{13}C]Ala-[^{15}N]Gly-OMe is considerably different from the σ_{22} values of the latter two peptides as listed in Table 2. This reflects the difference in the intermolecular interactions such as hydrogen bonding among these compounds and will be discussed below. The β_{DCN} values differ by 3° between the first two peptides and the third peptide.

Fig. 8 shows ^{13}C powder pattern spectra of Boc-[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac (A), Boc-GlyAla [1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac (B) and Boc-[1-^{13}C]Gly [^{15}N]Val-GlyAla-OPac (C). The difference between the first two spectra is small, indicating similar chemical shift tensor and β_{DCN} values. The results are summarized in Table 2.

5. Discussion

With the recent ability to relate the chemical shift tensor to a dipolar interaction that has a unique axis

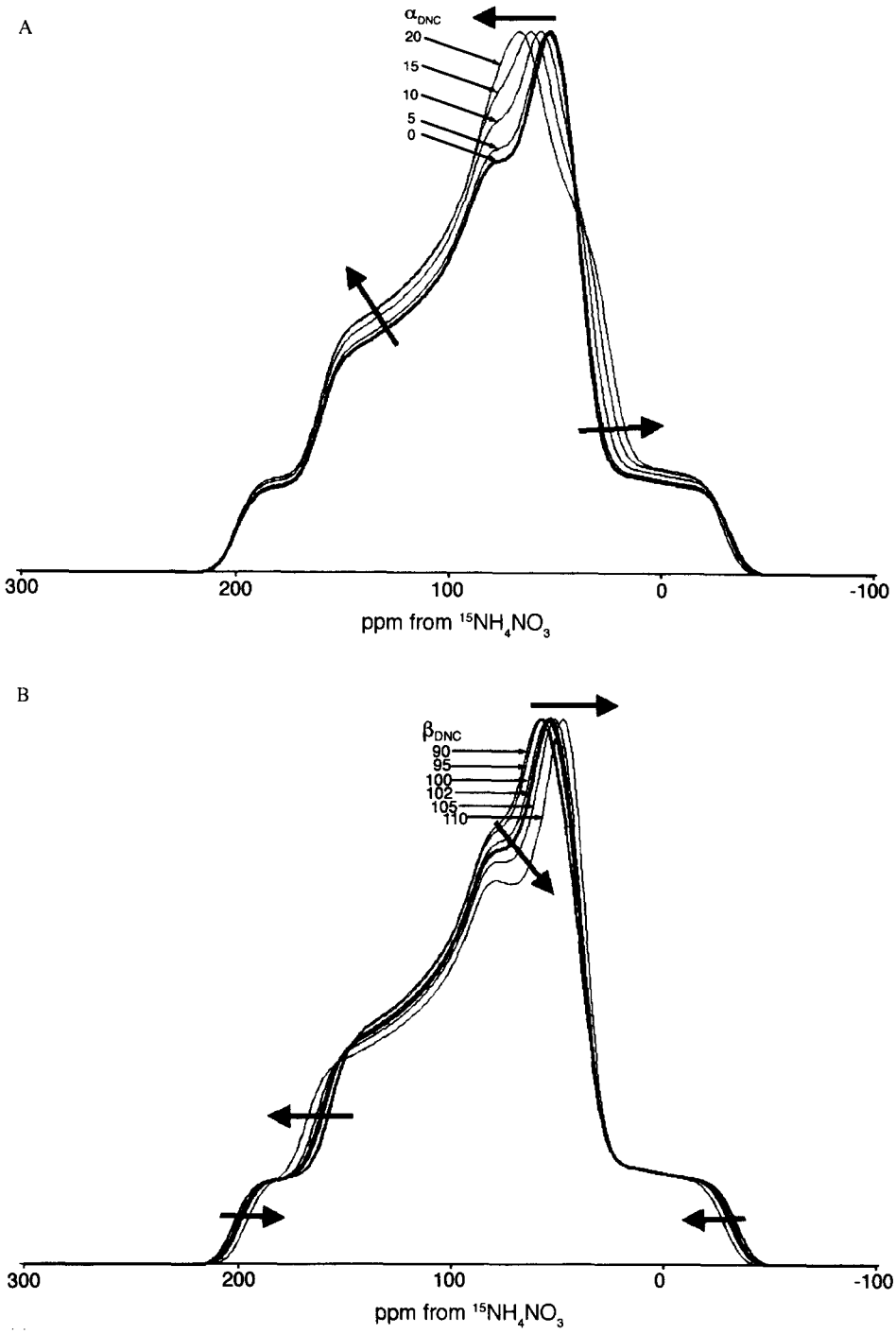

Fig. 5. The simulated 27.3 MHz ^{15}N CP NMR spectra of Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac as functions of the Euler angles, α_{DNC} and β_{DNC}, assuming σ_{11} = 12 ppm, σ_{22} = 60 ppm, and σ_{33} = 180 ppm. The spectra (A) were calculated with β_{DNC}; 102 deg as a function of α_{DNC}; 0, 5, 10, 15 and 20 deg. The spectra (B) were calculated with α_{DNC}; 0 deg as a function of β_{DNC}; 90, 95, 100, 105 and 110 deg. Bold lines correspond to α_{DNC} = 0° and β_{DNC} = 102°.

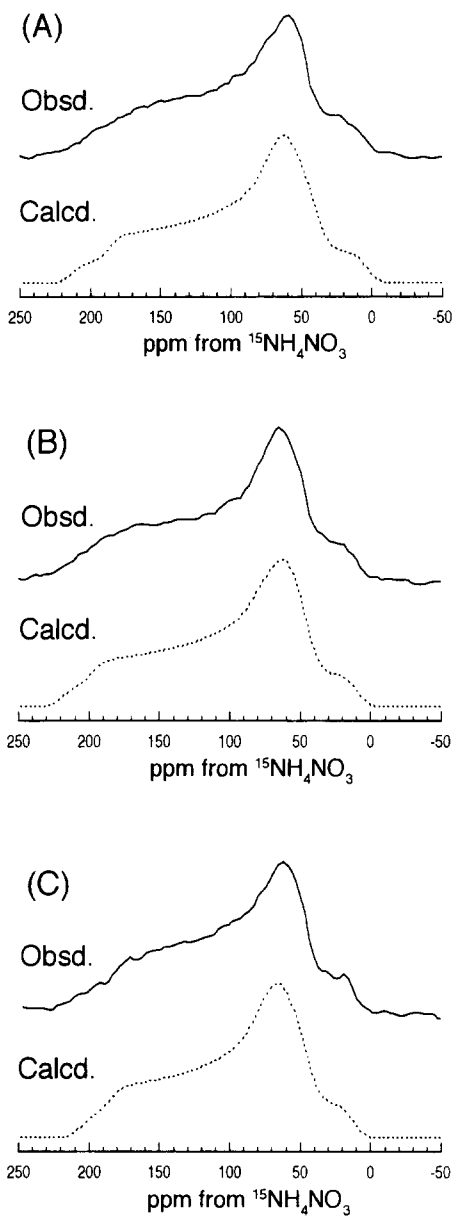

Fig. 6. Observed (solid line) and simulated (broken line) ^{15}N CP NMR spectra of Boc-[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac (A), Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac (B) and Boc-[1-^{13}C]Gly[^{15}N]Val-GlyAla-OPac (C) at 40.5 MHz.

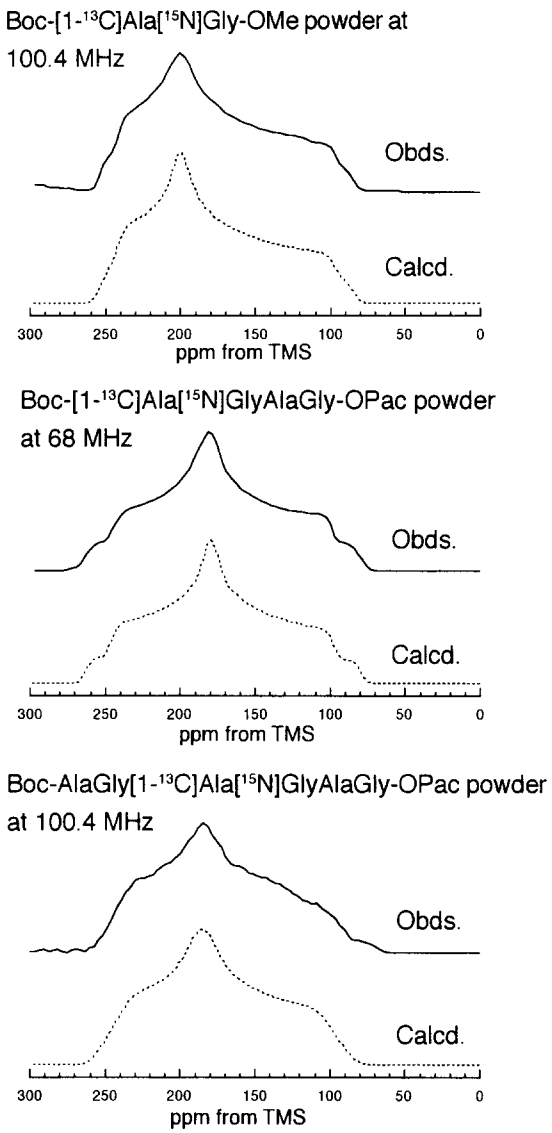

Fig. 7. Observed (solid line) and simulated (broken line) ^{13}C CP NMR spectra of Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe (upper) and Boc-AlaGly[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac (lower) powder at 100.4 MHz and Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac (middle) at 68 MHz.

fixed in the molecular frame, it is possible to consider a determination of the chemical shift tensor orientation for specific sites in the molecule of interest. The powder pattern fitting method used here has proven very sensitive to both the chemical shift tensors and the molecular orientation [15–22]. This method is as accurate as the single-crystal method because of the excellent agreement between the shift tensor values of GlyGly HCl determined by the single-crystal method and by the powder pattern fitting method of Oas et al. [17]. One advantage of the method is its instrumental simplicity.

Table 2
Chemical shift tensors and Euler angles relating to the MSA for ^{13}C PAS of various ^{13}C–^{15}N double labeled model peptides and ^{13}C single labeled *B. mori* silk fibroins (silk II) which were determined from spectral simulation with line broadening (lb)

Sample	σ_{11}	σ_{22}	σ_{33}	σ_{iso}	α_{DCN}	β_{DCN}	lb
Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe	91	202	247	180	90	36	4
Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac	89	183	253	175	90	36	4
Boc-AlaGly[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac	95	187	247	177	90	33	4
Boc-[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac	94	176	248	173	90	33	4
Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac	91	174	248	171	90	35	4
Boc-[1-^{13}C]Gly[^{15}N]ValGlyAla-OPac	88	168	240	165	90	35	8
[1-^{13}C]Ala-silk fibroin	96	186	242	175	–	–	
[1-^{13}C]Gly-silk fibroin	99	179	245	174	–	–	

The method was applied to ^{15}N and ^{13}C backbone sites of the model peptides of *B. mori* silk fibroin. The structures of all ^{13}C–^{15}N double labeled model peptides used here are basically β-sheet as judged from the Cβ chemical shifts of Ala residues in the ^{13}C CP/MAS NMR spectra [20–23]. However, there is a significant difference in the ^{15}N chemical shift tensors, σ_{11} and σ_{22}, for the Gly residue between Boc-[1-^{13}C]Ala-[^{15}N]Gly OMe and Boc-[1-^{13}C]Ala[^{15}N]Gly AlaGly-OPac as shown in Fig. 9(A). Oas et al. [18] showed that the values of the ^{15}N chemical shift tensors are affected by the lattice environment of the nucleus in several model dipeptides although there are no significant differences in the molecular orientations of these tensors. The hydrogen bonding effect on ^{15}N NMR chemical shifts of the Gly residue of several oligopeptides (X–Gly–Gly) in solid state has been studied by Kuroki et al. [32]; the decrease of the NH bond length leads to a linear increase in the ^{15}N shielding. Thus, the significant difference in the ^{15}N chemical shift tensors, σ_{11} and σ_{22}, is considered due to the difference in the intermolecular interactions such as hydrogen bonding between two peptide molecules. Contrary to the case of Oas et al., the molecular orientations of the tensors are also clearly changed; the difference in the angle β_{DNC} is 5°. Such a significant difference is also observed for the chemical shift tensor value, σ_{22}, of the carbonyl carbons of the Gly residues between Boc-[1-^{13}C]Ala-[^{15}N]Gly OMe and Boc-[1-^{13}C]Ala[^{15}N]Gly AlaGly-OPac (Fig. 9(B)). The ^{13}C chemical shift tensors have been determined for the Gly carbonyl carbon in a homologous series of peptides by Oas et al. [17]; there is no significant difference in σ_{11} among the five peptides used by them and this tendency is also observed for σ_{33}, but the σ_{22} values show a large variation among the peptides. Here the definition of the chemical shift tensors is the same as ours. Ando et al. [33] reported that the solid-state isotropic chemical shifts of the carbonyl carbons in Gly residues which are contained in a series of peptides move linearly downfield with a decrease in the hydrogen-bond length between nitrogen and oxygen determined by X-ray or neutron diffraction studies. Similarly, this tendency was also observed for the solid-state isotropic chemical shifts of the carbonyl carbons of Ala residues in several peptides by Asakawa et al. [34]. Among three chemical shift tensor values, such a change in the isotropic chemical shift predominantly arises from the change in the σ_{22} values. MacDermott also reported a linear relationship between the hydrogen bonding strength measured by IR and the σ_{22} values of the carbonyl carbons with well known crystal structures [35]. Thus, the difference in the σ_{22} values of the carbonyl carbons of the Gly residues between two peptides used here is due to the difference in the intermolecular interactions such as hydrogen bonding. This conclusion is the same as that obtained from ^{15}N solid state NMR mentioned above. Molecular orbital calculations of the paramagnetic contribution to the carbonyl carbon chemical shifts indicated that the variations observed in σ_{22} for different molecules are due primarily to variations in the excitation energy and molecular orbital coefficient of the n–π^* carbonyl electronic transition [36]. Takegoshi et al. [37] showed that a large part of the variation in the n–π^* carbonyl electronic transition may be due to different strengths of hydrogen bonds to the carbonyl oxygen.

As summarized in Fig. 9, there is no significant

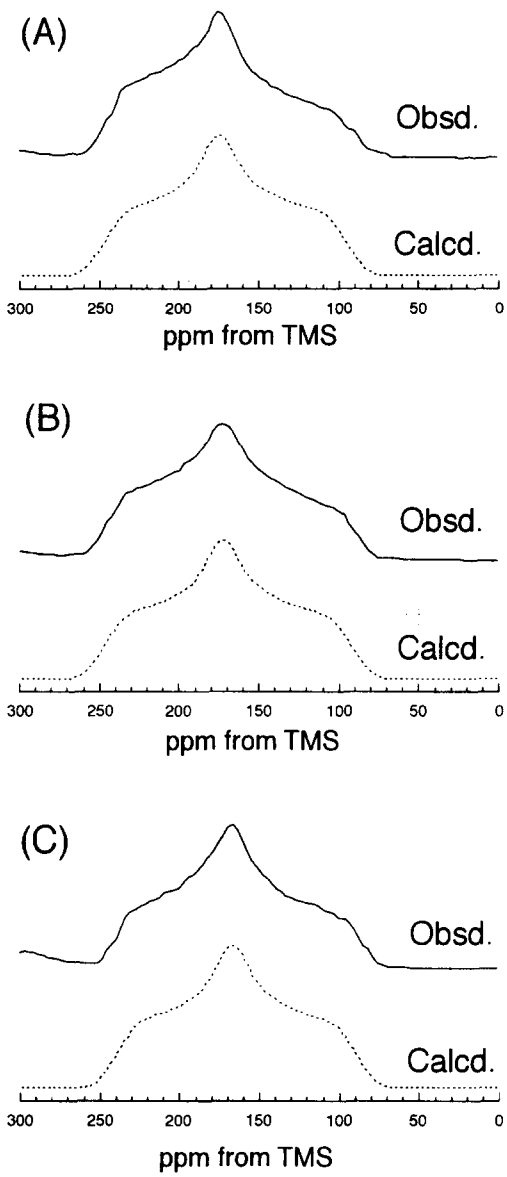

Fig. 8. Observed (solid line) and simulated (broken line) ^{13}C CP NMR spectra of Boc-[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac (A), Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac (B) and Boc-[1-^{13}C]Gly[^{15}N]Val-GlyAla-OPac (C) at 100.4 MHz.

difference in the ^{15}N chemical shift tensors of the [^{15}N]Gly residue between Boc-[1-^{13}C]Ala[^{15}N] GlyAlaGly-OPac and Boc-AlaGly[1-^{13}C]Ala-[^{15}N]GlyAlaGly-OPac. There is also no significant difference in the ^{15}N chemical shift tensors of the [^{15}N]Ala residue between Boc-[1-^{13}C]Gly[^{15}N] Ala-GlyAla-OPac and Boc-GlyAla[1-^{13}C]Gly[^{15}N] AlaGlyAla-OPac, and also the ^{13}C chemical shift tensors of the carbonyl carbon of Gly or Ala residue between four and six amino acid residue peptides.

The Euler angle, $\alpha_{DNC} = 0°$ determined here for ^{15}N nuclei is in agreement with previous values reported by Mai et al. [21], Oas et al. [18] and Hartzell et al. [19]. However, the angle, β_{DNC}, tends to be slightly larger by about 5° which is independent of the species of the amino acid residues. As mentioned above, the value of β_{DNC} obtained for Boc-[1-^{13}C]Ala-[^{15}N]Gly OMe is smaller than those for Boc-[1-^{13}C]Ala[^{15}N] GlyAlaGly-OPac and Boc-AlaGly [1-^{13}C]Ala[^{15}N [GlyAlaGly-OPac. Thus, the smaller angles, β_{DNC}, obtained for small peptides such as dipeptides [18,19] compared with those determined for larger peptides, tetrapetides or hexapeptides, used here might reflect differences in intermolecular interactions such as hydrogen bonding among dipeptides, tetrapeptides and hexapeptides.

On the other hand, systematic changes in the value of the angle, β_{DCN}, for the ^{13}C nuclei of several peptides were not observed when the length of the sequence was changed. The values determined here are in agreement with the reported values for individual amino acid residues [17,30]. Euler angles α_{DCN} and β_{DCN} for the Ala carbonyl carbon in Acetyl-AlaGly-NHMe, and for the Gly carbonyl carbons in Acetryl-GlyAla-NHMe and Ac-Gly Val-NHMe molecules, were calculated using the FPT-INDO method as 90.1–90.2° for α_{DCN} and 35.3–37.2° for β_{DCN} [22]. In the calculation, the peptides were assumed to have an antiparallel β sheet structure. Thus, the values determined from the spectral simulation for six peptides (Figs. 7 and 8), that is, 90° for α_{DCN} and 33–36° for β_{DCN} are in agreement with the calculated values. Hereafter, the Euler angles α_{DNC}, β_{DNC}, α_{DCN} and β_{DCN} determined here will be used for the determination of ϕ and φ angles of Gly, Ala and Val residues of *B. mori* silk fibroin with β-sheet structure.

In addition, recently, ^{15}N chemical shift anisotropy as well as the internal dynamics of the peptide backbone of protein has been reported from quantitative measurement of relaxation interference effects using solution NMR by Tjandra et al. [38]. It

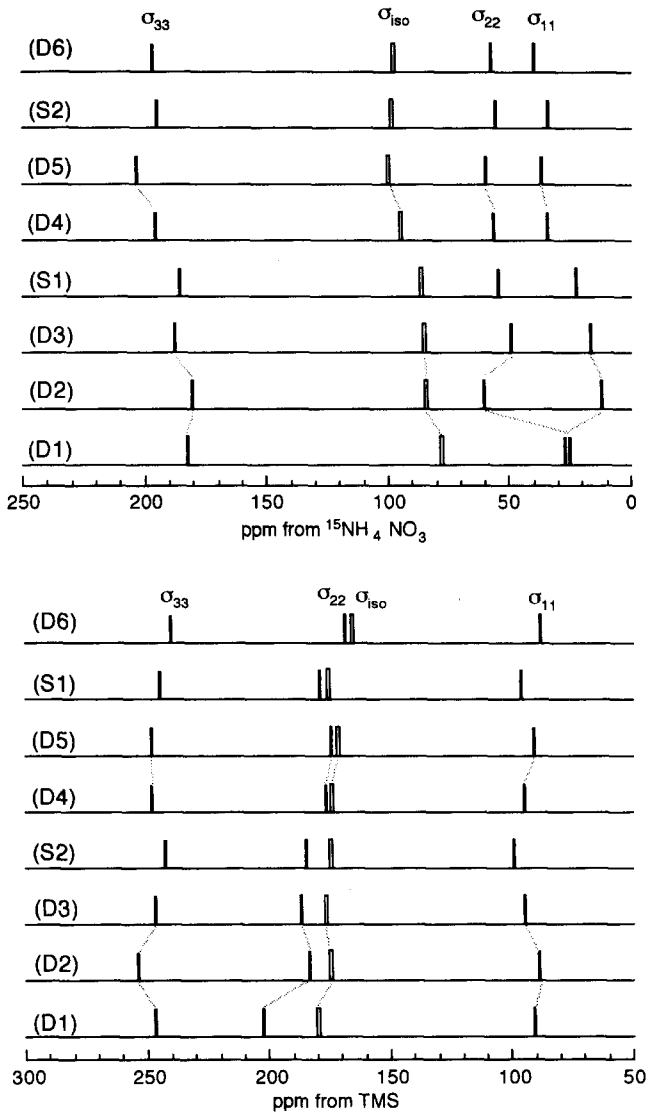

Fig. 9. ^{15}N (A) and ^{13}C (B) chemical shift tensors of various ^{13}C–^{15}N double labeled model peptides, Boc-[1-^{13}C]Ala[^{15}N]Gly-OMe (D1), Boc-[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac (D2), Boc-AlaGly[1-^{13}C]Ala[^{15}N]GlyAlaGly-OPac (D3), Boc-[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac (D4), Boc-GlyAla[1-^{13}C]Gly[^{15}N]AlaGlyAla-OPac (D5) and Boc-[1-^{13}C]Gly[^{15}N]ValGlyAla-OPac (D6), and ^{15}N Gly (S1), ^{15}N Ala (S2), ^{13}C Gly (S3), and ^{13}C Ala (S4) single labeled *B. mori* silk fibroins (silk II).

will be interesting to compare the chemical shift tensor information from solution NMR with that from solid state NMR.

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