

Determination of distance of intra-molecular hydrogen bonding in (Ala–Gly)₁₅ with silk I form after removal of the effect of MAS frequency in REDOR experiment

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Abstract

It is important to know the structure of silk I (*Bombyx mori* silk structure before spinning in the solid state) in order to understand the mechanism of fiber formation at the atomic level. In this study, ¹⁵N-dephased, ¹³C-observe REDOR has been carried out to determine the atomic distance of intra-molecular hydrogen bond between the ¹³C=O carbon of the 14th Gly residue and the ¹⁵N nitrogen of the 17th Ala residue of (AG)₆A[1-¹³C]GAG[¹⁵N]AG(AG)₆ with silk I form after removal of the effect of MAS frequency on the re-coupling. The distance was determined to be 4.3 Å, which confirmed the intra-molecular hydrogen bonding formation between these two atomic sites.

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1. Introduction

One of the most powerful capabilities of solid-state NMR is the measurement of inter-nuclear distances from dipolar couplings in solid samples. With the advance of routine magic-angle spinning (MAS) to enhance sensitivity and average chemical shift anisotropies, an analogous technique, rotational-echo double resonance (REDOR) [1–3], was developed for measuring hetero-nuclear dipolar couplings in rotating solids. Recently, the evaluation of long-distance (>5 Å) interactions using ¹³C–¹⁵N dephasing was reported by Arshava et al. [4]. They pointed out ¹⁵N-dephased, ¹³C-observe REDOR measurements are reliable for distances up to 6.0 Å, and the dipolar interactions can be detected for distances up to 7 Å. In the past few years, the REDOR techniques have been developed to determine inter-nuclear distances in biological solids such as peptides, proteins aggregates, and membrane proteins [5–13].

We recently used REDOR method along with 2D spin diffusion NMR, to determine the less stable silk I form [14,15]. The structure of silk I has remained poorly understood because attempts to induce orientation of the silk I form for studies by X-ray diffraction, electron diffraction, or solid-state NMR causes to convert from the silk I form to the more stable silk II form (the structure of *Bombyx mori* silk fibroin fiber after spinning) easily. Consequently, structural investigations of the silk I form have been based on model building of peptides such as (Ala–Gly)_n and comparison with limited experimental data, resulting in a number of conflicting models describing the structure of silk I [15–18]. The wide-angle X-ray scattering (WAXS) pattern calculated for these models could not reproduce the same intensity pattern as observed for silk I [19], and some of the models are in conflict with the ¹³C chemical shift data, used to predict the backbone conformation [20]. It is possible to prepare (Ala–Gly)_n with silk I form after the sample was dissolved in 9 M LiBr aqueous solution and then dialyzed against distilled water. The conformation of (Ala–Gly)₁₅ with silk I form has been clarified to be repeated type II β-turn structure with several solid-state NMR techniques coupled with stable isotope

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labeling of the peptide as shown in Fig. 1 [14]. This model can also reproduce the WAXS pattern of silk I very well [15].

In the repeated β -turn like structure, there are intra-molecular hydrogen bonds between the carbonyl oxygen atom of the i th Gly residue and the amide hydrogen atom of the $(i+3)$ th Ala residue, between the amide hydrogen atom of the $(i+1)$ th Ala residue and the carbonyl oxygen atom of the $(i-2)$ th Gly residue, and between the carbonyl oxygen atom of the $(i+2)$ th Gly residue and the amide hydrogen atom of the $(i+5)$ th Ala residue and so on. These hydrogen bonding distance between the carbonyl carbon and amide nitrogen in the model was calculated to be $4.0 \pm 0.1 \text{ \AA}$.

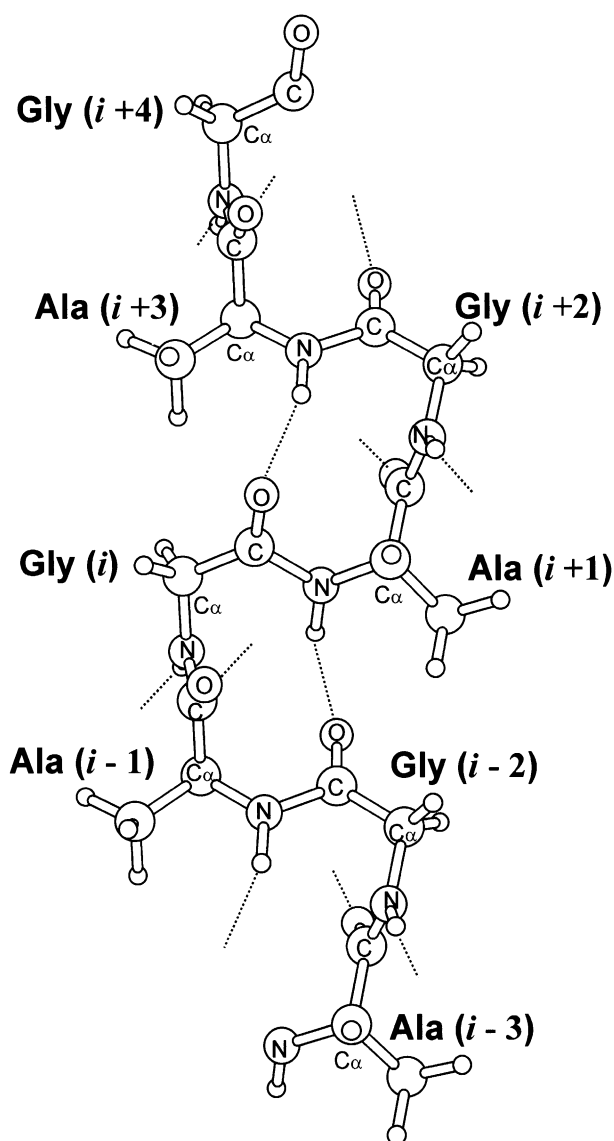


Fig. 1. The conformation of a repeated β -turn type II model proposed for silk I structure. There are intra-molecular hydrogen bonds between the carbonyl oxygen atom of the i th Gly residue and the amide hydrogen atom of the $(i+3)$ th Ala residue [14].

In this study, we observed REDOR spectra of $(\text{AG})_6\text{A}[1-^{13}\text{C}]\text{GAG}[^{15}\text{N}]\text{AG}(\text{AG})_6$ with silk I form at a variety of MAS frequencies to determine the distance of the intra-molecular hydrogen bond between the $^{13}\text{C}=\text{O}$ carbon of the 14th Gly residue and the ^{15}N nitrogen of the 17th Ala residue. From the extrapolation of MAS frequency to zero in the plot of $\Delta S/S_0$ vs. MAS frequency, we removed the effect of the MAS frequency on the REDOR experiments.

2. Methods

2.1. Materials

Selectively labeled peptide $(\text{AG})_6\text{A}[1-^{13}\text{C}]\text{GAG}[^{15}\text{N}]\text{AG}(\text{AG})_6$ (99 at.% ^{13}C : 99 at.% ^{15}N) was prepared by solid-phase synthetic methods [14]. The atomic positions of the ^{13}C and ^{15}N labels were chosen according to intra-hydrogen bond between Gly and Ala residues established previously. All selectively labeled peptides were dissolved in 9 M LiBr aqueous solution and then dialyzed against distilled water to prepare the silk I form.

2.2. REDOR NMR

All NMR experiments were performed on a Chemagnetics CMX Infinity 400 MHz spectrometer equipped with a solid-state accessory and a triple-channel magic-angle probe with a 4 mm–9 turns coil (HXY Triple Resonance T-3 Probe produced by Chemagnetics). The spinning frequencies for the REDOR experiments were 2500–8333 Hz. ^{13}C REDOR NMR spectra were obtained at 100.0 MHz following matched, ^1H – ^{13}C cross polarization contacts of 83 kHz. Contact times of 1.0 ms were used. ^1H decoupling fields were 93 kHz during the dipolar evolution period and 50 kHz during data acquisition. The π pulses for ^{13}C and ^{15}N channels were 6.0 and 8.2 μs , respectively. A recycle delay of 3.0 s was used throughout these experiments and the data were obtained with 64 or 128 transients. Phases of ^{15}N π pulses were cycled according to the XY-4 scheme to minimize off-resonance and pulse error effects [21,22]. REDOR evolution times ranged up to 24.0 ms. Values of $\Delta S/S_0 = 1 - S/S_0$ (where S_0 and S represent the reference and dipolar dephasing experiments, respectively) were computed as the ratios of peak heights in the REDOR spectra.

3. Results and discussion

REDOR experiments were done at $N_c T_r$ of 6.0, 12.0, 18.0, 21.6, and 24.0 ms, where N_c is the number of rotor cycles during the evolution period and T_r is the rotor period in MAS. Rotor cycles for each MAS frequency are listed in Table 1.

Table 1
The number of rotor cycles for each spinning frequency at given NcTr

| NcTr (ms) | Spinning rate (Hz) | | | | | | |
|-----------|--------------------|------|------|------|------|------|--|
| | 2500 | 3333 | 4000 | 5000 | 6666 | 8333 | |
| 6.0 | – | 20 | 24 | 30 | 40 | 50 | |
| 12.0 | 30 | 40 | 48 | 60 | 80 | – | |
| 18.0 | – | 60 | 72 | 90 | 120 | 150 | |
| 21.6 | 54 | 72 | – | 108 | 144 | 180 | |
| 24.0 | 60 | 80 | 96 | 120 | 160 | – | |

Representative ^{13}C -observe REDOR data for a $^{13}\text{C}/^{15}\text{N}$ -doubly labeled peptide of $(\text{AG})_6\text{A}[1-^{13}\text{C}]\text{GAG}$ [^{15}N] $\text{AG}(\text{AG})_6$ are displayed in Fig. 2, illustrating the

full-echo (S_0), rotational spin-echo with ^{15}N π pulse applied (S) and substantial echo (ΔS) amplitudes retained at 24.0 ms of evolution time and MAS frequency

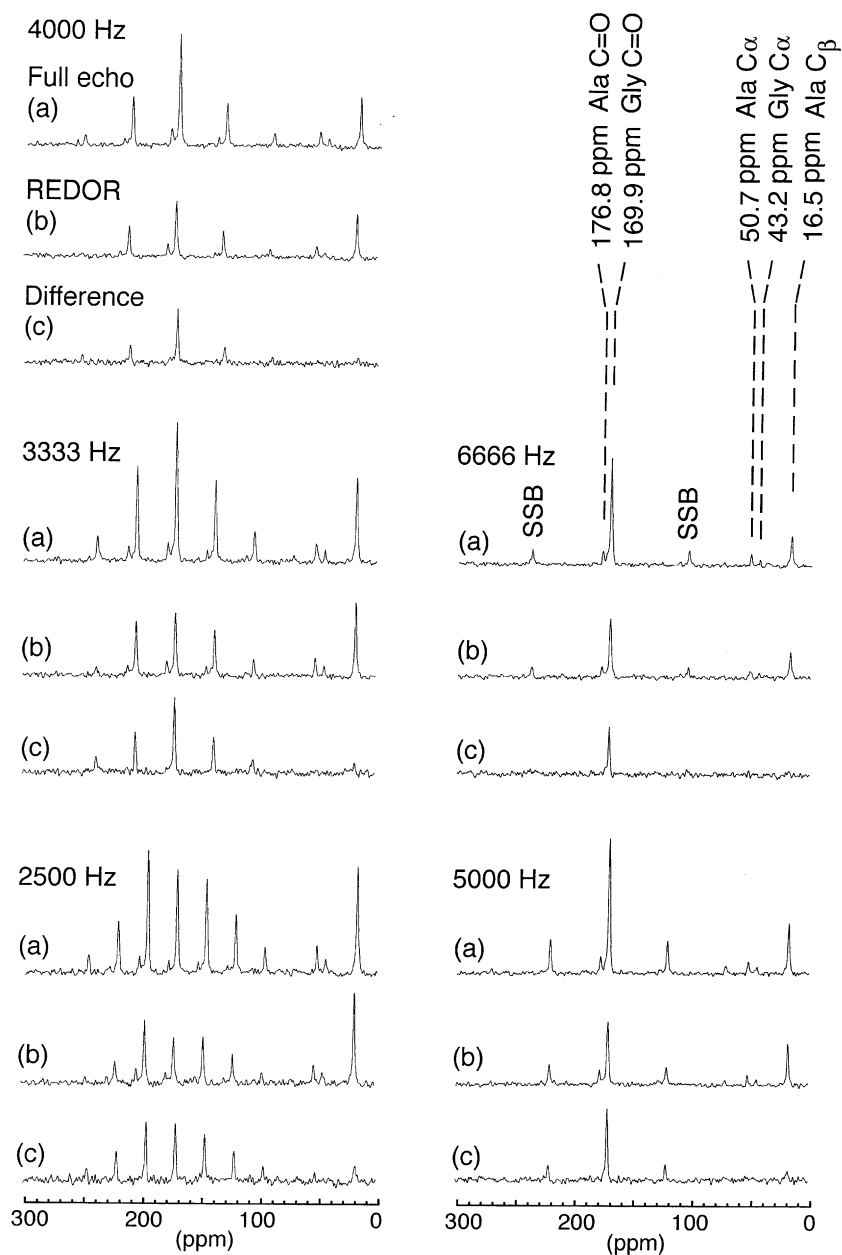


Fig. 2. ^{13}C NMR spectra for the $(\text{AG})_6\text{A}[1-^{13}\text{C}]\text{GA}[^{15}\text{N}]\text{G}(\text{AG})_7$. The full echo spectra (a), REDOR spectra (b), and the difference spectra (c) retained at 24.0 ms of evolution time and MAS frequency at 2500, 3333, 4000, 5000, and 6666 Hz.

at 2500, 3333, 4000, 5000, and 6666 Hz. For all of spectra, good signal-to-noise ratios were achieved.

The data of $\Delta S/S_0$ were obtained for a number of echo intervals by taking into account all of the sidebands to remove the orientation effects [6]. The $\Delta S/S_0$ for $N_c T_r$ of 6.0, 12.0, 18.0, 21.6, and 24.0 ms were plotted as a function of MAS frequency, shown in Fig. 3. The linear relation between $\Delta S/S_0$ and MAS frequency is evident. At given $N_c T_r$, an increase in MAS frequency from 2500 to 8333 Hz resulted in an increase in the $\Delta S/S_0$. Although, the REDOR measurements at $N_c T_r$ of 6.0, 12.0, 18.0, and 21.6 ms were carried out for the sample volume of 60 μl , the measurement at 24 ms was carried out for 60 and 20 μl in order to check the B_1 inhomogeneity [6]. Both of the samples were packed in the center part of a zirconia rotor. In Fig. 3, no difference was found between the sample volume of 60 and 20 μl at $N_c T_r$ of 24.0 ms, which indicates that contribution to the REDOR factor from B_1 inhomogeneity is insignificant in this study.

The pulse intervals for ^{15}N π pulses depend on the MAS frequency. The rotor periods at MAS frequency of 2500, 3333, 4000, 5000, 6666, and 8333 Hz are 400, 300, 250, 200, 150, and 120 μs , respectively. Therefore, the fraction of the 1/2 rotor period occupied by 8.2 μs of ^{15}N π pulse length at MAS frequency of 2500, 3333, 4000, 5000, 6666, and 8333 Hz are 4.1, 5.5, 6.6, 8.2, 10.9, and 13.7%, respectively. Such ^{15}N π pulse length is called the finite ^{15}N π -pulse length. The fraction of the rotor period occupied by ^{15}N RF pulses affect the REDOR

results. The first theoretical approach to calculate the effect of the finite ^{15}N π -pulses length on the REDOR was presented by Schmidt and Vega [23]. Naito et al. [6] considered it to be negligible as long as the pulse length is less than 10% of the rotor cycle. More recently, Jaroniec et al. [24] investigated the REDOR experiments for Gly-OH under conditions where a significant fraction of the rotor period (10–60%) is occupied by ^{15}N π -pulses and pointed out that the finite pulses give minor effect on the dipolar scaling factor expected for REDOR with ideal δ -function pulses. Using their derived analytical expressions, we calculated the $\Delta S/S_0$ factors at the dipolar coupling constant of 38.4 Hz (corresponding to a C...N distance of 4.30 \AA) as a function of the fraction of ^{15}N π pulse length (data not shown), which indicate that the effect of the finite π pulse length is a minor scaling of $\Delta S/S_0$.

Next, we considered the effect of cross relaxation [25] between ^{15}N and ^1H spins, which might be occurred because the ^1H decoupling field was close in strength to the ^{15}N RF field ($\omega_{\text{H}}^1/\omega_{\text{N}}^{15} = 1.52$). The effect of cross relaxation on REDOR would become larger as the numbers of inversion times of ^{15}N spin during evolution time increase. At given evolution time, the number of inversion time for ^{15}N spin increases with increasing MAS frequency. For example, at MAS frequency of 2500 and 6666 Hz, ^{15}N spins invert 118 and 318 times, respectively, during 24.0 ms evolution time. Therefore, it can be considered that the linear relation between $\Delta S/S_0$ and MAS frequency as shown in Fig. 3 responsible for the effect of $^{15}\text{N}\cdots^1\text{H}$ cross relaxation during evolution time. As the ^1H decoupling power is increased, the $^{15}\text{N}\cdots^1\text{H}$ cross relaxation is decreased, and its relaxation can be minimized by setting the RF field intensity for ^1H decoupling to around three times that for ^{15}N π -pulses, or stronger [25]. Indeed, the value of $\Delta S/S_0$ was affected by differing the level of the ^1H decoupling field [26,27]. When the ratio of $\omega_{\text{H}}^1/\omega_{\text{N}}^{15}$ changed from 1.32 to 1.57, the value of $\Delta S/S_0$ at 21.6 ms of evolution time and spinning rate at 8333 Hz was improved from 0.33 to 0.42.

The slope of the variation (k) of the $\Delta S/S_0$ against MAS frequency, depending on evolution time of $N_c T_r$, is shown in Fig. 4. It turns out that the slope of the variation of the $\Delta S/S_0$ against MAS frequency decreases with increasing $N_c T_r$, which suggests that the effect of MAS frequency on the $\Delta S/S_0$ increases with increasing $N_c T_r$. Therefore, the effect of MAS frequency expect to be non-negligible for the evaluation of weak ^{13}C - ^{15}N dipolar coupling, because the long evolution time of $N_c T_r$ for REDOR experiment is needed. The intercepts of the straight lines in Fig. 3 correspond to the MAS frequency of 0 Hz. Therefore, by extrapolating the data back to the MAS frequency of 0 Hz, the effect of the MAS frequency on the REDOR can be removed. The extrapolated values of $\Delta S/S_0$ for $N_c T_r$ of 6.0, 12.0, 18.0,

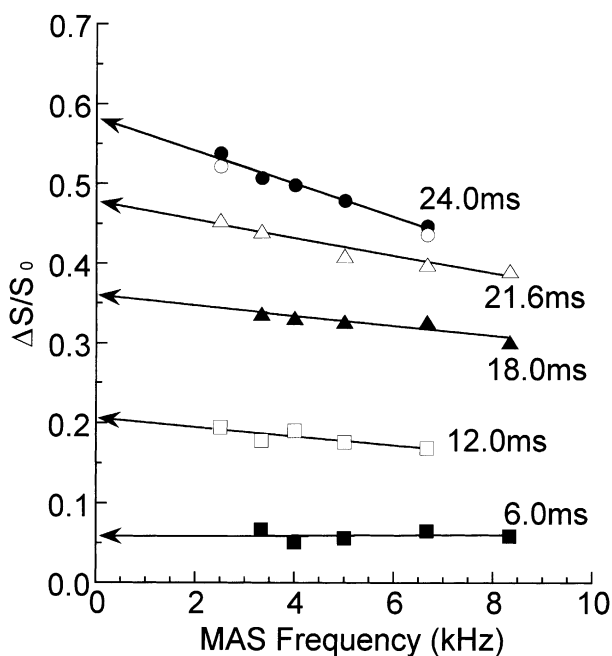


Fig. 3. The experimental ^{13}C REDOR difference of $\Delta S/S_0$ for $N_c T_r$ of 6.0 (■), 12.0 (□), 18.0 (▲), 21.6 (△), and 24.0 (sample volume of 60 μl ; ● and 20 μl ; ○) ms as a function of MAS frequency (kHz).

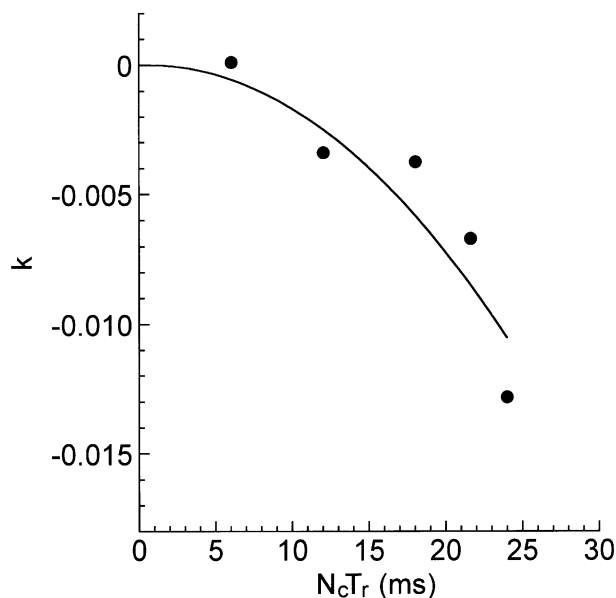


Fig. 4. The relation between the slope of the variation (k) of the $\Delta S/S_0$ against the MAS frequency derived from Fig. 3 and the evolution time of $N_c T_r$ (ms).

21.6, and 24.0 ms are 0.07, 0.27, 0.47, 0.62, and 0.74, respectively.

Although, we used 99% labeled peptides, the Gly residues in the sample of $(AG)_6 A[1-^{13}C]GAG[^{15}N]AG(AG)_6$ has 14 unlabeled C=O sites per labeled residue. These unlabeled spins have the same chemical shift as

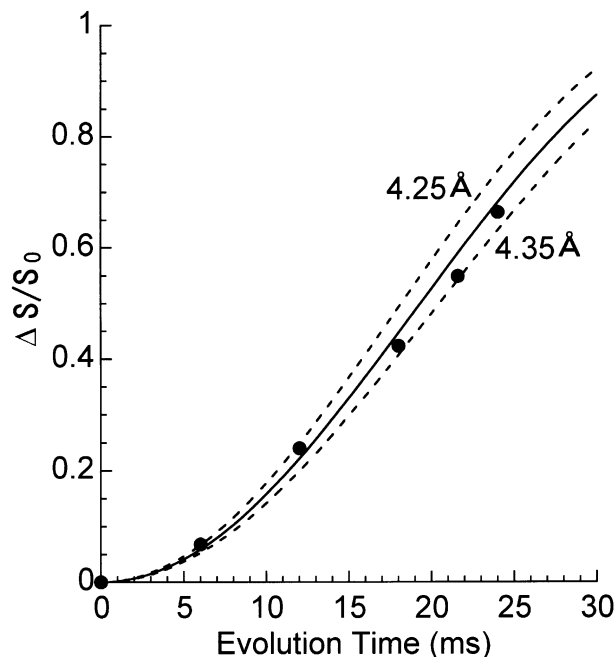


Fig. 5. The relation between the corrected REDOR $\Delta S/S_0$ data (see text) and the evolution time of $N_c T_r$ (ms) for $(AG)_6 A[1-^{13}C]GAG[^{15}N]AG(AG)_6$. Theoretical dephasing curves corresponding to the designated distances are shown.

the labeled (observe) nuclei. To measure distances accurately, REDOR data must be corrected for the effects of natural-abundance spins. Therefore, contributions to the S_0 and S intensities from natural-abundance spins by 12.8% were considered, and corrected data plotted, as shown in Fig. 5. The dotted lines show theoretical dephasing curves corresponding to the designated distances. From comparison between REDOR data and theoretical dephasing curves, the distance between the $^{13}C=O$ carbon of the 14th Gly residue and the ^{15}N nitrogen of the 17th Ala residue in $(AG)_6 A[1-^{13}C]GAG[^{15}N]AG(AG)_6$ was determined to be $4.3 \pm 0.5 \text{ \AA}$. Though the REDOR spectra in this study were observed for isotope-labeled compound without dilution of natural abundance in $(Ala-Gly)_{15}$, the distance was determined to be the same value for mixture of equivalent amount of this isotope-labeled compound and natural abundance [13]. Therefore, $^{13}C/^{15}N$ dipolar coupling with adjacent molecules are negligible. The backbone torsion angles (ϕ , φ) in the model peptide $(Ala-Gly)_{15}$, with the repeated β -turn type II like structure, were determined as $(-60^\circ \pm 5^\circ, -130^\circ \pm 5^\circ)$ and $(70^\circ \pm 5^\circ, 30^\circ \pm 5^\circ)$ for Ala and Gly residues, respectively. If the deviations of $\pm 5^\circ$ for ϕ and φ angles are considered, the predicted distance between the carbonyl carbon atom of the i th Gly residue and the amide nitrogen atom of the $(i+3)$ th Ala residue is 4.0–4.3 \AA . This predicted distance is in excellent agreement with the distance of 4.3 \AA obtained from present study.

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