Structure and Dynamics of Silk Fibroin Studied with ^{13}C, ^{15}N and ^{2}H Solid State NMR

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SUMMARY: [1-^{13}C]Gly, L-[1-^{13}C]Ala, [^{15}N]Gly, L-[^{15}N]Ala, [2,2-$^{2}H_2$]Gly, L-[3,3-$^{2}H_2$]Ser and [3,3,3-$^{2}H_3$]Ala labeled silk fibroin fibers from *Bombyx mori* and *Samia cynthia ricini* silkworms were prepared in order to analyze structure of backbone and dynamics of side chain. The torsion angles ϕ and ψ were determined from the angular dependent ^{13}C and ^{15}N solid state NMR spectra for uniaxially oriented fiber samples. In addition, the characteristic side chain dynamics of Ser residue determined from solid state ^{2}H NMR measurements was compared with those of Ala and Gly residues.

Introduction

Silkworms can produce silk fibroin with strong, stiff fibers at room temperature and from an aqueous solution, whereas synthetic materials with comparable properties must be processed at higher temperatures and/or from less benign solvents[1]. The fact that the impressive mechanical properties of natural silk can be achieved using mild processing condition indicates that global molecular alignment is easily generated and preserved while the fibers are being spun. Thus it is very important to study details of the molecular structure of the silk including the molecular alignment in order to clarify the origin of its impressive mechanical properties.

Orientation-dependent NMR interactions such as dipole-dipole, quadrupole and chemical shift yield structural information of polymers in solid state[2-10]. Namely, if these parameters are observed for each site in oriented polymers, NMR methods can give even the atomic co-ordinates of the polymers in the solid state. This is especially effective in determination of the atomic co-ordinates of peptides or proteins in fibrous proteins. On the other hand, the ^{2}H-NMR lineshape readily reveals molecular anisotropy and motional averaging at a segmental level. The one-dimensional lineshape itself carries much information

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about fast and intermediate dynamics, as well as molecular order in the sample.

In this paper, the solid state NMR study has been applied to elucidate atomic resolution details of the structures of highly ordered silk fibroin fiber. Selective isotope labeling of the sample is usually required in these NMR experiments for obtaining site-specific structural information. This is possible for silk fibroin fiber by oral administration of isotope labeled amino acids to silkworm or by cultivation of the silkglands, which produce silk fibroin in a medium containing isotope labeled amino acids. In addition, the repeating sequence that abounds in silk fibroin simplifies interpretation of the NMR data, since a majority of the observed intensity from isotope labeled amino acid residues will arise from chemically and structurally equivalent sites. The determination of the backbone torsion angles of oriented proteins is the main purpose in the analysis described here together with the molecular dynamics of silk fibers.

Experimental

Materials: The *Bombyx mori* and *Samia cynthia ricini* silk fibroins labeled with [1-^{13}C]Gly, L-[1-^{13}C]Ala, [^{15}N]Gly, L-[^{15}N]Ala, [2,2-$^{2}H_2$]Gly and L-[3,3-$^{2}H_2$]Ser (99 atom%) were obtained by the oral administration method. The [3,3,3-$^{2}H_3$]Ala labeled silk fiber was also prepared with $^{2}H_2O$ (99.8% atom%) by oral administration method because of the presence of the unique metabolic pathway in silkworm. Details of the labeling method used here were described in our previous paper[10]. The dried powder samples were used for powder pattern observation. The uniaxially aligned block samples of the isotope labeled silk fibers with 4.5 x 4.5 x 12 mm block size were prepared for the angular dependent solid state NMR experiment. Undrawn film samples for *B. mori* and *S.c.ricini* silk fibroins were prepared with the aqueous solution extracted from silkglands and cast on a polystyrene plate.

Methods: The static solid-state ^{15}N and ^{13}C NMR experiments were performed at 25°C on a JEOL GX400 spectrometer operating at 40.4 MHz and 100.4 MHz, respectively. The probe was equipped with a 7-mm inner diameter coil. Cross polarization was employed with high-power ^{1}H decoupling during the signal acquisition period. Typical NMR parameters were; 6

μs 90° pulse with a 3 ms mixing time and 7 s repetition delay for ^{15}N NMR and 5 μs 90° pulse with a 3 ms mixing time and 7 s repetition delay for ^{13}C NMR. The ^{15}N chemical shifts were referenced to $^{15}NH_4NO_3$ by setting the signal of solid $^{15}NH_4Cl$ to 18.0 ppm. The ^{13}C chemical shifts were referenced to tetramethylsilane by setting the ^{13}C signal of solid hexamehylbenzene to 17.3 ppm (CH_3).

The ^2H solid state NMR spectra were observed with JEOL GX-400 NMR spectrometer operating at 61.25 MHz equipped a solid state ^2H NMR observation unit. The quadrupole echo pulse sequence, $\pi/2_x$-τ-$\pi/2_y$-τ-echo, was used. The $\pi/2$ pulse was 3.4 μs. The interval between $\pi/2$ pulses, τ, was 30 μs and pulse delay time was 1 s. The fiber axis of the oriented block samples were placed parallel and perpendicular with respect to the applied magnetic field. The molecular dynamics spectral simulation of ^2H labeled site was performed using a program, MXQET, written by Vold[11].

Results and Discussion

Isotope Labeling of Silk Fibroin

Labeling was achieved biosynthetically through the use of an artificial diet supplemented with isotope labeled amino acid during 5th instar larval stage of *Bombyx mori*. For example, twenty mg of [1-^{13}C]Gly was dissolved in water and mixed with 2.7g of artificial diet. This was given to a 5th instar silkworm larva from day 4 to day 8. The labeled silk fibroin samples were obtained as cocoon. Fig. 1 is the solution ^{13}C NMR (expanded carbonyl region) spectra of natural abundance silk fibroin, and [1-^{13}C]Ala and [1-^{13}C]Gly labeled silk fibroins. The labeling ratio was high for both labeled samples and can be used for solid state NMR observation. In the [^{15}N] Gly labeling, a sample with sufficiently high labeling ratio was also obtained. However, the labeling ratio was low in the case of [^{15}N]Ala labeling of the sample. This is due to the high activity of the transaminations in silkworms. Thus, another isotope labeling method is required. Silk fibroin is exclusively synthesized in the posterior silk gland (each cell produces 1015 fibroin molecules, for example, about 80 mg during a period of only 3 to 4 days 5 th instar larva). Therefore, we tried to get [^{15}N]Ala silk

fibroin with high isotope labeling ratio using rotation culture of the posterior silk gland by adding [^{15}N] Ala to Grace medium which has been used as a medium for insect cell culture. The relative intensity of the ^{15}N Ala peak was estimated as 85 % of all NMR peaks. The ^{15}N enrichment of Ala residue was 20 times the natural abundance intensity, which was sufficiently high for solid state ^{15}N NMR analysis. The ^{2}H labeled silk fibroin samples were prepared through the use of an artificial diet supplemented with isotope labeled amino acid during 5th instar larval stage.

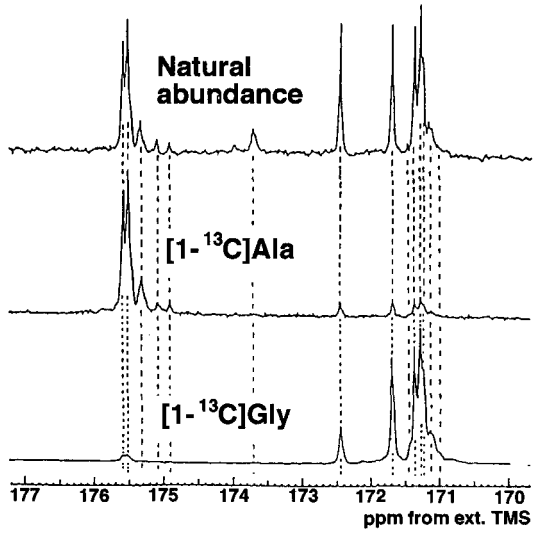


Fig.1: Solution ^{13}C NMR (expanded carbonyl region) spectra of natural abundance silk fibroin, [1-^{13}C]Ala and [1-^{13}C]Gly labeled silk fibroins from *B.mori*.

Determination of Torsion Angles, ϕ and ψ of Ala and Gly residues in *B.mori* Silk Fibroin Fiber

The torsion angles, ϕ and ψ of Ala and Gly residues in *B.mori* silk fibroin fiber can be determined by the solid state NMR. Orientation dependent ^{15}N and ^{13}C solid state NMR spectra of these isotope labeled silk fibroin fibers with silk II structure were observed when the fiber axis was placed parallel and perpendicular to the magnetic field direction. These data

were simulated based on the chemical shift anisotropy to determine the Euler angles from the principal axis system (PAS) to the fiber axis coordinate system.

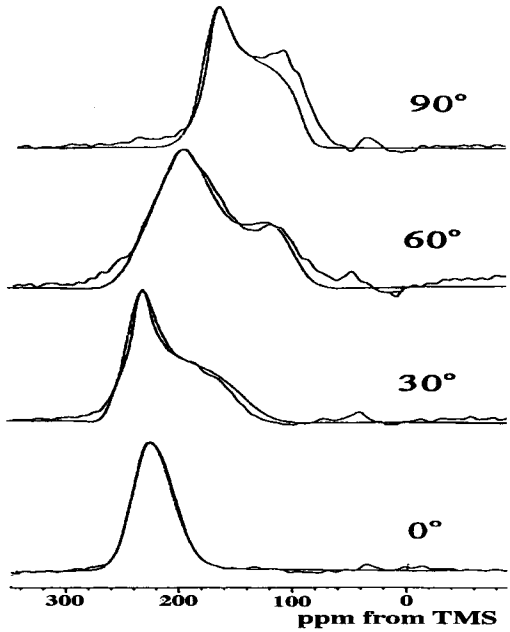
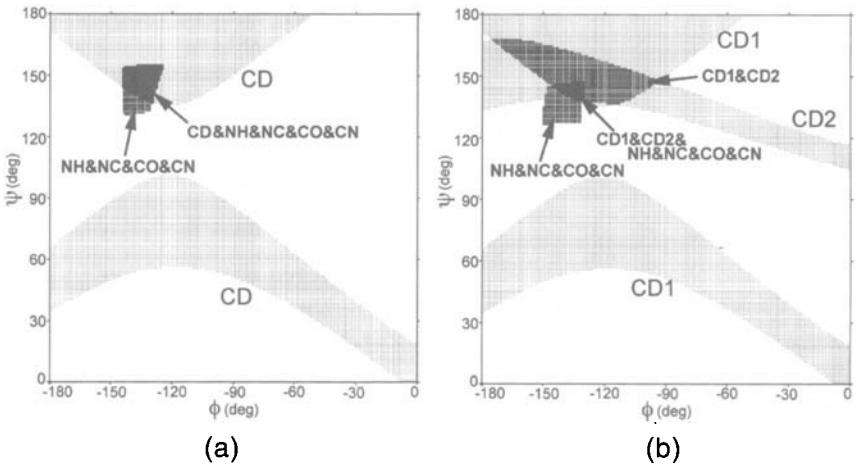


Fig.2: ^{13}C CP NMR spectra of the oriented block of [1-^{13}C]Gly fibroin fibers from *B. mori* as a function of the angles between the oriented fiber axis and the magnetic field Bo.

Fig. 2 shows the ^{13}C CP NMR spectra of the oriented block of [1-^{13}C]Gly fibroin fibers as a function of the angles between the oriented fiber axis and the magnetic field Bo as an example. The agreement between the simulated and observed spectra is good, indicating the high reliability of the structural parameters. Thus, the Euler angles for transforming the chemical shift anisotropy (CSA) principal axis systems (PAS) relative to the fiber axis system (FAS) were determined for carbonyl carbon sites of Gly residue. Similar observations and simulations were performed for ^{15}N Gly site as well as those for [1-^{13}C] and ^{15}N Ala sites to determine these Euler angles. Moreover, the dipolar modulated ^{15}N and ^{13}C powder pattern

spectra of ^{13}C-^{15}N double labeled silk fibroin model peptides are observed and simulated to determine the Euler angles for transforming the PAS to molecular symmetry axis (MSA) system. The specific orientations of N-H, N-C', C'=O, and C'-N bonds for Gly and Ala residues in the oriented silk fibroin fibers were determined with a combination of these Euler angles. Then, the conformational space for the Gly and Ala residues of the silk fibroin fibers was substantially reduced with these bond orientations and the known Cα(i-1)-Cα(i+1) vector orientation from fiber diffraction studies. The solid state ^{2}H NMR spectrum of uniaxially aligned ^{2}H labeled silk fibroin fiber also shows the structure directly *via* ^{2}H quadrupole splitting and can be used to determine the atomic co-ordinates along with the solid state ^{15}N and ^{13}C data of silk fibroin fibers.



Figs.3: Ramachandran plot of the allowed torsion angles (ϕ and ψ) of alanine (a) and glycine (b) in silk fibroin, as determined by solid state ^{13}C, ^{15}N and ^{2}H NMR of uniaxially aligned silk fibers from *B. mori*.

Figs. 3a and 3b are Ramachandran plots of the allowed torsion angles (ϕ and ψ) of Gly and Ala residues in *B.mori* silk fibroin, as determined by solid state ^{13}C, ^{15}N and ^{2}H NMR of uniaxially aligned silk fibers. The area NH & NC & CO & CN corresponds to the respective angles of the bond vectors relative to the fiber axis, as obtained from ^{15}N and ^{13}C NMR studies.

The two curves CD_1 and CD_2 in Figure 3 represent the Ca-$^2H_2$ bond vectors of Gly, and the curve, CD, in Figure 4 , the Cα-Cβ$^2H_3$ bond vector of the Ala methyl group by ^2H NMR. The width of the curves represents the experimental error. Finally, the best fit torsion angles within the reduced conformational space for Gly and Ala residues were determined as (-139 °, 135 °) and (-140 °, 142 °), respectively within experimental error (+/-5°). The unit cell length determined by solid state NMR is in excellent agreement with the fiber diffraction data.

Dynamics of Ala, Gly and Ser residues in Silk Fibroin Fibers

Dynamics of [2,2-$^2H_2$]Gly residues of *B. mori* silk fibroin fiber were analyzed from solid state ^2H NMR powder pattern as shown in Fig. 4. The splitting of Δn_Q = 117.9 kHz is slightly smaller than the expected rigid-lattice value of about 126 kHz. This indicates that methylene group of the glycine residue is essentially restricted in space and undergoes only some small-amplitude vibrational motion at room temperature.

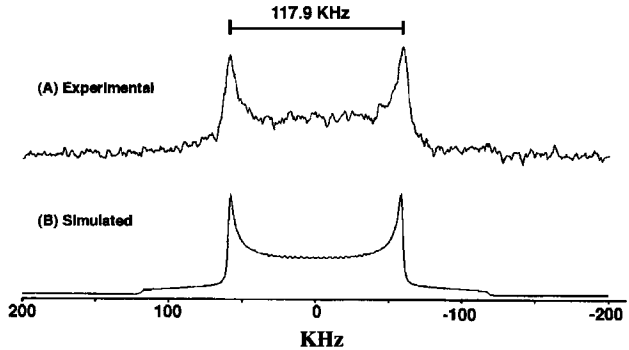


Fig. 4: Observed (A) and simulated (B) ^2H NMR powder pattern of [2,2-$^2H_2$]Gly labeled silk fibroin fiber from *B. mori*.

Fig. 4(B) shows the spectral simulation of [2,2-$^2H_2$]Gly residue by assuming the vibrational motion for Cα-^2H bonds under the amplitude = 12° with rate = 10^3 Hz. This result is in agreement with the prediction from the inter-molecular hydrogen bonding network in the silk fibroin backbone with an antiparallel β-sheet conformation. Figure 5A (upper) shows the ^2H

NMR powder pattern of [3, 3, 3-$^2H_3$]Ala labeled silk fibroin. This sample gives the splitting of Δn_Q = 37.9 kHz. The value of the powder splitting indicates a fast three-fold rotation of the alanine methyl group (10^8 Hz) about its Cα-Cβ axis with small angle libration (10°) (Fig. 5B upper).

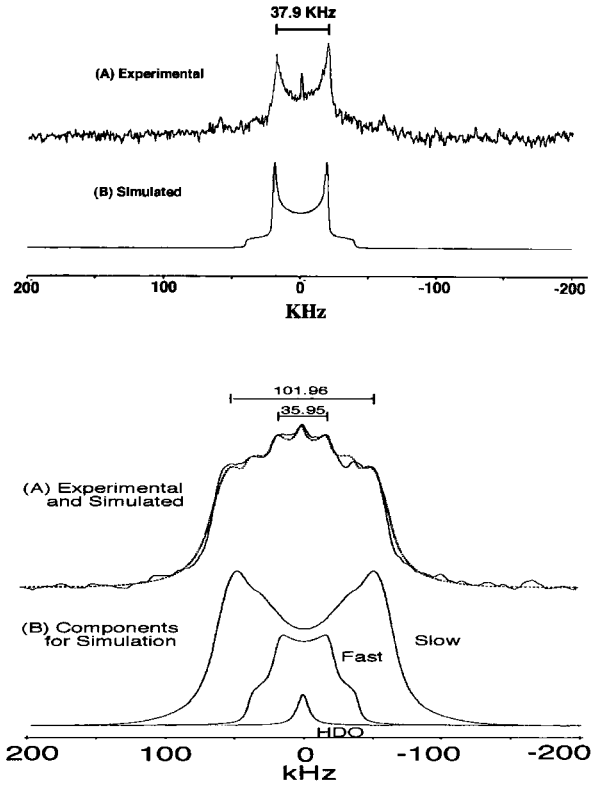


Fig. 5: ^2H NMR powder pattern of [3, 3, 3-$^2H_3$]Ala labeled (upper) and [2,2-$^2H_2$]Ser labeled (lower) silk fibroin from *B. mori*. .

This is also in agreement with the minimum in the spin-lattice relaxation time T_1, that was observed between -70 to -80 °C in a ^1H pulse NMR study of *B.mori* silk fibroin at 90 MHz.[13] A small additional doublet with Δn_Q = 118 KHz is seen in spectrum 5A upper, due to the

presence of a small amount of labelled glycine or serine, which is produced by feeding with ^2H_2O[12]. The single center peak is due to H^2HO.

The solid state ^{2}H-powder pattern NMR spectrum of [3,3-$^2H_2$]Ser labeled silk fibroin (Silk II) is shown in Fig. 5 lower. There is a large difference between this powder pattern and that of [2, 2-$^2H_2$]Gly and [3, 3, 3-$^2H_3$]Ala labeled silk samples as shown in Figs. 4 and 5 upper. By assuming a three fold jump motion with small angle libration such as methyl rotation of Ala residue, the side chain dynamics of Ser residue was found to be composed of two motional fractions, a slow component, ~10^4 Hz (77%) and a fast component, ~10^6 Hz (23%). In addition, the log-Gaussian rate distribution of the three fold jump motion for the slow component was smaller than that of the fast one, suggesting effect of the hydrogen bonding for Ser side chain. Similar results of side chain dynamics for *S. c.ricini* silk fibroins were obtained.

Conclusion

Structure as a conformational space of torsion angle ϕ and ψ for silk fibroin fiber from *B.mori* was determined based on the anaslysis of ^{13}C and ^{15}N chemical shift anisotropy of uniaxially aligned samples. The unit cell length as determined by solid state NMR is in excellent agreement with fiber diffraction data. The characteristic side chain motion for Ser residues in both *B. mori* and *S. c. ricini* silk fibroins having the fast and slow components was detected.

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