

SHORT COMMUNICATIONS

**Structure of *Bombyx mori* Silk Fibroin Studied by
REDOR NMR Spectroscopy**

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It has been confirmed that the solid-state conformation of silk fibroin from *Bombyx mori* is strongly dependent on sample preparation.¹ It is well known that the silk II form (anti-parallel β -sheet), obtained by drying at high temperature or under mechanical stress, is the most stable form. The metastable silk I form is obtained by drying from aqueous solution at room temperature. ^{13}C chemical shifts are sensitive to molecular conformation and have been used to show that silk I and silk II are structurally different.¹ Whereas the structure of silk II has been known for some time (for example, see ref 2), the structure of silk I has not been determined due, primarily, to the difficulty in obtaining high quality oriented samples. However, several models have been proposed for the silk I structure. These include the "crankshaft" model by Lotz and Keith² and three models by Asakura and Yamaguchi³ based on ^{13}C CP/MAS NMR, X-ray diffraction data and conformational energy considerations.

We are initiating efforts to obtain the backbone conformation of silk I using the

rotational-echo, double-resonance (REDOR) NMR technique to measure internuclear distances between specific ^{13}C - ^{15}N spin pairs.⁴⁻⁶ These measured distances will be used to determine the dihedral angles describing the backbone conformation.⁷ A particular advantage of our strategy is that oriented samples are *not* required and simple powder samples can be used. As a demonstration of the technique, we present initial REDOR results on a model compound with the well-characterized silk II conformation.

THEORETICAL BACKGROUND

Rotational-echo, double-resonance NMR is a high-resolution, solid-state MAS NMR technique designed to measure internuclear distances between heteronuclear spin pairs.⁴⁻⁶ REDOR is ideally suited for measuring specific internuclear distances between selectively labeled spin sites. In particular, selective labeling of proteins and peptides with ^{13}C and ^{15}N isotopes and measuring the internuclear separation between the labeled spin pair is

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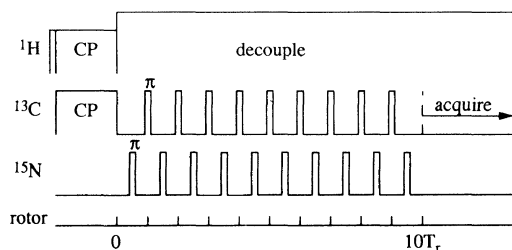


Figure 1. REDOR pulse sequence. The π -pulse trains use xy -8 phase cycling.

particular attractive due to the low natural abundance of these isotopes.

The internuclear distance, r_{CN} , between a ^{13}C - ^{15}N spin pair is related to the dipolar coupling constant, D , by

$$D = \frac{\gamma_{\text{C}}\gamma_{\text{N}}\hbar}{2\pi r_{\text{CN}}^3} \quad (1)$$

where the γ 's are the respective magnetogyric ratios; D is in Hz. Under usual MAS conditions, the heteronuclear dipolar interaction is coherently averaged to zero by the sample rotation. However, the REDOR experiment recovers the dipolar interaction by manipulating the spin coordinates of the dipolar interaction with rotor-synchronous π -pulse trains.⁵

REDOR is performed as a difference experiment. Figure 1 shows the ^{13}C -observe REDOR experiment used in this work.¹⁰ This experiment begins with cross-polarization of the ^{13}C spins *via* the protons. After cross-polarization, a strong rf decoupling field removes the proton-carbon dipolar interaction throughout the remainder of the experiment. The cross-polarization preparation is followed by a dipolar evolution period consisting of N_c rotor cycles (here $N_c = 10$). A compensated π -pulse train is applied on the ^{13}C channel during the dipolar evolution period to refocus isotropic chemical shifts prior to data acquisition, which immediately follows the evolution period. The *full* spectrum is obtained with no π pulses on the ^{15}N -channel. In this experiment, the dipolar interaction is averaged to

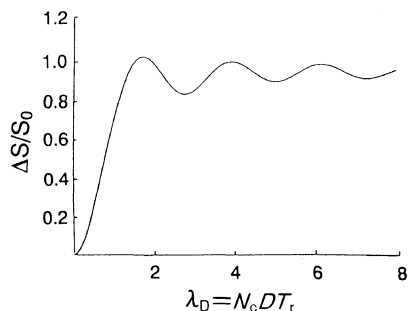


Figure 2. Calculated ratio of the REDOR *difference* signal to the *full-echo* signal.

zero by the sample rotation. However, when a train of π pulses is applied on the ^{15}N -channel, the ^{13}C magnetization is dephased by the ^{13}C - ^{15}N dipolar interaction. Two π pulses are applied per rotor cycle to ensure that dipolar dephasing is additive. This dephased signal is called the *reduced* signal. The *difference* signal is obtained by subtracting the *reduced* signal from the *full* signal. Only ^{13}C spins coupled to ^{15}N spins will appear in the *difference* spectrum.

The dipolar coupling constant and, consequently, the internuclear separation is obtained by comparing the measured ratio of the *difference* signal, ΔS , to the *full* signal, S_0 , to the calculated ratio. Figure 2 shows the calculated ratio of $\Delta S/S_0$ as a function of the product of the number of rotor cycles during the evolution period, N_c , the dipolar coupling constant, D , and the rotor period, T_r .⁵

EXPERIMENTAL

The ^{13}C - ^{15}N labeled hexapeptide used in this work is shown in Figure 3. The ^{13}C and ^{15}N labels are shown in the formula as **C** and **N**, respectively. To inhibit the side reaction, the OH group of the side chain of serine residue is changed to an OBzl group. The N- and C-terminus groups were synthesized with Boc- and -OBzl, respectively. A sample was also prepared without any isotopic labeling. This natural-abundance sample and the labeled

REDOR NMR of *Bombyx mori* Silk Fibroin

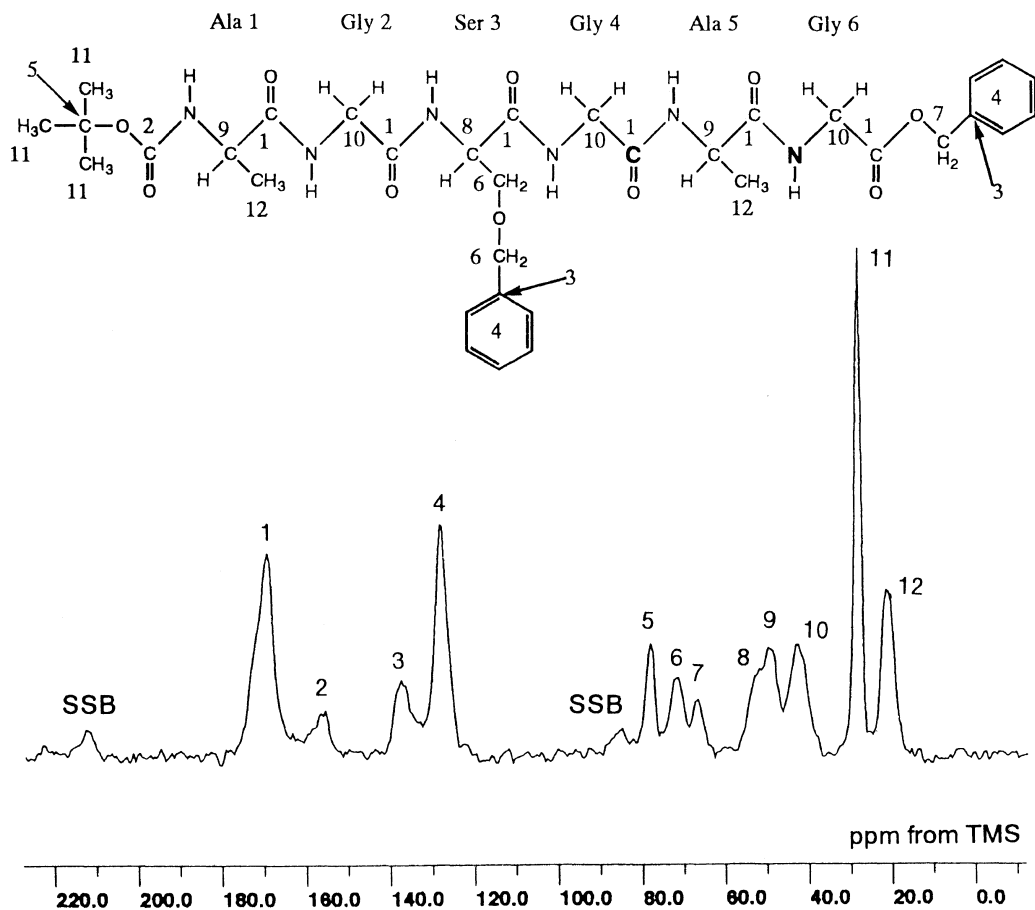


Figure 3. The structure of the labeled hexapeptide used in REDOR experiment (top) and ^{13}C CP/MAS spectrum of natural-abundance hexapeptide together with the assignment.

sample were recrystallized together in a ratio of 1 part labeled sample to 24 parts unlabeled sample to minimize intermolecular dipolar interactions. The measured ^{13}C - ^{15}N distance will place limits on the possible values of the ϕ , ψ dihedral angles of the Ala₅ residue.⁷

The REDOR experiments were performed at a ^{13}C frequency of 38.07 MHz at Florida State University on a home-built spectrometer. The 230 mg sample was centered in a zirconia rotor with Kel-F spacers. Sample spinning was done at 3205 Hz and $N_c = 34$. Cross-polarization and all carbon and nitrogen pulses were performed with an rf field strength of 38 KHz. Proton decoupling was performed at an rf field

strength of 110 KHz. The rotor-synchronous, compensated π -pulse trains used the xy -8 phase scheme to minimize off-resonance effects.⁸⁻¹⁰

RESULTS AND DISCUSSION

Figure 3 shows the ^{13}C CP/MAS NMR spectrum of natural-abundance hexapeptide with peak assignments. The C_β chemical shift of the alanine residue (*ca.* 20.0 ppm) suggests that this sample takes on the silk II form (antiparallel β -sheet).¹ In a previous study, the alanine torsion angles ϕ and ψ in the silk II form were reported as -142° and 145° , respectively.² Using these torsion angles, the

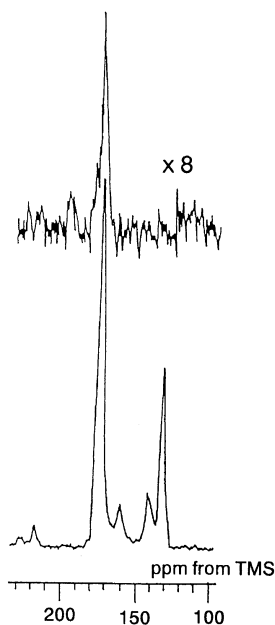


Figure 4. ^{13}C REDOR spectra of the carbonyl carbon region of the $[\text{}^{13}\text{C}\text{-}^{15}\text{N}]$ labeled hexapeptide. (bottom) echo spectrum collected in the absence of all ^{15}N pulses and (top) the REDOR difference spectrum.

distance between the ^{13}C and ^{15}N labels in the selectively labeled sample is 4.55 Å.

Figure 4 shows the ^{13}C -observe REDOR spectra of the carbonyl carbon region of the diluted hexapeptide sample. Figure 4 (bottom) shows the *full* spectrum collected in the absence of ^{15}N pulses and Figure 4 (top) shows the *difference* spectrum. In order to obtain $\Delta S/S_0$, several correlation must be made. First, natural-abundance contributions to the carbonyl carbon resonance from the unlabeled carbonyl carbon positions are taken into account to obtain S_0 . Second, corrections to ΔS from two sources must be made. The first correction to ΔS is to account for the coupling between the labeled carbonyl carbon and natural-abundance ^{15}N spins located closer to the ^{13}C label than the specific ^{15}N label. The second correction to ΔS accounts for other natural-abundance carbonyl ^{13}C spins coupled

to natural-abundance ^{15}N spins. Both corrections are done analytically assuming standard peptide geometry and bond lengths.⁷

After corrections, the experimentally determined ratio $\Delta S/S_0$ is 0.159. Reference to Figure 2 provides a $^{13}\text{C}\text{-}^{15}\text{N}$ distance of 4.32 Å (0.1Å). This internuclear separation is in good agreement with 4.55 Å distance predicted for silk II conformation. These results show that REDOR will be an effective tool in characterizing silk-like materials.

Further REDOR experiments with different labeling schemes are planned to probe the conformation of model compounds of silk I.

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