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Conformation Characterization of *Bombyx mori* Silk Fibroin in the Solid State by High-Frequency ^{13}C Cross Polarization–Magic Angle Spinning NMR, X-ray Diffraction, and Infrared Spectroscopy

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ABSTRACT: We recorded X-ray diffraction, ^{13}C CP–MAS NMR, and infrared spectra of silk fibroin samples from *Bombyx mori* in the solid state, prepared under different conditions, in order to compare conformational features obtained by the respective methods. These three methods provided consistent data as to the presence of the most stable silk II form. Silk I and random-coil forms were distinguishable only by the extent of line broadening in ^{13}C NMR peaks, although the chemical shifts are the same. The most obvious advantage of the ^{13}C CP–MAS NMR approach is that this method is intrinsically nondestructive and very convenient for evaluation of the relative proportion of silk I and II forms in the same sample. We found that powder formation even at liquid-nitrogen temperature caused a change from silk I to random-coil form, although the amount of silk II form remains unchanged during this procedure. In view of the sample preparation conditions as well as the similarity of the ^{13}C chemical shifts and infrared spectra between the silk I and random-coil forms, it is plausible that the random-coil form is a mixture of silk I type forms whose long-range crystalline packing is distorted to the extent to give halo X-ray diffraction.

Introduction

Two kinds of crystalline modifications, silk I and silk II, as well as the random-coil form, exist as dimorphs of silk fibroin from *Bombyx mori* in the solid state, depending on the sample preparation conditions.[1] On the basis of X-ray diffraction studies, the conformation of silk II has been established as the antiparallel β-sheet form by Marsh et al.[2] and later by Fraser et al.[3] The conformation of silk I,[4-9] however, appears to be not well-defined, as compared with that of silk II. The translations along the molecular axis calculated from the fiber-repeat distance were reported as 2.27–2.4 Å, which is longer than the corresponding value for the α-helix (1.5 Å) and shorter than the value for silk II type (3.5 Å). Lotz and Keith proposed a crankshaft model on the basis of the similarity of the diffraction pattern with that of (Ala-Gly)$_n$II.[9] There appears to be a conflict as to the classification of the random-coil form as viewed from the infrared absorption frequencies: Hayakawa et al.[10] proposed the silk I form for samples whose infrared frequencies are very similar to those of the random-coil form proposed by Miyazawa[11,12] and Magoshi et al.[8] We previously demonstrated that examination of the conformation-dependent ^{13}C chemical shifts as determined by high-frequency (75.46 MHz) cross polarization–magic angle spinning (CP–MAS) provides conformational characterization of the backbone moieties of peptides and polypeptides.[13-20] A similar approach has been applied to conformational analysis of polypeptides by Kricheldorf et al.[21-24] In particular, we showed that three forms of silk fibroins, silk I, silk II, and α-helix forms, are easily distinguished by the sizable displacement of the ^{13}C chemical shifts of the major amino acid residues: Ala, Gly, and Ser.[15,18]. However, we pointed out that new model building is required for the silk I form, because the Ala residue in the silk I form *does not* take torsion angles corresponding with the β-sheet form as proposed by the crankshaft model.

Our aim in this paper is to record and compare three sets of data, X-ray diffraction, ^{13}C CP–MAS NMR, and infrared spectra, of carefully prepared *B. mori* fibroin samples of silk I, silk II, and random-coil form. Our objective of this study is threefold. First, we clarify major advantages and disadvantages of these three methods and characteristic features inherent to the individual method

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Table I
Spacing (Å) and Intensities of the X-ray Diffraction Patterns of *B. mori* Silk Fibroin Samples Prepared under Different Conditions[a]

reflection	sample 1	sample 2	sample 3	sample 4	sample 5	sample 6b	sample 7	sample 8
R1	9.73 ms (II)	9.73 ms	9.73 ms	9.73 mw	9.97 mw	9.61 ms		9.67 ms (II)
R2	7.26 m (I)	7.36 m	7.26 ms	random coil/silk I + *silk II*	7.20 mw	7.26 ms	7.33 w	7.29 ms (I)
R3			5.59 w			5.45 w		5.61 w (I)
R4	4.69 vs (II)	4.63 vs	4.57 s	random coil/silk I	4.58 w	4.46 s	4.52 w	4.50 s (I)
R5	4.28 vs (II)	4.27 vs	4.26 s					
R6			4.01 w	random coil/silk I	4.02 w	4.11 m		4.02 w (I)
R7	3.65 ms	3.62 ms	3.66 m		3.59 w	3.62 m	3.77 w	3.62 m
R8			3.12 w			3.02 w		3.15 m (I)
R9	2.66 w (II)		2.75 w			2.64 w		2.73 w (I)
R10	2.25 w (II)		2.44 w			2.38 w		2.44 mw (I)
R11	2.04 w (II)		2.21 vw					2.24 vw (I)

[a] Intensities: vs, very strong; s, strong; ms, medium strong; m, medium; mw, medium weak; w, weak; vw, very weak.

in the conformational analysis of the solid-state fibroin. Second, we evaluate the relative proportion of these conformations in the same samples by CP-MAS NMR spectroscopy. Third, we delineate conformational features of the silk I and random-coil forms as defined by either the X-ray diffraction or infrared spectra. The CP-MAS NMR, X-ray diffraction, and infrared spectral data gave consistent results as to silk II samples. However, samples taking the silk I and random-coil forms as judged from the X-ray diffraction method yield the same ^{13}C chemical shifts, although the line widths of the samples classified as random-coil form are much broadened as compared with those of the silk I sample. Nevertheless, the major conformational features of the silk I and the random-coil forms are essentially the same as manifested by the similarity of chemical shifts. It appears that the difference is only the presence of conformers and/or segments whose torsion angles in the random-coil form slighlty deviate from those of the normal silk I form. This view seems to be reasonable because conditions for preparation of samples of silk I are not very different from those for the random-coil form.

Experimental Section

Materials. Silkworms of *Bombyx mori*, a hybrid between strains Nichi 140 and Shi 140, were reared with an artificial diet or with mulberry leaves in our laboratory. Silk glands were excised from the silkworms and the middle silk gland portion was collected. We prepared several kinds of fibroin samples with crystalline modifications, silk I, silk II, and random-coil forms, under several kinds of treatments as described below. After sericine, a silk protein, was removed, the remaining silk fibroin was immersed in methanol for 2 days and dried at 20 °C (sample 1). Sample 2 was the precipitate when the crystalline fraction of *B. mori* fibroin (Cp fraction) was dissolved in 9 M LiBr solution and dialyzed against distilled water.[18] Sample 3 was prepared from liquid silk stored in the middle silk gland portion of the silkworm. After sericine was removed, the remaining silk fibroin was dried at 4 °C for 2 days. Samples 4 and 5 were prepared by air-drying a 6% regenerated *B. mori* silk fibroin solution cast over films of poly(vinylidene chloride) at 27 and at 50 °C, respectively.[18] These films were crushed to powder at liquid-nitrogen temperature. Sample 6a was a film of silk fibroin, prepared in a similar manner to the method described above except for the temperature of casting, 20 °C. Sample 6a was allowed to stand in a desiccator in which was placed water (sample 6b). Thus, sample 6b was the same as sample 6a except for wetting with water. Sample 7 was the precipitate when *B. mori* fibroin was digested by alkaline phosphatase (Ap fraction).[18] Sample 8 was prepared by essentially the same method as that used for sample 3, except sample 8 was dried at 20 °C for several days.

Measurements. X-ray Diffraction. X-ray diffraction patterns were recorded on a Weissenberg camera at 35 kV and 20 mA, using a JEOL DX-GO-S apparatus with a copper target and nickel filter. X-ray diffraction patterns of samples 1, 3, and 8 and film sample 6b were directly observed. The powder samples, 2, 4, 5, and 7, were loaded into quartz glass capillaries of 1-mm diameter and then observed.

^{13}C CP-MAS NMR. ^{13}C CP-MAS NMR spectra (75.46 MHz) were recorded on a Bruker CXP-300 spectrometer equipped with a CP-MAS accessory. Samples were placed in an Andrew-Beams type rotor machined from perdeuterated poly(methyl methacrylate) and spun as fast as 3-4 kHz. Samples 1, 3, and 8 were cut into small pieces (2 × 2 mm) with a scissors. Film and other samples were directly loaded into the rotors. A contact time of 800 μs was chosen as will be described below. Repetition time was 2 s, and spectral width and data points were 30 kHz and 4K points, respectively. Spectra were usually accumulated 2000-3000 times. Chemical shifts were calibrated through external benzene and converted to the values from tetramethylsilane (Me_4Si).

IR. IR spectra were obtained with Shimadzu IR-435 and Jasco DS-701G infrared spectrometers at 4000-400 cm^{-1}. Film samples 6a and 6b were observed directly. KBr disks were prepared for powdered samples (samples 4 and 5) and for samples 2 and 7.

Results

X-ray Diffraction. First, we examined the X-ray diffraction pattern of several fibroin samples as reference data to be used for interpretation of the ^{13}C CP-MAS NMR and infrared spectra. Typical X-ray diffraction patterns of the silk I and silk II forms are illustrated in Figure 1. Intersheet spacings calculated by the Bragg equation are summarized in Table I. Obviously, the major conformation of samples 1 and 2 is silk II on the basis of the strong interference rings at 9.70, 4.69, and 4.30 Å; silk I is present as a minor form as seen from the observation at 7.25 Å.[4-7] On the other hand, silk I form is dominant for samples 3, 6b, and 8 as manifested from the strong interference rings at 7.25 and 4.50 Å. Silk II is also included as a minor component as manifested from the ring at 9.70 Å. By contrast, the intensities of the interference rings of samples 4, 5, and 7 are rather weak as compared with those of crystalline samples described above. Nevertheless, samples 4 and 5 contain silk I plus silk II, as seen from the rings at 9.70 and 7.25 Å, and sample 7 contains silk I as a minor component as found from the presence of the ring at 7.25 Å. These results are summarized in

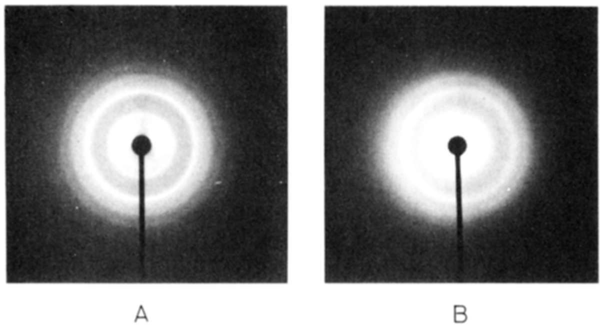

Figure 1. X-ray diffraction photographs of *B. mori* silk fibroins in the solid state: (A) sample 8, silk I; (B) sample 1, silk II.

Table II
Conformational Characterization of Solid-State *B. mori* Silk Fibroins with ^{13}C CP-MAS NMR, IR, and X-ray Diffraction

sample no.	physical state	^{13}C CP-MAS NMR % silk I[a]	^{13}C CP-MAS NMR % silk II	IR	X-ray diffraction
1	small piece	26[b]	74		silk I + *silk II*
2	precipitate	26	74	random coil/silk I + *silk II*	silk I + *silk II*
3	small piece	63	37		*silk I* + silk II
4	powder	79	21	random coil/silk I	silk I + silk II + *random coil*
5	powder	82	18	random coil/silk I	silk I + silk II + *random coil*
6a	film	83	17	random coil/silk I	
6b	film	70	30	*random coil/silk I* + silk II	*silk I* + silk II
7	precipitate	86	14	random coil/silk I	silk I + *random coil*
8	small piece	73	27		*silk I* + silk II

[a] ^{13}C CP-MAS NMR yields the same chemical shifts for silk I and random-coil conformations and thus, we could not distinguish these conformations by the chemical shifts. [b] We estimated that the relative proportion of silk I and silk II was ±4%.

Table II, in which dominant forms are indicated by italics.

^{13}C CP-MAS NMR. Prior to detailed analysis of the NMR data, we first examined the variation of the peak intensities as a function of contact times. The liquid silk sample from *B. mori* in the solid state was prepared under conditions similar to those used for sample 8, but the sample contains a small amount of sericine. The maximum peak intensity was achieved when contact times were chosen between 500 and 1000 μs (Figure 2). Thus, 800 μs was employed throughout this experiment.

As demonstrated previously,[18] distinction of the silk I and silk II forms is easy and straightforward by examination of the ^{13}C chemical shift values. Therefore, the major form of samples 8 and 1 (Figure 3) is straightforwardly determined as silk I and silk II, respectively, which agrees with X-ray diffraction data described above. In particular, the Ala and Ser C_β signals of the silk I form resonate upfield with respect to those of the silk II form. In addition, the significant downfield displacement of the carbonyl peak of the Ala residue as compared with that of silk II form (~4 ppm) is characteristic of the presence of silk I form.

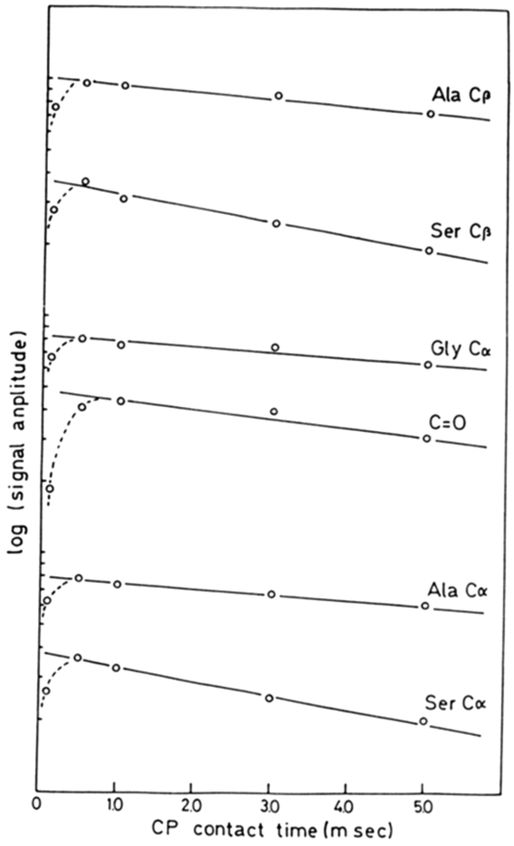

Figure 2. Plots of the signal intensities of C_α, C_β, and C=O carbons vs. CP contact time for liquid silk in the solid state extracted from *B. mori* silk gland.

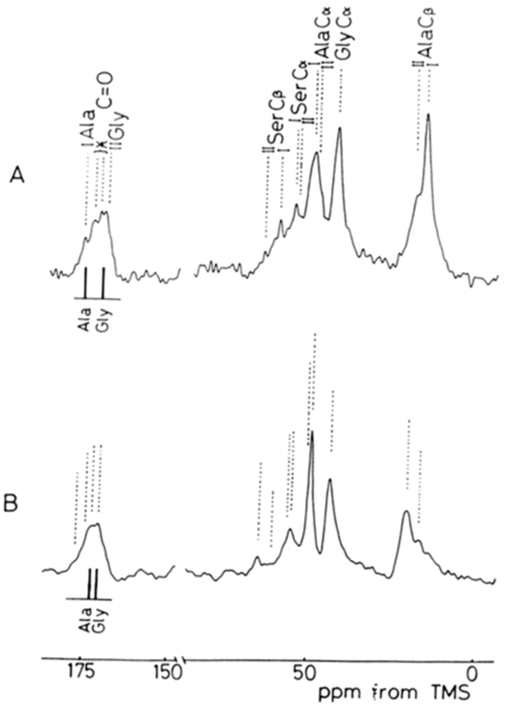

Figure 3. ^{13}C CP-MAS NMR spectra of *B. mori* silk fibroins in the solid state: (A) sample 8, silk I; (B) sample 1, silk II.

The relative proportion of the silk I and silk II forms in samples 1, 3, and 8 was evaluated by spectral simulation of the aliphatic region, by assuming Lorentzian line shapes and appropriate line widths[25] (Figure 4). The relative proportion determined from the Ala C_β peaks agrees with

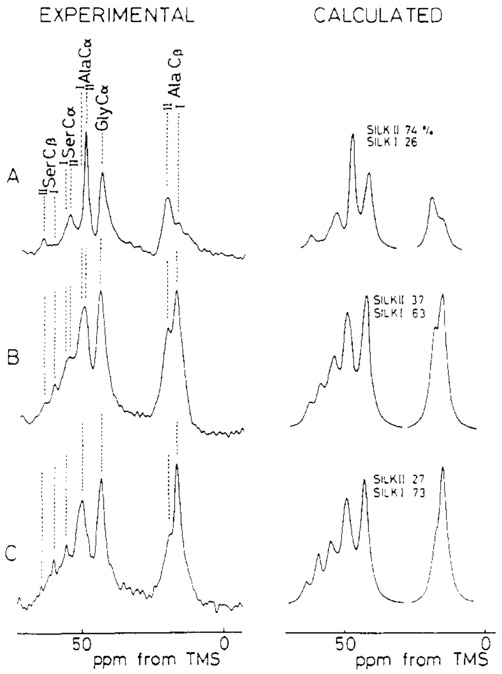

Figure 4. Experimental and calculated ^{13}C CP-MAS NMR spectra of liquid silk fibroins from *B. mori*: (A) sample 1; (B) sample 3; (C) sample 8.

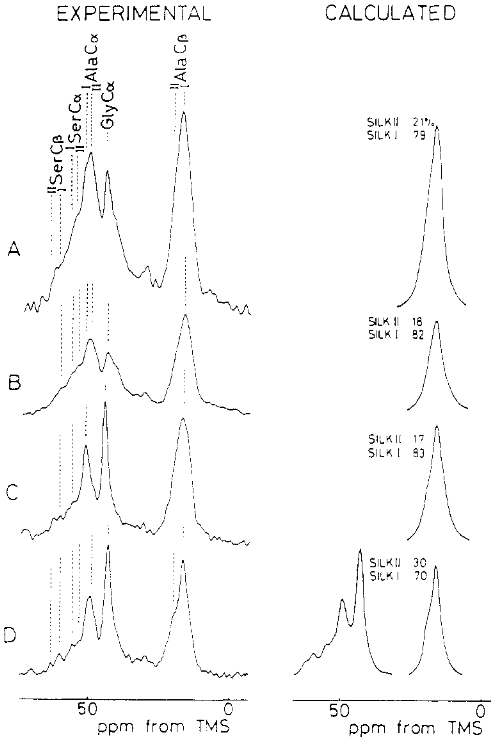

Figure 5. Experimental and calculated ^{13}C CP-MAS NMR spectra of regenerated silk fibroins from *B. mori*: (A) sample 4; (B) sample 5; (C) sample 6a; (D) sample 6b.

that determined from the Ser C_β area within ±4%. Thus, sample 8 (silk I) and sample 1 (silk II) contained 25–30% other forms as summarized in Table II. Dehydration by methanol causes the dramatic change from silk I to silk II (sample 3 → sample 1).

Interestingly, the ^{13}C NMR peaks are considerably broadened when silk I film is powdered (samples 4 and 5). As described above, the X-ray diffraction patterns of these samples were essentially halo. Therefore, the random-coil form as defined by X-ray diffraction gives rise to much broadened signals, although the ^{13}C chemical shifts are the same as those of silk I samples. The relative proportion of silk I plus random-coil forms was estimated as 80% for samples 4 and 5 on the basis of the spectral simulation at Ala C_β region (Figure 5). In addition, moisture absorption of the film causes an increase of the silk II form (sample 6a → sample 6b).

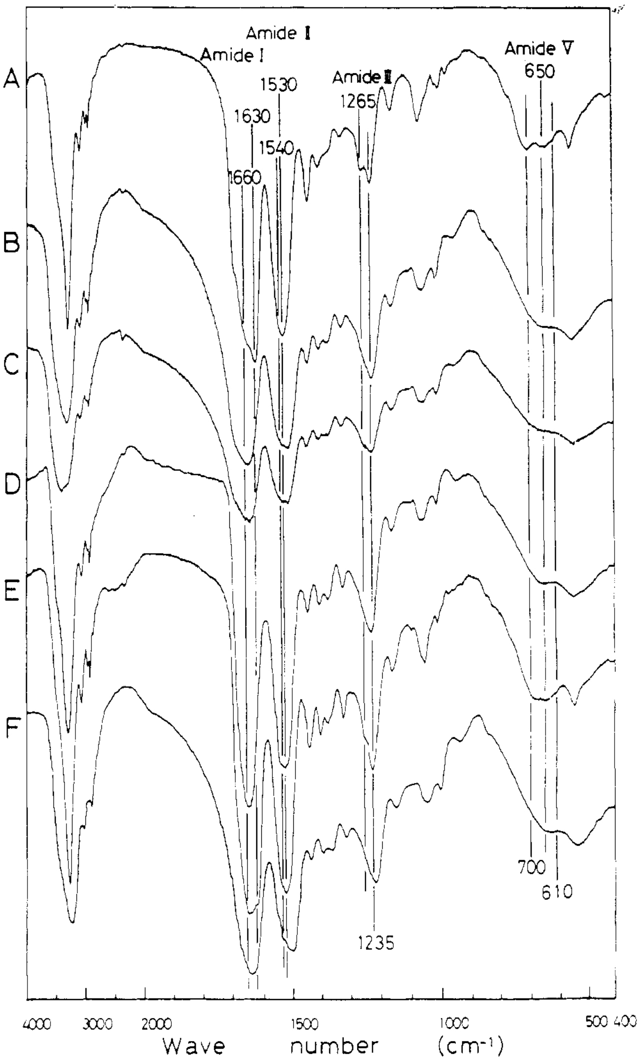

Figure 6. Infrared spectra of *B. mori* silk fibroins in the solid state: (A) sample 2; (B) sample 5; (C) sample 4; (D) sample 6a; (E) sample 6b; (F) sample 7.

Infrared Spectra. Figure 6 illustrates infrared spectra of samples 4–7 together with sample 2 (Cp fraction) as the reference of the silk II form. Obviously, characteristic absorption frequencies of the silk II form, 1630 (amide I), 1530 (amide II), 1265 (amide III), and 700 cm^{-1} (amide V), are clearly resolved for the sample 2. By contrast, samples 4–7 gave rise to absorption frequencies at 1660 (amide I), 1235 (amide III), and 650 cm^{-1} (amide V). These frequencies were previously ascribed to the silk I form by Hayakawa et al.[10] but were assigned later to the random-coil form by Magoshi et al.[8] The latter assignment is in agreement with that of Miyazawa et al.[11,12] Instead, Magoshi et al.[8] claimed that only an absorption at 610 cm^{-1} is ascribed to silk I form. The absorption at 610 cm^{-1}, however, is not clearly resolved in our present experiment. Clearly, distinction between the silk I and random-coil forms is impossible.

Discussion

None of the fibroin samples studied in this work consists of a single crystalline modification, as summarized in Table II, although analysis of a single component was made in our previous paper.[18] In fact, they contained 15–40% other form(s) (silk II or silk I/random coil) as minor component(s) (see Table II). This finding is reasonable because silk I and silk II are dimorphic structures of *B. mori* fibroin and are known to easily interconvert depending on the sample treatment. The presence of the silk II form is consistently identified by the three kinds of methods used in this work. The silk I form is readily distinguished from the silk II and random-coil forms by means of X-ray diffraction. On the other hand, distinction between the silk I and random-coil forms by infrared or NMR spectroscopy is either impossible or not easy, respectively. Nevertheless, it is emphasized that such distinction is possible in a qualitative manner in view of the extent of line broadening of the ^{13}C NMR peaks. Further, the relative proportion of the silk I (plus random-coil form) and silk II forms is easily evaluated by the ^{13}C CP–MAS NMR spectra from the comparison of peak intensities with the aid of spectral simulation.

It is noteworthy that samples claimed as the random-coil form by X-ray diffraction (samples 4, 5, and 7) are prepared by the procedure similar to that of silk I with or without minor modification. For samples 4 and 5, we powdered film samples of silk I form (cf. sample 6a) at liquid-nitrogen temperature. It is plausible that crystalline packing could deteriorate at the time of powder formation. However, it is unlikely that the conformations of the individual peptide chains are significantly altered at 77 K.

Taking into account the manner of sample preparation for the random-coil form, it is unlikely that the random-coil form of silk fibroin consists of mainly amorphous regions whose torsion angles of individual peptide chains are randomly populated. Previously, an attempt was made to prepare amorphous polypeptides whose line widths of ^{13}C chemical shifts were in the range 5–10 ppm.[26] We found that the line widths of fibroin samples judged as random coil are 4–5 ppm, but no significant change in the relative proportion of silk II form, which ensures the occurrence of conformational change, is noted between sample preparation of silk I and random coil (see Table II). Therefore, it is plausible that the conformation of the random-coil form is silk I type whose crystalline packing is distorted by the presence of conformers and/or segments in which torsion angles slightly deviate from those of normal silk I form. This view is consistent with our present infrared and ^{13}C CP–MAS NMR spectral data in which infrared frequencies and ^{13}C chemical shifts of the random-coil form cannot be distinguished from those of silk I form. In this connection, it is emphasized that the ^{13}C chemical shifts are sensitive to the conformation of individual molecular chains rather than the overall crystalline packing, which strongly influences the X-ray diffraction pattern.

The ^{13}C chemical shifts of the silk I and random-coil forms agree well with those of the liquid silk or of the regenerated silk fibroin in aqueous solution; the chemical shifts are essentially independent of concentration in the range 0.3–14.5%.[27] The CD pattern of the silk fibroin in dilute aqueous solution shows a typical random-coil pattern.[27] This finding indicates that random-coil form in aqueous solution can be considered as silk I type form in which conformational fluctuation around the torsion angles of silk I form gives rise to overall feature of random coil.[28]

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