^{13}C NMR of *Nephila clavipes* Major Ampullate Silk Gland

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ABSTRACT The major ampullate glands of the spider *Nephila clavipes* contain ~0.2 μl each of a highly concentrated (~50%) solution of silk fibroin. Therefore, the reservoir of silk in these glands presents an ideal opportunity to observe prefolded conformations of a protein in its native state. To this end, the structure and conformation of major ampullate gland silk fibroin within the glands of the spider *N. clavipes* were examined by ^{13}C NMR spectroscopy. These results were compared to those from silk protein first drawn from the spinneret and then denatured. The ^{13}C NMR chemical shifts, along with infrared and circular dichroism data, suggest that the silk fibroin in the glands exists in dynamically averaged helical conformations. Furthermore, there is no evidence of proline residues in U-^{13}C-D-glucose-labeled silk. This transient prefolded "molten fibril" state may correspond to the silk I form found in *Bombyx mori* silk. There is no evidence of the final β-sheet structure in the ampullate gland silk fibroin before final silk processing. However, the conformation of silk in the glands appears to be in a highly metastable state, as plasticization with water produces the β-sheet structure. Therefore, the ducts connecting the ampullate glands to the spinnerets play a larger role in silk processing than previously thought.

INTRODUCTION

The spider *Nephila clavipes* has two major ampullate glands that contain ~0.2 μl each of a highly concentrated (~50%; see below) solution of silk fibroin. The silk from these glands is then processed into dragline silk. The abundance of dissolved silk fibroin in the major ampullate glands presents an ideal opportunity to answer the question of whether there are prefolded conformations of a protein in its native state. In this manuscript we use the spectroscopic tools of solid and solution-state NMR, coupled with Fourier transform infrared spectroscopy (FTIR) and circular dichroism (CD) to address this question.

Silk production begins at the tail of the major ampullate gland, where 90% of the silk protein is produced (Bell and Peakall, 1969). The dragline silk fibroin is present as a dissolved high-molecular-weight polymer in the aqueous environment of the spider's silk glands. The most recent molecular weight data suggest that major ampullate gland silk fibroin has a M_n of 720,000 and a M_w/M_n of 1.03, and that its molecular weight degrades upon spinning and aging (Jackson and O'Brien, 1995). The dissolved protein must then pass through a long convoluted duct before reaching the spinnerets. After assembling in the sac and processing through the ducts, the silk protein changes from a dissolved polymer with a narrow molecular weight distribution to a very insoluble solid—dissolving only in harsh solvents such as 9 M LiBr (Mello et al., 1994), propionic acid/HCl (Michal et al., 1995), and concentrated formic acid—with about 30% lower molecular weight and greatly increased polydispersity (Jackson and O'Brien, 1995).

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The major ampullate gland silk protein is generally thought to comprise at least two proteins, spidroin I and spidroin II (Hinman and Lewis, 1992), also called MaSp 1 and MaSp 2. Spidroin I is composed primarily of a 30-amino acid repetitive motif that is rich in polyalanine regions. The polyalanine regions are composed of 4–10 consecutive alanine residues between glycine-rich regions. Early computer analysis based on globular proteins and attempts to relate the sequence to dragline silk's mechanical properties proposed that these polyalanine regions formed α-helical structures (Dong et al., 1991). However, solid-state ^{13}C NMR chemical shift data have rigorously established the presence of the polyalanine in β-sheets in the solid silk fiber (Simmons et al., 1994). This discrepancy in predicted and observed polyalanine structures could arise as a natural consequence of the biased globular protein data sets used to produce the predictive algorithms. It may also be possible that secondary structure(s) other than β-sheet could exist when the silk protein is synthesized and stored in the aqueous environment of the major ampullate glands.

As a first step toward understanding the early stages of in vivo spider silk processing, we have investigated the major ampullate gland silk fibroin (gland silk fibroin) while still in the glands using solution ^{13}C NMR and the known conformational sensitivity of the chemical shifts (Wishart and Sykes, 1994; Spera and Bax, 1991). These results were compared to data from four other samples: processed solid dragline silk in its native state (native dragline silk), denatured dragline silk in formic acid (denatured silk), flash-frozen and then lyophilized denatured dragline silk (lyophilized denatured silk), and flash-frozen lyophilized silk fibroin in the glands (lyophilized gland silk fibroin).

It has been established that ^{13}C chemical shifts of amino acids more than three residues from the end of a peptide are largely independent of sequence, except for proline neighbors (Howarth and Lilley, 1978). The conformational sensitivity of the α, β, and carbonyl carbons has allowed the

successful use of ^{13}C NMR chemical shifts to identify β-sheet and α-helical regions of proteins (Simmons et al., 1994). In addition, if an amino acid participates in more than one particular conformation, structural information may still be gleaned from the line-broadened spectrum due to chemical shift dispersion. The conformation of the polyalanine region of the silk protein as stored in the major ampullate gland is characterized by comparing ^{13}C NMR chemical shifts with established secondary structure chemical shifts for alanine in the processed dragline silk.

MATERIALS AND METHODS

Native dragline silk sample preparation

Both ^{13}C-labeled and unlabeled dragline silk samples from adult female *N. clavipes* spiders were mechanically drawn at a rate of 2.0 cm/s in a manner similar to that of Work and Emerson (1982). To prevent contamination from other silks, major ampullate gland silk was carefully collected under observation through a dissecting microscope. The isotopically labeled silk was prepared by hand-feeding spiders dropwise with a syringe. The spiders were fed a 10% w/v solution of U-^{13}C-D-glucose (C/D/N Isotopes, Pointe Claire, Québec, Canada) in Dulbecco's modified Eagle medium (GIBCO, Gaithersburg, MD) 10 min before and immediately after each 40-min silking interval. This feeding and silk collection protocol was repeated once a day for 5 consecutive days. Labeled and unlabeled silk samples (5.5 mg each) were dissolved separately in 0.5 ml of deuterated (99%) formic acid (C/D/N Isotopes) for ^{13}C NMR spectroscopy.

Gland preparation for solution NMR

After 5 days of feeding ^{13}C-labeled glucose and collecting silk, 17 major ampullate glands were neatly dissected from nine spiders. Dissections were performed in a solution containing 0.10 M sodium chloride and 0.015 M sodium citrate in deuterium oxide (Candelas and Cintron, 1981). A semicircular incision was made in the ventral side of the spider abdomen. The major ampullate glands, easily identified because of their location and gold color, were removed from the spider. The glands were immediately placed in a 5-mm NMR tube with 1 ml of 0.10 M NaCl, 0.015 M sodium citrate and ~0.2 ml of a 5% sodium azide solution (to prevent bacterial growth) in deuterium oxide.

Lyophilization

Fifteen major ampullate glands (unlabeled) were dissected from live spiders and individually and immediately flash-frozen in a liquid nitrogen bath and lyophilized. Native dragline silk (27 mg) was dissolved in 2.7 ml of 80% formic acid, and 0.05-ml droplets of solution were flash-frozen in a liquid nitrogen bath before lyophilization. The protein/formic acid solution exhibited pronounced freezing-point depression, so lyophilization was performed with the lyophilization flask immersed in a acetone/CO_2 bath maintained below −20°C at all times. Such treatment prevented melting during the lyophilization process.

Rehydration of lyophilized samples

After obtaining solid-state ^{13}C NMR spectra, the flash-frozen and lyophilized glands were rehydrated with ~5% w/w water, and the spectra were immediately reacquired.

^{13}C NMR spectroscopy

High-resolution solution-state ^{13}C NMR spectra were obtained at 25°C on a Varian VXR-400 operating at 100.6 MHz for ^{13}C. The solution spectrum of native dragline silk dissolved in formic acid (denatured silk) was obtained with a spectral width of 25 kHz and a 1.2-s relaxation delay for 5568 scans. The solution spectrum of silk fibroin in the major ampullate gland (gland silk fibroin) was obtained with the same parameters for a total of 4224 scans. Chemical shifts were referenced using an external chloroform sample and converted to ppm from tetramethylsilane (TMS).

Solid-state ^{13}C CP/MAS NMR spectra of native dragline silk, lyophilized gland silk fibroin, and lyophilized denatured silk were obtained with two different home-built spectrometers, one operating at 75.4 MHz for ^{13}C and employing a Chemagnetics probe. The other system operates at 90.56 MHz and uses a Doty probe. A 2.5-ms cross-polarization contact time was used with 80 kHz decoupling. All scans were taken with a spectral width of 55–60 kHz. An external adamantane sample spectrum referenced to TMS was used to calibrate chemical shifts.

FT infrared

FTIR spectra were recorded on a 2020 Galaxy Series Fourier transform infrared spectrometer using native silk or freshly dissected glands. The glands were placed between two NaCl plates and averaged over 16 scans unless otherwise specified. In some cases, mechanical shear was applied to the samples after initial spectra were obtained.

Circular dichroism

Glands were freshly dissected from live spiders and immediately placed between two quartz plates. CD spectra were recorded using a 0.01-mm path length cell on a Jasco J-720 spectopolarimeter. All spectra were recorded at room temperature with wavelengths from 180 to 260 nm and were averaged over 16 scans.

RESULTS AND DISCUSSION

^{13}C labeling and protein concentration in glands

The feeding of U-^{13}C-D-glucose labeled predominantly the alanine, glycine, serine, and glutamine residues in the dragline silk. There was no evidence of proline, as would be expected from standard metabolic pathways (Lehninger et al., 1993) and should be detected if the proline-containing MaSp2 were present in large amounts (Lewis, 1992). Degrees of labeling for glycine, alanine, and glutamine were calculated from peak integrals of the ^{13}C satellites in a proton spectrum (not shown) of the dissolved silk sample (Table 1). A comparison of the water and protein peak intensities lets us estimate that the protein concentration in the glands is 50%. Assignment of peaks was performed by the comparison of known peptide chemical shifts (Asakura et al., 1984; Saito, 1986) and known silk chemical shifts (Simmons et al., 1994). The nonuniform degree of labeling (Table 1) may be a consequence of the relatively short amount of time the spiders were fed the U-^{13}C-D-glucose solution. The absence of labeling of the tyrosine aromatic

TABLE 1 Percentage ^{13}C labeling obtained by feeding *N. clavipes* with U-^{13}C-D-glucose

Amino acid	% silk composition	% labeling
Glycine-CH_2	43.0	28
Alanine-CH_3	30.1	59
Glutamine-γCH_2	6.9	42

ring is expected and has been observed in other systems such as *B. mori*, in which tyrosine must be formed de novo from dietary phenylalanine (Takashima et al., 1964).

Spectroscopic studies

Table 2 shows the chemical shifts for native dragline silk, where the alanine residues are in antiparallel β-sheet structures (column 1). For comparison, it also shows reference chemical shifts for alanine in a β-sheet (column 2) and for alanine in an α-helix (column 3). A comparison of the chemical shifts of native dragline silk with the polyalanine ^{13}C chemical shifts verifies that the alanine in dragline silk is present in β-sheets, as expected (Simmons et al., 1994). For amino acid residues, the α-helix chemical shift of C_α is shifted downfield, whereas C_β is shifted upfield relative to the β-sheet chemical shifts.

We then compared a high-resolution spectrum of labeled denatured silk (Fig. 1 *a*) with the solution-state ^{13}C NMR spectrum of the intact, labeled gland silk fibroin (Fig. 1 *b*). (These data are tabulated in Table 2, columns 4 and 5.) Fig. 1, *a* and *b*, shows that the ^{13}C NMR spectrum of labeled gland silk fibroin is identical to that of the labeled denatured silk, with the exception of a shift in the position of the serine C_β. As serine C_β is likely to be involved in hydrogen bonding arrangements that are different in the two solvents, this difference is not substantial. A comparison of chemical shifts in Table 2 with the known chemical shifts for α-helix and β-sheet structures indicates that the polyalanines in the intact liquid silk are neither in β-sheets nor in α-helices on the NMR time scale.

These results from spider silk may be compared with studies performed by Asakura et al. (1984) on the ^{13}C chemical shifts of silk in the intact *B. mori* silk glands. The chemical shifts for the silk fibroin in the intact *B.mori* glands are shown in Table 2, column 6, where the results are very similar to those for the denatured silk and gland silk fibroin. These chemical shift values for *B. mori* gland silk

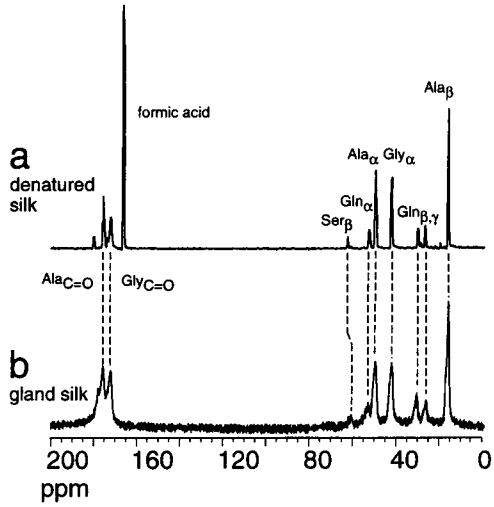

FIGURE 1 Solution ^{13}C NMR spectra. (*a*) U-^{13}C-D-glucose-labeled dragline silk in formic acid. (*b*) ^{13}C-labeled gland silk fibroin from dissected major ampullate glands.

were labeled as a silk I structure, which was been assigned to a loose fourfold helical conformation with a residue translation of 2.27 Å (Saito et al., 1984). However, silk I and random-coil forms may yield similar chemical shifts (Saito, 1986), except for ionizable groups such as the carbonyl carbons. For example, Asakura et al. (1985) found that silk I and random-coil forms yield similar ^{13}C chemical shifts, with broader linewidths for the random-coil form as compared with the silk I form. Therefore, the major conformational features of both forms may essentially be the same. This similarity can be explained in one of two ways: 1) either the native silk in the glands exists in a largely denatured state, or 2) the silk in the glands is undergoing interconversions between several conformational states, and these interconversions are rapid on the NMR time scale ($\tau_c < 10^{-7}$ s).

TABLE 2 ^{13}C alanine chemical shifts (ppm from TMS)

Amino acid	(1) Native dragline silk	(2) β-Sheet	(3) α-Helix	(4) Denatured silk	(5) *N. clavipes* gland silk fibroin	(6) *B. mori* gland silk fibroin	(7) Lyophilized gland silk	(8) Lyophilized denatured silk	(9) 3_1 helix
Alanine									
C_α	48.8	48.7	52.5	50.2	50.3	50.0	52.4 48.8	51.7	48.7
C_β	20.8	20.1	15.1	16.6	16.6	16.7	17.2	16.1	17.4
C=O	171.8*	171.9	176.5	175.4	175.5	175.5	172.2*	172.2*	172.1

(1) Solid-state NMR of *N. clavipes* native dragline silk.
(2) Polyalanine β-sheet chemical shift (Work and Emerson, 1982).
(3) Polyalanine α-helix chemical shift (Work and Emerson, 1982).
(4) Solution-state NMR of *N. clavipes* dragline silk denatured in formic acid.
(5) Solution NMR of intact *N. clavipes* major ampullate gland silk fibroin.
(6) Solution-state NMR of intact *B. mori* silk gland fibroin (Asakura et al., 1984).
(7) Solid-state NMR of lyophilized major ampullate gland silk fibroin.
(8) Solid-state NMR of lyophilized denatured silk.
(9) Alanine 3_1-helix chemical shift (Saito et al., 1984).
*Carbonyl regions are poorly resolved.

We performed a series of experiments to address the question of whether silk in the major ampullate glands exists in a random coil conformation or whether it is undergoing dynamic averaging on the NMR time scale in its native state. FTIR and CD, spectroscopic tools with a faster time scale than NMR, were used to take a "snapshot" of the protein. Solid-state NMR spectra of flash-frozen glands and denatured silk were used to probe the existence of conformational distributions.

FTIR can be a useful tool for probing protein secondary structure. Infrared vibrations occur at times (0.1 to 0.005 ps) that are short relative to translational motion, and the absorption envelope represents a summation of all existing states of the sample. The vibrational modes most widely used in structural studies are the amide I band (C=O stretching) and amide II band (N-H bending and C-N stretching) (Haris and Chapman, 1992). Studies with model polypeptides have shown that there is a good correlation between the amide I band frequency and type of secondary structure present (Susie and Byler, 1986). The amide I band in the spectral range 1620–1640 cm^{-1} is attributed to β-sheet structure, whereas the spectral range 1650–1658 cm^{-1} is attributed to the α-helical conformation (Haris and Chapman, 1992). Our FTIR spectroscopic data (Fig. 2) on freshly excised major ampullate glands had an amide I band centered at about 1648 cm^{-1} (n = 4, range 1639–1655 cm^{-1}), between what would be expected for the α-helical and β-sheet ranges. However, it is difficult to make firm conclusions based on these data because of the broadness of the peaks and the intense scattering arising from our inability to easily separate the lumen content from the surrounding gland tissue without drying and/or shearing the sample, which then caused large shifts in the spectra. In view of the known difficulties in the interpretation of FTIR spectra of dragline silk (Dong et al., 1991), we conclude only that these data are inconsistent with the silk being in either a well-defined α-helical or β-sheet conformation.

CD spectroscopy is sensitive to the secondary structure of proteins in an aqueous environment and has been used extensively to determine relative amounts of secondary structure. CD experiments were performed with gland samples sandwiched between quartz windows. Our CD spectra (Fig. 3) included a wide negative band between 210 and 240 nm that looked similar to CD spectra for the α-helix pure secondary structure (Brahms and Brahms, 1980); however, the spectrum was broad. The feature at 237–243 nm was assigned to the gland lumen rather than the silk fibroin by analysis of smaller and smaller gland fractions (wherein the lumen content concomittantly increased). An average of the results of an interpretive CD algorithm (Yang et al., 1986) yielded structure fractions of approximately 30% α-helix, 40% random coil, and 30% β-turn present in our sample. There was no indication of a β-sheet structure in any of the spectra.

Although suggestive of the presence of conformations other than random coil and β-sheet, neither the FTIR nor CD experiments were compelling. With hopes of "freezing in" possible averaging conformations on the NMR time scale in the gland silk fibroin, solid-state ^{13}C CP/MAS was performed on silk ampullate glands that were dissected, immediately flash-frozen, and lyophilized. This sample was compared to one in which formic acid-denatured silk was also flash-frozen and lyophilized. These results are shown in Fig. 4. Fig. 4 *a* shows, for purposes of comparison, the solid-state ^{13}C spectrum of native dragline silk, whose alanine residues have been established to be in β-sheet conformations (Simmons et al., 1994). (The dashed lines in Fig. 4 correspond to the denatured dragline silk chemical shifts for alanine and glycine from Fig. 1 *a*.) The spectrum of the native dragline silk (Fig. 4 *a*) is clearly different from that of the lyophilized gland silk fibroin (Fig. 4 *b*) and from the lyophilized denatured silk (Fig. 4 *c*).

We first examine the chemical shift dispersion in the alanine C_α peak of the lyophilized gland silk (Fig. 4 *b*). The C_α peak has a large peak at 52.4 ppm and another one upfield at 48.8 ppm. The two peaks indicate that at least two predominant conformations are present in the alanine regions, and these chemical shifts correspond to the value of alanine C_α chemical shift for an α-helix and a β-sheet (Table 2, columns 2 and 3).

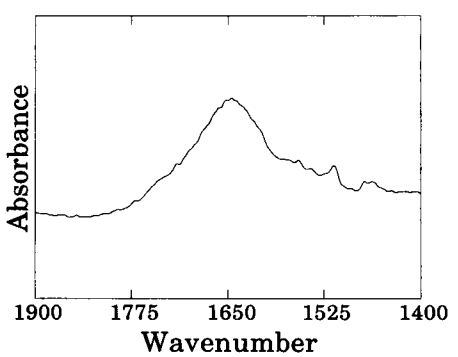

FIGURE 2 FTIR spectrum of freshly excised whole major ampullate gland sandwiched between two NaCl plates.

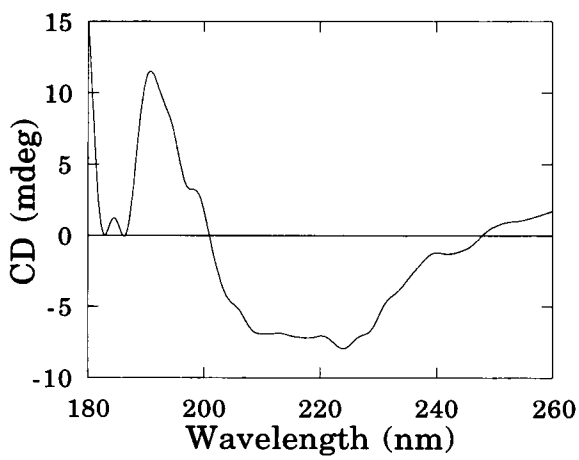

FIGURE 3 CD spectrum of freshly excised whole major ampullate gland sandwiched between quartz disks.

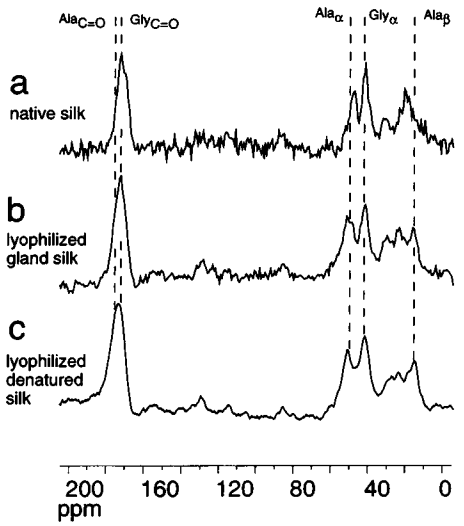

FIGURE 4 Solid-state ^{13}C CP/MAS NMR spectra obtained at a 75.4 MHz for ^{13}C and a spinning rate of 6.5 kHz. (*a*) Native dragline silk with 12,320 scans. (*b*) Flash-frozen and lyophilized major ampullate gland silk fibroin with 290,080 scans. (*c*) Flash-frozen and lyophilized denatured dragline silk with 347,488 scans. Dashed lines indicate denatured silk chemical shifts (from Fig. 1 *a*) for glycine and alanine.

However, an alanine C_α peak at 48.7 may also point to the possibility of the alanines in a 3_1 helix, as suggested by Kummerlen et al. (1995). Table 2, columns 9 and 2, shows that despite having the same C_α chemical shift value for a 3_1 helix and a β-sheet, the C_β chemical shift will be separated by 2.7 ppm. Fig. 4 *b* shows a distorted alanine C_β peak at 17.2 ppm, near the value of the 3_1 helix chemical shift, with a slight shoulder at 15.0 ppm, near the α-helix chemical shift. There is no peak near 20.1 ppm, which would otherwise indicate the presence of a β-sheet. CD spectra on individual dissected major ampullate glands lend support to this observation by indicating very little or no β-sheet secondary structure in the liquid gland silk. The flash-frozen denatured silk (Fig. 4 *c*) showed no similar dispersion about the alanine random coil chemical shifts, with the alanine C_α and C_β peaks both slightly shifted downfield and upfield in the direction away from a random coil toward an α-helix (Table 2, column 8).

The carbonyl region is relatively wide with a distorted lineshape and not well resolved in Fig. 4 as compared with Fig. 1. We do see, however, a slight shift downfield of the carbonyl carbons in the lyophilized gland silk fibroin and lyophilized denatured silk relative to the native dragline silk. This is consistent with the fact that ^{13}C carbonyl chemical shifts for β-sheets are located further upfield than that of the α-helix and random coil (Table 2, columns 2 and 3) and are separated from the α-helix by 4–5 ppm (de Dios and Oldfield, 1994).

As judged by solid-state ^{13}C NMR spectroscopy, the frozen-in conformation of the flash-frozen and then lyophilized silk in the major ampullate glands does not change upon the stresses induced by magic angle spinning or during storage for several months at room temperature. However, when this sample is rehydrated by plasticization with water (5% w/w), the alanine residues in the silk fibroin immediately assume a β-sheet conformation (Fig. 5). This result suggests that the conformation of the silk fibroin in the glands exists in a highly metastable state, and that hydrophobic interactions greatly affect the final conformation of the protein.

SUMMARY

The solution-state NMR data show that the alanine chemical shift in the liquid silk fibroin of the major ampullate glands match those of the silk I (loose helix) structure. The solid-state NMR spectrum of the lyophilized gland silk (Fig. 4 *b*) points to an averaging of helical conformations, in particular a 3_1-helix and an α-helix with no evidence of β-sheet structure in the gland silk fibroin. Although the alanine C_α peak for the lyophilized gland silk (Fig. 4 *b*) is wide, the C_β peak is consistent with the 3_1-helix and a shoulder suggesting an α-helix. We propose that this dynamic loose helical structure or similar form be termed a "molten fibril" in analogy to the molten globule state (Kuwajima, 1989). In the molten globule state, residual structure remains while the molecule is thermally unfolded, a feature that may be involved in protein translocation across membranes (Bychkova et al., 1988). We suggest that this molten fibril state is an important precursor for the final folded form of silk and prevents the protein from folding prematurely. It is clear from a comparison of Fig. 4 *a* and *b* that the gland silk fibroin is in a conformation dramatically different from that of the native dragline silk. Furthermore, hydration greatly affects the conformation of the silk (Fig. 5). The difference in native structure between the silk in the glands and the final dragline silk implies that the processing path from the ampullate gland to the spinneret plays an important role in forming silk's secondary structure. Our results show that the

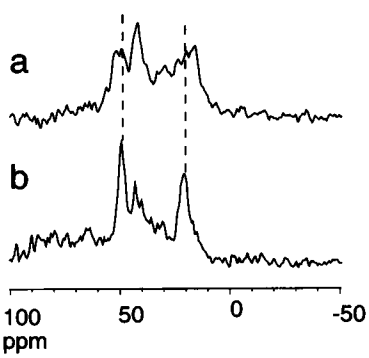

FIGURE 5 Expanded upfield region of the solid-state ^{13}C CP/MAS NMR spectra obtained at a 90.56 MHz for ^{13}C. (*a*) Flash-frozen and lyophilized major ampullate gland silk fibroin obtained with a 1-s repetition delay and 7400 scans. (*b*) Lyophilized and then rehydrated major ampullate gland silk fibroin obtained with a 1-s repetition delay and 14,000 scans. Dashed lines indicate the alanine C_α and C_β chemical shifts expected for dragline silk in the β-sheet conformation.

polyalanine β-sheets that are thought to provide the strength of the silk are not initially formed after translation, but require a special environment or processing path to induce β-sheet formation, which may then serve as nucleation sites for forming the rest of the silk's structure.

The long narrow ducts provide a way of changing the protein environment to induce the requisite large change in secondary structure. Because the ducts are five times longer than the length necessary to join the sac to the spinneret (Bell and Peakall, 1969), investigation of processing mechanisms may offer helpful clues to processing synthetically produced silk. It is known that the concentration of silk protein (Asakura et al., 1984) and the pH of the aqueous environment (Dabora and Marqusee, 1994) contribute to the final structure. A number of other conditions have been proposed that contribute to the processing of oriented silk such as dehydration (Tillinghast et al., 1984), shearing stress (Iizuka, 1983), and more recently, phosphorylation (Michal et al., 1995).

Knowledge of the initial secondary structure of the dragline silk before processing through the ducts will allow us to gain insight into the requirements of synthetic processing. In addition, the answer to this question may begin to explain how the spider is able to store the silk protein in high concentrations (50%) in the major ampullate gland, while maintaining it in a low-viscosity, liquid-like state. Projects are currently under way to further delineate the state and environment of the silk fibroin in the major ampullate gland and to investigate the role of the ducts in silk formation.

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