Solid-State ^{13}C NMR of *Nephila clavipes* Dragline Silk Establishes Structure and Identity of Crystalline Regions

Alexandra Simmons, Ed Ray, and Lynn W. Jelinski*

Center for Advanced Technology, Biotechnology Program, Cornell University, 130 Biotechnology Building, Ithaca, New York 14853-2703

Received April 1, 1994
Revised Manuscript Received July 6, 1994

Introduction. The dragline silk of the golden orb-weaving spider is a remarkable biopolymer: it possesses the unique combination of high tensile strength and large elongation to break.[1] Spiders have been spinning silk for nearly 400 million years;[2] yet, there is a dearth of information about the detailed structure of silk, and the source of its bulk properties is not understood. We are applying solid-state NMR to characterize the molecular-level structure and dynamics of silk in order to elucidate the origin of its exceptional mechanical properties. We present here solid-state ^{13}C spectra of dragline silk and examine some of its relaxation parameters.

The dragline silk of *Nephila clavipes* is produced by the ampullate glands and is drawn out of the spider from the spinnerets, which are located on the lower opisthosoma. The fiber originating in the major ampullate gland has been the most studied, because it is easily obtained through controlled silking of adult female spiders. The major ampullate silk consists of entirely of protein, and several reports on its composition have appeared. Average values from three reports[3-5] show that seven amino acids account for nearly 90% of the silk: glycine (42%), alanine (25%), glutamine (10%), leucine (4%), arginine (4%), tyrosine (3%), and serine (3%). The primary sequence of *N. clavipes* dragline silk protein has not yet been fully determined. Candelas *et al.* reported that the major ampullate gland of *N. clavipes* produces a single protein and used electrophoresis to obtain an estimated molecular weight of 320 000.[6] Lewis obtained clones about 2 kilobases long from the 3′ (carboxy) ends and determined the DNA sequence for two dragline silk proteins, Spidroin I and II.[7] Both proteins consist of repetitive sequences containing polyalanine regions in which the number of consecutive alanine residues varies from 4 to 10, sandwiched between sequences rich in glycine. Mello *et al.* recently observed a single protein of about 275 000 with a composition similar to that of Spidroin I.[8] Although there is consensus as to the nature of some repeating sequences in *N. clavipes* dragline silk, its composition is neither fully understood nor agreed upon in the literature at this point.

While some aspects of the primary structure of the major ampullate silk of *N. clavipes* are known, its secondary structure is still the subject of debate. Antiparallel β-sheet regions have been detected by X-ray diffraction[9] and Fourier transform infrared measurements.[10] This led to a proposed structure in which the glycine-rich regions of silk proteins adopt a β-sheet conformation, forming crystallites which give silk its strength.[10] The elasticity of silk would be provided by the polyalanine segments, which could adopt an α-helix conformation.[10] However, the intersheet spacing determined by X-ray diffraction matches that of an **alanine** β-sheet. A revised model in which the polyalanine regions form β-sheets which stack to form crystals in an "amorphous" glycine-rich matrix has been proposed.[7]

An understanding of the microstructure and dynamics of dragline silk and how they lead to its unique properties can only be reached by characterization of the material in the solid state. Not only is dragline silk insoluble in most solvents but it is unclear whether the solution structure would be the same as that of the solid. The inability to obtain single crystals of silk precludes the determination of its atomic level structure by X-ray diffraction. The success with which solid-state NMR has led to structure–property correlations in synthetic polymers suggests that it could yield significant information about dragline silk as well. This report, which presents the results of an initial ^{13}C CP/MAS study of major ampullate silk, shows this to be the case.

Experimental Section. Adult female *Nephila clavipes* spiders from central Florida were used. Silk production was initiated by manual stimulation at the spinneret, and the dragline fibers were collected on a takeup reel at 2 cm/s.[11] Constant observation under a microscope assured that no contamination by medial fibers (minor ampullate gland silk) took place. Up to 1 mg of silk could be collected from each spider at one silking.

Solid-state ^{13}C spectra of an unoriented sample of 45 mg of major ampullet fibers were obtained on a Bruker ASX-200 spectrometer operating at 50.307 MHz with cross-polarization (2.5 ms), high-power decoupling (67.5 kHz), and magic angle spinning (5 kHz). A total of 1024 scans were collected over a spectral width of 350 ppm, with a relaxation delay of 5 s. Chemical shifts were referenced to external adamantane and converted to ppm from TMS.

Relaxation experiments were performed on a Bruker CXP-200 operating at the above conditions, except that the sample was spun at about 3 kHz. Proton spin–lattice relaxation time constants in the rotating frame ($T_{1\rho}$) were measured using a pulse sequence with a variable delay (1–12 ms) following the cross-polarization pulse. A total of 1480 scans were collected for each point. Carbon spin–lattice relaxation time constants (T_1) were measured using the method of Torchia,[12] with 1200 scans collected for each point and a variable delay from 1 ms to 7 s.

Results and Discussion. The solid-state CP/MAS ^{13}C spectrum of the major ampullate fibers is shown in Figure 1, along with the assignments, made by comparing this spectrum with chemical shifts observed for peptides in solution[13] and the solid state.[14] An estimate of the composition of the sample was made by comparing the peak areas for each residue detected to the area of the carbonyl carbon peak. The integrated areas of peaks from glycine, alanine, glutamine, and tyrosine were 43%, 46%, 9%, and 4%, compared to values of 43%, 30%, 7%, and 4% obtained by amino acid analysis of a small piece of the same sample. The detection of alanine, as well as the expected amounts of glycine, glutamine, and tyrosine, shows that both the crystalline and amorphous regions of silk are detected by ^{13}C CP/MAS NMR. This must be the case, since no matter which model is used, the polyalanine segments are found in a different "phase" from the other amino acid residues. Fast spinning (5.0 kHz) eliminates sidebands in the aromatic region and allows the detection of the aromatic carbons of tyrosine, which makes up less than 5% of the silk. The larger-than-expected signal for alanine residues may be due to a higher cross-polarization efficiency, suggesting that the alanines are more static and are therefore in the crystalline regions of the silk. The observation of other amino acid residues in the expected proportions implies that, even in the amorphous phase, motion is restricted.

In the solid state, the chemical shifts of the α, β, and carbonyl carbons of amino acid residues in polypeptides are sensitive to their conformation.[14] The carbonyl and

0024-9297/94/2227-5235$04.50/0

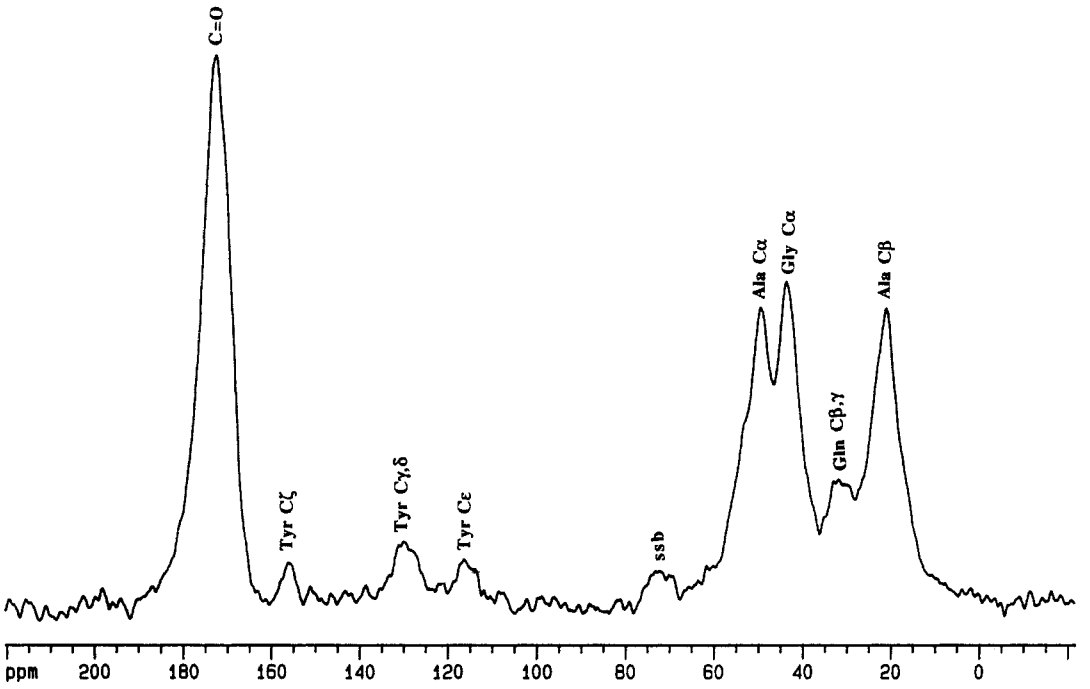

Figure 1. ^{13}C CP/MAS spectrum of major ampullate silk of *Nephila clavipes*.

Table 1. Chemical Shifts (ppm) for Carbons in *Nephila clavipes* Dragline Silk and for Amino Acid Residues in α-Helix and β-Sheet Conformations

carbon	dragline silk	β-sheet[11]	α-helix[11]
Ala C_β	20.6	20.1	15.1
Ala C_α	49.0	48.7	52.5
(Ala C=O)[a]	172.3	171.9	176.5
Gly C_α	43.2	43.7	
(Gly C=O)[a]	172.3	168.8	
Gln $C_{\beta,\gamma}$	31.6	29.4	25.7
(Gln C=O)[a]	172.3	171.6	175.5

[a] A single C=O peak was observed at 172.3 ppm.

Table 2. Relaxation Time Constants Measured for *Nephila clavipes* Dragline Silk

carbon	proton $T_{1\rho}$ (ms)	carbon T_1 (s)
Ala C_β	7.9	0.18 (40%) 2 (60%)
Ala C_α	8.5	12
Gly C_α	7.5	9
Glu $C_{\beta,\gamma}$	7.9	4
(C=O)[a]	7.8	20

[a] Single carbonyl signal containing all residues.

α-carbon in the α-helix are shifted downfield relative to those of a β-sheet, whereas the β-carbon is shifted upfield. This pattern has been used to show that the Silk II form of silkworm silk is predominantly β-sheet.[15] Table 1 lists the chemical shifts of major ampullate silk, as well as those for amino acid residues in peptides known to be in the α-helix or β-sheet conformation. The chemical shifts of alanine in dragline silk demonstrate without a doubt that alanine is present as a β-sheet. In fact, there is no evidence that any residues are present in the α-helix or random-coil conformation. This observation is consistent with a model in which the amorphous regions of dragline silk may be present in very small (too small to diffract) sheetlike structures.

The proton $T_{1\rho}$ and carbon T_1 values measured for major ampullate silk are listed in Table 2. Each set of proton $T_{1\rho}$ data was fit well by a single exponential. Backbone and side-chain carbons of glycine, alanine, and glutamine have the same relaxation time constant, about 8 ms. Observation of a single $T_{1\rho}$ for all sites indicates that the size of the inhomogeneities present in the silk fibers is limited to a few nanometers.[16] The short relaxation time constant of 8 ms suggests that spin diffusion results in the relaxation of all protons by a single efficient relaxation mechanism, probably the rotation of the alanine CH_3 group.

The carbon T_1s measured for dragline silk are of the same order of magnitude as those seen in silkworm silk.[17] An excellent fit to the glycine C_α and the glutamine ($C_{\beta,\gamma}$) peaks was obtained with a single exponential. The fact that glutamine CH_2 groups relax more quickly than the glycine CH_2 reflects the increased mobility of the side chains as compared to the backbone. A good one-component fit to the data for the carbonyl carbons was not obtained, but the number of points and reproducibility of the intensities was not sufficient to unambiguously define a two-component fit. It is clear that the carbonyl carbons relax very slowly, an expected result since they have no attached protons. A similar situation was encountered with the alanine C_α peak. The alanine CH relaxes almost as quickly as the glycine CH_2, even though it has only one attached proton. It appears, therefore, that the alanine CH relaxation rate is enhanced by the CH_3 rotation in that residue.

An excellent fit to the intensities from the alanine C_β (CH_3) was obtained by a two-component fit in which 40% of the carbons have a relaxation time constant of 0.18 s, and 60% relax more slowly, with a T_1 of 2 s. The observation of two T_1s indicates that alanine in dragline silk is present in two different motional environments. It is tempting to assign the slow-relaxing majority of the alanine carbons to the crystalline phase, while the remaining alanines present in the amorphous phase would be more mobile and relax faster. A calculation based on the amino acid sequence of Spidroin I[7] reveals that 68% of the alanines are present in polyalanine runs. The hypothesis that the polyalanine regions form the crystalline phase of dragline silk is compatible with our relaxation data which show that the majority of the alanines exhibit slow relaxation. This picture is consistent with the fact that the alanine C_α and carbonyl data could not be fit well

with a single exponential.

By obtaining ^{13}C CP/MAS spectra and measuring relaxation time constants in dragline silk of *N. clavipes*, we have demonstrated unambiguously that the alanine residues are present in the β-sheet conformation. Proton $T_{1\rho}$ data show that the heterogeneities in the silk must be small. Two motional environments have been detected for the alanine residues. These data verify that dragline silk is composed of crystalline regions of polyalanine β-sheets in a more flexible matrix. Studies on ^{13}C- and ^{2}H-labeled fibers are underway in our laboratory to characterize the structure, orientation, and dynamics of the amorphous and crystalline domains more fully. Then a correlation between the molecular-level structure and dynamics of dragline silk and its unique macroscopic properties will be developed. This will facilitate the design of materials which mimic or improve on those nature has taken 400 million years to evolve.

Acknowledgment. We acknowledge Dr. Almeria Natansohn, Department of Chemistry, Queen's University, Kingston, Canada, for assistance in obtaining the spectra. A.S. thanks NSERC Canada for funding through a Postdoctoral Fellowship, and we thank NSF (Grant MCB9303870) for financial support.

References and Notes

(1) Gosline, J. M.; Denny, M. W.; DeMont, M. E. *Nature* **1984**, *309*, 551.
(2) Shear, W. A.; Palmer, J. M.; Coddington, J. A.; Bonamo, P. M. *Science* **1989**, *246*, 479.
(3) Tillinghast, E. K.; Christenson, T. *J. Arachnol.* **1984**, *12*, 69.
(4) Work, R. W.; Young, C. T. *J. Arachnol.* **1987**, *15*, 65.
(5) Lombardi, S. J.; Kaplan, D. L. *J. Arachnol.* **1990**, *18*, 297.
(6) Candelas, G.; Candelas, T.; Ortiz, A.; Rodriguez, O. *Biochem. Biophys. Res. Commun.* **1983**, *116*, 1033.
(7) Lewis, R. V. *Acc. Chem. Res.* **1992**, *25*, 395.
(8) Mello, C. M.; Senecal, K.; Yeung, B.; Vouros, P.; Kaplan, D. Silk Polymers. *ACS Symp. Ser.* **1994**, *544*, 67.
(9) Warwicker, J. O. *J. Mol. Biol.* **1960**, *2*, 350.
(10) Dong, Z.; Lewis, R. V.; Middaugh, C. R. *Arch. Biochem. Biophys.* **1991**, *284*, 53.
(11) Work, R. W.; Emerson, P. D. *J. Arachnol.* **1982**, *10*, 1.
(12) Torchia, D. A. *J. Magn. Reson.* **1978**, *30*, 613.
(13) Asakura, T.; Watanabe, Y.; Uchida, A.; Minagawa, H. *Macromolecules* **1984**, *17*, 1075.
(14) Saitô, H. *Magn. Reson. Chem.* **1986**, *24*, 835.
(15) Saitô, H.; Iwanaga, Y.; Tabeta, R.; Narita, M.; Asakura, T. *Chem. Lett.* **1983**, 431.
(16) McBrierty, V. J. *Compr. Polym. Sci.* **1989**, *1*, 397.
(17) Saitô, H.; Ishida, M.; Yokoi, M.; Asakura, T. *Macromolecules* **1990**, *23*, 83.