are then presented sequentially in various combinations (that is, 1-1, 2-1, 1-2, and 2-2) with an interstimulus interval (ISI) interposed between the two tones. The subject is required to reproduce the sequence by pressing the panels in the correct order. The Computerized Repetition Test determines the threshold ISI at which sequences of two pure-tone stimuli of 150-, 75-, 40-, or 17-ms duration are perceived and reproduced with 75% accuracy. The ISIs vary from 500 to 0 ms. (v) R. Goldman and M. Fristoe, *Goldman-Fristoe Test of Articulation* (American Guidance Service, Circle Pines, MN, 1986). The Sounds-in-Words subtest was used to assess accuracy in speech articulation. Speech was elicited by having the child label a picture that depicted a common object or activity.

13. The speech and language exercises were developed as games to maintain attention and motivation over the course of the study. Tape recorded syllables, words, phrases, and sentences that had been acoustically modified with the speech algorithm developed for this study were presented to the child over headphones or free field. The games included acting out commands in a Simon Says format with props; pointing to pictures or colored blocks in response to commands; repeating verbatim syllables, nonsense words, real words, or sentences; and pointing to pictures corresponding to spoken words. Throughout training, commands of increasing length and grammatical complexity were used in these games. Careful attention was given in the design of the listening exercises to ensure that foils developed for each item would focus the attention of the child on the salient aspects of speech discrimination or receptive grammar being trained. In the listening games, regardless of the accuracy of the child's response, immediate nonverbal feedback was given after each response ("thumbs up" or "thumbs down"), followed by a repetition of the item with the correct response indicated by the clinician, so the child could have a second chance to process correctly. Each child won points for cooperation throughout the training, which were tallied daily and exchanged for prizes at the end of each week.
14. Changes from study 1 to study 2 included (i) increasing the duration of the laboratory sessions from 3 to 3.5 hours per day, (ii) providing homework solely in the form of recorded children's stories on tape [either acoustically modified (group A) or with natural unmodified speech (group B)] instead of computer games, (iii) increasing the number of computer game formats from two to four, and (iv) modifying the ratio of clinicians to children in each training session from one-to-one to usually one-to-one, but on occasion one-to-two. The children in study 1 and group A in study 2 received computer games that adaptively trained temporal processing and phoneme perception, whereas the children in group B study 2 received the same schedule of computer game training and reinforcement, but with games that did not contain temporally or phonetically adaptive stimuli.
15. Subjects were assigned to the two groups to minimize the differences between subjects on measures of performance IQ (PIQ) [*Wechsler Intelligence Scale for Children-III* (The Psychological Corporation, New York, 1991)] reported as mean (SEM) [PIQ, group A = 96.1 (2.6), group B = 96.6 (3.3)], and receptive language performance (Token Test Age scores) reported as mean (SEM) [group A = 5.4 (0.4), group B = 6.1 (0.7)].
16. Previous studies [P. Tallal and M. Piercy, *Neuropsychologia* **11**, 389 (1973)] have shown that the total signal duration of auditory stimulus patterns, as indexed by the relation between the duration and interval among stimulus elements, is critical for demonstrating the temporal processing deficits of LLI children. In the present investigation, temporal threshold values were calculated as the sum of the minimal tone durations (150-, 75-, 40-, or 17-ms tone pairs) and the average ISI based on an adaptive staircase (two-up and one-down) procedure to which subjects were able to reproduce pairs of tone sequences by pressing a response panel. A performance level of 75% or greater accuracy was required at a particular stimulus duration before a threshold would be calculated. The average pretraining thresholds by the LLI children were 491 ms in study 1 and 287 ms in study 2 (9). Normally developing children of a comparable age have been shown to require ISIs of less than 20 ms on this test (5).
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22. Informed consent was obtained from the parent or parents of each child after the potential risks and benefits of the studies were explained. We thank the therapists who referred subjects as well as the parents and children who participated. We thank A. Rubenstein, B. Glazewski, J. Flax, C. Roesler, K. Masters, J. Reitzel, T. Delaney, and P. Johnston for assistance in subject selection, stimulus preparation, and clinical testing and T. Realpe, I. Shell, C. Kapelyan, A. Katsnelson, L. Brzustowicz, C. Brown, A. Khoury, and S. Shapack for assistance in the experimental training. Valuable comments on the manuscript by I. Creese are appreciated. We thank the Charles A. Dana Foundation for supporting the research. For more information, see http://www.ld.ucsf.edu

29 September 1995; accepted 30 November 1995

Molecular Orientation and Two-Component Nature of the Crystalline Fraction of Spider Dragline Silk

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The molecular origin of the exceptional mechanical properties of spider silk is unclear. This paper presents solid-state ^2H nuclear magnetic resonance data from unoriented, oriented, and supercontracted fibers, indicating that the crystalline fraction of dragline silk consists of two types of alanine-rich regions, one that is highly oriented and one that is poorly oriented and less densely packed. A new model for the molecular-level structure of individual silk molecules and their arrangement in the fibers is proposed. These data suggest that it will be necessary to control the secondary structure of individual polymer molecules in order to obtain optimum properties in bio-inspired polymers.

Spider dragline silk is nature's high-performance fiber. A unique combination of tensile strength and elasticity gives the silk a higher energy to break than that of other natural or synthetic fibers, which is essential for its structural role in a spiderweb's frame and its function of supporting a dropping spider. It is known that dragline silk is a semicrystalline polymer, but the amount, composition, orientation, and structure of each of its phases remain the subject of debate. An early study of fibroins, including the silk of *Nephila madagascarensis*, showed that they could be grouped on the basis of their tensile behavior (*1*). The ratio of long-side-chain to short-side-chain amino acids in these protein polymers is similar for samples within a group. X-ray diffraction analysis of *N. madagascarensis* silk showed that the crystalline regions were composed of antiparallel pleated sheets (*2*). The relative amount of crystalline to amorphous content was thought to be high due to the preponderance of small amino acids, which pack efficiently. Warwicker placed the silk in a group containing fibroins with the same lattice spacing as β-polyalanine (*2*). He proposed that bulky residues reside in amorphous regions, as their side chains cannot be accommodated in the crystalline domains.

In 1977, Work discovered that wetting of unrestrained fibers of spider dragline silk at room temperature causes them to contract to half their initial length (*3*, *4*). In synthetic fibers, such supercontraction occurs only at extreme temperatures or in harsh solvents. Supercontraction of dragline fibers is accompanied by a decrease in tensile strength and an increase in elongation before breaking; fibers recover their original mechanical properties when dried. Interplanar spacings in crystalline regions of *N. clavipes* major ampullate silk do not change on supercontraction (*5*). Dry dragline silk has a predicted crystallinity of 30% (*6*).

Early data identified an ampullate protein of *N. clavipes* with a molecular weight greater than 200 kD (*7*, *8*), whereas more recent data (*9*) suggest that the freshly synthesized protein in the gland may be as large as 720 kD. Amino acid analysis has shown that, like silkworm silk, dragline silk contains not only

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large amounts of glycine (42%) and alanine (25%) but also some residues with bulky side groups such as glutamine and tyrosine (*7*). Xu and Lewis obtained the sequence of part of a *N. clavipes* major ampullate protein using a partial complementary DNA clone (*10*). The protein consists of repeating units in which a polyalanine run of five to seven residues is followed by a glycine-rich sequence containing the bulky residues. The first attempt to relate this sequence to dragline silk's mechanical properties proposed glycine-rich crystals connected by α-helical polyalanine segments that were purported to be the origin of silk's elasticity (*10*). Detection of helix formation in silk under tension was given as evidence to support this model (*11*). Later, alanine was proposed to be present as β sheets connected by glycine-rich regions containing β turns (*12*).

Termonia succeeded in modeling spider silk's mechanical properties with an amorphous rubberlike matrix containing 45% small crystallites by volume, which generate a surface layer of constrained chains that may be responsible for the toughness of dragline silk (*13*). The composition of the domains was not addressed. Thiel *et al.* interpreted analytical transmission electron microscopy of individual crystallites in dragline fibers as evidence of a crystalline phase of glycine and bulky residues, composing about 50% of the samples (*14*). Our ^{13}C cross-polarization–magic angle spinning (CP-MAS) nuclear magnetic resonance (NMR) data from silk fibers were not consistent with Thiel's findings. We showed that alanine is present as β sheets, that there are two motionally distinct alanine populations, and that none of the residues exhibit liquid-like mobility at room temperature (*15*).

The presence of polyalanine runs in the amino acid sequence of *N. clavipes* dragline silk, the x-ray diffraction data, and our ^{13}C NMR experiments point to alanine as composing the crystalline domains. In the work presented here, we wished to determine the orientations of the two alanine populations with respect to the fiber axis, to investigate their behavior under tension, and to understand their role in supercontraction. The sensitivity of ^2H NMR line shape and relaxation time to orientation and dynamics was used to selectively study the alanine-containing regions of silk. This paper presents the results of these studies and discusses their implications for the detailed structure of spider dragline fibers.

The ^2H spectrum (*16*) of polycrystalline *l*-alanine-3,3,3-$^2H_3$ (Fig. 1A) displays an axially symmetric powder pattern, narrowed by rapid methyl group reorientation. In the simulation of the line shape, which was corrected for fall off of pulse power with frequency (*17*), the quadrupole coupling constant and the width of the Gaussian broadening were the only adjustable parameters. In unoriented dragline silk fibers from spiders that had been fed deuterated alanine (*18*) (Fig. 1B), the methyl deuterons were undergoing fast reorientation, as in the alanine (Fig. 1A). The spectrum also contains a small component with a splitting that is representative of static deuterons (Fig. 1B) with perpendicular singularities at ±60 kHz. The spectrum could be satisfactorily simulated with the use of the sum of a methyl component that contributes 90% of the intensity and a static component that contributes 10% of the intensity. The repetition delay of 0.5 s in these spectra was insufficient to allow the nonmethyl component to relax fully. A spectrum of the fully relaxed component showed that 20% of the ^2H in the silk was in sites other than the alanine methyl groups, which indicated that some scrambling of deuterons had occurred in the spiders' metabolism.

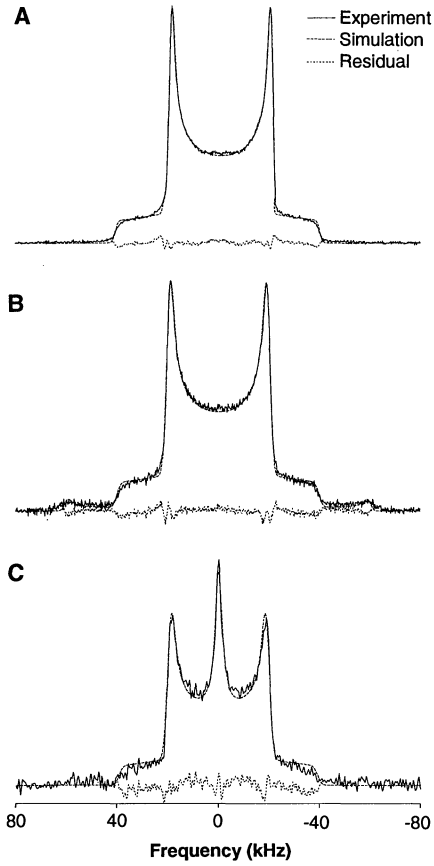

Fig. 1. (**A**) Solid-state ^2H spectrum of polycrystalline *l*-alanine-3,3,3-$^2H_3$ with best fit simulation using quadrupole splitting ($\omega_Q/2\pi$) = 40.4 kHz and FWHM = 2100 Hz, with residual. (**B**) Spectrum of labeled *N. clavipes* dragline silk. Methyl component (90%) was simulated with $\omega_Q/2\pi$ = 39.9 kHz, FWHM = 2700 Hz. Nonmethyl component (10%) was simulated with splitting of 120 kHz. (**C**) Spectrum of supercontracted silk. Methyl component (86%) was simulated as in (B); nonmethyl component (14%) was simulated by a Gaussian distribution centered at zero frequency.

The ^2H spectrum of supercontracted silk (*19*) (Fig. 1C) shows that the methyl component is unchanged upon wetting. However, the static signal is replaced by a peak centered at zero frequency, which is typical for averaging of the quadrupolar coupling by fast isotropic reorientation. The static component is plasticized by water and must be in the amorphous domain. Complete recovery of the initial spectrum (Fig. 1B) occurred after the silk was dried for 2 hours in air at room temperature.

To obtain spectra of oriented silk fibers, the fiber axis of a bundle of about 90,000 spider dragline silk fibers was oriented parallel to the magnetic field (*20*). The line shape of the oriented bundle (Fig. 2A) is very different from that of the unoriented fibers (Fig. 1B). The alanine methyl groups are not randomly oriented in the fibers, but are preferentially oriented at 90° to the fiber axis, as would be observed for alanine in β sheets with the chain axis parallel to the fiber axis.

We performed a quantitative analysis by calculating spectra based on proposed orientation distributions. Angular spreads and proportions of each distribution were adjustable parameters. The best fit was found with the use of the NL2SOL algorithm, implemented in routines from the PORT library (AT&T Bell Laboratories, Murray Hill, New Jersey) (*21*). The best fit to the experimental

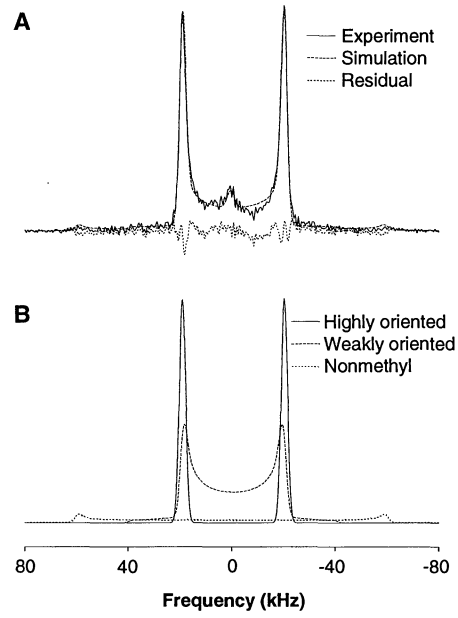

Fig. 2. (**A**) Solid-state ^2H NMR spectrum of a bundle of 90,000 *N. clavipes* dragline silk fibers oriented parallel to the static field. The simulation reflects a methyl group orientation distribution of two Gaussians centered at 90° with a FWHM of 5° (37%) and 75° (63%). (**B**) Components of the simulation, including a nonmethyl portion (11%) with a splitting of 120 kHz. The small central peak, which contributes approximately 2% of the total spectral intensity, was modeled in (A) as a Gaussian peak and was omitted from (B) for clarity.

data is shown in Fig. 2A; the individual components of the simulation are shown in Fig. 2B. The simplest distribution that fits the experimental spectra (see Table 1) consists of two Gaussian distributions, both centered at 90° to the fiber axis. One Gaussian distribution, representing about 40% of the alanines, has a full width at half maximum (FWHM) of 5° (+8°/–2°). This distribution is at least as narrow as the distribution recently observed for the synthetic high-performance fiber Kevlar (12° FWHM) (*22*). The other Gaussian distribution, representing about 60% of the alanines, has a much poorer degree of orientation (FWHM of 75° ± 5°). If only a single Gaussian population distribution is allowed, the best fit simulation qualitatively resembles the curve labeled "highly oriented" in Fig. 2B and is inadequate because it lacks significant intensity outside of the strong peaks.

Comparison with x-ray diffraction data ascertains that both of these alanine components represent crystalline regions. From x-ray diffraction data, Work calculated an orientation function, $\langle\cos^2\phi\rangle$, of 0.776 for *Araneus marmoreus* and 0.788 for *N. cruentata* (*3*) dragline silk. These yield average crystallite orientations of 28.2° and 28.1°. The two-population orientation distribution calculated from our NMR data yields $\langle\cos^2\phi\rangle$ of 0.760 and an average crystallite orientation of 29.3° for *N. clavipes* dragline silk, which is in excellent agreement with Work's data. The similarity between the average orientations from x-ray diffraction and NMR analyses suggests that both alanine populations contribute to the x-ray scattering, which implies that both environments are crystalline. This is the first time that the presence of both a highly oriented and a poorly oriented crystalline phase has been considered or detected in dragline silk.

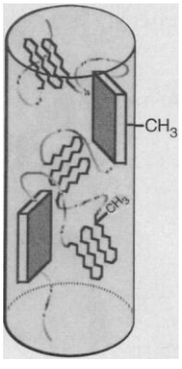

Fig. 3. Cartoon of the proposed model for the molecular arrangement of the alanine residues in a dragline silk fiber. Highly oriented alanine-rich crystals of β sheets (rectangles) and weakly oriented yet crystalline unaggregated sheets (canted sheetlike structures) are depicted in an amorphous glycine-rich matrix (curved lines). In reality, the glycine-rich matrix composes about 70% of the fiber; in this drawing it has been largely supressed for clarity.

Table 1. Orientation of alanine methyl residues in *N. clavipes* dragline fibers.

Silk sample	Highly oriented		Less oriented	
	Distribution width (FWHM)	Alanines (%)	Distribution width (FWHM)	Alanines (%)
Dry, oriented silk	5°(+8°/−2°)	37	75°(±5°)	63

We monitored the effect on the alanine residues of stretching the oriented silk sample by performing eight different ^2H NMR experiments, increasing the stress on the fibers until the breaking point was reached. All spectra obtained under tension (*20*) were indistinguishable from that obtained before stretching. No increase in the amount or orientation of the crystallites due to stretching was observed. Portions of the fiber bundle broke at 15% elongation and an average tension of 400 MPa. The elongation at the breaking point is typical of that reported for *N. clavipes* dragline silk (*23, 24*), but the tensile strength is somewhat lower than that observed for single fibers (*23, 24*), due to the difficulty of maintaining an equal load on all the fibers in a 90,000-fiber bundle.

We observed above that the ^2H line shape of polycrystalline *l*-alanine-3,3,3-$^2H_3$ is narrowed by fast methyl reorientation, as expected. The spin-lattice relaxation time, T_1, of deuterons can be used to obtain information about the reorientation rate. Inversion-recovery measurements (*25*) made at the perpendicular and parallel parts of the line shape give T_1's of 3.6 and 2.8 ms. This agrees with measurements by Batchelder *et al.* who noted that this orientation dependence of T_1 is consistent with a three-site jump model for methyl reorientation (*26*). Fast relaxation of methyl deuterons in alanine is due to a long (2-ns) correlation time for reorientation. Tight packing of the methyl groups in alanine crystals and their lack of additional lattice motion results in a high activation energy for reorientation and increases the correlation time.

We found that alanine-labeled silk has the same orientation dependence of relaxation times, indicating that the three-site jump model applies to the methyl groups in silk. However, the relaxation data could not be fitted to a single exponential. With the use of two exponentials, a fit could be obtained with a T_1 (^2H) of 66 ms for about 60% of the methyl deuterons and a T_1 (^2H) of 16 ms for about 40% (Table 2) (the sum of squares of residuals dropped by a factor of 36 upon addition of the second exponential to the fit). These T_1 values correspond to jump rates of 0.05 and 0.2 ns, respectively. Methyl reorientation in all the silk deuterons thus proceeds more quickly than in crystalline alanine, indicating less dense packing or increased mobility in the silk or both. Detection of two dynamic environments for alanine methyl groups in dragline silk complements the two fractions of alanines observed in the orientation-dependent data

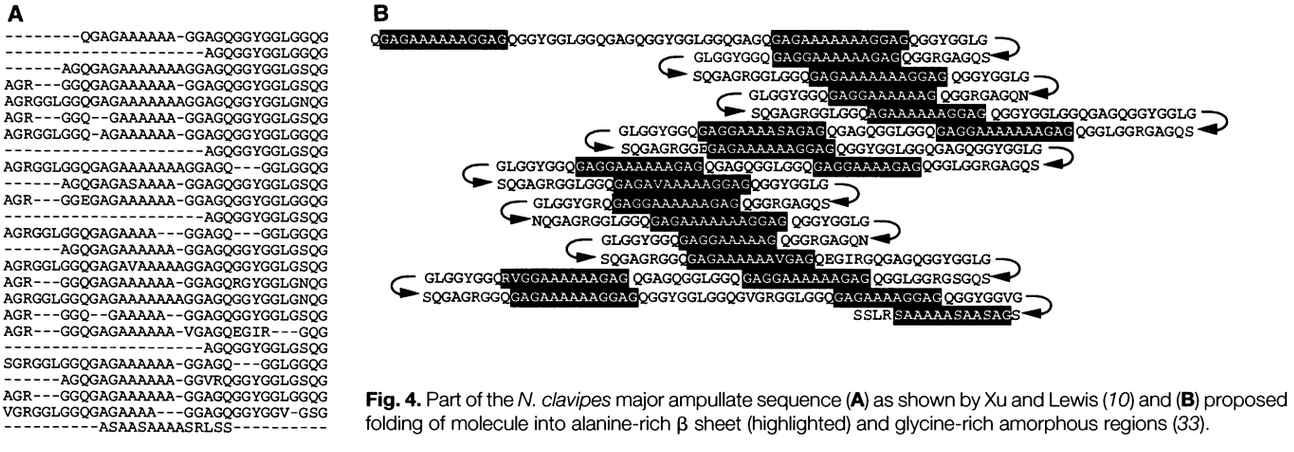

Fig. 4. Part of the *N. clavipes* major ampullate sequence (**A**) as shown by Xu and Lewis (*10*) and (**B**) proposed folding of molecule into alanine-rich β sheet (highlighted) and glycine-rich amorphous regions (*33*).

Table 2. Dynamics of alanine methyl residues in *N. clavipes* dragline fibers.

Silk sample	Highly oriented		Less oriented	
	T_1 (ms)	Alanines (%)	T_1 (ms)	Alanines (%)
Dry silk (^{2}H NMR)	16	40	66	60
Supercontracted (wet) silk (^{2}H NMR)	15	33	60	67
Dry silk (^{13}C NMR) [from (*15*)]	180	40	2000	60

presented above. We conclude that about 40% of the alanine methyl groups are present in β sheets that are highly oriented parallel to the fiber axis. The other 60% are poorly oriented and have a larger volume available for unimpeded methyl reorientation. The existence of two motional and structural environments for the methyl groups is also supported by our previous ^{13}C CP-MAS NMR measurements of the T_1 (^{13}C) of the methyl group in dragline silk (*15*). Those experiments also detected a majority of slowly relaxing methyl groups, with about 40% relaxing more quickly (Table 2). As observed for the ^{2}H nuclei, methyl ^{13}C relaxation in silk is slower than in crystalline alanine (*27*), reflecting the increased ease of methyl reorientation in dragline silk.

T_1 (^{2}H) was also measured for supercontracted dragline silk. Table 2 shows that the T_1 (^{2}H) of the methyl groups in wet silk is similar to that in the dry fibers. Along with the fact that the line shape is unchanged from that of the dry silk, this confirms that the dynamic environment of the alanine side chains in supercontracted silk is identical to that in the dry silk. This in turn implies that the alanine side chains are inaccessible to water; that is, both of the alanine motional and structural environments must be part of some kind of crystalline domain. This is also consistent with Work's observation that the crystallites in dragline silk rotate upon supercontraction but are otherwise unaffected by the presence of water (*3*).

A possible model for dragline silk is that some of the residues are present in a classical crystalline phase while the remainder are in protocrystals, possibly preformed β sheets. The internal structure of such a fiber may resemble that depicted in the cartoon in Fig. 3. The weakly oriented β sheets may account for the compressive strength of silk, as they provide reinforcement at a variety of angles. This hypothesis requires that individual protein molecules possess β-sheet conformations before fiber formation. The sequence for a part of a *N. clavipes* major ampullate protein (*10*) provides some insight into the possible origin of such structural preorganization. Because of the common occurrence of glycine (G), serine (S), or asparagine (N) residues in β bends (*28*), we propose that the chain direction reverses at the points in the sequence (Fig. 4A) where GX occurs (where X is S or N), to produce the secondary structure of Fig. 4B. The alanine-rich regions do not all line up to form a continuous β-sheet domain; instead, small staggered sections of sheet are present. We believe that the highlighted residues form the crystalline fraction of dragline silk. Some glycine residues are easily accommodated by the alanine intersheet spacing; however, adjacent glutamine residues are not. The function of these flanking glutamines, along with the GGX sequences that appear beside them [which are not stable β-sheet formers (*29*)], would be to prevent further growth of the β sheet in the chain direction. In the second dimension, the sequence is designed to induce the formation of small β-sheet domains in order to aggregate into the small crystallites envisioned by Termonia (*13*) and suggested by our preliminary synchrotron x-ray data (*30*).

In this model, 90% of the alanine residues would be in the β sheets, and 70% of the glycines would be in the amorphous regions. We conclude that all of the crystalline fraction of dragline silk is composed of alanine-rich sequences, which exist in two distinct environments. The 35% crystallinity of dragline silk predicted by this model agrees with Gosline's estimate (6) of crystallinity. That these two environments are not simply surface and interior sites as in the synthetic polymer poly (*p*-phenyleneterephthalamide) (*22*) is confirmed by the line shape data, which indicate that the majority site is poorly oriented. These poorly oriented crystallites may be important in effectively coupling the highly oriented crystalline domains and the amorphous regions, thereby producing a biomaterial with exceptional toughness.

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18. Adult female *N. clavipes* spiders were hand-fed a solution of 10% w/v of *l*-alanine-3,3,3-$^2H_3$ (Cambridge Isotopes, Woburn, MA; or C/D/N, Pointe-Claire, Quebec) in Dulbecco's modified Eagle medium (DMEM) (Gibco). On the basis of the amino acid composition of silk (*7*) and a comparison of spectral intensity with an alanine standard, we estimate that the labeling level of the alanine residues in the dragline silk is 4%. Fibers of major ampullate silk were obtained by controlled silking at 1 to 2 cm/s as described elsewhere (*32*).
19. We prepared supercontracted silk by soaking fibers in deuterium-depleted H_2O (MSD Isotopes, Rahway, NJ) for 12 hours, then blotting them with filter paper.
20. Fiber bundles were mounted into cylindrical Delrin discs and fastened in place with epoxy. The discs were held in a homemade probe with Vespel collars that attached the sample to fixed supports within the probe. The length of the sample was altered by the turning of an aluminum screw at the bottom of the magnet. The sample length could be adjusted with a resolution better than 0.02 mm. The probe incorporated a nonmagnetic load cell (A. L. Design, Buffalo, NY) for monitoring the applied tension. The load cell had a maximum capacity of 10,000 N, with a resolution of about 50 N. The load cell was calibrated on an Instron tensile strength tester. The entire probe was contained in an aluminum and brass tube that fitted into the 54-mm diameter magnet.
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34. The authors acknowledge the National Science Foundation (MCB-9303870) for financial support and a grant from Cambridge Isotope Laboratories. A.H.S. and C.A.M. thank the Natural Sciences and Engineering Research Council of Canada for a postdoctoral fellowship and a 1967 Science and Engineering Scholarship, respectively. R. Do, S. Lee, and R. Rosado assisted with sample collection.

1 August 1995; accepted 9 October 1995