Articles

NMR Characterization of Native Liquid Spider Dragline Silk from *Nephila edulis*

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Solid spider dragline silk is well-known for its mechanical properties. Nonetheless a detailed picture of the spinning process is lacking. Here we report NMR studies on the liquid silk within the wide sac of the major ampullate (m.a.) gland from the spider *Nephila edulis*. The resolution in the NMR spectra is shown to be significantly improved by the application of magic-angle spinning (MAS). From the narrow width of the resonance lines and the chemical shifts observed, it is concluded that the silk protein within the wide sac of the m.a. gland is dynamically disordered throughout the molecule in the sense that each amino acid of a given type senses an identical environment, on average. The NMR data obtained are consistent with an isotropic liquid phase.

1. Introduction

Spinning silk from liquid dope to virtually insoluble fibers, as mastered by many insects and spiders, is still a poorly understood process. Recently, considerable progress toward the characterization of the spinning process has been reported,[1−3] and a number of partially folded intermediates, some of them forming liquid-crystalline phases, have been postulated. Nevertheless, a detailed molecular model of the spinning process is still missing although it may be key to the biomimetic production of natural or engineered silk.

NMR offers a wealth of methods to study, on a molecular as well as on a macroscopic level, the structure and function of biological macromolecules.[4] Here we report progress toward high-resolution ^{1}H and ^{13}C NMR spectra of spider "silk dope". Here, "silk dope" stands for the native liquid silk stored within the lumen of the wide sac of a major ampullate (m.a.) silk gland.[5] The silk dope consists of a concentrated protein solution, over 30% of the mass being protein[6,7] and is stored in specialized glands.[8,9] On its way from the storage along the spinning duct to the spinnerets, the silk proceeds through one or several phase transitions to be extruded as a solid thread.[1,2,5] Silk spun from the m.a. gland is mainly used in draglines and web support such as radii and has received much attention due to its impressive mechanical properties.[10,11]

Spider dragline contains predominantly silk proteins (spidroins) composed of more than 60% of alanine and glycine[12,13] organized in highly repetitive segments traditionally divided into "amorphous" and "crystalline" domains.[14,15] Solid-state NMR and X-ray studies have shown that the alanine-rich regions form predominately β-sheets,[14−20] whereas the glycines are only partially found in β-sheets but contribute mostly to helical structures showing a 3-fold symmetry.[20]

The molecular structure of silk dope and of intermediates during the transition from the soluble form into the solid thread along the silk production pathway still needs to be characterized.[5,21,22] Based on CD and NMR spectroscopy a secondary structure, called "dynamic loose helical structure", was postulated for the spiders silk dope[7] in analogy to the silk I structure postulated for solid silk from the domesticated silkworm *Bombyx mori*.[23,24]

Results from CD spectroscopy suggest a secondary structure poor in α-helices and β-sheets for the silk dope in the upstream end of the m.a. gland of the spider *Nephila clavipes*, the so-called "A-zone",[5] whereas in the downstream end ("B-zone"), β-sheet structures were detected.[22] In the silk dope from the silkworm *Bombyx mori* no classical secondary structure was found,[25,26] but for the silk dope of the wild silkworm *Samia cynthia ricini*, α-helical structures were encountered.[25−29]

This article describes NMR spectroscopic studies on silk dope from the spider *Nephila edulis*. Magic-angle sample spinning (MAS) is shown to lead to a considerably enhanced resolution compared to previously described spectra,[7] which allows for an almost complete assignment of the carbon, as well as the proton spectrum, and forms the basis for future NMR experiments. The reduction in line width is ascribed to averaging susceptibility effects. We conclude that the viscous silk dope forms an isotropic liquid and that the

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Table 1. Amino Acid Composition (Mol %)[a] of the Solid Dragline Silk of *Nephila edulis* as Determined by Standard Amino-Acid Analysis

amino acid	amount (mol %)	amino acid	amount (mol %)
Ala	32 (1)	Lys	0.3 (1)
Arg	2.1 (2)	Met	0.2 (2)
Asp/Asn	1.8 (5)	Phe	0.3 (3)
Gly	39 (4)	Pro	4 (1)
Glu/Gln	10 (1)	Ser	3.6 (5)
His	0 (0)	Thr	0.6 (1)
Ile	0.6 (1)	Tyr	3 (1)
Leu	2.7 (3)	Val	0.7 (2)

[a] The values are given with their standard deviation corrected for student-t distribution.

polypeptide chains assume no well-defined secondary structure.

Liquid silk stored within the gland attracts attention not only because it is the starting material for the interesting spinning process under ambient temperature, low pressure, and with water as solvent[21,30] but also because the spider manages to keep its highly concentrated and stress-sensitive viscous silk dope in a liquid state, avoiding the formation of insoluble structures.

2, Materials and Methods

Sample Preparation. To produce isotopically labeled samples, mature female *Nephila edulis* spiders were kept for two weeks on a diet of 1−2 mealworms per week, supplemented with two daily doses of 6−8 droplets of an aqueous amino acid solution (85 mg/mL) reflecting the amino acid composition of m.a. silk (Table 1), of which a particular amino acid was uniformly ^{13}C-labled. The spiders were silked twice during the first week to empty their m.a. gland from unlabeled silk.[31] This procedure yields a gland containing liquid silk uniformly ^{13}C-enriched (3−10%) in the particular amino acid. To obtain silk enriched at every site, subsequently called "fully labelled" silk, all amino acids in the feedstock were uniformly ^{13}C-labelled and, in addition, daily 6−8 droplets of an aqueous solution of uniformly ^{13}C-labelled glucose (50 mg/ml) was fed. This leads to a material where roughly 10% of all amino acids are uniformly enriched, more or less irrespective of the amino acid type.

For the experiments on silk dope, the m.a. gland and its associated structures were dissected in Schartau and Leidersher's spider Ringer solution.[32] After cutting off its duct, the tail was removed from the gland and the remaining wide sac was gently drawn into a 2.5 mm MAS rotor made of ZrO (Bruker, Karlsruhe) using a syringe.[5]

To perform the measurements on denatured silk dope one to two wide sacs were placed overnight in approximately 50 μL of 7.7 M urea containing 0.05% (w/v) NaN_3. The sac was disrupted by 5 cycles of sonication (in a ultrasonic bath) and vortexing. The sample was subsequently clarified by centrifugation (10 min, 9000 g), and the resulting supernatant was loaded into a 2.5 mm MAS rotor made of ZrO.

The amino acid composition of the solid dragline silk was determined using a standard amino acid analysis.

NMR Experiments. NMR spectra were obtained on a Bruker DMX 400 Avance Spectrometer at 400.13 MHz for ^{1}H using a Bruker 2.5 mm double-resonance magic-angle spinning (MAS) probe head. A carbon- and proton field strength of 35 kHz was applied for the hard pulses. The field strength was reduced to 4.5 kHz for carbon decoupling and to 15 kHz for proton decoupling. The decoupling was performed using the GARP decoupling scheme.[33] For proton-detected spectra, water was presaturated with a field strength of 65 Hz. The recycle delays for proton and carbon spectra were set to 2 and 10 s, respectively. The experiments were carried out at a temperature of 296 K and a MAS frequency (stable to ±10 Hz) between 1.0 and 1.2 kHz. From 1D spectra it was judged that, under these conditions, samples were stable for approximately 4 days.

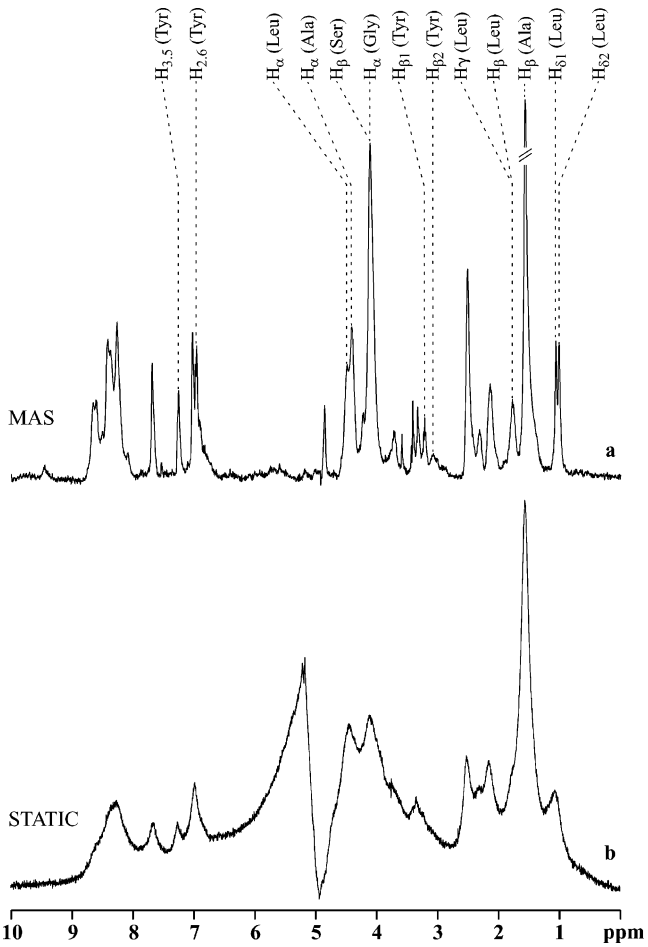

Figure 1. One-pulse ^{1}H spectra of silk dope from *Nephila edulis* recorded with (a) and without (b) MAS. The resonance for (Ala)H_β in (a) has been scaled by a factor of 3. For both spectra, 4 scans were taken.

The chemical-shift scale was referenced to an external TMS sample. Internal TMS was shown to yield an identical reference.

3. Results and Discussion

To preserve, as closely as possible, the "native" environment of liquid spider silk stored in the wide sac of the m.a. gland prior to the spinning process (silk dope), only freshly dissected glands were used for all experiments.

Typical one-pulse proton spectra of silk dope are shown in Figure 1. Upon spinning the sample about the magic angle,

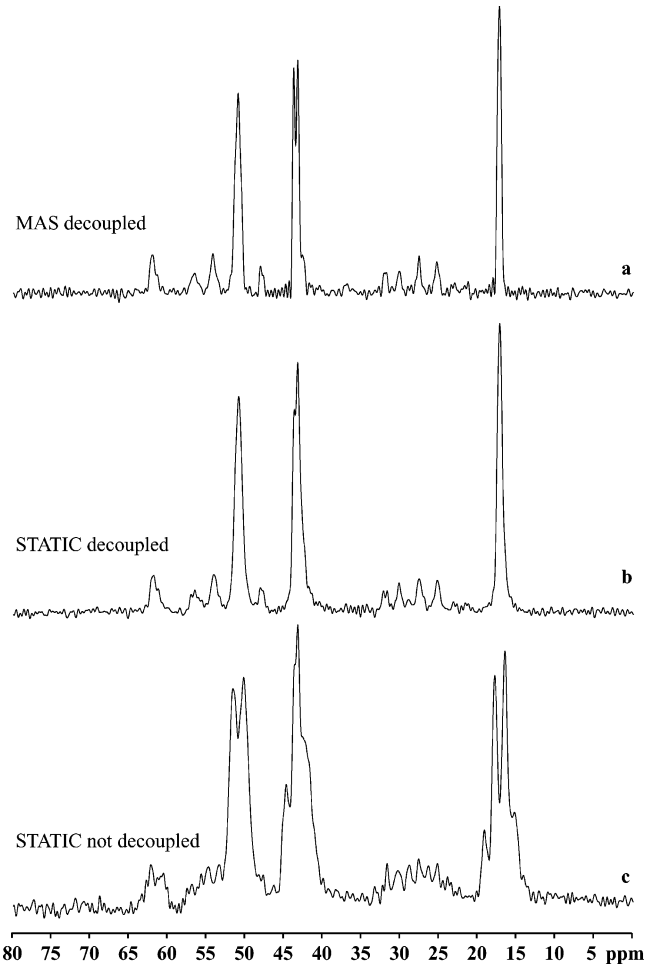

Figure 2. Aliphatic region of one-pulse ^{13}C spectra of "fully labeled" silk dope from *Nephila edulis*. Spectrum (a) was recorded with slow MAS. Proton decoupling was applied in the two upper spectra (a, b). The three strongest resonances are assigned to (Ala)C_α (51 ppm), (Gly)C_α (43 ppm), and (Ala)C_β (17 ppm). The $^1H-^{13}C$ J-coupling leads to the splitting of the resonances by about 135 Hz in c). $^{13}C-^{13}C$ J-coupling (50 Hz) is seen for (Gly)C_α (a, b). The spectra are individually scaled. The number of co-added transient was 1230, 2970, and 4380 in (a), (b), and (c), respectively.

a reduction in linewidth by a factor of 4 is observed (Figure 1a).

The aliphatic portion of the static ^{13}C one-pulse NMR spectra of "fully labeled" silk dope of the spider *Nephila edulis* are shown in Figure 2 in the presence (b) and absence (c) of proton decoupling. The splittings in the spectrum presented in Figure 2c can be fully explained by the expected $^1H-^{13}C$ J-coupling constants for the alanine and glycine residues and no sign of the presence of residual dipolar couplings are observed. As seen in Figure 2, the line width of the proton-decoupled ^{13}C spectrum (b) is significantly reduced by the application of MAS (a), whereas no changes in the line positions were observed. The resolved splitting of the glycine C_α resonance at 43 ppm in Figure 2a is due to the $^{13}C-^{13}C$ J coupling, the remaining line width in the MAS spectrum is probably dominated by nonresolved homonuclear J couplings. The experimental spectra are fully consistent with the assumption that the silk dope is an isotropic liquid and no indication for liquid-crystallinity was observed. For a liquid crystalline sample, one would expect the appearance of residual dipolar couplings and residual

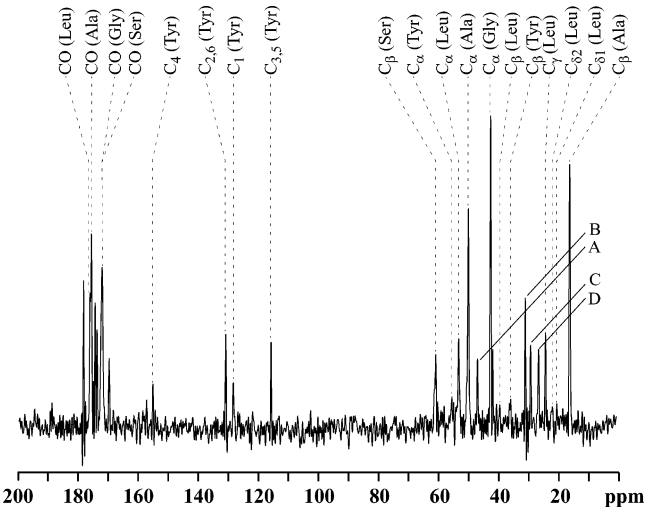

Figure 3. Proton-decoupled one-pulse ^{13}C spectra of silk dope from *Nephila edulis*. with natural isotopic abundance (4096 scans). The peaks marked with letters are not assigned and may belong to other molecules than the silk protein.

chemical-shielding anisotropy (CSA) effects which, without MAS, would lead to the appearance of additional residual dipolar line splittings and a variation in the line position away from the isotropic shift. We have found no indications for these effects in our spectra and attribute the reduction in line width observed under MAS to the averaging of macroscopic susceptibility effects arising predominantly from the inhomogeneous sample distribution within the partially filled rotor and possibly to the presence of particles of epithelium tissue. Liquid-crystalline phases occurring in other specific locations along the whole silk production pathway are still conceivable.[2,21,34,35]

The ^{13}C MAS spectrum of silk dope with natural isotopic abundance is given in Figure 3. The spectrum shows resonances arising from sidechain and backbone sites within the protein. Because of the absence of homonuclear J couplings, the lines are narrower (~40 Hz FWHH) than for spectra from ^{13}C labelled liquid silk and allow the assignment of the resonances to spins within the individual amino acids.

This assignment was obtained from ^{13}C one-pulse experiments of liquid m.a. silk enriched specifically with a uniformly ^{13}C-labeled amino acid (Figure 4). Each spectrum shows a significantly increased resonance intensity with respect to the natural abundance spectrum (Figure 3) almost exclusively at sites that can be assigned to the specific amino acid. The level of isotropic scrambling is low in accordance with earlier findings.[17,36] The resulting assignment listed in Table 2 and indicated in Figure 3 was obtained on the basis of the distinct chemical shifts, the observed $^{13}C-^{13}C$ J-couplings and previously published chemical shifts for peptide systems.[37]

In Table 4, the chemical shifts of the native liquid silk within the wide sac of the m.a. gland are compared with both the chemical shifts of its denatured form, representing the random-coil structure of liquid silk, and with C_α and C_β chemical shifts from various databases.[37,38,40] The spectra of liquid silk denatured in 7.7 M urea (spectra not shown) are similar in appearance to the spectra of native liquid silk with only small (<2 ppm) upfield shift of the resonance lines.

Table 2. ^{13}C Chemical Shift (ppm)[a,b] Assignment of Native Liquid Silk within the m.a. Gland of *Nephila edulis* and Its Liquid Silk Denatured in 7.7 M Urea (in Brackets)

	C_α	C_β	C_γ	C_δ	C=O	other
Ala	51.0 (50.0)	17.3 (16.7)			176.3 (−[c])	
Gly	43.5 (42.7)				172.3 (−[c])	
Leu	53.8 (53.4)	40.5 (40.0)	25.1 (24.6) 23.0 (22.5)	21.6 (20.9)	176.0 (−[c])	
Ser	−[c]	62.1 (61.4)			172.4 (−[c])	
Tyr	56.4 (56.0)	37.2 (36.4)			−[c] (−[c])	129.0 [C_1] 131.4 [$C_{2,6}$] (130.8) 116.3 [$C_{3,5}$] (115.9) 155.5 [C_4]

[a] The accuracy of the chemical shift is 0.2 ppm. [b] The chemical shifts refer to TMS. [c] Could not be determined unambiguously.

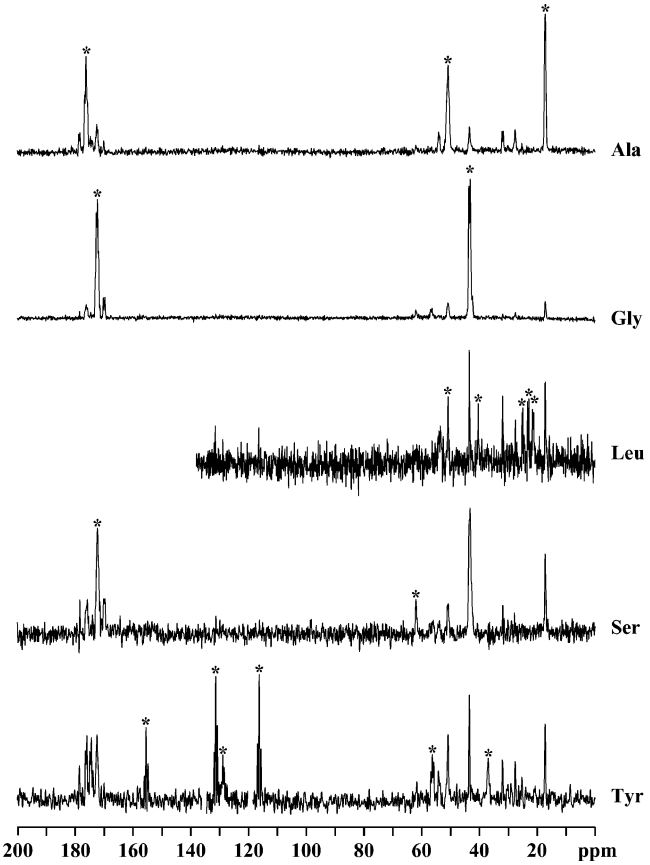

Figure 4. Proton-decoupled one-pulse ^{13}C spectra of silk dope from *Nephila edulis* enriched specifically with uniformly ^{13}C-labeled amino acids. Asterisks denote the resonances enhanced by labeling that are assigned to sites within the labeled amino acids. The two resonances around 30 ppm are not assigned and may belong to other molecules than silk. They occur in all spectra and are unaffected by the various isotopes labels applied here. The number of co-added transients was 1024 for alanine, glycine, and serine, 2048 for tyrosine, and 4096 for leucine.

The values obtained in this study, within experimental accuracy, match the range of random-coil shifts employed by these databanks although these are strongly biased toward globular proteins.

The chemical shifts and the absence of any detectable distribution in the resonances together with the relatively narrow lines indicates that all amino acids of a given type see, on the NMR time scale, the same environment. These results strongly suggest that native liquid silk within the wide sac of the m.a. gland is a dynamically disordered protein without a well-defined secondary structure. However, it should be noted that we cannot exclude the presence of other secondary structures having chemical shifts similar to random coil values.

Table 3. ^1H Chemical Shift (ppm)[a,b] Assignment of Native Liquid Silk within the m.a. Gland of *Nephila edulis* and Its Liquid Silk Denatured in 7.7 M Urea (in Brackets)

	H_α	H_β	H_γ	H_δ	other
Ala	4.43 (4.36)	1.56 (1.47)			
Gly	4.12 (4.05)				
Leu	4.51 (4.43)	1.78 (1.70)	1.78 (1.70)	1.02 (0.94) 1.05 (0.99)	
Ser	−[c] (−[c])	4.04 (3.97)			
Tyr	−[c] (−[c])	3.07 (−[c])			7.27 (7.19) [H2,6]
		3.22 (−[c])			6.95 (6.89) [H3,5]

[a] The accuracy of the chemical shifts is 0.20 ppm. [b] The chemical shifts refer to TMS. [c] Could not be determined unambiguously.

Table 4. Chemical Shift (ppm) Distributions for C_α and C_β in Secondary Structure Elements[c] for Particular Amino Acids

		Ala	Gly	Leu	Ser	Tyr
C_α	sheet	49.6−52.1	42.4−44.9	52.4−54.9	55.5−58.0	55.4−57.9
	helix	54.4−56.4	47.2−49.2	57.2−59.2	60.3−62.3	60.2−62.2
	rc	50.8−53.1	43.5−45.3	52.8−55.6	56.6−58.5	55.8−57.7
	gland[b]	51.0	43.5	53.8	*a*	56.4
	urea[b]	50.0	42.7	53.6	*a*	*a*
C_β	sheet	19.3−23.1		42.6−46.4	63.5−67.3	39.1−42.9
	helix	17.8−19.5		41.1−42.8	62.0−63.7	37.6−39.3
	rc	17.7−19.0		40.5−42.4	62.3−63.8	37.0−39.1
	gland[b]	17.3		40.5	62.1	37.2
	urea[b]	16.6		40.0	61.4	*a*

[a] Could not be determined unambiguously. [b] The chemical shifts refer to external TMS and the accuracy is 0.2 ppm. [c] Sheet: β-sheet, helix: α-helix, rc: random coil. The values for random coil are taken from refs 38−40. Gland: silk dope, urea: silk dope denatured in urea.

A proton spectrum of the silk dope recorded under MAS is shown in Figure 1a. The lines are narrow (~20 Hz FWHH) enabling the resolution of many resonances. The assignment of these resonances (Figure 1a and Table 3) was obtained from the assigned ^{13}C resonances in $^1H-^{13}C-$HSQC experiments[41] performed on samples enriched specifically with a uniformly ^{13}C-labeled amino acid (Figure 5). The proton shifts corroborate the conclusions drawn from the carbon shifts that silk dope is stored without a preferred secondary structure.

The unassigned peaks in the aliphatic region (indicated A−E) may conceivably belong to other molecules than silk. However, to date, no experimental data exists from liquid m.a. gland silk of *Nephila edulis* enriched specifically with further uniformly ^{13}C-labeled amino acids.

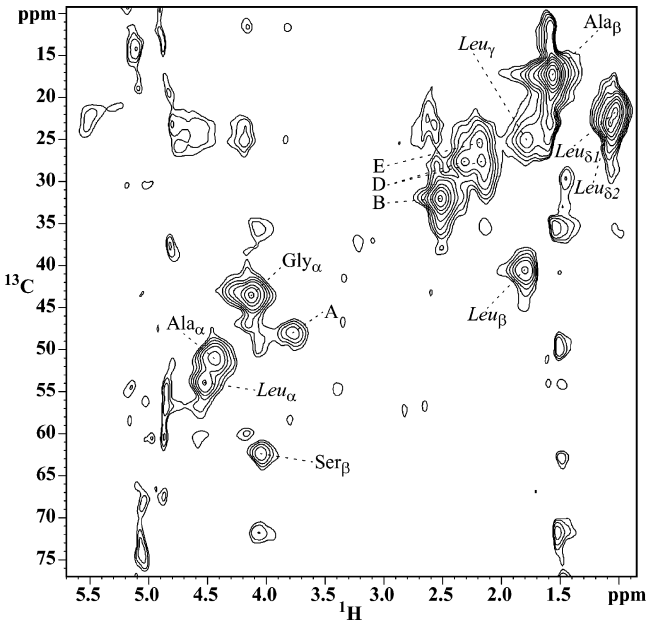

Figure 5. Example of a ^{1}H−^{13}C−HSQC spectrum of silk dope from *Nephila edulis* enriched specifically with uniformly ^{13}C-labeled leucine. The peaks marked with a single letter are not assigned and may belong to other molecules than silk. The number of co-added transient was 32 with 1024 data points in the direct and 512 data points in the indirect dimension. The aquisition time was 51.2 ms with an INEPT delay of 1.7 ms.

4. Conclusions

Both the ^{13}C and proton spectra of the liquid silk stored within the wide sac of the m.a. gland of *Nephila edulis* recorded under MAS are well resolved and have permitted an assignment of most resonances to particular amino acid residues. The observation of a single narrow line instead of a distribution of resonance frequencies indicates that identical amino acids, irrespective of their position in the primary structure, observe a homogeneous local environment (on a molecular scale). This is an indication for the absence of partial prefolding of the protein. The similar chemical-shift values found for native liquid silk and its denatured form, representing the random-coil structure, suggests that the molecule is not stored with well-defined secondary structure. Comparable results were found for silk dope from the silkworm *Bombyx mori*,[25] whereas the silk dope from the silkworm *Samia cynthia ricini* forms an α-helical structure.[27,29] CD spectroscopy on silk dope from the spider *Nephila clavipes* suggests β-sheet structures are only present in the small volume at the downstream end of the m.a. gland.[22] Other work on spider silk dope[7] suggested a "dynamic loose helical structure" similar to the silk I structure occurring in solid *Bombyx mori* silkworm silk. More recently two somewhat similar models were independently postulated for silk I.[23,24] Those models are thought to be only stable for $(\text{Ala-Gly})_x$ sequences and seem to be unlikely for the poly-Ala sequences found in spider silk. As mentioned above, our results are comparable with a random coil arrangement but a different structure, uniform about the entire macromolecule, cannot be excluded.

No signature of liquid crystallinity for the spider's silk dope was detected. With the improved spectral resolution and the assignment of the resonances available, we are in a position to investigate other intermediates occurring during spinning and which may indeed be liquid crystalline and partially folded.

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