Transition to a β-Sheet-Rich Structure in Spidroin in Vitro: The Effects of pH and Cations†

Cedric Dicko,*,‡,||,⊥ John M. Kenney,§,|| David Knight,‡ and Fritz Vollrath‡,⊥

Department of Zoology, Oxford University, OX1 3PS, Oxford, U.K., Department of Physics, East Carolina University, North Carolina 27858, Institute for Storage Ring Facilities, University of Aarhus, 8000 Aarhus C., Denmark, and Department of Zoology, University of Aarhus, 8000 Aarhus C., Denmark

Received August 3, 2004; Revised Manuscript Received September 2, 2004

ABSTRACT: Unlike man-made fibers, the silks of spiders are spun from aqueous solutions and at atmospheric pressure in a process still poorly understood. The molecular mechanism of this process involves the conversion of a highly concentrated, predominantly disordered silk protein (spidroin) into β-sheet-rich structures. To help store and transport the spidroins in solution, as well as probably control their conversion, a liquid crystalline arrangement is established in the storage region in the ampulla and persists into the duct. Although it has been suggested that changes in the concentration of hydrogen and metal ions play a role in the formation of the solid thread, there is no reported evidence that these ions influence the secondary structure of native spidroin in solution. Here, we demonstrate that pH values between approximately 3.5 and 4.5 induce a slow change of conformation from the disordered to the β-sheet-rich form. We also report that Al^{3+}, K^+, and Na^+ ions induce similar changes in structure, while Ca^{2+} and Mg^{2+} stabilize the predominantly disorder state of the protein. Cs^+ and Li^+ have no apparent effect. Direct volumetric and spectrophotometric titration showed a p*I* of 4.22 ± 0.33 and apparent p*K* values of 6.74 ± 0.71 and 9.21 ± 0.27, suggesting a mechanism for the effect of low pH on the protein and a rationale for the observed reduction in pH in the duct. We discuss the importance of these findings for the spinning process and the active role played by the spider to alter the kinetics of the transition.

The spider dragline silk is one of the toughest materials known. It is an extraordinary achievement for a spider to draw protein fibers from aqueous solution at room temperature and atmospheric pressure to produce mechanical properties comparable to and often better than man-made fibers (*1*). Although the composition of the silk is not fully known, partial gene sequences (*2*−*5*) from *Nephila clavipes* suggest that spider dragline silk contains two large structural proteins: spidroin 1 and spidroin 2. The complexity of the silk gland suggests that the control of the spinning process relies on a precise sequence of tightly controlled events (storage−transport−fibril formation) that take place along the production pathway (*5*). During this process, the spider actively controls the conversion of the soluble and highly concentrated aqueous solution (up to 30% in weight) of silk protein(s) into an insoluble fiber. Physiological parameters, such as pH and ions, appear to play key roles in this control by affecting the folding and conformational transitions of spidroin (*6*−*10*).

Studies on both silkworm and spider have demonstrated the presence of proton pumps thought to acidify the silk proteins as they flow through the spinning duct (*5*, *10*, *11*). In a recent study, we demonstrated the existence of a pH gradient in the ampulla of the *Nephila* spider, indicating an apparent pH of about 7.2 for storage and a pH of about 6.4 for spinning (*10*). We also demonstrated in vitro conformational changes produced by a similar reduction in pH. Rheological data by Chen et al. (*12*) indicated that in spiders both pH and salt content influenced strain dependence during shear. Terry et al. (*13*) demonstrated a sol/gel transition on changing the pH of native *Bombyx mori* silkworm fibroin from 11 to 2. This transition was reversible provided that the material was not stored under acid conditions for more than a few minutes. These findings are of relevance because it is now agreed that pH-induced gelation is accompanied by an increase in β-sheet structures. The isoionic point (p*I*) of fibroin [about pH 4−5 (*14*)] has been found to be the optimal value for gel formation and β-sheet conversion (*15*, *16*), suggesting that electrostatic forces may regulate the solubility of spidroin and hence its precipitation. There are no definite links between precipitation and β-sheet formation, although it is generally accepted that β-sheet formation leads to precipitation. Other factors can influence precipitation of silk proteins. For example, the addition of alcohol to native spidroin leads to the formation of flakes and β-sheet structure (*17*, *18*).

† This work was supported by the British Biological and Engineering Research Council UK-BBSRC (S12778), UK-EPSRC (GR/NO1538/01), the European Commission (G5RD-CT-2002-00738), and the Danish Natural Sciences Research Council (SNF) for financial support. The Institute for Synchrotron Radiation, ISA, Aarhus, Denmark is acknowledged for use of their CD facility through an EC-Human Potential Program Transnational Access to Major Research Infrastructures (EU contract no HPRI-CT-2001-00122).

* To whom correspondence should be addressed. Telephone: +44-1865-271216. Fax: +44-1865-281253. E-mail: cedric.dicko@zoology.ox.ac.uk.

‡ Oxford University.

§ East Carolina University.

|| Institute for Storage Ring Facilities, University of Aarhus.

⊥ Department of Zoology, University of Aarhus.

10.1021/bi0483413 CCC: $27.50
Published on Web 10/16/2004

The ionic balance as much as the pH is likely to be of great importance during the spinning process. Knight et al. (*7*) detected Na, K, S, P, and Cl, in the thread and lumen of the spinning duct using cryo-SEM EDX. Ca and Mg ions were not detected consistently and were often close to the detection limit. Although absolute ionic concentrations could not be determined, there was an increase in K^+ and a decrease in Na^+ ion concentrations, suggesting that those ions may be important in fiber formation. Rheological data and the study of spidroin aggregation supported this view (*12*). In silkworm, Ca^{2+} and Mg^{2+} ions have been found (*19, 20*) to play a determinant role in the swelling and gelation of fibroin prior to fiber formation (*21, 22*). Ochi et al. (*23*) suggested that Ca^{2+} and Mg^{2+} maintained the gel network of fibroin by bonding to the carboxylic groups of fibroins.

The complexity of the ionic balance and charge interactions in silk proteins is not well-understood and probably underestimated because of their large size. It is therefore important to examine separately the role of stabilizing and destabilizing forces to understand how their interaction is involved in storage and fiber formation in silk proteins. In this study, we used synchrotron radiation circular dichroism (SRCD)[1] to monitor the stabilizing and destabilizing effects of salts and pH on the secondary structure in dilute aqueous solutions of spidroin.

We show in this study that the conformational transition from the predominantly random-coil starting structure to the final β-sheet-rich state happens at remarkably specific pH and ionic conditions. We consider these conditions in the light of the Hoffmeister series and the direct measures of the p*K* values and equivalent point in native spidroin. We describe the changes in spidroin secondary structure inferred from SRCD spectroscopy (*17*). We also discuss variation in the starting solution structure leading to variation in kinetics of conversion and its implication for the spinning process.

MATERIALS AND METHODS

Sample Preparation. Mature female "Golden Silk" spiders, *Nephila edulis* (tetragnathidae), were reared free range in an environmentally controlled room, They were fed *Musca domestica* flies ad libitum every other day, and their webs were sprayed with tap water. The proteins from the major ampullate gland were obtained as follows: the opisthosoma (abdomen) of the spider was cut away from the cephalothorax and immediately dissected, and the major ampullate (Ma) gland was collected in 10 mM phosphate buffer at pH 7. The epithelium was gently stripped off, and the gland material was gently blotted and collected in preweighed Eppendorf tubes. After the material was reweighed, it was then dissolved overnight at room temperature, 20 ± 2 °C in twice distilled water. Stock solutions at 2% blotted weight/weight (wt/wt) were prepared. Henceforth, wt %/wt will be shortened to wt %. Care was taken to avoid shearing the protein solutions at all times, because this material is very sensitive to strain, particularly when concentrated (*12*).

Constrained Spiders. To discover whether storage of the protein in vivo affected the ability of the silk protein to undergo the structural transition, mature female *Nephila edulis* spiders, reared as above, were constrained for 2 weeks within a cylindrical plastic pot 20 cm high and 10 cm wide. They were fed a mealworm every other day ad libitum and sprayed with tap water. Samples were prepared as described above. Surprisingly, the amino acid composition from constrained and free-range spiders was similar (data not shown).

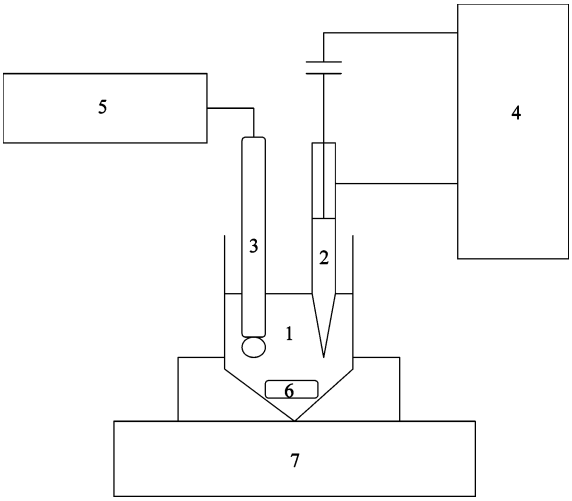

FIGURE 1: Experimental setup for the volumetric titration. (1) Protein solution in an eppendorf tube, (2) microsyringe with titrant, (3) pH minielectrode, (4) micropump, (5) pH-meter, (6) 3-mm long magnetic bar, and (7) magnetic stirrer.

Volumetric Titration. To fully ionize the protein, 400 μL of 1 wt % Ma gland solution was treated with either 5 μL of hydrochloric acid (2 M) or 5 μL of sodium hydroxide (2 M) and left to equilibrate overnight at 5 °C. The acid solutions were titrated with 0.15 M NaOH, and the basic solutions were titrated with 0.15 M HCl.

The setup (Figure 1) consisted of a pH semimicroelectrode (model 911600, Orion), a standard pH meter (PHM80; Radiometer, Copenhagen), and a Micro Syringe pump controller [model Micro 4, World Precision Instrument (WPI)] fitted with a 1 mL syringe type F (WPI).

The titrants were delivered in steps of 2−10 μL at a rate of 500 nL/s. The temperature of titration was not controlled but measured to be around 22 °C. The addition of the titrant was hand-controlled, and at each addition, the syringe was removed from the solution (to prevent diffusion of the titrant into the solution) and the pH values were recorded once a stable reading was reached (from 30 min to 4 h depending on the pH value). A back titration was also performed on both acidic and basic samples. Although at a different concentration (approximately 0.5% for the back titration), the two titration curves were identical. The p*K* and equivalent points (p*E*) were determined using a second derivative analysis. Direct titration of water without the protein gave a small response, leaving us to assume that dissolution of carbon dioxide into the solution under test was negligible.

Photometric Titration. UV spectra of 0.5 wt % aqueous solutions of Ma gland proteins in 1 cm rectangular quartz cell were collected using a Perkin−Elmer UV−visible spectrophotometer model Lambda 20 (Winlabs software) fitted with a water bath thermostatically controlled temperature held at 22 °C. A total of 500 μL of the protein solution was adjusted with 10, 1, 0.1, and 0.01 M HCl and NaOH and left to equilibrate overnight at 5 °C. Prior to collecting

[1] Abbreviations: SRCD, synchrotron radiation circular dichroism; CD, circular dichroism; Ma, major ampullate.

the UV spectra, the pH of each solution was recorded using the semimicroelectrode described above. Changes in absorption at 250, 274, 294, 355, and 440 nm were extracted and normalized using the following equation:

$$f = \frac{A - A_{\min}}{A_{\max} - A_{\min}} \quad (1)$$

where f is the fraction of the change, A is the observed absorption at a pH value, and $A_{\max}$ and $A_{\min}$ are the largest and smallest absorptions observed, respectively. The titration curves were analyzed by linearization of the ionization equation

$$f = \frac{1}{1 + \frac{K}{[H^+]}} \quad (2)$$

to give

$$\frac{f}{[H^+]} = \frac{1}{K} - \frac{f}{K} \quad (3)$$

where K is the ionization constant, $[H^+]$ is the hydrogen ion concentration and, f is the fraction of the change. Most samples yielded a function made up of two or three linear segments. A corresponding ionization constant could be then calculated for each of these segments.

The Effect of pH. We studied the effect of pH on the conformation of spidroin at a concentration of 1 wt % in the presence of 50 mM buffer. A total of 2 wt % spidroin samples were prepared (see above) and mixed with 0.1 M buffer solutions in a 1:1 ratio. We used four different buffer systems: acetic acid/acetate (pH 2.5−5), citric acid/phosphate (pH 2.5−8), phosphate monobasic/phosphate dibasic (pH 6−9), and bicarbonate/carbonate (pH 9−10.2), covering a pH range from 2.5 to 10.2. The mixtures were left to equilibrate overnight prior to recording the circular dichroism (CD) spectra.

The pH of 1 wt % spidroin in water was directly adjusted with 2 M/0.1 M/0.01 M HCl and 2 M/0.1 M/0.01 M NaOH (see photometric titration above), and the CD spectra were collected after allowing them to equilibrate overnight.

The kinetic of the change in conformation was monitored at its optimal point, i.e., about pH 4.34. Mixtures of spidroin at 1 wt % in either 0.5 M acetic acid/acetate or citric acid/phosphate were prepared (see above) and monitored with time by CD. The fractions of the change f were calculated as followed:

$$f = \frac{\theta - \theta_{\min}}{\theta_{\max} - \theta_{\min}} \quad (4)$$

where θ is the ellipticity at a certain wavelength, and $\theta_{\max}$ and $\theta_{\min}$ are the largest and smallest ellipticities measured, respectively.

We defined the r value as the ratio of the ellipticities of the plateau at 217 nm (θ_{217nm}) and the negative band at 199−200 nm (θ_{199nm})

$$r = \frac{\theta_{217nm}}{\theta_{199nm}} \quad (5)$$

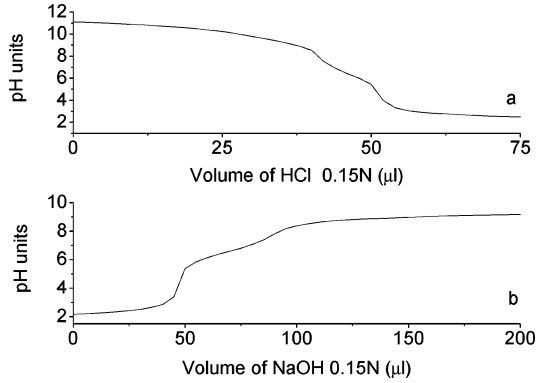

FIGURE 2: Volumetric titration. (a) Titration with 0.15 M HCl of 400 μL of spidroin 1 wt % after alkaline adjustment. (b) Titration with 0.15 M NaOH of 400 μL of spidroin 1 wt % after acidic adjustment. The apparent pK values were determined by second derivative analysis.

Salt Samples. To study the effect of different cations on the conformation, we mixed equal volumes of 2 wt % spidroin solution and 1 or 0.5 M solutions of each of the following salts in turn: KCl, NaCl, LiCl, CsCl, $CaCl_2$, $MgCl_2$, and $AlCl_3$. All salts were purchased from Sigma (at least 99% purity). The protein−salt mixtures were left to equilibrate at room temperature, prior to recording the CD spectra. The pH of the protein−salt mixtures was not actively controlled but only measured, with the pH values lying between 5.9 and 6.2, except with Al^{3+}, where the pH was approximately 2. CD spectra were recorded as described above.

We also used more dilute solutions (125 mM final concentration) of the above salts to study the kinetics of the change in conformation of 1 wt % spidroin (final concentration); the acquisition of CD spectra was initiated immediately after mixing of the protein with the salt. The fractions of the change were calculated as in eq 4.

CD. Solution samples for CD were transferred to a 0.1 mm path-length Suprasil quartz sandwich cell (Hellma 124-QS). Spectra were collected at 20 °C on the synchrotron radiation-based CD facility at ISA, Aarhus, Denmark. The loaded cell was checked for bubbles and voids. Insoluble fibers of silk produced by shear are strongly birefringent; thus, a polarizing microscope was used to check that the contents of the sandwich cell had not been sheared. The control voltage that determines the high-tension (HT) dynode voltage of the photomultiplier tube (PMT) was recorded with each CD spectra as an indication of the reliability of each CD spectra. In all of our measurements, the control voltage was −5 V (corresponding to a 600 V HT) for a highly transmitting sample and +5 V (1600 V HT) for a highly absorbing sample (for which the CD signal is useless). The samples were scanned 3 times with a 3 s/nanometer accumulation time, and the scans were repeated at least 3 times.

RESULTS

Volumetric Titration and Spectrophotometric Titration. To determine the ionization characteristics and the groups involved in buffering spider Ma gland proteins, we titrated a solution of spidroin at 1 wt %. Figure 2 shows the volumetric titration by acid and base addition.

The starting solutions were fully protonated (in acid) or deprotonated (in base), allowing all ionizable groups to be

Table 1: pK and Equivalent Points of Spidroin in Water Determined by Volumetric and Spectrophotometric Titration

	volumetric titration	spectrophotometric titration		
		240 nm	294 nm	440 nm
pK_1	6.74 ± 0.71	4.4 ± 0.28		4.12 ± 0.2
pK_2	9.21 ± 0.27	9.19 ± 0.1	9.47 ± 0.14	
pE_1	4.22 ± 0.33			
pE_2	7.63 ± 0.2			
pE_3	10.32 ± 0.25			

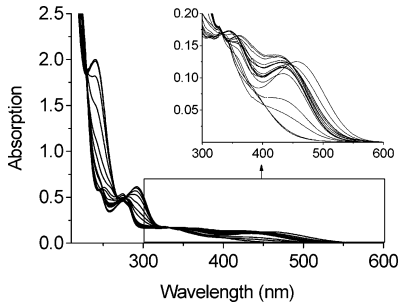

FIGURE 3: Photometric titration. UV-absorption spectra of 0.5 wt % spidroin at different pH values in a 1-cm quartz cuvette (see the Materials and Methods).

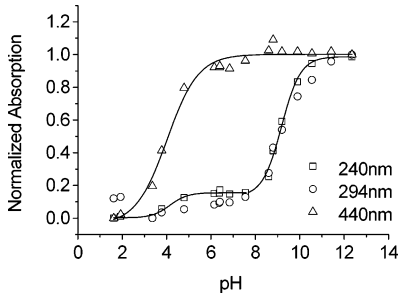

FIGURE 4: Photometric titration curves. Normalized fraction at 440, 294, and 240 nm (see the Materials and Methods). The fractions were linearized using eq 3 to determine the pK values and then fitted with a combination of eq 2 for each corresponding pK value.

titrated. The results in Table 1 showed that acid or base titration were equivalent, leading to the same apparent pK and equivalent points.

Unfortunately, the complexity of the titrated mixture made it difficult to attribute with confidence the equivalent point to a pI. However, we observed two apparent pK values at 6.74 ± 0.71 and 9.21 ± 0.27. A direct titration of spidroin in water with acid and base without prior pH adjustment yielded the same results (data not shown).

Figure 3 shows the change in the absorption spectra with a change in the pH of the solution.

We identified three major changes in absorbance with pH: first, at 440 and 355 nm, we observed an increase in the normalized absorption at this wavelength with pH (see Figure 4), correlated with a change in the color of the solution (from colorless to yellow); second, the tyrosine titration band at about 294 nm; and third, a band at 240 nm. This last band because of its close similarity in shape and behavior with tyrosine at 294 nm suggested a higher energy tyrosine band. Figure 4 shows the fraction at all three wavelengths as a function of pH, and Table 1 summarizes the pK values obtained by spectrophotometric titration.

We noted that at low pH the fractions at 294 and 240 nm showed a decrease and then an increase, suggesting that in

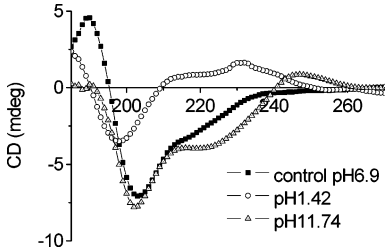

FIGURE 5: Reversible acid and base denaturation of spidroin. CD spectra of 1 wt % spidroin in a 0.1-mm sandwich cell after incubation in acidic (pH 1.42) and alkaline (pH 11.74) conditions compared to the control in water (pH 6.9).

a highly acidic condition (below pH 2) the environment of the tyrosine, hence, the tertiary structure of spidroin, was different than at pH values above 2. Further, direct evidence of this from CD spectra is given below. The apparent pK values at 440 nm showed that the solution changed coloration at approximately pH 4.12 ± 0.2. At 294 and 240 nm, the titration showed two apparent pK values at 4.4 ± 0.28 and 9.47 ± 0.14, representing about 15 and 85% of the change in the fraction, respectively. This suggested that the tyrosine residues were predominantly exposed and readily titratable, except perhaps at very low pH, where they might be partially buried. The pK values obtained from volumetric and spectrophotometric titrations could be correlated only in alkaline conditions, with volumetric titration giving a pK of 9.21 ± 0.27 and spectrophotometric titration giving pK values of 9.19 ± 0.1 and 9.47 ± 0.14 for 240 and 294 nm, respectively (see Table 1). The observed pK values between 9.2 and 9.5 were attributed to tyrosine residues. These results also indicated that pK of 4.12 from observations at 440 nm (see Table 1) was from the yellow pigment present in MA.

pH-Induced Conformational Change. Figure 5 shows the CD spectra of spidroin in very acidic and alkaline conditions at pH 1.42 and 11.74, respectively.

When compared to the control at pH 6.9 in water, both acid and alkaline treatment produced dramatic changes in the structure. In acidic conditions, the spectrum was similar to the classical random-coil-type CD spectrum with a positive band at about 230 nm and a strong negative band at 199 nm. On the other hand, in alkaline conditions, the CD spectrum was closer in shape to the control except for a stronger negative signal at 220 nm and the presence of a positive band at about 250 nm. We were not able to interpret these differences. We noted that, after either acid or alkaline treatment, adjusting the pH back to approximately 7 produced a complete recovery of the CD spectral shape. Remarkably, we also noted that adjusting the dilute spidroin solutions to pH values between 2 and 11 did not result in the above-mentioned alteration. Instead, we observed a conformational transition from the random-coil-type structure to a β-sheet-rich structure. Figure 6 shows the effect of pH on the CD spectra of spidroin plotted from the ratio of the intensities of the band at 217 nm to the band at 199 nm (r value).

We used the r value because it was a good measure for the conformational change experienced by the proteins and was independent of the solution concentration, hence allowing the comparison of the different structures regardless of protein precipitation. We noted three regions in Figure 6: first, a flat region corresponding to no significant change in the fold, with a typical r value between 0.2 and 0.3; second,

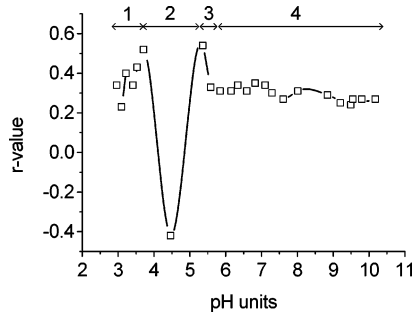

FIGURE 6: Effect of pH on the conformational change. Plot of the *r* value (ratio of the ellipticity at 217 nm to the ellipticity of the negative band at about 199 nm) as a function of pH (see the Materials and Methods). Note the four different zones: (1 and 3) onset and increase in order prior to the conformational change, respectively; (2) optimal pH region for the conformational change; and (4) region of conformational stability with pH.

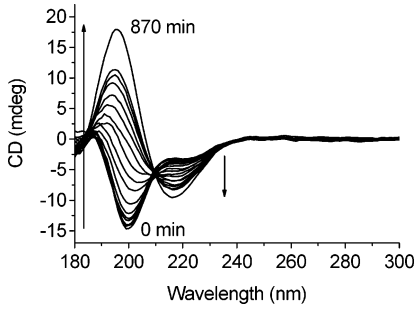

FIGURE 7: Time-resolved CD of the pH-induced conformational transition in 1 wt % spidroin in the presence of 0.5 M acetic acid/acetate buffer at pH 4.34.

a region where the *r* value increased suddenly and dramatically to values typically between 0.3 and 0.6 [This region corresponded to an increase in the order in the CD spectra and was at a position close to that of the measured p*I* (see above)]; and third, a conversion/aggregation region where the *r* value became negative. We noted in Figure 6 that the optimum value to convert the starting structure to the β-sheet-rich structure corresponded to a pH value centered at about 4.34, and on each side of the conversion region, we observed a change in the order (onset regions, see above). We noted that this depended on the sample and also the equilibration time, and thus the onset region could stretch from a pH of 4.4−6.2. We repeated the same experiment by adjusting the spidroin solution only with HCl and NaOH (see the Materials and Methods) and observed a similar behavior except that it was much slower, suggesting that the buffer solution chosen (acetic/acetate or citric acid/phosphate) interacted with spidroin.

To clarify the kinetics of the pH-induced transition, we recorded the CD spectra of spidroin in the presence of acetic acid/acetate buffer as a function of time. Figure 7 shows the time evolution of the transition.

Microscopy using crossed polarizers (see Figure 8) of the flakes formed by prolonged treatment at pH 4.34, suggested that the conversion to the β-sheet-rich structure caused the spidroin to phase separate.

The fractions of change at 217 and 200 nm are plotted in Figure 9.

We attributed the increase in β-sheet structure to the band at 217 nm and the decrease of disorder to the band at 200 nm (*17*, *24*). The fraction of the change at 217 and 200 nm

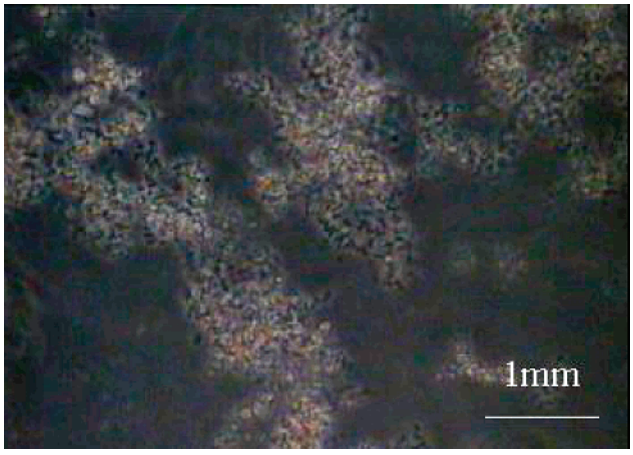

FIGURE 8: Micrograph taken between crossed polarizers of a solution of 1 wt % spidroin after at least 24 h of incubation at pH 4.34. Similar structures were observed in the presence of K^+ and Na^+. Al^{3+} caused the solution to form a gel.

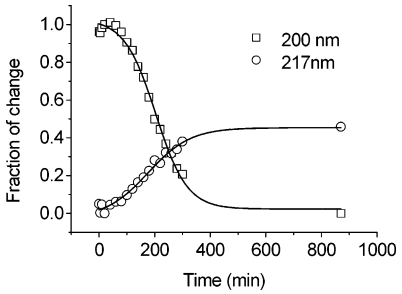

FIGURE 9: Kinetics of the fraction of the change for the pH-induced transition. The fractions were extracted from the CD spectra of Figure 7, using eq 4.

could be fitted by a sigmoid. Although we did not provide a physical interpretation of the sigmoids, they revealed a complex conversion mechanism. We noted that the change of the fraction at 217 nm was delayed and slower compared to the one at 200 nm. Similar kinetic behavior was obtained in citric acid/phosphate buffer, and in the presence of HCl, the latter yielding to a much slower kinetic, suggesting, as mentioned above, that the buffers used may interact preferentially with the proteins.

Cations Induced Conformational Change. We noted that in the presence of the cations (0.5 M) the CD signal of spidroin decreased in intensity without a change in the shape compared to the control, suggesting a decrease in the concentration most likely associated with a precipitation of the proteins (*17*). We observed that in the presence of Al^{3+} the signal disappeared completely, a sign of a total precipitation, and that the solution in the test tube was gellike after only a few minutes. The strong acidity (pH 2) in the presence of Al^{3+} compared to pH 5.9−6.2 with the other salts might be one reason for Al^{3+} potency. It is worth noting that Al^{3+} is known to have a strong precipitant action on other proteins.

The effectiveness of reducing the CD signal without causing a change in the fold of the remaining soluble protein was $Al^{3+} \gg K^+$, $Na^+ > Mg^{2+}$, Cs^+, Li^+, and Ca^{2+}. After an overnight incubation, all of the salts yielded precipitation except Ca^{2+} and Mg^{2+} (data not shown). With 1 M salts, we noted a stronger and faster precipitation compared with 0.5 M salts (data not shown). In Figure 10, the *r* values of spidroin in the presence of 0.5 M salts are plotted against

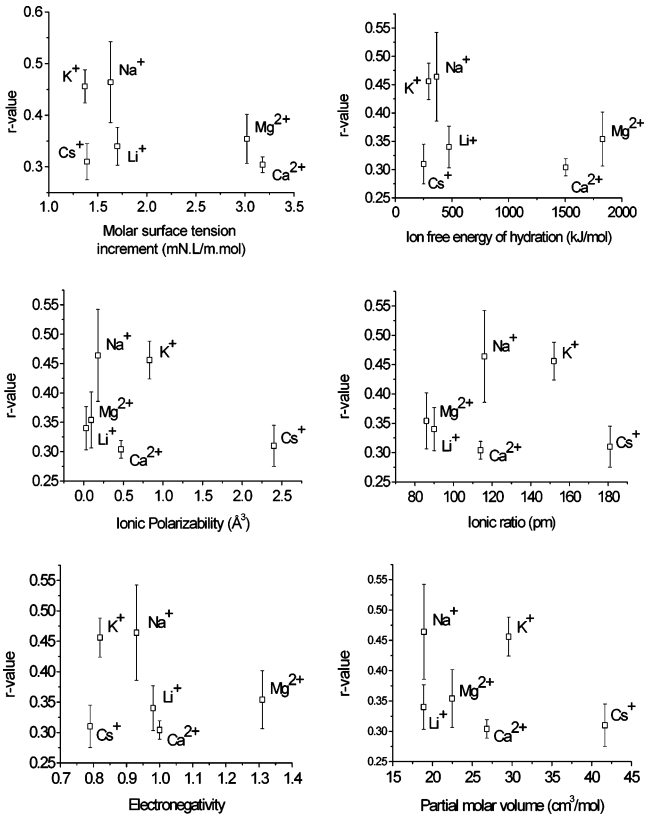

FIGURE 10: Relationships between the change in spidroin fold (*r* values) and the physical and chemical properties of specific ions. None of the plots showed a correlation between the change in the fold and the properties of the cations.

various properties of the salts. The *r* values were collected and pooled from samples that were left to incubate for no more than 2 h. A one-way ANOVA at 5% significance level showed that the means of the *r* values were not significantly different (Levene test of equal variance, number of repeats per salts $n = 5$, $p = 0.3117$). A mean comparison using Tukey test at 5% significance level showed that the *r* values from K^+ and Na^+ were significantly different from *r* values of the other salts but that K^+ and Na^+ were not significantly different from each other. Similarly, the *r* values of Ca^{2+}, Mg^{2+}, Li^+, and Cs^+ were not significantly different from each other.

The six panels of Figure 10 show no evidence of a correlation between the changes in the fold represented by the *r* value and the specific properties of the cations investigated. We noted in particular the absence of any correlation with the molar surface tension. Generally, phenomena that are dominated by hydrophobic effects show a significant dependence on the molar surface tension (*25*, *26*). This led to the conclusion that Al^{3+}, K^+, and Na^+ ions played a key role in the conformational change perhaps by destabilizing spidroin, whereas the other salts would stabilize spidroin. Interestingly we found that the change in the fold of the spidroin and its solubility did not follow the Hoffmeister series.

To understand the conformational change in the presence of salts, we monitored the change in CD spectra of 1% spidroin in the presence of 125 mM K^+ (data not shown). We observed a change in conformation from a predominantly disordered structure to the β-sheet-rich state. Interestingly, we noted that the signal intensity at 217 nm hardly changed

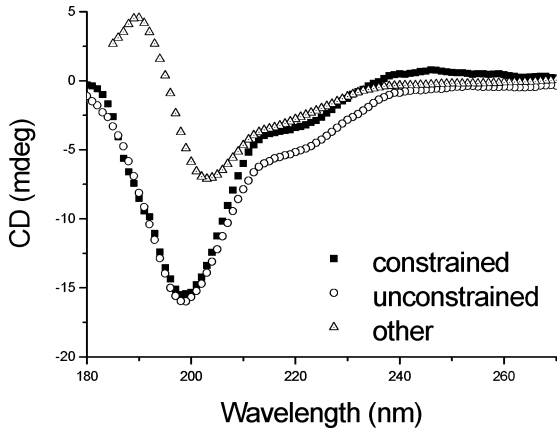

FIGURE 11: Variability in the CD spectra for the starting structure of spidroin at 1 wt % in water. The structure from the constrained spider had an *r* value of about 0.24 ± 0.04 ($n = 16$); the favorable structure (other), an *r* value of 0.5 ± 0.1 ($n = 15$); and the most commonly occurring type of structure, an *r* value of about 0.31 ± 0.03 ($n = 35$). The three *r* values were significantly different (see the text).

compared to that of Figure 9, suggesting that the potassium ions did not alter the β-sheet content of the protein but reordered the disordered structures and favored protein aggregation. Unsurprisingly, we observed in some instances a very fast kinetic of transition at about 240 min (data not shown) compared to that at 870 min and pH 4.34 (Figure 9), highlighting a great variability in the sample extracted from native spider glands. A set of other experiments was performed (data not shown) to investigate the change in the CD spectra of 1 wt % spidroin in the presence of salts in phosphate-buffered solution in the physiological region of pH 6−8. We observed only a smaller reduction of the signal compared to spidroin in water, but no complete precipitation was observed after several days (except in the presence of Al^{3+}).

Variability. The variability observed in the kinetics and amplitude of signals is not easily explained. However, we were able to establish a circumstantial link between the kinetics and the shape of the CD spectra of spidroin in solution. Figure 11 shows three CD spectra with different kinetic behavior. The first was obtained from constrained spiders (see the Materials and Methods) and consistently yielded no changes in the conformation with pH, salts (data not shown), alcohols, and detergents (*18*). In the second and third, free-range spiders were used (see the Materials and Methods). The second shows a "favorable structure", which yielded the fastest response with pH and salts (data not shown), and for the third, a structure that was present most of the time yielded the slower kinetics described above.

We calculated the *r* value for these three types of structures and found that the structure from the constrained spider had an *r* value of about 0.24 ± 0.04 ($n = 16$); the favorable structure, an *r* value of 0.5 ± 0.1 ($n = 15$); and the most commonly occurring type of structure, an *r* value of about 0.31 ± 0.03 ($n = 35$). A one-way ANOVA of the three *r* values at 5% significance level showed that the three *r* values were significantly different (Levene test for equal variance $p = 0$). A mean comparison using a modified Tukey test for unequal sample size at 5% significance level showed that the three *r* values were significantly different pair wise ($p < 0.001$). Moreover, we noted that structures with an *r* value

of 0.5 ± 0.1 were more ordered (see Figure 10) and hence more likely to undergo a faster conversion (*27*). Although it seems likely that the protein in the constrained spider was stored in a safe form with slow kinetics and resistance to conformation change, it is unclear why the two free-range spiders reared under similar conditions contain silk protein with such different *r* values and hence different structures.

DISCUSSION

In this study, apparent p*K* values were determined by two different methods. Only the alkaline p*K* (9.21) was consistent with both methods. The macroscopic p*K* determined from volumetric titration at 6.74 ± 0.71 suggested a contribution of p*K* values from different groups. The spectrophotometric titration suggested the contribution from tyrosine residues and a yellow chromophore. The p*K* value of 6.74 ± 0.71 was consistent with the average value of pH (6.73 ± 0.23 in the middle of the gland), which we measured in the ampulla using a pH microelectrode (*10*).

The observed p*E* suggested that the p*I* of spidroin could be 4.22, similar to the degummed silk fibroin (pH 4.8−5) (*14*). This value was comparable to the predicted p*I* values of 5.79 and 4.56 for the central repetitive portion of spidroin I in the sequenced fragments P46804 and AAK30609 in *N. clavipes* and *N. senegalensis*, respectively. These values are very different from the predicted p*I* 10.32 for the central repetitive sequence plus the strongly basic C-terminal fragment (*28*) in *Nephila* spidroin I. This suggests that the p*I* of the whole spidroin I molecule may be governed by a very large central repetitive region with its relatively low density of negative charges rather than the proportionally minute C terminus with its relatively high density of basic amino acids.

The optimal pH value of 4.34 for the conformational transition (Figure 6) was remarkably close to the value of 4.22 found by volumetric titration as the p*I* value for spidroin. This suggested that the net negative charge on the molecule at pH values above p*I* prevented the conformational transition and could provide a repulsive force preventing the close approach of the molecular chains required for the formation of β sheets. Furthermore, the availability of the tyrosine residues and their position on loops (*29*) may favor tyrosine−tyrosine interactions between different chains, thus promoting β-sheet structure formation.

The effect of pH on the kinetics of the secondary-structure transition change helps to explain the key role of pH in the natural (and biomimetic) spinning process. We demonstrated elsewhere (*10*) that for highly concentrated spidroin solutions (20 wt %), the optimal pH for the conformational change to a β-sheet-rich state in 10 mM phosphate buffer was relatively high (pH 6.2) compared with pH 4.34 for dilute solutions in the same buffer. This difference is most likely linked to the change in the protein−protein interaction at higher concentrations (*27*), with a more dilute solution requiring greater suppression of ionization to induce sufficient protein−protein interactions than a more concentrated solution. The complex kinetics of the transition in dilute spidroin solutions at pH 4.34 were similar to those induced by methanol or ethanol (*17*, *18*). However, the absence of the initial red shift in the negative band at 199 nm indicated a weaker type of interaction. The kinetics for the acid-induced transition were dependent on the acid used (HCl, acetic acid, or citric acid; data not shown). The hydrophobic nature of acetic acid and citric acid as well as their higher polarizability may account for the faster kinetics produced by these acids, suggesting that an enhancement of hydrophobic interaction, for example, would promote the conformational change in spidroin. We have previously suggested that a similar enhancement of hydrophobic interactions accounts for the observation that the conformational transition was faster at 20 °C as compared to 5 °C (*17*).

We were not able to suggest a precise mechanism for the action of pH on the conversion to the β-sheet-rich structure other than the already suggested hydrodynamic gelation reported in the silkworm silk system (*9*, *16*, *30*) and spider silk (*12*).

The effects of salts on spidroin are very complex as illustrated by Figure 10. Hence, we decided to look at the effect of salts in simple and general terms. We considered that salts, which destabilize the native folded form of a macromolecule, also increase macromolecular solubility of the unfolded form. Conversely, salts that stabilize the folded conformation by strengthening intramolecular interactions decrease macromolecular solubility of the unfolded form. This argues for the role of salts in storing silk proteins in a soluble form and then converting them to insoluble fibers. In spiders, as well as in silkworm, it has been demonstrated that the silk protein lowest energy conformations are β-sheet-rich structures. In this context, Al^{3+}, K^+, and Na^+ favored the formation of the β-sheet-rich state by strengthening intramolecular contacts and decreasing macromolecular solubility according to our interpretation. This suggested that a progressive dehydration of the protein promoted the phase separation, which occurred on β-sheet structure formations.

We observed that concentrations of salts ranging from 0.5 to 1 M produced effects on the relatively low concentration (1 wt %) of spidroin. We could not establish if, at the salt concentration studied, the electrostatic interactions were saturated or partially shielded and how critical the ratio of salt/protein was. Furthermore, we found no relation between the ability of ions to induce structural changes in spidroin and their position in the Hoffmeister series, suggesting that the bindings of specific ions were the predominant forces involved in the conformational change instead of solvent-reordering effects. This was consistent with the finding that the kinetics of the conformational change in spidroin films was dependent on the K^+ concentration [biphasic at low concentrations and monophasic at high concentrations (*8*)].

Finally, uncertainty about the net charge of spidroin makes it more difficult to interpret the effects of pH and cations, although this protein is likely to have a net positive charge below the apparent p*K* of 6.74 ± 0.71.

CONCLUSION

Our results showed that spiders, in the same manner as *B. mori*, control and promote the conformational change to β-sheet-rich structures in spidroin by acidification of the protein mixture down to their apparent p*I* (measured value of 4.22 ± 0.33 for spidroin; measured value for fibroin of 4−5), thus, contributing to fiber formation. The observed p*K* value of 6.74 ± 0.71 supports our direct measurement of pH in the lumen of the ampulla and confirmed this value as the optimal storage pH. Although the contributions to the

apparent p*K* values and p*I* were not fully resolved, we observed that the higher p*K* value (9.47 ± 0.14), associated with the tyrosine residues, suggested that tyrosines were not buried, hence, directly accessible for titration. Interestingly, the mobility and accessibility of tyrosines residues support the hypothesis that spider protein aggregation may occur via tyrosine−tyrosine interactions.

We found that the conformational transition was significantly dependent on the amount of structural order (as measured by our *r* values) in the starting material. Structures with higher order showed a rapid conformational change, whereas less ordered structures delayed or prevented the conformational change. Similar results were found by increasing the concentration of spidroin (*27*), suggesting that at low concentrations spidroin was not monodisperse and instead formed large soluble aggregated structures of partially extended chains. Differences in the ability of salts to induce the conformational change in spidroin were found not to depend on the Hoffmeister series, suggesting that the stability and conformation of the spidroin structure is at least in part dependent on the specific cation interaction and perhaps a weaker solvent-ordering effect. Our results imply the important roles that pH and ion affinity play for understanding the way in which spiders and silkworms store and spin tough threads under ambient conditions.

ACKNOWLEDGMENT

We thank the British Biological and Engineering Research Councils (BBSRC-S12778, EPSRC-GR/NO1538/01), the European Commission (F5-G5RD-CT-2002-00738), AFSOR of the United States of America (F49620-03-1-0111), and the Danish Natural Sciences Research Council (SNF-21-00-0485) for financial support. We further thank the Institute for Synchroton Radiation, ISA, Åarhus, Denmark for use of their CD facility through an EC-Human Potential Program Transnational Access to Major Research Infrastructures (EU Contract no. HPRI-CT-2001-00122). We are grateful to Dr. Ann Terry and Dr. David Porter for reading the manuscript and for helpful discussion and Else Rasmussen for technical support.

REFERENCES

1. Gosline, J. M., DeMont, M. E., and Denny, M. W. (1986) The structure and properties of spider silk, *Endeavour 10*, 31−43.
2. Lewis, R. V. (1992) Spider silk—The unraveling of a mystery, *Acc. Chem. Res. 25*, 392−398.
3. Hinman, M. B., and Lewis, R. V. (1992) Isolation of a clone encoding a 2nd dragline silk fibroin—*Nephila clavipes* dragline silk is a 2-protein fiber, *J. Biol. Chem. 267*, 19320−19324.
4. Winkler, S., and Kaplan, D. L. (2000) Molecular biology of spider silk, *Rev. Mol. Biotechnol. 74*, 85−93.
5. Vollrath, F., and Knight, D. P. (2001) Liquid crystalline spinning of spider silk, *Nature 410*, 541−548.
6. Valluzzi, R., Szela, S., Avtges, P., Kirschner, D., and Kaplan, D. (1999) Methionine redox controlled crystallization of biosynthetic silk spidroin, *J. Phys. Chem. B 103*, 11382−11392.
7. Knight, D. P., and Vollrath, F. (2001) Changes in element composition along the spinning duct in a *Nephila* spider, *Naturwissenschaften 88*, 179−182.
8. Chen, X., Knight, D. P., Shao, Z. Z., and Vollrath, F. (2002) Conformation transition in silk protein films monitored by time-resolved Fourier transform infrared spectroscopy: Effect of potassium ions on *Nephila* spidroin films, *Biochemistry 41*, 14944−14950.
9. Hossain, K. S., Ochi, A., Ooyama, E., Magoshi, J., and Nemoto, N. (2003) Dynamic light scattering of native silk fibroin solution extracted from different parts of the middle division of the silk gland of the *Bombyx mori* silkworm, *Biomacromolecules 4*, 350−359.
10. Dicko, C., Vollrath, F., and Kenney, J. (2004) Spider silk refolding is controlled by changing pH, *Biomacromolecules 5*, 704−710.
11. Vollrath, F., Knight, D. P., and Hu, X. W. (1998) Silk production in a spider involves acid bath treatment, *Proc. R. Soc. London, Ser. B 265*, 817−820.
12. Chen, X., Knight, D. P., and Vollrath, F. (2002) Rheological characterization of *Nephila* spidroin solution, *Biomacromolecules 3*, 644−648.
13. Terry, A. E., Knight, D., Porter, D., and Vollrath, F. (2004) pH induced changes in the rheology of middle division fibroin extract of *Bombyx mori* silkworm, *Biomacromolecules 5*, 768−772.
14. Eloed, E., and Pieper, E. (1928) Studien üder beiz-und färbevorgänge, *Angew. Chem. 41*, 16−19.
15. Hanawa, T., Watanabe, A., Tsuchiya, T., Ikoma, R., Hidaka, M., and Sugihara, M. (1995) New oral dosage form for elderly patients—Preparation and characterization of silk fibroin gel, *Chem. Pharm. Bull. 43*, 284−288.
16. Magoshi, J., Magoshi, Y., and Nakamura, S. (1994) Mechanism of fiber formation of silkworm, in *Silk Polymers*, (Kaplan, D., Wade, W. W., Farmer, B., and Viney, C., Eds.) Vol. 544, pp 292−310, American Chemical Society: Washington, D.C.
17. Dicko, C., Knight, D., Kenney, J., and Vollrath, F. (2004) Structural conformation of spider silk proteins: A synchrotron radiation circular dichroism study, *Biomacromolecules 5*, 758−767.
18. Dicko, C., Knight, D., Kenney, J., and Vollrath, F. Conformational polymorphism, stability, and aggregation in spider dragline silk proteins, manuscript to be submitted.
19. Zhang, H., Magoshi, J., Becker, M., Chen, J. Y., and Matsunaga, R. (2002) Thermal properties of *Bombyx mori* silk fibers, *J. Appl. Polym. Sci. 86*, 1817−1820.
20. Liu, Y., Yu, T., Yao, H., and Yang, F. (1997) PIXE analysis of silk, *J. Appl. Polym. Sci. 66*, 405−408.
21. Magoshi, J., Magoshi, Y., Kato, M., Becker, M. A., and Nakamura, S. (1997) The role of calcium ions in the silk fibroin fiber formation process, *Abstr. Pap. Am. Chem. Soc. 214*, 209−PMSE.
22. Magoshi, J., Magoshi, Y., Kato, M., Becker, M. A., Zhang, H., and Nakamura, S. (1999) Biospinning—The control of calcium ions, *Abstr. Pap. Am. Chem. Soc. 217*, 274−PMSE.
23. Ochi, A., Hossain, K. S., Magoshi, J., and Nemoto, N. (2002) Rheology and dynamic light scattering of silk fibroin solution extracted from the middle division of *Bombyx mori* silkworm, *Biomacromolecules 3*, 1187−1196.
24. Kenney, J. M., Knight, D., Wise, M. J., and Vollrath, F. (2002) Amyloidogenic nature of spider silk, *Eur. J. Biochem. 269*, 4159−4163.
25. Baldwin, R. L. (1996) How Hofmeister ion interactions affect protein stability, *Biophys. J. 71*, 2056−2063.
26. Bostrom, M., Williams, D. R. M., and Ninham, B. W. (2001) Surface tension of electrolytes: Specific ion effects explained by dispersion forces, *Langmuir 17*, 4475−4478.
27. Dicko, C., Kenney, J., and Vollrath, F. Major and minor ampullate, flagelliform and cylindrical glands secondary structures. Concentration and temperature effects, *Biomacromolecules*, in press.
28. Xu, M., and Lewis, R. V. (1990) Structure of a protein superfiber—Spider dragline silk, *Proc. Natl. Acad. Sci. U.S.A. 87*, 7120−7124.
29. Hayashi, C. Y., Shipley, N. H., and Lewis, R. V. (1999) Hypotheses that correlate the sequence, structure, and mechanical properties of spider silk proteins, *Int. J. Biol. Macromol. 24*, 271−275.
30. Hossain, K. S., Nemoto, N., and Magoshi, J. (2000) Rheological study on aqueous solutions of silk fibroin, *13th International Congress on Rheology* (Binding, D. M., Hudson, N. E., Mewis, J., Piau, J.-M., Petrie, C. J. S., Townsend, P., Wagner, M. H., and Walters, K., Eds.) pp 1−371, Cambridge, U.K.

BI0483413