Further Investigation on Potassium-Induced Conformation Transition of Nephila Spidroin Film with Two-Dimensional Infrared Correlation Spectroscopy

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We used two-dimensional (2D) correlation infrared spectroscopy to study further the potassium-induced conformation transition in Nephila spidroin films. It provided increased resolution and important new information on the sequence of events in the conformation transition process, showing that β-sheet formed from the helical component before they formed from random coil. It also showed more evidence that formation of the 1691 cm⁻¹ (turn/bend) peak did not proceed with the same kinetics as the 1620 cm⁻¹ (antiparallel β-sheet component) one, so we attribute the 1691 cm⁻¹ peak to turns which formed with different kinetics as the antiparallel β-sheets. We present a single coherent and detailed hypothesis for the assembly and secondary structural transition of silk proteins in vivo and in vitro based on our findings and on evidence from other laboratories.

Introduction

Spiders, one of the earliest terrestrial invertebrates, have spun silk for some 400 millions years.¹ The outstanding mechanical properties²–⁶ of spider dragline silk have motivated study of the primary and secondary structure of the silk protein (spidroin) in recent decades. Interest has also focused on the natural silk spinning process⁵ as a result of the poor mechanical properties of the silk fibers obtained by several different artificial spinning methods.⁷–¹¹ Moreover, artificial spinning involves extreme processing conditions, such as high pressure and toxic solvents in sharp contrast to the mild processing conditions in vivo.¹² There is good evidence that the excellent properties of silk fibers depend as much or more on the spinning process¹³–¹⁵ as on the protein sequence and structure.¹⁶–¹⁸ It is widely accepted that the spinning process involves a conformation transition in the silk protein (fibroin or spidroin) from random coil/helical conformations to β-sheet conformation produced by shearing and/or rapid extensional flow converting a highly aqueous concentrated solution of silk protein into an insoluble silk fiber (refs 19 and 20, and the references therein). Therefore, further study of the conformation transition in spidroin would be helpful to understand the natural spinning mechanism and, in particular, the way that the spinning dope is transformed into an extremely tough and viscoelastic dragline silk fiber.

Several attempts have been made to study the conformational changes of spinning dope and silk in spiders;²⁻⁴,²¹–²⁴ however, these studies are static rather than dynamic as they only describe the conformations of the protein before and after natural spinning. In our previous work,²⁵ we provided dynamic data using time-resolved FTIR to study the conformation transition process induced by potassium ions in dried Nephila spidroin film. This system may provide a good model for understanding the conformation transition in vivo as potassium ions are thought to be involved in the spinning of spider silk.¹⁹ Although we proposed a mechanism for the conformation transition in vitro based on the kinetics data, several details remained to be investigated including the assignment of the 1691 cm⁻¹ absorption band. Here we use two-dimensional (2D) FTIR correlation spectroscopy to further investigate the conformation transition.

Two-dimensional correlation spectroscopy, a novel technique integrating spectroscopy and 2D correlation techniques, has been developed in the past 20 years.²⁶ Noda first introduced the 2D correlation technique into infrared spectroscopy in 1986. In 1993, he developed the concept of 2D correlation spectroscopy to generalized 2D correlation spectroscopy and extended this technique from the infrared spectra to Raman, near-infrared, and fluorescence spectra.²⁷–³⁰ Despite some debates,³¹–³⁵ 2D spectroscopy has developed rapidly and is widely used to study inter- and intramolecular interactions.³⁶–³⁹

The improved resolution of 2D FTIR correlation spectroscopy compared with conventional FTIR spectroscopy has made it a powerful tool for studying secondary structure transitions in proteins.⁴⁰–⁴³ such as serum albumin,⁴⁴ ovalbumin,⁴⁵ centrin,⁴⁶ bacteriorhodopsin,⁴⁷ and lactalbumin.⁴⁸ The 2D asynchronous correlation analysis is particularly useful to separate badly overlapping amide bands of proteins

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frequently seen in conventional FTIR spectra. Another merit of 2D correlation spectroscopy is that it can probe the effects of variables such as time, temperature, and pressure, on the order of appearance of certain absorption bands. 49

In the present article, we describe the use of 2D correlation techniques to study time-resolve FTIR spectra data. We discuss the assignments of the absorption bands involved in the conformation transition induced by potassium ions in Nephila spidroin films. We also discuss our results in relation to recent work from other laboratories on the structure and conformation transitions of silk proteins using different observational techniques.

Experimental Section

Preparation of Nephila Spidroin Films. The preparation of Nephila spidroin films is as described in our previous work. 19,25 Final instar female Nephila senegalensis were killed and the ampullae (sac) of the major ampullate gland were removed. The epithelium was removed to expose the concentrated dope. To make the thin film for FTIR measurement, the dope was diluted to 2% (w/w) dope concentration by adding deionized water. Care was taken to avoid shearing while dilution. The dope completely dissolved in a plastic micro-centrifuge tube when left overnight at room temperature (approximately 20 °C) with occasional slight rotation of the tube. Films were cast by allowing 1 mL aliquots of the resulting solution to dry overnight at approximately 20 °C and 50% relative humidity in 3 cm × 3 cm plastic weighing boats. The thickness of the film was approximately 5 μm.

Conformation Transition Measurement of Nephila Spidroin Film with Time-Resolved FTIR Spectroscopy. The time-resolved FTIR spectra of spidroin films induced by 1.0 mol/L KCl/D2O solution were collected in rapid scan mode using a Nicolet Magna 550 FTIR spectrometer equipped with a MCT detector cooled by liquid nitrogen. The resolution of the spectra was 4 cm⁻¹ and the minimum time interval between successive spectra was 0.57 min. Details of the time-resolved FTIR measurement can be found in our previous publications.25,50,51

Two-Dimensional Correlation Analysis. According to our previous work,25 the potassium induced conformation transition of spidroin film is a bimodal process that has two time constants τ1 (~10 min) and τ2 (~60 min). Thus, we divide the conformation transition into three stages based on τ1 and τ2. The first stage (stage I) ran from the start to 10 min, containing 18 spectra each lasting 0.57 min; the second stage (stage II) from 10 to 60 min (22 spectra each lasting 2.28 min); and the third stage (stage III) from 60 min to the end (240 min; 19 spectra each lasting 10 min). The data treatment was performed using MATLAB software (The Math Works Inc.). During the calculation, we applied the generalized 2D correlation formalism developed and modified by Noda.47 The time-averaged spectrum was applied as a reference. Since 2D correlation is a function of two independent wavenumbers, the correlation peaks are represented as pairs (ν1/ν2). In all 2D correlation plots, the negative peaks are shaded; thus, the remaining (unshaded) peaks are positive. The peaks in 2D correlation spectra which developed before the noise peaks appear are considered as significant. The cross-peaks we discuss are in the upper left part in both synchronous and asynchronous correlation maps.

Protein Secondary Structure Prediction. We used GOR IV (http://us.expasy.org/tools/) to predict the random coil contents in the repetitive central region within the published Stageial sequences for the principal silk proteins spidroin I (SPD1_NEPL; P19837) and spidroin II (SPD2_NEPL; P46804) present in the major ampullate silk gland of Nephila clavipes. This species is closely related to Nephila senegalensis used for the preparation of spidroin films in this study.

Results

The conventional FTIR spectra of stage I of the transition are shown in Figure 1. The intensity at 1620 cm⁻¹ (β-sheet) increased with the time, whereas the changes in other bands could not be resolved. The synchronous 2D correlation spectra (Figure 2A), however, showed three peaks, an auto-peak at 1620 cm⁻¹, a positive cross-peak at 1691/1620 cm⁻¹, and a negative cross-peak at 1658/1620 cm⁻¹. The asynchronous spectra (Figure 2B) showed three positive cross-peaks at 1658/1642, 1658/1620, and 1642/1620 cm⁻¹ and one negative cross-peak at 1691/1642 cm⁻¹.

The conventional FTIR spectra for stage II of the transition (Figure 3) revealed the further development of the 1620 cm⁻¹ peak and the emergence of a new band at 1691 cm⁻¹. The synchronous 2D correlation spectra (Figure 4A) were almost the same as in stage I but showed an increase in intensity of the cross-peak 1691/1620 cm⁻¹. The asynchronous spectrum showed more cross-peaks during stage II (Figure 4B) than in stage I. Seven prominent cross-peaks were seen: four positive ones at 1655/1620, 1648/1620, 1642/1620, and 1658/1642 cm⁻¹ and three negative ones at 1691/1620, 1691/1648, and 1691/1642 cm⁻¹.

In stage III, the conventional FTIR spectra showed that the β-sheet band (1620 cm⁻¹) became the strongest within the broad amide I envelope (Figure 5). The synchronous spectra at this stage (Figure 6A) showed a large new auto-peak around 1650 cm⁻¹, whereas asynchronous spectra were remarkably simple with a single positive peak at 1691/1650 cm⁻¹.
cm\(^{-1}\) and a single negative one at 1650/1620 cm\(^{-1}\) (Figure 6B).

**Discussion**

**Relationship between Time-Resolved FTIR Data and 2D Correlation Analysis.** The time-resolved FTIR spectra in our previous work\(^\text{25}\) on the potassium ion-induced conformation transition in spidroin provided some information on how the amide I absorption band changed with time. Two major changes were observed: the increase of 1620 cm\(^{-1}\) (assigned to \(\beta\)-sheet structure) and the decrease of a broad 1650 cm\(^{-1}\) band (assigned to random coil and/or helical structure). Here, two auto-peaks at 1620 and 1650 cm\(^{-1}\) in synchronous 2D correlation spectra agree well with our previous time-resolved FTIR data. The increase at 1691 cm\(^{-1}\) (previously assigned to antiparallel \(\beta\)-sheet and the turns or bends associated with this antiparallel \(\beta\)-sheet) in time-resolved FTIR was not detected in synchronous spectra as a result of the much lower intensity change here compared to other two major peaks (1620 and 1650 cm\(^{-1}\)). The increase...
of the 1620 and 1690 cm\(^{-1}\) bands and the decrease of a broad 1650 cm\(^{-1}\) band were also reflected by a positive cross-peak at 1690/1620 cm\(^{-1}\) and a negative cross-peak at 1650/1620 cm\(^{-1}\) in the synchronous spectra.

Unlike synchronous 2D correlation spectra, asynchronous spectra can provide detail not seen in conventional FTIR spectra.\(^{26}\) In our previous work,\(^{25}\) we assigned the original conformation of the *Nephila* spidroin film to random coil and/or helical structure based on the broad envelope around 1650–1660 cm\(^{-1}\). Fourier self-deconvolution and second derivative methods did not provide further information on the existence of sub-components in this broad peak. However, in the present study, the asynchronous 2D correlation spectra (Figure 2B), showed four cross-peaks (1658/1642, 1642/1620, and 1691/1642 cm\(^{-1}\)) clearly indicating there were at least two components (1642 and 1658 cm\(^{-1}\) absorption bands) in the spidroin film before treatment with potassium ions. According to the classic assignment,\(^{52-55}\) the absorption band at 1642 cm\(^{-1}\) was attributed to random coil, whereas the peak at 1658 cm\(^{-1}\) was attributed to helical conformation (\(\alpha\)-helix). This provided strong evidence that both random coil and helical structures existed in the *Nephila* spidroin film air-dried from dilute spidroin aqueous solution. Subsequently, we used PeakFit 4.1 (SPSS Inc.) as a peak separation tool to determine the content of these two conformations. The result showed that the content of the random coil and helical structure was 59.9 ± 6.0% and 40.1 ± 6.0%, respectively, remarkably close to the values of 59.3% and 40.7% we calculated from the repetitive part of *Nephila* spidroin I in the chain model\(^{36}\) proposed on the basis of solid-state \(^{1}\)H NMR. The GOR IV prediction of random coil contents of 63% and \(\alpha\)-helix of 32% in the same material were reasonably close to these values.

The assignment of the 1691 cm\(^{-1}\) band in silk protein was uncertain in our previous research. Traditionally, this band has been assigned to a high-frequency band of antiparallel \(\beta\)-sheet;\(^{57}\) however, finding that the kinetics of the formation of the 1691 cm\(^{-1}\) band were different from those of 1620 cm\(^{-1}\) (classical \(\beta\)-sheet) band, we suggested that turns or bends associated with the antiparallel \(\beta\)-sheet structure contributed to the 1691 cm\(^{-1}\) band.\(^{25}\) This view is supported in the present paper by our application of Noda theory\(^{26}\) to the asynchronous correlation spectra. The negative sign of the correlation peaks \(\psi(v_1/v_2)\) indicates that the band \(v_1\) changes after the band \(v_2\). The clear negative cross-peak at 1691/1620 cm\(^{-1}\) in Figure 4B indicates that the 1691 cm\(^{-1}\) band (turns and bends) formed more slowly than the 1620 cm\(^{-1}\) band (\(\beta\)-sheet). This strongly supports our hypothesis that the absorption band at 1691 cm\(^{-1}\) is not the high-frequency band of antiparallel \(\beta\)-sheet conformation and may arise from bends or turns. In this connection, it is interesting to note that Michal and Jelinski\(^{58}\) have presented evidence from REDOR NMR spectroscopy that the sequence LGXG present in spidroin I forms turns in *Nephila* silk thread.

**Mechanism of the Conformation Transition Induced by Potassium Ions in Nephila Spidroin Film.** We proposed a mechanism for the potassium-induced conformation transition in *Nephila* spidroin film based on kinetics data obtained from time-resolved FTIR measurement.\(^{25}\) We suggested that the transition had two phases represented by two time constants (\(\tau_1, \tau_2\)). The fast phase (\(\tau_1\)) represented an initial formation of \(\beta\)-sheets probably arising from the rapid adjustment of individual segments of spidroin molecules within films. We suggested that after this, the remaining potential \(\beta\)-sheet-forming domains undergo a slow intermolecular rearrangement (\(\tau_2\)) to complete the formation of \(\beta\)-sheet structure. A bimodal process is well confirmed by 2D correlation infrared spectra. During stage I (from the beginning to \(\sim\tau_1\)), the synchronous spectra (Figure 2A) showed a single auto-peak at 1620 cm\(^{-1}\) representing an initial rapid formation of \(\beta\)-sheet structure. During stage III (from \(\sim\tau_5\) to the end), a broad auto-peak at 1650 cm\(^{-1}\) showed that much of the change of random coil/helical conformation did not take place until this stage. We suggested that this delay resulted from a slow rearrangement of whole macromolecular chains rather than local segments or from a slow improvement in the packing of the previously formed \(\beta\)-sheet structure.\(^{25}\) The latter hypothesis is in line with wide-angle X-ray scattering data on the development of the silk II pattern in hydrogels based on regenerated *Bombyx mori* fibroin.\(^{59}\)

A study of the two positive cross-peaks at 1642/1620 cm\(^{-1}\) and 1658/1620 cm\(^{-1}\) and a negative one at 1691/1642 cm\(^{-1}\) in asynchronous spectra of stage I (Figure 2B) gives more...
information about the order of events in the conformation transition. These cross-peaks suggest that random coil (1642 cm$^{-1}$) and helical structure (1658 cm$^{-1}$) are converted into $\beta$-sheet and related structures (1620 and 1691 cm$^{-1}$). The positive cross-peak at 1658/1642 cm$^{-1}$ indicates that the helical structures are converted into $\beta$-sheets before domains with random coil conformation.

The asynchronous spectra (Figure 4B) for stage II of the transition (from $\sim\tau_1$ to $\sim\tau_2$) showed a more complex pattern than that for stage I with more components in the random coil/helical conformation region (1642, 1648, and 1655 cm$^{-1}$). These probably resulted from both segmental adjustment and more drastic rearrangement of the whole macromolecule. During stage III of the conformation transition, there were only two broad cross-peaks (1650/1620 and 1691/1620 cm$^{-1}$) in the asynchronous spectra (Figure 6B). These two cross-peaks indicated that the random coil and/or helical component (1650 cm$^{-1}$) varied before the change of $\beta$-sheets (1620 cm$^{-1}$) and turns or bends (1691 cm$^{-1}$) components. We could imagine that most of $\beta$-sheet structures were formed in this stage, so mainly the chain movement belonged to random coil and helical structures.

Thus, taken together, our evidence from time-resolved FTIR$^{25}$ and from 2D correlation spectroscopy presented above suggests a two component model in which the transition starts with a rapid initial conversion of the $\alpha$-helical component into $\beta$-sheets followed by a slower incorporation of the random coil into $\beta$-sheets. It seems likely that the polyalanine domains give rise to the fast component as they are thought to be present in an initial $\alpha$-helical conformation in *Nephila* silk dope$^{3,60}$ and transform into the principal $\beta$-sheet component$^{24,61}$ of the formed silk. We note the evidence that QGAGQ domains and glycines immediately adjacent to the ends of the polyalanine chains are also thought to be incorporated into $\beta$-sheet crystallites, so these may represent the slower component. The less favorable position and energetics of these components may retard their incorporation into $\beta$-crystallites.

A believable view has recently emerged on the molecular structures of *Nephila* spidroin and insect fibroins in the natural spinning dopes (silk-I-like structure) and in silk threads (silk II structure). Sikorski and Atkins$^{62}$ have used dynamic molecular modeling to derive a nylon-66-like model for *Bombbyx* fibroin in the natural silk dope (silk-I-like state). In their model, more-or-less fully extended chains lacking an ordered secondary structure are repeatedly folded back on themselves in loose hairpin loops to give a multi-lamellar rod-shaped molecule with the lamellae arranged transverse to the long axis of the rod. Introducing the $\alpha$-helical polyalanine domains to major and minor ampullate spidroin I of spider and wild silk moth in place of only random coil chains make no appreciable difference to the model. Phase separation of highly hydrophobic domains$^{63}$ is likely to assist in forming this structure. We also note that a similar structure is seen in the silk-like protein fibronectin. Thus, the structure of the silk molecules in the dope is thought to prefigure and define the antiparallel $\beta$-sheet and turn/beaded necklace structure$^{65}$ for the silk II nanofibril deduced from AFM images and AFM force spectroscopy. Simultaneous observation of small angle and wide-angle X-ray scattering pattern of fibroin hydrogels$^{59}$ supports this hypothesis. Thus it, requires a simple, probably staggered side-to-side aggregation of elongated rod-shaped molecules without radical uncoiling or drastic change in chain alignment to produce the silk II nanofibril from the silk-I-like molecule.

Therefore, combining our FTIR data and view discussed above, we suggest the following hypothesis for the conversion of silk I into silk II. Figure 7 shows a hypothetical model illustrating nanofibril formation and conformation change in vivo. Potassium ions may form coordination complexes between chains$^{66}$ facilitating their aggregation$^{69}$ into nanofibrils. The closer approach and chain alignment produced by aggregation may in turn help trigger conversion of the $\alpha$-helical domains into $\beta$-sheets. In vivo, this effect may be greatly enhanced by elongational flow$^{15}$ acting on an easily aligned main chain liquid crystal polymer. This should result in a close approach and alignment of the $\alpha$-helix initiating their transition to $\beta$-sheets. The reduction in pH in vivo is likely to produce an increased chain interaction indicated by fibroin and spidroin gelling in vitro$^{67}$ and this may account for the promotion of $\beta$-sheet formation at low pH.$^{19}$ An implication of this model for the precursor molecule and final assembly state in the thread is that the diameter of the nanofibril is likely to be reduced as $\beta$-sheet formation proceeds and this has been noted in regenerated silk hydrogels.$^{59}$ A close approach of the resulting $\beta$-sheets from the $\alpha$-helix may initiate nucleation$^{68}$ by providing a thermodynamically favorable environment for further $\beta$-sheet formation. Thereafter, as suggested above, some domains with random coil conformation may be slowly incorporated at the surface of the $\beta$-crystallites. Subsequently, turn formation may be initiated as the structure becomes more compact forcing the ends of loose loops in the random coil conformation to collapse into turns as we know type II $\beta$-turns are likely to be favored as these produce an advantageous 180° reversal of the direction of the chains. Further collapse in the interface between the crystallites within the nanofibril may result in the formation of more compact structures.$^{70}$

**Conclusion**

Our findings show that the 2D correlation technique is a powerful technique for processing data from time-resolved FTIR spectroscopy to study the potassium-induced conformation transition in *Nephila* spidroin films. It provides increased resolution and important new information on the sequence of events in the conformation transition, in particular further evidence that the 1691 cm$^{-1}$ band is not the high-frequency band of antiparallel $\beta$-sheet but represents turns and/or bends. It also provided stronger evidence that random coil and $\alpha$-helical conformations coexist in the original spidroin film unresolved in our previous attempts to analyze FTIR spectra. It revealed that $\beta$-sheet formation from helix occurred before random coil in the early stage of the conformation transition.

Finally, our observations support a liquid crystalline spinning mechanism$^{5}$ for natural silks as ordered structures
are thought to adopt a "random coil" conformation while the latter from the more hydrophobic polyalanine blocks. The former blocks separation of the more hydrophilic glycine-rich blocks (dotted lines) the hairpin structure. Folding is assisted by a lamellar phase antiparallel hairpin structure similar to that of nylon 66. This conformation nanofibrils in vivo based on the present paper and studies using other Figure 7. Illustration of a model for the assembly of spidroin
blocks adjacent to the polyalanine blocks are also more slowly incorporated into the β-sheets (see text for evidence).
produced by flow alignment in a calamitic (based on rod-shaped units) liquid crystal are likely to favor β-sheet formation.

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