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Reversible Assembly of β -Sheet Nanocrystals within Caddisfly Silk

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Abstract

Nuclear magnetic resonance (NMR) and X-ray diffraction (XRD) experiments reveal the structural importance of divalent cation–phosphate complexes in the formation of β -sheet nanocrystals from phosphorylated serine-rich regions within aquatic silk from caddisfly larvae of the species *Hesperophyla consimilis*. Wide angle XRD data on native caddisfly silk show that the silk contains a significant crystalline component with a repetitive orthorhombic unit cell aligned along the fiber axis with dimensions of $5.9 \text{ \AA} \times 23.2 \text{ \AA} \times 17.3 \text{ \AA}$. These nanocrystalline domains depend on multivalent cations, which can be removed through chelation with ethylenediaminetetraacetic acid (EDTA). A comparison of wide angle X-ray diffraction data before and after EDTA treatment reveals that the integrated peak area of reflections corresponding to the nanocrystalline regions decreases by 15–25% while that of the amorphous background reflections increases by 20%, indicating a partial loss of crystallinity. ³¹P solid-state NMR data on native caddisfly silk also show that the phosphorylated serine-rich motifs transform from a rigid environment to one that is highly mobile and water-solvated after treatment with EDTA. The removal of divalent cations through exchange and chelation has therefore caused a collapse of the β -sheet structure. However, NMR results show that the rigid phosphorus environment is mostly recovered after the silk is re-treated with calcium. The ³¹P spin–lattice (T_1) relaxation times were measured at 7.6 ± 3.1 and 1 ± 0.5 s for this calcium-recovered sample and the native silk sample, respectively. The shorter ³¹P T_1 relaxation times measured for the native silk sample are attributed to the presence of paramagnetic iron that is stripped away during EDTA chelation treatment and replaced with diamagnetic calcium.

INTRODUCTION

Caddisfly larvae (order Trichoptera) utilize aquatic silk fibers to stitch together available debris into elaborate underwater structures.^{1,2} Although the caddisfly is closely related to Lepidopteran insects, including the domesticated silkworm *Bombyx mori*,^{3,4} their fibers do

not contain runs of poly(Ala) or poly(Gly-Ala). These repetitive motifs form rigid β -sheet regions in spider and silkworm silks, contributing greatly to the impressive mechanical properties of the fibers.⁵⁻⁸ Instead of alanine-rich repetitive motifs, caddisfly fibers contain phosphorylated (SX)₄ repeat regions in the H-fibroin primary protein sequence.^{9,10} This phosphate-rich motif is colored red in Figure 1, which shows a portion of the caddisfly H-fibroin protein sequence labeled the D-repeat. Caddisfly silk fibers also contain a significant crystalline component;¹¹⁻¹⁴ thus, it was recently suggested that phosphorylated (SX)₄ repeats form insoluble β -sheet structures through Ca²⁺-phosphoserine complexes.¹⁵ Such regions may serve as a structural replacement for β -sheet-forming poly(Ala) or poly(Gly-Ala) domains in spider and silkworm silks.

There is strong conceptual and experimental support for this hypothesis that has been laid out previously.¹⁴⁻¹⁶ To summarize, the nanocrystalline regions must arise from some sheet-forming motifs other than poly(Ala) or poly(Gly-Ala), and (pSX)₄ repeats fit the mold. A conserved proline-glycine motif, often seen in β -turn structures,¹⁷ exists shortly after every (pSX)₄ repeat region in the H-fibroin protein sequence (underlined in Figure 1).^{9,10} The X residue in the (SX)₄ motif is often valine or isoleucine, which both have strong tendencies to form β -sheet structures.^{18,19} Elemental analysis reveals significant levels of multivalent cations, including calcium, magnesium, and iron.^{15,20} Computer simulations of (pSX)₄ repeats show a dramatic increase in strength when calcium cations are present.²¹ Multiphosphorylated peptides have previously been shown to form β -sheets when calcium cations are present; tangle this formation mechanism in Alzheimer may be 'related' to disease to patients.²²⁻²⁴ Our recent ¹³C and ³¹P solid-state nuclear magnetic resonance (NMR) data for caddisfly larval silk showed that valine and phosphoserine residues from phosphorylated (SX)₄ motifs exist in β -sheet structures, and that caddisfly silk contains a rigid phosphorus environment that is likely stabilized by divalent cations.¹⁴ Moreover, it was recently shown that both the tensile properties of individual caddisfly silk fibers and the overall β -sheet content of the silk are diminished after the removal of cations via ethylenediaminetetraacetic acid (EDTA) chelation.²⁵

While a strong interaction between negatively charged phosphates and di- and trivalent cations is no surprise, its prevalence within caddisfly larval silk is very unique within silk-based biopolymers. In this work, we further demonstrate that multivalent cations are absolutely essential to the structural integrity of caddisfly silk. NMR and X-ray diffraction (XRD) results show the reversible deformation and formation of β -sheet nanostructures within the phosphorylated serine-rich regions of caddisfly silk, through depletion and exposure of ■ divalent cations, respectively.

MATERIALS AND METHODS

Silk Collection

Native caddisfly silk was obtained from larvae of the species *Hesperophylax consimilis* from the upper Red Butte creek in Salt Lake County, Utah. The insects were removed from their original stone cases and were given blocks of polytetrafluoroethylene (PTFE) as fodder to construct new cases. After 3 days, they were removed from PTFE cases and were placed back into their original stone cases for feeding. Silk was separated from the PTFE cases

using fine forceps and stored in tap water at 4 °C. This process was repeated multiple times to obtain a sufficient sample for solid-state NMR (SSNMR)

Preparation of Phosphoserine Cation Salts

Samples were prepared by dissolving L-*O*-phosphoserine (Sigma-Aldrich) in deionized water, adding equimolar amounts of cation chloride salts, and adjusting the pH to 8.3 using dilute NaOH. The solution was then flash-frozen and lyophilized. The cation mixture sample containing paramagnetic iron (Figure 6E) was prepared using a 10:6:3:1 phosphoserine:Ca²⁺:Mg²⁺:Fe³⁺ ratio, a ratio similar to that observed in native caddisfly silk.¹⁵

Scanning Electron Microscopy (SEM)

Both native and exchanged silk samples were placed onto conductive carbon tape and then coated with gold using a Denton vacuum sputter coater desk II for 180 s at a deposition rate of 5 nm/min. SEM was performed using a XL30 Environmental SEM-FEG instrument built by FEI. The secondary electron (SE) detector was used for imaging. Measurements were taken under a vacuum pressure of $<9 \times 10^{-5}$ mbar with a beam current of 5.00 kV.

Wide Angle X-ray Diffraction

Caddisfly larval silk was mounted onto a cardboard washer for wide angle X-ray diffraction (WAXD) measurements, and a small amount of super glue was used to secure the fibers to the washer at either end. The WAXD experiments were conducted at the 14-BM-C beamline at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL) using a beam energy of 12.6 keV. The exposure time was fixed at 60 s for each of five averaged exposures, and the nine-panel CCD array detector was placed at a distance of 450 mm in both cases. The ADSC Quantum-315 detector with a beam size of approximately $130 \mu\text{m} \times 340 \mu\text{m}$ (full width at half-maximum) was used. The sample was aligned such that the fibers were parallel to the axis of the beamstop. Cerium dioxide (CeO₂) was used as a calibrant in the FIT2D X-ray processing software to analyze the two-dimensional (2D) diffraction patterns. Ethylenediaminetetra-acetic acid (EDTA), a known metal ion chelator, was then utilized to remove cations from the silk fibers through chelation. Identical XRD experimental conditions were used for both the native and EDTA-exchanged silk samples. To prepare the exchanged sample, the native sample was carefully treated with a cation exchange solution containing 10 mM Tris (pH 8.1), 2 mM EDTA, and 10 mM NaCl for 6 h. Agitation was kept to a minimum so that fiber alignment was not disrupted by turbulence.

Cation Exchange Treatment

For NMR studies, a bundle of native caddisfly silk fibers was added to a solution containing 10 mM Tris (pH 8.1), 2 mM EDTA, and 10 mM NaCl. This solution was stirred overnight with a magnetic stir bar to ensure complete EDTA treatment. After NMR data had been collected, this sample was similarly treated with a solution containing 10 mM Tris (pH 8.1) and 10 mM CaCl₂. The sample was stirred with a magnetic stir bar overnight, and silk was recovered for solid-state NMR experiments.

³¹P Solid-State NMR

³¹P solid-state NMR experiments were conducted on a 400 MHz Varian wide-bore instrument equipped with a 3.2 mm triple-resonance MAS probe. All samples were spun at the magic angle at 4 kHz. ³¹P chemical shifts were referenced externally to crystalline ammonium phosphate at 0.8 ppm, and the same reference sample was used to optimize ¹H → ³¹P cross-polarization under magic angle spinning (CP-MAS) conditions. For CP experiments, the following typical experimental conditions were used: an initial 2.6 μs proton $\pi/2$ pulse, a 1 ms ramped (~10%) spin-lock pulse on both the ¹H and ³¹P channels near 80 kHz, a 100 kHz spectral width, a 10 ms acquisition time, a 5 s relaxation delay, and either 2048 or 4096 scan averages. Direct detection under magic angle spinning (DD-MAS) experiments on caddisfly silk samples utilized a 4.1 μs $\pi/2$ pulse, a 10 ms acquisition time, a 100 kHz spectral width, a 5 s relaxation delay, and 2048 scan averages. All experiments were conducted using 100 kHz TPPM²⁶ proton decoupling during acquisition.

³¹P T₁ Measurements

The ³¹P T₁ relaxation times were measured for native and exchanged caddisfly silks (Figure 4) and for phosphoserine in the presence of various cations (Figure 5) using the progressive saturation method.²⁷ The following typical experimental parameters were used: a 4.1 μs $\pi/2$ pulse, a 10 ms acquisition time, a 100 kHz spectral width, a minimum of eight scans, and a 4 kHz MAS spin rate. Two-pulse phase-modulated²⁶ (TPPM) proton decoupling at 100 kHz was applied during acquisition. The recycle delay was varied to obtain a buildup curve dependent on T₁ relaxation properties. To obtain T₁ relaxation time constants, spectra from each recycle delay were baseline corrected, and the center band of the chemical shift anisotropy (CSA) powder pattern was integrated and normalized. Normalized peak areas were plotted against recycle delay, and the curve was fit to the equation

$$M(t) = A_1 [1 - \exp(-t/T_1)] + A_2$$

where A₁ is a normalization constant and A₂ is the y-intercept necessary for obtaining good fits. All T₁ values are listed in Table 1, and errors are reported as the 95% confidence intervals from the fits.

RESULTS AND DISCUSSION

Caddisfly silk from *H. consimilis* was studied via WAXD and solid-state NMR techniques, and the importance of multivalent cations within the silks was investigated. EDTA was introduced to caddisfly silk to remove divalent cations from the fibers. Figure 2 shows SEM micrographs of caddisfly silk, demonstrating the effects of removing divalent cations. The natural silks from *H. consimilis* are a fusion of two fibrils into flat, ribbonlike fibers. The surface detail seen in Figure 2B shows that the fibers are composed of approximately 120 nm nanofibrils aligned along the fiber axis. When the silk is treated with a cation exchange solution [10 mM Tris (pH 8.1), 2 mM EDTA, and 10 mM NaCl], the fiber morphology is completely destroyed. Instead of 5–10 μm wide ribbons, the silk becomes warped and inconsistent, and the nanofibrils are exposed and disorganized (Figure 2C,D).

The substantial changes to the appearance of the fiber seen in the SEM images occur upon removal of divalent cations via EDTA chelation. These cations are potentially being removed from phosphoserine–cation complexes; thus, we probed any structural changes caused by EDTA treatment through wide angle X-ray diffraction (WAXD). Many of the reflections have been assigned using an orthorhombic unit cell; here the peaks labeled 1–4 correspond to (123), (004), (120), and (100) reflections, respectively. The d spacings associated with each peak are indicated in Figure 3B. The assignments yield a repetitive orthorhombic unit cell aligned along the fiber axis, with dimensions of $5.9 \text{ \AA} \times 23.2 \text{ \AA} \times 17.3 \text{ \AA}$.

This crystalline units are not caused by the traditional poly(Ala) or poly(Gly-Ala) found in spider and silkworm fibers; instead, they arise from phosphorylated serine-rich motifs stabilized by calcium, magnesium, and iron cations.^{14,15} To further investigate any structural changes upon removal of cations from the silk, the native caddisfly silk sample used for Figure 3A was carefully treated with a cation exchange buffer [10 mM Tris (pH 8.1), 2 mM EDTA, and 10 mM NaCl] for 6 h. Agitation was minimal so that the sample alignment was not altered. The sample was rinsed and air-dried, and the WAXD experiment was then conducted again (Figure 3B). Both the 2D diffraction pattern and the equatorial 2θ integration profiles clearly show an increase in the background and a decrease in the number of aligned repetitive reflections. The integrated peak area of the amorphous background signal, seen as the large broad lines, increased by 20% after cation removal. Similarly, the intensities of the diffraction peaks associated with axially aligned repetitive β -sheet units decreased after EDTA treatment; the peak areas for each of the three equatorial reflections (1, 3, and 4) are diminished by 15–25%. The WAXD data clearly indicate that removal of divalent cations from caddisfly silk disrupts the repetitive β -sheet structures. The percent crystallinity was calculated for the native and treated samples using methods described by Grubb et al.,²⁸ resulting in 18 and 16% crystallinity, respectively. We associate the decrease in crystallinity with a partial disruption of the cation–phosphoserine nanocrystalline β -sheets within caddisfly larval silks. While this is only a minor change in crystallinity, we note that the sample agitation was kept to a minimum, so a full disruption of the β -sheets is not expected.

A more complete EDTA treatment was performed on nonoriented caddisfly silk bundles, and changes in phosphorus environments were probed with ^{31}P NMR. Initially, both ^{31}P DD-MAS and CP-MAS spectra were recorded for caddisfly silk in its natural, hydrated state. The ^{31}P solid-state NMR data provide clear evidence of rigid phosphate environments. Both the DD-MAS and CP-MAS spectra in panels A and B of Figure 4 show broad powder patterns with an isotropic chemical shift (σ^{iso}) at 1.5 ppm. The ^{31}P chemical shift anisotropy (CSA) pattern mapped out by the spinning side bands exhibits a negative skew parameter (skew = -0.4), which is characteristic of a rigid phosphate carrying a charge of -2 .^{29,30} One might expect charged phosphoserine residues to be highly mobile and water-solvated; however, the observation of a strong $^1\text{H} \rightarrow ^{31}\text{P}$ cross-polarization signal implies that the phosphates are surrounded by a rigid proton dipolar coupling network.

Rigid phosphoserine environments are found in caddisfly silk even when the fibers are wet; however, this is lost upon EDTA treatment. Both direct and CP spectra were again collected

(Figure 4C,D) after the silk had been treated with the cation exchange buffer. The lack of signal from the CP-MAS spectrum implies that ^{31}P nuclei are no longer in strong dipolar contact with rigid protons. The direct ^{31}P spectra have collapsed to a single isotropic resonance as opposed to a broad powder pattern, indicating that molecular motion is now sufficiently fast to average away the chemical shielding tensor. The isotropic phosphorus chemical shift in the direct spectra now falls at 3.1 ppm, shifted downfield from that of the natural silk as indicated by the dotted line. This noticeable shift is consistent with our recently published data collected on phosphoserine–cation salts; the isotropic ^{31}P chemical shift from phosphoserine moves downfield when sodium is present as opposed to either calcium or magnesium.¹⁴ Furthermore, the observations of a dramatically increased intensity of the signal and the clearly sharpened line shape in the direct spectra are again indicative of a mobile environment. Therefore, upon EDTA-mediated removal of divalent cations from caddisfly silk, a rigid phosphoserine environment is replaced by one that is highly mobile and solvent-exposed.

Both our WAXD and NMR data on native versus exchanged caddisfly silk are in excellent agreement with recently published mechanical and FTIR results.²⁵ When individual silk fibers were treated with EDTA and multivalent cations were replaced with Na^+ , the fiber's tensile properties (stiffness, strength, and energy-dissipating hysteresis) were all destroyed. The loss of mechanical properties is directly correlated to a decrease in the overall β -sheet content of the silk, which is evident in a decrease in the intensity of the β -sheet component of the amide I band in the FTIR spectra after EDTA treatment. Amazingly, the mechanical properties of the original fiber and the overall β -sheet content are both reestablished after calcium cations are reintroduced to the material.

Our NMR results also show that one can reestablish rigid calcium–phosphoserine β -sheet regions from cation-depleted caddisfly silk after reintroducing calcium cations. The ^{31}P NMR data shown in Figure 4 reveal that when the exchanged silk was treated with a solution containing 10 mM CaCl_2 , rigid phosphorus environments are partially recovered. Although the CP-MAS signal intensity is diminished in Figure 4F with respect to that of the natural silk spectra shown in Figure 4B, the broad powder pattern is clearly recovered. The chemical shift in the CP spectra moves from 3.1 ppm back to the original value of 1.5 ppm, again in agreement with a phosphoserine–divalent cation interaction.¹⁴ The direct spectrum shows a faint sharp component at 3.1 ppm, similar to that observed for the EDTA-treated silk; however, the signal intensity is dramatically decreased. Both spectra were collected on the same amount of sample and with the same NMR acquisition parameters; thus, the loss of direct ^{31}P signal intensity is explained by mobile phosphates being reincorporated into rigid, water-inaccessible calcium–phosphoserine complexes. Interestingly, the native silk exhibits strong and broad DD-MAS and CP-MAS signals (Figure 4A,B), but after removal of cations and reintroduction of Ca^{2+} , this broad DD-MAS signal is weak as compared to the CP spectrum (Figure 4E,F). In fact, for the native silk sample, the direct signal is actually stronger than that from the CP with the same relaxation delay (5 s) and the same number of scan averages (2048) but is much weaker than that from the CP after calcium is reintroduced as the stabilizing cation. This difference is intriguing and deserves an explanation. We

attribute the difference between the direct spectra in panels A and E of Figure 4 to differences in T_1 relaxation properties.

To further investigate the difference in relaxation properties between the native and calcium-exchanged silk samples, spin–lattice relaxation times were measured using the progressive saturation method,²⁷ and trends were compared to model systems of phosphoserine in the presence of various cations. The ^{31}P T_1 relaxation times for native and calcium-exchanged caddisfly silks were measured at 1 ± 0.5 and 7.6 ± 3.1 s, respectively (Figure 5 and Table 1), confirming that the weakened signal observed in Figure 4E as compared to that in Figure 4A is simply a result of incomplete spin–lattice relaxation. We propose that the likely explanation for this curious difference is that native caddisfly silk contains paramagnetic Fe^{3+} .¹⁵ The data are shown in Figure 6, and -1 values are summarized in Table 1. We observe ^{31}P relaxation times in the range of 70 s for the phosphoserine samples when iron is not present, which is very common for rigid phosphate materials.^{31,32} However, in the mixture, the phosphorus spin–lattice relaxation time is reduced ~10-fold (Figure 6 and Table 1). While the relaxation rates are certainly different between the hydrated silk materials and the powder phosphoserine model systems, it is the difference between samples with and without paramagnetic iron that is important. While this paramagnetic effect is likely just a coincidence and does not directly improve the properties of silk, this observation provides additional support that multivalent cations are being removed from the silk fibers with EDTA. This result further highlights the importance of these cations within caddisfly larval silks.

CONCLUSIONS

Figure 7 provides a summary of our findings. ^{31}P solid-state NMR data reveal that when cations are removed from caddisfly silks via EDTA chelation, rigid phosphate environments transform into solvated, highly mobile regions. When calcium is reintroduced to the fibers, rigid calcium–phosphoserine complexes are partially recovered. A paramagnetic effect caused by the presence of Fe^{3+} cations within the silk was lost after EDTA treatment, adding further support to the idea that removal of multivalent cations from the silk fibers is responsible for the structural changes observed after EDTA treatment. Additionally, WAXD results show that removal of divalent cations disrupts repetitive β -sheet nanocrystallites within the silk. The data highlight the structural necessity of di- and trivalent cations in caddisfly larval silks and strongly support a structural motif in which repetitive $(\text{pSX})_4$ motifs interact with calcium, magnesium, and iron cations to form rigid β -sheet structures.¹⁵

Acknowledgments

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D Repeat:
VSISRSVSIERIVTPGVTKISRSSSVSVEGG

Figure 1.

Extended D-repeat from the H-fibroin protein sequence from *Hesperophylax* sp.²⁵ Phosphorylated (SX)₄ regions are colored red, and a conserved, underlined proline-glycine turn is seen after both motifs. Calcium, magnesium, and iron cations are thought to stabilize these phosphorylated serine-rich motifs to form rigid β -sheet structures within caddisfly larval silks.

Figure 2. Scanning electron microscopy (SEM) images of natural caddisfly silk (A and B) and silk treated with EDTA (C and D). The fibers are destroyed when Ca^{2+} is removed through EDTA chelation, exposing the small (≈ 120 nm) nanofibrils.^{16,21} The images were collected on gold-coated samples using an XL30 Environmental SEM-FEG instrument built by FEI.

Figure 3.

Wide angle X-ray diffraction (WAXD) profiles of axially aligned caddisfly silk before (A) and after (B) EDTA treatment. Integrations from the equatorial wedges (indicated in panel A) are shown below each profile, and the data were fit to four Gaussian peaks (1–4) and one background peak. After EDTA treatment, the intensity of the amorphous background peak is increased while the intensities of peaks 1, 2, and 4 are reduced. The data show a decrease in crystallinity after cation removal through EDTA chelation.

Figure 4.

^{31}P DD-MAS and CP-MAS NMR data on natural (A and B), EDTA-treated (C and D), and CaCl_2 -treated (E and F) caddisfly silk fibers. Direct spectra were acquired using 2048 scan averages and a 5 s recycle delay, while CP spectra were collected using 2048 (B and F) or 4096 (D) scan averages, a 1 ms CP contact time, and a 5 s recycle delay.

Figure 5.

^{31}P T_1 relaxation curves for native caddisfly silk (A), silk after cations had been removed with EDTA (B), and silk after reincorporation of Ca^{2+} (C). Data points are colored blue, the resulting fits shown as solid red lines, and the 95% confidence intervals shown as red dashed lines. The difference in T_1 relaxation rates between native silk and Ca^{2+} -exchanged silk is due to the removal of paramagnetic iron from the native sample.

Figure 6.

^{31}P T_1 relaxation curves for phosphoserine in the presence of various cations. (A–E) pSer with Li^+ , Na^+ , Mg^{2+} , Ca^{2+} , and the Fe^{3+} mixture, respectively. (F) ^{31}P signal buildup for pSer with Ca^{2+} (red) and for the Fe^{3+} mixture (black). Data points are colored blue, the resulting fits shown as solid red lines, and the 95% confidence intervals shown as red dashed lines. The 10-fold enhancement in relaxation rates when iron is present as opposed to absent is similar to that observed in native vs exchanged caddisfly silk.

Figure 7.

Rigid cation–phosphate regions from (pSX)₄ repeat motifs in native caddisfly silk (A) are stripped of cations through EDTA chelation. Multiple negatively charged phosphates now reside in the proximity. Water is able to penetrate into these regions and solvate the phosphates, increasing the level of molecular motion to a near liquid-like dynamic regime (B). However, when calcium cations are reintroduced to the silk, a rigid phosphorus environment is recovered as phosphorylated (SX)₄ repeat regions collapse back into calcium-stabilized β -sheet nanocrystalline structures (C). The phosphoserine side chains are visible, and the protein backbone is represented by yellow tubes.

Table 1
 T_1 Relaxation Times for Native and Exchanged Caddisfly Silks and for Phosphoserine in the Presence of Various Cations

sample	T_1 (s)
caddisfly native	1 ± 0.5
caddisfly EDTA/NaCl	1.7 ± 0.7
caddisfly CaCl_2	7.6 ± 3.1
pSer with 2LiCl	88 ± 8
pSer with 2NaCl	61 ± 3
pSer with MgCl_2	54 ± 5
pSer with CaCl_2	69 ± 6
pSer and a mixture ^a	7.5 ± 2.5

^a Sample prepared with a 10:6:3:1 pSer: Ca^{2+} : Mg^{2+} : Fe^{3+} ratio.