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The Role of Mn(II) in Silk Fibroin Based on EPR and NMR Spectroscopy

Yi-Bin Deng¹, Dan Ji¹, Ping-Chuan Sun², David Knight³, Jian-Hua Cai¹, and Ping Zhou¹

¹The Key Laboratory of Molecular Engineering of Polymers, Department of Macromolecular Science, Fudan University, Shanghai, P.R. China
²Department of Chemistry and Department of Physics, Nankai University, Tianjin, P.R. China
³Department of Zoology, University of Oxford, U.K.

ABSTRACT The formation of the natural silk fiber depends on the conformational transition of silk fibroin (SF) from a soluble random coil and/or helix state (Silk I) to an insoluble β-sheet state (Silk II). Previous investigations have shown that many factors including H⁺, K⁺, Na⁺, Ca²⁺, Cu²⁺, and Fe³⁺ have an effect on the transition. In the present investigation, we report that ¹³C nuclear magnetic resonance (NMR) spectroscopy indicates that Mn²⁺ ion does not promote the conformational transition of silk fibroin. Using electron paramagnetic resonance (EPR), we show that Mn(II) ions are likely to exist in the silk fibroin molecules in two states: a six-coordination Mn(II) = SF complex and a Mn(H₂O)²⁺₆ complex. The binding of Mn(II) to the amino acid residues located in the hydrophilic spacers of the silk fibroin heavy chain might favor the Silk II state, while the Mn(H₂O)²⁺₆ complex might counter this effect by stabilizing the Silk I state. pH change has no considerable influences on the conformation transition of the silk fibroin because of the Mn(H₂O)²⁺₆ complex existence.

KEYWORDS conformation, EPR, Mn(II) ion, NMR, silk fibroin

INTRODUCTION

Natural silk has been used extensively as a textile for 1000 years.[¹–²] More recently, its good mechanical performance and biocompatibility has attracted interest in its potential as a biomedical material.[³–⁴] The properties of silk fibers are related to the amino acid sequences, secondary and higher ordered structures of the protein. The heavy-chain fibroin with molecular weight of 391 kDa accounts for about 90% by weight of the protein content in the degummed silk filament as calculated from the published sequences of FIBH and FIBL BOMM[⁵] in the Bombyx mori (B. mori) silk. The amino acid sequence of heavy-chain fibroin contains hydrophobic domains, largely composed of multiple repeats of the peptides, including GAGAGS, GAGAGY, GAGAGVGY, and 11 well-conserved hydrophilic spacers, GTGSSGFGPYVA(N/H)GGYSGYEYAWSSESDFGT.[⁵] The hydrophobic domains form intra- and inter-molecular β-sheet crystallites contributing greatly the high strength and thermal stability of the silk fiber.[⁶] In the liquid state, the hydrophobic domains are predominantly in a random coil and/or helix conformation (a helix-form, so-called silk I) along with the repeated type II β-turns,[⁷] and the conformation is readily converted into the
crystalline $\beta$-sheet (a $\beta$-form, so-called Silk II) during natural spinning. The formation of Silk II is thought to undergo a nucleation-dependent aggregation process and is accelerated by the increase in fibroin concentration, shearing and/or extensional flow, high temperature, low pH, high ionic strength, metal ions, and other factors. Ions including $H^+$, $K^+$, $Na^+$, $Ca^{2+}$, $Cu^{2+}$, and $Fe^{3+}$ are thought to play a part in influencing the secondary structure of silk fibroin as previously investigated by our group. The results showed that low pH and some metal ions could promote the conformational transition from helix form to $\beta$ form. Zong et al. found that $Cu^{2+}$ could coordinate with the regenerated silk fibroin in different modes at different pH values. At basic and neutral pH, the polypeptide AHGGYSGY in the hydrophilic domain of silk fibroin sequence may form a 1:1 complex with Cu(II), which may make the interaction between two adjacent hydrophobic polypeptide chains more difficult, leading to the formation of an amorphous structure. Instead under weakly acidic conditions, an intermolecular His(N$_{y}$)-Cu(II)-His(N$_{y}$) bridge is thought to be formed, which may favor the interaction between hydrophobic polypeptide chains, leading to the formation of $\beta$-sheet structure and fibroin aggregation. The mechanism is strikingly similar to that of the cellular prion protein (PrP$^\text{C}$) transition to a $\beta$-sheet-rich isof orm scrapie prion (PrP$^\text{Sc}$) and the aggregation of amyloid-$\beta$ peptides (A$\beta$), both of which cause neurodegenerative diseases. The formation of the fibrils among A$\beta$, PrP$^\text{Sc}$, and silk fibroin share a common aggregation mechanism, a nucleation-dependent growth. However, the fibrils of A$\beta$ and PrP$^\text{Sc}$ form the cross-$\beta$-sheet structures, while those structures in the silk fibers are also formed in addition to the structures of $\beta$-sheets or $\beta$-strands arranged in parallel and in antiparallel with respect to the fiber axis. The difference in assembly probably results from the much larger molecular weight of the latter protein. Therefore, the silk fibroin available in large quantities from $B. mori$ fibers might serve as a model protein to explore the assembly of $\beta$-sheet proteins with relevance to those important processes in neurodegenerative diseases.

Manganese was found to be present in $B. mori$ silk at concentrations of $9.0 \pm 1.5 \mu g/g$ in silk dope, $3.1 \pm 0.1 \mu g/g$ in dry unreeeled cocoon silk, and $0.4 \pm 0.1 \mu g/g$ in degummed silk, which are very low concentrations compared with other metal elements. Zhou et al. studied the effect of Mn$^{2+}$ ions on the concentrated silk fibroin solutions by Raman spectroscopy, showing that Mn$^{2+}$ ions had no detectable effect on the silk fibroin conformation. However, the possible role of Mn(II) ions in the silk fibroin and the coordination of Mn(II) ion with silk fibroin is still unclear.

Electron paramagnetic resonance (EPR) is a very powerful method used to detect the environment of transitional metallic elements and has often been applied to study Mn(II) interaction with proteins. EPR can provide information about the coordination between Mn(II) and proteins or enzymes. Some work on EPR was reviewed by Reed and Markham. For instance, Mn(II) in pyruvate kinase acts as a cofactor, participating in the binding of substrates and in catalysis in glycolysis. Mn(II) in alkaline phosphatase acts as a modulating factor influencing the affinity of the phosphate substrate for the second active site in the allosteric control of this enzyme activity. In addition, the ability of Mn(II) to substitute for Mg(II) has made Mn(II) popular as a spectroscopic probe for the Mg(II) site in many enzyme complexes.

However, the EPR spectroscopy of Mn(II) binding to silk fibroin is still poorly understood. In this work, we attempted to use EPR as well as $^{13}$C cross polarization/magic angle spinning nuclear magnetic resonance ($^{13}$C CP/MAS NMR) to investigate the interaction and coordination between Mn(II) and silk fibroin with the aim of shedding light on the possible function of Mn(II) ions in this protein.

**MATERIALS AND METHODS**

**Preparation of Regenerated Silk Fibroin**

$B. mori$ cocoons were treated with boiling aqueous Na$_2$CO$_3$ (0.5 wt%) solution for 0.5 hr to remove the sericin gum, and this process was repeated twice. The degummed silk fibers were washed with hot ultra-pure water (resistivity $\sim 18.2 \text{ M}\Omega \cdot \text{cm}$, pH $= 7.5$) and then air-dried at 60°C. The regenerated silk fibroin solution was prepared by dissolving 8 g of degummed dry silk fibers in 100-ml aqueous 9.3-M LiBr solution for 2 hr at room temperature.
The resultant solution was filtered through analytical grade filter paper, and then dialyzed against 2 L of ultra-pure water for 3 days at ambient temperature to remove the LiBr. During dialysis, the ultra-pure water was refreshed every 4 hr, and a ~3-wt% regenerated silk fibroin stock solution was obtained.

For removing the intrinsic trace metal ions in the regenerated silk fibroin, 20-ml 3-wt% silk fibroin stock solution was dialyzed against 0.01-M EDTA (ethylene diamine tetraacetic acid) solution for 2 days and then against ultra-pure water for 2 more days at ambient temperature. Both EDTA solution and ultra-pure water used for dialysis were refreshed every 3 hr, and then an EDTA-treated silk fibroin was obtained.

**Preparation of Mn(II)/SF Samples**

The manganese element content in the regenerated silk fibroin is about 0.3 μg/g as measured herein by atomic absorption spectroscopy (AAS). We investigated a series of samples with added Mn(II) contents of 0, 0.5, 2.0, 4.0, 10.0, and 40.0 μg/g with respect to the regenerated silk fibroin. The samples were prepared by mixing defined volumes of fresh aqueous MnCl₂ solution and 3-wt% silk fibroin stock solution, resulting in a series of Mn(II)-containing 2-wt% silk fibroin solutions. pHs of the solutions were adjusted to the desired values between pH 5.2 and 7.5 by adding 0.01-M hydrochloric acid or sodium hydroxide. All the water used in the sample preparations was ultra-pure water with resistivity of ~18.2 MΩ·cm.

Fibroin films cast on a smooth polyester surface were prepared from the above Mn(II)/SF solutions and allowed to dry for 4 days at ambient temperature.

**EPR Spectroscopy**

\[ ^{55}\text{Mn(II) (3d}^5\text{)} \text{is characterized by a nuclear spin } I \text{ and a fundamental electronic spin } S \text{, both of which were 5/2. The magnetic spin levels of Mn(II) can be described by a Hamiltonian spin function:} \]

\[ H = g\beta B \cdot S + A I \cdot S + D[S_x^2 - S(S + 1)/3] + E[S_x^2 - S_y^2] \]

\[ (1) \]

Where \( g, \beta, B, S, \) and \( I \) are respectively g-factor, Bohr magneton, magnetic field strength, electronic spin, and nuclear spin. The first term is the Zeeman interaction of electronic spin in the applied magnetic field \( (B) \), and the second term is the interaction of electronic spin and nuclear spin. The constant \( A \) is the hyperfine-coupling constant, which indicates the extent of the covalency of the metal-ligand bond. The third and fourth terms result from the zero-field splitting (ZFS) interaction induced by the ligand field surrounding the central metal. Whenever Mn(II) is bound in an environment with a coordination symmetry lower than cubic, the asymmetry imposed by the ligand field lifts the degeneracy of the electron spin levels to three Kramer doublet degeneracy levels in the absence of any applied magnetic field, causing a zero-field splitting,\(^{[28]}\) \( D \) and \( E \) respectively weight the axial and rhombic parts of the ZFS interaction, giving information regarding the coordination numbers, ligand field strength, and symmetry of the central metal in the complex. A large \( D \) value indicates a large splitting interaction. The \( E/D \) ratio is related to the local symmetry of the central ion and \( 0 \leq |E/D| \leq 1/3 \) in a proper axis system.\(^{[31]}\) The value of \( |E/D| \) is either 0 or 1/3, indicating an axial or fully rhombic symmetry, respectively. In addition to the degeneracies lifted by the zero-field splitting, the remaining degeneracies are lifted in the applied magnetic field to the six nondegeneracy levels, and therefore the five allowed transition bands \( (\Delta I = 0, \Delta S = \pm 1, i.e., \pm 5/2 \rightarrow \pm 3/2, \pm 3/2 \rightarrow \pm 1/2, +1/2 \rightarrow -1/2) \) are produced. Furthermore, every allowed transition band is lifted into sextet splitting due to the hyperfine coupling between electron spin \( S \) and nuclear spin \( I \). Therefore, a total of 30 allowed transitions may occur in the spectrum, creating a distinguishing signature of Mn(II)-EPR signals. These spectral features are much dependent on the environment of Mn(II) ions.\(^{[52]}\) Besides, the forbidden transitions \( (\Delta S = \pm 1, \Delta I = 1) \) are usually observed in the solid state EPR spectra of Mn(II).\(^{[50]}\) However, the 30 allowed transitions of Mn(II) in the X-band (microwave frequency of ~9.5 GHz) spectrum are often not fully resolved from each other in the solid state or when Mn²⁺ ion is bound to a macromolecular protein\(^{[29]}\) due to the spread by ZFS interaction and the random orientational distribution of the molecules.\(^{[33]}\) Fortunately, a resolved spectrum of the electronic spin transition \( +1/2 \rightarrow -1/2 \) along with a sextet hyperfine coupling is usually dominant when \( D \ll B_0 \) and is easily observed in the X-band even at room temperature.\(^{[54]}\)
In this work, EPR spectra were recorded using a Bruker EMX 8/2.7 ESR spectrometer (Bruker Co., Germany) with the parameters as follows: X-band microwave frequency of 9.45 GHz, modulation frequency of 100 kHz, modulation amplitude of 4 Gauss, microwave power of 2 mW, time constant of 163.84 ms, conversion time of 40.96 ms, field sweep of 1500 Gauss, measurement temperature of 100 K, and accumulation of 3 scans. Every sample was packed with the same weight into a 4-mm cylindrical quartz tube. The measured g-values were referred to that of DPPH (diphenylpicrylhydrazyl, \( g = 2.0036 \)) as an external standard. The EPR spectra were simulated to extract the paramagnetic parameters in Eq. (1) with EasySpin software, which represents a powerful, highly flexible and integrated analysis and simulation environment.\[35\]

\[\text{\textit{13C CP/MAS NMR and Spectral Deconvolution}}\]

\[\text{\textit{13C CP/MAS NMR experiments were performed on a Bruker DSX 300 NMR spectrometer with \text{\textit{13C}} resonance frequency of 75.5 MHz, pulse repeat time of 2 s, }^{1}H\text{ 90° pulse width of 4.23 (μs, }^{1}H\text{-}{\text{\textit{13C}}}\text{ cross polarization magnitude of 62.5 kHz, contact time of 1.1 ms, }^{1}H\text{ decoupling magnitude of 62.5 kHz, and over 1000 scans to ensure a good signal-to-noise (S/N) ratio. The methine peak of adamantane observed at 38.5 ppm was used as an external reference. Samples were routinely spun at 5 kHz in a magic angle spinning rotor with a diameter of 4 mm. The NMR peak of the }C_\beta\text{ in alanine residue at chemical shift between 6 and 26 ppm was simulated using the Gaussian function to analyze quantitatively the relevant contents based on our previous DFT chemical shift calculations, which indicated that the deconvoluted components in this peak provided very sensitive discrimination among the secondary structures of Silk I (17 ± 0.5 ppm, helix), Silk I-like (15 ± 0.5 ppm, helix-like), Silk II (20 ± 0.5 ppm, β-sheet), and Silk II-like (22 ± 0.5 ppm, β-sheet-like) in silk fibroin.}^{12,13,36–39}\]

\[\text{\textit{RESULTS}}\]

\[\text{\textit{Concentration-Dependent Mn(II) EPR Spectra}}\]

Figure 1 shows the EPR spectra of the Mn(II)/SF samples with different Mn(II) contents prepared at pH = 7.5. From Fig. 1a to 1c, the added Mn(II) contents in the Mn(II)/SF samples increase from 4.0 to 40.0 μg/g. It is clearly observed that the EPR spectra change considerably with the Mn(II) content increase. Figure 1a shows a low and broad spectral intensity, indicating a large ZFS and low symmetry.\[40–42\] We deduce that the spectrum is derived from the Mn(II), which binds the silk fibroin at the very low Mn(II) content of 4.0 μg/g. Figure 1c shows a typical Mn\((\text{H}_2\text{O})_2^{2+}\) EPR pattern with sextet splitting\[43–45\] when the Mn(II) content was increased to 40.0 μg/g. The small broad peaks around B = 2800 Gauss (marked by asterisks) originated from the background of the sample tube (data not shown) and was removed from Fig. 1c by subtracting the spectrum of Fig. 1b, giving rise to Fig. 1d. Therefore, Fig. 1d is thought to represent predominantly a Mn\((\text{H}_2\text{O})_6^{2+}\) signal.

In addition, there is a sharp signal at B = 3373 Gauss (g = 2.002, marked by arrow), derived from free radicals present in the samples.\[29\] To determine the origin of free radicals, we recorded the EPR spectra for a series of samples including regenerated SF, EDTA-treated SF and Mn(II)/SF mixtures (Fig. 2). All the samples gave free radical signals, indicating that the free radicals are continuously present in the silk fibroin. The free radicals have also been found in wool keratin and soybean proteins.\[29,46\]
We will discuss the free radical role in the silk fibroin in a following paper. The signals around $B = 3250$ G, 3308 G, and 3320 G (marked by double asterisks) are partially derived from the intrinsic Cu(II) ions present in the silk fibroin as demonstrated by our previous study based on EPR of Cu(II) in silk fibroin.[13] Comparison of the EPR spectrum of the EDTA-treated SF (Fig. 2c) with that of the untreated regenerated SF sample (Fig. 2b) shows that EDTA is able to remove some of the Mn(II) and Cu(II) signal. However, we can still observe the signals of Mn(II)/SF complex in Fig. 2c, indicating that there is an interaction between Mn(II) and SF.

**pH-Dependent EPR Spectra**

Figure 3 shows the EPR spectra of Mn(II)/SF samples, which were prepared from samples with an initial pH of 7.5, 6.0, and 5.2. All three spectra shown in Fig. 3c with added Mn(II) contents of 40.0 μg/g were almost identical, indicating that the environments of the Mn(II) ions were not pH dependent. Figure 3d shows typical sextet splitting along with double peaks between the adjacent peaks, very similar to that of MnCl$_2$ in 12-M HCl aqueous solution and in methyl alcohol solution at frozen state (T = 90 K),[43] where Mn$^{2+}$ ion is in the Mn(H$_2$O)$_6^{2+}$ complex. However, Fig. 3a and 3b with Mn(II) contents of 4.0 and 10.0 μg/g, respectively, show some influence of pH; the sextet splitting becomes evident as pH decreases from 7.5 to 5.2.

**EPR Parameters from Spectra of Mn(II)/SF Samples**

Based on Eq. (1), EPR spectra were simulated for extracting the paramagnetic parameters related to the atomic components and structural symmetry. Due to the low signal intensity of Mn(II) EPR and the influence from free radical and Cu(II) as well as the interaction between Mn(II) and SF in Fig. 3A-a, it is difficult to simulate the spectra of silk fibroin containing low contents of Mn(II). However, we deduced that the $D$ values of Fig. 3A-a and Fig. 3A-b as well as Fig. 2c were smaller than 500 G, because the larger $D$ value resulted in the simulated spectrum far away from the experimental spectra (data not shown). The simulated spectrum for Fig. 3A-d is shown in Fig. 4. The parameters extracted from Fig. 3A-d are $g = 2.000 \pm 0.001$, $A = 96 \pm 2$ G, Gaussian broadening = $18 \pm 2$ G, and $D$ value with a Gaussian distribution of a half-width $\sigma = 140 \pm 20$ G centered at an average value of $D_{av} = 155 \pm 10$ G, i.e., $D/B_0 = 0.046 \pm 0.004$. Once the

![FIGURE 2](image-url)

**FIGURE 2** EPR spectra of solid state Mn(II)/SF samples: (a) Mn(II) contents of 4.0 μg/g prepared at pH = 7.5, (b) pure regenerated SF and (c) EDTA-treated SF. The peaks marked with single and double asterisks originate from the background signals of sample tube and residual Cu(II), respectively, while the peaks marked with arrows originate from free radical signals. The six stick-lines on the top of figure indicate the sextet splitting of Mn(II) with $A = 96$ G. All spectra were recorded at 100 K, $v = 9.45$ GHz, sweep width = 1500 G.

![FIGURE 3](image-url)

**FIGURE 3** Dependence of EPR spectra of Mn(II)/SF samples with different added contents of Mn(II) upon pH. (a), (b), (c) represent the added Mn(II) contents of 4.0, 10.0, 40.0 μg/g under pH of 7.5 (A), 6.0 (B), 5.2 (C), respectively. The peaks marked with arrows are free radical signals, respectively. The six stick-lines on the top of figure indicate the sextet splitting of Mn(II) with $A = 96$ G. All spectra were recorded at 100 K, $v = 9.45$ GHz, sweep width = 1500 G.
deviations of the simulated parameters exceeded the standard deviation, the simulated spectrum departed markedly from the observed spectrum. During the simulation, changes in the $E/D$ value between 0 and 1/3 did not sensitively influence the EPR features, resulting in the uncertainty of $E/D$ value, which is perhaps due to the larger $D$ values of the studied samples.\[28\]

\[181\] The Role of Mn(II) in Silk Fibroin Based on EPR and NMR Spectroscopy

$^{13}$C CP/MAS NMR of silk fibroin was studied to investigate the effect of Mn(II) ions on the conformational transition of silk fibroin. Figure 5A shows the effect of pH values ranging from 7.5 to 5.2 on the solid-state $^{13}$C CP/MAS NMR spectra of Mn(II)/SF samples with Mn(II) contents of 4.0 μg/g. The spectra are essentially identical, indicating that pH over this range have no considerable influence on the secondary structure of silk fibroin. The conformation sensitive alanyl $C_{\beta}$ peaks at chemical shifts between 6 and 26 ppm were deconvoluted for the samples containing a series of Mn(II) contents at pH values of 7.5, 6.0, and 5.2. Figure 5B shows the deconvoluted examples for the samples with Mn(II) content of 4.0 μg/g at pH values of 7.5, 6.0, and 5.2. Figure 6 shows the dependence of total Silk II contents (including Silk II and Silk II-like conformations) upon the added Mn(II) content and pH. The total Silk II maximum content of 32 ± 2% was observed at the added Mn(II) content of 4.0 μg/g and at pH of 7.5, while the content is much lower compared with the content of 54 ± 2% obtained by adding cupric ions to the regenerated SF.\[13\] Besides, the EDTA-treated silk fibroin had almost the same NMR...
spectrum as pure silk fibroin (data not shown). These observations indicate that Mn(II) had no detectable effect on the promotion of the conformation transition in SF.

What we are interested in is the role of Mn(II) in the silk fibroin from the above observation and what could be inferred from the data.

DISCUSSION

Formation of Mn(H₂O)₆²⁺ Complex in Silk Fibroin

When the added Mn(II) contents were 40.0 mg/g, the EPR spectra (Fig. 3c) of Mn(II)/SF samples showed typical features of Mn(H₂O)₆²⁺ complex described elsewhere.[43,47] The spectrum obtained from one part of Fig. 3d gives the parameters of g = 2.000 ± 0.001, A = 96 ± 2 G, Dav = 155 ± 10 G, with σ = 140 ± 20 G, and is practically identical to the EPR spectrum of Mn(II) in aqueous solution at a microwave frequency of ~9 GHz and temperature of 77 K.[54] A change in the EPR spectrum from large anisotropic sextet splitting to isotropic sextet splitting on increasing Mn(II) concentration has also been observed in the apoenzyme alkaline phosphatase, indicating the formation of the Mn(H₂O)₆²⁺ complex.[48] The change accounts for the fact that Mn(II) binds one or more water molecules in the active sites of the enzymes[49] and then binds six water molecules in aqueous solution.[45] For the studied samples, thermo gravimetric analysis (TGA) showed that the samples of Mn(II)/SF contained an average of 5.2 ± 0.5% H₂O (data not shown), which is sufficient to allow the formation of the hexahydrated Mn(II) complex. In addition, the sextet splitting becomes evident as pH decreased from 7.5 to 5.2 in Fig. 3b, indicating that the Mn(II)/SF complex is somewhat replaced by the Mn(H₂O)₆²⁺ complex at the lower pH value.

Mn(II) Coordination in Mn(II)/SF Complex

The fact that the EPR spectrum of regenerated SF treated with the strong chelating agent EDTA still shows Mn(II) signals indicates a strong and selective coordination between Mn(II) and silk fibroin (Fig. 2). When the added Mn(II) content is less than 10.0 μg/g, Mn(II) ions interacted selectively first with silk fibroin and then with H₂O. In this connection, Lawrence et al.[41] investigated the Mn(II) EPR spectra of native 3,4-dihydroxyphenylacetate 2,3-dioxygenase as well as the complex of the enzyme with the substrate 3,4-dihydroxyphenylacetate (ES). The former showed a typical sextet splitting of Mn(II), while the latter exhibited distinct anisotropic features similar to the spectrum in Fig. 2c, indicating that Mn(II) ions were bound in a low symmetric environment.[41] Vetting and coworkers[50] studied the X-ray crystal structures of 3,4-dihydroxyphenylacetate 2,3-dioxygenase and the Mn(II) complex with the substrate. They found that the substrate 3,4-dihydroxyphenylacetate coordinated with the Mn(II), leading to a change of environment around Mn(II) from a high symmetry to a distorted one. Accordingly, the electronic symmetry of Mn(II) in Mn(II)/SF complex might also be lower compared with that in Mn(H₂O)₆²⁺, as indicated by the increase in D values we observed. Mantel et al.[51] studied the D values of several enzymes ranging from 200 to 4000 G. They found that the five-coordinated Mn(II) complexes had especially large D values (≥2000 G), while the six-coordinated Mn(II) enzymes had small D value (≤800 G). Therefore, our observation of D values smaller than 500 G for the Mn(II)/SF complex probably indicates that the Mn(II) is present in SF as a six-coordination complex.

Mn(II)’s Role in Silk Fibroin

Figure 6 shows that Mn(II) ions over the concentration range studied show no detectable effect on
The Role of Mn(II) in Silk Fibroin Based on EPR and NMR Spectroscopy

The role of Mn(II) in silk fibroin is discussed based on EPR and NMR spectroscopy. It is shown that Mn(II) ions, along with other ions such as Cu(II) and Fe(III), can induce the silk fibroin conformation transition from Silk I (helix-form) to Silk II (β-sheet form). The presence of Mn(II) ions at certain concentrations may facilitate the silk fibroin conformation transition from Silk I to Silk II; however, the evidence presented here suggests that Mn(II) ions do not. The lower β-sheet content and the reduced sensitivity to pH induced-conformation transition observed in presence of low concentrations of Mn(II) suggest that this ion may actually prevent the transition to Silk II. The Mn(II) ion effect which may selectively bind to specific amino acids in the silk fibroin tending to promote the silk fibroin conformation to Silk II, may be counteracted by an inhibitory effect of hydrated Mn(II) ions, Mn(H2O)62+ on the transition. In this connection, the Mn(II) content in the extruded silk fiber is much lower than that in the fibroin stored in the lumen of silk gland, suggesting that Mn(II) ions may be removed from the dope in the gland duct. The lower conformation transition in silk, Mn(II) ions appear to have a weak effect. Our results indicate that there are two types of Mn(II) complexes present in the silk fibroin: a six-coordinated Mn(II)/SF complex when the content of Mn(II) is small (less than 10.0 μg/g) and a Mn(H2O)62+ complex that predominates at higher concentrations.

**Possible Coordination Sites of Mn(II) in Silk Fibroin**

In silk fibroin, some amino acid residues with appropriate stereo space, affinity, and hydrophilicity could ligate the Mn(II) ions. Gaggelli et al. studied the interaction of the human prion PrP (106-126) sequence with Mn(II) ions, showing that a six-coordination octahedral or distorted octahedral Mn(II)-PrP complex was formed and that Gaggelli et al. also studied the binding affinities of transitional metals to the human prion protein. Their results demonstrated that Cu(II) ions bound in the N-terminal octapeptide-repeat segment had a low dissociation constant Kd (~10^-14 M), while Ni(II), Zn(II), and Mn(II) were bound more loosely with dissociation constants that were three or more orders higher. Thus proteins like silk fibroin readily capable of forming β-sheet-containing fibrils might be stabilized in a helix-form (Silk I state) in solution by Mn(II) ions holding H2O molecules in the first water shell and might be prevented from the complete dehydration during the SF conformation transition.

In the natural extrusion of silkworm, Cu^{2+}, Fe^{3+}, Ca^{2+}, and K^+ ions at certain concentrations may facilitate the silk fibroin conformation transition from Silk I to Silk II; however, the evidence presented here suggests that Mn(II) ions do not. The lower β-sheet content and the reduced sensitivity to pH induced-conformation transition observed in presence of low concentrations of Mn(II) suggest that this ion may actually prevent the transition to Silk II. The Mn(II) ion effect which may selectively bind to specific amino acids in the silk fibroin tending to promote the silk fibroin conformation to Silk II, may be counteracted by an inhibitory effect of hydrated Mn(II) ions, Mn(H2O)62+ on the transition. In this connection, the Mn(II) content in the extruded silk fiber is much lower than that in the fibroin stored in the lumen of silk gland, suggesting that Mn(II) ions may be removed from the dope in the gland duct. The lower conformation transition in silk, Mn(II) ions appear to have a weak effect. Our results indicate that there are two types of Mn(II) complexes present in the silk fibroin: a six-coordinated Mn(II)/SF complex when the content of Mn(II) is small (less than 10.0 μg/g) and a Mn(H2O)62+ complex that predominates at higher concentrations.

**CONCLUSIONS**

Although H^+, Cu^{2+}, Fe^{3+}, Ca^{2+}, and K^+ ions appear to have a large effect on the conformation transition in silk, Mn(II) ions appear to have a weak effect. Our results indicate that there are two types of Mn(II) complexes present in the silk fibroin: a six-coordinated Mn(II)/SF complex when the content of Mn(II) is small (less than 10.0 μg/g) and a Mn(H2O)62+ complex that predominates at higher concentrations.
Mn concentrations. The six-coordinated complex may form on Asp, Glu, and His residues in the hydrophilic spacers promoting the Silk II conformation. In contrast, the Mn\((\text{H}_2\text{O})_{\text{6}}^{2+}\) complex might stabilize the first water shell, thereby tending to maintain the silk fibroin Silk I conformation, therefore leading to the pH change, hardly influencing the conformation transition of the silk fibroin. Mn(II) ions, existing in the silk gland,[27] may play a role in maintaining the appropriate balance of the secondary structure components including helix-form and \(\beta\)-form to keep the silk fibroin stable in liquid state in the secretory pathway. Finally it is interesting to note that Mn(II) also has only a weak effect on proteins like \(\alpha\)-synuclein,[52] prions[53–54,60], and non-pathological variants of ataxin-3.61 The similarities in behavior between (a) fibroin and (b) \(\alpha\)-synuclein and prions suggest that the former may make a useful model system for studying the folding and assembly of \(\beta\)-sheet proteins with relevance to the latter.

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**REFERENCES**

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