Tightly winding structure of sequential model peptide for repeated helical region in *Samia cynthia ricini* silk fibroin studied with solid-state NMR

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(RECEIVED November 7, 2002; FINAL REVISION December 18, 2002; ACCEPTED December 18, 2002)

Abstract

There are many kinds of silks from silkworms and spiders with different structures and properties, and thus, silks are suitable to study the structure-property relationship of fibrous proteins. Silk fibroin from a wild silkworm, *Samia cynthia ricini*, mainly consists of the repeated similar sequences by about 100 times where there are alternative appearances of the polyalanine (Ala)_{12–13} region and the Gly-rich region. In this paper, a sequential model peptide, GGAGGGYGGDGG(A)₁₂GGAGDGYGAG, which is a typical sequence of the silk fibroin, was synthesized, and the atomic-level conformations of Gly residues at the N- and C-terminal ends of the polyalanine region were determined as well as that of the central Ala residue using ¹³C 2D spin diffusion solid-state nuclear magnetic resonance (NMR) under off-magic angle spinning. In the model peptide with α -helical conformation, the torsion angle of the central Ala residue, the 19th Ala, was determined to be $(\phi, \psi) = (-60^\circ, -50^\circ)$, which was a typical α -helical structure, but the torsion angles of two Gly residues, the 12th and 25th Gly residues, which are located at the N- and C-terminal ends of the polyalanine region, were determined to be $(\phi, \psi) = (-70^\circ, -30^\circ)$ and $(\phi, \psi) = (-70^\circ, -20^\circ)$, respectively. Thus, it was observed that the turns at both ends of polyalanine with α -helix conformation in the model peptide are tightly wound.

Keywords: Structure of *Samia cynthia ricini* silk fibroin; 2D spin diffusion NMR under off-magic angle spinning; α -helix of polyalanine region; determination of torsion angles

Silks are a wide class of fibrous proteins with properties that intrigue scientists ranging from structural engineers to polymer chemists, physicists, and biomedical researchers (Gosline et al. 1999). There are many kinds of silks from silkworms and spiders with different structures and properties, and thus, silks are suitable to study the structure-property relationship of fibrous proteins. *Samia cynthia ricini* is a wild silkworm and the amino-acid composition of the silk fibroin is different from that of the silk fibroin from the domesticated silkworm, *Bombyx mori*. The sum of Gly and Ala residues is 82%, which is similar to *B. mori* silk fibroin

(73%), but the relative composition of Ala and Gly is reversed (Asakura and Murakami 1985; Zhou et al. 2000); the proportion of Gly residues is greater in *B. mori* silk fibroin, while the content of the Ala residues is greater in *S. c. ricini* silk fibroin. *S. c. ricini* silk fibroin mainly consists of about 100 times repeated similar sequences with alternative appearances of the (Ala)_{12–13} region and the Gly-rich region (Asakura et al. 1999a). Here, the polyalanine sequences such as (Ala)_{12–13} or (Ala)₁₂ are defined as poly-Ala in this paper. This primary structure is similar to the structure of dragline (major ampullate) silk from spider but the length of poly-Ala is shorter: (Ala)_{5–6} (Xu and Lewis 1990; Hinman and Lewis 1992).

From ¹³C solution nuclear magnetic resonance (NMR) studies of *S. c. ricini* silk fibroin, it is evaluated that ~90% of the Ala residues form a poly-Ala region with α -helical structure, and fast exchange in the NMR time scale between

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Article and publication are at <http://www.proteinscience.org/cgi/doi/10.1110/ps.0239203>.

helix and coil forms in the poly-Ala region has been observed with increasing temperature (Asakura and Murakami 1985; Asakura et al. 1988). On the other hand, the Gly carbonyl peaks, which were split into the primary structure, did not change during the transition, suggesting that the solution structure of the Gly-rich region is mainly in random-coil state. However, we recently found that the under-scored Gly residue in the Gly-Gly-(Ala)₁₂₋₁₃ sequence of *S. c. ricini* silk fibroin was incorporated into the helix structure (Nakazawa and Asakura 2002). A more detailed structure of both poly-Ala and Gly-rich regions of the silk fibroin before spinning is required to clarify the fiber formation mechanism.

In general, X-ray diffraction is one of the most practical methods to obtain the detailed structure for single-crystal samples, and this method has provided the molecular structures of numerous proteins in atomic level. However, it is necessary to prepare a single crystal of proper size for the purpose and thus X-ray diffraction is not suitable to determine the structure of amorphous and un-oriented samples in the solid state. On the other hand, the chemical shift interaction and dipolar interaction, which are mainly observed in solid state NMR for half-spin nuclei, have been used for the determination of solid-state structures. Especially, two dimensional (2D), spin-diffusion, solid-state NMR is a powerful method to obtain the relative orientation of two chemical shift tensors of ¹³C-labeled sites in the local molecular framework. When two carbonyl carbons of the neighboring residues in peptide were ¹³C labeled, the torsion angles, ϕ and ψ , of the amino-acid residue could be determined. Actually, the 2D spin-diffusion NMR under off-magic angle spinning has been used to determine the torsion angles of Ala and Gly residues of the peptide, (Ala-Gly)₁₅ as a model peptide of the crystalline domain of *B. mori* silk fibroin and a new structural model for *B. mori* silk fibroin before spinning in the solid state has been proposed (Asakura et al. 2001).

In this paper, solid state NMR, especially ¹³C, 2D, spin-diffusion, solid-state NMR under off-magic angle spinning, was used for the determination of the torsion angles, ϕ and ψ of the Gly residues at the N- and C- terminal ends of the poly-Ala region along with that of the central Ala residue in the sequential model peptide, GGAGGGYGGDGG(A)₁₂-GGAGDGYGAG, which is a typical sequence containing the poly-Ala region of the silk fibroin.

Results and discussion

¹³C CP/MAS NMR spectra of *S. c. ricini* silk fibroin film and GGAGGGYGGDGG(A)₁₂-GGAGDGYGAG

Figure 1 shows ¹³C CP/MAS (cross polarization/magic angle spinning) NMR spectra of the model peptide of *S. c.*

Figure 1. ¹³C CP/MAS nuclear magnetic resonance spectra of the model peptide of *S. c. ricini* silk fibroin, GGAGGGYGGDGG(A)₁₂GGAGDGYGAG, after dialysis of the 9M LiBr solution against water (A) and after TFA treatment of the same peptide (B), together with *S. c. ricini* silk fibroin film (C).

ricini silk fibroin, GGAGGGYGGDGG(A)₁₂GGAGDGYGAG after dialysis of the 9M LiBr solution against water (Fig. 1A) and after trifluoroacetic acid (TFA) treatment (Fig. 1B), together with the film of *S. c. ricini* silk fibroin prepared from the silk fibroin stored in the silk gland (Fig. 1C). Here “TFA treatment” means that the peptide was dissolved in TFA and then precipitated in diethylether. The chemical shifts of the spectrum (Fig. 1A), 20.2 ppm for Ala Cβ, 48.7 ppm for Ala Cα, and 171.9 ppm for Ala carbonyl carbons, clearly show that the conformation is β-sheet structure (Asakura et al. 1999b). On the other hand, the model peptide after TFA treatment takes α-helical conformation judging from the chemical shifts of the Ala residues in the spectrum (Fig. 1B), 15.3 ppm for Ala Cβ, 52.3 ppm for Ala Cα, and 175.9 ppm for Ala carbonyl carbons. The latter chemical shift values are the same as those of *S. c. ricini* silk fibroin film (Fig. 1C). Thus, it is concluded that both conformations of the silk fibroin film and the sequential model peptide after TFA treatment are α-helix.

Determination of the torsion angle of the 19th Ala residue in GGAGGGYGGDGG(A)₅[1-¹³C]A₁₈[1-¹³C]A₁₉(A)₅GGAGDGYGAG

In Figure 2, the 2D spin-diffusion NMR spectra (only the carbonyl region is expanded) of the model peptide, GGAGGGYGGDGG(A)₅[1-¹³C]A₁₈[1-¹³C]A₁₉(A)₅GGAGDGYGAG, after TFA treatment (Fig. 2A) and after dialysis of the 9M LiBr solution against water (Fig. 2B) are shown, together with the calculated spectra of the Ala residue with the torsion angles $(\phi, \psi) = (-60^\circ, -50^\circ)$, (Fig. 2C), and $(\phi, \psi) = (-150^\circ, 150^\circ)$ (Fig. 2D). For the calculation of the 2D spin-diffusion NMR spectra, it is necessary to obtain the principal values of the chemical shift tensors of the Ala carbonyl carbon atoms and therefore these values were determined using slow MAS experiments described elsewhere (Asakura et al. 2001).

The remarkable change was observed between the spectra (Fig. 2A,B); the spectrum (Fig. 2A) has strong off-diagonal intensity compared with the spectrum (Fig. 2B), which emphasizes the diagonal intensity. To examine the dependence of the spectral pattern on the torsion angle of the Ala residue, the spectra were calculated as a function of the torsion

angles ϕ and ψ of the Ala residue for each 30° in the region of $-180^\circ < \phi < 0^\circ$ and $-180^\circ < \psi < 180^\circ$ as shown in Figure 3. The calculated spectral patterns change remarkably depending on the ϕ and ψ values, suggesting that the torsion angles can be determined with high precision. Through more detailed spectral calculations in typical α -helical or β -sheet regions of a Ramachandran map, the torsion angles (ϕ, ψ) of the Ala₁₉ residue at the center of the poly-Ala region could be determined to be $(\phi, \psi) = (-60^\circ, -50^\circ)$ and $(-150^\circ, 150^\circ)$, respectively, as shown in Figure 2, C and D. The error in the angle determination was $\pm 5^\circ$ and $\pm 10^\circ$, respectively. The torsion angles of the Ala₁₉ residue in the model peptide with α -helical form determined here are essentially the same as the reported angles, $(\phi, \psi) = (-60^\circ, -45^\circ)$ of the Ala residue for [1-¹³C]Ala-silk fibroin film from *S. c. ricini* using the 2D DOQSY NMR measurements (van Beek et al. 2000).

Determination of the torsion angles of the 12th Gly residue in GGAGGGYGGD[1-¹³C]G₁₁[1-¹³C]G₁₂(A)₁₂GGAGDGYGAG and the 25th Gly residue in GGAGGGYGGDGG(A)₁₁[1-¹³C]A₂₄[1-¹³C]G₂₅GAGDGYGAG.

Recently, the solution ¹³C NMR has been applied to analysis of the conformation of Gly residues, which are located closely at the poly-Ala region in [1-¹³C]Gly- *S. c. ricini* silk fibroin extracted from the silkgland (Nakazawa and Asakura 2002). It was observed that some of such Gly residues were incorporated into the α -helix conformation of the poly-Ala region. Therefore, 2D spin-diffusion NMR was also applied to determination of the torsion angles of these Gly residues of the peptide, GGAGGGYGGDGG(A)₁₂GAGDGYGAG as a model of the silk fibroin structure before spinning.

As shown in Figure 4, 2D spin diffusion NMR spectra were observed for two kinds of the model peptides, GGAGGGYGGD[1-¹³C]G₁₁[1-¹³C]G₁₂(A)₁₂GGAGDGYGAG (Fig. 4A) and GGAGGGYGGDGG(A)₁₁[1-¹³C]A₂₄[1-¹³C]G₂₅GAGDGYGAG (Fig. 4B), respectively, after TFA treatment. The spectral patterns are slightly different from that of the 19th Ala residue in GGAGGGYGGDGG(A)₅[1-¹³C]A₁₈[1-¹³C]A₁₉(A)₅GGAGDGYGAG (Fig. 2A). To clarify the origin of the difference, the 2D spin-diffusion NMR spectra were calculated as a function of torsion angles ϕ and ψ of the Gly residue for each 30° in the region of $-180^\circ < \phi < 0^\circ$ and $-180^\circ < \psi < 180^\circ$ (data not shown). However, the Ramachandran maps of the calculated spin-diffusion patterns were similar to Figure 3. Thus, the difference between Figures 2A and 4A or 4B is a result of the difference in the torsion angles rather than the difference between Gly and Ala residues. There are four candidates that satisfy the observed spectra, Figure 4, A and B;

Figure 2. Two-dimensional spin-diffusion, ¹³C, solid-state nuclear magnetic resonance spectra (only the carbonyl region is expanded) of the model peptide, GGAGGGYGGDGG(A)₅[1-¹³C]A₁₈[1-¹³C]A₁₉(A)₅GGAGDGYGAG, after TFA treatment (A), and after dialysis of the 9-M LiBr solution against water, (B) are shown together with the calculated spectra of the Ala residue with the torsion angles $(\phi, \psi) = (-60^\circ, -50^\circ)$ (C), and $(\phi, \psi) = (-150^\circ, 150^\circ)$ (D).

Figure 3. Ramachandran map of the two-dimensional spin-diffusion nuclear magnetic resonance spectral patterns calculated as a function of torsion angles ϕ and ψ of the Ala residue for each 30° in the region of $-180^\circ < \phi < 0^\circ$ and $-180^\circ < \psi < 180^\circ$.

Figure 4. Two-dimensional spin diffusion nuclear magnetic resonance spectra of two kinds of the model peptides: (A) GGAGGGYGGD [1-¹³C]G₁₁[1-¹³C]G₁₂(A)₁₂GGAGDGYGAG and (B) GGAGGGYGGDGG(A)₁₁[1-¹³C]A₂₄[1-¹³C]G₂₅GAGDGYGAG. The calculated spectra of the Gly residues with the torsion angles (C) (ϕ, ψ) = ($-70^\circ, -30^\circ$) and (D) (ϕ, ψ) = ($-70^\circ, -20^\circ$), respectively, are also shown.

(ϕ, ψ) = ($-60^\circ, -30^\circ$), ($60^\circ, 30^\circ$), ($-120^\circ, 90^\circ$) and ($120^\circ, -90^\circ$). It is necessary to select the torsion angles of the Gly residues by considering further structural information. In our previous papers (Asakura et al. 1999b; Ashida et al. 2002), the Gly C α chemical shift was used to select possible torsion angles of Gly residues. By using the observed Gly C α chemical shift value, 44 ppm, only the torsion angle, (ϕ, ψ) = ($-60^\circ, -30^\circ$) was selected among the four candidates. Then, in order to determine the torsion angle more precisely, the root-mean-squared deviations (RMSD), χ^2 , between the observed and calculated 2D spin-diffusion NMR spectra, were calculated as a function of the torsion angles, ϕ and ψ of the Gly residue in the α -helical region such as (ϕ, ψ) = ($-50^\circ \sim -90^\circ, -10^\circ \sim -50^\circ$) (data not shown) (Ashida et al. 2002). Finally, the torsion angles of the Gly₁₂ and Gly₂₅ residues were determined to be (ϕ, ψ) = ($-70^\circ, -30^\circ$) and (ϕ, ψ) = ($-70^\circ, -20^\circ$), respectively, as shown in Figure 4. The error in the angle determination was $\pm 10^\circ$. Thus, the torsion angles of the Gly residues at both N- and C-terminals of the poly-Ala region were slightly different from the torsion angle of the Ala residue at the center of the poly-Ala region in the model system before spinning. It has been observed that the final turn at the ends of the α -helix

is tightly wound because of the appearance of the conformation such as 3_{10} helix; (ϕ, ψ) = ($-49^\circ, -26^\circ$) (Creighton 1993). The ψ values of the Gly residues, -30° or -20° , determined here are the same as that of 3_{10} helix, but the ϕ value, -70° seems to be significantly different from -49° of 3_{10} helix. Thus, it is likely that the N- and C-terminal ends of Poly-Ala with typical α -helix conformation in *S. c. ricini* silk fibroin have tightly wound conformation.

After dialysis of the 9M LiBr solution of the model peptide against water, the peptide was precipitated. The 2D spin-diffusion NMR spectra of the peptides, GGAGGGYGGDGG(A)₁₁[1-¹³C]G₁₁[1-¹³C]G₁₂(A)₁₂GGAGDGYGAG and GGAGGGYGGDGG(A)₁₁[1-¹³C]A₂₄[1-¹³C]G₂₅GAGDGYGAG show typical patterns of β -sheet structure as shown in Figure 2B. Thus, the Gly residues at both N- and C-terminals of the poly-Ala region were incorporated into β -sheet structure of the poly-Ala region. In our previous paper (Asakura et al. 1999a), the structure of ¹³C- and ¹⁵N-labeled *S. c. ricini* silk fibers after spinning was determined with ¹³C and ¹⁵N CP NMR for the blocks of the oriented silk fibers by changing the angles of the oriented silk fiber axis and the magnetic field. 65% of Gly residues in the silk fiber was in the β -sheet structure. Thus, the incorporation of the Gly residues at the terminal ends into β -sheet structure of the poly-Ala region that was observed for the model peptide is in agreement with the previous structural study on *S. c. ricini* silk fiber.

Materials and methods

The model peptides of *S. c. ricini* silk fibroin containing the poly-Ala region were synthesized by the solid phase method.

GGAGGGYGGDGG(A)₁₂GGAGDGYGAG, GGAGGGYGGDGG(A)₅[1-¹³C]A₁₈[1-¹³C]A₁₉(A)₅GGAGDGYGAG, GGAGGGYGGD[1-¹³C]G₁₁[1-¹³C]G₁₂(A)₁₂GGAGDGYGAG, GGAGGGYGGDGG(A)₁₁[1-¹³C]A₂₄[1-¹³C]G₂₅GAGDGYGAG.

After synthesis, "TFA treatment" was tried in order to transform the structure of the peptide into the structure of the silk fibroin before spinning. Namely, the model peptides were dissolved in TFA and then precipitated in diethylether. The peptides were dried under vacuum at room temperature. The 2D spin-diffusion NMR spectra were obtained with Varian Unity INOVA 400 NMR spectrometer and 7-mm ϕ , Jakobsen-type, double-tuned MAS probe at off-magic angle condition ($\theta_m = 7^\circ$) and sample spinning of 6 kHz at room temperature. Therefore, the scaling factor of the 2D spin-diffusion spectra is $1/2 (3 \cos^2 [\theta_m - 7^\circ] - 1) = 0.198$. Other NMR experimental conditions are the same as those in our previous paper (Asakura et al. 2001).

Acknowledgments

T.A. acknowledges support from the Program for Promotion of Basic Research Activities for Innovative Biosciences, Japan.

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References

- Asakura, T., and Murakami, T. 1985. NMR of silk fibroin. 4. Temperature- and urea-induced helix-coil transition of the $-(\text{Ala})_n-$ sequence in *philosamia cynthia ricini* silk fibroin protein monitored by ^{13}C NMR spectroscopy. *Macromolecules* **18**: 2614–2619.
- Asakura, T., Kashiba, H., and Yoshimizu, H. 1988. NMR of silk fibroin. 8. ^{13}C NMR analysis of the conformation and the conformational transition of *Philosamia cynthia ricini* silk fibroin protein on the basis of Bixon-Scheraga-Lifson theory. *Macromolecules* **21**: 644–648.
- Asakura, T., Ito, T., Okudaira, M., and Kameda, T. 1999a. Structure of alanine and glycine residues of *Samia cynthia ricini* silk fibers studied with solid-state ^{15}N and ^{13}C NMR. *Macromolecules* **32**: 4940–4946.
- Asakura, T., Iwadata, M., Demura, M., and Williamson, M.P. 1999b. Structural analysis of silk with ^{13}C NMR chemical shift contour plots. *Int. J. Biol. Macromol.* **24**: 167–171.
- Asakura, T., Ashida, J., Yamane, T., Kameda, T., Nakazawa, Y., Ohgo, K., and Komatsu, K. 2001. A repeated β -turn structure in poly(Ala-Gly) as a model for silk I of *Bombyx mori* silk fibroin studied with two-dimensional spin-diffusion NMR under off magic angle spinning and rotational echo double resonance. *J. Mol. Biol.* **306**: 291–305.
- Ashida, J., Ohgo, K., Komatsu, K., Kubota, A., and Asakura, T. 2002. Determination of the torsion angles of alanine and glycine residues of model compounds of spider silk $(\text{AGG})_{10}$ using solid-state NMR methods. *J. Biomol. NMR* (in press).
- Creighton, T.E., 1993. *Proteins. Structures and molecular properties*. 2nd ed., p. 187–188. W.H. Freeman and Company, NY.
- Gosline, J.M., Guerette, P.A., Ortlepp, C.S., and Savage, K.N. 1999. The mechanical design of spider silks: From fibroin sequence to mechanical function. *J. Exp. Biol.* **202**: 3295–3303.
- Hinman, B.M. and Lewis, R.V. 1992. Isolation of a clone encoding a second dragline silk fibroin. *J. Biol. Chem.* **267**: 19320–19324.
- Nakazawa, Y. and Asakura, T. 2002. Heterogeneous exchange behavior of *Samia cynthia ricini* silk fibroin during helix-coil transition studied with ^{13}C NMR. *FEBS Lett.* **529**: 188–192.
- van Beek, J.D., Beaulieu, L., Schafer, H., Demura, M., Asakura, T., and Meier, B.H. 2000. Solid-state NMR determination of the secondary structure of *Samia cynthia ricini* silk. *Nature* **405**: 1077–1079.
- Xu, M., and Lewis, R.V. 1990. Structure of a protein superfiber: Spider dragline silk. *Proc. Natl. Acad. Sci.* **87**: 7120–7124.
- Zhou, C., Confalonieri, F., Medina, N., Zivanovic, Y., Esnault, C., Yang, T., Jacquet, M., Janin, J., Dugué, M., Perasso, R., et al. 2000. Fine organization of *Bombyx mori* fibroin heavy chain gene. *Nucleic Acids Res.* **28**: 2413–2419.