FEBS Letters 529 (2002) 188–192 FEBS 26540

# Heterogeneous exchange behavior of *Samia cynthia ricini* silk fibroin during helix–coil transition studied with $^{13}C$ NMR

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Received 28 June 2002; revised 12 August 2002; accepted 13 August 2002

First published online 6 September 2002

Edited by Thomas L. James

**Abstract The structure and structural transition of the glycine residue adjacent to the N-terminal alanine residue of the poly(L-alanine), $(Ala)_{12–13}$, region in *Samia cynthia ricini* silk fibroin was studied using $^{13}C$ nuclear magnetic resonance (NMR). Most of the glycine carbonyl peaks in the $^{13}C$ solution NMR spectrum of [1-$^{13}C$]glycine-silk fibroin could be assigned to the primary structure from the comparison of the $^{13}C$ chemical shifts of seven glycine-containing tripeptides. The slow exchange between helix and coil forms in the NMR time scale was observed with increasing temperature exclusively for the underlined glycine residue in the Gly-Gly-$(Ala)_{12–13}$ sequence during fast helix–coil transition of the $(Ala)_{12–13}$ region.**
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*Key words:* Nuclear magnetic resonance; Helix–coil transition; Poly(L-alanine); $^{13}C$ isotope labeling of silk; *Samia cynthia ricini* silk fibroin

## 1. Introduction

There are many kinds of silks from silkworms and spiders with different structures and properties [1]. Thus, silks are suitable to study the structure–property relationship for molecular design of fibers with high strength and high elasticity [2,3]. *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 (71%), but the relative composition of Ala and Gly is reversed [4]. Namely, the proportion of Gly residues is greater in *B. mori* silk fibroin, while the content of Ala residues is greater in *S. c. ricini* silk fibroin. The primary structure of the silk fibroin from *S. c. ricini* has recently been determined by Yukuhiro et al. (personal communication), and it is very similar to the structure of silk fibroin from *Antheraea pernyi* [5,6]. Namely, the silks mainly consist of about 100 times repeated similar sequences with alternative appearance of the poly(L-alanine) (PLA), $(Ala)_{12–13}$, region and Gly-rich region. This is similar to the primary structure of dragline (major ampullate) silk from spider but the length of PLA is shorter: $(Ala)_{5–6}$ [7,8]. From $^{13}C$ and $^{15}N$ nuclear magnetic resonance (NMR) studies of *S. c. ricini* silk fibroin in aqueous solution, it is evaluated that about 70% of Ala residues form α-helices, while the conformation of the other Ala residues is random coil [9–13]. The fast exchange between helix and coil forms in the PLA region has been observed because of the gradual high field shift of the *single* main peak assigned to the carbonyl carbons of PLA during helix to coil transition with increasing temperature [11]. The helicity of each Ala residue in the PLA region was calculated theoretically according to the Bixon–Scheraga–Lifson theory [14] for the helix–coil transition of PLA including the hydrophobic side- chain interactions [12]. The change in the NMR spectrum of the Ala carbonyl region due to the temperature-induced helix–coil transition was interpreted in terms of the change in the statistical weight parameter $\omega$, where $\omega$ is related to the formation of an intramolecular hydrogen bond. On the other hand, the Gly carbonyl peaks which were split in the primary structure spectra did not change significantly during the transition although the signal–noise ratio of the Gly carbonyl carbon region was relatively poor [11]. This means that the Gly-rich region is basically in the random coil state. However, if the assignment of the Gly carbonyl carbon peak in the primary structure and better spectra are obtained, more detailed structural information of the Gly-rich region will be obtained. It is important to clarify the structure of such a Gly-rich region in understanding the properties such as elasticity of the silk [2,3].

In this paper, the assignment of the Gly carbonyl peaks in the $^{13}C$ NMR spectrum of [1-$^{13}C$]Gly-*S. c. ricini* silk fibroin was performed by comparison with the chemical shifts of the Gly carbonyl peaks of seven Gly-centered tripeptides which were selected from the primary structure (Yukuhiro et al., personal communication). Then the behavior of the Gly carbonyl peaks was monitored by $^{13}C$ NMR during the helix–coil transition that occurred in the PLA region with changing temperature.

## 2. Materials and methods

### 2.1. Materials

*S. c. ricini* larvae were reared in our laboratory [10,15]. 100 μl of 10% (w/v) [1-$^{13}C$]Gly (99.9% $^{13}C$ enrichment, Mastrace, Woburn, MA, USA) in aqueous solution was given by oral administration to 3–6-day-old 5th instar larvae for 4 days: two times, morning and evening, per day [10,11,16]. The middle silk glands were pulled out from an anesthetized 7-day-old 5th instar larva. The silk glands containing [1-$^{13}C$]Gly silk fibroins were then washed twice in ice-cold 1.15% (w/w) potassium chloride solution. The center of the silk gland was cut and the effluent was collected into an NMR sample tube of 8 mm diameter and 30 mm length immersed in a beaker of distilled

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*Abbreviations:* NMR, nuclear magnetic resonance; PLA, poly(L-alanine)

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PII: S0014-5793(02)03332-X

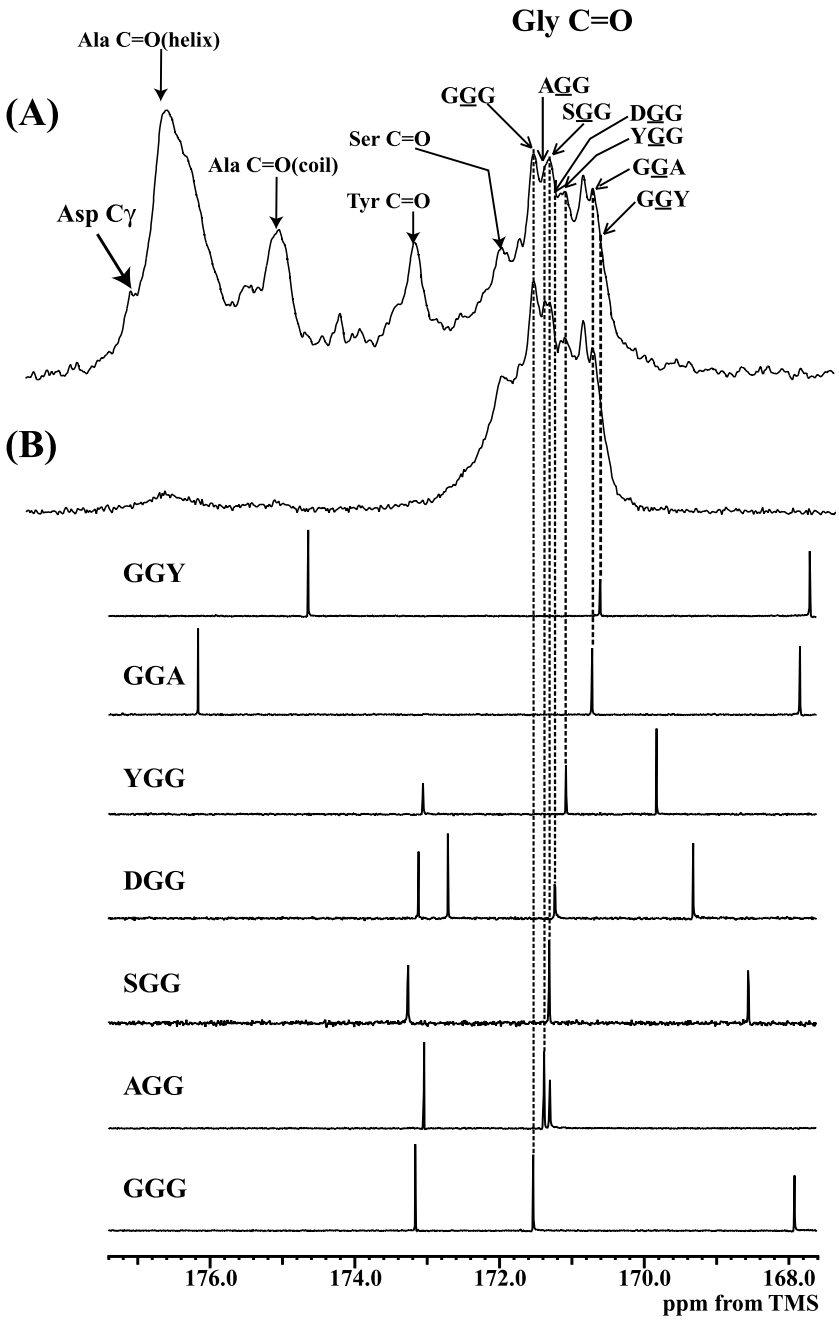


Fig. 1. $^{13}C$ NMR spectra of the carbonyl region of (A) non-labeled and (B) [1-$^{13}C$]Gly-labeled *S. c. ricini* silk fibroins along with the assignment. The spectra of seven Gly-centered tripeptides are also shown for assignment of the Gly carbonyl carbon peaks of the silk fibroin in the primary structure.

water to remove the soluble protein, sericin. Most of the sericin is removed after this treatment. The seven tripeptides (GGY, GGA, YGG, DGG, SGG, AGG and GGG) were synthesized by solid phase method with Pioneer Peptide synthesizer (Applied Biosystems, Foster City, CA, USA).

### 2.2. NMR measurement

The $^{13}C$ NMR measurements as a function of temperature were performed using a JEOL (Akishima, Tokyo, Japan) EX-400 NMR spectrometer, operating at 100 MHz for $^{13}C$. The experimental mode adopted was gated decoupling without nuclear Overhauser effect and tube size was 10 mm outer diameter. The number of acquisitions was 2000 and pulse delay was 5 s. The $^{13}C$ chemical shifts were calibrated indirectly through the 1,4-dioxane peak observed at 66.5 ppm relative to tetramethylsilane. The intensity of each Gly carbonyl peak was calculated by Lorentzian deconvolution.

## 3. Results and discussion

### 3.1. Assignment of Gly carbonyl peaks in the $^{13}C$ NMR spectrum of S. c. ricini silk fibroin

The $^{13}C$ NMR (expanded carbonyl region) spectra of natural abundance (A) and [1-$^{13}C$]Gly-*S. c. ricini* silk fibroin (B) are shown in Fig. 1. High isotope labeling of the carbonyl carbon of Gly residues was attained and thus Gly carbonyl peaks are clearly observed in Fig. 1B. The splitting of the peak reflects mainly the primary structure [11]. In order to assign each peak, Gly-centered tripeptides selected from the primary structure (Yukuhiro et al., personal communication) were synthesized. Except for the tripeptide DGG,

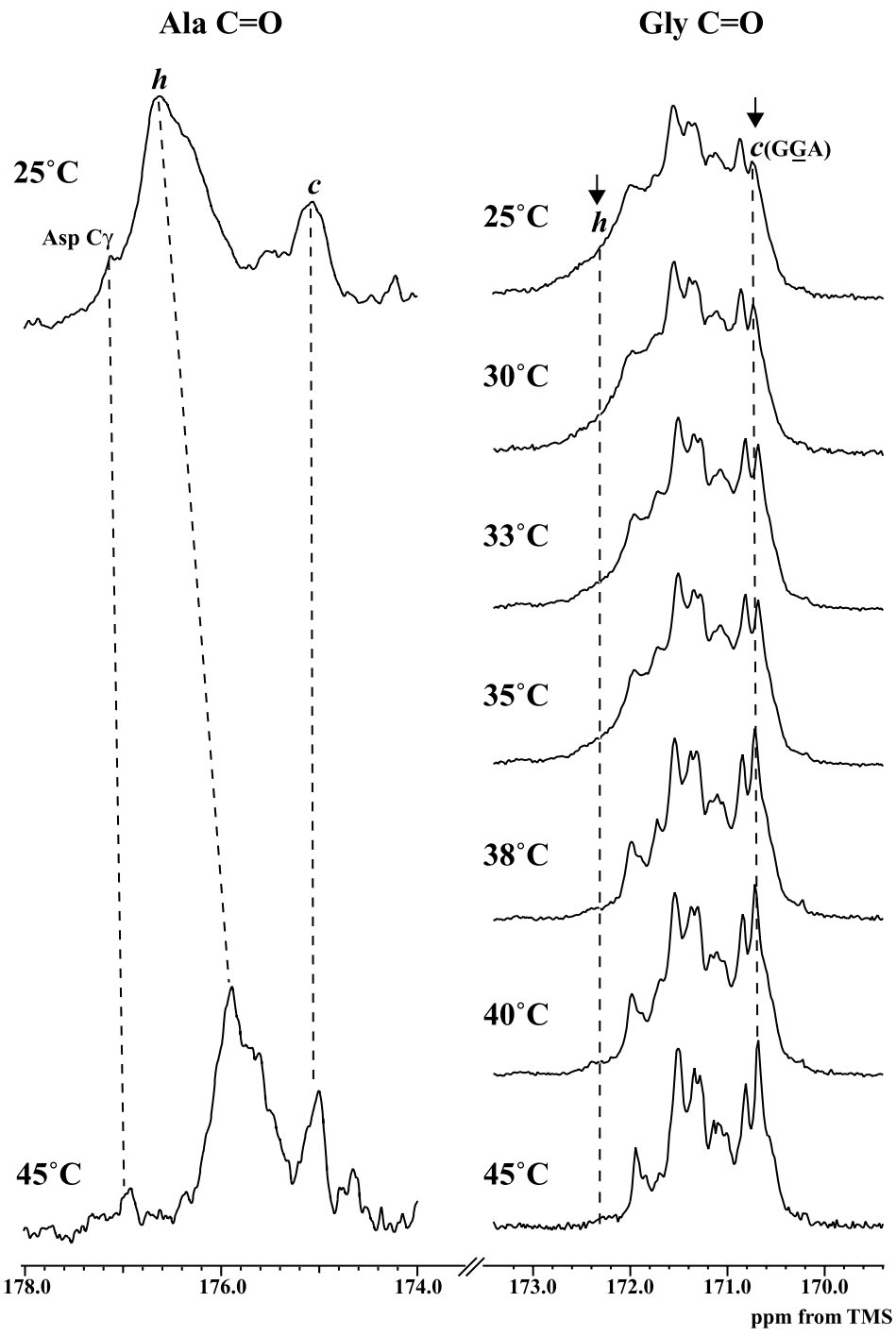


Fig. 2. $^{13}C$ NMR spectra of the Ala carbonyl region of non-labeled *S. c. ricini* silk fibroin at 25°C and 45°C are shown on the left side. On the right side, $^{13}C$ NMR spectra of the Gly carbonyl region of [1-$^{13}C$]Gly-labeled *S. c. ricini* silk fibroin are also shown as a function of temperature.

which has one carbonyl carbon at the side chain, three carbonyl peaks were observed. The carbonyl peaks of the N-terminal and C-terminal residues are easily assigned and thus the central peaks are assigned to the Gly residue at the center of the tripeptides. By comparing the peak position, most of the Gly carbonyl peaks of *S. c. ricini* silk fibroin could be assigned in the primary structure as shown in Fig. 1A.

### 3.2. Temperature dependence of $^{13}C$ NMR spectra of [1-$^{13}C$]Gly-S. c. ricini silk fibroin

The carbonyl carbon spectra of *S. c. ricini* silk fibroin were observed as a function of temperature from 25°C to 45°C. As shown on the left side of Fig. 2, the main peak of Ala carbonyl carbons of natural abundance *S. c. ricini* silk fibroin shifts upfield with increasing temperature, suggesting fast exchange with respect to the chemical shift between helix and coil con-

formations in the PLA region, as reported previously [11,12]. The carbonyl carbon peak of the Ala residues in the random coil form, (*c*), and the carboxyl carbon peak of the side chain of Asp residue do not shift. Each peak of the Gly residue in [1-$^{13}$C]Gly-*S. c. ricini* silk fibroin reduced the line width due to an increase in molecular motion of the silk fibroin (right side of Fig. 2).

Greater change was observed in the peak intensities rather than the chemical shift. In the spectrum of 25°C, it is noted that there is a broad peak at 172.3 ppm. The carbonyl carbon chemical shifts of different Gly helix peaks have been reported by Ando et al. using $^{13}$C solid state NMR (the values for $3_1$-helix, α-helix and ω-helix are 171.8 ppm, 172.1 ppm and 171.1 ppm respectively) [17,18]. The chemical shift for α-helix conformation is in good agreement with the value of the resonance (***h***) in the Gly carbonyl region of Fig. 2 and thus, (***h***) can be assigned to the Gly carbonyl carbon in the α-helix conformation. This resonance was broader than other Gly carbonyl peaks, indicating that the motion of such a Gly residue is restricted in ordered structures such as α-helix. With increasing temperature, the intensity of the resonance, (***h***), gradually decreased and conversely, the underlined Gly peak, (*c*), of the GGA sequence gradually increased. Other Gly carbonyl peaks did not change. Thus, the conformation of the Gly residue adjacent to the N-terminal Ala residue of the PLA region changed to random coil during the conformational change from helix to random coil of the PLA region with increasing temperature. From previous NMR experiments, changing the observed temperature from −5°C to 50°C [11,12], the helix–coil transition already occurred at 25°C. We could assume 100% helix at −5°C and 0°C because the helix content evaluated from both the chemical shifts and relative intensities of the Ala peak, (***h***), was in agreement with the helix content determined from circular dichroism and there was no shift between these two temperatures [11]. By using the chemical shift value of 100% helix reported previously and the amino acid composition of Ala in the silk fibroin [4], the helix content at 25°C to 45°C was calculated. Fig. 3 shows the change in the content of helix of the PLA region (right side scale): a decrease from 26% (at 25°C) to 10% (at 45°C). The helix and coil contents of the Gly residue adjacent to the N-terminal alanine residue of the PLA region were also calculated from the relative intensities of the helix and coil peaks, and the amino acid composition of Gly in the silk fibroin. The change in the content is shown in Fig. 3 (left side scale). A decrease from approximately 6% (at 25°C) to 1% (at 45°C) in the helix content and an increase from approximately 2% (at 25°C) to 5% (at 45°C) in the random coil content were evaluated. The change is slightly larger between 35°C and 38°C.

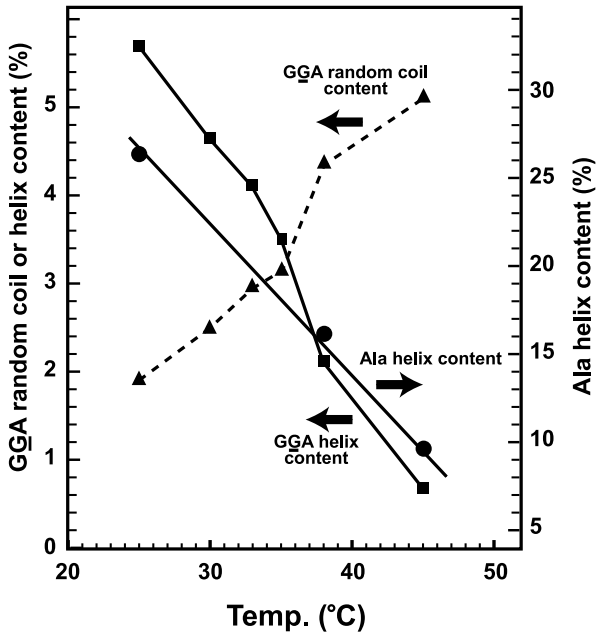


Fig. 3. Change in the helix content of the PLA region (●: right side scale) calculated from both the $^{13}$C chemical shift of the Ala residues and the amino acid composition of Ala in *S. c. ricini* silk fibroin. The helix (■: left side scale) and coil (▲: left side scale) contents of the Gly residue adjacent to the PLA region were calculated from the relative intensities of the helix and coil peaks by Lorentzian deconvolution, and the amino acid composition of Gly in the silk fibroin.

### 3.3. Heterogeneous helix–coil exchange in S. c. ricini silk fibroin

The helix–coil exchange behavior of the silk fibroin chain during helix–coil transition is interesting. The difference in the chemical shift of Ala residues in the PLA region between helix and coil forms has been reported to be 2.10 ppm [11]. From the chemical shift difference, $\delta_{hc}$, between the two peaks, 210 Hz, the exchange was calculated to be faster than 466 Hz ($(\pi/\sqrt{2})\times\delta_{hc}$) [19]. Contrary to the case [19], the chemical shift difference between helix and coil forms is temperature-independent for the Gly residue adjacent to the PLA region (GGA sequence). The chemical shift difference was 1.55 ppm, which means that the exchange is slower than 344 Hz. Thus, a difference in the exchange rate was observed between the PLA region and the adjacent Gly residue. In the PLA region, the helix–coil exchange rate is speculated to be slower towards the N-terminal end. For other Gly residues, the conformation is considered to be random coil because there was no chemical shift change during the helix–coil transition of the PLA region. As reported previously [11], the $^{13}$C spin-lattice relaxation time of the Gly C$^{\alpha}$ peak of the silk fibroin observed at 25°C was 0.09 s, indicating very fast motion of the order of $10^{-10}$ s as the averaged correlation time in the random coil forms [20].

We are now trying to determine the torsion angles of the Gly residues adjacent to the PLA region together with those of Ala residues in the region using several $^{13}$C and $^{15}$N isotope-labeled model peptides of *S. c. ricini* silk fibroin in the solid state with $^{13}$C spin-diffusion NMR and rotational echo double resonance techniques [21,22]. Preliminary data indicate that the N-terminal Ala residue and the Gly residue adjacent to the N-terminal residue in the PLA region are wound more strongly than a typical α-helix of central Ala residues in the PLA region. These results seem in agreement with the position-dependent exchange rate during the helix–coil transition of *S. c. ricini* silk fibroin observed in this paper. These dynamic characteristics seem to be similar to those for the case of the helix–coil transition of *A. pernyi* [23].

*Acknowledgements:* T.A. acknowledges support from the Bio-oriented Technology Research Advancement Institution.

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