NMR of Silk Fibroin. 9. Sequence and Conformation Analyses of the Silk Fibroins from *Bombyx mori* and *Philosamia cynthia ricini* by ^{15}N NMR Spectroscopy

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ABSTRACT: ^{15}N NMR spectra of the silk fibroin proteins from *Bombyx mori* and *Philosamia cynthia ricini* are reported. The silk fibroins were labeled biosynthetically with [^{15}N]glycine or [^{15}N]alanine. In the glycine resonance region, there are roughly three peaks for *B. mori* silk fibroin and four peaks for *P. c. ricini* silk fibroin. These peak splittings reflect the amino acid sequences in the main chain. There are two peaks in the alanine resonance region of the *P. c. ricini* silk fibroin spectrum. The higher field peak is assigned to the sequence of only alanine residues, where fast interconversion between α-helix and random coil forms occurs, and the lower field peak to the random coil form. The latter peak coincides in position with the single alanine peak observed in the *B. mori* silk fibroin spectrum. The temperature-induced helix–coil transition occurring in the alanine sequence portion of *P. c. ricini* silk fibroin permits structural monitoring by the ^{15}N NMR method. The changes in the ^{15}N NMR spectra differ slightly from those observed by the ^{13}C NMR method. The values of the ^{15}N NMR relaxation parameters, i.e., spin–lattice relaxation time and nuclear Overhauser effect, determined for [^{15}N]Gly *B. mori* silk fibroin, indicate very fast segmental motion (ca. 5×10^{-10} s at 25 °C) of the chain.

Introduction

Silk is a fibrous protein having very desirable properties for textiles.[1] In addition, it has been shown that silk fibroin is also an excellent material for the immobilization of several kinds of enzymes.[2,3] Since these advantages of silk are essentially caused by its unique amino acid composition and by its primary and higher order structures, structural analysis has fundamental significance.

In our previous papers,[4-17] the conformational characterizations of the silk fibroins from *Bombyx mori* and *Philosamia cynthia ricini* have been studied in aqueous solution, in the middle silk gland of the living silkworm, and in the solid state by means of the ^{13}C NMR method including solid-state high-resolution ^{13}C CP/MAS NMR[4,8,9] and the spin-label ESR method.[16] The conformation of *B. mori* silk fibroin in aqueous solution is essentially a random coil.[5-7,12,15] On the other hand, two conformations, that is, random coil and α-helix, coexist in the aqueous solution of *P. c. ricini* silk fibroin. The latter α-helical form appears in the $-(Ala)_{22}-$ sequence in the chain.[5,10,17] These differences in the conformational characteristics of these two kinds of silk fibroins in aqueous solution are also retained in the solid state,[4,8] and thus these silks have different functional properties such as permeability, separation ability, enzyme immobilization, etc.

In this paper, ^{15}N NMR spectroscopy has been applied to the sequence and conformational analyses of *B. mori* and *P. c. ricini* silk fibroins. On the basis of the ^{15}N NMR chemical shift data for several copolypeptides in trifluoroacetic acid, Kricheldorf et al.[18] have proved that the wide chemical shift distribution arising from the sequence in the chain is a merit of the ^{15}N NMR approach for sequence analysis compared with ^{13}C and ^{1}H NMR. Also, ^{15}N nuclei are frequently located at the interaction sites of proteins; for example, amide nitrogens in the peptide are the key to maintaining the peptide backbone conformation by hydrogen bonding.[19]

Experimental Section

Materials. Cocoons from *B. mori* reared on an artificial diet in our laboratory were used (sample 1) for ^{15}N NMR observation after degumming.[6] [^{15}N]Gly cocoons from *B. mori* were prepared biosynthetically by feeding [^{15}N]glycine[7] (95% enrichment, Shoko Co., Ltd., Tokyo) in addition to providing an artificial diet to the silkworm during the fifth instar, 6-day-old larvae to the fifth instar 8-day-old (mature larvae).[20] The [^{15}N]Gly *B. mori* silk fibroin (sample 2) in aqueous solution was prepared from the [^{15}N]Gly cocoon after degumming.[7] The [^{15}N]Gly *B. mori* silk fibroin (sample 3) labeled for whole days during the fifth larval stage was also prepared. The [^{15}N]Ala *P. c. ricini* silk fibroin (sample 4) was obtained from the silk gland portion of *P. c. ricini* mature larvae that were reared on an artificial diet containing 10–20 mg of [^{15}N]alanine (95% enrichment, Shoko Co., Ltd., Tokyo). The [^{15}N]Gly *P. c. ricini* silk fibroin was prepared in a similar manner (sample 5). The ^{15}N labeling of these silk fibroin samples is summarized in Table I.

Table I
^{15}N Labeling of *B. mori* and *P. c. ricini* Silk Fibroins

sample	silk fibroin	labeling period
1	nonlabeled *B. mori*	
2	[^{15}N]Gly-labeled *B. mori*	5th instar, 6-day-old larvae–mature larvae
3	[^{15}N]Gly-labeled *B. mori*	5th instar, whole days
4	[^{15}N]Ala-labeled *P. c. ricini*	5th instar, 6-day-old larvae–mature larvae
5	[^{15}N]Gly-labeled *P. c. ricini*	5th instar, 6-day-old larvae–mature larvae

Measurement. Proton-decoupled ^{15}N NMR spectra were observed at 25 °C by using a JEOL FX-90Q NMR spectrometer operating at 9.08 MHz. For [^{15}N]Gly *B. mori* silk fibroin (sample 3), the ^{15}N spin–lattice relaxation time, T_1, was determined from a series of partially relaxed ^{15}N NMR spectra, and the ^{15}N nuclear Overhauser enhancement, NOE, was obtained from the difference in the peak area between the ^{1}H-decoupled and ^{1}H-gated decoupling spectra as described elsewhere.[6] The spin–spin coupling constant, $^{1}J_{NH}$, was obtained by the insensitive nuclei enhancement by polarization transfer (INEPT) NMR technique. The typical ^{15}N NMR experimental conditions were as follows: 8k data points; 2k–30k accumulations; frequency range 2 kHz; acquisition time plus pulse delay 2–10 s. The ^{15}N NMR chemical shift is represented in parts per million downfield from external $^{15}NH_4NO_3$.

Results and Discussion

***B. mori* Silk Fibroin.** Figure 1 shows the proton-decoupled ^{15}N NMR spectra, A–C, of *B. mori* silk fibroins corresponding to samples 1–3, respectively. The chemical shifts and the relative peak areas for spectrum A are summarized in Table II. The assignment of the peaks to the Ala, Tyr, Ser, and Gly residues in *B. mori* silk fibroin was readily performed from both the relative peak area

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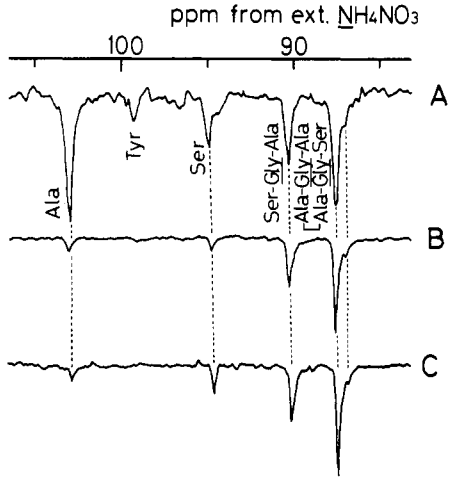

Figure 1. ^{15}N NMR spectra of *B. mori* silk fibroin in aqueous solutions: A, sample 1; B, sample 2; C, sample 3. Samples 2 and 3 are [^{15}N]Gly *B. mori* silk fibroins (see Table I).

Table II
^{15}N NMR Chemical Shift, Relative Peak Area, and Assignment of the Amide Nitrogen of *B. mori* and *P. c. ricini* Silk Fibroins

chemical shift,[a] ppm	relative peak area,[b] %	assignment
	B. mori	
103.1	32.9	Ala
99.5	5.7	Tyr
94.9	15.6	Ser
90.3	16.1	Ser–*Gly*–Ala
87.6		Ala–*Gly*–Ser + Ala–*Gly*–Ala
	29.7	
87.0		Gly[c]
	P. c. ricini	
103.0	9.7	Ala(c)[d]
101.0	39.7	Ala(h)[e]
99.5	3.7	Tyr
94.8	7.8	Ser
90.1	5.6	Gly
89.5	4.4	Gly
87.7	25.6	Gly
86.2	3.4	Gly

[a] In ppm downfield from external NH_4NO_3. [b] These values were determined by cutting out and weighing traces of the peaks for *B. mori* silk fibroin spectrum and by the peak simulation assuming the Lorentzian for *P. c. ricini* silk fibroin spectrum. [c] This peak is attributable to the ^{15}N nucleus of the Gly residue, but further assignments were not performed. [d] This peak was assigned to the isolated Ala and/or the $-(Ala)_n-$ sequence, whose lengths are too short to form α-helix (see text). [e] This peak was assigned to fast exchange between the helical and random coil states of $-(Ala)_{22}-$ sequence (see text).

and a comparison of the spectra of the nonlabeled and [^{15}N]Gly-labeled silk fibroins. In spectra B and C of the [^{15}N]Gly-labeled silk fibroins, which were labeled with [^{15}N]Gly in the fifth larval stage of the silkworm, the relative peak area of the Ser residue in spectrum C increased compared with that of spectrum B. This is due to increase of the ^{15}N-labeling of the Ser residue through partial conversion of [^{15}N]Gly to [^{15}N]Ser by a biosynthetic process in vivo[7] (Table I). The assignment of the Gly peaks to the Ser–*Gly*–Ala, Ala–*Gly*–Ala, and Ala–*Gly*–Ser sequences in the chain was performed from both the relative peak area and the peak position.[7] The wide chemical shift distribution arising from the sequence in the chain is a merit of the ^{15}N NMR approach for sequence analysis compared with ^{13}C or ^{1}H NMR.[18] The main ^{15}N peaks were essentially unchanged when the temperature was raised to 80 °C or the pH of the aqueous solution was changed (pH 11).

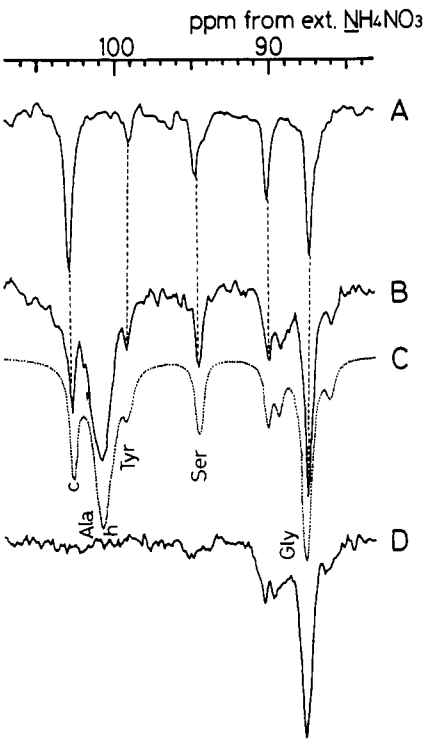

Figure 2. ^{15}N NMR spectra of *B. mori* and *P. c. ricini* silk fibroins in aqueous solutions (sample 3): A, *B. mori* silk fibroin (sample 1); B, [^{15}N]Ala *P. c. ricini* silk fibroin (sample 4); C, simulation of spectrum B; D, [^{15}N]Gly *P. c. ricini* silk fibroin (sample 5). In sample 4, averaging of the [^{15}N]Ala over all amino acid residues of the silk fibroin occurs biosynthetically, in the silkworm (see text).

The spin–spin coupling constant, $^{1}J_{NH}$, for the [^{15}N]Gly *B. mori* silk fibroin (sample 3) was determined. The value obtained, 89 Hz, indicates a trigonal structure for the ^{15}N nuclei.[21] This is independent of the sequence for the Gly peak. Long-range spin–spin couplings were not detected under these experimental conditions.

The ^{1}H-gated decoupling and ^{1}H-decoupling ^{15}N NMR spectra of [^{15}N]Gly *B. mori* silk fibroin (sample 3) were also observed. From the determination of the relative peak area, the NOE value for the Gly residues, Ser–*Gly*–Ala, and Ala–*Gly*–Ala + Ala–*Gly*–Ser peaks was determined as −3.9 (the minus sign indicates negative enhancement), which is independent of sequence within experimental error. This indicates that the correlation time for the segmental motion of *B. mori* silk fibroin is less than ca. 5×10^{-10} s.[19]

In addition, the spin–lattice relaxation times, T_1, of the two ^{15}N NMR peaks, Ser–*Gly*–Ala and Ala–*Gly*–Ala + Ala–*Gly*–Ser of the [^{15}N]Gly *B. mori* silk fibroin (sample 3) were determined from a series of partially relaxed ^{15}N NMR spectra. The T_1 values for these peaks are 0.68 s at 25 °C, and the correlation time for segmental motion is determined as ca. 5×10^{-10} s.[19] Such very fast segmental motion is the same as found for *B. mori* silk fibroin from ^{13}C relaxation.[6] The T_1 values are sequence-independent as are also the NOE values, implying that the dipole–dipole interaction between the ^{15}N and directly bonded ^{1}H nuclei is the dominant mechanism for the ^{15}N relaxation.

***P. c. ricini* Silk Fibroin.** Figure 2 shows ^{15}N NMR spectra of *P. c. ricini* silk fibroins, B–D, together with that of *B. mori* silk fibroin, A. The ^{15}N NMR spectrum of *P. c. ricini* silk fibroin is relatively complex compared with that of *B. mori* silk fibroin. For assignment of the peaks

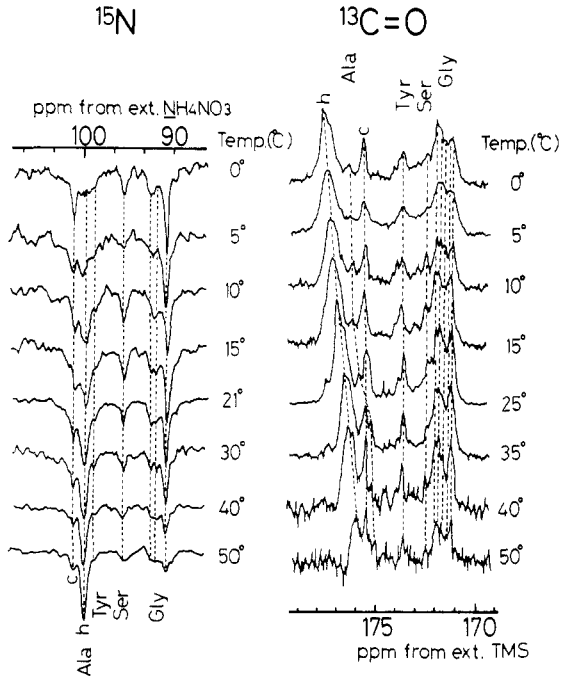

Figure 3. ^{15}N and $^{13}C{=}O$ NMR spectra of *P. c. ricini* silk fibroins as a function of temperature.

to the Gly residues, the ^{15}N NMR spectrum of [^{15}N]Gly-labeled *P. c. ricini* silk fibroin is also observed as shown in Figure 2D. There are at least four peaks in the Gly resonance region, and two of them coincide with the resonance positions of the sequences Ser–*Gly*–Ala and Ala–*Gly*–Ala + Ala–*Gly*–Ser in the *B. mori* silk fibroin spectrum. [^{15}N]Ala was given to *P. c. ricinic* silkworm to assign the alanine resonance region. [^{15}N]Ala was given to *P. c. ricini* silkworm to assign the alanine resonance region. The ^{15}N NMR observation was performed for the silk fibroins obtained from the silkworm injected with [^{15}N]Ala (spectrum B), but it is clear that averaging of the [^{15}N]Ala over all amino acid residues of the silk fibroin occurs biosynthetically in the silkworm taking into account the relative peak area (Table II). Actually, when ^{15}N NMR of usual *P. c. ricini* silk fibroin solution with the same concentrations was observed, no spectrum was obtained within the same accumulation times. In spectrum B two peaks are observed in the Ala resonance region, and one of them coincides in position with the single Ala peak in the *B. mori* silk fibroin spectrum. Another peak is assigned to the Ala residues in the $-(Ala)_{22}-$ sequence as mentioned below. The ^{15}N NMR chemical shifts, the peak assignments, and the relative peak area are summarized in Table II. In the determination of the relative peak area, the peak simulation was performed as shown in Figure 2C. Good agreement in the amino acid composition of *P. c. ricini* silk fibroin between the ^{15}N NMR and amino acid analyses reported previously[1] is obtained, supporting the ^{15}N NMR assignment. A comparison of the ^{15}N and ^{13}C (carbonyl region[10]) NMR spectra of *P. c. ricini* silk fibroin was performed (Figure 3). Several points are similar between them: (1) the order of the peaks i.e., Ala, Tyr, Ser, and Gly to higher field; (2) a complex peak splitting due to the sequence in the Gly resonance region: (3) appearance of mainly two peaks in the Ala resonance region. In our previous papers,[10,17] a detailed assignment of the Ala carbonyl region was reported. The peak h was assigned to the Ala residues in the $-(Ala)_{22}-$ sequence whose length is long enough to form an α-helix, where fast exchange with respect to the chemical shift between the helical and random coil states occurs. Strictly speaking, the helicity of each residue in the $-(Ala)_{22}-$ sequence increases gradually as the residue is present at the inner position of the sequence. The calculation of helicity has been performed in terms of the statistical thermodynamic treatment for the helix–coil transition of polypeptides.[17] On the other hand, peak c was assigned to the isolated Ala residue and/or $-(Ala)_n-$ sequence which is too short to form an α-helix. This assignment is essentially applicable to two peaks in the alanine resonance region of the ^{15}N NMR spectrum although the relative positions of two peaks are reversed between the ^{13}C and ^{15}N NMR spectra, as judged by the relative peak areas, summarized in Table II. With increasing temperature, the conformational transition α-helix to random coil occurs locally in the $-(Ala)_{22}-$ sequence portion. The characteristics of the temperature-dependent behavior in the spectra differ considerably between the ^{15}N and ^{13}C NMR. Above 40 °C, the relative ^{15}N NMR peak intensities decrease except for peak h of the Ala residue. It has been reported that most of the random coil domain in the *P. c. ricini* silk fibroin membrane obtained from liquid silk was easily converted into the antiparallel β-structure by methanol treatment, but the α-helical domain of the $-(Ala)_{22}-$ sequence was unaltered by such treatment.[2] Essentially, the occurrence of a similar situation would be expected when the temperature is raised. Thus, it seems that the random coil domain is partly converted into the antiparallel β-structure above 40 °C, and the observable domain with random coil state decreases. The peak attributable to the $-(Ala)_{22}-$ sequence is sensitive to the observed temperature. Actually, considerable broadening occurs below 15 °C. Within this ^{15}N NMR experimental condition, the displacement of peak h is scarcely observed when the temperature is changed. Thus, this temperature-dependent ^{15}N NMR behavior of *P. c. ricini* silk fibroin is different from the ^{13}C NMR behavior as reported previously,[10] although the helix–coil conformational transition is observed by both methods. Thus, different information is obtained from the ^{15}N and ^{13}C NMR spectra concerning the helix–coil transition of *P. c. ricini* silk fibroin.

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Macroporous Gels. 4. An NMR Study of the Formation of Macroporous Gels Containing Trimethylolpropane Trimethacrylate

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ABSTRACT: The radical polymerization of trimethylolpropane trimethacrylate (TRIM) and the radical copolymerization of TRIM and methyl methacrylate (MMA) in solution were studied by ^{1}H NMR in situ measurements. The conversion at the point of gelation was determined. A method based on line-width measurements of an inert component in the system is suggested for the estimation of the gel point of the system. An investigation of the influence of the polymerization time and temperature on the amount of unreacted methacrylate groups has also been performed. The number of unreacted double bonds decreased with increasing polymerization time and temperature even after 100% monomer conversion. From this investigation it was possible to divide the polymerization of TRIM into three discernible steps.

Introduction

Polymerization of trimethylolpropane trimethacrylate (TRIM) in a solvent results in a macroporous gel.[1,2] When toluene was used as pore-forming agent, the resulting macroporous gel had two pore size distributions, one consisting of small pores (radius < 50 Å) and one of large pores (radius > 50 Å).[1] The pore size distribution of small pores was very narrow and probably caused by the structure of the monomer.[1] Different pore size distributions were obtained by changing the solvent and/or by copolymerization of TRIM with a suitable comonomer.[2,3]

In this paper we present an ^{1}H NMR study of the course of polymerization for both the TRIM and the TRIM–methyl methacrylate (MMA) systems in two different solvents (ethyl acetate and toluene) as well as the level of conversion at which the gel formation occurs in the former system.

The progress of the polymerization process can be followed by analyses of the monomer consumption in the system. One way to do this to interupt the polymerization at different stages and analyze the unreacted monomer–solvent mixture. Due to the effect of gel formation this method can be difficult to perform. Another way is to use an FT–NMR spectrometer and make the polymerization in situ while collecting spectra.

Highly cross-linked polymers can be analyzed by IR spectroscopy[4] or by solid-state ^{13}C FT-NMR (CP-MAS-DD).[1] The latter method was used to determine the residual amount of unreacted carbon–carbon double bonds as a function of time and temperature.

Experimental Section

Polymerization (NMR Kinetics). The mixture of trimethylolpropane trimethacrylate (TRIM, technical quality containing more than 98% TRIM from Merck AG or Alfa Products), a solvent (toluene or ethyl acetate, analytical grade), and in copolymerizations methyl methacrylate (MMA, analytical grade) was transferred into a 50-mL hypo-vial containing 0.1% (w/w) AIBN (analytical grade). The mixture used consisted of 30% monomer and 70% solvent. In the copolymerization experiments the monomer was a mixture of 20% (v/v) MMA and 80% TRIM. Nitrogen gas was bubbled through the solution for at least 1 min before the vial was sealed. A sufficient amount for analysis was then transferred into an NMR sample tube and 1–2 droplets of C_6D_6 were added.

Polymerization (Double-Bond Consumption). A polymerization experiment was performed as follows. AIBN, 20 mg, was weighed into a 50-mL hypo-vial. The vial was sealed, evacuated, and filled with nitrogen. Then 20 mL of a solution containing monomer (TRIM 30%) and solvent (toluene 70%) was added. The vial was placed in a shaking bath at elevated temperature (73 °C) and was kept there for the specified time. The resulting polymers were ground and dried at 60 °C in vacuum for at least 24 h before the determination of unreacted double bonds was made.

Kinetic Measurements with NMR. The in situ ^{1}H NMR experiments were carried out at different temperatures with a Varian XL-200 spectrometer operating at 200 MHz. The pulse angle used was 81° and the waiting time between the pulses was at least $3T_1$. T_1 measurements were performed for all hydrogen atoms in the TRIM monomer according to the inversion–recovery sequence with delay times longer than $5T_1$ of the slowest relaxing hydrogen. All chemical shifts are reported in parts per million downfield from TMS. An NMR spectrum was recorded before heating was commenced and the others at the reaction temperature. The interval between the spectra was 1 s and the time for one spectrum to be collected was 73 s.

Determinations of Unreacted Carbon–Carbon Double Bonds. The amount of unreacted carbon–carbon double bonds was determined by high-resolution solid-state ^{13}C NMR spectroscopy using cross-polarization (CP), magic-angle spinning (MAS), and high-power decoupling (DD) (Varian XL-200 with solid sample accessories). Carbonyl groups conjugated with a double bond have a lower chemical shift (166 ppm versus TMS) than the unconjugated, reacted, ones (176 ppm versus TMS). The difference was large enough to almost completely resolve the two peaks. This together with nearly similar contact times makes quantitative measurements possible.[1]

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