NMR of Silk Fibroin. 4. Temperature- and Urea-Induced Helix–Coil Transitions of the $-(Ala)_n-$ Sequence in *Philosamia cynthia ricini* Silk Fibroin Protein Monitored by ^{13}C NMR Spectroscopy

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ABSTRACT: ^{13}C NMR spectra have been obtained for *Philosamia cynthia ricini* silk fibroin protein in aqueous solution as functions of temperature and urea concentration. The spin–lattice relaxation times have also been determined. Peak doublings were observed not only for the C_α and carbonyl but also for the C_β carbons of the alanine residue, where the assignment of the carbonyl resonance was performed from a comparison among [1-^{13}C]alanine-labeled, [1-^{13}C]glycine-labeled, and unenriched silk fibroin spectra. In the "double peaks", the low-field peaks of the C_α and carbonyl carbons shift upfield 1.0 and 1.5 ppm, respectively, while the upfield peak of the C_β carbon shifts downfield 0.5 ppm as the temperature is increased from −5 to +50 °C. However, other peaks show no temperature dependence. In addition, exchanges between the individual peaks do not occur. We conclude that the temperature-dependent peaks are attributable to the $-(Ala)_n-$ sequences whose lengths are long enough to form an α-helix and reflect fast exchange with respect to the chemical shift between the helix and coil conformations. On the other hand, the temperature-independent peaks are attributable to isolated Ala residue and/or $-(Ala)_n-$ sequences whose lengths are too short to form an α-helix and which reflect the random coil state. The helix content of *P. c. ricini* silk fibroin determined from the ^{13}C NMR spectra as a function of temperature on the basis of these assignments is in *quantitative* agreement with values reported for the CD spectra for *Antheraea pernyi* silk fibroin, whose amino acid composition resembles that of *P. c. ricini* silk fibroin very closely, supporting strongly the assignment of the ^{13}C NMR spectra performed here. A similar trend was observed for the urea-induced helix–coil transition of the protein. Thus, the conformational change is local and occurs only for the "$-(Ala)_n-$ sequence", 34% of all residues; other residues remain unaffected by the change. The spin–lattice relaxation data yield fast segmental motion of the backbone chain of 3.5×10^{-10} s at 25 °C, which is of the same order as determined for *B. mori* silk fibroin.

Introduction

^1H and, later, ^{13}C NMR experiments have provided many valuable insights concerning the solution structure and conformational transitions in polypeptides and proteins.[1,2]

In previous papers,[3,4] we reported conformational characterizations of silk fibroin proteins stored in the silk glands of intact *Bombyx mori* and *Philosamia cynthia ricini* silkworms using ^{13}C NMR directly. Emphasis was on the appearance of "double peaks" for individual carbons of the Ala residues of *P. c. ricini* silk fibroin. This peak doubling is believed to reflect specific structural features because the presence of α-helical segments in the protein has been confirmed from ORD and CD spectra.[5,6] A large number of low molecular weight polypeptides also exhibit peak doubling of the $C_\alpha H$ and NH protons and the C_α, C_β, and carbonyl carbons during helix–coil transitions.[1,2,7] In general, observation of separate helix and coil peaks implies that the lifetime of each form should be approximately 10^{-3} s or greater. However, short (10^{-6} s or faster) lifetimes for the helix–coil transition have been reported by other kinds of experiments: temperature jump, ultrasonic attenuation, dielectric dispersion, electric field jump, and electric field pulse method.[1] In this case, only a single NMR peak should be observed. Most of this discrepancy was interpreted in terms of polydispersity of the polypeptides, which yield "double peak" spectra.[8-12]

In order to clarify the origin of the "double peaks", we will report here the temperature and urea-induced helix–coil transition of *P. c. ricini* silk fibroin. In particular, since the amino acid residue of interest is alanine, which has no ionizable groups, and since strong acids such as trifluoroacetic acid[1,13-15] were not used as solvents, an examination of the spectral changes of *P. c. ricini* silk fibroin during the helix–coil transition is, therefore, of interest. Inhomogeneity in the local conformation and the mechanism of the helix–coil transition of the protein are clarified, including dynamic insights.

Experimental Section

Materials. Rearing of *P. c. ricini* silkworm was performed with an artificial diet or with *Ailanthus glandulosa* leaves in our laboratory. [1-^{13}C]Alanine-labeled silk fibroins from *P. c. ricini* were prepared biosynthetically by feeding [1-^{13}C]alanine (90% enrichment, Dai-ichi Pure Chemicals Co., Tokyo) in addition to an artificial die to silworms of the fifth instar for a whole day.[16] [1-^{13}C]Glycine-labeled silk fibroins from *P. c. ricini* were obtained in a manner similar to that for the [1-^{13}C]alanine-labeled sample (enrichment was 92.6% for [1-^{13}C]glycine, Merck and Co., Inc. Rahway, NJ). The *B. mori* silkworm was reared with mulberry leaves.

^{13}C NMR Measurement. ^{13}C NMR spectra were recorded with a JEOL FX-200 NMR spectrometer operating at 50.3 MHz equipped with a temperature controller. The liquid silk sample was collected in an NMR sample tube as follows. The middle silk gland divisions were excised from the mature larvae. After washing with distilled water, the center of the middle silk gland was cut with scissors. The effluent was collected in an NMR sample tube of 8-mm diameter and ca. 30-mm length immersed in a beaker of distilled water. Five to eight silkworms were necessary to obtain a liquid silk sample for each NMR measurement. The NMR sample tube was stoppered and inserted in turn into a 10-mm NMR tube containing D_2O plus a small amount of dioxane. In NMR measurements below 5 °C, $(CD_3)_2C{=}O$ was used instead of D_2O. A sample was prepared for each separate NMR measurement except the spectra observed at −5, 0, +5, and +15 °C, which were recorded with the same sample. In order to avoid gelation of the fibroin, urea was added to the liquid silk after the pH value was adjusted to 7.5 with NaOH. In most ^{13}C NMR measurements, spectral conditions were the following: 3000–35 000 pulses, 45° pulse angle (10 μs); 1.5–2.5-s delay between pulses; 12 000-Hz spectral width; 16K data points; ^1H noise decoupling; no sample spinning. The measurements at 25 °C were performed using the NNE mode, i.e., ^1H gated decoupling only during ^1H irradiation.[17] Chemical shifts were measured in ppm relative to dioxane and converted to an external

0024-9297/85/2218-2614$01.50/0

Table I
^{13}C NMR Chemical Shifts (δ^a) and Spin-Lattice Relaxation Times (T_1, s) of both *P. c. ricini*[b] and *B. mori* Liquid Silks[c] and ^{13}C NMR Chemical Shifts (δ) of Pentapeptides Gly-Gly-X-Gly-Gly[d] at 25 °C

	P. c. ricini		*B. mori*		GGXGG
carbon	δ	T_1, s	δ	T_1, s	δ
Ala C_α	50.21 (c)[e]	0.17	49.96	0.19	50.0–50.2
	51.42 (h)[f]	0.15			
Ala C_β	16.20 (c)	0.32	16.52	0.30	16.7–17.0
	15.50 (h)	0.29			
Gly C_α	42.60	0.09	42.63	0.11	42.2–42.8
Ser C_α	55.85	0.14	55.83	0.19	55.8–55.9
Ser C_β	61.14	0.09	61.26	0.11	61.3–61.5
Tyr C_α	55.39	0.17	55.42		55.4–55.8
Tyr C_β	36.09	0.09	36.18	0.11	36.5–36.8
Tyr C_γ	127.83		127.92		127.9–128.6
Tyr C_δ	130.40	0.17	130.43	0.20	130.6–130.9
Tyr C_ϵ	115.42	0.17	115.48	0.19	116.0
Tyr C_ζ	154.64		154.55		154.8–155.0
Asp C_α	51.42				51.7–51.9
Asp C_β	38.83				38.7–39.0
Asp C_γ	177.56				177.6
Phe C_α	56.18				55.7–56.0
Phe C_β	35.60				35.8–36.2
Phe C_γ	136.06				136.4–136.8
Phe C_δ	129.26				129.5–129.6
Phe C_ϵ	129.00				129.3
Phe C_ζ	126.66				127.7
Arg C_α	53.33				53.8–53.9
Arg C_β	27.85				28.0–28.2
Arg C_γ	24.29				24.5–25.0
Arg C_δ	40.55				40.9
Arg C_ζ	156.71				156.7–156.8
His C_β	26.31				26.3,[g] 28.5[h]
His C_γ	135.02				134.1, 136.4
His $C_{\delta 2}$	117.26				118.1, 117.6

[a] Values are represented in ppm from external Me_4Si. [b] Concentration, 18.3% (w/v). [c] Concentration 24.2% (w/v). [d] X = Ala, Gly, Ser, Tyr, Asp, Phe, Arg, and His. Data taken from ref 20–22. [e] Peak c was assigned to random coli. [f] Peak h was assigned to a fast exchange between the helical and random coil states. [g] Imidazolium form. [h] Imidazole form.

Me_4Si reference. The spin–lattice relaxation times, T_1, measured only for the protonated carbons, were made by the inversion–recovery method.[4]

Results

^{13}C NMR Spectra. Figure 1 shows the ^{13}C NMR spectrum of the liquid silk from *P. c. ricini* mature larva together with that of *B. mori* liquid silk. As expected from the previous spectrum of intact *P. c. ricini* mature larva,[3] the spectrum of the liquid silk is sharp, which enables us to clarify the solution structure from the NMR spectrum in detail. Major amino acids of *P. c. ricini* silk fibroin are Ala and Gly, which are the same as those of *B. mori* silk fibroin, but the content of these residues is in reverse ratio.[18,19] This difference would be expected to yield a difference in the conformation, which is reflected in their ^{13}C NMR spectra. In Figure 1, attention should be focused on the "double peaks" not only for the C_α and carbonyl but also for the C_β carbons of the Ala residue of *P. c. ricini* liquid silk, in contrast to the singlet observed for the Ala resonances of the *B. mori* liquid silk spectrum.[3,4,17] The assignment, except for the carbonyl carbon resonance, was performed by reference to chemical shift data of the pentapeptides, Gly-Gly-X-Gly-Gly, where X equals the specified residue,[16,20–23] and the amino acid composition. The chemical shift data are summarized in Table I together with the relaxation data.

The carbonyl carbon resonance region of the *P. c. ricini* spectrum is expanded in Figure 2, together with the spectra of [1-^{13}C]alanine-labeled and [1-^{13}C]glycine-labeled *P. c. ricini* silk fibroins and *B. mori* silk fibroin.[16] The chemical shifts are listed in Table II. At least 15 peaks, a–o, are observed, although some peaks show further splittings. Peaks b–d are attributable to the Ala carbonyl carbons, as can be seen by comparing spectra A and C.[23] Peak a is attributable to the C_γ carbon of the Asp residue from its chemical shift and intensity, which is supported from the lack of temperature dependence of this peak throughout the helix–coil transition, in contrast to the temperature dependence of peaks b and c, as will be described below. Similarly, peaks k–o are attributable to the Gly carbonyl carbons from a comparison of spectra B and C. These peaks show no temperature dependence, as will

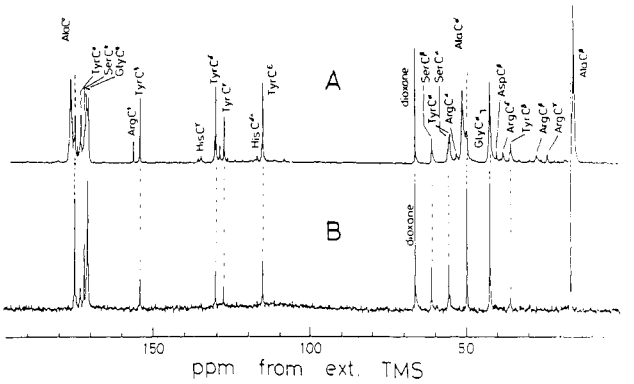

Figure 1. ^{13}C NMR spectra of (A) *P. c. ricini* (sample concentration 18.0% (w/v), 35 000 pulses) and (B) *B. mori* (sample concentration 12.1% (w/v), 17 000 pulses) liquid silks at 25 °C. The ^1H nuclei were decoupled only during the sampling time of 0.68 s.

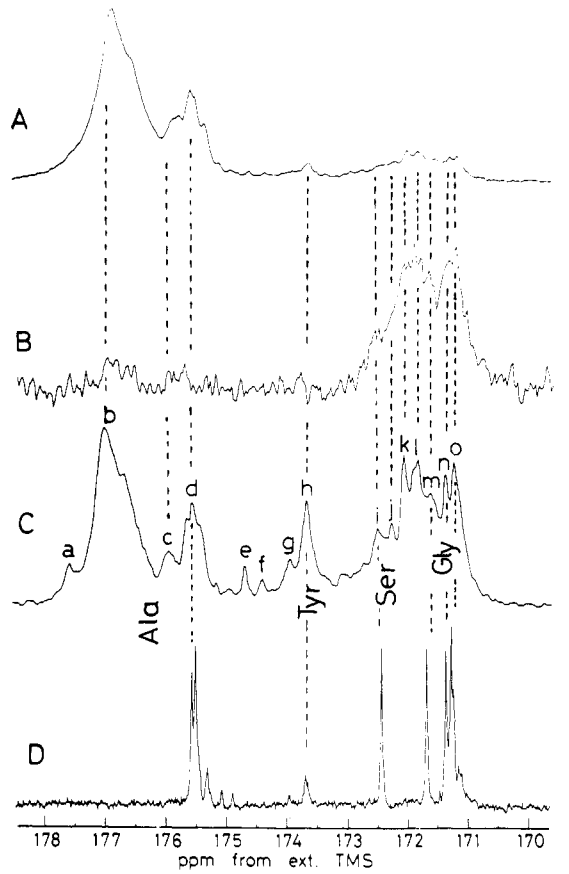

Figure 2. Comparison of ^{13}C NMR spectra of the carbonyl region among (A) [1-^{13}C]alanine-labeled (10 000 pulses), (B) [1-^{13}C]-glycine-labeled (14 000 pulses), and (C) unenriched (35 000 pulses) *P. c. ricini* liquid silks at 25 °C. The spectrum of regenerated *B. mori* silk fibroin in aqueous solution (D) is also shown.[16]

Table II
Chemical Shifts and Assignments of the Carbonyl Carbons of *P. c. ricini* Liquid Silk

peak[a]	chem shift[b]	residue
a	177.56	Asp C_γ
b	177.00	Ala
c	175.92	Ala
d	175.54	Ala
e	174.67	
f	174.40	
g	173.91	
h	173.64	Tyr
i	172.45	Ser
j	172.21	Ser
k	172.01	Gly
l	171.78	Gly
m	171.57	Gly
n	171.34	Gly
o	171.19	Gly

[a] Peaks were labeled a–o toward higher field in spectrum C, Figure 2. [b] Values are represented in ppm from external Me_4Si (25 °C).

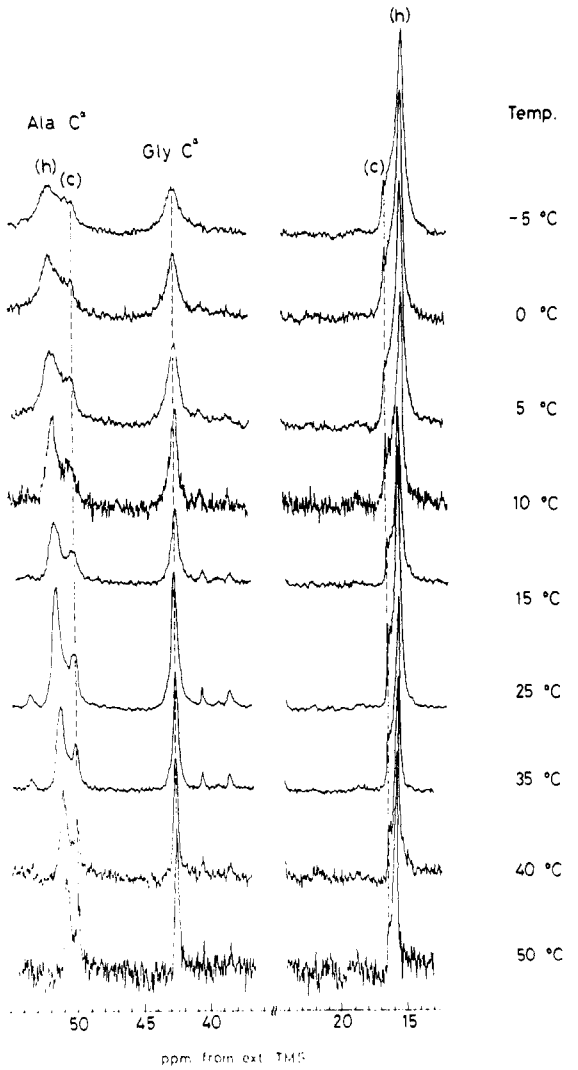

Figure 3. ^{13}C NMR spectra of *P. c. ricini* liquid silk in the aliphatic region as a function of temperature.

be described below, and therefore are attributable to the Gly carbonyl carbons of specified sequences in *P. c. ricini* silk fibroin protein as well as in *B. mori* silk fibroin.[16] Since the primary sequence of the former protein has not been reported yet except for the presence of the $-Ala_n-$ sequence,[19] the sequence assignment is difficult. However, by referring to the sequence assignments performed for

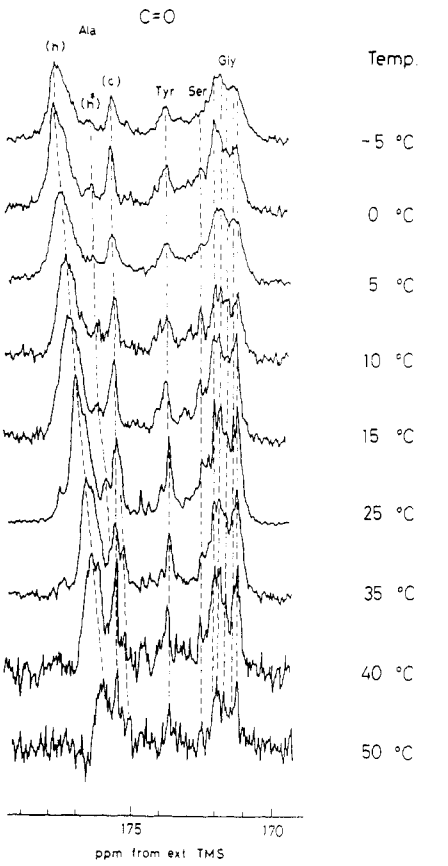

Figure 4. ^{13}C NMR spectra of *P. c. ricini* liquid silk in the carbonyl region as function of temperature.

the Gly carbonyl resonances of *B. mori* silk fibroin,[16] peaks m, n, and o may be attributed to the Ala-*Gly*-Ser, Ser-*Gly*-Ala, and Ala-*Gly*-Ala sequences.

Temperature Dependence. The spectra of *P. c. ricini* silk fibroin protein were observed between −5 and +50 °C as a function of temperature. Figure 3 shows the Ala C_α, Gly C_α, and Ala C_β resonance regions. All peaks become sharp with increasing temperature, indicating an increase in the segmental motion of the protein. In addition, a reduced signal-to-noise ratio between 40 and 50 °C indicates a partial formation of aggregates with β structure (no signal appears for the the aggregates).[3] Most striking is the dramatic change observed in the Ala resonance region. The upfield component of the Ala C_β peak marked h shifts gradually downfield 0.5 ppm and the downfield component h of the Ala C_α peak shifts upfield 1.0 ppm as the temperature is increased from −5 to +50 °C, but other peaks marked c and the Gly C_α peak show almost no temperature dependence. A similar spectral change was also observed in the carbonyl region (Figure 4). The peaks marked h, h*, and c of the Ala residue in Figure 4 are the same as peaks, b, c, and d in Figure 2, respectively. Peak h shows a gradual upfield shift of 1.8 ppm as the temperature is increased from −5 to +50 °C, but peak c shows almost no temperature dependence. This behavior corresponds to that observed for the Ala C_α and C_β resonances. One exception is the temperature-dependent peak h*, as will be described below. When exchange between the helical and coil states does not occur in peak h, the chemical shift differences between the h and c peaks are 2.3 ppm for the carbonyl carbon, 1.9 ppm for the C_α carbon, and 1.1 ppm for the C_β carbon. The individual Gly carbonyl resonances reflecting the sequence show no temperature dependences as also do the Tyr and Ser carbonyl carbon resonances. The change in the chemical shift is summarized in Figure

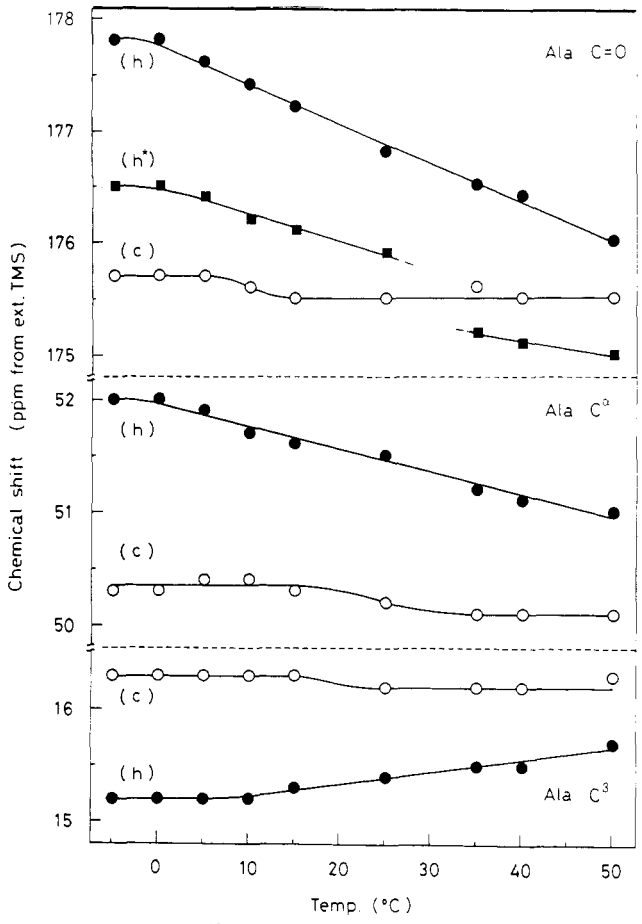

Figure 5. Plots of ^{13}C NMR chemical shifts of the Ala C_α, C_β, and carbonyl carbons of *P. c. ricini* liquid silk vs. temperature.

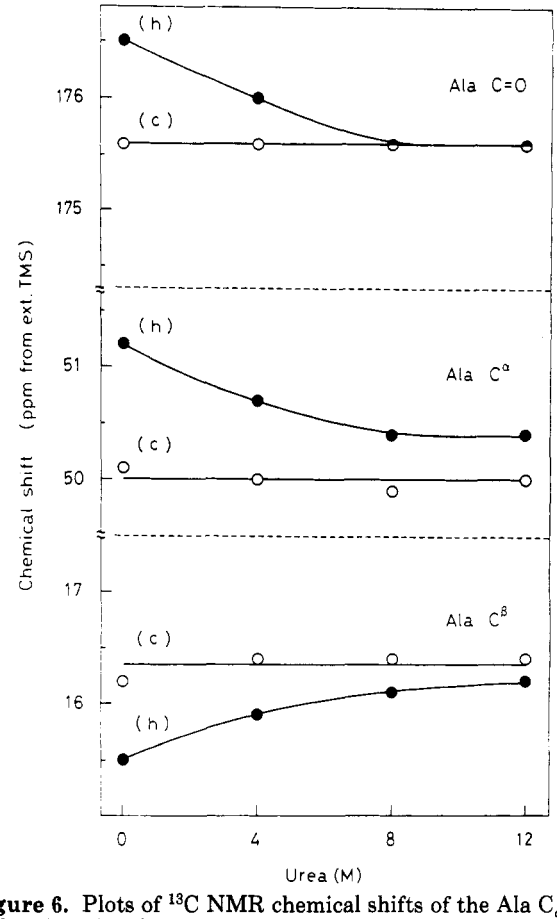

Figure 6. Plots of ^{13}C NMR chemical shifts of the Ala C_α, C_β, and carbonyl carbons of *P. c. ricini* liquid silk vs. urea concentration.

5 as a function of temperature.

Urea Concentration Dependence. In our previous paper,[3] urea was added as a denaturing regent to the liquid silk of *P. c. ricini* in order to assign the ^{13}C NMR spectra. These spectra, whose signal-to-noise ratio and resolution are much improved, are reported in this paper.

Similar to the temperature-induced helix–coil transition of the protein, with increasing urea concentration, the peaks marked h assigned to the carbonyl and C_α carbons of the Ala residue shift upfield, peak h of the C_β carbon shifts downfield, but peaks c of the Ala carbons show no shift. The magnitude of the shifts of the individual peaks h was 0.9 ppm for the carbonyl carbon, 0.8 ppm for the C_α, and 0.7 ppm for the C_β at 35 °C for urea solutions between 0 and 12 M (Figure 6). No shift was observed for the resonances assigned to other amino acid residues on changing the urea concentration, in conformity with the temperature-induced conformational transition. Thus, it is concluded that urea induces the helix–coil transition locally in the $-Ala_n-$ sequences as well as temperature. Even in 12 M urea, coalescence of the "double peaks" was not observed in the Ala C_α and C_β resonance regions although coalescence was obtained for the carbonyl resonance.

Spin–Lattice Relaxation Time. In order to discuss the conformation of *P. c. ricini* silk fibroin from the viewpoint of molecular dynamics, the ^{13}C NMR spin–lattice relaxation times were observed. The plots of $M_0 - M_z$ vs. τ are essentially single exponential, where M_0 is the equilibrium amplitude of the fully relaxed spectrum, M_z is the amplitude of a partially relaxed spectrum, and τ is the delay time between the 180° and 90° pulses. The T_1 values are listed in Table I, together with those of *B. mori* liquid silk. All T_1 values of *P. c. ricini* are somewhat smaller than those of *B. mori* silk fibroin. In addition, the T_1 value of peak c is slightly larger than that of peak h for both the Ala C_α and C_β carbons, which supports previous assignments of the peak doubling. From both the NT_1 value averaged over the Ala, Gly, and Ser C_α carbons, where N is the number of hydrogen atoms attached to the specified carbon atom directly, and the NOE value, 2.2, assumed by reference to previous data concerning *B. mori* silk fibroin, the average correlation time for the segmental motion of the protein was determined with a log χ^2 distribution model[24] for segmental motion. The value was 3.5×10^{-10} s, compared with 2.2×10^{-10} s for *B. mori* silk fibroin[4] at 25 °C. Thus, the segmental motion of the *P. c. ricini* silk fibroin chain is very fast as is also the case for *B. mori* fibroin, which is typical of a random coil polymer.

Discussion

Most of the information concerning the helix–coil transition of polypeptides has been obtained with systems in which other important factors, in addition to the conformational changes, were playing a role; these are the ionization of side-chain functional groups (in polylysine[25] and poly(glutamic acid)[7]) and the extensive solvation of peptide bonds by strong acids such as trifluoroacetic acid used as solvents.[1,11,13–15] Our sample, *P. c. ricini* silk fibroin protein, including the solvent system water,[26] is excellent for monitoring the helix–coil transition. In particular, the residue of interest is alanine, which is common in proteins.[27]

Assignment of the "Double Peaks". Here, we will assign the h and c peaks observed in the Ala resonance region to the conformation of *P. c. ricini* liquid silk. The

presence of an α-helix in the protein has been confirmed from ORD measurement by Iizuka;[5] the α-helix content was reported as 20%, but the temperature was not shown. Kataoka et al.[6] reported the temperature dependence of the helix content in the liquid silk of *A. pernyi*, whose amino acid composition resembles that of *P. c. ricini* very closely. For example, the Ala and Gly contents of *A. pernyi* are 48.6% and 28.3%, respectively, compared with the corresponding values of 48.4% and 33.2% for *P. c. ricini*. In addition, we observed previously the ^{13}C CP–MAS NMR spectrum of *P. c. ricini* silk fibroin in the solid state prepared from the liquid silk by drying. The chemical shifts of the Ala C_α, C_β, and carbonyl carbons agreed with those of poly(L-alanine) with an α-helical conformation.[28,29] Generally, "double peaks" observed for polypeptides have been assumed to correspond to helical and coil peaks whose chemical shift difference in hertz permits determination of minimum limits for the lifetime of the carbons in the two environments. However, the peak doublings observed for *P. c. ricini* liquid silk differ from those of polypeptides. Peak h is temperature-dependent, but peak c is nearly temperature-independent concerning the peak position. In addition, the relative intensity shows no temperature dependence. Thus, it is clear that an exchange between individual peaks does not occur. We conclude that the temperature-dependent peak h may be assigned to Ala residues in the $-Ala_n-$ sequences whose lengths are great enough to form an α-helix, where fast exchange with respect to the chemical shift between the helical and random coil states occurs. That is, the peak position reflects a weighted average of α-helical and random coil peaks. On the other hand, peak c is assigned to the isolated Ala residue and/or $-Ala_n-$ sequences whose lengths are too short to form an α-helix; the conformation is random coil.[11] In addition, the chemical shifts of the Ala carbonyl and C_α peaks marked h remain constant below 0 °C, indicating complete α-helix formation of the $-Ala_n-$ sequences h under the environments. Minor chemical shift changes of <0.2 ppm were observed throughout the helix–coil transition for the carbonyl carbon marked c at 10 °C and for the C_α and C_β carbons c at 20 °C.

Next, attention was focused on peak h* in the carbonyl resonance region. This peak is attributable to the Ala residue, as can be seen by comparing spectra A and C in Figure 2, and the ratio of the area of peak h* to the total area of the Ala carbonyl peak is approximately 10%. The temperature dependence is complex. With increasing temperature from −5 to +35 °C, the peak shifts gradually upfield. Then, coalecence of peaks c and h* occurrs at 35 °C. However, another peak whose intensity is nearly the same as that of peak h* appears at higher field than peak c in the Ala carbonyl region and shifts upfield with increasing temperature from 35 to 50 °C.

Determination of α-Helix Content. It is possible to evaluate the α-helix content [θ] of *P. c. ricini* silk fibroin at ambient temperature, *t*, (°C), from both the ^{13}C NMR chemical shifts and relative intensities using the following equations:

$$[\theta]_t^{C=O} = \frac{\delta_t^{C=O} - \delta_c^{C=O}}{\delta_h^{C=O} - \delta_c^{C=O}} \frac{A_{C=O}^{h+h^*}}{A_{C=O}^{tot}} F_A = \frac{\delta_t^{C=O} - 175.7}{177.8 - 175.7} \times 0.341$$

$$[\theta]_t^{C_\alpha} = \frac{\delta_t^{C_\alpha} - \delta_c^{C_\alpha}}{\delta_h^{C_\alpha} - \delta_c^{C_\alpha}} \frac{A_{C_\alpha}^{h}}{A_{C_\alpha}^{tot}} F_A = \frac{\delta_t^{C_\alpha} - 50.3}{52.0 - 50.3} \times 0.73 \times 0.438$$

$$[\theta]_t^{C_\beta} = \frac{\delta_t^{C_\beta} - \delta_c^{C_\beta}}{\delta_h^{C_\beta} - \delta_c^{C_\beta}} \frac{A_{C_\beta}^{h}}{A_{C_\beta}^{tot}} F_A = \frac{-(\delta_t^{C_\beta} - 16.3)}{-(15.2 - 16.3)} \times 0.88 \times 0.438$$

Table III
α-Helix Content [θ] of *P. c. ricini* and *A. pernyi* Liquid Silks Determined with ^{13}C NMR and CD Methods, Respectively, as a Function of Temperature

	P. c. ricini				*A. pernyi*
temp, °C	$[\theta]^{C=O}$	$[\theta]^{C_\alpha}$	$[\theta]^{C_\beta}$	$[\theta]^{av\,a}$	[θ]
−5	34.1	32.0	38.5	34.9	
0	34.1	32.0	38.5	34.9	
5	30.9	28.1	38.5	32.5	33.3
10	29.2	24.3	38.5	30.7	
15	27.6	24.3	35.1	29.0	
21					26.5
25	21.1	24.3	28.1	24.5	
30					22.0
35	16.2	20.8	24.7	20.6	
40	14.6	18.9	24.7	19.4	

[a] $[\theta]^{av}$ represents the value averaged over $[\theta]^{C=O}$, $[\theta]^{C_\alpha}$, and $[\theta]^{C_\beta}$. [b] Data taken from ref 6.

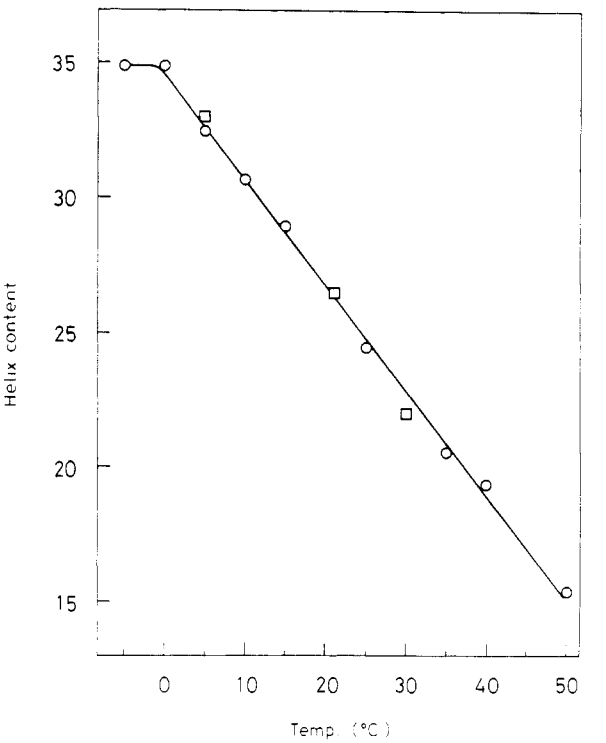

Figure 7. Temperature dependences of the α-helix contents [θ] in *P. c. ricini* (○) and *A. pernyi* (□)[6] liquid silks determined with ^{13}C NMR and CD methods, respectively.

where $[\theta]_t^{C=O}$ is the α-helix content at *t* (°C), determined from the carbonyl resonance. The chemical shifts, in ppm, of the Ala carbonyl carbons in the helical state (at −5 °C), in the coil state, and at *t* are represented as $\delta_h^{C=O}$, $\delta_c^{C=O}$, and $\delta_t^{C=O}$, respectively. Notations for the chemical shifts of the C_α and C_β carbons are similar to those of the carbonyl carbon. $A_{C=O}^{h+h^*}$ and $A_{C=O}^{tot}$ are the area of the Ala carbonyl peaks h + h* and the total area of the Ala carbonyl peaks, respectively. F_A is the alanine content determined from the carbonyl peak region. The peak areas were determined from the NMR spectrum with no NOE effect observed at 25 °C, as described in the Experimental Section. Table III lists the α-helix content of *P. c. ricini* silk fibroin determined from the ^{13}C NMR spectra as a function of temperature together with those of *A. pernyi* silk fibroin determined from the CD spectra.[6] The helix content of *P. c. ricini* obtained from every carbon resonance differs somewhat from each other, which arises mainly from the differences in the temperature dependence of the chemical shifts among them, as described above. However, the average values, $[\theta]_t^{av}$ are in *quanti-*

tative agreement with those of *A. pernyi*, obtained from the CD spectra, at any temperature (Figure 7). Taking into account the close similarity in the amino acid composition of the silk fibroins between *P. c. ricini* and *A. pernyi*, this quantitative agreement between the ^{13}C NMR and CD data concerning the α-helix content supports strongly our assignment of the *P. c. ricini* silk fibroin spectra, including that of the peak doubling. Thus, it is concluded that the helix–coil transition of *P. c. ricini* silk fibroin protein occurs locally in the $-Ala_n-$ portion whose chain lengths are great enough to form an α-helix. Actually, Kricheldorf et al.[30,31] reported the ^{13}C CP–MAS NMR chemical shifts of the Gly and Tyr residues involved in the α-helix conformation as 172.1 and 176.7 ppm, respectively. We could not observe such peaks in the corresponding resonance region of *P. c. ricini* silk fibroin (see spectra B and C in Figures 2 and 4). The change in the helix content over the range 0–40 °C is linear rather than sigmoidal, indicating "weak cooperativity" throughout the helix–coil transition.[32]

Acknowledgment. This work was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan.

References and Notes

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