Registry No. SPS, 28325-75-9; IPS, 25086-18-4; APS, 9003-53-6.

References and Notes

(1) Ishihara, N.; Seimiya, T.; Kuramoto, M.; Uoi, M. *Macromolecules* **1986**, *19*, 2464.
(2) Natta, G.; Danusso, F.; Moraglio, G. *Makromol. Chem.* **1958**, *28*, 166.
(3) Natta, G.; Corradini, P.; Bassi, I. W. *Nuovo Cimento, Suppl.* **1960**, *15*, 68.
(4) Kobayashi, M.; Nakaoki, T.; Uoi, M. *Rep. Progr. Polym. Phys. Jpn.* **1988**, *31*, 481; *Polym. Prepr. Jpn.* **1988**, *37*, 2552.
(5) Immirzi, A.; de Candia, F.; Iannelli, P.; Zambelli, A.; Vittoria, V. *Makromol Chem., Rapid Commun.* **1988**, *9*, 761.
(6) Vittoria, V.; de Candia, F.; Iannelli, P.; Immirzi, A. *Makromol. Chem., Rapid Commun.* **1988**, *9*, 765.
(7) Kobayashi, M.; Nakaoki, T.; Ishihara, N., to be published in *Macromolecules*.
(8) Shimane, Y.; Ishioka, T.; Chatani, Y.; Ijitsu, T. *Polym. Prepr. Jpn.* **1988**, *37*, 2534.
(9) Natta, G.; Pasquon, I.; Corradini, P.; Peraldo, M.; Pegorano, M.; Zambelli, A. *Atti Accad. Naz. Lincei, Cl. Sci. Fis., Mat. Nat., Rend.* **1960**, *28*, 539.
(10) Natta, G.; Peraldo, M.; Allegra, G. *Makromol. Chem.* **1964**, *75*, 215.
(11) Tadokoro, H.; Kobayashi, M.; Kobayashi, S.; Yasufuku, K.; Mori, K. *Rep. Progr. Polym. Phys. Jpn.* **1966**, *9*, 181.
(12) Kobayashi, M.; Akita, K.; Tadokoro, H. *Makromol. Chem.* **1968**, *113*, 324.
(13) Jasse, B.; Koenig, J. L. *J. Polym. Sci., Polym. Phys. Ed.* **1979**, *17*, 799.
(14) Jasse, B.; Chao, R. S.; Koenig, J. L. *J. Raman Spectrosc.* **1979**, *8*, 244.
(15) Girolamo, M.; Keller, A.; Miyasaka, K.; Overbergh, N. *J. Polym. Sci., Polym. Phys. Ed.* **1976**, *14*, 39.
(16) Atkins, E. D. T.; Issac, D. H.; Keller, A.; Miyasaka, K. *J. Polym. Sci., Polym. Phys. Ed.* **1977**, *15*, 211.
(17) Atkins, E. D. T.; Issac, D. H.; Keller, A. *J. Polym. Sci., Polym. Phys. Ed.* **1980**, *18*, 71.
(18) Painter, P. C.; Kessler, R. E.; Snyder, R. W. *J. Polym. Sci., Polym. Phys. Ed.* **1980**, *18*, 723.
(19) Kobayashi, M.; Tsumura, K.; Tadokoro, H. *J. Polym. Sci., Polym. Phys. Ed.* **1968**, *6*, 1493.
(20) Schaufelle, R. F. *J. Chem. Phys.* **1968**, *49*, 4168.
(21) Snyder, R. G. *J. Chem. Phys.* **1982**, *76*, 3921.
(22) Snyder, R. G.; Schlotter, N. E.; Alamo, R.; Mandelkern, L. *Macromolecules* **1986**, *19*, 621.
(23) Snyder, R. G.; Wunder, S. L. *Macromolecules* **1986**, *19*, 496.
(24) Snyder, R. G.; Krause, S. J.; Scherer, J. R. *J. Polym. Sci., Polym. Phys. Ed.* **1978**, *16*, 1593.
(25) Peraldo, M.; Cambini, M. *Spectrochim. Acta* **1965**, *21*, 1509.
(26) Schachtschneider, J. H.; Snyder, R. G. *Spectrochim. Acta* **1965**, *21*, 1527.

Dynamic Features of Side Chains in Tyrosine and Serine Residues of Some Polypeptides and Fibroins in the Solid As Studied by High-Resolution Solid-State ^{13}C NMR Spectroscopy

Hazime Saitô,*,† Mika Ishida,‡ Motoko Yokoi,† and Tetsuo Asakura‡

Biophysics Division, National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan, and Faculty of Technology, Tokyo University of Agriculture and Technology, Nakamachi 2-chome, Koganei, Tokyo, Japan.

Received November 29, 1988; Revised Manuscript Received April 5, 1989

ABSTRACT: We have recorded high-resolution ^{13}C NMR spectra of silk fibroins in the solid state from *Bombyx mori* and *Philosamia cynthia ricini* and appropriate model peptides and polypeptides to gain insight into the dynamic features of side-chain groups of Tyr and Ser residues. First, ^{13}C NMR peaks of Tyr residues were identified, except for the peak of C_β, by comparing ^{13}C NMR peaks of the *B. mori* fibroin with those of the crystalline fraction whose Tyr residue is reduced to less than 2%. It turned out that the phenolic ring of Tyr residues undergoes a flip-flop motion with a rate constant of $>10^2$ s^{-1}, as inferred from the coalesced single peaks of Tyr C_ϵ. The aromatic side chains of $(Tyr)_n$ and $(Phe)_n$, however, undergo rotational diffusion with a correlation time on the order of 10^{-8} s, as manifested from selective reduction of the ^{13}C spin–lattice relaxation time of the laboratory frame (T_1^C). The shortened T_1^C values (<1 s) of Ala C_β in $(Ala)_n$ and fibroins were ascribed to the presence of C_3 rotation. The T_1^C of Ser C_β in $(Ser)_n$ and fibroins appears to arise from two different relaxation processes, a shorter (0.6–1 s) and a longer (10–40 s) component. The former and latter were obviously ascribed to the presence of free hydroxymethyl groups and those hydrogen bonded to either carbonyl or other hydroxyl groups, respectively.

Introduction

It has been demonstrated[1-4] that the ^{13}C NMR signals of Ala, Gly, and Ser residues in silk fibroins are well resolved in the solid state, and the characteristic displacements of individual residues can be used as a convenient means for distinction of polymorphic structures in *Bombyx mori* and *Philosamia cynthia ricini* fibroins. This view is based on the fact that relative displacements of ^{13}C chemical shifts from individual amino acid residues in peptides, polypeptides, and proteins are in many instances independent of amino acid sequence and significantly vary (up to 8 ppm) with the local conformation of the amino acid residue under consideration.[5-16] The major advantage in using this solid-state NMR approach for conformational characterization is that NMR spectroscopy is a nondestructive means and can be equally applied to systems of noncrystalline as well as crystalline samples.

To make this NMR methodology more effective, it is essential to resolve and assign as many ^{13}C NMR signals as possible. No assignment of peaks, however, has been

† National Cancer Center Research Institute.
‡ Tokyo University of Agriculture and Technology.

0024-9297/90/2223-0083$02.50/0

made for the Tyr residue, despite the fact that Tyr plays an important role as an active site in the development of the silk fibroin fiber with a covalently immobilized enzyme[17,18] and also as a reaction site with the azo dyes such as Orange II.[19] This is because its relative proportion is low (4.8%)[20] as compared with those of Ala, Gly, and Ser residues in *B. mori* fibroin, and many of its aromatic peaks were overlapped with the spinning sidebands of the carbonyl peaks. The latter contribution, however, is now easily removed by the pulse sequence of the total suppression of spinning sidebands (TOSS).[21]

Furthermore, this NMR approach can be extended to analyze dynamic features of silk fibroins, especially the manner of side-chain orientation or motion, either by examination of line shape or by means of relaxation parameters of NMR spectroscopy. Analysis of the molecular motion of the fibroin in the solid is very important in relation to understanding their physical state as mentioned above. To this end, we have previously showed on the basis of an ^2H NMR study of [2-*Ser*-$^2H_2$]- and [2-*Ala*-$^2H_3$]-fibroin that the majority of the Ser C_β hydroxymethyl group is hydrogen bonded to either hydroxyl or carbonyl groups, whereas the C_β methyl group of Ala residues undergoes rapid C_3 rotation on the NMR time scale.[22] Examination of the ^2H NMR line shape of the powder pattern permits one to study dynamic features on the intermediate time scale of 10^{-5}–10^{-6} s such as in the flip-flop motion of the phenolic ring in Tyr.[23,24] Much slower motion can be detected by chemical exchange of isotropic peaks.[25] On the other hand, more rapid motion such as rotational diffusion with a time scale of 10^{-8} s can be monitored by ^{13}C spin–lattice relaxation times of the laboratory frame.

In this paper, we recorded ^{13}C NMR spectra of a variety of fibroin samples as well as a number of model peptides and polypeptides to examine the presence or absence of flip-flop motion in the phenolic ring of Tyr residues. We further extended this NMR approach to study main-chain or side-chain motions of fibroins and some polypeptides by examination of ^{13}C spin–lattice relaxation times (T_1^Cs) of the laboratory frame.

Experimental Section

Poly(L-tyrosine) (Sigma Type II, P-8523), poly(L-phenylalanine) (Sigma, P-8254) and poly(L-serine) (Sigma, P-5887) were used without further purification. Preparation and spectral characterization of poly(L-alanine) (PLA-200) and Z-(L-Ala)$_8$-NH-(CH$_2$)$_3$CH$_3$ were described previously.[9] L-Tyrosylglycine (T-5254), *N*-acetyl-L-tyrosine ethyl ester (A-6751), *N*-acetyl-L-tyrosinamide (A-6626), and L-tyrosinamide (T-3879) were purchased from Sigma Chemical Co. Preparation of *N*-acetyl-L-tyrosine methylamide was previously described.[26]

Cocoons of *B. mori* and *P. c. ricini* were obtained in our laboratory. The *B. mori* fibroin was degummed with 0.5% citric acid solution. The crystalline (Cp) fraction was obtained by digestion of the aqueous solution of the degummed fibroin by chymotrypsin, followed by collection of the resulting precipitates.[2,27] An oligopeptide model of the Cp fraction, Boc-(Gly-Ser-Gly-Ala-Gly-Ala)$_2$-OBzl,[1] was a generous gift from Professor Mitsuaki Narita of Tokyo University of Agriculture and Technology. Highly crystalline silk I preparation was obtained from precipitates resulting from the dialysis of a 9 M LiBr solution of the Cp fraction against distilled water containing CH$_3$COOH (pH <5). The silk I form was confirmed by X-ray powder diffraction.[3] The α-helical *P. c. ricini* fibroin was obtained by casting liquid silk directly taken from the posterior silk gland over PMMA plates.[28]

The 75.46-MHz single contact cross polarization–magic angle spinning (CP-MAS) ^{13}C NMR spectra were recorded on a Bruker CXP-300 spectrometer equipped with a Bruker CP-MAS accessory. Powdered samples or film cut into small pieces was placed in an alumina syrindrical rotor and spun as fast as 2.8 kHz. The 90° pulse, contact, and repetition times were 3.5 μs, 1 ms, and 4 s, respectively. Chemical shifts were calibrated indirectly through the ^{13}C chemical shift of the glycine carboxyl peak (176.03 ppm) with respect to tetramethylsilane. Spinning sidebands were removed by the method of TOSS,[21] except for the T_1^C measurements. ^{13}C spin–lattice relaxation times of the laboratory frame were measured by a pulse sequence of the proton-enhanced sequence of the T_1^C measurements by Torchia.[29] T_1^C values are evaluated by plotting log $M_{cp}(t)$ vs t (delay time) based on the following formula

$$M_{cp}(t) = 2M_{cp}(0)\exp(-t/T_1^C) \quad (1)$$

where $M_{cp}(t)$ and $M_{cp}(0)$ stand for the ^{13}C NMR peak intensities at the times t and 0, respectively. Spectra were usually accumulated 700–1500 times, except for those used for the measurements of the spin–lattice relaxation times. For the latter, 80–150 transients were accumulated for each trace with a different delay time.

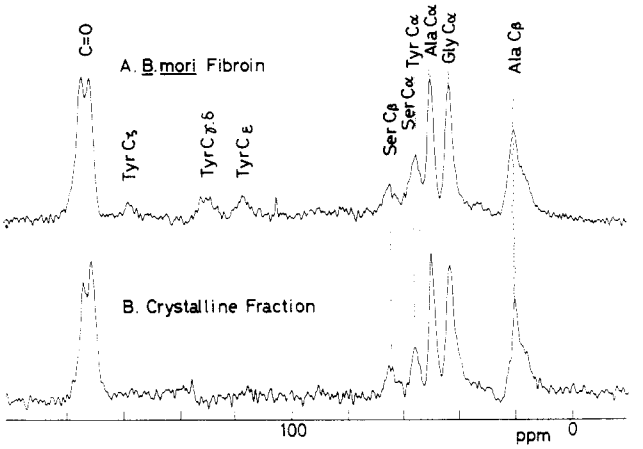

Figure 1. 75.46-MHz ^{13}C NMR spectra of the *B. mori* fibroin (A) and its crystalline (Cp) fraction (B) in the solid state.

Results

Figure 1 illustrates ^{13}C NMR spectra of the *B. mori* fibroin (A) and the Cp fraction (B) whose Tyr residue was reduced to less than 2% as a result of digestion by chymotrypsin at the Tyr residue.[2,27] The peak positions of Tyr C_α and C=O (downfield portion of well-resolved doublet) are evident as shown by significantly decreased peak height of Ser/Tyr C_α in Figure 1B as compared with that in Figure 1A. The other peaks $C_{\gamma\delta}$, C_ϵ, and C_ζ are straightforwardly assigned on the basis of the peak assignment in the solution state,[30,31] although C_β signals are invisible.[32] Figure 2 illustrates ^{13}C NMR spectra of (Tyr)$_n$ and related Tyr-containing peptides and amino acid derivatives. The ^{13}C chemical shifts of the aromatic side chain in these compounds and their peak separation were summarized in Table I. It is easy to distinguish the Tyr C_δ and C_ϵ peaks because they are split into doublet peaks as seen in Tyr-Gly, Tyr-NH$_2$, Ac-Tyr-NH$_2$, Ac-Tyr-NHMe (spectrum not shown) and Tyr-OH (see also Table I). These peaks, however, were singlets in (Tyr)$_n$ and Ac-Tyr-OEt. Note that the C_δ signal is resonated at a higher field than that of C_γ signal in Ac-Tyr-OEt.

Figure 3 illustrates a typical example of a stacked plot of ^{13}C NMR peak intensities of (Ser)$_n$ as obtained by the proton-enhanced sequence of T_1^C measurements as proposed by Torchia[29] together with the delay times indicated on the right-hand side. Semilog plots of the peak intensities $M_{cp}(t)$ vs t gave rise to single straight lines, except for Ser C_β (Figure 4). The plot of Ser C_β signals from both (Ser)$_n$ and silk I is not composed of a single

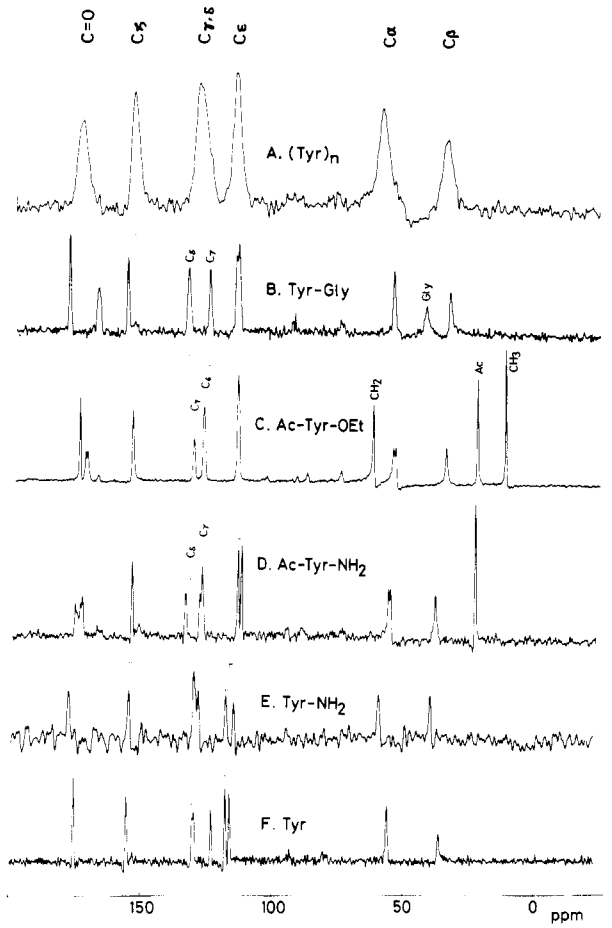

Figure 2. 75.46-MHz ^{13}C NMR spectra of $(Tyr)_n$ (A) and Tyr-containing peptides (B–F).

Table I
^{13}C Chemical Shifts (ppm) of Aromatic Side Chains of Tyr Residues in the Solid State

	C_γ	C_δ	Δ^a	C_ϵ	Δ^a	C_ζ
$(Tyr)_n$	128.4	128.4		115.0		154.4
Tyr-Gly	126.0	136.0		115.9	0.9	157.5
				115.0		
Ac-Tyr-OEt	132.1	128.5		115.4		155.3
Tyr-NH_2	128.4	130.0		114.7	2.9	154.8
				117.6		
Ac-Tyr-NH_2	128.1	129.0	5.4	112.7	1.4	154.5
		134.4		114.1		
Ac-Tyr-NHMe	125.9	130.1	2.7	112.7	4.3	154.0
		132.8		117.0		
Tyr-OH	123.6	130.1	0.6	116.4	1.6	155.4
		130.7		118.0		

[a] Separation of peaks.

line but a composite of two lines with shorter and longer T_1^C. The shorter T_1^C was obtained by a plot of peaks (closed circles) after contributions of the longer T_1^C were subtracted (closed squares). The remaining peaks, however, were found to decay as approximated by single lines. We measured the T_1^C values of $(Ala)_n$, $(Phe)_n$, and $(Tyr)_n$, adopting either α-helical or β-sheet forms, as summarized in Table II. The T_1^C values of *B. mori* and *P. c. ricini* fibroins and model oligopeptides of the crystalline fraction of the *B. mori* fibroin are summarized in Table III. In all cases, the T_1^C curves of Ser C_β are not approximated by a single line but by a composite of two lines, with shorter and longer T_1^C values.

Discussion

Slow Side-Chain Motions of Phenolic Rings in Tyr Residues. It is noteworthy that the C_δ and C_ϵ signals of

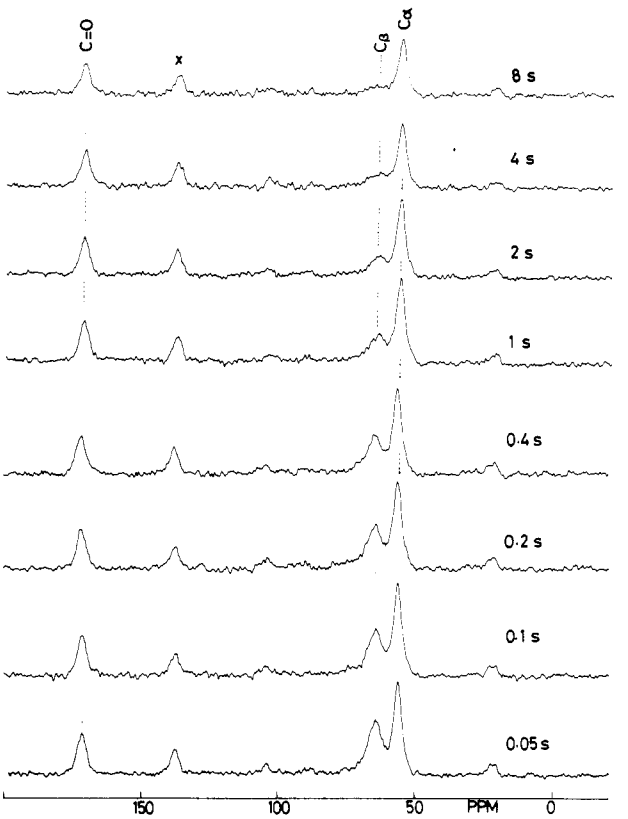

Figure 3. Stacked plot of ^{13}C NMR spectra of $(Ser)_n$ recorded by the cross-polarization enhanced pulse sequence for T_1^C measurements.

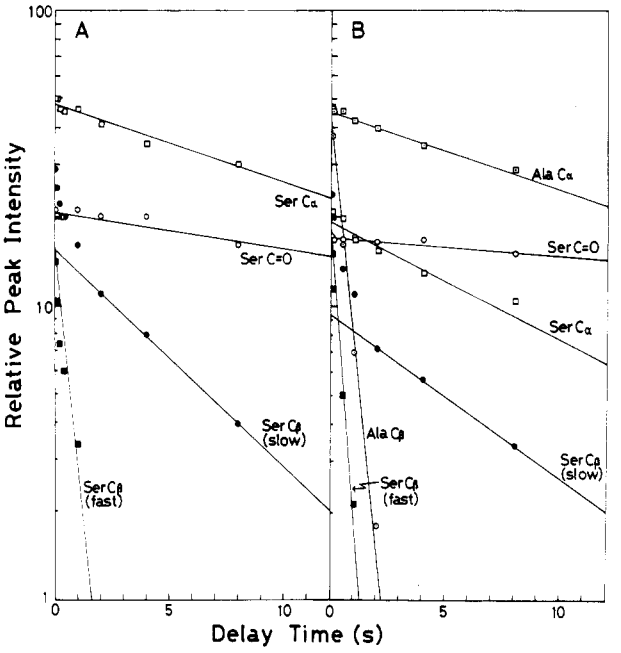

Figure 4. Plot of relative ^{13}C peak intensities of $(Ser)_n$ (A; Figure 3) and silk I form fibroin (B) vs delay time. Plots of peak intensities for Gly residue were omitted in Figure 4B. For separation of Ser C_β into two components, see text.

Tyr-OH, Ac-Tyr-NH_2, Ac-Tyr-NHMe, Tyr-NH_2, and Tyr-Gly are split into doublets, the separations of which being 0.9–4.3 ppm and 0–5.4 ppm, respectively, depending upon a variety of molecular structures.[31] Similar peak splittings were previously noted for a number of para-substituted methoxybenzenes and phenols[33] as well as poly(phenylene oxide).[34] These splittings, however, are averaged to give single lines in solution because of the rapid

Table II
^{13}C Spin-Lattice Relaxation Times (s) of the Laboratory Frame in Some Polypeptides in the Solid State

	conformatn[a]	C_α	C_β	C_γ	C_δ	C_ϵ	C_ζ	C=O
$(Ala)_n$	α-helix	14	0.52					32
	β-sheet	13	0.84					46
$(Ser)_n$	β-sheet	16	0.63 6.1					33
$(Tyr)_n$	α-helix	10	8.7	*b*	*b*	4.1	20	*b*
$(Phe)_n$	α-helix	15	8	7.8	2.4[c]	2.4[c]	2.4[c]	*b*

[a] Determined by characteristic displacements of ^{13}C chemical shifts (ref 6). [b] Overlapped with spinning sidebands from quartanary carbons. [c] Not resolved.

Table III
The Observed T_1^C Values (s) of Silk Fibroins from Various Sources

	C_α			C_β		C=O		
	Ala	Gly	Ser	Ala	Ser	Ala	Gly	Ser
silk I								
Cp fraction	15	25	13	0.54	0.50[a] 8.5[b]	48	57	38
silk II								
Cp fraction	14	20	11	0.53	0.56[a] 31[b]	20[c]	46	20[c]
lyophilized	17	22	18	0.33	0.63[a] 37[b]	32[c]	51	32[c]
Boc-$(GSGAGA)_2$-OBzl[d]	11	15	12	0.38	1.1[a] 25[b]	14[c]	36	14[c]
α-helix (*P. c. ricini*)								
cast film	16	13		0.98		24	33	
β-sheet (*P. c. ricini*)								
Cp fraction	15	17		0.82		19[c]	19[c]	19[c]

[a] Shorter T_1^C component. [b] Longer T_1^C component. [c] Not resolved. [d] Boc-(Gly-Ser-Gly-Ala-Gly-Ala)$_2$-OBzl.

internal rotation of the C–O bond or of the aromatic ring. The cause of such splitting was previously explained in terms of a compression shift due to a van der Waals interaction between one of the ortho carbons and the methyl (or methylene) carbon.[25] However, it is difficult to account for the existence of a peak separation between two meta carbons (see Table I) by means of this type of steric interaction. In this connection, we have previously pointed out, on the basis of an ab initio molecular orbital calculation, that a nonequivalence in the electron distribution between two carbons in ortho and metal positions caused by the methoxyl or hydroxyl groups partly accounts for the above-mentioned peak splittings.[33] Nevertheless, the observation of such differential peak separations in Tyr C_δ and C_ϵ signals of a variety of compounds as mentioned above suggests that differences in the stereochemical environment around the Tyr residue play an important role in determining their ^{13}C chemical shifts from both steric interaction and nonequivalence in the electron distribution.

Such separation of peaks would diminish or disappear, even in the solid state, when the flip-flop motion or continuous diffusion of the aromatic ring occurs. The presence of the former motion can be visualized by the observation of coalescence of the doublet pattern into a single peak, if the exchange rate (k) is faster than the frequency of the peak separation δ_{AB}[35]

$$k > \pi/\sqrt{2}\delta_{AB} \quad (2)$$

Accordingly, the rate constant for the flip-flop motion of the Tyr side chain is estimated to be larger than 10^2 s^{-1}, if the peak splitting of Tyr C_δ or C_ϵ carbons without such motion is assumed to be 1–5 ppm as in the cases of the data in Table I. Hence, the observed sharp singlet peak in C_δ and C_ϵ carbons of Ac-Tyr-OEt can be accounted for by this view. Undoubtedly, the presence (Ac-Tyr-OEt) or absence (Ac-Tyr-NH_2) of such motion depends on the manner of molecular crowdedness around the Tyr residues. In a similar manner, we previously noted that the Tyr C_ϵ peak is observed as a doublet peak in the extended form of [Met^5]- and [Leu^5]-enkephalins in the solid state, whereas these peaks were coalesced into a singlet peak in the β-bend form of [Leu^5]-enkephalin,[36] suggesting the presence of such flip-flop motion in the crystalline state.

Rapid Motions As Studied by ^{13}C Spin-Lattice Relaxation Times. There appears no significant difference in the ^{13}C T_1^C values of $(Ala)_n$ between the α-helix and β-sheet forms (Table II). In addition, the T_1^C values of Ala and Ser C_α carbons are very similar (10–18 s) among the polypeptides and fibroins used, irrespective of the difference in the primary and secondary structures (Tables II and III). Further, the T_1^C values of Gly C_α in fibroin are slightly longer (20–25 s) than those of Ala and Ser residues. This is not unexpected because there exists no rapid backbone motion whose correlation time is on the order of 10^{-8} s, which is effective as a relaxation pathway. Much slower motion, if any, cannot be detected by this sort of relaxation times of the laboratory frame. Therefore, spin–lattice relaxation times of backbone carbons in these polypeptides and proteins are mainly determined by dipolar couplings with proton(s) undergoing rapid intramolecular reorientation such as the C_3 rotation of the methyl group. The effectiveness of this relaxation pathway vary with a function of r^{-6} where r is the distance between carbon and proton in question. Therefore, this effect can be visualized by the observation of a gradual reduction of the T_1^C values from the carbons near the backbone to the site nearer to the methyl or other groups undergoing internal motions.[36] The observation of such a T_1^C gradient is a very useful means for confirming assigned peaks.[36]

It is noteworthy that the C_ϵ T_1^C of $(Tyr)_n$ is very short (4 s) as compared with that of C_ζ (Table II). Obviously,

the existence of such shorter T_1^C values in the aromatic side chain as compared with those of the backbone is well explained by the onset of rapid rotational diffusion about the C_γ–C_ζ axis with a correlation time on the order of 10^{-8} s. The C_ζ T_1^C is indifferent to the presence of this motion because no proton is attached to this carbon. In a similar manner, the T_1^C values of C_δ and C_ϵ carbons (not resolved) in $(Phe)_n$ are substantially reduced (2.4 s) as compared with that of C_γ (7.8 s).[38] Thus, it appears that intermolecular interactions between aromatic side-chain groups are very weak in the α-helix form so as to allow continuous diffusion in $(Tyr)_n$ or $(Phe)_n$. Nevertheless, such rapid motion of the side chain in Tyr and Phe residues does not occur in amino acids or oligopeptides of better crystalline packing. In fact, such reduction of ^{13}C T_1^C was noted in the phenolic ring in Tyr in the case of Ac-Tyr-OEt (data not shown), although flip-flop motion results in an averaging of the chemical shift nonequivalence, as described above.[39]

As summarized in Table II and III, the T_1^C values of Ser C_β are observed as a composite of two components in all samples of fibroin and $(Ser)_n$, irrespective of the differences in the conformations.[40] Undoubtedly, the component, which gives rise to a longer T_1^C value, is ascribed to hydroxymethyl groups participating in various types of hydrogen bonds because the spin–lattice relaxation times in the solid are at the low-temperature site. On the basis of the survey of X-ray diffraction data on many globular proteins, Janin et al.[41] and Gray and Matthews[42] showed that approximately 70% of the Ser residue have their O_γ atom within hydrogen-bonding distance (3.5 Å) of at least one carbonyl group, with an acceptable angular geometry for the OH···O bond. The presence of such hydroxymethyl groups in the Ser residue in the *B. mori* fibroin was previously ascribed to a component that gives rise to a static ^2H powder pattern spectra of [*Ser*-^2H]-fibroin with a quadrupole splitting of 125 kHz (~70%).[22,43] On the other hand, the remaining component (~30%), giving rise to reduced quadrupole splittings of less than 35 kHz in ^2H NMR spectra, was ascribed to the Ser hydroxymethyl group which undergoes rapid internal rotation about an axis of C_β–C_γ bond and is not involved in any type of hydrogen bonding. Therefore, it is now clear that the above-mentioned rapidly relaxing component of the Ser residue is undoubtedly ascribed to the Ser group not involved in any type of hydrogen bonding. The relative proportion of this component is roughly estimated as less than 50% on the basis of the relative peak intensity extrapolated at time zero (see Figure 4), although quantitative evaluation of the relative proportion from this plot is not easy because of several types of uncertainty involved. In any case, it is interesting to note that such an estimated value is roughly in accordance with the data from ^2H NMR powder pattern of [*Ser*-^2H]-fibroin, as described above.

The presence of such a free Ser hydroxymethyl group as a component giving rise to rapidly relaxing species, however, seems to be very important as a site for hydration in stabilizing the silk I form. In other words, a solvent-induced conformational change could be associated with the destabilization of the silk I or silk I type form as a result of dehydration, as will be discussed in more detail in the following paper.[44]

Concluding Remarks

We found that examination of ^{13}C line-shape and spin–lattice relaxation times of silk fibroin and related model polypeptides gives us a clue about slow or rapid flip-flop motion or continuous diffusion of the phenolic side chain of the Tyr residue in the solid. The spin–lattice relaxation times of fibroin, however, is found to be determined by dipolar coupling with protons rapidly undergoing internal rotation such as the methyl group of the Ala residue or the hydroxymethyl group of the Ser residue and is not by any type of backbone motion. The presence of the latter type of side chain in the Ser residue was observed in both forms of the *B. mori* fibroin, which might play an important role in the stabilization or destabilization of the silk I type form, leading to a solvent-induced conformational transition.

Registry No. Tyr-Gly-OH, 673-08-5; Ac-Tyr-OEt, 840-97-1; $Tyr-NH_2$, 4985-46-0; $Ac-Tyr-NH_2$, 1948-71-6; Ac-Tyr-NHMe, 6367-14-2; Tyr-OH, 60-18-4; $BOC-(GSGAGA)_2-OBzl$, 123621-17-0; Ser-OH, 56-45-1; Tyr homopolymer, 25619-78-7; Tyr (SRU), 25667-16-7; Ala homopolymer, 25191-17-7; Ala (SRU), 25213-34-7; Ser homopolymer, 25821-94-7; Phe homopolymer, 25191-15-5; Phe (SRU), 25248-59-3.

References and Notes

(1) Saitô, H.; Iwanaga, Y.; Tabeta, R.; Narita, M.; Asakura, T. *Chem. Lett.* **1983**, 431.
(2) Saitô, H.; Tabeta, R.; Asakura, T.; Iwanaga, Y.; Shoji, A.; Ozaki, T.; Ando, I. *Macromolecules* **1984**, *17*, 1405.
(3) Asakura, T.; Kuzuhara, A.; Tabeta, R.; Saitô, H. *Macromolecules* **1985**, *18*, 1841.
(4) Kricheldorf, H. R.; Muller, D.; Ziegler, D. *Polym. Bull. (Berlin)* **1983**, *9*, 284.
(5) Saitô, H.; Tabeta, R.; Shoji, A.; Ozaki, T.; Ando, I.; Asakura, T. In *Magnetic Resonance in Biology and Medicine*; Govil, G., Kheterapal, C. L., Saran, A., Eds.; Tata McGraw-Hill: New Delhi, 1985; pp 195–215.
(6) Saitô, H. *Magn. Reson. Chem.* **1986**, *24*, 835.
(7) Saitô, H.; Ando, I. *Annu. Rep. NMR Spectrosc.* **1989**, *21*, 209.
(8) Taki, T.; Yamashita, S.; Satoh, M.; Shibata, A.; Yamashita, T.; Tabeta, R.; Saitô, H. *Chem. Lett.* **1981**, *1803*.
(9) Saitô, H.; Tabeta, R.; Shoji, A.; Ozaki, T.; Ando, I. *Macromolecules* **1983**, *16*, 1050.
(10) Saitô, H.; Tabeta, R.; Ando, I.; Ozaki, T.; Shoji, A. *Chem. Lett.* **1983**, 1437.
(11) Ando, I.; Saitô, H.; Tabeta, R.; Shoji, A.; Ozaki, T. *Macromolecules* **1984**, *17*, 457.
(12) Shoji, A.; Ozaki, T.; Saitô, H.; Tabeta, R. Ando, I. *Macromolecules* **1984**, *17*, 1472.
(13) Saitô, H.; Tabeta, R.; Shoji, A.; Ozaki, T.; Ando, I.; Miyata, T. *Biopolymers* **1984**, *23*, 2279.
(14) Muller, D.; Kricheldorf, H. R. *Polym. Bull. (Berlin)* **1983**, *9*, 284.
(15) Kricheldorf, H. R.; Muller, D. *Macromolecules* **1983**, *16*, 615.
(16) Kricheldorf, H. R.; Mutter, M.; Mazer, F.; Forster, D. *Biopolymers* **1983**, *22*, 1357.
(17) Asakura, T. *Bioindustry* **1987**, *4*, 36.
(18) Asakura, T.; Kanetake, J.; Demura, M. *Polym.-Plast. Manufact. J.* **1989**, *28*, 453.
(19) Asakura, T.; Ishida, M. *J. Colloid Interface Sci.* **1989**, *130*, 184.
(20) Shimura, K. In *Zoku Kenshi no Kozo (Structure of Silk Fibers)* Hojo, N., Ed.; Shinshu University: Ueda, 1980.
(21) Dixon, W. T. *J. Chem. Phys.* **1982**, *77*, 1800.
(22) Saitô, H.; Tabeta, R.; Kuzuhara, A.; Asakura, T. *Bull. Chem. Soc. Jpn.* **1986**, *59*, 3383.
(23) Rice, D. M.; Witterbort, R. J.; Griffin, R. G.; Meirovitch, E.; Stimson, E. R.; Meinwald, Y. C.; Freed, J. H.; Scheraga, H. A. *J. Am. Chem. Soc.* **1981**, *103*, 7707.
(24) Gall, G. M.; DiVerdi, J. A.; Opella, S. J. *J. Am. Chem. Soc.* **1981**, *103*, 5039.
(25) Garroway, A. N.; Ritcheny, W. M.; Moniz, W. L. *Macromolecules* **1982**, *15*, 1051.
(26) Asakura, T.; Watanabe, Y.; Uchida, A.; Minagawa, H. *Macromolecules* **1984**, *17*, 1075.
(27) Asakura, T.; Watanabe, Y.; Itoh, T. *Macromolecules* **1984**, *17*, 2421.
(28) Asakura, T.; Murakami, T. *Macromolecules* **1985**, *18*, 2614.
(29) Torchia, D. A. *J. Magn. Reson.* **1978**, *30*, 613.
(30) Howarth, O. W.; Lilley, D. M. J. *Prog. Nucl. Magn. Reson. Spectrosc.* **1978**, *12*, 1.
(31) Peak positions of C_ϵ and C_δ are greatly displaced among the compounds studied. Such displacement of peaks might be affected by a relative orientation of the phenol ring with respect to the C_α–C_β bond and/or the rest of the backbone moiety.

(32) The reason why the peak intensity of the Tyr C_β signal is so low is at present not clear. However, it is probable that such a loss of peak intensity is associated with the presence of molecular motion at the Larmor frequency of the rotating frame corresponding to the amplitude of the proton decoupling field (VanderHart, D. L.; Earl, W. L.; Garroway, A. N. *J. Magn. Reson.* **1981**, *44*, 361). It is also pointed out that no appreciable Tyr C_β signal was observed due to freezing of such motion at a temperature of 226 K, whereas the peak intensity of the Ala C_β signal was substantially decreased owing to the partial freezing of the C_3 rotation (Ishida, M. MS thesis, Tokyo University of Agriculture and Technology, 1989).
(33) Saitô, H.; Yokoi, M.; Aida, M.; Kodama, M.; Oda, T.; Sato, Y. *Magn. Reson. Chem.* **1988**, *26*, 155.
(34) Schaefer, J.; Stejskal, E. O.; Buchdahl, R. *Macromolecules* **1977**,*10*, 384.
(35) Pople, J. A.; Scheider, W. G.; Bernstein, H. J. *High-resolution Nuclear Magnetic Resonance*; McGraw-Hill: New York, 1959.
(36) Saitô, H.; Tabeta, R. In *Peptide Chemistry 1987*; Shiba, T., Sakakibara, S., Eds.; Protein Research Foundation: Osaka, 1988; pp 53.
(37) Saitô, H.; Tabeta, R.; Yokoi, M. *Magn. Reson. Chem.* **1988**, *26*, 775.
(38) The C_ζ signal is also not resolved from the C_δ and C_ϵ signals.
(39) Measurements of Tyr C_ϵ in fibroin samples were not easy because of lower amino acid composition (ca. 4%) and accidential overlap with sidebands from an intense carbonyl signal. The ^{13}C T_1^C value of lyophilized silk II sample turned out to be on the order of 28 s. This finding indicates that the Tyr residue in fibroin is located in an environment of a more packed state than in $(Tyr)_n$. In fact, the rate of flip-flop motion of this sample, if any, is much longer than 10^{-8} s but shorter than 10^{-2} s.
(40) This sort of deflection in the peak intensity of $(Ser)_n$ is not ascribed to any instrumental artefact because there exists no such deflection in the similar plot of Ala C_β whose T_1^C is of the same magnitude with that of Ser C_β.
(41) Janin, J.; Wodak, S.; Levit, M.; Maigret, B. *J. Mol. Biol.* **1978**, *125*, 357.
(42) Gray, T.; Matthews, B. W. *J. Mol. Biol.* **1984**, *175*, 75.
(43) Asakura, T.; Yamaguchi, T. *J. Serc. Soc. Jpn.* **1987**, *56*, 300.
(44) Ishida, M.; Asakura, T.; Yokoi, M.; Saitô, H. *Macromolecules*, following paper in this issue.

Solvent- and Mechanical-Treatment-Induced Conformational Transition of Silk Fibroins Studied by High-Resolution Solid-State ^{13}C NMR Spectroscopy

Mika Ishida,† Tetsuo Asakura,† Motoko Yokoi,‡ and Hazime Saitô*,‡

Faculty of Technology, Tokyo University of Agriculture and Technology, Nakamachi 2-chome, Koganei, Tokyo, Japan, and Biophysics Division, National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo, Japan. *Received November 29, 1988; Revised Manuscript Received April 5, 1989*

ABSTRACT: We have recorded ^{13}C NMR spectra of silk fibroins from *Bombyx mori* and *Philosamia cynthia ricini* to examine solvent- (or diluent-) or mechanical-treatment-induced conformational transition. First, ^{13}C NMR spectra of silk I and II forms of the crystalline (Cp) fraction of *B. mori* fibroin and of α-helix and β-sheet forms of *P. c. ricini* fibroin were recorded under improved spectral resolution than those previously reported. For the latter fibroin, two kinds of α-helical domains (stable and less stable α-helical domains consisting of longer and shorter Ala sequences, respectively) were distinguished in view of the substantial difference in the stability of helices between the solid and the solution. Hydration of *B. mori* fibroin resulted in a stabilization of silk I forms, as manifested from the significant narrowing of peaks without inducing conformational transition. In *P. c. ricini* fibroin, however, hydration caused a partial conformational change from the less stable α-helix to the β-sheet form, without any change in line widths. Furthermore, solvent-induced conformational change of *B. mori* fibroin from the "random coil" to the silk II form was examined and explained in terms of ease of dehydration by the solvent used. In addition, conformational change by mechanical treatments such as drawing and compression was also examined.

Introduction

Crystalline silk fibroins are known to exist in one of the polymorphs, either silk I or silk II and either α-helix or β-sheet forms, depending on the species of silkworms, *Bombyx mori* and *Philosamia cynthia ricini*, respectively.[1-4] Besides, the "random coil" form can be distinguished from the above-mentioned polymorphs in fibroins of noncrystalline samples. Previous works showed that a number of solvents (or diluents) or mechanical treatments induce conformational transition from the less stable "random coil" or silk I form to the more stable silk II (β-sheet) form.[5-7] Extensive study of such a process is very important to gain a better understanding of the relative stability of the respective forms as well as a plausible mechanism of induced conformational transition.

Recently, Asakura and co-workers attempted to utilize this sort of conformational transition of silk fibroins to develop an immobilized enzyme support system without inducing inactivation of entrapped enzymes.[8-11] Here, entrapment is facilitated by the partial formation of effective physical "cross-links" of polymers from aggregates of ordered β-sheet sequences. Effectiveness of this procedure is based on the fact that such conformational transition is induced by a gentle process such as solvent or mechanical treatment. For this purpose, it is important to clarify and to control various types of conformational

† Tokyo University of Agriculture and Technology.
‡ National Cancer Center Research Institute.

0024-9297/90/2223-0088$02.50/0