Conformational Characterization of Polypeptides in the Solid State As Viewed from the Conformation-Dependent ^{13}C Chemical Shifts Determined by the ^{13}C Cross Polarization/Magic Angle Spinning Method: Oligo(L-alanine), Poly(L-alanine), Copolymers of L- and D-Alanine, and Copolymers of L-Alanine with *N*-Methyl- or *N*-Benzyl-L-alanine

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ABSTRACT: ^{13}C chemical shifts of oligo(L-alanines) and poly(L-alanines) of various chain lengths were measured by the cross polarization/magic angle spinning (CP/MAS) method. It is found that ^{13}C chemical shifts of the C_α, C_β, and carbonyl carbons are significantly displaced depending on conformation, such as disordered, β-sheet, and α-helix forms, the conformations of which were also characterized by infrared spectroscopy and X-ray diffraction. The relative ^{13}C chemical shifts of the α-helix with respect to those of the β-sheet forms, Δ, are 4.2, −5.0, and 4.6 ppm for the C_α, C_β, and C=O carbons, respectively, and are qualitatively in agreement with the values obtained for $(Leu)_n$, $(Val)_n$, and $(Ile)_n$. Further, conformations of block and random copolymers consisting of L-alanine and D-alanine and also random copolymers of L-alanine with *N*-methyl- or *N*-benzyl-L-alanine were well characterized by examining the conformation-dependent ^{13}C chemicals shifts. These results were consistent with our findings by the infrared and X-ray diffraction methods. Especially, it was found for the random copolymers of L- and D-alanines that their microconformation in the solid state may be ascertained from the ^{13}C chemical shifts by the CP/MAS method. We have also compared the ^{13}C chemical shifts of the α-helix form with those of the random coil form obtained in CF_3COOD solution.

Introduction

^{13}C NMR spectroscopy has proven to be a very useful tool to probe the conformation and dynamics of proteins and peptides in solution.[1,2] It is essential to have reference data concerning the conformation-dependent ^{13}C chemical shifts of the individual amino acid residues under consideration and to establish how and to what extent ^{13}C chemical shifts are displaced upon folding. Therefore measurements of ^{13}C chemical shifts of homopolypeptides with particular conformations such as α-helical and β-sheet forms obviously provide one excellent source of reference data. Such a study in solution, however, has been limited to several α-helical polypeptides[3-11] because of solubility, and no data are available for β-sheet polymers. To overcome this limitation, we previously showed[12] that measurements of ^{13}C chemical shifts of solid polypeptides ($(Val)_n$, $(Ile)_n$, and $(Leu)_n$) by cross polarization/magic angle spinning (CP/MAS) NMR spectroscopy[13-16] offer an excellent alternative means to explore the conformation-dependent ^{13}C chemical shifts. The most obvious advantage of recording ^{13}C chemical shifts in the solid state is that conformation-dependent ^{13}C chemical shifts free from any conformational fluctuations are obtained from samples whose conformations are determined by X-ray diffraction[17] and by infrared and Raman spectroscopy.[18]

As a continuation of this approach, we report here a ^{13}C CP/MAS NMR study of oligo(L-alanines) and poly(L-alanines) to elucidate the conformation-dependent ^{13}C chemical shifts as a novel means to probe the conformational behavior of Ala residues in proteins and peptides in aqueous solution. Poly(L-alanines) are one group of polypeptides whose conformations have been extensively studied by various methods.[20-32] In particular, the conformations of the α-helix, β-sheet, and disordered forms in the solid state have been established by many authors.[20-27] In addition, solution properties of these compounds have been characterized by ^1H NMR and other techniques.[28-32] Therefore it is possible to correlate the ^{13}C chemical shifts of oligo(L-alanines) and poly(L-alanines) with particular conformations. Furthermore, we have extended this approach to probe conformational features of block and random copolymers consisting of L- and D-alanines and random copolymers of L-alanine with *N*-methyl- or *N*-benzyl-L-alanine. Recently, Müller and Kricheldorf published preliminary ^{13}C CP/MAS NMR spectra of solid polypeptides, including $(Ala)_n$ with $\overline{DP}$'s of 10, 20, 50, and >100.[19] The assignments of peaks are in agreement with ours, although no detailed chemical shifts were given.

Experimental Section

Materials. A series of oligo(L-alanines), Z-$(L\text{-}Ala)_n$-NH-$(CH_2)_3CH_3$ (n = 1–8, containing an n-butylamide group at the C-terminal residue and a benzyloxycarbonyl (Z) group at the N-terminal residue), and HBrH-$(L\text{-}Ala)_n$-$NH(CH_2)_3CH_3$ (n = 1–8) were synthesized according to the procedure of Fujie et al.[27] The synthetic route is shown in the following scheme.

$$\text{Z-(L-Ala)-OH} + CH_3(CH_2)_3NH_2 \xrightarrow[N\text{-methylmorpholine}]{(CH_3)_2CHCH_2OCOCl} \underset{\text{I}}{\text{Z-(L-Ala)-NH}(CH_2)_3CH_3} \xrightarrow{HBr/CH_3COOH} \underset{\text{I}'}{\text{HBr·H(L-Ala)-NH}(CH_2)_3CH_3} \quad \text{(i)}$$

$$\text{Z-(L-Ala)-OH} + \text{I}' \xrightarrow[N\text{-methylmorpholine}]{(CH_3)_2CHCH_2OCOCl} \underset{\text{II(1,1)}}{Z\text{-(L-Ala)}_2\text{-NH}(CH_2)_3CH_3} \xrightarrow{HBr/CH_3COOH} \underset{\text{II}'(1,1)}{HBr\cdot H\text{-(L-Ala)}_2\text{-NH}(CH_2)_3CH_3} \quad \text{(ii)}$$

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$$\text{Z-(L-Ala)-N}_3 + \text{II}'(1,1) \xrightarrow{(C_2H_5)_3N} \text{Z-(L-Ala)}_3\text{-NH(CH}_2)_3\text{CH}_3 \xrightarrow[\text{III}(1,2)]{\text{HBr/CH}_3\text{COOH}} \text{HBr·H-(L-Ala)}_3\text{-NH(CH}_2)_3\text{CH}_3 \text{ (iii)}$$
III′(1,2)

$$\text{Z-(L-Ala)-N}_3 + \text{III}'(1,2) \xrightarrow{(C_2H_5)_3N} \text{Z-(L-Ala)}_4\text{-NH(CH}_2)_3\text{CH}_3 \xrightarrow[\text{IV}(1,3)]{\text{HBr/CH}_3\text{COOH}} \text{HBr-H-(L-Ala)}_4\text{-NH(CH}_2)_3\text{CH}_3 \text{ (iv)}$$
IV′(1,3)

$$\text{Z-(L-Ala)}_3\text{-N}_3 + \text{II}'(1,1) \xrightarrow{(C_2H_5)_3N} \text{Z-(L-Ala)}_5\text{-NH(CH}_2)_3\text{CH}_3 \xrightarrow[\text{V}(3,2)]{\text{HBr/CH}_3\text{COOH}} \text{HBr·H-(L-Ala)}_5\text{-NH(CH}_2)_3\text{CH}_3 \text{ (v)}$$
V′(3,2)

$$\text{Z-(L-Ala)}_3\text{-N}_3 + \text{III}'(1,2) \xrightarrow{(C_2H_5)_3N} \text{Z-(Ala)}_6\text{-NH(CH}_2)_3\text{CH}_3 \xrightarrow[\text{VI}(3,3)]{\text{HBr/CH}_3\text{COOH}} \text{HBr·H-(L-Ala)}_6\text{-NH(CH}_2)_3\text{CH}_3 \text{ (vi)}$$
VI′(3,3)

$$\text{Z-(L-Ala)}_2\text{-N}_3 + \text{V}'(3,2) \xrightarrow{(C_2H_5)_3N} \text{Z-(LAla)}_7\text{-NH(CH}_2)_3\text{CH}_3 \xrightarrow[\text{VII}(2,5)]{\text{HBr/CH}_3\text{COOH}} \text{HBr·H-(L-Ala)}_7\text{-NH(CH}_2)_3\text{CH}_3 \text{ (vii)}$$
VII′(2,5)

$$\text{Z-(L-Ala)}_3\text{-N}_3 + \text{V}'(3,2) \xrightarrow{(C_2H_5)_3N} \text{Z-(L-Ala)}_8\text{-NH(CH}_2)_3\text{CH}_3 \xrightarrow[\text{VIII}(3,5)]{\text{HBr/CH}_3\text{COOH}} \text{HBr·H-(L-Ala)}_8\text{-NH(CH}_2)_3\text{CH}_3 \text{ (viii)}$$
VIII′(3,5)

All the peptides were purified by careful recrystallization.

Oligo(L-leucine) Z-(L-Leu)$_6$-OC$_2$H$_5$ was prepared by coupling Z-(L-Leu)$_3$-N$_3$ with HBr·H-(L-Leu)$_3$-OC$_2$H$_5$ in a manner similar to that described above. The peptide was recrystallized from dimethylformamide/*n*-butyl alcohol and subsequently from dimethylformamide.

Poly(L-alanines) and D,L copolymers having sharp molecular weight distributions were prepared by heterogeneous polymerization of L- or D-alanine *N*-carboxyanhydride (NCA) and copolymerization of the NCAs in acetonitrile with *n*-butylamine as initiator.[28] Random copolymers[33] of L-alanine and *N*-methyl-L-alanine (or *N*-benzyl-L-alanine) were prepared by heterogeneous copolymerization of L-alanine-NCA with *N*-methyl-L-alanine-NCA (or *N*-benzyl-L-alanine-NCA) in acetonitrile with *n*-butylamine as initiator.[32] Table I summarizes the samples used in this study together with some physical data.

Methods. Single-contact ^{13}C CP/MAS NMR spectra were recorded at 75.46 MHz with a Bruker CXP-300 spectrometer equipped with a CP/MAS accessory. Samples (ca. 300 mg) were contained in an Andrew–Beams type rotor machined from perdeuterated poly(methyl methacrylate) and spun as fast as 3–4 kHz. Contact time was 1 ms (not optimized, but chosen to avoid build up of strong signals from residual carbon signals from the rotor and probe assembly) and repetition time was 2 s. Spectral width and data points were 30 kHz and 4K, respectively. Resolution enhancement was performed by the method of Gaussian multiplication.[34] ^{13}C chemical shifts were calibrated indirectly through external benzene (128.5 ppm relative to Me$_4$Si).

Infrared (IR) spectra (4000–250 cm^{-1}) were obtained for KBr disks with a Model 260–50 Hitachi infrared spectrophotometer. X-ray powder diffraction patterns were recorded with a Rigaku-Denki Rota Flex RU-3 X-ray diffractometer.

Results

Oligo(L-alanines). Figure 1 shows ^{13}C CP/MAS NMR spectra of a series of monodisperse oligo(L-alanines) in the solid state: Z-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$ (Figure 1A) and HBr·H-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$ (Figure 1B). Signals from the L-alanine residues are easily identified and assigned[19] as given in Figure 1 with reference to published data for the monomer and residues in the proteins and peptides.[1,2] In addition, ^{13}C signals from *n*-butylamide groups are easily discriminated by observing that the intensities of these peaks gradually decrease with increasing number of L-Ala residues in the oligomers. They are assigned in a straightforward manner as shown in Figure 1. Signals designated by SSB (and also by the arrows) and "+" are ascribed to the spinning sidebands of the carbonyl and quaternary carbon signals and arise owing to an insufficient magic angle spinning rate compared with the widths of the chemical shift anisotropy[15] and residual ^{13}C signals from the deuterated PMMA rotor and probe assembly, respectively. The peak at 44.2 ppm, unassigned by Müller and Kricheldorf,[19] is obviously to be ascribed to one of the signals marked by the "+" (44.3 ppm) from the rotor.

It is obvious from a plot of ^{13}C chemical shifts against DP$_n$ (degree of polymerization) (Figure 2) that the C$_\beta$ and C=O signals of the monomer and dimer of Z-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$ are slightly displaced downfield relative to those of the trimer and higher oligomers. The C$_\alpha$ signals of the dimer and trimer, especially of the oligomer with a benzyloxycarbonyl group at the N-terminal residue, are split into two peaks arising from the N-terminal residue and other residues. The assignment of the former peak is straightforward because the position of this peak is almost unchanged on going from the monomer to the tetramer and the peak intensity gradually decreases with increasing number of residues (Figure 1). Parallel with these changes, the ^{13}C chemical shifts of the *n*-butylamide group at the C-terminal residue are considerably changed on going from the monomer to the trimer and reach constant values at the tetramer, as shown in Figure 3. These displacements of peaks, except for those of C$_3$(C$_3'$), are almost the same between the two types of oligomers (Figure 3). However, the C$_3$ signals of the oligomers are significantly displaced upfield (up to 8 ppm) and superimposed on the C$_4$ signal. The cause of such large displacements is not clear at present; however, it is clear that these displacements of peaks in the *n*-butylamide group arise from conformational changes of the N-terminal residues. Therefore we conclude that a unique conformation of these oligomers is achieved by the tetramer and higher oligomers. It is interesting to note that the conformational properties of the oligomers examined are not strongly affected by the N- or C-end groups, in contrast with the previous result of Sutton and Koenig.[25]

We also examined infrared spectra and the X-ray powder diffraction of the same materials. As shown in Figure 4, the peaks at 1630 and 430–445 cm^{-1}, characteristic of the β-sheet form,[23] appear at the tetramer of the oligomers. We also observed X-ray diffraction patterns characteristic of the β-sheet forms at $2\theta = 16.7°$ (the (020) reflection) and 20.2° (the (110) reflection) arising from the orthorhombic crystal with $a = 4.79$, $b = 10.7$, and $c = 6.88$ Å[20,21,27] for the tetramer and higher oligomers. These results are in good agreement with the NMR data, as mentioned above.

Disordered conformations in the dimer or trimer may arise from the presence of residue(s) adopting conformational angles slightly different from those of the β-sheet form, as concluded from the displacement of the ^{13}C signals.

Poly(L-alanines). Poly(L-alanines) of higher molecular weight (PLA-1, PLA-5, PLA-50, and PLA-200 for molecular weight 1200, 4700, 50 000, and 200 000, respectively)

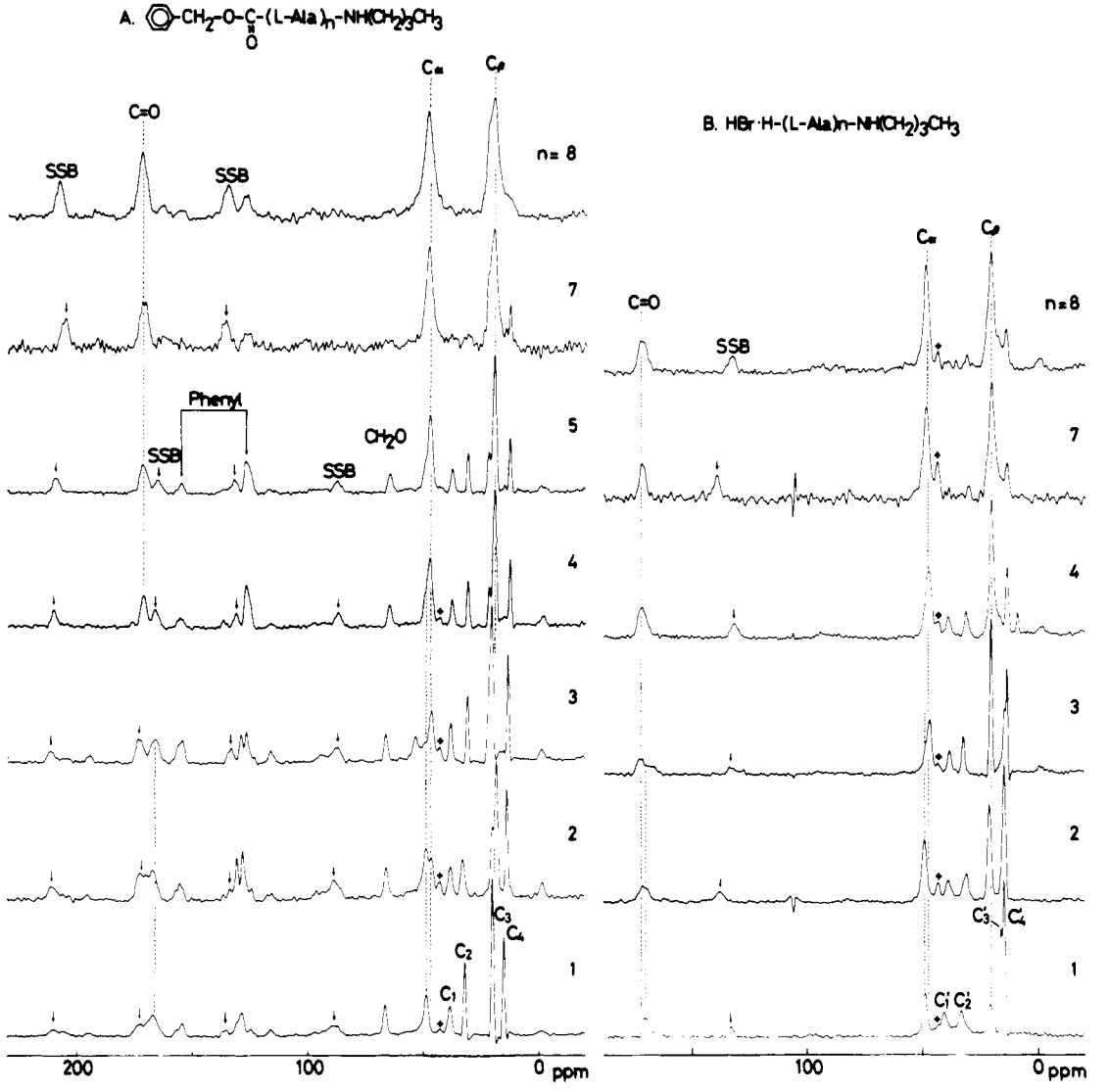

Figure 1. 75.46-MHz ^{13}C CP/MAS NMR spectra of monodisperse oligomers of L-alanine in the solid state. Signals marked by SSB and arrows are from spinning sidebands. 500–1000 transients. (A) Z-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$; (B) HBr·H-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$.

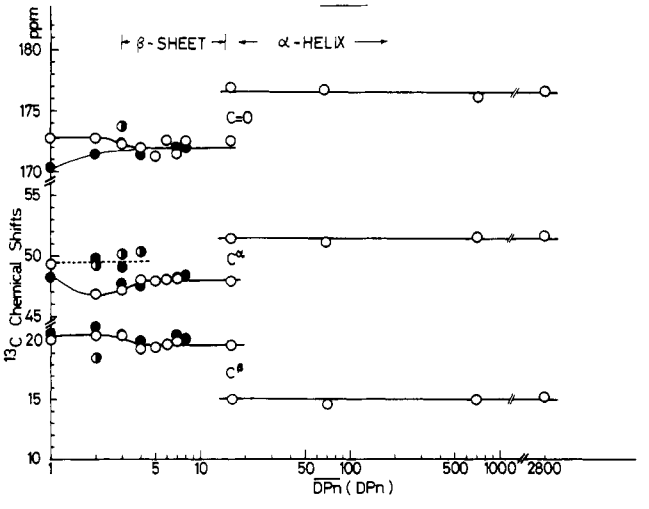

Figure 2. Plot of the ^{13}C chemical shifts of oligo(L-alanines) and poly(L-alanines) ((Ala)$_n$) vs. DP$_n$ (or $\overline{DP}_n$): (●) HBr-H-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$; (○) Z-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$ and poly(L-alanines); (◑) minor peak of Z-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$.

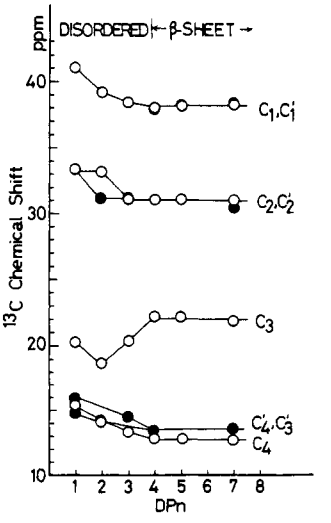

Figure 3. Plot of the ^{13}C chemical shifts of the *n*-butylamide moiety of oligo(L-alanines): (●) HBr·H-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$ (carbons with the prime); (○) Z-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$.

(Figure 5) give rise to ^{13}C chemical shifts of the α-helix form,[20-23] as manifested by characteristic bands in the infrared spectrum and X-ray diffraction: 1656 and 370 cm^{-1} (infrared)[18,23,27] and $2\theta = 11.5°$ (the (100) reflection), 20.8° (the (110) reflection), and 24° (the (200) reflection) from the hexagonal crystal with $a = b = 8.55$ Å and $c = 70.3$ Å (X-ray diffraction).[20,22,27] The ^{13}C chemical shifts of poly(L-alanines) are also plotted against the $\overline{DP}_n$ (num-

Table I
Characteristics of the Samples Used

sample[a]	$\overline{DP}_n$[b] (DP_n)	L composition,[c] %	*N*-methyl- or *N*-benzyl-L-Ala content, %
Z-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$	1–8		
HBr·H-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$	1–4, 7, 8		
Z-(L-Leu)$_6$-OC$_2$H$_5$	6		
PLA-1	16		
PLA-5	65		
PLA-50	700[d]		
PLA-200	2800[d]		
PDLA-RL90	120	91	
PDLA-RL25(RD75)	120	22	
PDLA-RL60	130	59	
PDLA-RL50	120	50	
PDLA-BL90	150	82	
PDLA-BL75	150	76	
PDLA-BL50	140	50	
copoly(L-Ala,*N*-methyl-L-Ala)			
M-1	90[e]		1[f]
M-5	80[e]		4[f]
M-25	58[e]		14[f]
copoly(L-Ala,*N*-benzyl-L-Ala)			
B-10	50[e]		6[g]
B-20	28[e]		9[g]

[a] Abbreviations: Z, benzyloxycarbonyl; PLA, poly(L-alanine); PDLA-R, random copolymer of L- and D-alanine; PDLA-B, block copolymer of L- and D-alanine; M, copolymer of L-alanine and *N*-methyl-L-alanine; B, copolymer of L-alanine and *N*-benzyl-L-alanine. [b] Calculated from the concentration of the NH_3^+ end group (by ^1H NMR) and in CF_3COOH. For poly(L-alanines), a linear relation between the degree of polymerization (DP_n) by ^1H NMR and intrinsic viscosity [η] was obtained (see refs 28 and 29). [c] Calculated from $[\alpha]_{365}$ by the optical rotatary dispersion method in CF_3COOH. [d] Theoretical number-average degree of polymerization ($\overline{DP}_n^o$), equal to the ratio A/I, where A and I denote the concentration of monomer and initiator, respectively. [e] Calculated from [2 × intensity(α-CH)/intensity(-NHCH_2-)] as determined by ^1H NMR spectra. [f] *N*-Methyl-L-alanine content (%) was calculated from [100 × intensity(NCH$_3$)/{3 × intensity(α-CH)}] as determined by ^1H NMR spectra. [g] *N*-Benzyl-L-alanine content (%) was calculated from [100 × intensity(NCH$_2$C$_6H_5$)/{5 × intensity(α-CH)}].

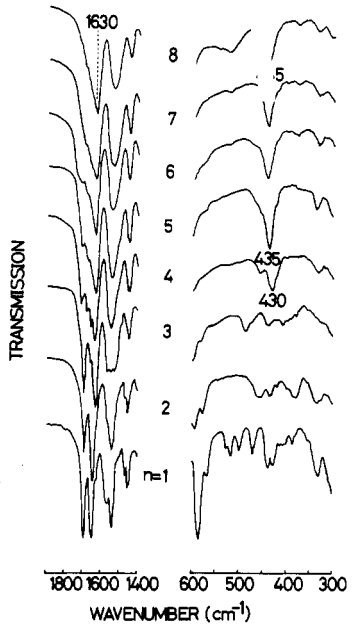

Figure 4. Infrared spectra of Z-(L-Ala)$_n$-NH(CH$_2$)$_3$CH$_3$.

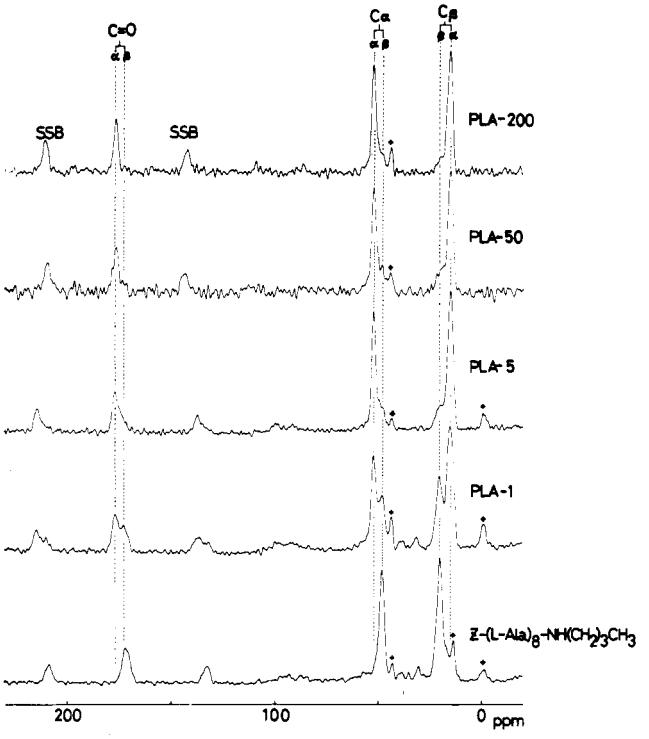

Figure 5. 75.46-MHz ^{13}C CP/MAS NMR spectra of poly(L-alanines) with various molecular weights (see Table I). 500–1000 transients.

ber-average degree of polymerization) (Figure 2). Clearly, ^{13}C chemical shifts of (Ala)$_n$ ($n \geq 16$) are unchanged among the polypeptides of various DP_n's within experimental error and can serve to characterize the α-helix form. The ^{13}C chemical shifts of the C_α and carbonyl carbons of the α-helix are significantly displaced downfield (4.2 and 4.6 ppm, respectively) relative to those of the β-sheet form, while the ^{13}C shift of the C_β carbon of the α-helix is displaced upfield with respect to that of the β-sheet form (−5.0 ppm). These data are summarized in Table II. Interestingly, the magnitude of the displacements in the C_β carbons at the side chain is larger than that of the backbone C_α carbons..

The ^{13}C signals of the minor β-sheet present in the major α-helix are easily identified and are marked by β in Figure 5. The proportion obtained by comparison of the peak

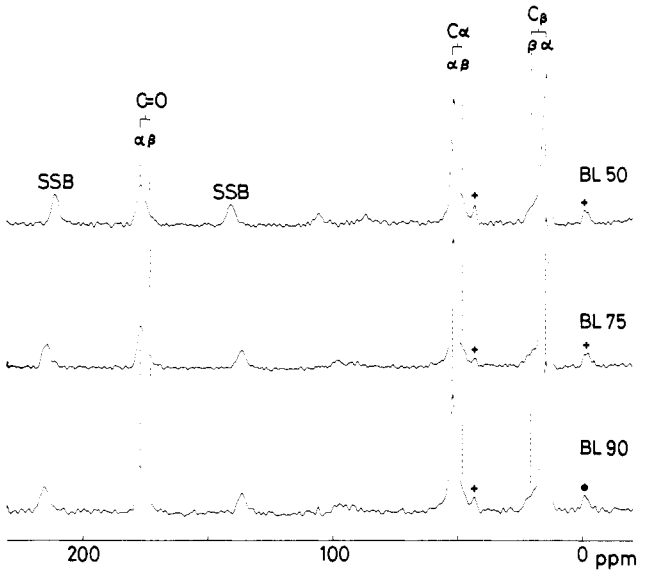

Figure 6. 75.46-MHz ^{13}C CP/MAS NMR spectra of block copolymers of L- and D-alanines (see Table I).

intensities (ca. 20%) is roughly consistent with that obtained by infrared and X-ray diffraction data (not shown).[35,36] The ^{13}C signals of PLA-1 consist of two kinds of signals arising from the α-helix and β-sheet forms. This might be caused by a plausible distribution of the chain length. Thus the α-helix conformation is formed for the oligomers between $n = 9$ and $n = 16$.

Copolymers Consisting of L- and D-Alanines. Figure 6 illustrates ^{13}C CP/MAS NMR spectra of block copolymers of L- and D-alanines; the proportion of L-alanine is 50, 76, and 82% for BL50, BL75, and BL90, respectively, as summarized in Table I. We found that the ^{13}C chemical shifts of these block copolymers are identical with those of the homopolypeptides. As expected, no difference in chemical shift was observed between the right- and left-handed α-helices. Furthermore, signals from the junctions between right- and left-handed helices are too small to be observed.

In the random copolymers, however, the intensities of additional peaks, designated by the asterisks in Figure 7, become prominent when the proportion of L-alanine (or D-alanine) is increased, besides peaks from the α-helix conformation. For the sample PDLA-RL90, containing 91% L-alanine and 9% D-alanine, the ^{13}C signals are predominantly ascribed to the right-handed α-helix. When 25% L-alanine residues are randomly copolymerized with 75% D-alanine residues, the peak intensities of the asterisked peaks are anomalously distributed among the C_α, C_β, and carbonyl peaks; the intensity of the asterisked peak in the carbonyl region is almost the same as that of the α-helix peak, and the intensity of the asterisked peak in the C_α region is about 60% of that of the α-helix peak. On the contrary, the intensity of the asterisked peak in the C_β region is very small. Such anomalies in the distribution of the peak intensities of the asterisked peaks are also seen for samples RL50 and RL60.

The ^{13}C chemical shifts of the asterisked peaks are very close to those of the β-sheet forms. Assignment of these peaks to the β-sheet form, however, is ruled out in view of the anomalous peak intensities and also infrared and X-ray diffraction data, where no characteristic bands of the β-sheet form are seen. Itoh et al.[23] proposed that the peaks at 420 and 478 cm^{-1} in the far-infrared spectra can be ascribed to D-alanine residues incorported into the right-handed α-helix or L-alanine reisdues incorporated in

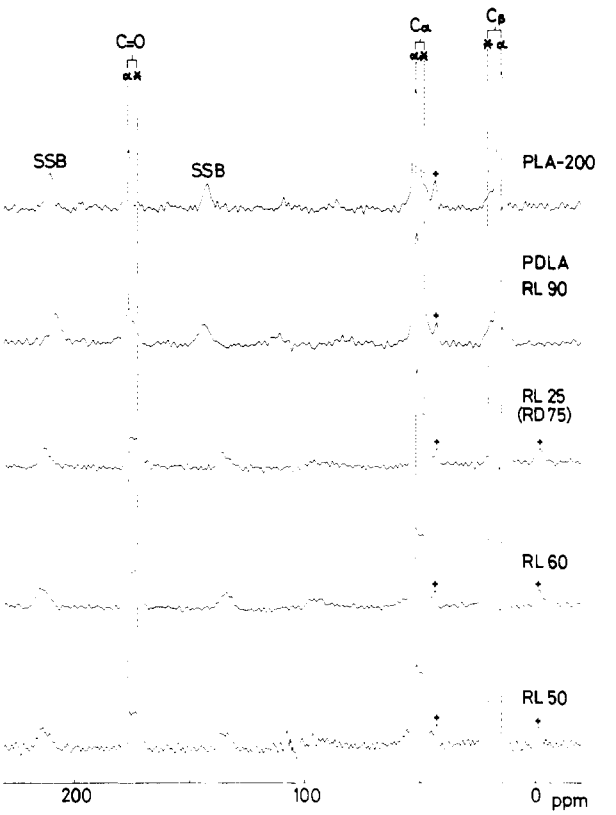

Figure 7. 75.46-MHz ^{13}C CP/MAS NMR spectra of random copolymers of L- and D-alanines (see Table I). 500–100 transients.

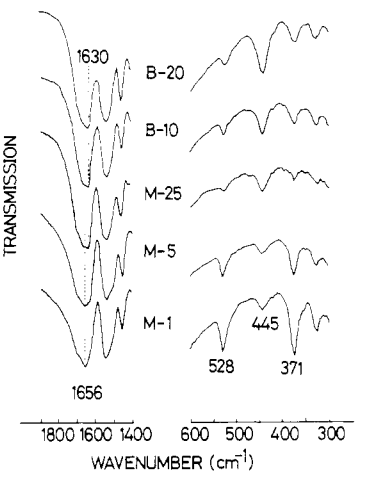

Figure 8. Infrared spectra of copoly(L-Ala,*N*-methyl-L-Ala)s and copoly(L-Ala,*N*-benzyl-L-Ala)s.

the left-handed α-helix. The pronounced NMR result observed for PDLA-R is consistent with the far-infrared result mentioned above. Thus it is noteworthy that random copolymers consisting of L- and D-alanines seem to be disturbed to form the β-sheet form and that ^{13}C chemical shifts may provide some information as to microconformation (or local conformation) of polypeptides in the solid state.

Copolymers of L-Alanine with *N*-Methyl- or *N*-Benzyl-L-alanine. Incorporation of *N*-methyl-L-alanine or *N*-benzyl-L-alanine residues into random copolymers with L-Ala residues would result in disruption of some of NH···O=C hydrogen bonds which play an important role in maintaining the α-helical conformation. As judged from the far-infrared spectra illustrated in Figure 8, a peak characteristic of the β-sheet form (445 cm^{-1}) increases at the expense of the α-helix peak (375 cm^{-1}) when the pro-

Table II
^{13}C Chemical Shifts of Polypeptides Characteristic of α-Helix, β-Sheet, and Random Coil Forms (± 0.5 ppm from Me_4Si)

	$(Ala)_n$				$(Leu)_n$				$(Val)_n$ [a]				$(Ile)_n$ [a]			
	α-helix	β-sheet	random coil [b]	Δ [c]	α-helix [a]	β-sheet [d]	random coil [b]	Δ [c]	α-helix	β-sheet	random coil [b]	Δ [c]	α-helix	β-sheet	random coil [b]	Δ [c]
C_α	52.4	48.2	51.1	4.2	55.7	50.5	55.2	5.2	65.5	58.4	61.2	7.1	63.9	57.8	61.1	6.1
C_β	14.9	19.9	15.7	-5.0	39.5	43.3	39.7	-3.8	28.7	32.4	31.7	-3.7	34.8	39.4	37.1	-4.6
C=O	176.4	171.8	176.1	4.6	175.7	170.5	175.7	5.2	174.9	171.8	174.4	3.1	174.9	172.7	175.8	2.2

[a] Data taken from ref 12. [b] CF_3COOD solution (a few drops of concentrated H_2SO_4 was added in the cases of $(Ile)_n$ and $(Leu)_n$. [c] Difference between the ^{13}C chemical shift of the α-helix form and that of the β-sheet form. [d] Data from Z-(L-Leu)$_6$-OC_2H_5.

portion of *N*-methyl-L-alanine or *N*-benzyl-L-alanine is increased (for the proportion, see Table I). In agreement with this result, ^{13}C peaks from the β-sheet form increase together with increasing proportion of *N*-methyl-L-alanine or *N*-benzyl-L-alanine (Figure 9).[37,38] No deviation of chemical shifts corresponding to the α-helix and β-sheet forms is observed with respect to those obtained from the homopolypeptides and oligomers. It is interesting to note that B-10 and B-20 have a substantially higher β-sheet content than M-25 despite the lower content of comonomer in the former two compounds relative to the latter. This can be explained by enhanced steric hindrance of the *N*-benzyl group as compared with the *N*-methyl group in forming α-helix.

***Z*-(L-Leu)$_6$-OC_2H_5.** The ^{13}C chemical shifts of solid Z-(L-Leu)$_6$-OC_2H_5 taking the β-sheet were also measured and are included in Table II. In agreement with the results of $(Ala)_n$, $(Val)_n$, and $(Ile)_n$, the ^{13}C shifts of the β-sheet forms are significantly displaced relative to those of the α-helix.

Trifluoroacetic Acid Solution. To obtain the ^{13}C chemical shifts of the random coil form, we recorded high-resolution ^{13}C NMR spectra of poly(L-alanines) and oligo(L-alanines) in CF_3COOD solution. No chemical shift difference was observed in solution among samples with different chain lengths. The ^{13}C chemical shifts are summarized in Table II.

Discussion

Conformation-Dependent ^{13}C Chemical Shifts. We found that the ^{13}C chemical shifts of the C_α, C_β, and carbonyl carbons of Ala residues are substantially displaced, depending on the conformation of the residue, e.g., disordered, β-sheet, or α-helix form (Figure 2 and Table II). In addition, exactly the same displacements of the ^{13}C shifts as those of the homopolypeptides are seen for the block and random copolymers of L- and D-alanines and the random copolymers of L-alanine with *N*-methyl- or *N*-benzyl-L-alanine. We emphasize that such conformation-dependent ^{13}C chemical shifts are seen not only for $(Ala)_n$ but also for $(Leu)_n$, $(Val)_n$, and $(Ile)_n$ (see Table II). A major advantage of the ^{13}C CP/MAS NMR method over X-ray diffraction and infrared spectroscopy is that detailed conformational characterization, involving elucidation of the conformation of individual amino acid residues, is feasible. As demonstrated previously, conformational study is still possible even for samples in an imperfect crystalline state, although line widths are broadened by dispersion of chemical shifts arising from slightly different conformations.[39] Moreover, conformational differences between solid and solution states can be directly compared by examining the conformation-dependent ^{13}C chemical shifts, as described in more detail later.

These conformation-dependent ^{13}C shifts are explained by the change of electronic state with the dihedral angles (ϕ and ψ). To prove this view, theoretical calculation of the ^{13}C chemical shifts on the basis of the electronic states obtained by quantum chemical methods is necessary. In addition, ^{13}C chemical shifts values of conformers with lower stability are easily obtained and can be related to solution-state ^{13}C chemical shifts, which are time-averaged values of ^{13}C shifts from several conformations in many instances. Such an attempt, employing the FPT-INDO method,[40,41] is in progress in our laboratories and will be published shortly. With regard to other lines of evidence concerning the existence of conformation-dependent ^{13}C chemical shifts in biopolymers, we have previously showed that the ^{13}C chemical shifts of C-1 and C-4 at the glycosidic linkages of the backbone of solid cyclohexaamyloses in-

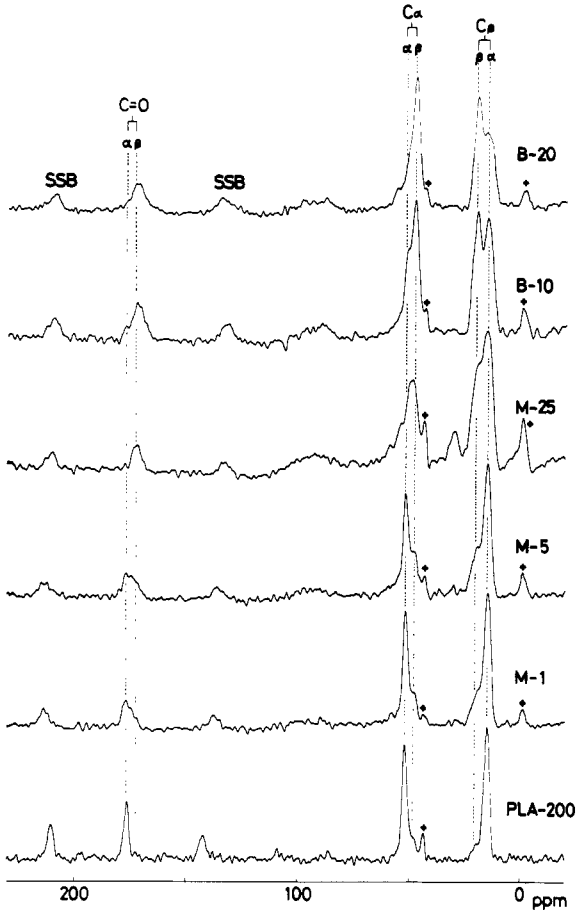

Figure 9. 75.46-MHz ^{13}C CP/MAS NMR spectra of copoly(L-Ala,*N*-methyl-L-Ala)s and copoly(L-Ala,*N*-benzyl-L-Ala)s (see Table I). 500–1000 transients.

corporating several kinds of guest molecules may be successfully related to the similar dihedral angles of C-1–O_{gly} (ϕ) and O_{gly}–C-4 (ψ).[42] We also showed that the ^{13}C chemical shifts of carbons at the glycosidic linkages for a number of solid polysaccharides are substantially displaced downfield relative to those of aqueous solution (up to 5 ppm).[39,43-45] Such downfield shifts were explained in terms of rapid conformational isomerism about the glycosidic linkages in solution state as a result of the random coil form.

It is worthwhile to compare the conformation-dependent ^{13}C chemical shifts of $(Ala)_n$ in more detail with those of other polypeptides examined so far:[12] $(Leu)_n$, $(Val)_n$, and $(Ile)_n$. It is meaningless to compare the absolute ^{13}C chemical shifts, except for those of C=O carbons (175.5 ± 1 and 171.7 ± 1 ppm for the α-helix and β-sheet forms, respectively), because the C_α and C_β carbon chemical shifts are mainly determined by the chemical structure rather than the conformation of the individual amino acid residues. To compensate for the former contribution, we may utilize the relative ^{13}C shifts of the α-helix with reference to those of the β-sheet form, Δ, as summarized in Table II, for comparison. It is clear from Table II that the Δ values of the carbonyl carbons in $(Ala)_n$ and $(Leu)_n$ (4.6 and 5.2 ppm, respectively) are significantly larger than those in $(Val)_n$ and $(Ile)_n$ (3.1 and 2.2 ppm, respectively). On the other hand, the Δ values of the C_α carbons in the latter polypeptides (7.1 and 6.1 ppm). Such differences of Δ values should be ascribed to the conformational differences in either the α-helix or β-sheet, or both, between the two types of polypeptides. In accordance with this expectation, Yamashita et al.[46] showed that a large contraction of the *c* axis (fiber axis) occurs in $(Val)_n$ and

Table III
Displacements of ^{13}C Shifts due to α-Helix Formation (ppm)

	neutral polypeptides[a]				basic polypeptides[a,b]	
	$(Ala)_n$	$(Leu)_n$	$(Val)_n$	$(Ile)_n$	$(Lys)_n$	$(Arg)_n$
C_α	−1.3	−0.5	−4.3	−2.8	−3.1	−2.8
C_β	0.8	0.2	3.0	2.3	0.6	0.6
C=O	−0.3	0	−0.5	0.9	−2.0	−2.4

[a] ^{13}C shifts of the random coil forms of the neutral and basic polypeptides are taken in CF_3COOD solution and in aqueous solution (pH 7), respectively. [b] α-Helix formed in the presence of neutral salt at pH 7.

$(Ile)_n$ in the β-sheet form (c = 6.59 and 6.6 Å, respectively) relative to that of β-sheet $(Ala)_n$ (c = 6.89 Å). Such a contraction of the backbone was attributed to the formation of stable van der Waals contacts among the bulky hydrophobic side chains. On the other hand, no such large contraction occurs in β-sheet $(Ala)_n$, since the methyl side chains are too small. Although no detailed conformational study of α-helical $(Val)_n$ and $(Ile)_n$ was achieved by X-ray diffraction, it is likely that the appreciable differences of the Δ values can be partly ascribed to the slight conformational change of the backbone moiety.

Comparison of Solid-State ^{13}C Shifts with Those of Solution. To probe conformational features of segments involving Ala residues of proteins and peptides in solution, it is necessary to have a knowledge of displacements of ^{13}C chemical shifts with respect to those of the random coil conformation. As a reference for the ^{13}C NMR shifts in the random coil, we used the values obtained in CF_3COOD solution.[47] Displacements of the ^{13}C chemical shifts of α-helical $(Ala)_n$ with respect to those of the random coil form are summarized in Table III, together with the values for $(Leu)_n$, $(Val)_n$, $(Lys)_n$, and $(Arg)_n$. The displacements of the latter two polypeptides were previously obtained in our solution NMR data.[11] Except for the case of the carbonyl ^{13}C shifts of $(Leu)_n$ and $(Ile)_n$, in which the ionization effect is dominant over the conformational contribution because of the addition of a few drops of H_2SO_4 to solubilize the samples, there appears a general trend that the C_α and carbonyl carbons of the α-helix are shifted downfield relative to those of the random coil form, while the C_β shifts are displaced upfield. In accordance with this observation, we previously showed that the folding behavior of calf thymus histones is well monitored by observing the upfield displacements of the C_β signals of several amino acid residues, especially those of Ala, involved in the α-helix.[48] These results show that ^{13}C chemical shifts arising from the random coil conformation appear between those of the α-helix and β-sheet forms and are quite reasonable since those signals arise from rapid conformational isomerism. Nevertheless, the displacements of the ^{13}C chemical shifts of the C_α carbons of $(Ala)_n$ and $(Leu)_n$ are appreciably smaller than those of other polypeptides. It is probable that the population of the conformers near the α-helical region is strongly favored in $(Ala)_n$ and $(Leu)_n$ even in the random coil conformation,[49] although those conformations are not favored in $(Val)_n$ and $(Ile)_n$, in which the α-helix conformation is destabilized because of the presence of disubstitution at the C_β group.

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Registry No. Z-(L-Ala)$_n$-$NH(CH_2)_3CH_3$, 85851-51-0; H-(L-Ala)$_n$-$NH(CH_2)_3CH_3$·HBr, 85881-52-3; poly(L-alanine), 25191-17-7;

L-alanine–D-alanine copolymer, 9051-60-9; L-alanine–*N*-methyl-L-alanine copolymer, 35268-87-2; L-alanine–*N*-benzyl-L-alanine copolymer, 85851-52-1; (*S*)-poly[imino(1-methyl-2-oxo-1,2-ethanediyl)], 25213-34-7.

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(33) Here we simply use the term "random" to denote randomly polymerized copolymers. D,L-random copolymers might have a *random block sequence* rather than the *random sequence* achieved by Linderstrøm-Lang and characterized by the method of digestion with carboxypeptidase (Linderstrøm-Lang, K. *Acta Chem. Scand.* **1958**, *12*, 851). We have previously shown that copolymers with *N*-alkylalanines take a *random-block sequence* as manifested by conformational analysis in the solution state.[32]
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(35) The peak intensity is determined by either the contact time or repetition time, which are governed by the motional states of the individual polymers. For quantitative analysis, it is necessary to optimize these parameters.
(36) There remains a possibility that a considerable proportion of the β-sheet conformation in PLA-5, -50, and -200 is ascribable to the presence of lower molecular weight oligomers owing to a bimodal distribution of molecular weights, as pointed out by a reviewer. This view, however, is easily ruled out on the basis of the following three pieces of evidence, and such a proportion of the β-sheet conformation is formed by a conformational change during the course of crystallinization, washing, and/or drying. First, the infrared spectrum of the PLA-200 film, obtained by casting a dichloroacetic acid solution of the polymer on a glass plate, exhibited only the α-helix conformation (no β-sheet bands), whereas that of a sample in a KBr disk showed both the α-helix (major) and β-sheet peaks. Second, we have observed for PLAs that a dramatic conformational change from the α-helix to the β-sheet form occurred by rolling those samples in a mortar with a pestle over 2 h (unpublished observation), inconsistent with the previous finding by Frushour and Koenig (Frushour, B. G.; Koenig, J. L. *Biopolymers* **1974**, *13*, 455). In addition, the amount of the β-sheet increases with increasing moleculr weight, suggesting that the β-sheet form is more stable in the higher molecular weight polymer. Third, poly(γ-methyl-L-glutamate), prepared from heterogeneous polymerization of NCAs in acetonitrile, exhibited almost the same amount of the α-helix and β-sheet forms as determined by infrared spectra but showed the α-helix form only when a film sample casts from DCA solution was examined.
(37) The molecular weights of these copolymers decrease slightly as the proportion of comonomers increases (Table I). In relation to the discussion in footnote 36, there might remain again a possibility that the β-sheet conformation in the copolymers is simply explained by the reduction of the molecular weights. However, note that conformational features are not affected by the molecular weight of poly(L-alanines) as long as their DP's are larger than 50, as described in the text. This situation applies in the copolymers except for B-20 (DP = 28). Nevertheless, it is clear that conformational stability plays a dominant role over the molecular weight difference in view of the data shown in Figures 8 and 9.
(38) It is very important to have knowledge about the cis/trans isomerism about the peptide bonds and its subsequent effect on secondary structure, as discussed in the previous paper,[32] in the solution state, on the basis of NCH_3 ^1H NMR signals. In this connection, the peak at 30.3 ppm in the M-25 sample could be assigned to the NCH_3 carbon of the trans peptide bond, because this peak position is in good agreement with that of the *cis*-CH_3 (as viewed from the CH_3 group) of *N*,*N*-dimethylformamide (DMF) (31.3 ppm). No peak is seen for the NCH_3 carbon of the cis peptide bond (36.8 ppm of the *trans*-CH_3 signal in DMF) because of insufficient S/N ratio for this purpose. Elucidation of this problem is under way with use of appropriate model peptides.
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(47) In comparing the ^{13}C chemical shifts of a solid sample with those obtained in the solution state, one should take the following three points into account: (1) solvent effect, (2) magnetic susceptibility, and (3) choice of chemical shift standard. As to the solvent effect, CF_3COOH, used here, is known to be a strong proton donor and to lead protonation. Such an effect is more prominent when a few drops of H_2SO_4 is added as the in the case of $(Ile)_n$ and $(Leu)_n$.[12] For this reason, the displacement of carbonyl ^{13}C chemical shifts was not included in the discussion. The solvent effect on the C_α and C_β carbons, however, seems to be less significant, because displacements of ^{13}C shifts between the α-helix and random-coil forms obtained here are consistent with the values observed in histone sample[48] and silk fibroin (Watanabe, Y.; Asakura, T. *Polym. Prep., Jpn.* **1982**, *31*, 1865), where random-coil data were obtained at *neutral* pH. Magnetic susceptibility is significant only when molecules with large anisotropic susceptibilities such as benzene molecules are involved (Earl, W. L.; VanderHart, D. L. *J. Mang. Reson.* **1982**, *48*, 35. VanderHart, D. L.; Earl, W. L.; Garroway, A. N. *Ibid.* **1981**, *44*, 361) and can be disregarded as long as the experimental errors are claimed to be as large as 0.5 ppm. Chemical shifts were calibrated by examining ^{13}C shifts of aqueous benzene at the same magnetic field. The error from these sources is not significant because we found that chemical shifts in the solid state are in good agreement with those observed in aqueous solution as far as ^{13}C shifts that do not exhibit conformation-dependent change are concerned.[39,43-45]
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