^{13}C-NMR Study of the Solvent Effect of the *N*-Acetyl-L-alanine Methyl Ester

Tetsuo ASAKURA* and Atsuo NISHIOKA

Department of Polymer Chemistry, Tokyo Institute of Technology, Ookayama, Meguro-ku, Tokyo 152

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The ^{13}C-NMR spectra of the *N*-acetyl-L-alanine methyl ester in solution were observed in order to clarify the solvent effect. The assignment of the two kinds of carbonyl groups was carried out on the basis of the difference in the long-range ^{1}H–^{13}C coupling patterns and the spectral change through the hydrolysis of the ester group in a D_2O solution. The internal rotational angle around the N–C$^{\alpha}$ bond was estimated as $-94°$ from the observed value of $^{3}J_{C'-N-C^{\alpha}-H}$, using Bystrov equation. In a trifluoroacetic acid solution, the formation of strong hydrogen bonds accompanying partial protonation between the *N*-acetyl-L-alanine methyl ester and the solvent was suggested. Moreover, the acetyl C=O group tends to interact with trifluoroacetic acid more strongly than the alanyl C=O group.

High resolution NMR spectroscopy has been extensively used to study the helix-coil transition of some synthetic polypeptides induced by such organic acids as trifluoroacetic acid.[1,2]

As the cause of the chemical shift change of the α-CH proton in the backbone chain through the helix-coil transition, two possibilities have been mainly considered. One possibility is a change in the intramolecular shielding effect which is intrinsic to the polypeptide conformation, and the other one is a solvent effect of the peptide residue by the organic acids in the coil state.[1]

On the basis of a theoretical chemical-shift calculation of poly(L-alanine), taking into account only the intramolecular shielding contribution, we have suggested that the latter is more probable, since the α-CH peak in the coil state was predicted to resonate upfield slightly (by 0.1 ppm) from the helical peak position; this is, however, contrary to the observed spectral behavior.[3,4]

In order to clarify such a polypeptide-solvent interaction in solution, it seems useful to investigate the solvent effect of the dipeptide model compounds, since the flexibility of the polypeptide backbone chain originates essentially from the rotation about the N–C$^{\alpha}$ and C$^{\alpha}$–C′ single bonds adjacent to the α-carbons; also, polypeptide-solvent interaction can be expected to occur in a relatively short range along the polypeptide chain.

In our previous paper,[5] we have studied the modes of the self-association of the *N*-acetyl-L-alanine methyl ester (Ac–Ala–OMe) and *N*-acetyl-L-alanine methylamide (Ac–Ala–NHMe) in deuteriochloroform, which is widely used as a helix-supporting solvent of polypeptides, using ^{1}H and ^{13}C-NMR spectroscopies.

In this paper, the ^{13}C-NMR spectra of Ac–Ala–OMe were measured in several kinds of solvents, including trifluoroacetic acid, which is used as a coil solvent of polypeptides, in order to study the solvent effect.

Especially, the ^{13}C-NMR resonances of the carbonyl groups give useful information on the peptide-solvent interaction. However, there is a considerable problem in assigning the carbonyl resonances, since the range of the chemical shifts is relatively small and since the resonance positions of the carbonyl groups depend strongly on the solvent. Thus, the ^{13}C assignment of the acetyl and alanyl carbonyl resonances in Ac–Ala–OMe was carried out on the basis of the difference in the long-range ^{1}H–^{13}C coupling patterns[6,7] and the spectral change through the hydrolysis of the ester group in deuterium oxide.

Experimental

The Ac–Ala–OMe sample was synthesized as has been described previously.[5] The carbon tetrachloride (CCl_4), sulfuric acid (H_2SO_4), and trifluoroacetic acid(CF_3COOH) were purchased from the Tokyo Kasei Company, Ltd. The benzene-d_6 (C_6D_6), dimethyl-d_6 sulfoxide (Me_2SO-d_6), deuteriochloroform ($CDCl_3$), and deuterium oxide (D_2O) were purchased from CEA, France.

The ^{1}H-decoupled ^{13}C-NMR spectra were mostly recorded at 25 °C with a JEOL PS-100 spectrometer equipped with PFT-100 Fourier transform units operating at 25.14 MHz. As for the measurement of the ^{1}H-coupled spectrum, a JEOL FX-60 spectrometer was used operating at 15.01 MHz at 40 °C. The chemical shifts were represented in ppm downfield from internal tetramethylsilane (Me_4Si) except for the D_2O solution. In the D_2O solution, only the partial spectra of the carbonyl region were observed.

Results and Discussion

Assignment of the Carbonyl Resonances in D_2O. Figures 1a) and b) show the ^{1}H-decoupled and -coupled ^{13}C-NMR spectra of the carbonyl carbons of Ac–Ala–OMe in D_2O respectively. The use of D_2O as a solvent leads to a simplification of the ^{1}H-coupled spectrum because the coupling between an exchangeable NH proton and every ^{13}C nucleus in this molecule disappears when the NH proton is exchanged with deuterium. On the basis of the difference in the long-range ^{1}H–^{13}C coupling patterns between the two kinds of carbonyl resonances, the higher-field resonance can easily be assigned to the acetyl C=O group, and the lower-field resonance, to the alanyl C=O group. The long-range ^{1}H–^{13}C coupling constants obtained from the first-order analysis of the spectrum are given as follows:

Acetyl C=O $^{2}J_{C'-C-H} = 5.9\pm0.1$ Hz

$^{3}J_{C'-N-C^{\alpha}-H} = 2.5\pm0.1$ Hz

Alanyl C=O $^{2}J_{C'-C^{\alpha}-H} = 8.1\pm0.2$ Hz

$^{3}J_{C'-C^{\alpha}-C-H} = 4.3\pm0.2$ Hz

$^{3}J_{C'-O-C-H} = 4.3\pm0.2$ Hz

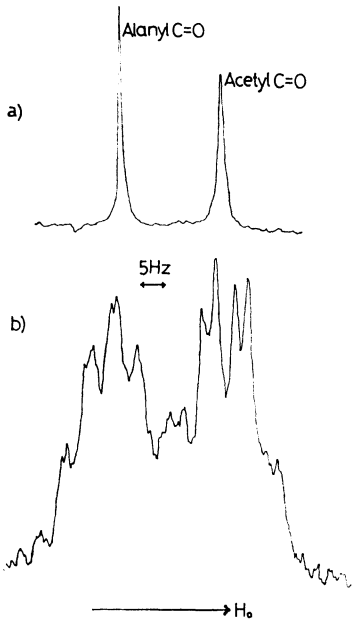
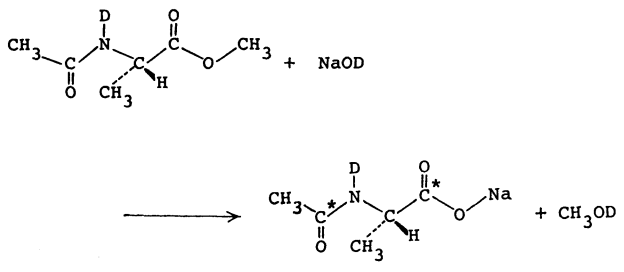


Fig. 1. ^{1}H-decoupled a) and -coupled b) ^{13}C-NMR spectra for the carbonyl region of *N*-acetyl-L-alanine methyl ester at 15.01 MHz (35 v/v % in D_2O at 40 °C). 500 and 2500 transients were collected to obtain the spectra a) and b), respectively. Pulse interval of 30 s was used.

The relative shielding order of these resonances is consistent with that in neat liquid, although the chemical-shift difference between the two peaks becomes small in D_2O (1.68 ppm in 50 v/v% D_2O, 2.16 ppm in neat liquid).[8]

It should be noticed that the acetyl C=O resonance is broader than the alanyl C=O resonance in the ^{1}H-decoupled spectrum (Fig. 1a)). This might come from the coupling between the ^{14}N nucleus and the *N*-linked acetyl C=O nucleus and also the coupling between the amide deuterium and the acetyl C=O nucleus.

Moreover, these carbonyl resonances were also assigned on the basis of the spectral change through the hydrolysis of the ester group in Ac-Ala-OMe. When a trace of NaOD is added to the D_2O solution, the following hydrolysis occurs:



As is shown in Fig. 2, the C=O peaks of the expected hydrolysis product marked by an asterisk appear after the addition of a trace of NaOD to the solution. The peak shifted remarkably to a low field by the hydrolysis can be assigned to the alanyl C=O resonance. This assignment is in agreement with that carried out on

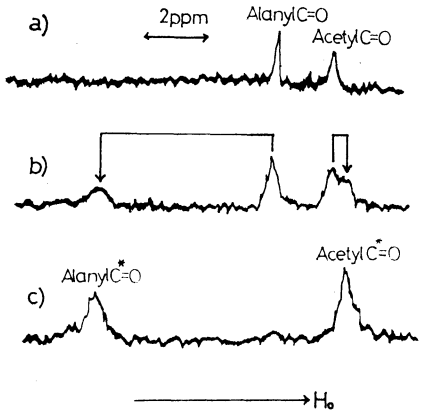


Fig. 2. ^{1}H-decoupled ^{13}C-NMR spectra for the carbonyl region of *N*-acetyl-L-alanine methyl ester at 25.14 MHz at 25 °C.
a) 50 v/v % in D_2O. b) After addition of a trace of NaOD to the solution a). c) Same as b) except for the increase of the amounts of NaOD in the solution.

the basis of the difference in the long-range ^{1}H–^{13}C coupling pattern in the ^{1}H-coupled spectrum.

Conformation of Ac-Ala-OMe in D_2O. Comparing the long-range ^{1}H–^{13}C coupling constants of acetyl C=O and alanyl C=O resonances in the Ac-Ala-OMe spectrum with the corresponding ^{1}H–^{13}C coupling constants of *N*-methylacetamide[7] and methyl acetate[9] respectively, it is found that the values of the corresponding coupling constants are almost the same except for the $^{2}J_{\underline{C}'-C-\underline{H}}$ of the alanyl C=O group. The value of the $^{2}J_{\underline{C}'-C-\underline{H}}$ of alanyl C=O in Ac-Ala-OMe is larger than that in methyl acetate[9] by about 1 Hz.

Especially, the $^{3}J_{\underline{C}'-N-C^{\alpha}-\underline{H}}$ constant is known to obey the general Karplus relation and can be used for determining the internal rotational angle around the N–C^{α} bond, ϕ.[10,11] This value of $^{3}J_{\underline{C}'-N-C^{\alpha}-\underline{H}}$, 2.5 Hz in Ac-Ala-OMe is coincident with the value of $^{3}J_{\underline{C}'-N-C^{\alpha}-\underline{H}}$, 2.5 Hz in Ac-Ala-NDMe in methanol-d_4[10] and corresponds to four angles, ϕ,[12] −94°, −146°, −6°, and 126°, using the Karplus-like equation reported by Bystrov.[10] However, Ingwall and Goodman[13] have reported a conformational energy map for Ac-Ala-OMe in which the low-energy areas enclosed by the 1 kcal mol^{-1} energy contours are spread over the range of $-50° < \phi < -180°$. Thus $\phi = -6°$ and 126° can clearly be eliminated. In our previous paper,[5] we have observed the vicinal coupling constant, $^{3}J_{\underline{H}-N-C^{\alpha}-\underline{H}}$, between the NH and C^{α}–H protons in the ^{1}H-NMR spectra of Ac-Ala-OMe as a function of its concentration in $CDCl_3$. $^{3}J_{\underline{H}-N-C^{\alpha}-\underline{H}}$ increased from 6.1 Hz in neat liquid to 6.9 Hz in 23 v/v% in a $CDCl_3$ solution with dilution. Using the Karplus-like equation reported by Ramachandran *et al.*,[14] the increase in $^{3}J_{\underline{H}-N-C^{\alpha}-\underline{H}}$ from 6.1 Hz to 6.9 Hz corresponds to the variation in ϕ from −75° to −81°. Since the value of $\phi = -94°$ is near to the value obtained from $^{3}J_{\underline{H}-N-C^{\alpha}-\underline{H}}$ in a 23 v/v% $CDCl_3$ solution, $\phi = -94°$ was chosen for the internal rotational angle around the N–C^{α} bond. Taking into account the energy map by Ingwall and

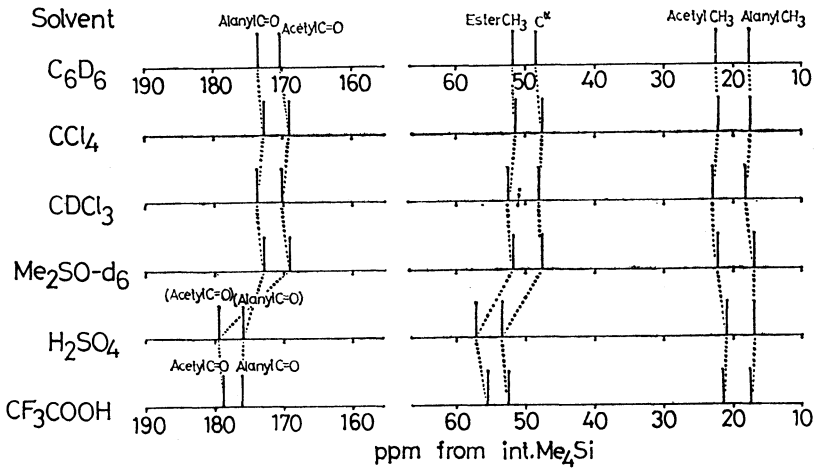


Fig. 3. ^{13}C-NMR stick spectra of *N*-acetyl-L-alanine methyl ester observed in several kinds of solvents (10 v/v %, at 25 °C). Chemical shifts were represented from internal Me_4Si.

Goodman, the value of $\phi = -94°$ may be regarded as ϕ averaged over the low-energy conformations.

Solvent Effect. The ^{13}C-NMR spectra of Ac–Ala–OMe observed in several kinds of solvents are shown in Fig. 3 as stick spectra.

In C_6D_6, CCl_4, and $CDCl_3$ solutions, the resonance positions of the ^{13}C nuclei are scarcely changed among these solvents. A similar spectrum of Ac–Ala–OMe was observed in Me_2SO-d_6. Thus, the amount of the shift induced by Me_2SO-d_6 is very small, although this solvent is well known as a good hydrogenbond-accepting solvent.[5,14–17]

On the other hand, two C=O resonances shift markedly to a low field in the H_2SO_4 solution. Since the peak positions of the acetyl and alanyl C=O groups are reversed in the CF_3COOH solution relative to those in the $CDCl_3$ solution, as will be discussed below, the relative shielding order of these peaks might be reversed in the H_2SO_4 solution compared with that in nonpolar solvents. In this solvent, protonation to the carbonyl groups of Ac–Ala–OMe would occur[18] and the ^{13}C chemical shifts of every ^{13}C nucleus in Ac–Ala–OMe would be influenced by the polarization of these carbonyl groups.[19–23] In truth, the ester CH_3 and C^α carbon resonances shift remarkably to a low field, whereas the acetyl CH_3 and alanyl CH_3 resonances shift slightly a high field.

A ^{13}C peak behavior similar to that of the H_2SO_4 solution was observed in the CF_3COOH solution. However, the amounts of the induced ^{13}C chemical shift are smaller in the CF_3COOH solution than in the H_2SO_4 solution. Thus, it is likely that strong hydrogen bonds with partial protonation[18,24–29] are formed between the solvent and the carbonyl groups of Ac–Ala–OMe in the CF_3COOH solution.

Moreover, the ^{13}C-NMR chemical shift of Ac–Ala–OMe is shown in Fig. 4 as a function of the CF_3COOH composition in the CF_3COOH–$CDCl_3$ solvent systems. With an increase in the CF_3COOH composition, the two carbonyl, ester CH_3 and C^α peaks shift gradually to a low field, while the acetyl CH_3 and alanyl CH_3 carbons shift to a high field in the region of 0—30

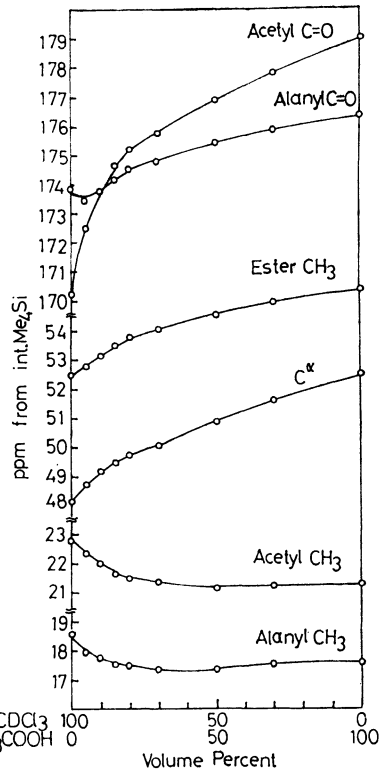


Fig. 4. ^{13}C-NMR chemical shift of *N*-acetyl-L-alanine methyl ester represented as a function of the CF_3COOH composition in $CDCl_3$–CF_3COOH solvent system (10 v/v %, at 25 °C). Chemical shifts were represented from internal Me_4Si.

v/v% CF_3COOH compositions, but do not shift upon a further increase in the CF_3COOH composition. In the two carbonyl resonances, the acetyl C=O peak shift is larger than that of the alanyl C=O peak with an increase in the CF_3COOH composition; as a result, the relative resonance position in the $CDCl_3$ solution is reversed in the CF_3COOH solution. The amount of the solvent-induced shift of the acetyl C=O carbon in Ac–Ala–OMe is comparable with that of the acetyl C=O carbon in

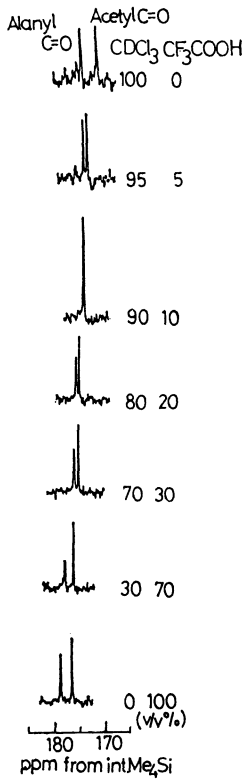


Fig. 5. ^{13}C-NMR spectra for the carbonyl region of *N*-acetyl-L-alanine methyl ester observed in $CDCl_3$–CF_3COOH solvent system (10 v/v %, at 25 °C). Chemical shifts were represented from internal Me_4Si.

N,N-diethylacetamide in the $CDCl_3$–CF_3COOH solvent system.[23] As is shown in Fig. 5, the acetyl C=O peak becomes broader in the region of 20—70 v/v% CF_3COOH composition and again becomes sharp in the 100% CF_3COOH solution, contrary to the alanyl C=O peak behavior. Thus, the acetyl C=O group tends to interact with the CF_3COOH molecule more strongly than the alanyl C=O group. A similar difference in the solvent effect by CF_3COOH is observed among the internal alanyl C=O groups and the *N*-terminal acetyl C=O group in the *N*-acetyl-L-alanyl-L-alanine methyl ester from the ^{13}C-NMR measurements.[30]

These results suggest that the interaction between trifluoroacetic acid and the internal peptide groups of poly(L-alanine) is weaker than that between the acid and simple amide molecules where protonation occurs.[23,26–28] That is, a hydrogen-bond interaction might take place.

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