^{13}C n.m.r. relaxation study of poly(aspartic acid)

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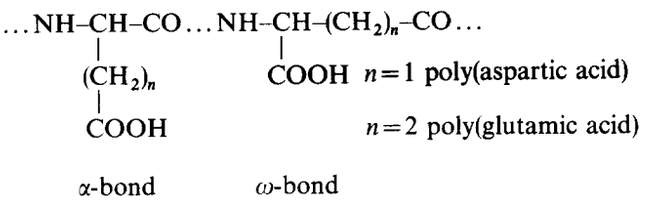
^{13}C n.m.r. relaxation parameters (T_1, line widths and NOE factors) and chemical shifts were measured in dependence on pH for several samples of poly(α-L-Asp) differing in molecular mass and polydispersity, and for samples of poly(α,β-D,L-Asp) differing in molecular mass. Poly(α-L-Asp) dissolved at pH 9 and acidified to pH 6–4 can appear in two different forms, A and B. In n.m.r. spectra these forms differ mainly by the width of the bands of side chain carbons which is larger in form A. Conditions for a reproducible generation of either of the two forms were not found although the effect of molecular mass ($3–6 \times 10^4$), polydispersity, sample concentration (2.0–0.2 mol l^{-1}), temperature of dissolution (4°C–37°C), concentration of NaCl (0–4 mol l^{-1}) or rate of acidification (1 pH unit s^{-1} $week^{-1}$) and rate of mixing were investigated. The dynamics of poly(α,β-D,L-Asp) is almost unaffected by change of pH. The relaxation parameters of poly(α-L-Asp) at pH 9 and 4 differ more in form A than in form B. Analysis of relaxation data for the methine carbon of poly(α-L-Asp) in form A by means of the isotropic model with a log χ^2 distribution of correlation times yields a correlation time of 1 ns at pH 4. This indicates that the dynamics of the backbone is dominated by rapid segmental motions even at pH 4. The dependence of chemical shifts on pH indicates that the chemical shift values are determined mainly by the ionization of the carboxyl group in the side chain rather than by a conformational transition. The evaluated relaxation parameters suggest, when compared with those of polypeptides with known conformational behaviour, that the two forms of poly(α-L-Asp) differ in conformation (α-helix, β-structure).

(Keywords: polypeptide dynamical behaviour; poly(aspartic acid) structure and dynamics; ^{13}C n.m.r. relaxation parameters)

INTRODUCTION

From a large number of physical measurements it is known that in poly(glutamic acid), conformational transitions between the α-helix, β-conformation and the random coil can be induced by changes of various factors[1]. Contrary to this, the nearest structural homologue, poly(aspartic acid) [poly(Asp)] is supposed to exist predominantly or exclusively in the form of the random coil (Ref. 2 and literature cited therein).



This polymer is important in studies of proteins, of biological evolution and in some medical applications[3,4].

In our preceding papers[3,4] it was shown that the previously studied samples of poly(Asp) contained not only α-, but also ω-peptide bonds (in the case of poly(Asp) the latter are further designated as β-bonds). Therefore we have studied the conformational structure of polymers which did not contain any β-bonds[2]. It was found that at pH ~4, a conformational transition takes place in dilute solutions of poly(Asp) (~10^{-3} mol aspartic acid residue/l), and at still lower pH a precipitate separates. The polymer in the precipitate is probably predominantly in helical form. We suppose that this transition has not been observed previously because of insufficient regularity of the structure of the peptide bonds[5].

In this paper we continue our studies of the structure of poly(Asp), concentrating on measurements of internal mobility in solution by means of ^{13}C n.m.r. spectroscopy. The more conventional methods of ^1H n.m.r. spectroscopy could not be applied because of the overlap of the methine proton band by the band of HOD.

EXPERIMENTAL

Materials

The samples of poly(α-L-Asp) were prepared by debenzylation of poly(β-benzyl-L-aspartate) by HBr in trifluoroacetic acid[3]: sample I by debenzylation of the polymer obtained by polymerization of β-benzyl-L-aspartate initiated by sodium methoxide (molar ratio of initiator:monomer = 1:200); for the preparation of sample II the same poly(β-benzyl-L-aspartate) was used, but its molecular mass was increased by means of dicyclohexylcarbodiimide[7]; sample III is the central (fifth) fraction obtained by fractionation of sample I into 12 fractions by means of gel permeation chromatography (g.p.c.) on a preparatory column[7] filled by a mixture of Sepharose 6B and Sephadex G-200. The g.p.c. elution curves (analytical column with the same stationary phase[7]) have shown that the mean molecular mass of both polymers is identical, but sample III has a considerably narrower distribution of molecular mass. The preparation

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Table 1 Studied samples of poly(Asp)

Sample	Configuration	Mole fraction of β-bonds	$M_W \times 10^{-4}$	Ref.
Poly(α-L-Asp)I	L	0	3.0	3, sample IX
Poly(α-L-Asp)II	L	0	5.9	this work
Poly(α-L-Asp)III	L	0	3.0*	this work
Poly(α, β-D, L-Asp)IX	D, L	0.65	1.8	4
Poly(α, β-D, L-Asp)XI	D, L	0.59	3.7	4

*Fraction of poly(α-L-Asp)I with a narrowed distribution of molecular weights (g.p.c. fractionation)

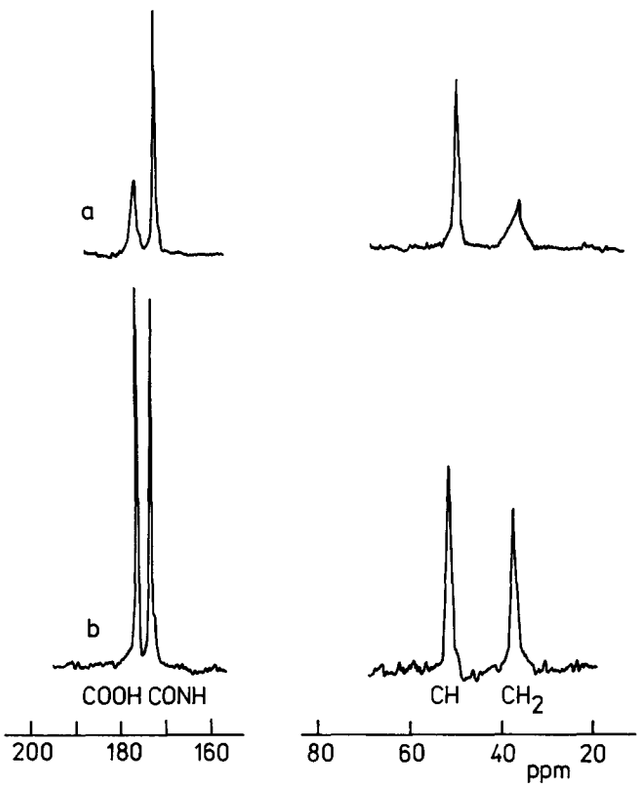

Figure 1 ^{13}C n.m.r. spectra of poly(α-L-Asp)II; (a) form A, pH 4.4; (b) form B, pH 4.2

of the samples of poly(α,β-D,L-Asp) has been described previously[4]. All prepared samples were analysed and characterized by previously described methods[2-5]. The results of all analyses confirmed the desired structure. Some characteristics of the samples of poly(Asp) are summarized in *Table 1*. For more detailed characterization see refs. 3 and 4.

Preparation of samples

Concentration of poly(Asp) is given in moles of aspartic acid residue (Asp) per litre. The samples were dissolved in aqueous NaOH (4 mol l^{-1}) and water so as to obtain the final concentration of Asp 3 mol l^{-1}, and final pH 9.0. The solution was then further diluted by water to the desired concentration and the pH adjusted by aqueous (4 mol l^{-1}) HCl. The lowering of pH was performed at controlled temperature at a controlled rate. In solutions with pH 6.5–4, poly(α-L-Asp) generated one of two different forms (designated further as A and B) which differed by their n.m.r. spectra (see further). We tried to find the conditions necessary for the generation of one or the other of the two forms: pH was lowered at 4°C, 25°C and 37°C at a concentration of Asp 1 mol l^{-1} at the rate of 1 pH min^{-1}; at 25°C and concentration of Asp 1 mol l^{-1} at the rates 1 pH per hour, day and week, and at 25°C at the rate 1 pH per min at the concentrations of Asp 2, 1 and 0.5 mol l^{-1}. Under all the cited conditions, either of the two forms was generated in repeated experiments with all three studied samples of poly(α-L-Asp). Any effect of the rate of mixing during the lowering of pH could also not be observed. Conditions for a reproducible generation of either of the two forms were not found. Either of the two forms was stable at 25°C for at least 3 weeks. Transition of one into the other could not be induced by change of temperature (0°C–37°C), concentration (2–0.2 mol l^{-1}) or concentration of added NaCl (0–4 mol l^{-1}). A transition of one form into the other could be induced by an increase of pH to 9 and subsequent acidification, or by isolation of the sample and subsequent dissolution. At temperatures above 60°C the sample was irreversibly damaged, as indicated by i.r. spectra after isolation, or n.m.r. spectra measured at pH 9, probably by degradation of the backbone.

Poly(α,β-D,L-Asp) generated only a single form at low pH.

N.m.r. spectra

^{13}C n.m.r. spectra were measured on the FX-60 (JEOL) spectrometer at 15 MHz. Spectra were measured with proton noise decoupling with a bandwidth of 1 kHz. The T_1 relaxation times were measured automatically by the inversion recovery method, with a pulse repetition rate of 10 s, with an accuracy of 10%. The T_2 relaxation times were determined from δ, the line widths measured at half-maximum height ($T_2 = 1/\pi\delta$) with an accuracy of ±15%. The NOE factors were measured by the gated decoupling technique, with a pulse repetition rate up to 20 s, and were evaluated from the integrated line intensities. NOE factors could be determined with an accuracy of 10–20%.

RESULTS

N.m.r. spectra

Figure 1 shows the n.m.r. spectra of both forms A and B of poly(α-L-Asp) II at pH 4. These measurements have shown that both forms have identical chemical shifts of all bands in the measured range of pH (*Figure 2*), but differ by shape of n.m.r. spectra (*Figure 1*). While the spectrum of form B is little affected by change of pH, form A exhibits considerable broadening of the bands of the side-chain carbons (CH_2 and COOH) of poly(α-L-Asp) at decreasing pH.

From *Figure 2* it is evident that with decreasing pH of the solutions a shift of all carbons of poly(α-L-Asp) to high field is observed in n.m.r. spectra, but the shift of the backbone carbons is very small in the whole measured

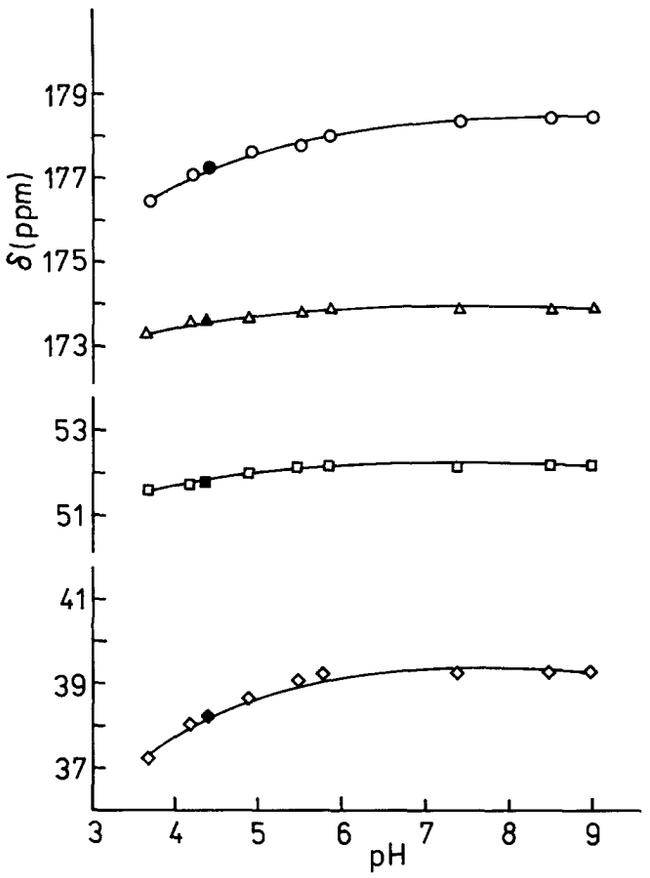

Figure 2 pH dependence of the ^{13}C chemical shift δ of poly(α-L-Asp)II. (◆) CH_2; (■) CH; (▲) CONH; (●) COOH; form A. (◇) CH_2; (□) CH; (△) CONH; (○) COOH; form B

range of pH. For the side chain carbons the total change of chemical shift is larger than for the backbone carbons. In poly(α,β-D,L-Asp) the chemical shifts behave similarly.

Relaxation parameters

The measurements of relaxation parameters of aqueous solutions of the sample poly(α-L-Asp) II are summarized in *Figures 3–5*. In *Table 2*, the relaxation parameters of both forms of the sample poly(α-L-Asp) II are compared with the parameters of poly(α,β-D,L-Asp) IX at pH 4 and 9.

Relaxation time T_1: The pH dependence of T_1 and NT_1 of all carbons of both forms of poly(α-L-Asp) II in aqueous solution is shown in *Figure 3*. By decreasing the pH from 9 to 6, the relaxation times decrease only very little, with the exception of T_1 of COOH in form A. A more pronounced decrease of the relaxation times of all carbons is only observed at pH 4, with the exception of the carboxyl carbon of form A whose relaxation time does not further change from pH 6. From *Figure 3* and *Table 2* it can be seen that a pronounced difference of relaxation times of the two forms is only observed for the carboxyl carbons; for the other carbons, the relaxation times of both carbons of poly(α-L-Asp) exhibit very little difference.

The relaxation times of all ^{13}C carbons of poly(α,β-D,L-Asp) at pH 9 are higher than those of the carbons of poly(α-L-Asp) II. Contrary to the latter, the relaxation times of CH and CH_2 group carbons are independent of pH (*Table 2*).

Line widths δ: The pH dependence of line widths in ^{13}C n.m.r. spectra of both forms of poly(α-L-Asp) II is shown in *Figure 4*. It can be seen that all the lines of form B are little affected by pH of the solution; they undergo slight broadening at pH 5. In spectra of form A, lowering of pH in the whole measured range is accompanied by a pronounced broadening of the lines of side chain carbons, whereas the lines of the backbone carbons start to show broadening only at pH 6. The effect of concentration (in the range 2.0–0.2 mol l^{-1}) of the sample on the shape and width of the lines was followed in aqueous solutions of poly(α-L-Asp) I in form B at pH 4. Hardly any change of line width was observed even when the sample changed from gel to liquid state. The measurement of the concentration dependence of form A produced a similar result. In spectra of poly(α,β-D,L-Asp) the width of lines does not depend on the pH of the solution, as demonstrated by the values T_2 in *Table 2*.

NOE factor: The pH dependence of the NOE factor for poly(α-L-Asp) II is shown in *Figure 5*. It can be seen that the NOE factors of carbons of the polymer in form A are lower than in form B, at all values of pH. In consequence of the strongly increasing width of lines in spectra of form A at decreasing pH also the error in the NOE values increases, leading to a considerable scatter of the points on the curves in *Figure 5*. Consequently a detailed comparison of the values of NOE factors of poly(α-L-Asp)

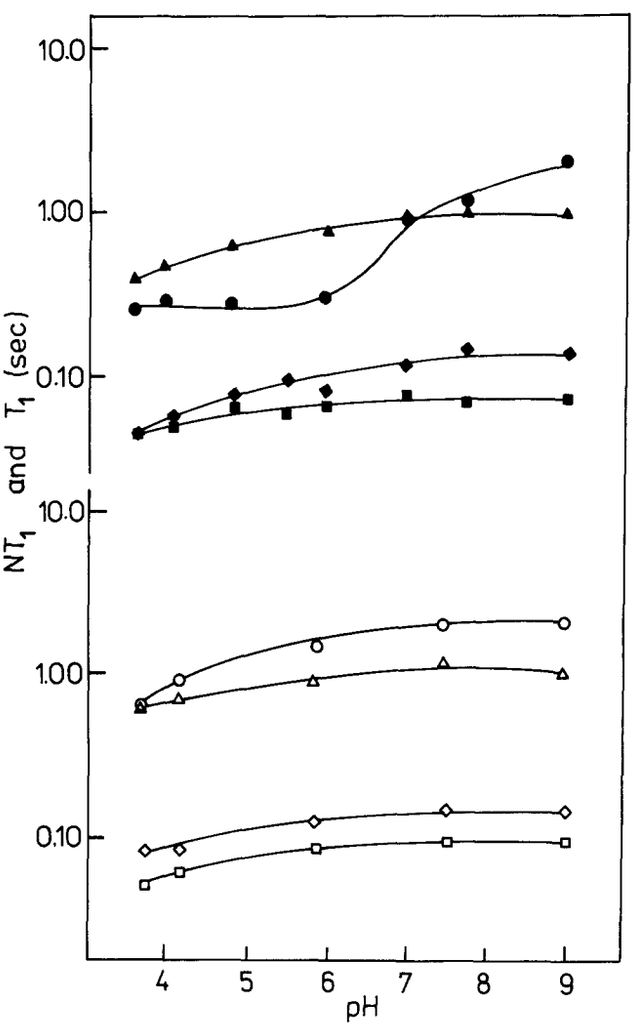

Figure 3 pH dependence of ^{13}C T_1 and NT_1 of poly(α-L-Asp)II (◆) CH_2; (■) CH; (▲) CONH; (●) COOH form A. (◇) CH_2; (□) CH; (△) CONH; (○) COOH, form B

II and of poly(α,β-D,L-Asp) cannot be made. Nevertheless, data of *Table 2* indicate, that the NOE factors of carbons in poly(α,β-D,L-Asp) are much less dependent of the pH of the solutions as compared to poly(α-L-Asp) II.

Effect of molecular mass and polydispersity: The relaxation parameters of poly(α-L-Asp) of lower molecular mass and narrower distribution of molecular mass (samples I and III—*Table 1*) do not differ from the values found for poly(α-L-Asp) II. Any differences between poly(α,β-D,L-Asp) IX and XI were also not detected.

DISCUSSION

^{13}C chemical shifts and relaxation parameters T_1, T_2 and NOE were measured for several samples of poly(α-L-Asp) differing by molecular mass and polydispersity, and for samples of poly(α,β-D,L-Asp) differing by molecular mass (*Figures 2–5 Table 1*). These measurements have shown that n.m.r. spectra and relaxation parameters of samples of poly(α-L-Asp) of molecular mass above 30000 are

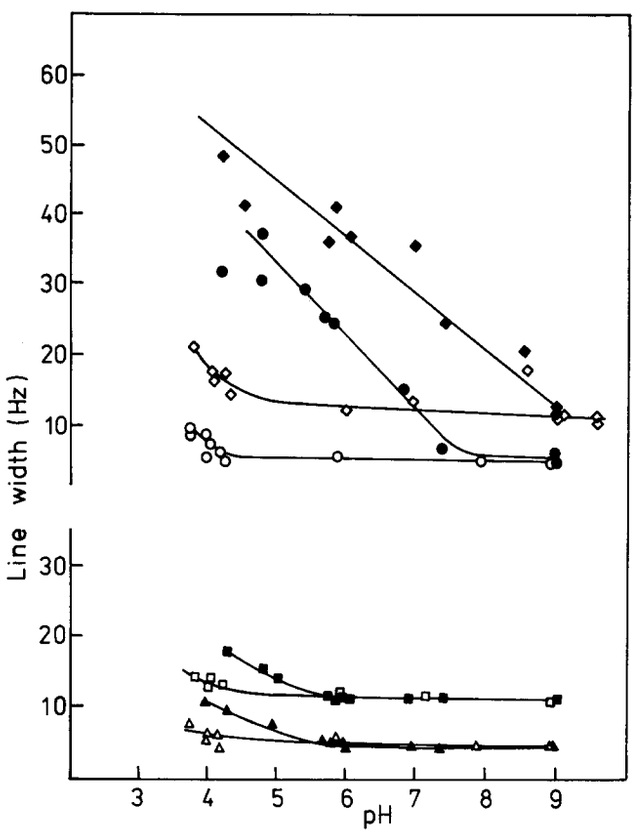

Figure 4 pH dependence of line widths in ^{13}C n.m.r. spectra of poly(α-L-Asp)II (◆) CH_2; (■) CH; (▲) CONH; (●) COOH, form A. (◇) CH_2; (□) CH; (△) CONH; (○) COOH, form B

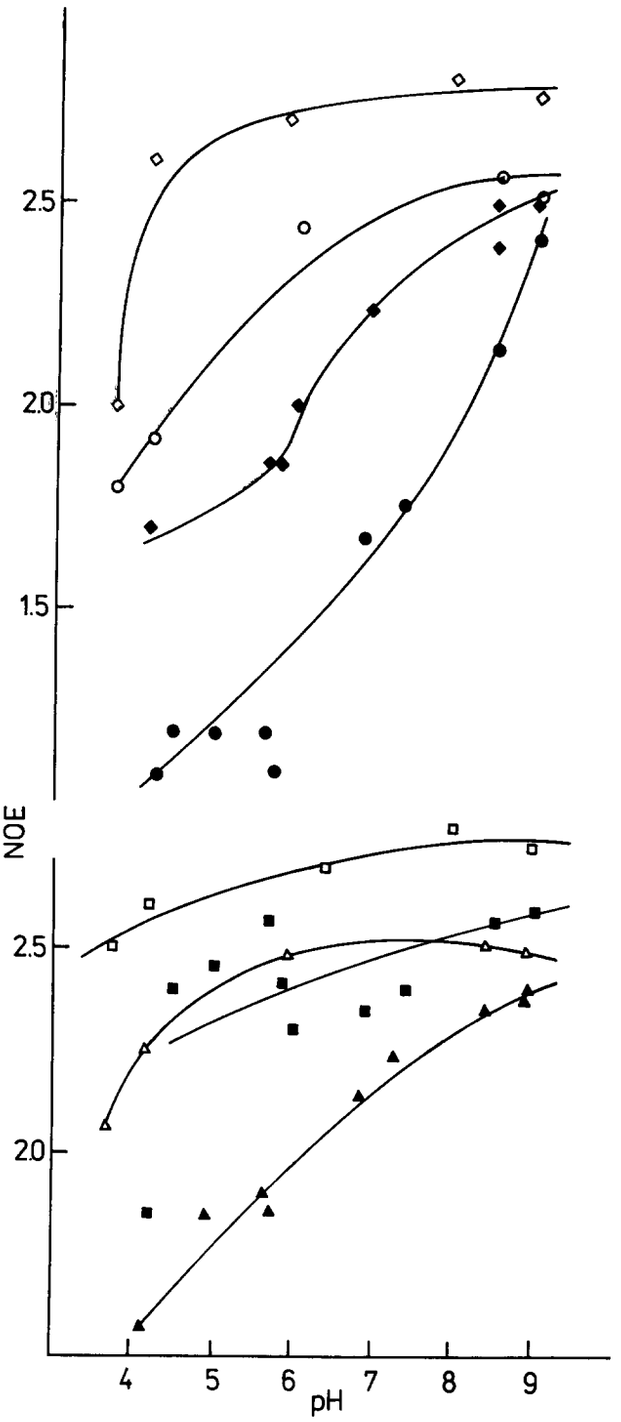

Figure 5 pH dependence of ^{13}C NOE factors of poly(α-L-Asp)II (◆) CH_2; (■) CH; (▲) CONH; (●) COOH, form A. (◇) CH_2; (□) CH; (△) CONH; (○) COOH, form B

Table 2 Relaxation parameters T_1 (s), T_2 (s), NOE of poly(Asp)

Sample	pH	COOH			CONH			CH			CH_2		
		T_1	T_2	NOE	T_1	T_2	NOE	T_1	T_2	NOE	T_1	T_2	NOE
Poly(α, β-D, L-Asp)IX	9	3.16	0.056	2.8	1.55	0.056	2.6	0.10	0.028	2.7	0.07	0.020	2.7
Poly(α, β-D, L-Asp)XI	4	1.20	0.050	2.6	0.90	0.056	2.5	0.12	0.028	2.7	0.07	0.020	2.7
Poly(α-L-Asp)II	9	2.01	0.056	2.5	0.91	0.060	2.5	0.08	0.028	2.7	0.06	0.025	2.7
Poly(α-L-Asp)II form A	4	0.24	0.010	1.2	0.54	0.032	1.6	0.05	0.016	2.2	0.03	0.008	2.1
Poly(α-L-Asp)II form B	4	0.90	0.056	2.2	0.76	0.056	2.1	0.06	0.023	2.6	0.04	0.019	2.6

Table 3 Experimental and calculated values of the relaxation parameters of the methine carbon of poly(Asp)

Sample	pH	Experimental			Calculated				
		T_1 (s)	T_2 (s)	NOE	T_1 (s)	T_2 (s)	NOE	p	$\bar{\tau}$ (ns)
Poly(α, β-D, L-Asp)IX	9	0.10	0.03	2.7	0.105	0.030	2.29	12	0.2
Poly(α, β-D, L-Asp)XI	4	0.12	0.03	2.7	0.105	0.030	2.29	12	0.2
Poly(α-L-Asp)II	9	0.08	0.03	2.7	0.077	0.030	2.25	14	0.4
Poly(α-L-Asp)II form A	4	0.05	0.02	2.2	0.053	0.020	2.12	16	1.0
Poly(α-L-Asp)II form B	4	0.06	0.02	2.6	0.061	0.019	2.16	14	0.7

independent of sample polydispersity, and that the pH dependence of the relaxation parameters is much more affected by the appearance of form A or B than by the molecular mass of the sample. Many measurements of n.m.r. spectra have further shown that lowering of pH of a solution of poly(α-L-Asp), irrespective of the concentration of the polymer and of the concentration of NaCl, leads to the generation of either one, or the other of the two forms A and B (presence of small amounts of the second form cannot be excluded due to equality of ^{13}C chemical shifts of the bands of both forms). The large scatter of the widths of CH_2 and COOH bands in the spectra of form A at low pH, sharp peaks of the broad CH_2 bands in some spectra of form A, or broad line wings in some spectra of form B indicate that the second form sometimes may be present in amounts of 10–20%. The shape of the broad bands may also be affected by the formation of aggregates[2] which in solutions of poly(α-L-Asp) begin to appear at pH < 4. All measurements were made in only a limited pH range, because at pH < 4 the polymer begins to form a precipitate.

The pH dependence of the chemical shifts of backbone carbons is often used in studies of conformational transitions of polypeptides[8]. The small change of chemical shift and a general high field shift of the methine carbon of poly(α-L-Asp) as compared to other polypeptides[9-11] indicate that the carbon chemical shifts of poly(Asp) are determined mainly by the ionization of the carboxyl group and not by a conformational transition.

From *Table 2* it is evident that the relaxation parameters of poly(α,β-D,L-Asp) are practically independent of pH. This is probably caused by the random[4] arrangement of α and β bonds and the D,L configuration of this polymer which prevents formation of a regular conformational structure. In poly(α-L-Asp), with decreasing ionization of the carboxyl group, the relaxation parameters change due to decreasing mobility. As can be seen from *Table 2*, at pH decreasing from 9 to 4, the values of the relaxation parameters of the backbone carbons decrease, but not more than by a factor of 2 (with the exception of T_2 of the CH_2 group). Analysis of the measured relaxation parameters of poly(Asp) by means of the model assuming isotropic motion of the nuclei leads to large differences in the correlation times determined from the experimental parameters T_1, T_2 and NOE. Deviations from ideal isotropic behaviour are usually interpreted by means of a distribution of correlation times[12]. For the distribution[13] $\log \chi^2$ the resulting correlation times for the CH carbon of poly(Asp) are shown in *Table 3* together with the experimental and calculated values of the relaxation parameters. From the Table it can be seen that this model gives best results for poly(α-L-Asp) for A at pH = 4. In other cases, differences between experimental and calculated values are exhibited especially for the NOE factors. However, the results shown in *Table 3* are not unique; another solution can be found giving good agreement of experimental and calculated data for T_2 and NOE, but with larger differences for T_1. As the T_1 values are subject to lowest experimental error, only the solution with best agreement for the T_1 and T_2 parameters is shown in *Table 3*.

As could be expected, the correlation time 0.2 ns of the CH carbon of poly(α,β-D,L-Asp) is independent of pH, indicating that the backbone segmental motion does not change in this range of pH (*Table 3*).

The effective correlation time is most affected by change of pH with poly(α,L-Asp) form A; its value increases from 0.4 ns at pH 9 to 1 ns at pH 4 (with form B only to 0.7 ns). This relatively small change of correlation time indicates that the backbone motion is dominated by segmental mobility even at pH 4. Similarly also measurements of deuterium line widths of α- and γ-deuterated samples of poly(L-glutamic acid)[14] indicate a relatively high segmental mobility with a correlation time $0.1 < \tau_{eff} < 0.5$ ns at room temperature, both in the random coil and helix forms of the polymer.

A simpler analysis of ^{13}C relaxation data of poly(D-glutamic acid) in aqueous solution by means of the ideal isotropic approximation yielded[9] for the CH carbon $\tau_{eff} = 0.65$ ns at pH = 7.5 and 2.8 ns at pH = 4.9. It is known that in this range of pH, a coil-helix transition takes place in aqueous solutions of poly(Glu), which contrary to poly(Asp) can be followed by means of chemical shifts of CH and CONH carbons; the correlation time 2.8 ns can therefore be regarded as characteristic of the helix structure. Although with poly(Asp) formation of helical structure cannot be followed by means of chemical shifts, the tabulated data indicate that the pH trend of correlation times is similar for both polypeptides. Therefore although the structure of poly(α-L-Asp) cannot be directly determined from n.m.r. spectra at low values of pH its relaxation parameters are similar as for polypeptides of known conformational structure [poly(glutamic acid), poly(lysine)][9,11], indicating the presence of analogous conformations (α-helix, β-conformation).

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