^{13}C NMR Relaxation Times of a Tripeptide Methyl Ester and Its Polymer-Bound Analogues

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ABSTRACT: Carbon-13 spin–lattice relaxation times of the tripeptide methyl ester Boc-Gly-Pro-Pro-OMe and its polymer-bound analogues are reported. Line widths of the signals of α-carbon atoms of Pro(2) and Pro(3) are also measured and an interpretation of the line broadening is presented. As polymeric macromolecules the following supports were employed: insoluble cross-linked polystyrene (PS), soluble poly(oxyethylene) (POE), and the graft copolymer poly(oxyethylene)–polystyrene–divinylbenzene (POE–PS). The molecular motions of the peptide moieties were determined by analysis of the relaxation time T_1. The peptide methyl ester was synthesized classically and after saponification of the ester group it was coupled to the polymers according to known procedures. In the present studies for the measurements of the T_1 values only the signals of the trans forms of the X-Pro sequence have been considered. The investigations revealed that the T_1 values and hence the mobility of the C atoms of the peptide esters decrease in the sequence methyl ester > POE ester ≥ POE–PS ester > PS ester.

Introduction

The molecular motions of molecules are closely related to their energy level and thus to their chemical reactivities. Therefore, not only are measurements of the molecular motions interesting from the point of view of theoretical chemistry but they also possess considerable experimental significance. One of the most important techniques to measure molecular motion is NMR spectroscopy.

Carbon-13 nuclear magnetic resonance has been used extensively to investigate molecular motions of numerous polymers in solution or as amorphous solids and those of peptides.[1-3] Moreover, polymers or short chains adsorbed on the surface or grafted to the solids have been the subject of theoretical or experimental studies.[4]

A large body of information is normally obtained by analysis of the spin–lattice and spin–spin relaxation times T_1 and T_2 as well as the nuclear Overhauser enhancement of the ^{13}C NMR spectra.[2] ^{13}C spin–lattice relaxation times of protonated carbons of large molecules are relatively easy to relate to the dynamic properties of the molecules. This is due to the fact that the ^{13}C relaxation of these carbons is largely dominated by the ^{13}C–^{1}H dipolar interaction with directly bonded hydrogen(s).[5]

Cross-linked polystyrenes and chloromethylated polystyrenes with different degrees of cross-linking have also been analyzed by high-resolution ^{13}C NMR spectroscopy.[6] These studies have revealed that there is a dependence of the line width and the detectable peak areas on the percent of DVB cross-linking.[7,8] Although spectra of the least cross-linked gel have narrow lines resembling those of polymer solutions, spectra of the more highly cross-linked gels show broad lines similar to the spectra of amorphous solids.[8]

Our interest in the molecular motions of the polymeric materials focuses on peptides and peptides bound to polymers. Thus we have attached the amino-protected tripeptide Boc-Gly-Pro-Pro-OH to three different types of polymeric supports and studied the effects that are exerted on the molecular motion of the peptide by these polymers and compared these motions with those of the tripeptide methyl ester. The employed macromolecular supports include soluble poly(oxyethylene) (POE), insoluble cross-linked polystyrene–divinylbenzene (PS), and the graft copolymer poly(oxyethylene)–polystyrene–DVB (POE–PS). These polymers are widely used as supports in organic syntheses such as peptide synthesis[9-13] but also for chromatographic purposes, transition-metal and phase transfer catalysts, etc.

The proline-containing peptide was chosen due to the fact that several ^{13}C spin–lattice relaxation studies have been carried out on proline-containing peptides.[14,15] The spin–lattice relaxations on proline and peptides containing this amino acid have been interpreted in terms of contributions of internal motion and overall molecular motion.[14,16-18] Moreover, the X-Pro peptide bond is a useful NMR probe for conformational studies of linear and cyclic peptides and for the establishments of the equilibrium between cis and trans forms of X-Pro peptide bonds.[19-21]

Even though polystyrene–divinylbenzene is the most predominantly employed polymeric support for the stepwise peptide synthesis, many investigators consider that this carrier is probably not the most appropriate polymer for this purpose.[22-24] Among the most interesting approaches to overcome the shortcomings ascribed to the cross-linked polystyrene resins, the introduction of the soluble poly(oxyethylene)[11] and the graft copolymer poly(oxyethylene–polystyrene)–DVB as polymeric supports merit particular attention.[12,13,25,26]

To investigate the suitability of graft copolymers and poly(oxyethylene) in comparison with cross-linked polystyrene, several studies have been initiated. We present herein the use of ^{13}C spin–lattice relaxation time measurements to study the molecular motion of the peptide attached to these macromolecules.

Experimental Section

All spectra were recorded on a Bruker WM 400 spectrometer at 100.6 MHz using proton broad-band decoupling at 303 K. The spectra contained 16K data points over a 24-KHz frequency range with 2–4K acquisitions. Relaxation data were obtained by using the inversion–recovery 180°–τ–90° pulse sequence.[27] Repetition time between two acquisitions was 10 s for the tripeptide methyl ester and 3 s in the case of peptide

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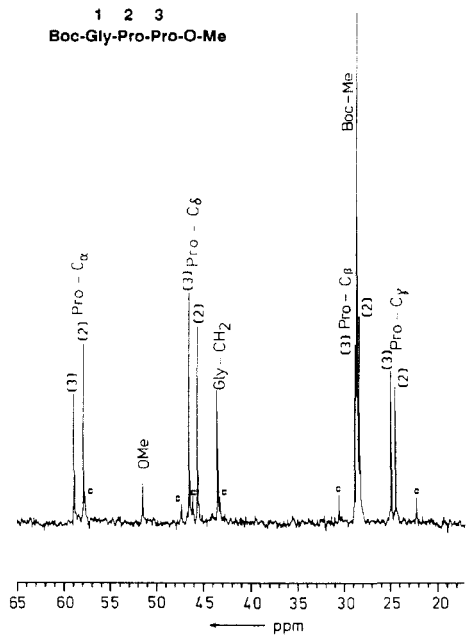

Figure 1. ^{13}C NMR spectrum of Boc-Gly-Pro-Pro-OMe in C_6D_6 at 100.6 MHz with assignments.

polymers. The spin–lattice relaxation times were determined from the relaxation data by using a regression analysis that was incorporated in the T_1 routine of the Bruker acquisition and processing program (DISNMR, version 870101.1) and given by the expression

$$Y = A_3 + A_2 e^{(-\tau/T_1)}$$

in which A_3 and A_2 are constants and τ represents the delay time between the 180° and 90° pulses. For the calculation of T_1 we used the relative intensities of the ^{13}C signals at 12–16 different τ values in an appropriate range; standard deviations were in the range ±0.007 to ±0.086 s.

All NMR spectra were determined in C_6D_6.

The following abbreviations were used: PS = $-CH_2C_6H_4-$polystyrene–divinylbenzene; Boc = *tert*-butyloxycarbonyl; POE = poly(oxyethylene); POE–PS = poly(oxyethylene)–polystyrene–divinylbenzene graft copolymer.

POE_{3000} was supplied by Hüls, Marl, Federal Republic of Germany. Chloromethylated polystyrene (Bio-Beads S-X1, cross-linked with 1% divinylbenzene, 200–400 mesh) was purchased from Bio-Rad Laboratories, München, Federal Republic of Germany. POE_{3000}–PS was prepared from Bio-Beads according to the procedure of Bayer and Rapp.[25] The average molecular mass of the poly(oxyethylene) chain was 3000 Da.

The rate constants of peptide couplings were determined according to Bayer et al.[37] Amino acid analysis, according to Spackman et al.[28] was carried out on a Biotronik LC 6000 E amino acid analyzer. The peptide samples were hydrolyzed with 12 N aqueous HCl propionic acid (1:1) for 20 h at 110 °C. Enantiomeric purity was controlled by GC using a Chirasil-Val capillary column.[29-32] Ester hydrolysis was achieved with an autotitrator Metrohm Impulsomat E 473.

The tripeptide Boc-Gly-Pro-Pro-OMe was classically synthesized by using the DCCI/HOBT method described by König and Geiger.[33] Coupling of Boc-Gly-OH and H-Pro-OMe gave the corresponding dipeptide, the ester was hydrolyzed with 0.5 N NaOH at pH 9.8. The resulting Boc-Gly-Pro-OH was coupled to H-Pro-OMe, giving the protected tripeptide with 75% yield, mp 130 °C.

Amino acid analysis: Gly 1.00, Pro 2.05

Anal. Calcd for ($C_{18}H_{29}N_3O_6$, 383.45): C, 56.38; H, 7.62; N, 10.96, O, 25.04. Found: C, 56.40; H, 7.32, N, 10.83.

Racemization test: percent of D-Pro, 2.0 (2.5). The value in parentheses shows the extent of racemization that takes place during acidic hydrolysis and derivatization.[29-32]

Boc-Gly-Pro-Pro-O-PS. A solution of 0.57 g of Boc-Gly-Pro-Pro-OH (1.55 mmol) and 0.253 g of cesium carbonate (0.775 mmol) in 5 mL of water was freeze-dried, and in the meantime 5.0 g of chloromethylated polystyrene–divinylbenzene (capacity 1.37 mequiv/g) was washed several times with CH_2Cl_2, acetic acid, ethanol, and diethyl ether, in that order. The tripeptide cesium salt was dissolved in 20 mL of DMF, added to the polymer, and shaken for 20 h at 50 °C. After filtration and thorough washing with DMF, water, DMF, ethanol, and dry ether, the compound was dried and analyzed.

Amino acid analysis: Gly 1.00, Pro 2.27; loading 0.155 mequiv/g.

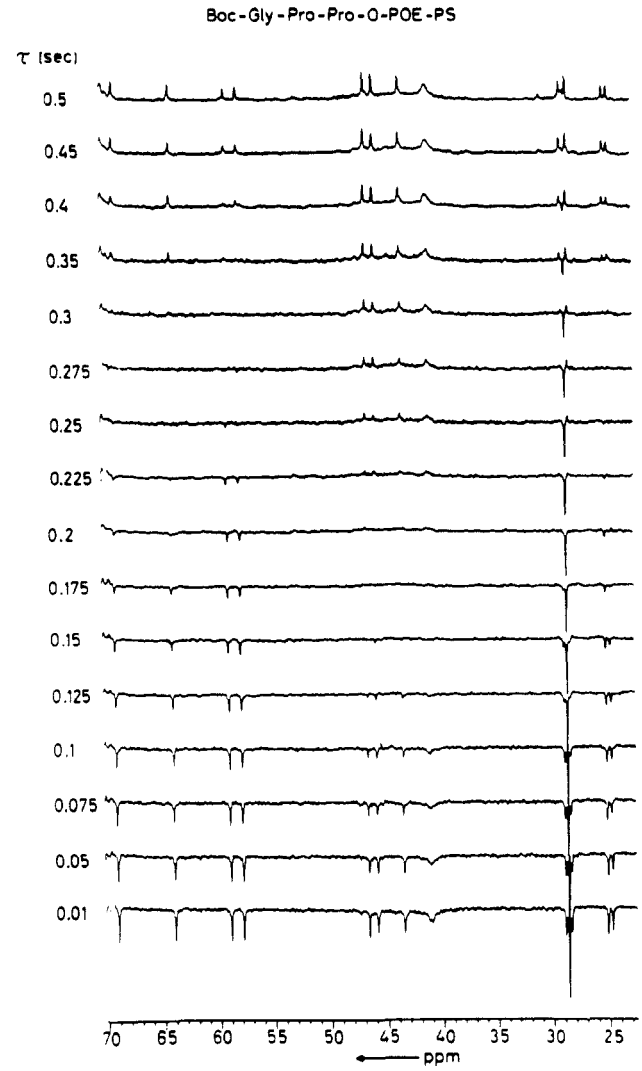

Figure 2. Inversion–recovery experiments of Boc-Gly-Pro-Pro-O–POE–PS in C_6D_6 by ^{13}C NMR at 100.6 MHz, 303 K, under conditions of complete decoupling of ^1H. The delay time is indicated (180°–τ–90°).

Boc-Gly-Pro-Pro-O($CH_2CH_2O)_n CH_2C_6H_4$–PS. To a solution of 3.02 g of Boc-Gly-Pro-Pro-OH (8.19 mmol) in 20 mL of CH_2Cl_2 were added 1.1 g of 1-hydroxybenzotriazol (in 8 mL of warm tetrahydrofuran) and 8.2 mL of 1 M DCCI solution (CH_2Cl_2). After 1 h at 0 °C, the precipitated dicyclohexyl urea was filtered off and the filtrate was added to 2.0 g of POE_{3000}–PS (capacity 0.24 mequiv/g), 20 mL of CH_2Cl_2, and 12 drops of pyridine. The mixture was refluxed and shaken for 5 days, the peptide–polymer was then filtered off and washed three times with CH_2Cl_2, three times with methanol, twice with CH_2Cl_2, and three times with dry ether, in that order.

Amino acid analysis: Gly, 1.00, Pro 1.98; loading 0.101 mequiv/g.

To get a higher loading (comparable to the peptide–polystyrene) it was necessary to repeat the coupling with another 1.3 g of tripeptide acid (3.52 mmol) for another 3 days.

Amino acid analysis: Gly, 1.00, Pro 2.01; loading 0.148 mequiv/g.

Table I
Spin-Lattice Relaxation Times T_1 of the Tripeptide Esters[a]

	T_1 (stand. deviation), s						
	Boc-Gly-Pro-Pro-					H-Gly-Pro-OH	
	OMe	O-POE	O-PS	O-POE-PS	H-Pro-Gly-OH	trans	cis
Boc	1.290 (0.008)	0.892 (0.032)	0.683 (0.007)	1.030 (0.012)			
Gly-C_α	0.726 (0.015)	0.418 (0.086)	0.179 (0.017)	0.259 (0.033)	2.14	0.92	0.98
Pro(2)-C_α	1.199 (0.027)	0.634 (0.082)	0.333 (0.038)	0.645 (0.029)			
					1.59	0.79	0.78
Pro(3)-C_α	1.445 (0.027)	0.506 (0.053)	0.358 (0.056)	0.546 (0.037)			
Pro(2)-C_β	0.922 (0.013)	0.391 (0.057)	not res.	0.328 (0.038)			
					1.63	1.42	1.24
Pro(3)-C_β	1.051 (0.019)	0.582 (0.029)	not res.	0.381 (0.044)			
Pro(2)-C_γ	1.112 (0.022)	0.545 (0.076)	0.353 (0.056)	0.408 (0.050)			
					2.19	1.68	1.48
Pro(3)-C_γ	1.330 (0.020)	0.558 (0.059)	0.360 (0.039)	0.569 (0.038)			
Pro(2)-C_δ	0.786 (0.026)	0.436 (0.055)	0.172 (0.028)	0.274 (0.049)			
					1.60	0.88	1.02
Pro(3)-C_δ	0.767 (0.020)	0.446 (0.053)	0.217 (0.022)	0.248 (0.054)			

[a] The T_1 values of two dipeptides from the literature are also given for comparison.[14]

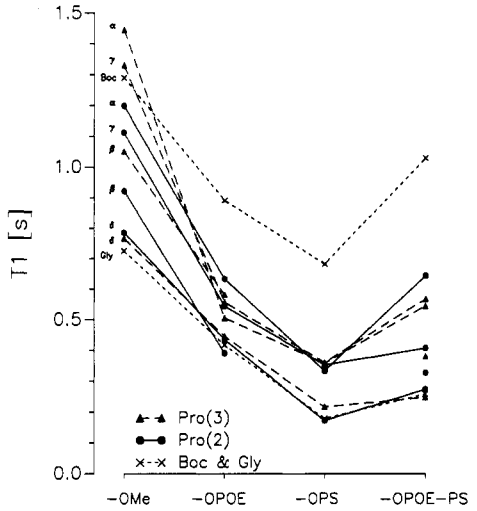

Figure 3. ^{13}C NMR spin-lattice relaxation times of the carbon atoms of the tripeptide esters.

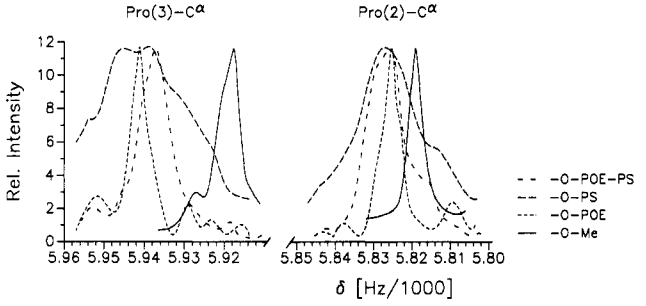

Figure 4. Line width of the signals of α-carbon atoms of Pro(2) (57.8–57.9 ppm) and Pro(3) (58.8–59.1 ppm) of the tripeptide esters (at 100.6 MHz).

Boc-Gly-Pro-Pro-O-POE$_{3000}$. A solution of the active ester of 1.34 g of Boc-Gly-Pro-Pro-OH (3.62 mmol) and 0.49 g of HOBT, prepared as reported above, was added to a mixture of 0.4 mL of 4-methylmorpholine (3.62 mmol) and 5.44 g of POE$_{3000}$ (3.62 mequiv) in 50 mL of CH_2Cl_2 and the mixture was stirred for 2 days at room temperature. The peptide-containing poly(oxyethylene) was precipitated out by addition of 300 mL of dry diethyl ether and stirring 30 min at 0 °C. It was purified by repeated precipitation from CH_2Cl_2 with ether.

Amino acid analysis: Gly, 1.00, Pro 1.67; loading 0.20 mequiv/g.

Results and Discussion

The ^{13}C spectrum of Boc-Gly-Pro-Pro-OMe in C_6D_6 is given as an example in Figure 1. Peak assignments are performed according to the literature.[34-36] As is known, peptides containing the sequence of X-Pro exist in the cis and trans forms.[34-36] The ratio of cis/trans depends also on the solvent.[34,35] In our solvent system, as seen, the trans form is the predominant form. The signals marked with "c" correspond to the cis form. In the present investigation for the measurements of the T_1 values only the signals of the trans forms have been considered because of the low intensity of the cis signals.

As an example, Figure 2 shows the T_1 measurement of the aliphatic region of the tripeptide–POE–PS in deuteriobenzene-d_6 with the inversion–recovery method. By comparing the ^{13}C spectrum of the tripeptide methyl ester with that of tripeptide–POE, it was observed that this substitution had only a very small effect on the chemical shifts and line widths of the signals. The T_1 values are reduced by a factor of about 2, which is due to the attachment of the tripeptide to poly(oxyethylene) (Table I). One could expect that the T_1 values should decrease even more drastically, considering the increase of the molecular mass from 300 to more than 3000 Da. As the molecular mass increases, the overall molecular motion of the molecule decreases. But the relaxation is the sum of the

Table II
Line Widths (Hz) in the ^{13}C NMR Spectra of the Tripeptide Esters at 100.6 MHz[a]

	W, Hz	
ester	Pro(2)-C_α (57.8–57.9 ppm)	Pro(3)-C_α (58.8–59.1 ppm)
OMe	5	6
OPOE	5	5
O-POE-PS	9	9
O-PS	19	32

[a] Digital resolution: 2.9 Hz.

Table III
Reaction Rate Constants of the Aminolysis of Boc-Gly-ONP with H-Gly-OR[a]

R	k, dm^3 mol^{-1} s^{-1}
POE$_{2000}$	0.016
POE$_{4000}$	0.017
POE$_{2000}$-PS	0.037
POE$_{3300}$-PS	0.037

[a] Solvent, acetonitrile; 25 °C; starting concentration, 10^{-5} M.

slow overall polymer rotation and the segmental motion. Therefore due to the flexibility of the poly(oxyethylene), the molecular motion of the peptide linked to this polymer is not lowered as drastically as one otherwise might expect. The line widths of the ^{13}C resonances of the peptide–POE–PS still are comparable to those of the soluble peptide and peptide–polymer, confirming a great freedom of mobility. In the spectra of the polystyrene-bound peptide, the signals are substantially broadened. Moreover, the T_1 values are reduced.

The spin–lattice relaxation times of the carbon atoms of the amino acid residues in the tripeptide methyl ester and polymer esters are summarized in Table I. As is seen, the T_1 values and hence the mobility of the C atoms of the peptide esters decrease in the sequence methyl ester > POE ester ≥ POE–PS ester > PS ester. For comparison, the T_1 values of the peptides H-Pro-Gly-OH and H-Gly-Pro-OH are also given.[14] Figure 3 depicts these results in a graphic form. It is interesting to note that the C atoms of the peptide attached to PS support show lower mobility than those bound to POE–PS, despite the fact that the interstitial space in the swollen beads is larger in the case of the PS in comparison to the POE–PS support (determined by measuring the swelling ratio in benzene: PS, 5.2; POE–PS, 3.6). The polystyrene in the graft copolymer must additionally accommodate the poly(oxyethylene) to an extent of approximately 75% of the total weight of the copolymer. Even if there is still ample space in the interstitial volume of the copolymer, one could expect a greater restriction of the peptide mobility in the POE–PS. However, the reverse is found. The higher mobility of the peptide chain in peptide–POE–PS in comparison with peptide–PS can plausibly be attributed to the presence of the spacing moiety POE. The obtained results also show that the ^{13}C NMR spectral line widths vary depending on the nature of the polymer support. Thus, the signals at about 57.8 and 58.8 ppm (α-carbon atoms of Pro(2) and Pro(3)) show broadening in the sequence Me ≤ POE < POE–PS < PS, as shown in Figure 4. The results are summarized in Table II. This phenomenon indicates that the whole peptide polymer becomes more rigid when the peptide is directly attached to the cross-linked resin in comparison with the peptide–POE–PS. This finding is in accordance with the report that as the cross-linking of polystyrenes increases, the carbon signals become broader.[8] Parallel to the increased mobility of the peptide–POE–PS (in comparison with peptide–PS) shown by the NMR data, the peptide coupling rate constants also increase when POE is used instead of PS,[37] whereas the kinetics of peptide coupling with POE–PS supports are comparable to those of POE.[38] While the peptide coupling rates (*o*-nitrophenyl active ester method) for cross-linked PS supports are lower than the corresponding rates without carrier,[39] the reverse is true when POE–PS carriers are employed[38] (Table III).

In summary, in the POE–PS graft copolymers, two important properties, the NMR relaxation behavior as well as the reaction rates, are dominated by the linear grafted poly(oxyethylene) chain more than by the cross-linked polystyrene matrix. On the basis of the NMR relaxation data and in accordance with the kinetic studies, we conclude that peptides terminally bound to POE–PS graft copolymers behave more like the homogeneous soluble POE–peptides.

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