AGGREGATION OF PRO–LEU–GLY–NH_2 IN AQUEOUS SOLUTION

Roderich WALTER*, Roxanne DESLAURIERS and Ian C. P. SMITH
**Department of Physiology and Biophysics, University of Illinois at the Medical Center, Chicago, IL 60612, USA* and *Division of Biological Sciences, National Research Council of Canada, Ottawa, K1A OR6 Canada*

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1. Introduction

Deuterated dimethylsulfoxide [1] and in the crystalline state, Pro–Leu–Gly–NH_2, have been proposed to form a β-turn structure [2], characterized by a 10-membered ring closed by a hydrogen bond between one of the primary carboxamide protons and the carbonyl oxygen of proline. In an X-ray diffraction study this very structure was found for the Pro–Leu–Gly–NH_2 monomer [3]. Subsequent theoretical [4–6] and an experimental study of spin-lattice relaxation time measurements [7] showed Pro–Leu–Gly–NH_2 to be a flexible molecule with a fair number of conformations, as would be expected for a linear, low molecular weight peptide. We reported evidence suggesting that Pro–Leu–Gly–NH_2, but not Pro–Leu–Gly–$N(CH_3)_2$, can aggregate in dimethylsulfoxide [7] – an observation further expanded upon in [8,9].

The present ^{13}C NMR study shows that Pro–Leu–Gly–NH_2 also aggregates in D_2O, albeit slowly, over a period of 3–7 days, and that this process is quickly reversed upon protonation of the secondary amino group of the proline residue.

2. Materials and methods

Carbon-13 NMR spectra were obtained using Varian XL-100 and CFT-20 spectrometers, operating in the Fourier transform mode at 25.12 MHz and 20 MHz in 12 mm and 10 mm o.d. tubes, respectively, at 32°C. Spectra were obtained with complete proton noise decoupling. Chemical shifts are referenced to tetramethylsilane (TMS) contained in a 5 mm tube concentric with the sample tube. Spin-lattice relaxation times (T_1) were determined with an accuracy of 15% by the inversion recovery method in [10]. The width of a 90° pulse is 14 μs on the XL-100 and 22 μs on the CET-20 spectrometer. Crystalline Pro–Leu–Gly–$NH_2 \cdot \frac{1}{2}H_2O$ [11] was dissolved at 40 mg/ml D_2O. Samples were titrated with CD_3COOH or with HCl, NH_4OH and NaOH diluted in D_2O. The pH values reported are measured in D_2O and uncorrected for the deuterium isotope effect [12]. The pK_a values are those obtained in D_2O. The differences in activities between hydrogen and deuterium at the glass electrode are reported to be approximately equal and opposite to the difference in activity of these ions with respect to the titratable groups [13].

3. Results

When two identical 50 mg samples of Pro–Leu–Gly–$NH_2 \cdot \frac{1}{2}H_2O$ were dissolved in 1 ml D_2O, the resultant solutions read pH 9.4. In one sample the pH was decreased from 9.4–1.8 by gradual addition of dilute HCl. In the second sample the pH was increased by addition of NH_4OH to 11.1. The app. pK_a for the transition of proline from the charged to uncharged species was about 8.5 (the pK_a of free proline is 10.6 [14]) as monitored by chemical shift changes in the ^{13}C NMR spectrum of the proline moiety. The carbonyl carbon, C_β and C_γ of the proline residue as well as the carbonyl carbon and C_α of the leucine residue were most strongly perturbed by titration of Pro–Leu–Gly–NH_2. The chemical shift changes caused by going from the protonated to the deprotonated form are listed in table 1.

Table 1
Effect of titration of the proline moiety on the ^{13}C chemical shifts of Pro–Leu–Gly–NH_2[a]

Residue	C=O	C_α	C_β	C_γ	C_δ
Prolyl	+7.0	+0.3	+0.8	+1.7	0.0
Leucyl	+0.6	−0.7	+0.2	0.0	0.0
					0.0
Glycyl	+0.2	0.0			

[a] A plus (+) sign indicates a downfield shift of the ^{13}C NMR resonance line from external TMS upon deprotonation of the proline moiety. Changes indicated in parts per million (ppm)

When the Pro–Leu–Gly–NH_2 sample was kept in this aqueous solution at 32°C and pH 9.4 or higher, a number of secondary resonances appeared. These peaks of the minor component can account for up to 50% of those resonances identified with the uncharged, monomeric Pro–Leu–Gly–NH_2 (fig.1). The addition of CH_3COONH_4 or NaSCN at a 10:1 molar ratio to Pro–Leu–Gly–NH_2 had no detectable effect on the 'mixed' spectrum shown in fig.1. The proline exhibited the largest difference in chemical shifts between the resonances of the major and minor species. The major resonances perturbed were those of the α and β carbons of proline, which show differences of 1.4 ppm and 0.7 ppm between the major and minor species (table 2). The next most perturbed resonances were those of the leucyl residue. Addition of suffient acid to the sample with the 'mixed' spectrum to protonate the proline moiety of the tripeptide yielded a unique

Table 2
Differences in ^{13}C chemical shifts between major and minor species of Pro–Leu–Gly–NH_2[a]

Residue	C=O	C_α	C_β	C_γ	C_δ
Prolyl	+0.2	+1.4	+0.1	0.0	+0.7
Leucyl	+0.2	−0.3	−0.8	−0.2	+0.1
					−0.4
Glycyl	+0.2	0.0			

[a] Sample kept for 7 days at 32°C in a D_2O solution of a pH meter reading of 9.4. A plus (+) sign indicates a shift to lower field of the ^{13}C resonances of the minor species. Changes indicated in ppm

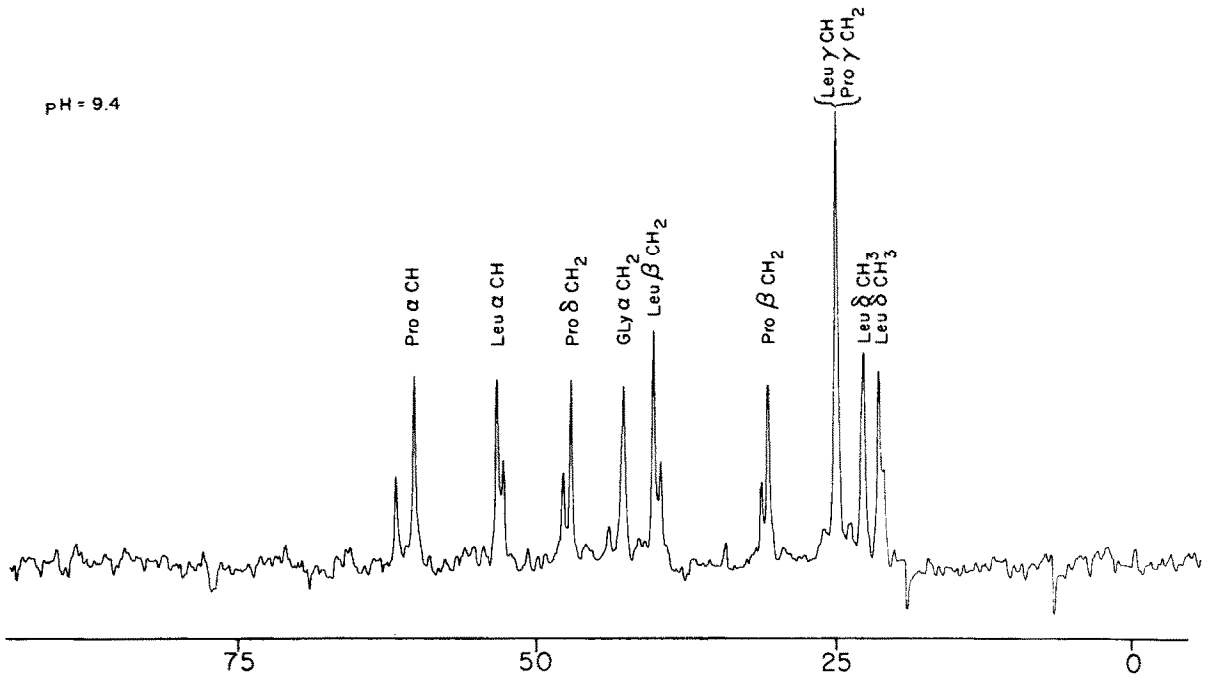

Fig.1. Carbon-13 NMR spectrum of Pro–Leu–Gly–NH_2 after having been kept for 3–7 days in aqueous D_2O at a meter reading of pH 9.4 at 32°C. The resonances for both the major as well as minor component are readily visible; for discussion see text.

set of resonance peaks for each of the carbons. However, titration of this solution with either NaOH or NH_4OH above pH 9 and storage of the solution at 32°C for several days, again resulted in the typical 'mixed' spectrum shown in fig.1. These spectral changes reveal the reversibility of the phenomenon.

4. Discussion

Pro–Leu–Gly–NH_2, which is thought to be formed enzymatically from its precursor molecule oxytoxin [15,16], is of biological interest in view of its ability to inhibit pituitary MSH [14] and ACTH [16] as well as its broad spectrum of biological effects on brain function [17–19]. Pro–Leu–Gly–NH_2 also has drawn considerable attention as a proline-containing model for theoretical and experimental conformational studies [1,3–9], since this molecule was the first biologically-active linear tripeptide for which a preferred structure was proposed [1].

This report describes observations that seem to indicate that uncharged Pro–Leu–Gly–NH_2 aggregated in a time-dependent fashion in aqueous solution. A number of secondary resonances are observed, which can reach an intensity of 50% of the major component (fig.1), when Pro–Leu–Gly–NH_2 is kept in aqueous solution for 3–7 days at pH values (~pH 9–11) where the peptide is in an uncharged state. The peaks of the minor component are not due to degradation of the peptide, as addition of sufficient acid to protonate the proline moiety yields a unique set of peaks for each of the carbons in the ^{13}C NMR spectrum of the tripeptide.

Because one set each of resonances are observed for the major and minor components in basic solution, the interconversion between species must be slow on the ^{13}C NMR time scale. This contrasts with the phenomenon generally observed during the titration of an N-terminally located proline, where only one resonance is ever observed for each carbon resonance (and provided no *cis–trans* isomerism is possible about an X–Pro bond which may be present in the peptide; X = amino acid or peptide). Thus, during titration of proline the rate of exchange between the charged and uncharged species must be fast relative to the separation between the resonances [20]. Furthermore, the chemical shift differences observed between

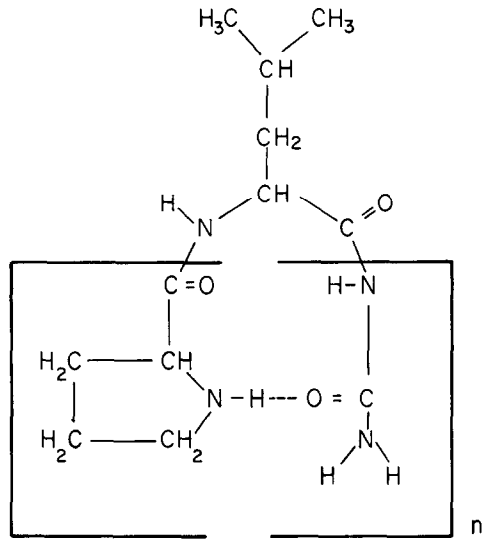

Fig.2. A model for the Pro–Leu–Gly–NH_2 aggregate.

the major and minor species of Pro–Leu–Gly–NH_2 are in several instances larger than those caused by pH titration of the prolyl residue (for comparison see data presented in tables 1 and 2).

One explanation for the appearance of a separate set of resonances for the minor species would be the presence of constraints imposed by tertiary and quarternary structures on Pro–Leu–Gly–NH_2. Figure 2 depicts the simplest possibile model, and it does not rule out significant possible populations of other structures. However, the ^{13}C T_1 measurements performed on the resonances which showed significant doubling did not reveal any gross changes in T_1 values indicating that in aqueous solutions above pH 9 no high molecular weight oligomers of Pro–Leu–Gly–NH_2 are formed.

Acknowledgements

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