CONFORMATIONAL ANALYSIS OF LINEAR PEPTIDES BY ^{15}N NMR SPECTROSCOPY USING THE ENKEPHALIN-RELATED FRAGMENT TYR–GLY–GLY–PHE AS A MODEL COMPOUND

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1. Introduction

The studies of the receptor-recognition processes of hormones or neuromodulators, such as enkephalins, and their structure–activity relationships require a preliminary knowledge of the conformational states of these peptidic molecules. ^{1}H NMR spectroscopy is the most commonly used method to study the structural behavior of peptides in solution [1,2]. For instance the formation of β-bends in oligopeptides can be evidenced by the smaller temperature dependencies of the amide protons involved in internal hydrogen bonds. Obviously the nitrogen atoms of these peptide bonds are affected by such bonding effects. Consequently the ^{15}N NMR spectroscopy has been shown useful as an alternative method for the conformational studies in cyclopeptides [3,7].

Here we extend this method to the conformational analysis of linear peptides using as a model Tyr–Gly–Gly–Phe [1] which adopts a well-defined folded conformation both in DMSO solution and in the solid-state [8,9]. This structure is characterized by a β-turn involving the CO and NH groups of the Tyr^{1} and Phe^{4} residues, reinforced by an electrostatic interaction.

The ^{15}N amide group chemical shift differences observed on changing the solvent (DMSO, H_2O) and during the titration of the ionizable groups were compared for compound [I] and its N protected form *t*.boc-Tyr–Gly–Gly–Phe [II]. The structure already proposed for [I] in DMSO is confirmed and the results are extended:

In water peptides [I] and [II] are unfolded irrespective of their ionization state;

In DMSO, the protected compound [II] exhibits an unfolded structure, whereas the structure of the free peptide [I] depends on its ionic state.

2. Materials and methods

Peptides [I] and [II] were synthesized using ^{15}N enriched amino acids (glycine 30%; phenylalanine 50%, from the CEA, Saclay) through liquid phase method, following the procedure in [8]. Cationic (C) and anionic (A) forms were directly obtained by addition of DCl or NaOD to the neutral peptidic solutions.

The spectra were recorded at 30°C on a Bruker WH 270 spectrometer equipped with a ^{15}N probe operating at 27.37 MHz in the FT mode.

For each spectrum about 2000–4000 scans were accumulated on 32 k (memory size). $^{15}NH_4{}^{15}NO_3$ (1 M) in 0.4 M HNO_3 was taken as an external reference.

3. Results and discussion

3.1. *Titration effects*

In order to eliminate a possible occurence of self-aggregation of the peptides, in this exploratory study, the three amide bonds of peptides [I] and [II] were ^{15}N enriched so that the ^{15}N spectra could be obtained from solutions of similar concentrations ($<10^{-1}$ M) as for ^{1}H NMR studies. The ^{15}N NMR spectra of [I] in DMSO-d_6 exhibit large chemical shift variations for the amide protons as a function of the ionic state of the peptide (fig.1). The results summarized in table 1 for [I] and its N-protected derivative [II] show that

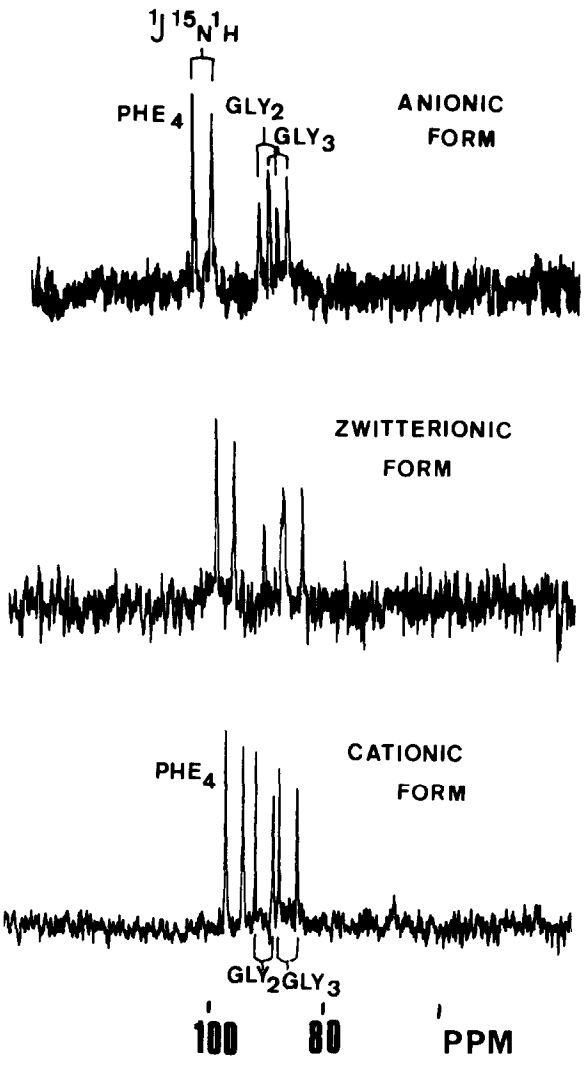

Fig.1. ^{15}N NMR spectra of Tyr–Gly–Gly–Phe [I] in DMSO-d_6 as anionic zwitterionic and cationic forms.

Table 1
^{15}N Chemical shifts for Tyr^1–Gly^2–Gly^3–Phe^4 [I] and *t*.boc-Tyr^1–Gly^2–Gly^3–Phe^4 [II] in ppm from $^{15}NH_4$-$^{15}NO_3$ (10^{-4} M), HNO_3 (4×10^{-4} M) in D_2O as an external reference

Compound	Residue	Solvent	δC	δZ	δA
	Gly^2		90.4	89.3	88.0
[I]	Gly^3	DMSO	85.8	85.9	85.9
	Phe^4		96.2	98.1	100.9
	Gly^2		91.8	91.8	91.6
[I]	Gly^3	H_2O	87.7	88.1	87.5
	Phe^4		99.1	103.6	103.6
			δN		δA
	Gly^2		85.0		85.6
[II]	Gly^3	DMSO	84.5		85.1
	Phe^4		95.7		100.9
	Gly^2		89.8		89.8
[II]	Gly^3	H_2O	87.8		87.6
	Phe^4		100.0		103.7

The spectra were recorded at 30°C on a Bruker WH 270 spectrometer, equipped with a ^{15}N probe operating at 27.37 MHz in the FT mode. δ in ppm (±0.2 ppm) are downfield from $^{15}NH_4^+$ resonance of $^{15}NH_4$ $^{15}NO_3$ (10^{-4} M), HNO_3 (4×10^{-4} M) in D_2O as an external reference for A, Z, C and N, the anionic, zwitterionic, cationic and neutral forms, respectively.

in DMSO, the deprotonation of the carboxylic group of [II] affects only the chemical shift of the terminal ^{15}N amide resonance ($\Delta\delta Phe^4 = -5.2$ ppm). The value of this downfield shift is quite similar to that observed ($\Delta\delta \simeq -6$ ppm) in small random-coil peptides [10,12], a result in favour of an unfolded structure for the peptide [II]. Similarly, the transition from the cationic (C) to the anionic (A) form of Tyr–Gly–Gly–Phe in solution in DMSO causes a ^{15}N deshielding of –4.7 ppm for the amide group of the Phe^4 residue. This downfield shift, similar to that obtained for the protected analog [II] may be interpreted as resulting from the existence of averaged extended conformations for both the (A) and (C) forms of [I]. During the titration, the ^{15}N Gly^2 resonance is slightly shielded by the deprotonation of the ammonium group.

Starting from these results, one would expect similar chemical shifts for the ^{15}N Phe^4 signal in both the A and Z forms of [I] since the carboxylic group remains negatively charged. To the contrary, the ^{15}N Phe^4 resonance is shielded ($\Delta\delta = +2.8$ ppm) in the zwitterionic form compared to the anionic one (and occurs at an intermediate chemical shift between those of the C and A forms). This feature can be related to the $\beta_{1\to4}$ bend preferential structure of [I] as zwitterionic state. In this conformation, the NH of Phe^4 involved in an internal hydrogen bond with the CO Tyr^1 is protected from the deshielding influence of the basic solvent DMSO.

In aqueous solution, the ^{15}N terminal residue Phe^4 only, is sensitive to the deprotonation of the carboxylic moiety in [I] as well as in [II] (table 1). Moreover, no significant change occurs on the Gly^2 and Gly^3 ^{15}N amide resonances during the titration of

the ammonium group. Such behaviors have been already reported for small unfolded peptides [10,12] and support the existence of averaged conformational structures in both compounds, regardless of their ionization states.

3.2. *Solvent effects*

Our data may be interpreted in terms of solvent effects, following the change from DMSO to H_2O. The solvent dependencies of the chemical shifts may be tentatively rationalized in terms of inter- versus intramolecular interactions [4,5,14].

Protic solvents (H_2O) favour the delocalization of the nitrogen lone pair through hydrogen bond with the carbonyl of the peptide bond, inducing a deshielding of the ^{15}N signals. Likewise, a ^{15}N downfield shift, smaller than the latter occurs through the formation of hydrogen bond between the NH group and basic solvents (DMSO). These two types of interactions must be taken into account to explain the ^{15}N deshielding obtained on going from DMSO to H_2O (fig.2).

In DMSO, the amide proton of the residue Phe^4 in the Z form of [I] involved in an hydrogen bond with the CO of Tyr^1, is protected from the solvent. Then, on going from DMSO to H_2O an important deshielding of the ^{15}N Phe^4 is obtained, due to the formation of intermolecular hydrogen-bond between the CO of Gly^3, linked to the NH of Phe^4, and water ($\Delta\delta = -5.5$ ppm) (fig.3). A smaller deshielding ($\Delta\delta = -2.2$ ppm) is observed on the ^{15}N Gly^3 resonance because its amide bond (CO and NH) is solvent-exposed in both the solvents. A small deshielding is also measured for the ^{15}N Gly^2 ($\Delta\delta = -2.5$ ppm) showing that this NH is solvent-exposed in DMSO and its peptide-linked Tyr^1 CO is also exposed in water. This clearly indicates that the Tyr^1 CO linked to the Gly^2 NH and

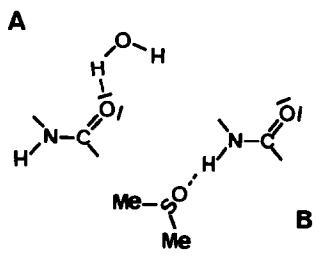

Fig.2. Solvent interactions with the peptide bond. Both water and DMSO provide ^{15}N deshielding effects ($\Delta\delta_{H_2O} > \Delta\delta_{DMSO}$).

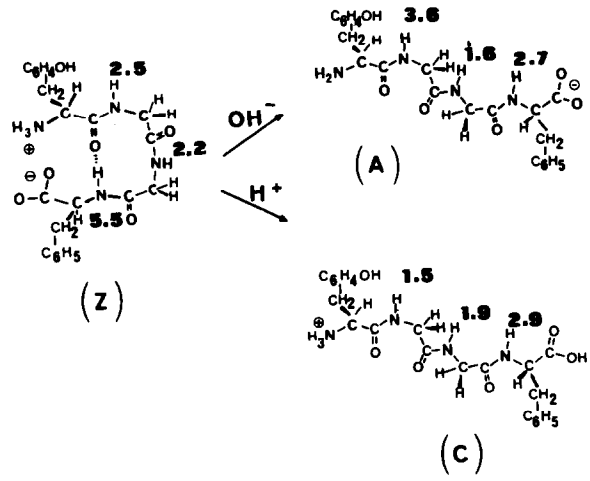

Fig.3. Schematic representation of the conformational states of [I] in different ionic states. The values reported are the ^{15}N deshieldings (in ppm) on going from DMSO to water.

involved in an hydrogen-bond with the Phe^4 NH proton in DMSO becomes solvent-exposed in water. All these results strongly support the unfolding of peptide [I] in H_2O.

In the anionic (A) and cationic (C) forms, similar deshieldings are observed for all the ^{15}N resonances accompanying the solvent change from DMSO to H_2O. This suggests that all the amide-bonds are solvent-exposed in both the solvents, and consequently that the peptide is flexible in its cationic and anionic forms. Obviously several other factors, such as nitrogen electronic density changes provided by a head to tail ($NH_3^+ \ldots {}^-OOC$) interaction may add up to these effects.

In conclusion, the conformational behaviors of Tyr–Gly–Gly–Phe [I] and *t*.boc-Tyr–Gly–Gly–Phe [II] as cationic, zwitterionic and anionic forms were determined as well in DMSO as in water. This work demonstrates the possible extension of the ^{15}N NMR spectroscopy to the conformational study of linear peptides in several solvents, and especially in water.

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