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Does a 2 ← 5 β-turn structure exist in enkephalins?

Study of a fully protected Leu-enkephalin in organic solution by ^{17}O-NMR

E. Moret, I.P. Gerothanassis, R.N. Hunston and J. Lauterwein

Institut de Chimie Organique, Université de Lausanne, rue de la Barre 2, CH-1005 Lausanne, Switzerland

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The ^{17}O chemical shifts of the Gly-2 and Gly-3 oxygens of a fully protected Leu-enkephalin were measured to be identical in acetone solution. This allows the conclusion that neither of these peptide oxygens is hydrogen bonded and that no specific 2 ← 5 β-turn structure exists to an appreciable extent.

^{17}O-NMR; Enkephalin; β-Turn structure

1. INTRODUCTION

Enkephalins are endogenous brain pentapeptides with morphine-like activity which, during the 15 years since their discovery [1], have been studied with a variety of spectroscopic methods [2], probably more extensively than any other biologically active peptide. Although their conformational state in solution is still highly controversial, with numerous debates in the literature, there is apparently accumulating evidence that in organic solution Leu-enkephalin exists in a folded 2⟵5 intramolecular hydrogen bonded β-turn structure [3–5]. Interestingly, the 2⟵5 structure has also been proposed as the dominant conformation of enkephalins when interacting with lipids [6], which is the biologically more relevant experiment. Recently, however, the formation of a 2⟵5 structure of Leu-enkephalin in both aqueous and organic solution was excluded from the analysis of ^{17}O-NMR chemical shift and relaxation time measurements [7]. On the other hand, a very strong intermolecular head-to-tail interaction was reported in organic solution even at relatively low concentrations and depending on the ionic state of the terminal groups. Therefore we extended this work to the case of a fully protected Leu-enkephalin, *t*-Boc-Tyr(OBz)-Gly-Gly-Phe-Leu-OMe, selectively enriched to 10% ^{17}O at either of the positions Gly-2 or Gly-3, in an effort to elucidate differences in the hydrogen bonding environment at these positions without the interference of the effects of the terminal carboxyl and amino groups, or the tyrosine hydroxyl group.

Correspondence address: J. Lauterwein, Organisch-Chemisches Institut, Universität Münster, Orléans-Ring 23, D-4400 Münster, FRG

2. MATERIALS AND METHODS

2.1. *Synthetic part*

The synthesis of fully protected ([^{17}O]Gly-2,Leu-5)- and ([^{17}O]Gly-3,Leu-5)-enkephalin was carried out by the mixed anhydride method in solution. *t*-Boc-Tyr(OBz)-[^{17}O]Gly-Gly-Phe-Leu-OMe was obtained by coupling *t*-Boc-Tyr(OBz)-[^{17}O]Gly, activated by reaction with ethyl chloroformate, with Gly-Phe-Leu-OMe. Fully protected Leu-enkephalin, selectively enriched in Gly-3, was prepared in a similar fashion by coupling *t*-Boc-Tyr(OBz)-Gly-[^{17}O]Gly with Phe-Leu-OMe. Enrichment of the carboxyl group in *t*-Boc-Tyr(OBz)-[^{17}O]Gly and *t*-Boc-Tyr(OBz)-Gly-[^{17}O]Gly was performed by saponification of the corresponding methyl esters by Na^{17}OH in methanol.

2.2. *^{17}O-NMR measurements*

The ^{17}O-NMR spectra were obtained at 48.8 MHz using a Bruker WH-360 instrument equipped with a high resolution probe accepting 10 mm sample tubes. Solute concentrations, temperature and acquisition/processing parameters are given in table 1 and fig.1 captions. No field/frequency lock was used. The resonance lines were fitted to a Lorentzian line-shape function. In order to alleviate acoustic ringing problems the spectra were recorded with a spin-echo type pulse sequence [8].

3. RESULTS AND DISCUSSION

The quantitative and, to a lesser extent, qualitative interpretation of the chemical shifts of the nuclei involved in a peptide bond is greatly complicated because of the contribution of several factors such as hydrogen bonding, field effects, type of substitution, magnetic anisotropy effects, etc. [9]. It has recently been suggested that when discussing ^{17}O-NMR chemical shifts in model amides and peptides, hydrogen bonding is of predominant importance [7,10,11]. Thus, the ^{17}O chemical shifts of the amide resonances of dipeptides involving heterochiral sequences, Ac-L-Pro-D-Y-NHMe (Y ≡ amino acid), at dilute concentrations are strongly dependent on the formation of intramolecular

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Table 1

^{17}O-NMR chemical shifts of *t*-Boc-Tyr(OBz)-Gly-Gly-Phe-Leu-OMe in various solvents at 40°C

Residue	Concn (M)	Methanol		Acetone		$\Delta\delta^b$
		δ (ppm)	$\Delta\nu_{1/2}$ (Hz)[a]	δ (ppm)	$\Delta\nu_{1/2}$ (Hz)[a]	
[^{17}O]Gly-2	0.025	302.0	2160	323.3	1480	21.3
	0.007	298.5	2260	324.5	1350	26.0
[^{17}O]Gly-3	0.025	303.0	2000	325.5	1605	22.5
	0.007	302.2	1925	324.4	1477	22.2

[a] Linewidths of the resonances at half-height corrected for the line broadening factors. Estimated error ± 10%

[b] Chemical shift changes ongoing from methanol to acetone solutions

$i \longleftarrow i+3$ (β-turn) structures [11]. To a good approximation, the difference in the ^{17}O chemical shift between the open and folded *trans* isomer in methylene chloride solution was estimated to be 20 ppm. This large change in the chemical shift on formation of a β-turn appears surprising since X-ray structural data indicate hydrogen bonding interactions of only medium to weak strength (N...O distances are between 2.97 and 3.10 Å [12]).

In acetone, which is a weakly basic solvent lacking proton donor properties and which has a lower dielectric constant than DMSO, the typical solvent employed in the NMR investigation of enkephalins, the only hydrogen bonds which can be formed by the Gly-2 peptide oxygen are either intermolecular or intramolecular with the NH groups. A study of the concentration dependence of the oxygen chemical shifts and linewidths was performed in acetone between 0.006 and 0.02 M. Table 1 shows that concentration has only a negligible effect confirming the absence of intermolecular interactions (in contrast, a sizeable effect on the ^{17}O linewidths was observed in $CHCl_3$ solution, indicating intermolecular hydrogen bonding interactions and prohibiting accurate estimation of spectral parameters). Once intermolecular self-association has been eliminated, the only possible hydrogen-bonding mechanism in acetone is the formation of a β-turn structure. If f is the fraction of molecules with a β-turn conformation stabilized by a $2 \longleftarrow 5$ hydrogen bond, one can write:

$$\delta_{obs} = f\delta_{turn} + (1 - f)\delta_{open}$$

where δ_{obs} is the observed chemical shift of the Gly-2 peptide oxygen, δ_{turn} is the chemical shift when Gly-2 is

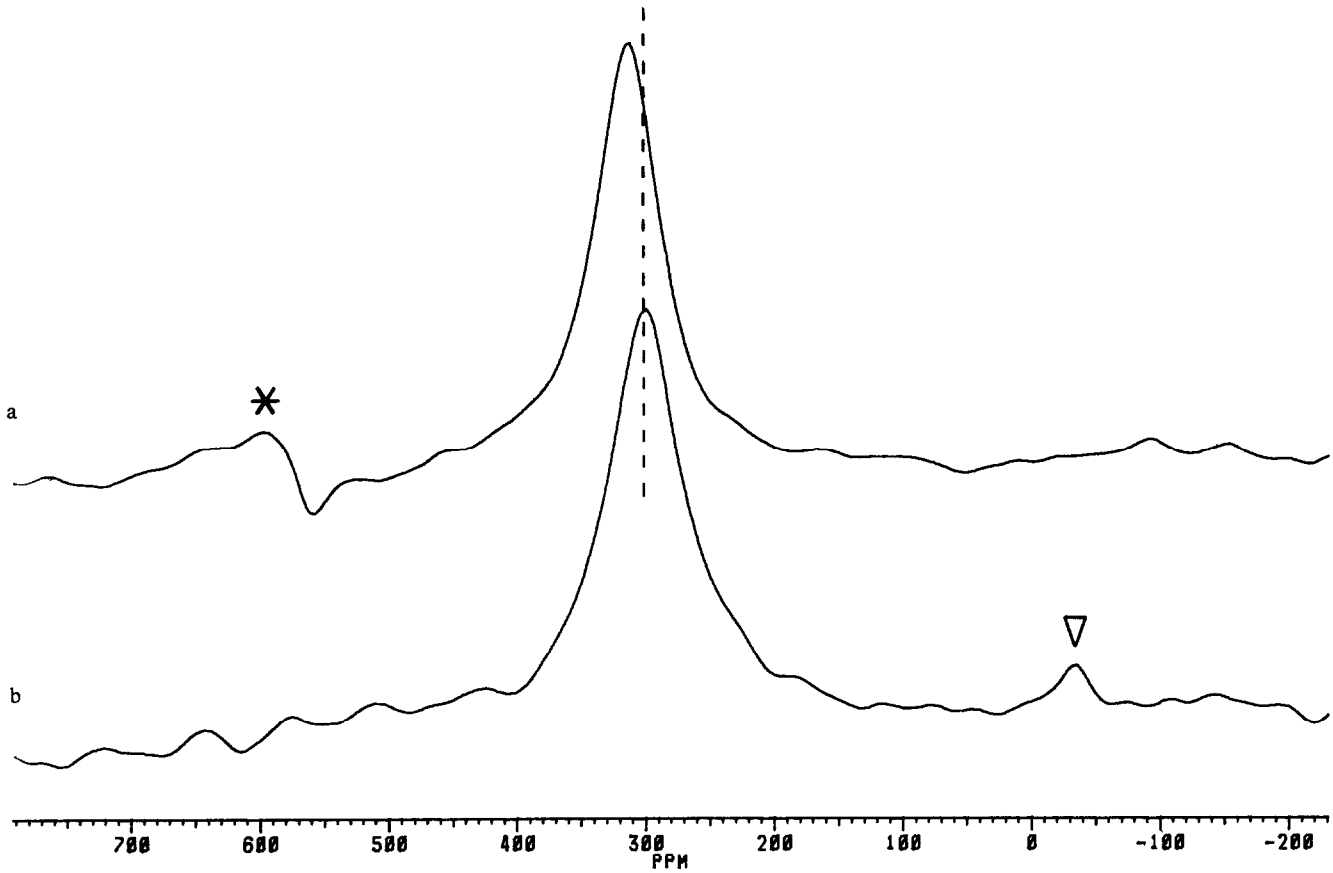

Fig.1. 48.8 MHz ^{17}O-NMR spectra of t-Boc-Tyr(OBz)-Gly-[^{17}O]Gly-Phe-Leu-OMe (10% enrichment) in (a) acetone and (b) methanol. Solution concentration 7 mM; T = 40 ± 1°C. Acquisition/processing parameters: spectral width = 50 kHz, 90°C pulse = 32 μs, quadrature detection, acquisition time = 5 T_2, no relaxation delay, LB = 1.5 kHz, zero-filling up to 2 K before FT. (*) and (▽) mark the solvent resonances of acetone and methanol, respectively.

exclusively in the β-turn and δ_{open} is the chemical shift corresponding to the open conformations of Gly-2. The quantitative evaluation of f requires the knowledge of δ_{turn} and δ_{open}. In a good approximation $\delta_{turn} = \delta_{open} + 20$ (ppm) and δ_{open} can be considered equal to the chemical shift of the Gly-3 peptide oxygen which is assumed to not participate in a folded β-turn structure. From table 1 it can be seen that the chemical shift differences measured for the peptide oxygens of the Gly-2 and Gly-3 residues are negligibly small in acetone solutions. This predicts that $f \approx 0$, and, consequently, that both oxygen sites experience similar hydrogen bonding environments.

On the other hand, the average chemical shift difference of the peptide oxygens of both Gly residues was measured to be ≈23 ppm in acetone and methanol (fig.1). This value is smaller than that found for the same oxygens in (unprotected) Leu-enkephalin where an acetonitrile/DMSO (4:1) mixture and water were used as solvents ($\Delta\delta \approx 29$ ppm) [7]. Since the existence, in acetone, of intermolecular hydrogen bonding of the C=O...NH type was excluded, the most probable reason for the smaller chemical shift difference appears to be a decreased solvation and/or an inferior hydrogen bonding strength of methanol with respect to water. This conclusion is further substantiated by considering that the oxygens of the Gly residues of enkephalins in water are largely monohydrated because of steric crowding [7].

From the present results it is clear that a 2←—5 β-turn intramolecular hydrogen bond between Gly-2 (C=O) and Leu-5 (NH) in enkephalins should be excluded in acetone. ^{17}O-NMR chemical shift data appear to be advantageous relative to those of other NMR-active nuclei in the case of small or medium-sized peptides where the problems of selective enrichment and large ^{17}O linewidths are relatively minor.

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