^{1}H AND ^{13}C NMR STUDIES OF CONFORMATIONAL BEHAVIOUR OF LEU-ENKEPHALIN

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1. Introduction

Since the isolation from brain tissues by Hughes et al. [1] of two pentapeptides (Met-enkephalin and Leu-enkephalin) which interact with opiate receptors, a number of other endogenous peptides have been shown to share this property [2].

All these compounds (except Leu-enkephalin) are fragments of β-lipotropin and have in common the Met-enkephalin 61–65 N-terminal part of this peptide.

We have recently studied by ^{1}H and ^{13}C NMR the preferential conformation of Met-enkephalin in DMSO-d_6 solution [3,5]. Our results and those of Gibbons obtained indepently [6] are in favour of a folded conformation (β_I-turn) bearing a structural analogy between morphine and the N-terminal part of the peptide.

Many synthetic derivatives of enkephalins have been prepared and their affinities for opiate receptors tested [7–9]. Few structural modifications are allowed and decisive features for receptor interaction seem to be the presence and location of the aromatic side chains of the tyrosine and phenylalanine residues.

On the other hand the inactivation of enkephalins has been shown to take place through cleavage of the Tyr–Gly amide bond. From this knowledge, two groups [10,11] have prepared the D.Ala_2 Met-enkephalin which is an enzyme-resistant analog and elicits a potent long-lasting analgesia.

Compared to Met-enkephalin, the natural peptide Leu-enkephalin exhibits a lower affinity for opiate receptors in vitro [9].

In order to obtain informations about the structure activity-relationships between the two natural peptides, we have studied the preferential conformation of Leu-enkephalin in DMSO-d_6 by ^{1}H and ^{13}C NMR.

2. Materials and methods

2.1. *Synthesis of Leu-enkephalin*

Large quantities of Leu-enkephalin were prepared by standard liquid-phase synthesis as previously described for Met-enkephalin [4] using L.Leu.*O*Me in place of L.Met.*O*Me. Leu-enkephalin (F : 216°C) was purified by crystallization ($MeOH/H_2O$ – 9/1).

2.2. *Spectroscopic measurements*

^{1}H Spectra were recorded on a Cameca 250 spectrometer operating at 250 MHz in CW mode and equiped with decoupler unit and variable temperature accessories.

^{13}C Spectra were recorded on a Varian XL-100 spectrometer operating at 25.2 MHz in FT mode. Measurements of the spin-lattice relaxation time T_1 were made with the 180°C–τ–90° sequence (ten values of τ between 10 ms and 300 ms). The T_1-values were obtained by regression analysis ($0.95 < r < 0.99$). T_1 (± 20%) of the carbonyl and aromatic non-protonated carbons were measured by progressive saturation method.

All the chemical shifts are given with respect to external Me_4Si.

The analysis of the $^3J_{NH-CH_2}$ coupling constants of the glycine residues were obtained through simulation of the spectra.

3. Results

3.1. *^{1}H NMR results*

3.1.1. Analysis of Leu-enkephalin ^{1}H spectra in DMSO-d_6

Figure 1 shows the ^{1}H spectrum of Leu-enkephalin (4.5×10^{-2} M) in DMSO-d_6 (100%) at 40°C. Unambiguous proton assignments were obtained from selective irradiations performed at different temperatures for avoiding overlaps. The assignment of Gly NH-signals was based on the same features as used for Met-enkephalin [4].

The experimental data (δ, J) measured at 40°C are reported in table 1 and the derived conformational parameters in table II.

The conformational θ and ϕ dihedral angles [12] are derived from their relationships with the ^3JNH–Hα vicinal coupling constants [13,14]. The most probable ψ values have been evaluated from steric energy-maps [15]. The rotameric populations of the side chains residues (Tyr, Phe, Leu) around χ_1-angle and called '*a,b,c*' were extracted from the side chain coupling constants ^3JCH$_\alpha$–CH$_\beta$ according to Pachler [16].

3.1.2. Temperature-dependence of the NH chemical-shifts

Figure 2 shows the chemical shift variations $\Delta\delta$ of the amide protons versus temperature. Compared to the NH of Gly_2 and Phe_4, the amide protons of Gly_3 and especially of Leu_5 ($\Delta\delta = 3.3 \times 10^{-3}$ ppm.d^{-1}) exhibit smaller temperature dependencies. It is worth noting that a small temperature-dependence of an NH proton is often evidence for a hydrogen bond [17] involving this proton but it could only indicate that the NH is buried [18]. In any case the smaller $\Delta\delta$ for the NH of Gly_3 and moreover of Leu_5 are in favour of either a hydrogen bond or a buried position for these protons.

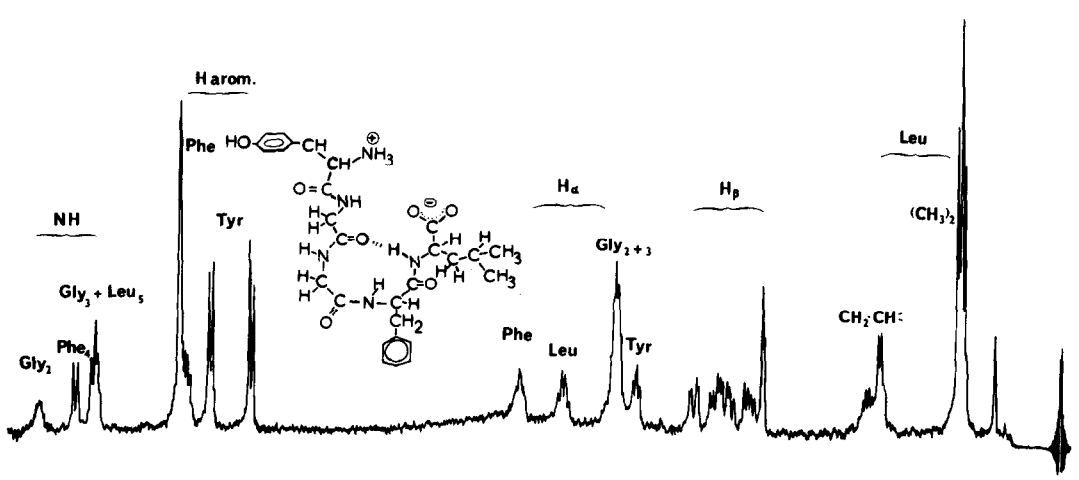

Fig.1. ^{1}H Spectrum of Leu-enkephalin (4.5×10^{-2} M) in DMSO-d_6 at 40°C.

Table 1
Chemical-shifts and J-coupling in Leu-enkephalin at 40°C

Residue	Shifts (ppm/TMS)				Coupling constants (J in Hz)				
	H_α	H_β	NH	Other	$^2J_{\alpha_1\alpha_2}$	$^3J_{\alpha\beta}$	$^2J_{\beta_1\beta_2}$	$^3J_{NH-H_\alpha}$	Other
		2.9		Arom.		5.0			
Tyr_1	3.5_5			7.0 H_m			13.5		$J_{o.m} = 8.5$
		2.6		6.7 H_o		8.0			
Gly_2	3.5		8.4					~4.5[a] (twice)	
Gly_3	3.6		7.9		17.8[a]			5.5–6[a] (twice)	
		3.1		Arom.		4.0			
Phe_4	4.5		8.1				13.8	8.5	
		2.8		7.2		9.5			
				$CH-(CH_3)_2$		12			$JCH-CH_3 = 6.5$[b]
Leu_5	4.1	1.5	7.9_5				14	7.8	$JCH-H_{\beta I} = 5.5$[b]
				1.6 0.9		1.0			$JCH-H_{\beta 2} = 7.5$[b]

[a]Computed values
[b]Computed values at 90°C

Chemical-shifts and J-coupling in Leu-enkephalin at 40°C

3.1.3. Determination of the preferential conformation of Leu-enkephalin

From the conformational angles determined in table 2 we can reject a γ-turn [19,20] involving the CO of Gly_3 and NH of Leu_5 for the profit of a β-turn.

As for Met-enkephalin [3,4] we suggest a β_I-bend to be the lowest energy-chain reversal conformation for Leu-enkephalin. The angular parameters required for this type of bend [18] ($\phi_{i+1} = -60°$, $\psi_{i+1} = -30°$ and $\phi_{i+1} = -90°$, $\psi_{i+2} = 0°$) are satisfied when residues $i + 1$ and $i + 2$ are identified with Gly_3 and Phe_4. Consequently such a β_I-turn does involve the

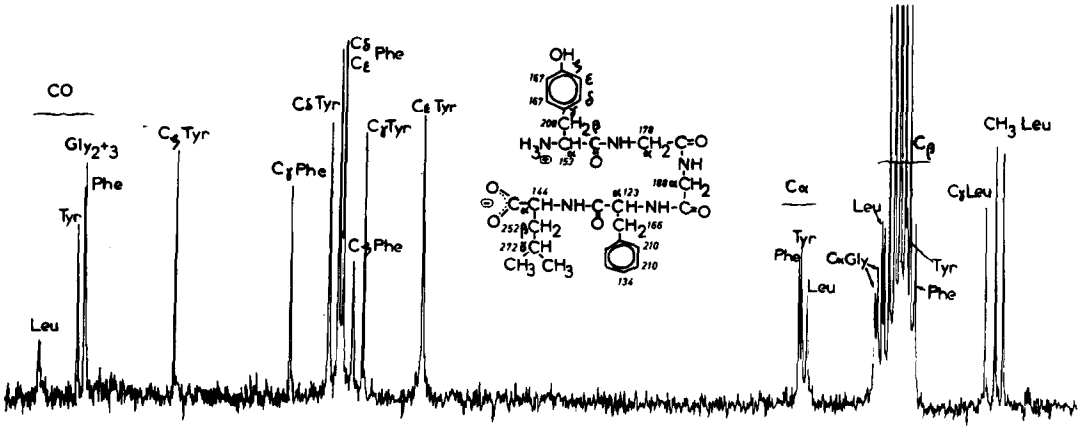

Fig.2. Plots of δNH (ppm) versus temperature (°C).

Table 2
Conformational parameters in Leu-enkephalin at 40°C

Residue	$\theta°$	$\phi°$	$\psi°$	Rotamers population		
				a	*b*	*c*
Tyr			± 60[a]	0.49[b]	0.22[b]	0.29
Gly_2		+ 90	+ 30 and −60 to −120			
	± 30	+ 30	~ + 60			
	± 120	− 60	~ − 30			
		± 180	± (60 to 180)			
Gly_3	± 10	+ 50	+ 30 and −120			
	± 130	± 70	± 30 and −120			
		− 170	± (60 to 180)			
Phe_4	+ 150	− 90	0 ± 30	0.63[b]	0.10[b]	0.27
	+ 210	− 150	−60 and 120 ± 40			
Leu_5	+ 145	− 85		~ 1.0[b]	~ 0.0[b]	~ 0.0
	+ 215	− 155				

[a] Most probable value
[b] The attribution of rotamers *'a'* and *'b'* may be reversed if the signal locations for the β-protons pro *R* and pro *S* are opposite

Conformations quoted *'a'*, *'b'* and *'c'* are those with C_β–C_R bond antiperiplanar to respectively the carbonyl O=O, the peptide-NH and C_α–H bond.

Gly_2, Gly_3, Phe_4 and Leu_5 residues with a hydrogen bond between the CO of Gly_2 and NH of Leu_5 in accordance with the small temperature variation for the NH of Leu_5. A $\beta_{II'}$-type bend, although predicted [18,21] to be less energetically favoured than a type I cannot be rejected definitively because the values determined for Gly_3 and Phe_4 (table 2) are closed to those described in a $\beta_{II'}$-type bend (ϕ_{i+1} = +60°, ψ_{i+1} = −120°, ϕ_{i+2} = −80°, ψ_{i+2} = 0°) with these residues at the $i+1$ and $i+2$ positions of the turn [18].

For the residues not included in the chain reversal we propose to take the values (ϕ_2 = 180°, ϕ_5 = −155°) for Gly_2 and Leu_5 which favour a final folded structure with a head-to-tail interaction between NH_3^+ and COO^- as proposed in Met-enkephalin [4].

The somewhat smaller temperature dependence of the NH of Gly_3 cannot be explained by a γ-turn between the CO of Tyr_1 and NH of Gly_3 because the ϕ, ψ values (table 2) are not in accordance with those required [19,20] for such a bend.

Except for Gly_2, the coupling constants are not largely modified by an increase of temperature indicating that the conformation of the backbone is well defined. At 80°C, the coupling constant ^3JNH–CH_2 in Gly_2 becomes < 0.8 Hz Nevertheless this decrease is not necessarily related to conformational changes. The exchange of the NH-proton following the NH_3^+ of the first amino acid rises with temperature ($\tau < 1/J$) which prevents the existence of coupling constant [22,23].

The side-chain rotamer populations listed in table 2, show that for Tyr and Phe, the two possible *trans*-gauche rotamers are favorised but like in Met-enkephalin the others have significant populations. On the opposite in the residue Leu only one *trans*-gauche rotamer around χ_1 is populated.

3.2. ^{13}C NMR chemical-shifts and T_1 relaxation times

Figure 3 shows the ^{13}C spectrum of Leu-enkephalin (0.1 M in DMSO-d_6/D_2O, 1/1) at 38°C. The assignments of all the ^{13}C-resonances were made by comparison with those of Met-enkephalin [5]. The experi-

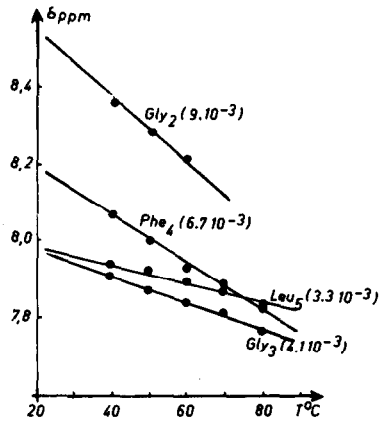

Fig.3. ^{13}C Spectrum of Leu-enkephalin.

Table 3
^{13}C Chemical-shifts and relaxation-time of Leu-enkephalin in DMSO–D_2O

Tyr	CHα	55.2	0.143
	$CH_2\beta$	37.1	0.208
	Cγ	126.1	1.5
	CHδ	131.6	0.167
	CHε	116.6	0.167
	Cζ	156.4	1.0
	C=O	171.9	1.5
Gly	$CH_2\alpha^a$	43.4	0.178
	$C=O^b$	170.9	2.0
Gly	$CH_2\alpha^a$	43.0	0.188
	$C=O^b$	170.8	1.7
Phe	CHα	55.5	0.123
	$CH_2\beta$	38.1	0.166
	Cγ	137.8	1.6
	CHδ	129.4	0.210
	CHε	130.1	0.210
	CHζ	127.7	0.134
	$C=O^b$	170.6	0.9
Leu	CHα	55.0	0.144
	$CH_2\beta$	42.2	0.253
	CHγ	25.5	0.272
	$CH_3\delta$	23.9	1.5
	$CH_3\delta$	22.6	1.7
	C=O	178.2	0.8

[ab]These assignments may be reversed

mental data (δ, T_1) are reported in table 3. All the protonated ^{13}C exhibit a maximum NOE and thus their relaxation is only due to a dipole–dipole mechanism.

The T_1-values for the CH_α of the backbone are of the same order and these results imply the same correlation time for the motions of all these carbons including those of the terminal residues. Phe_4 and Leu_5 side-chains have significant internal rotational freedom contrasting with that of Tyr_1-residue which exhibits no reorientation about its C_γ–C_ζ axis. All these results were found in Met-enkephalin [5] and the behaviour of Tyr has been attributed to a restricted motion with respect to the backbone and related to the mechanism of ligand–receptor interaction for this peptide [24].

In fact the smaller NT_1-values of Tyr-residue were also found in the peptide precursors of enkephalin (to be published) and these results show that the T_1 measurements of Tyr should be interpreted with care in terms of conformational behaviour.

4. Conclusion

As shown by the NH temperature-dependence the conformational preference of the backbone of Leu-enkephalin is not as well defined as in Met-enkephalin.

In Leu-enkephalin it seems that the energy difference between at least two β-bends is small and there could exist a rapid equilibrium between these forms. However, in all cases it appears that Leu-enkephalin exhibits a highly folded structure as shown by the conformational preference of the Leu_5 side-chain which should not be restricted in a time averaged rotational conformation.

Many structural analogs of the enkephalin were prepared but it seems that the $L.Tyr_1$- and $L.Phe_4$-residues are essential for activity [7–9]. Thus the topological requirements between these aromatic rings are certainly of great importance particularly if the Phe-ring is involved in a folded part of the peptide. On the other hand, the replacement of Gly_2 by $D.Ala_2$ leads to a more active compound because it is not cleaved by the brain enzymes. The inactivation is easily obtained in the natural peptide by the relative freedom of the *N*.Tyr–Gly moiety. Due to the presence of many conformational rotamers for Tyr and Phe

side-chains, there may not be a simple relationship between the rotamers populations and the side-chain conformations at the receptor, although the *trans*-gauche rotamer of Tyr which presents the best geometrical analogy with morphine is also the most populated. Hence the differences in analgesic potencies between Met-enkephalin and Leu-enkephalin could be related to the life-time of the ligand–receptor complex but cannot be easily explained by our conformational study. Nevertheless such relationships between conformation and affinity for the receptor should be reinvestigated on the various classes of binding sites of enkephalins recently evidenced in rat-striatum [25,26].

Acknowledgements

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