PREFERENTIAL CONFORMATION OF THE ENDOGENOUS OPIATE-LIKE PENTAPEPTIDE MET-ENKEPHALIN IN DMSO-D_6 SOLUTION DETERMINED BY HIGH FIELD ^{1}H NMR.

C. GARBAY-JAUREGUIBERRY, B.P. ROQUES and R. OBERLIN

ERA (CNRS) n° 613 - ECOLE POLYTECHNIQUE
17, rue Descartes - 75230 PARIS CEDEX 05. FRANCE

M. ANTEUNIS and A.K. LALA

Laboratory for NMR Spectroscopy - UNIVERSITY OF GENT
Krigslaan 271 - GENT B-9000.BELGIUM

Received May 14,1976

SUMMARY :

A preferential conformation of the endogenous opiate-like pentapeptide Met-Enkephalin in DMSO-d_6 solution was proposed from high field ^{1}H NMR experiments at variable temperature and complete analysis of the coupling constants in relation with conformational energy steric maps.

This conformation is characterized by a highly folded secondary structure with a β_I turn involving a head-to-tail interaction and a quasi-axial position of the methionine side chain. The N-terminal Tyr-Gly moiety which exhibits a relative degree of freedom shows all the steric requirements found in opiates for a stereospecific interaction with the receptor. All these results are discussed in relation with the physico-chemical and biological properties of opiate-like peptides.

The mechanism of action of morphine and opiates has been for many years an unsolved problem in neuropharmacology. However, two years ago, the existence of opiate receptor has been directly demonstrated (1) and very recently two endogenous peptides (enkephalins) with morphine-like action have been isolated by Hughes et al from brain extracts (2). The sequence of the most active peptide Met-Enkephalin was determined as H-Tyr-Gly-Gly-Phe-Met-OH (2). Morphine and opiates have very rigid structures and exhibit fairly stringent structural requirements for activity. So, it seems of great importance to determine the structural analogy between opiates and enkephalins, which belong to very different classes of compounds. Although for low molecular peptides many conformations are possible, it seems that the great hydrophobic character of enkephalins, due to the nature of their amino-acids components, could lead to a preferential conformation in solution exhibiting relationships with opiates. ^{1}H NMR spectroscopy is one of the most appropriate methods to investigate

the conformational behaviour of such a peptide in solution (3).

Met-Enkephalin was studied in DMSO-d_6 solution at 300 MHz and its preferential conformation determined by use of temperature variation and complete analysis of coupling constants in relation with Neel's curves (4) and conformational energy steric maps (5). The large differences in the $^3J(NH-CH_\alpha)$ coupling constants permitted to exclude a random-coil structure and to propose a preferential conformation for Met-Enkephalin in solution, *e.g.* a highly folded secondary structure, probably a β_I turn, characterized by a head-to-tail interaction. This structure shows all the steric requirements found in morphine for a stereospecific interaction with the receptor (6). The relative freedom of the N-terminal peptide Tyr-Gly and a quasi-axial position of the methionine side chain are the most characteristic features of the proposed conformation and could account for some of the physicochemical and biological properties of enkephalins and others endorphins (peptides with opioid activity).

MATERIAL AND METHOD.

Synthesis of Met-Enkephalin. Large quantities of Met-Enkephalin were prepared by standard liquid phase synthesis. Materials were Gly-Gly-L.Phe, t.boc-L.Tyr, L-Met.OMe (Sigma) ; triethylamine, trifluoroacetic acid (TFA), dicyclohexylcarbodiimide (DCC) (Merck).

After protection by t.boc, the Gly-Gly-Phe was coupled with L.Met.OMe (DCC method) and after deprotection by TFA of the so-formed tetrapeptide the resulting compound was finally coupled with t.boc-Tyr (DCC). The free Met-Enkephalin was obtained by successive deprotections by saponification (C-terminal group) and by use of TFA (N-terminal group). All the intermediates and the Met-Enkephalin (F:210°C) were purified by crystallization. All the compounds displayed correct elemental analyzes and their structures were confirmed by ^1H NMR.

Spectroscopic determinations. The spectra were recorded on a VARIAN HR 300 spectrometer operating in CW mode equiped with a SC 8525-2 decoupler unit and monitored by observing the splitting in ethylene glycol. Chemical shifts were measured from internal reference (TMS) and reliable to ±0.01 ppm.

RESULTS.

Description of the spectra.

Figure 1 represents the spectrum of Met-Enkephalin (10^{-1}M) in DMSO-d_6 (100 %) at 19°C. Unambiguous proton-assignments were obtained from selective irradiations and the δ, J parameters are reported in table I. A problem raised as to assign the Gly-signals to the correct residues. This was possible based on the fact that the near N-terminal peptide NH-proton in oligopeptides displays a broadened signal as a result of the proximity of NH_3^+ (7). This phenomenon is general feature as well in water (8) as in DMSO-d_6, as shown in a separated study of di- to tetrapeptides including

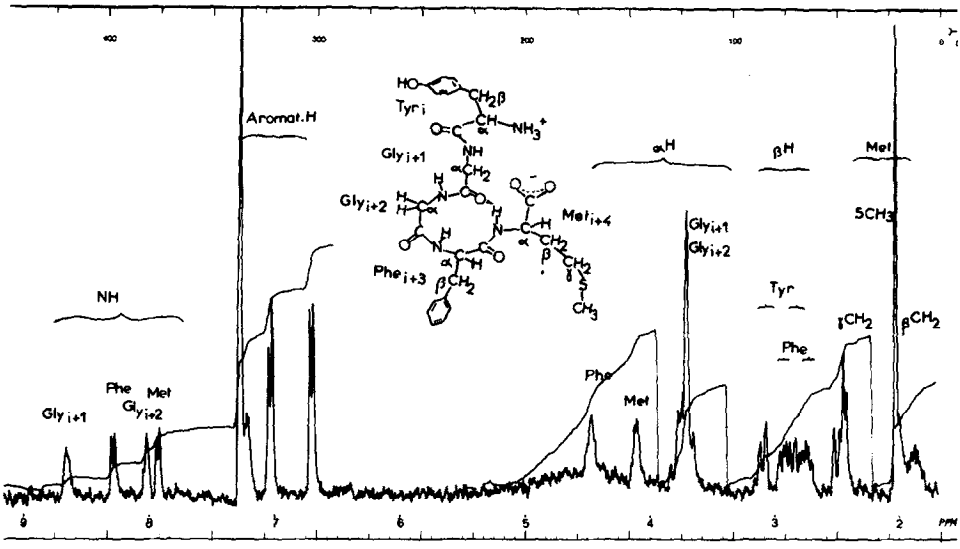

Figure 1. 300 Mhz spectrum of Met-Enkephalin (10^{-1} M) in DMSO-d_6 at 19°C. TMS as internal reference.

TABLE I

	Shifts (δppm/TMS)				Coupling constants (J in Hz)				
Residue	δH_α	δH_β	δNH	other	$^2J_{\alpha_1\alpha_2}$	$^3J_{\alpha\beta}$	$^2J_{\beta_1\beta_2}$	$^3J_{NH,H_\alpha}$	other
Tyr_i	3.7	2.7 2.9		arom. 7.0 H_m 6.7 H_o		2.9 5.8	13.7		arom. $J_{o.m}$ = 8.5
Gly_{i+1}	3.8 3.6		8.6		16.6			~ 1.5 (twice)	
Gly_{i+2}	3.7		8.0					5.2 (mean)	
Phe_{i+3}	4.4	3.1 2.8	8.2	arom. 7.2		3.8 9.9	13.9	8.6	
Met_{i+4}	4.1	2.0 1.8	7.9	γCH_2 SCH_3 2.4 2.0		7.4		7.4	

Chemical shifts and J couplings of Met-Enkephalin in DMSO-d_6 at 19°C.

the precursors of Met-Enkephalin (*e.g.* Tyr-Gly, Tyr-Gly-Gly, Tyr-Gly-Gly-Phe (9)).

Determination of the preferential conformation.

Due to the nature of the residues, we have accepted only trans-peptide bonds to be present in Met-Enkephalin. If, for instance, a mixture of cis and trans conformations would be present, we expect the observation of separate signals corresponding to each one.

The experimental data are gathered in table I, and from these, the conformational parameters χ and ϕ values (10) (table II) were obtained. The most probable ψ values have been evaluated from steric energy maps (5). ϕ_i value was experimentally not accessible and therefore we propose $\psi_i = \pm 60°$ as follows from the most probable region in the ϕ,ψ maps. It fits well with our final folded from for Met-Enkephalin.

We reject definitively the possibility of $\phi_{i+1} = 0$ (eclipsed strain).

A value of $\theta_{i+1} \simeq 120°$, giving $\phi_{i+1} \simeq 180°$ shoud be rejected if the Neel's refinement of the Bystrov curve is used (4), because this would result in a valence angle of $CH_2^{\alpha}{}_{i+1} > 120°$. Accepting a certain zone relationships (11), one should be aware of the uncertainties in angle values, especially near minima and maxima of the (J,θ) curves. However, although this ϕ_{i+1} value of 180° is not in a deep well energy (5), it could represent an averaged value of *e.g.* two favorable conformations having ϕ-values of opposite sign.

The ϕ_{i+2} values of -65°, +65° correspond to minimum energy conformations in relation with ψ_{i+2} of -30° and +30° in contrast to ϕ_{i+2} values of ± 140° that should be less favorable. The ϕ_{i+3} and ϕ_{i+4} are easily extracted because the J-values are such that we are out of the degenerated region of the Neel's graph (4).

From table II, it can be seen that next to some specific conformations whose existence cannot be completely eliminated, the β_I type bend is one of the best candidates (12). This turn, involving to CO of j and NH of j+3 residues is characterized (12) by (ϕ,ψ) values for j+1 and j+2 residues of respectively (-60°, -30°) and (-90°, 0°) to be identified with Gly_{i+2} and Phe_{i+3}. Consequently, such a β_I turn does involve the Gly_{i+1}, Gly_{i+2}, Phe_{i+3} and Met_{i+4} residues with an H-bond between the CO of Gly_{i+1} and NH of Met_{i+4}.

. NH exchange.

The proposed secondary structure is confirmed by the temperature variation of the NH chemical shifts (δ). As shown in figure 2, the $Met_{i+4}NH$ exhibits the smaller variation $\Delta\delta$ in fonction of temperature (figure 2).

TABLE II

Residue	θ°	φ°	ψ°	rotamers population a	b	c
Tyr_i			± 60 •	0.48 ✡	0.29 ✡	0.23
Gly_{i+1}	± 55	0 ± 5				
	± 120	+ 120				
		+ 180	from 60 to 180			
Gly_{i+2}		± 65	± 30 ✡✡			
		± 140	from + 60 to + 180 and -60			
Phe_{i+3}	+ 150	- 90	~	0.67 ✡	0.11 ✡	0.22
	+ 210	- 150	-60 or 130 ± 40			
Met_{i+4}	+ 140	- 80	0 to -60	0.44	0.22	0.34
	+ 220	- 150	-60 or 120 ± 40			

Conformations quoted "a", "b" and "c" are those with $C\beta$-C_{ar} bond anti periplanar to respectively the carbonyl C=O, the peptide NH and C^{α}-H bond.
✡ Analysis of the spin system of C^{α}, C^{β} protons do not allow in DMSO-d_6 to extract rotamer populations. This attribution may be reversed if the signal location of the pro R and pro S β-protons of the aromatic residues are opposite.
• Not extracted from the spectrum but temptatively obtained from considerations of energy plots.
✡✡ Most probable value, especially for poly-Gly flexible model (5).

Although this fact does not necessarily disclose hydrogen bonding, it does indicate that the Met_{i+4}NH is buried. It should be noted that the typical CO_j-NH_{j+3} hydrogen-bond is not a definitive requirement for a β-turn as recently suggested by Scheraga et al (12). In any case, the preferential broadening for the two Gly NH protons on heating (and not for Met and Phe) might show a relative preferential screening for at least the Met NH.

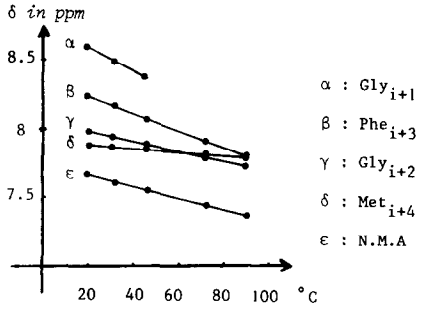

Figure 2. Chemical shifts variation (ppm) of the NH protons of Met-Enkephalin and N-Methyl acetamide (NMA) in DMSO-d_6 versus temperature.

DISCUSSION.

From molecular model-building of Met-Enkephalin we prefer the ϕ_{i+4} value of -150° because the value of -80° would bring in strong steric interactions the Met-side chain and the head of the peptide backbone (table II). Then, the Met-side chain appears in a pseudo-axial position. The behaviour around χ^1_{i+4} suggests a preference for certain rotational isomers (see table II). In the folded structure adopted by Met-Enkephalin in solution (6) the two aromatic residues (Tyr and Phe) are pseudo-equatorial to the mean plane of the molecule and their χ^1 values show that they have some degree of freedom. These points are very interesting because they show that the three hydrophobic chains (Tyr-Phe-Met) are directed, away from the backbone in accordance with the low water solubility of Met-Enkephalin.

The minor changes in coupling constants with the increase of temperature indicate that the conformation is well defined. This is further corroborated by the analogy in conformation of Met-Enkephalin in DMSO and after adding more than 60 % H_2O (13). The addition of D_2O does not induce any noticeable change in the chemical shifts of the aromatic protons, excluding any intra or intermolecular stacking between the two aromatic rings.

The proposed conformation of Met-Enkephalin in solution is different to that advanced by Bradbury et al (14) and characterized by a β-turn involving an H-bond between the CO of the Tyr residue and the NH of the Phe residue.

Structure-activity relationships.

From our results, some physico-chemical and biological properties of

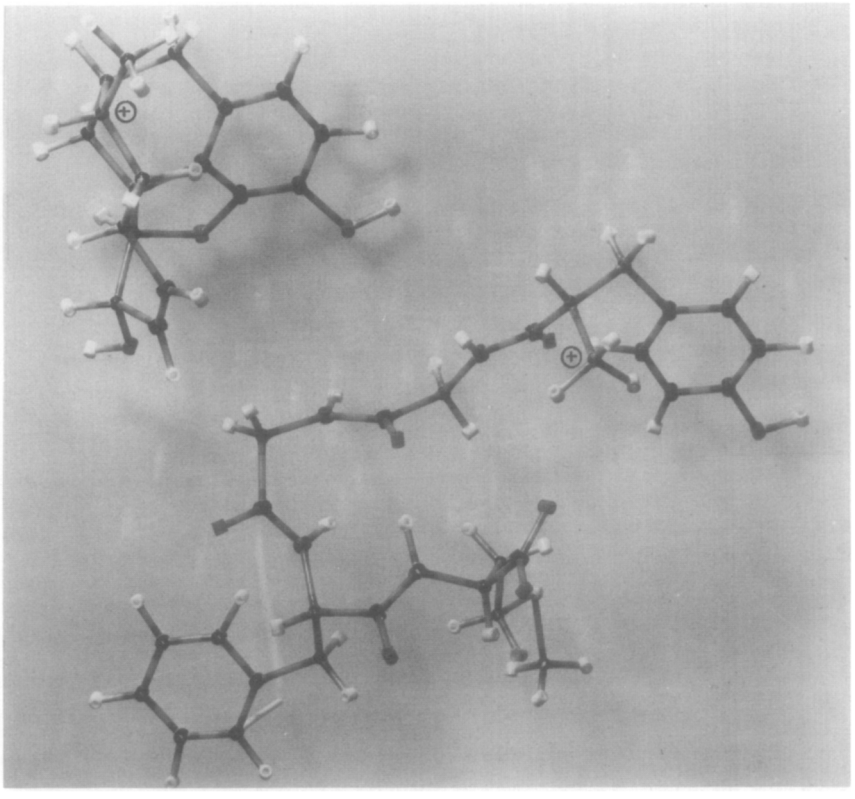

Figure 3. Framework molecular models of the proposed conformation of Met-Enkephalin (right) and of the morphine structure (left).

opiates could be explained. A three points contact is necessary to explain enantiomeric binding selectivity. The most evident analogy between morphine and enkephalins therefore concern the phenol ring linked to an ethylamine chain which is represented in enkephalins by the N-terminal Tyr residue (15) charged at physiological pH (figure 3).

All the opioid peptides like enkephalins, lipotropin C and synthetic peptides have in common the opioid N-terminal Tyr-Gly residues, but differ by the hydrophobic rest of the molecule. It can be assumed that in all these compounds the Tyr-Gly moiety is in a state of relative freedom allowing the primary attachment of the opioid part and the secondary adaptation of the different hydrophobic moieties of these peptides to the receptor. Thus, the relative differences in affinity between all these compounds could be due to the secondary interactions. According to this hypothesis, the

Tyr-Gly-Gly-Phe peptide (16) and Leu-Enkephalin (2) exhibit smaller activity than Met-Enkephalin. Moreover this assumption affords an explanation to the loss of activity for the opioid peptide H-Tyr-$(Gly)_3$-Lys-Met-Gly-OH synthesized by Golstein (15), when the ammonium terminal residue group is protected by N-Ac-Ser residue. The synthesis of Enkephalin derivatives offers an unique possibility to explore the receptor structure and to design an analgesic without addictive properties (17). Several of these problems are now in progress in our laboratory.

ACKNOWLEDGMENTS.

This work was supported by the CNRS, the Université René Descartes and the Université of GENT.

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