AVERAGING OF ϕ_2 AND ϕ_3 IN [5-LEUCYL]-ENKEPHALIN

NMR study of two isotopic isomers

Alan J. FISCHMAN, Mark W. RIEMEN and David COWBURN
The Rockefeller University, New York, NY 10021, USA

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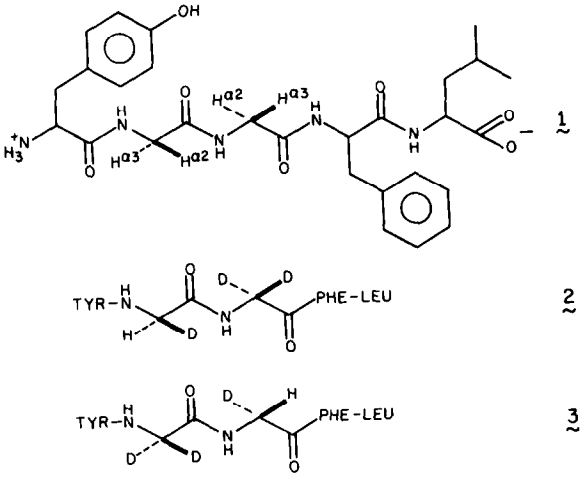
1. Introduction

Speculation, e.g. [1], concerning the possible conformational similarities between enkephalin and opiates might be resolved by detailed NMR studies of the conformations of the peptide. Studies to date [2–4] have demonstrated that proton NMR spectra of enkephalin in aqueous solution are most consistent with a number of rapidly interconverting conformers. Analysis of $^3J(H'-H^{\alpha})$ [2–4] for glycyl residues 2 and 3 have heretofore been of limited validity because only the sums of $^3J(H'-H^{\alpha 2})$ and $^3J(H'-H^{\alpha 3})$ were available. Observation of the individual $H'-H^{\alpha}$ couplings of these two residues requires NMR measurements of the isotopic isomers of the peptide synthesized with stereochemically deuterated glycines [5,6].

2. Experimental

Solid phase synthesis of 2 and 3 were carried out using a typical manual procedure [7]. Boc-L-leucine was esterified to chloromethylated polystyrene co-1% divinyl benzene by the cesium salt procedure [8]. The degree of substitution of the resin by leucine was 0.19 mmol/g, as determined by amino acid analysis. Trifluoroacetic acid (50%) in methylene chloride (20 min) was used for deprotection. Diisopropylethylamine (5%) in methylene chloride (10 min) was used for neutralization. Coupling was done with 3 equiv. Boc-amino acid and 3 equiv. dicyclohexylcarbodiimide (DDC) in methylene chloride: the Boc-amino acid was equilibrated for 10 min with the resin before the addition of DCC: coupling was allowed to proceed for 1 h. Single couplings were performed with isotopic isomers of glycine and with tyrosine, and double couplings were done with phenylalanine. The hydroxyl group of tyrosine was unprotected in each synthesis. At the end of a synthesis the peptide-resin was treated with benzylamine (10%) in DMF for 30 min at room temperature to remove any unwanted esters that might have formed at the hydroxyl of tyrosine. Peptides were removed from the resin by treatment for 1 h at 0°C with liquid hydrogen fluoride containing 10% anisole as a cation scavenger. The peptides were extracted from the resin with acetic acid (5%) in water and lyophilized.

The structures of [5-leucyl]-enkephalin and of the isotopic isomers are shown in fig.1. The unsubstituted



1 2 3

Fig.1. Structures of enkephalins studied in this report.

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material, 1, was purchased from Vega-Fox. The products 1–3 were > 90% pure on the basis of total material in a single peak on a Dowex 50 chromatogram, using ninhydrin detection.

(*R*)-[α-^{2}H]Glycine was prepared by a modification of the procedure in [6] which will be published elsewhere. [α-$^2H_2$]Glycine was prepared by the method in [9]. Glycines were protected for peptide synthesis using di-*t*-butyldicarbonate (Fluka) according to the manufacturer's instructions. The NMR methods used are described in [10,11].

3. Results

For comparison with previous studies in dimethylsulfoxide (DMSO), fig.2 shows 220 MHz proton NMR spectra of 1–3 in DMSO, under conditions in which the zwitterion (NH_3^+-Tyr–Gly–Gly–Phe–Leu-COO^-) is expected to exist [4]. Changes in coupling patterns seen at 8.4, 7.8, and around 3.5 ppm downfield from

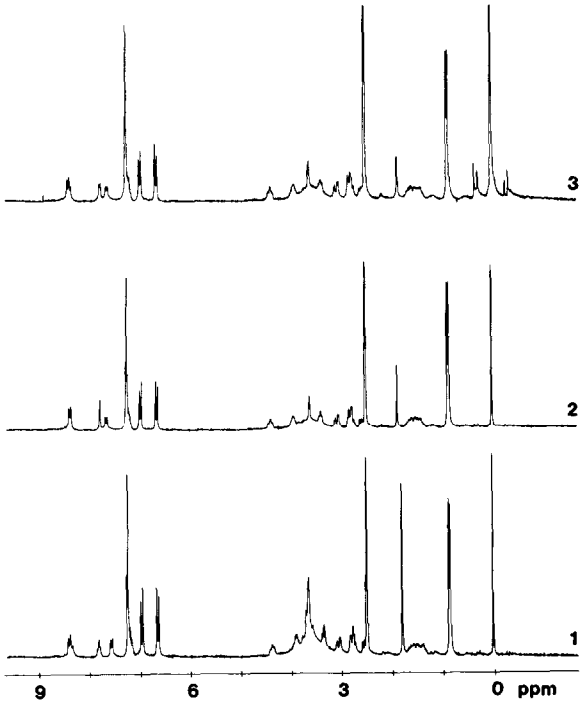

Fig.2. 220 MHz proton NMR spectra of the 1, 2 and 3 in [$^2H_6$]dimethylsulfoxide.

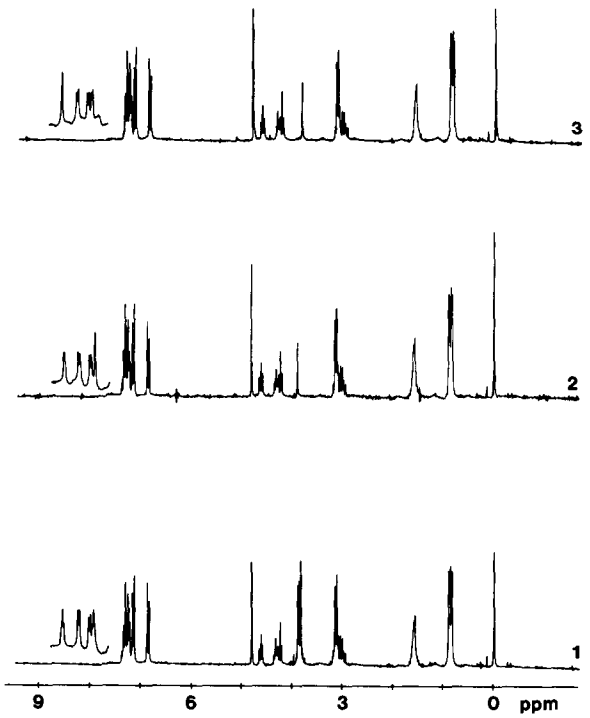

Fig.3. 220 MHz proton NMR spectra of 1, 2 and 3 in D_2O and, in the inserts, in H_2O.

TMS are consistent with previous assignments [4] of the glycyl residues.

In fig.3, the spectra in D_2O and H_2O (insets) are shown. Tabulations of chemical shifts and coupling constants from these figures are in table 1. At pH 7, in agreement with the observations in water/DMSO solution [2], the H_2' resonance is broadened beyond detection, and H_3' resonances are buried by the Phe^4 H' signal.

4. Discussion

The use of $^3J(H'–H^\alpha)$ to investigate the angle ϕ is well known [13]. Whether or not ϕ is averaged is difficult to determine when only a single $^3J(H'–H^\alpha)$ is available, but in the case of glycyl residues a pair of $^3J(H'–H^\alpha)$'s is available for the ϕ angle. For a fixed dihedral angle, the value of ^3J is expected to fall on the appropriate Karplus curve [12,13], and for the two α protons of a glycyl residue, the two ^3J's should

Table 1
Chemical shifts and coupling constant of [5-leucyl]-enkephalin from the compounds studied, 1–3

Residue	Chemical shift		Coupling constants	
	Proton	ppm downfield from reference[a]	Between protons	Value (Hz)
In [$^2H_6$]dimethylsulfoxide				
Glycyl 2	Amide	8.39		
Glycyl 3	Amide	7.82	Amide–α2[b]	5.1
			Amide–α3	5.1
Phenylalanyl 4	Amide	8.41	Amide–α	8.3
Leucyl 5	Amide	7.57	Amide–α	7.6
In H_2O/D_2O at pH 3				
Glycyl 2	Amide	8.94	α2–α3	−16.8
	α2	3.87	Amide–α2	5.5
	α3	3.92[c]	Amide–α3	4.7
Glycyl 3	Amide	8.32	α2–α3	−17.3
	α2	3.86	Amide–α2	5.5
	α3	3.83_5[c]	Amide–α3	4.8
Phenylalanyl 4	Amide	8.40	Amide–α	7.3
Leucyl 5	Amide	7.42	Amide–α	8.6

[a] Reference was TMS in [$^2H_6$]DMSO and TSP in aqueous media
[b] The α2 proton in glycine, named from the rules in [18], is the (*pro*$_2$–*R*) α proton, and is in the equivalent position to the deutron of (*R*)-[α-^{2}H]glycine
[c] In 2 and 3, a small isotope substitution shift, ~0.01 ppm upfield, in seen in these α3 protons

fall, at the same value of the torsion angle ϕ, on two such curves displaced by about 120°. Although the exact dependence of $^3J(H'-H^\alpha)$ on a dihedral angle, θ, is not known, fairly precise values of the constants of the equation:

$$^3J(H'-H^\alpha) = A\cos^2\theta + B\cos\theta + C \qquad (1)$$

have been proposed [12,13]. If any of the observed pairs of ^3J's of the glycyl residues (table 1) are used in conjunction with these Karplus curves, then, for one proposed set of constants [12], no satisfactory paired intersections are found. If the other set [13] is used, a pair of ^3J's for each residue at ~6 Hz could be the result of a fixed ϕ either at about +75° or −75°. The question as to whether these fixed ϕ angles can explain the observed glycyl couplings of table 1 centers around the numerical difference between the predicted and observed values. Two compounds, gallichrome [13] and cyclo(Gly–Pro–Gly–D-Ala–Pro) [14] having glycyl ϕ's in this area have had crystal structures determined and NMR spectra analyzed. In both cases, the vicinal coupling constants found are significantly larger than those observed here in enkephalin. The possibility that fixed values of ϕ_2 or ϕ_3 exists in [5-leucyl]-enkephalin thus seems to be small (see fig.4).

A recent report of the crystal structure of [5-leucyl]-enkephalin determined the ϕ_2 and ϕ_3 angles to be 59° and 97°, respectively [15]. These values are moderately close to the +75° value found from the intersections on the proposed Karplus curves, but none the less, it seems more probable that averaging is occurring in solution for the following reasons. For both glycyl residues one of the $^3J(H'-H^\alpha)$'s predicted by the intersection of the crystal structure angle on the proposed curve (fig.4) always differs from the other coupling by > 2 Hz, significantly greater than

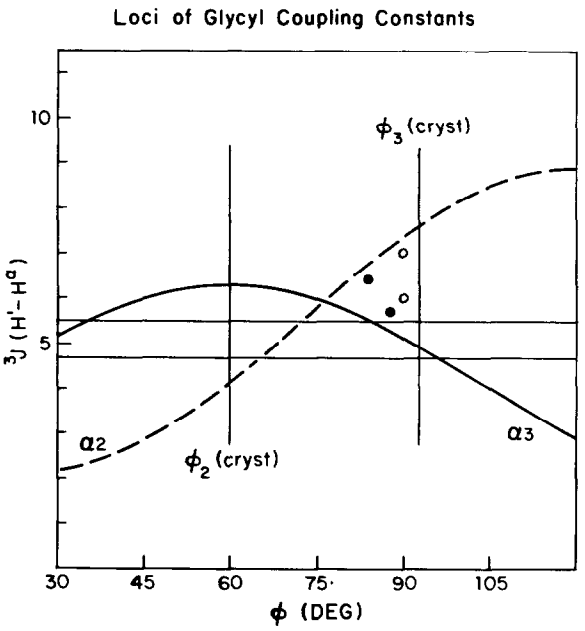

Fig.4. A quadrant of the angular dependence of $^3J(H'-H^\alpha)$ upon ϕ, showing the predicted curves from [14]. The curve for $^3J(H'-H^{\alpha 2})$ is continuous and for $^3J(H'-H^{\alpha 3})$ is dashed. Two sets of observed couplings (•) [13] and (○) [14] are shown. The largest and smallest $^3J(H'-H^\alpha)$ observed in enkephalin in this study are plotted as straight horizontal lines. The vertical lines are for values of ϕ found in the crystal structure of [5-leucyl]-enkephalin [15].

any observed difference in couplings in solution. In addition, for Gly^3 the predicted $^3J(H'-H^{\alpha 2})$ for a fixed ϕ would be larger than the $^3J(H'-H^{\alpha 3})$ predicted, whereas the reverse would hold for the pair of couplings for Gly^2. In fact, this is not observed in solution; both couplings to α2 are slightly larger. Although it would be very desirable to have more precise values for these fitted curves of ^3J as a function of ϕ, within the limits of experimental error, the possibility seems small that ϕ_2 and ϕ_3 of [5-leucyl]-enkephalin in solution are fixed either at the angles observed in the crystal structure or at other positions.

Little intramolecular hydrogen bonding has been demonstrated [2–4] in solution, whereas the crystal structure [15] contains two such bonds, and this, together with the coupling constant information presented here, makes it appear most unlikely that the solution structure resembles the crystal structure in its major features.

What can be said about the probable averaging about the glycyl angles in enkephalin in solution? Using empirical calculations of conformational energy, the values of averaged coupling constants, ^3J, have been calculated to be 5.8–6.1 [16] or 6.2–6.7 [17] for glycyl residues in peptides. A similar calculation can be made by assuming that $\phi \simeq +60°$, $+180°$ or $-60°$, for equal amounts of time, and then $^3J = A/2 + C$ from eq. [1]. Then ^3J would be expected to be 5.1 [13] or 4.8 [14] or, if a correction for electronegativity is included, 4.9 or 4.6 [13]. Given the limitations of the methods involved, the observed glycyl coupling constants appear to be very close to these expected values. In DMSO, the equality of $^3J(H'-H^{\alpha 2})$'s and $^3J(H'-H^{\alpha 3})$'s further supports the notion of averaging. The lack of such equality in aqueous solution at pH 3.0 would then reflect slight favoring of one sector of the rotation about ϕ over others.

In conclusion, there appears to be no evidence from this study that a particularly favored conformation of this backbone region of residues 2 and 3 of enkephalin exists in aqueous solution. The possible conformational resemblance of enkephalin to opiates may nonetheless exist, but such similarities might arise after binding of the effector to its appropriate receptor; presumably entropic costs inherent in such ordering would be borne by the binding energy of that complex.

Acknowledgements

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