NOE data at 500 MHz reveal the proximity of phenyl and tyrosine rings in enkephalin

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Met^5-enkephalin – a pentapeptide (Tyr-Gly-Gly-Phe-Met) – can exist in two possible folded arrangements with a rigid two-hydrogen-bonded network. In one arrangement, a Gly 2-Gly 3 β-bend is formed and in the other a Gly 3-Phe 4 β-bend. The two conformations are distinguished by the spatial relation of Tyr 1 and Phe 4: in the Gly 2-Gly 3 β-bend, Tyr 1 and Phe 4 can be brought close to each other while in the Gly 3-Phe 4 β-bend they are far apart (>5 Å). We have utilized one-dimensional (1D) nuclear Overhauser effect (NOE) measurements between the ring protons of Tyr 1 and Phe 4 to determine their proximity. The NOE data clearly show that a pair protons, one each from Tyr 1 and Phe 4, are as close as 3.3 Å while other inter-proton distances are beyond 4.5 Å. Therefore, we propose the presence of a Gly 2-Gly 3 β-bend (in which Tyr 1 and Phe 4 are spatially close) for Met^5-enkephalin in solution. The structure of Met^5-enkephalin in solution is very similar to the single crystal structure of Leu^5-enkephalin and tends to explain the biological activity data of several modified enkephalins.

Nuclear Overhauser effect *Enkephalin* *Met^5-enkephalin* *Conformation*

1. INTRODUCTION

Enkephalin, an endogenous morphine-like substance, is a mixture of two pentapeptides, i.e. Tyr-Gly-Gly-Phe-Met (Met^5-enkephalin) and Tyr-Gly-Gly-Phe-Leu (Leu^5-enkephalin) [1]. In view of the fact that enkephalin operates at the same receptor sites as natural morphine-like opiates [2], it is of interest to examine whether it has a very rigid structure like morphine [3]. Although there has been an accumulation of considerable data from both theoretical [4–11] and experimental [12–16] studies, there is a general lack of agreement regarding the solution conformation of Met^5/Leu^5-enkephalin. Models of enkephalin proposed so far [4–16] fall into two distinct types, both involving a β-bend but one with Gly 2 and Gly 3 at the corners of the β-bend in which Tyr 1 and Phe 4 are spatially close (fig.1) whereas the other has a Gly 3-Phe 4 bend in which Tyr 1 and Phe 4 are far apart (fig.2). Note that in both models, 4 of the 5 amino acids are rigidly held by a two-hydrogen-bonded network (figs 1,2). Our goal was to determine which model is the most likely in solution.

2. EXPERIMENTAL

The distinguishing feature of the two models is the spatial relation of Tyr 1 and Phe 4, i.e. in one model (fig.1) they are close to each other whereas in the other (fig.2) they are far apart. The proximity of the ring protons of Tyr 1 and Phe 4 can be monitored by observing the primary NOE at the Tyr 1 ring protons from Phe 4 ring protons and vice versa. We conducted our NOE experiments at 20°C for a dilute solution of Met^5-enkephalin (1 mM, pH 7.0) in D_2O such that intermolecular aggregation was prevented but at the same time intramolecular hydrogen bonds were retained (ensured by monitoring the amide protons of enkephalin in water). All the NMR experiments

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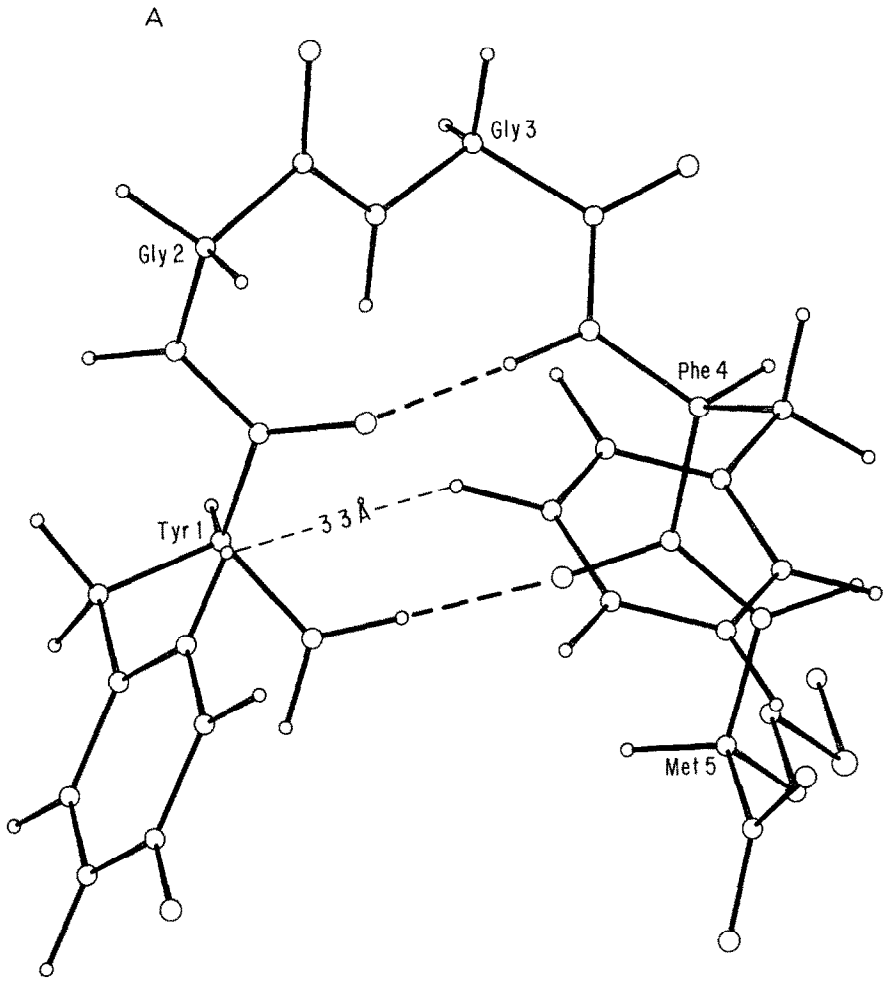

were conducted at 500 MHz. Assignment of all the non-exchangeable protons belonging to the 5 amino acids of Met^5-enkephalin was done by use of 2D COSY experiments (not shown) and a discussion of these is beyond the scope of this paper.

3. RESULTS

3.1. *NOE results*

For our experimental design, the aromatic protons of Tyr 1 and Phe 4 were of interest. As shown in fig.3A, 5 ring protons of Phe 4 appear at lower field as a multiplet (2:1:2) centered at 7.34, 7.30 and 7.26 ppm and Tyr 1 ring protons as a set of two doublets (1:1) centered at 7.15 and 6.86 ppm. When the signal of Tyr 1 at 7.15 ppm was irradiated a strong NOE was observed at 7.26 ppm of Phe 4 (fig.3B). Weak but noticeable NOEs were observed at 7.30 and 7.34 ppm of Phe 4. This could originate either as a secondary NOE from the site at 7.26 ppm or as a primary NOE between sites at 7.15 ppm of Tyr 1 and at 7.30 and 7.34 ppm of Phe 4 (distance >4 Å); however, the important conclusion from fig.3B is that the proton of Tyr 1 at 7.15 ppm and that of Phe 4 at 7.26 ppm are close in space (~3 Å). This point was re-confirmed by performing a reciprocal NOE experiment (fig.3C) in which the signal at 7.26 ppm (Phe 4) was irradiated. It was interesting to observe that NOE was observed only at 7.15 ppm (but not at 6.86 ppm) of Tyr 1. This clearly revealed that the proton of Phe 4 at 7.26 ppm is close to that of Tyr 1 at 7.15 ppm and the proton of Tyr 1 at

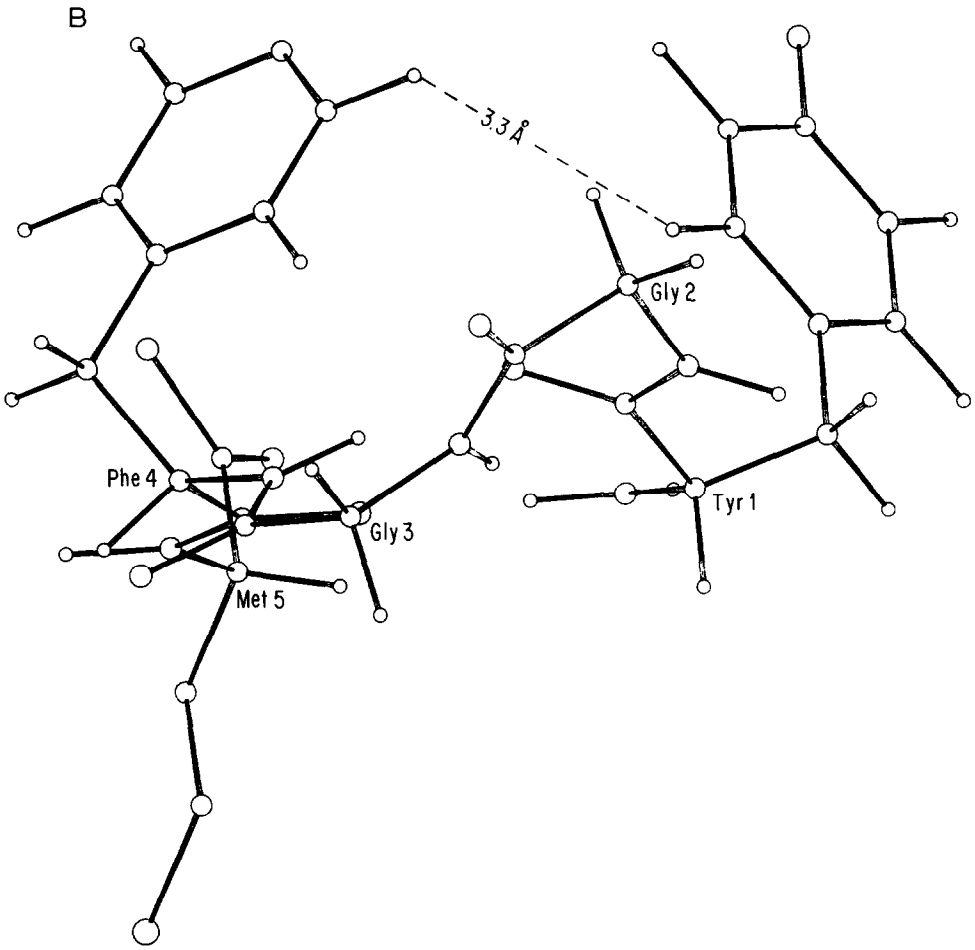

Fig.1. Two mutually perpendicular projections of Met^5-enkephalin in the type I′ β-bend arrangement. In projection A, the two-hydrogen-bonded peptide skeleton lies in the plane of the paper while in B the peptide skeleton is perpendicular. The α-atoms of the 5 amino acid residues are indicated. The torsion angles (designated as ϕ_i and ψ_i (°) around the N–C_i^α and C_i^α–C bonds) for this folded structure are: $\phi_2 = 60$, $\psi_2 = -20$; $\phi_3 = 130$, $\psi_3 = 5$; $\phi_4 = -135$, $\psi_4 = 180$. Note that, in this folded arrangement, in addition to two straight hydrogen bonds between Tyr 1 and Phe 4, there is a bifurcated hydrogen bond between -C = O of Tyr 1 and N-H of Gly 2 which might render additional stability to the molecule. For clarity, the bifurcated hydrogen bonds are not shown. Also, no hydrogen bonds are shown in B. The most interesting feature of this structure is the proximity of the two ring protons, one each from Tyr 1 and Phe 4, which is consistent with the NOE data of fig.3. In this structural arrangement, Tyr 1 and Phe 4 lie on one side of the peptide skeleton while Met 5 is on the opposite side. The hydrogen bonding schemes shown here (A) and in fig.2A are those proposed from extensive studies [4–16] and we have made attempts to reinvestigate these.

6.86 ppm is far away from the Phe 4 ring protons (which was also re-affirmed by irradiating the signal at 6.86 ppm and observing no NOE at Phe 4 ring protons; not shown).

3.2. *Molecular model building*

The NOE data unequivocally rule out the possibility of a structure in fig.2 involving a Gly 3-Phe 4 β-bend which keeps the Phe 4 and Tyr 1 far apart and suggests a β-bend arrangement centered at Gly 2 and Gly 3 (fig.1) either as a type I β-bend or its mirror image type I′ [17] in which Tyr 1 and Phe 4 are spatially close. In the type I β-bend, the torsion angles are $\phi_2 \sim -60°$, $\psi_2 \sim -30°$ and ϕ_3 ~90°, ψ_3 0° (fig.1). Since glycine is an achiral amino acid, both type I and type I′ β-bends

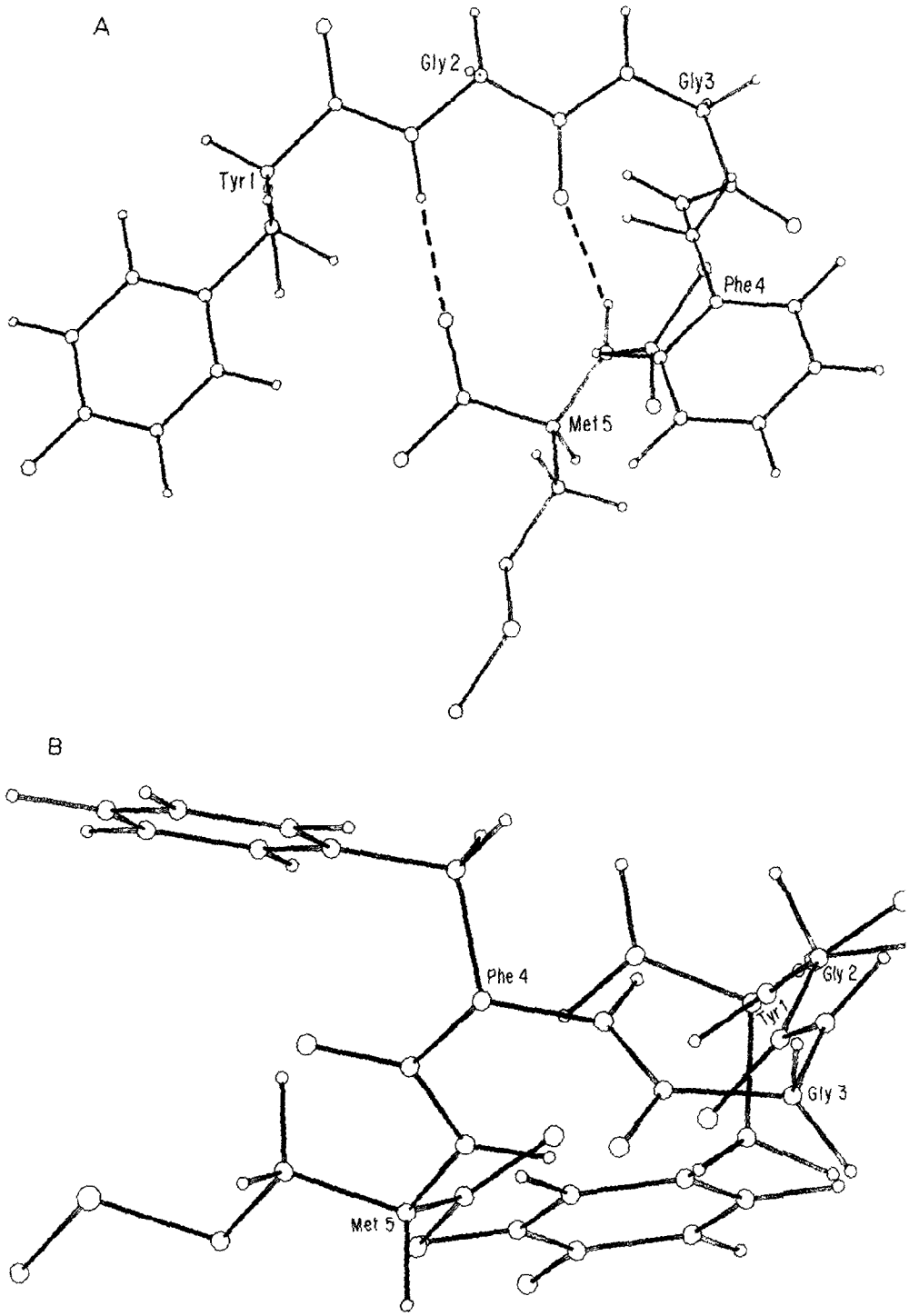

Fig.2. Two mutually perpendicular orientations of Met^5-enkephalin in the type I β-bend. In projection A, the two-hydrogen-bonded peptide skeleton lies in the plane of the paper while in B it is perpendicular. Torsion angles (°) for the folded structure: $\phi_2 = 178$, $\psi_2 = 170$; $\phi_3 = -57$, $\psi_3 = -5$; $\phi_4 = -112$, $\psi_4 = 8$; $\phi_5 = 125$, $\psi_5 = 184$. Note that, in this model too, in addition to the hydrogen bonds shown there is a possibility of a bifurcated hydrogen bond between -C=O of Gly 2 and Met 5. However, the ring protons of Tyr 1 and Phe 4 are always far apart and thus such a structural model does not agree with the NOE data.

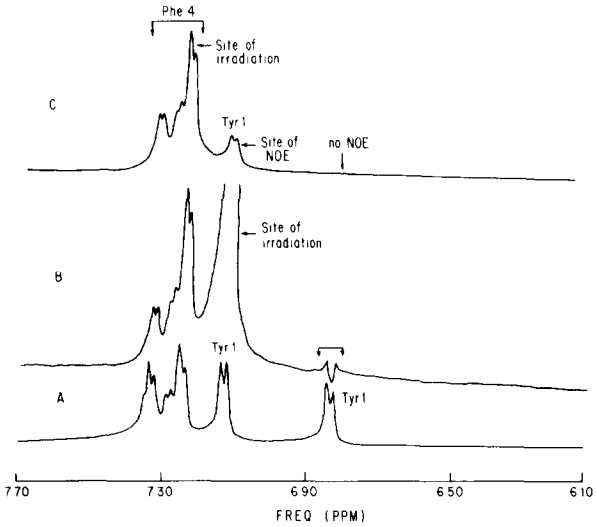

Fig.3. Aromatic region of the 500 MHz ^{1}H-NMR spectra of Met^5-enkephalin in D_2O at 20°C; peptide concentration 1 mM; pH 7.0. (A) Aromatic proton region of Met^5-enkephalin showing 5 protons of Phe 4 and 4 of Tyr 1. Note that 5 protons of Phe 4 appear as multiplets (2:1:2) centered at 7.34, 7.30 and 7.26 ppm while 4 protons of Tyr 1 appear as two doublets (1:1) centered at 7.15 and 6.86 ppm. (B) NOE difference spectrum of Met^5-enkephalin when the signal at 7.15 ppm (Tyr 1) is irradiated for 300 ms. Note that the signal at 7.26 ppm (Phe 4) is the most prominent site of NOE, suggesting that the proton at 7.15 ppm (of Tyr 1) is close in space with the proton at 7.26 ppm (of Phe 4). (C) NOE difference spectrum of Met^5-enkephalin when the signal at 7.26 ppm (Phe 4) is irradiated for 300 ms. Note that there is a strong NOE at 7.15 ppm of Tyr 1 (and at other ring protons of Phe 4 as expected) but no trace of NOE at 6.86 ppm of Tyr 1. This suggests that only the proton at 7.15 ppm (of Tyr 1) and not the one at 6.86 ppm (of Tyr 1) is close to the proton at 7.26 ppm (of Phe 4). When the peak at 7.15 ppm (Tyr 1) is irradiated (spectrum B), NOE is expected at 6.86 ppm of Tyr 1 which is present in the spectrum but skewed due to loss of a data point.

are stereochemically equally probable in fig.1. However, for reasons given below, we prefer the type I′ β-bend for Met^5-enkephalin. The type I′ β-bend has been observed for Leu^5-enkephalin in the single crystal [16] and it has been well demonstrated that Leu^5/Met^5-enkephalin show similar activity towards opiate receptors [1–3]. Substitution of D-Ala for Gly 2 resulted in similar biological activity to that of the parent compound Met^5/Leu^5-enkephalin [18]. In view of the fact that D-Ala tends to prefer stereochemically the type I′ β-bend, it is not unlikely that Met^5-enkephalin in the active conformation adopts the type I′ β-bend. Models were generated for Met^5-enkephalin in the type I′ β-bend conformation subject to the following constraints:

(i) stereochemically acceptable hydrogen bonds were formed between Tyr 1 N-H and Phe 4 C=O and between Tyr 1 C=O and Phe 4 N-H, i.e. N...O ~2.8 Å and N-H---O < 10°;

(ii) Tyr 1 and Phe 4 aromatic rings were oriented in such a way that the distances between the two ring protons were consistent with the NOE data;

(iii) it was ensured that no model had any steric compression.

Fig.1 shows the final model of Met^5-enkephalin; note that only one proton belonging to Tyr 1 is close to only one proton of Phe 4 (distance ~3.3 Å); all other inter-proton distances between the two rings are ≥4 Å. In fig.2, another possible model of enkephalin is shown; this involves a type I β-bend with Gly 3 and Phe 4 at the corners of the bend and thus with Phe 4 and Tyr 1 far apart.

4. CONCLUSIONS

NOE measurements aided by computer model building enable us to suggest that Met^5-enkephalin forms a type I′ β-bend in solution like Leu^5-enkephalin in the crystal [16]. It turns out that in our model only D-amino acids can be substituted for Gly 2 such that there is no steric compression; this is interesting in view of the fact that replacement of Gly 2 by D-Ala/D-Thr is known to retain/enhance the biological activity of the molecule [18–20]. It may also be mentioned that in our model only Gly could be accommodated stereochemically at the 3rd position – it has been reported that the Gly substitution in Leu^5/Met^5-enkephalin results in the loss of biological activity [19,20]. Thus, our solution model of Met^5-enkephalin based upon NOE is stereochemically acceptable and appears to explain the biological activity of the molecule on a structural basis.

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