

# Nuclear Overhauser effects in linear peptides

## A low-temperature 500 MHz study of Met-enkephalin

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Met<sup>5</sup>-enkephalin was studied in 1 mM solutions in <sup>2</sup>H<sub>2</sub>O at room temperature and in a cryoprotective mixture (DMSO-d<sub>6</sub>/<sup>2</sup>H<sub>2</sub>O, mole fraction of DMSO 0.49) in the temperature range 265–298 K. Small positive effects were observed between the *ortho* and *meta* protons of Tyr in aqueous solution at room temperature. Intraresidue effects can be made strong and negative by increasing the viscosity of the medium with a combination of cryoprotective mixtures and low temperatures. The use of mixtures with properties very close to water is very promising for conformational studies of enkephalins and of other small linear peptides.

Nuclear Overhauser effect; Met<sup>5</sup>-enkephalin; Cryoprotective mixture; Conformation

### 1. INTRODUCTION

Theoretical studies [1–5] on the conformational preferences of enkephalins (Tyr-Gly-Gly-Phe-Met/Leu) indicate that the most stable conformations are various types of  $\beta$ -turns. Detection of these turns in water or in DMSO solutions has been claimed by several authors [6–9], mainly on the basis of low temperature coefficients of NH or CO chemical shifts. A careful work by Higashijima et al. [10], however, has shown that enkephalin amides in DMSO do not contain an appreciable population of folded conformations whereas zwitterionic enkephalins have but a small fraction of disordered folded forms, owing to the strong head-to-tail electrostatic interaction.

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**Abbreviations:** DMSO, dimethylsulfoxide; NOE, nuclear Overhauser enhancement; NMR, nuclear magnetic resonance; TSP, sodium 3-trimethylsilyl propionate

The best way to clarify this issue would be the detection of long-range NOEs. All attempts have failed [11], probably owing to the unfavourable value of  $\omega\tau_c$ , to the flexibility of the molecules and/or to the small fractional population of folded conformers. A recent paper [12] claims detection of strong negative NOEs between the aromatic protons of Tyr<sup>1</sup> and Phe<sup>4</sup> in a 1 mM aqueous solution of Met-enkephalin at 500 MHz. We have been unable to confirm their results but sought conditions that can maximize NOEs for this kind of flexible molecule. Satisfactory results, albeit limited to intra-residue effects, were obtained at subzero temperatures, in a mixture of <sup>2</sup>H<sub>2</sub>O and DMSO-d<sub>6</sub> ( $X_{\text{DMSO}} = 0.49$ ), a typical cryoprotective solvent that at this temperature has a dielectric constant similar to that of water at room temperature [13].

### 2. MATERIALS AND METHODS

[Met<sup>5</sup>]enkephalin was purchased from Sigma (St. Louis, MO) and used directly, without further purification, since its NMR spectrum showed no detectable impurity. <sup>2</sup>H<sub>2</sub>O (99.9% atom <sup>2</sup>H) and

DMSO- $d_6$  (99.8% atom  $^2H$ ) were purchased from C. Erba (Milan).

NMR spectra were obtained at 500 MHz in the Fourier mode, with quadrature detection, using a Bruker WM-500 spectrometer. Chemical shifts are reported as  $\delta$  values, in ppm from deuterated TSP. The Overhauser experiments were run, in the difference mode, by pre-irradiating a transition for a fixed time (indicated in the figure legends) before sampling. The frequency setting of the pre-irradiation pulse was alternated between on- and off-resonance spectral positions every 16 scans.

### 3. RESULTS AND DISCUSSION

The *ortho* and *meta* protons of Tyr<sup>1</sup> are separated by 0.248 nm, a distance that in rigid systems should lead to strong NOE enhancements. Irradiation of the two doublets of the aromatic protons of Tyr is thus well suited for checking the magnitude and sign of NOEs in different experimental conditions. Fig.1 shows the nuclear Overhauser experiments performed on a 1 mM solution of Met-enkephalin in  $^2H_2O$  at 500 MHz, using conditions that allow the measurement of steady-state Overhauser effects with sufficient selectivity to avoid accidental irradiation of adjacent peaks. Irradiation of the high-field doublet (at 6.86 ppm, fig.1C) causes a small positive NOE enhancement of the *ortho* doublet at 7.15 ppm (~5%); whereas irradiation of this doublet causes a slightly larger effect (~13%) at 6.86 ppm (fig.1D) but no effect on the adjacent Phe<sup>4</sup> resonances. Selectivity was checked by irradiation at 7.45 ppm, a position separated from Phe resonances as much as the *ortho* Tyr doublet but where there is no resonance (fig.1A). All attempts to observe negative NOEs on the Phe resonances, like those reported by Gupta et al. [12], failed, as long as we used irradiation powers of sufficient selectivity and long enough irradiation times to ensure steady-state conditions. It is possible however to generate apparent negative NOEs by using power levels comparable to those used in decoupling experiments.

Fig.2D shows that irradiation at 7.15 ppm for 300 ms with a power setting 8-times higher than that used for the experiments of fig.1 has no effect on the other Tyr doublet at 6.86 ppm, but induces a large perturbation on the adjacent Phe resonance

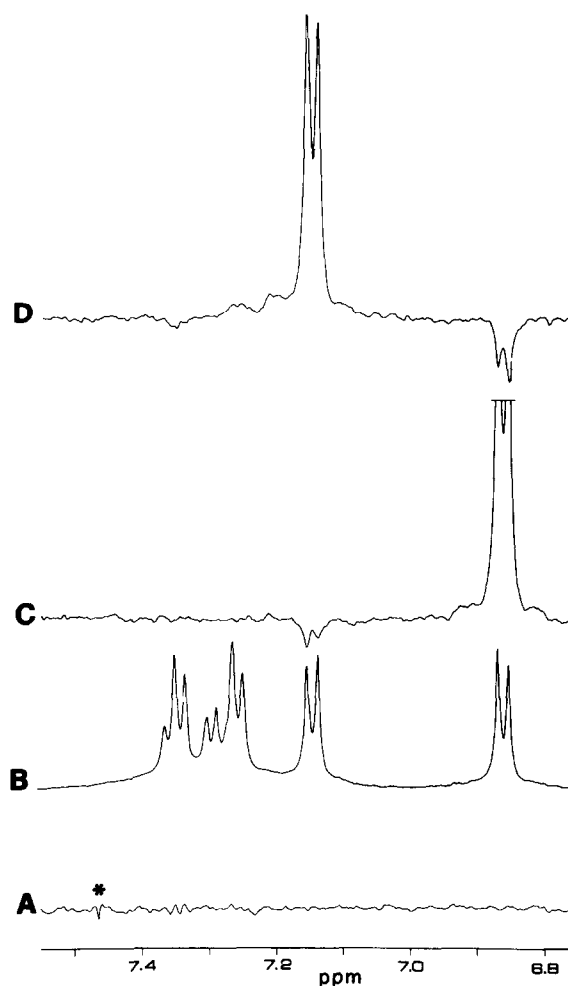


Fig.1. 500 MHz proton Overhauser experiments of  $1 \times 10^{-3}$  M Met<sup>5</sup>-enkephalin in  $^2H_2O$  at 297 K and pH\* 6.5 (uncorrected for isotope effect). (B) Normal spectrum of the aromatic region; (C,D) NOE difference spectra obtained by irradiating the Tyr<sup>1</sup> *meta* and *ortho* signals at 6.86 and 7.15 ppm, respectively. Irradiation time 10 s, using sufficient power to saturate fully the signals in about 0.1 s; 3.5 s recycling time. Each difference spectrum represents an average of 800 scans off-resonance minus 800 scans on-resonance, so that positive NOEs are shown as peaks of opposite sign with respect to those irradiated. (A) Control experiment in which a blank position, indicated by an asterisk, at about 50 Hz downfield from Phe<sup>4</sup> resonances, is irradiated.

as a direct consequence of the large bandwidth of the r.f. used, i.e. a predictable 'spill-over effect' of irradiation power. A similar effect is obtained by

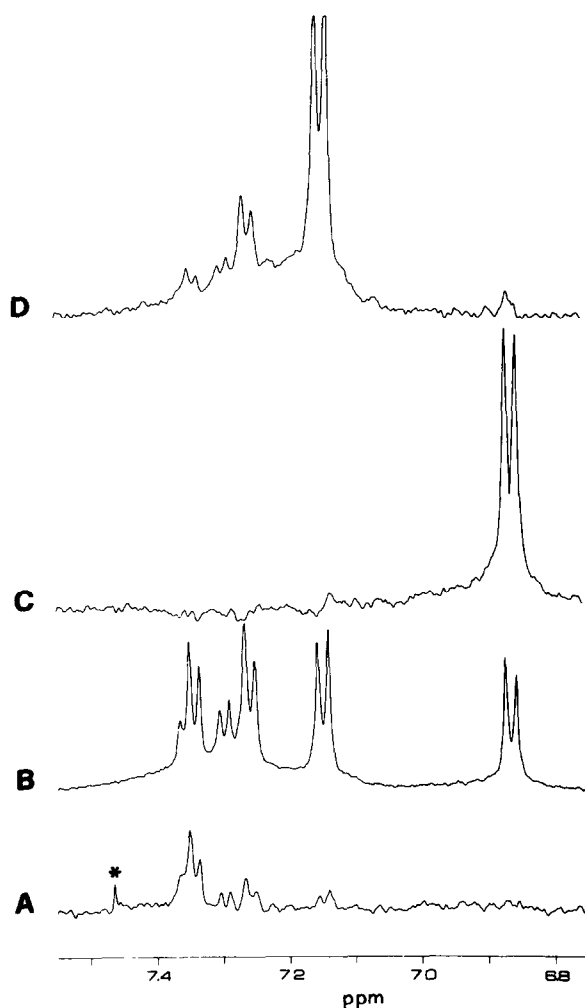


Fig.2. 500 MHz proton Overhauser experiments of  $1 \times 10^{-3}$  M Met<sup>5</sup>-enkephalin in  $^2\text{H}_2\text{O}$  at 297 K and pH\* 6.5 (uncorrected for isotope effect). (B) Normal spectrum of the aromatic region; (C,D) NOE difference spectra obtained by irradiating the Tyr<sup>1</sup> *meta* and *ortho* signals at 6.86 and 7.15 ppm, respectively. The resonances were irradiated for 0.3 s with an 8-fold increase of power with respect to the experiments of fig.1. Spill-over effects were monitored by a control experiment (A), irradiating at 50 Hz from the closest downfield peak of Phe<sup>4</sup>.

irradiation at 7.47 ppm (fig.2A), a point 50 Hz downfield from the lowest Phe resonance, where there are no resonances and consequently no true NOE can possibly be generated. These data are similar to those reported in [12].

The small values of the positive NOEs of fig.1C,D can be attributed in part to the fact that

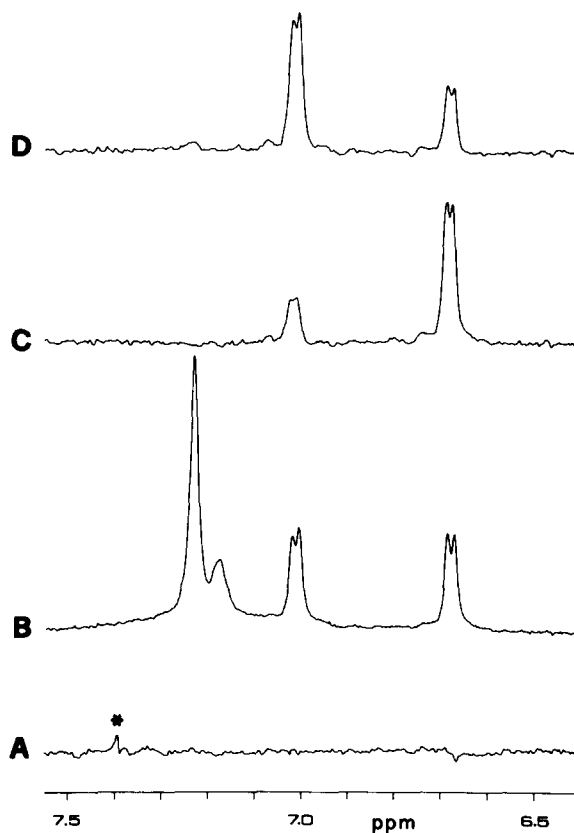


Fig.3. 500 MHz proton Overhauser experiments of  $1 \times 10^{-3}$  M Met<sup>5</sup>-enkephalin in  $^2\text{H}_2\text{O}/\text{DMSO}_{d6}$  ( $X_{\text{DMSO}} = 0.49$ ) 265 K. (B) Normal spectrum of the aromatic region; (C,D) NOE difference spectra obtained by irradiating the Tyr<sup>1</sup> *meta* and *ortho* signals at 6.68 and 7.02 ppm, respectively. The resonances were irradiated for 1.0 s with enough power to null the peaks in 0.05 s. Recycling time 3.5 s. A control experiment (A), irradiating at the blank position indicated by the asterisk, was run to check the selectivity of the irradiation bandwidth, ruling out undesired spill-over effects. The difference spectra average 800 scans off-resonance minus 800 scans on-resonance, thus showing negative effects with the same sign of the irradiated peaks.

$\omega^2\tau_c^2$  for a molecular mass of 577 Da is probably very close to unity. It is possible to change this condition by changing  $\tau_c$  since its value depends almost linearly on the value of the viscosity of the medium [14]. A substantial increase in viscosity of the solution could thus change the small positive NOEs observed into large negative ones [14]. We

chose to increase the viscosity by means of a combination of solvent composition and temperature. It has been shown that mixtures of water and DMSO can be used to study enzyme-catalyzed reactions at subzero temperatures since their properties (notably the dielectric constant) remain similar to those of water even at very low temperatures [13]. Moreover, the viscosity of these mixtures changes rapidly with temperature [15]; accordingly it is easy to select a mixture and a temperature such that  $\omega^2\tau_c^2 \gg 1$ , keeping acceptable linewidths for all resonances.

Fig.3 shows the 1-D NOE experiments obtained from a 1 mM solution of Met-enkephalin in a mixture of DMSO<sub>d6</sub> and <sup>2</sup>H<sub>2</sub>O (with a mole fraction  $X_{\text{DMSO}} = 0.49$ ) at 265 K. At this temperature the viscosity of the solvent is ~10 cP, i.e. an order of magnitude larger than that of water and 5-times larger than that of DMSO at room temperature [15]. This condition shifts the value of  $\tau_c$  to a range typical of small proteins and ensures that we can observe negative NOEs at least for the rigid parts of the molecule. The effects observed are now as large as expected for the distance of 0.248 nm that separates the *ortho* and *meta* protons of Tyr. In fact, they are 29% for the experiment of fig.3C and 47% for that of fig.3D. Other small intraresidue effects were also observed but it was not possible to detect any interresidue effect. A preliminary NOESY experiment of a concentrated solution (20 mM) of enkephalin at 243 K in the same solvent mixture used for the 1-D NOE experiments of fig.3 showed small interresidue effects but it is possible that they arise from associated species. Experiments on dilute samples in <sup>1</sup>H<sub>2</sub>O/DMSO<sub>d6</sub> mixtures are now in progress in order to evaluate possible contributions of folded structures at low temperatures.

#### 4. CONCLUSIONS

NOE enhancements in aqueous solutions of enkephalins at room temperature are very small and positive, even for protons rigidly held at a short distance, as previously observed by several laboratories. This observation is consistent with the absence of a single rigid conformation and with the fact that correlation times typical of molecular

masses comprised between 500 and 1500 Da, at high fields, make  $\omega^2\tau_c^2 = 1$ . The recent observation [12] of a strong negative NOE between the aromatic protons of Tyr<sup>1</sup> and Phe<sup>4</sup> was shown to be an artifact. One of the causes of the small values of the NOEs in these molecules, i.e. the unfavourable correlation time, can be effectively eliminated using a cryoprotective mixture [13] and a suitable combination of solvent composition and temperature. This method can prove very useful in the study of bioactive peptides since it can ensure favourable conditions for the measurement of NOEs in media resembling water in many respects (Temussi, P. et al., in preparation).

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