CONFORMATIONAL STATES OF ENKEPHALINS IN SOLUTION

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SUMMARY: Two pentapeptides with opiate activity, [Met_5] enkephalin and [Leu_5] enkephalin, were studied by means of PMR, CMR, UV and CD spectroscopies in different solvents and at different concentrations. The primary result which we report is the demonstration of a concentration dependence. Spectral properties which are characteristically used to evaluate conformation are shown to differ at different concentrations. This provides an explanation for conflicting results of previous studies.

Two conformational states of enkephalins which are consistent with the data are considered: i) A monomeric form, containing a β-turn with Gly_3 and Phe_4 at the corners, a 7-atom H-bond and the folding of the Tyr_1 aromatic side chain over the molecule stabilized by an interaction of its OH proton with the Gly_3 C=O. ii) An associated form with an antiparallel cross-β-structure stabilized by four intermolecular H-bonds and with a "head to tail" interaction.

INTRODUCTION

The recent discovery of two naturally occurring pentapeptides (enkephalins) with morphine-like action (1) has raised much interest among research workers (2-12) in elucidating structure-function relationships. The sequence of these peptides are H-L-Tyr_1-Gly_2-Gly_3-L-Phe_4-L-Met_5-OH, ([Met_5] enkephalin), and H-L-Tyr_1-Gly_2-Gly_3-L-Phe_4-L-Leu_5-OH, ([Leu_5] enkephalin). The enkephalins, being small peptides, can adopt different conformations in solution while morphine has a very rigid structure. Realizing this, several research groups (5-12) have studied the secondary structure of these peptides with the purpose of arriving at a structural framework which would provide the basis of competition with morphine for the brain opiate receptor. Nuclear magnetic resonance (NMR) methods have been used mainly to investigate the conformational characteristics of [Met_5] enkephalin in solution (7-10). Significantly,

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TABLE I

TEMPERATURE COEFFICIENTS ($d\delta/dT$) OF PEPTIDE PROTONS OF ENKEPHALINS

MOLECULE	AMINO ACID RESIDUE	$d\delta/dT \times 10^{-3}$ ppm/°C in DMSO-d_6 (0.001M)	DMSO-d_6 (0.1M)	H_2O (<0.01M)	TFE (0.01M)	MeOH (.01M)
[Met_5] Enkephalin / [Leu_5] Enkephalin	Tyr_1	——	——	——	——	——
	Gly_2	-7.9 / -8.8	-7.6 / -9.1	-8.1 / -6.8	-7.6 / -7.0	-8.2 / -7.4
	Gly_3	-4.4 / -4.6	-3.4 / -3.9	-5.5 / -4.5	a / 4.7	-5.2 / 4.8
	Phe_4	-6.3 / -6.7	-4.7 / -4.9	-8.5 / -7.9	-7.2 / 6.6	-8.2 / -7.5
	Met_5 / Leu_5	-4.1 / -4.8	-1.8 / -2.4	-5.9 / -5.8	a / 5.6	-5.4 / -5.7

a) Values could not be obtained due to the overlapping with the aromatic proton signals.

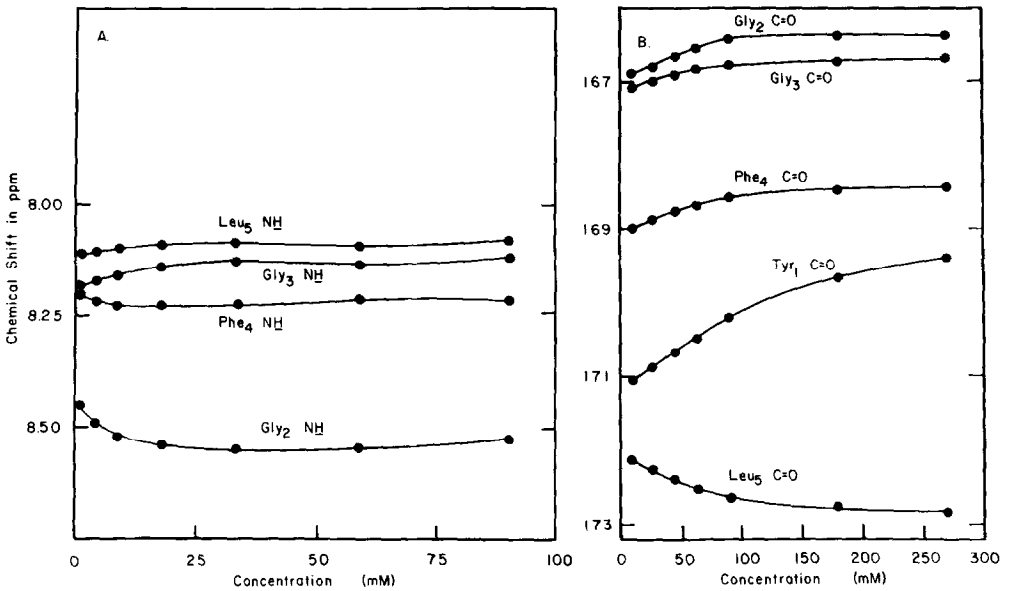

FIGURE 1: Concentration dependence of [Leu$_5$] enkephalin in DMSO-d_6 A) NH proton chemical shifts as a function of concentration. B) C=O carbon chemical shifts as a function of concentration.

however, one finds in these reports discrepancies and ambiguities in signal assignments and also in proton resonance parameters. The results of extensive investigations in our laboratory on both enkephalins by NMR, CD and UV prompt us to report findings on the concentration dependence of enkephalins which heretofore had been overlooked and which provide the basis for resolving some of the differences in previous results. While the many details of signal assignments and of parameters giving rise to secondary structural information on the enkephalins will be presented subsequently, here we briefly present temperature dependence of peptide NH chemical shifts and discuss two conformational states which are consistent with the data.

MATERIALS AND METHODS

Both [Leu$_5$] and [Met$_5$] enkephalins were purchased from Bachem, Inc. Fine Chemicals and were used without further purification. The UV and CD measurements were carried out on Cary-14 and Cary-60 (Model 6001 CD accessory) spectrometers, respectively, at room temperature. The PMR spectra were obtained on Varian 220 MHz and JEOL PS-100 MHz spectrometers. ^{13}C spectral

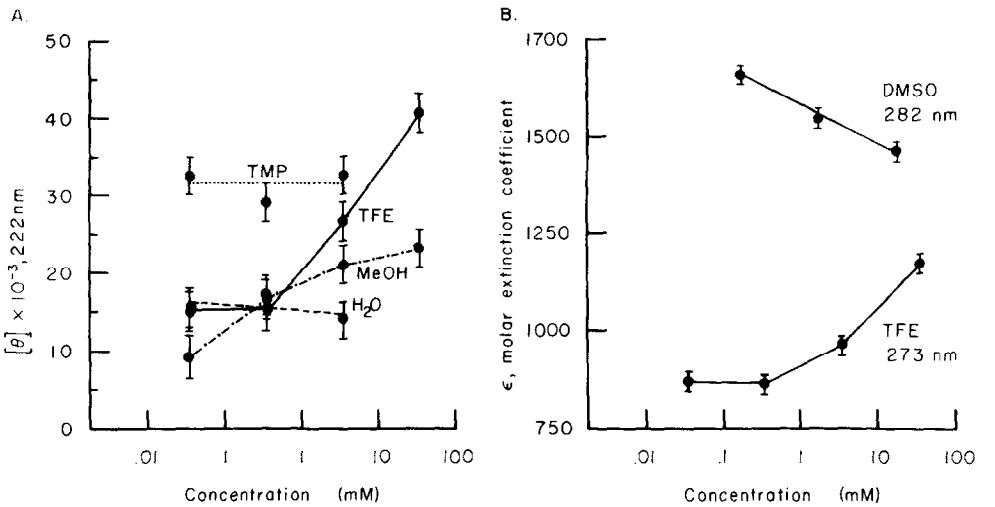

FIGURE 2: Concentration dependence of [Leu_5] enkephalin in different solvents. A) Molar ellipticity at different concentrations. B) Aromatic region, concentration dependence of molar extinction coefficient.

measurements were obtained on a JEOL PFT-100 spectrometer using a 1 sec repetition time and a 40 μs pulse width for 90° tilt of the magnetization vector. The pH values were determined by means of a Radiometer pH Meter 25.

RESULTS AND DISCUSSION

A study of the temperature dependence of the peptide NH proton chemical shifts of enkephalins at a concentration of 0.1 M in DMSO-d_6 resulted in the values given in Table I. These values are similar to those of Jones, et al. (9) but differ significantly from those of Bleich, et al. (7,8). Stepwise dilution of the sample to a concentration of .001 M demonstrated a strong concentration dependence of the chemical shifts of the peptide NH and C=O resonances (see Figure 1). As both [Leu_5] and [Met_5] enkephalins show similar concentration dependence, only the data of [Leu_5] enkephalin is included. It can be seen in Figure 1 that the concentration dependence is observed down to the concentration limit of our NMR instrumentation which was 0.3 mg/ml. Interestingly, one observes differing behavior of NH proton and C=O carbon chemical shifts. The Gly_3 NH and Leu_5 NH protons shift downfield as the solution is diluted, whereas the Phe_4 NH and Gly_2 NH protons shift upfield.

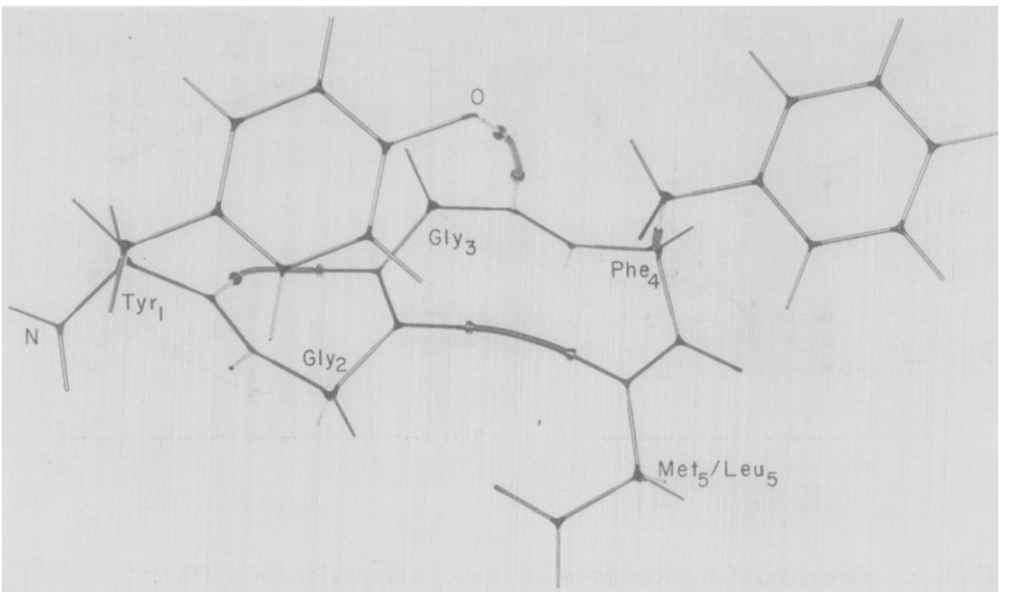

FIGURE 3: Dreiding molecular model of enkephalins. Note that the three H-bonds are shown by the flexible spring connections (see text for discussion).

The upfield shift of the Phe_4 N<u>H</u> and Gly_2 N<u>H</u> resonances on dilution is consistent with these moieties being involved in intermolecular interactions. In the CMR curves the Gly_3 <u>C</u>=O carbon resonance shifts downfield less than the other <u>C</u>=O resonances except for the carboxyl carbonyl which shifts upfield on dilution.

The concentration effects were also investigated using UV and CD spectroscopies in several different solvents. The results are presented in Figure 2. The ellipticity at 222 nm (Figure 2A) shows little concentration dependence of enkephalins in TMP (tetramethyl phosphate) and H_2O while in TFE there is a large concentration effect which is no longer observed below a concentration of 0.2 mM. The aromatic region of the [Leu_5] enkephalin was observed in DMSO and TFE using UV spectroscopy (see Figure 2B). The concentration dependence is observable in DMSO throughout the accessible concentration range whereas in TFE the concentration effect is as observed in the CD studies (compare Figure 2A and 2B).

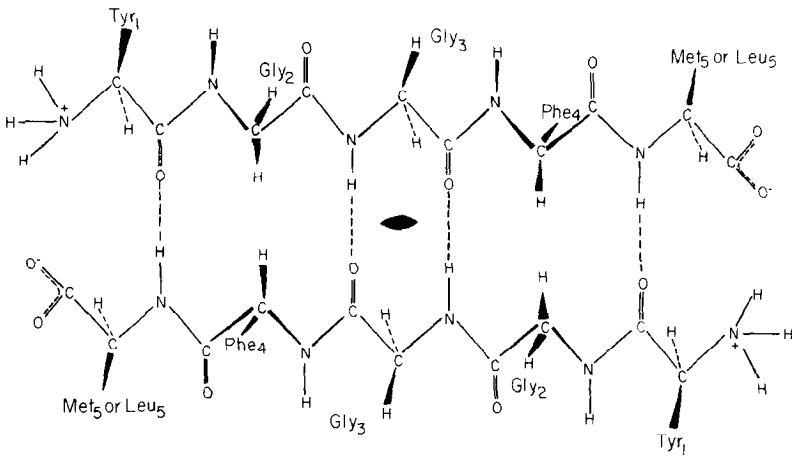

FIGURE 4: Secondary structure of enkephalins in dimer form showing an extended antiparallel β-pleated sheet structure.

PMR measurements were made in H_2O, TFE and MeOH at .01 M or less as tractable concentrations. The temperature coefficients, $d\delta/dT$ obtained in these solvents and in DMSO-d_6 at two different concentrations (.001M and .1M), are given in Table I where it can be seen that low $d\delta/dT$ values for the Gly_3 NH and Met_5/Leu_5 NH protons indicate more shielding of these protons than of the Gly_2 NH and Phe_4 NH protons. Gly_3 NH exhibits the smallest $d\delta/dT$ in H_2O, TFE and MeOH whereas in DMSO-d_6 at higher concentration (0.1M, Table I) the NH of residue 5 has the smaller temperature dependence. At a lower concentration (.001M) in DMSO-d_6, however, the trend is towards the values obtained in H_2O, TFE and MeOH (see Table I). A solvent titration of the peptide NH protons going from DMSO-d_6 to TFE showed the Gly_3 NH and Leu_5 NH protons to be less perturbed. From these experiments it is evident that the Gly_3 NH is the most shielded and that the Met_5/Leu_5 NH is also shielded.

Based on the temperature dependences and solvent perturbation of the peptide NH protons, a possible secondary structure for monomeric enkephalin is depicted in Figure 3. This structure contains a β-turn with Gly_3 and Phe_4 at its corners, as proposed by previous workers (9,10), a 7-membered H-bond

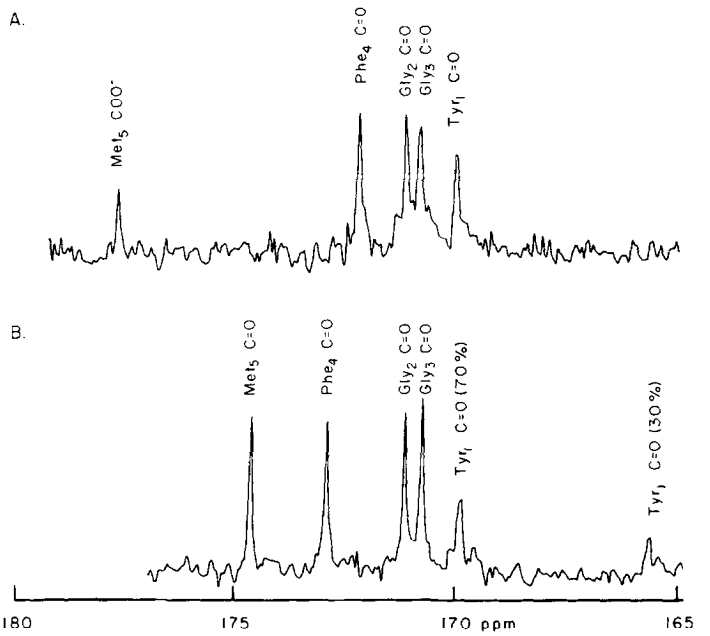

FIGURE 5: 25.15 MHz CMR spectra of [Met_5] enkephalin (C=O region) A) in H_2O (pH = 4.78) without adding HCl or NaOH, B) at pH 0.87 by adding (2N) HCl.

between the Gly_3 NH and the Tyr_1 C=O and a folding of the Tyr_1 aromatic side-chain over the molecule which is stabilized by an interaction of the phenolic OH proton with the Gly_3 C=O. This folding of the Tyr_1 side-chain is consistent with the high stability of its gauche-trans conformation at elevated temperatures (9). Also in this conformation the distance between the Tyr_1 N atom and the oxygen atom of Tyr_1 side chain OH group maintains a distance of ~7 Å which is similar to that of many morphine derivatives as noted by Horn and Rodgers (11).

An associated form of the molecule can also explain the above discussed peptide NH shielding data. The dimeric cross-β-structure in Figure 4 shows the Gly_3 NH and the Leu_5/Met_5 NH to be shielded from the solvent by hydrogen bonding. While the structure is shown to be in a planar antiparallel pleated sheet conformation, it should also be appreciated that the Gly_3 residue allows

for inversion of the pleat at the central α-carbon which would result in a partial turn of a β-helix (13). Consistent with such an association is the observation of a head to tail interaction wherein the Tyr C=O resonance is found to be split at low pH but to titrate to a single resonance following the pK of the residue 5 carboxyl group. Relevant CMR spectra are given in Figure 5.

In a very recent theoretical study on the monomeric state of enkephalin (14) several conformations of similar energy were found. The two most probable of which relate to the structures in Figures 3 and 4. The calculations, which show the extended conformation to be most favored naturally lead, by association, to the type of cross-β-structure shown in Figure 4.

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