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Comparative study of conformational behaviour of leucine and methionine enkephalinamides by ^{1}H-nuclear magnetic resonance spectroscopy

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Abstract. The conformational proclivity of leucine and methionine enkephalinamides in deuterated dimethyl sulphoxide has been investigated using proton magnetic resonance at 500 MHz. The resonances from the spin system of the various amino acid residues have been assigned from the 2-dimensional correlated spectroscopy spectra. The temperature variation of the amide proton shifts indicates that none of the amide proton is intramolecularly hydrogen-bonded or solvent-shielded. The analysis of vicinal coupling constants, $^3J_{HN.C^2H}$,along with temperature coefficients and the absence of characteristic nuclear Overhauser effect cross peaks between the NH protons reveal that there is no evidence of the chain folding in these molecules. However, the observation of nuclear Overhauser effect cross peaks between the NH and the $C^{\alpha}H$ of the preceding residue indicates preference for extended backbone conformation with preferred side chain orientations particularly of Tyr and Phe in both [Leu^5] - and [Met^5]-enkephalinamides.

Keywords. Enkephalinamides; nuclear magnetic resonance; structure-activity relationship; endogenous peptides; temperature coefficients.

Introduction

Leucine and methionine enkephalins (H_2N-Tyr^1-Gly^2-Gly^3-Phe^4-Leu^5/Met^5-COOH) are endogenous pentapeptides (Hughes *et al.,* 1975) that have been shown to possess analgesic properties similar to those associated with alkaloid opiates (morphine) and their agonists and antagonists (Belluzi *et al.,* 1976; Bradbury *et al.,* 1976). Since opiates and enkephalins belong to different classes of compounds, there has been a spate of NMR investigations of structure-activity relationships (Roques *et al.,* 1976; Jones *et al.,* 1977; Khaled *et al.,* 1977; Zetta and Cabassi, 1982). The results of such investigations have been reviewed by Schiller (1984). Based on the proton coupling constants and temperature coefficients, a preferred backbone conformation for [Met^5]-enkephalins in hexadeutero dimethyl sulphoxide (DMSO-d_6) has been proposed, while on the basis of concentration and temperature dependence of C^{a}-resonances, a conformational equilibrium has been proposed for the same molecule in the same solvent (Garbay-Jaureguiberry *et al.,* 1976; Jones *et al.,* 1976; Higashijima *et al.,* 1979). However, in aqueous solution the conformation of [Met^5]-enkephalin has been a controversial issue. Based on results from one-dimensional nuclear Overhauser (NOE) effect experiments, Gupta *et al.* (1986) have proposed a folded conformation for the molecule but this has been recently refuted by Motta *et al.* (1987). The results of NMR Studies on [Leu^5] -enkephalin have suggested the

Abbreviations used: [Leu$_5$]-/[Met$_5$]-enkephalin, Enkephalin with leucine/methionine at position 5; DMSO-d_6, hexadeutero dimethyl sulphoxide; NOE, nuclear Overhauser; TSP, sodium 3-(trimethylsilyl) propionate; COSY, correlated spectroscopy; NOESY, NOE effect spectroscopy.

possibility of a less defined backbone conformation in DMSO-d_6 (Garbay-Jaureguiberry *et al.,* 1977; Fischman *et al.,* 1978).

The introduction of D-Ala and D-Ser in place of Gly_2 in [Met_5]-enkephalin and [Leu^5]-enkephalin-threonine (a synthetic hexapeptide), respectively, results in relatively more potent analogues with rigid backbones as demonstrated by proton spin-lattice relaxation and 2D NOESY studies (Niccolai *et al.,* 1980; Dhingra and Saran, 1987). Similarly, the amidation of [Met^5]-enkephalin has also been demonstrated to impart rigidity to the backbone (Gairin *et al.,* 1981) as well as enhanced potency to the peptide (Chang *et al.,* 1976). Comparative conformational studies by NMR on [Met^5]-enkephalin and [Met^5]-enkephalinamide in DMSO-d_6 revealed that the native peptide adopts a folded backbone conformation while there is no folding in [Met_5]-enkephalinamide (Higashijima *et al.,* 1979). Furthermore, a circular dichroism (CD) study on [Met^5]-enkephalin in methyl alcohol and trifluoroethanol indicated considerable flexibility (Sudha and Balaram, 1981) but no attempt was made to ascertain the nature of conformational changes subsequent to alteration in solvent conditions.

In this paper, we report the results of high field (500 MHz) proton NMR investigation on [Leu^5]- and [Met^5]-enkephalinamide acetates in DMSO-d_6. The proton-proton vicinal coupling constants, temperature coefficient of the amide protons and NOE effect in 2-D mode have been utilised to probe the conformational behaviour of these molecules in solution. The effect of amidation on the conformation of native pentapeptides and the conformation-activity relationship are discussed.

Materials and methods

Leucine and methionine enkephalinamide acetates were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. The compounds (2·5 mg) were dissolved in 0·5 ml of 99·8% DMSO-d_6. The solvent used had a trace of water and the signal arising from it has been identified.

One and two-dimensional proton magnetic resonance measurements were carried out on an AM-500 Bruker FT-NMR spectrometer. The temperature of the sample was maintained at 300 K. The resonance peak of DMSO was used as internal reference and the chemical shifts were converted relative to sodium 3-(trimethylsilyl) propionate (TSP) by adding 2·6 ppm to the observed shift values. ^1H spectra of [Leu^5]- and [Met^5]-enkephalinamides are shown in figure 1. ^1H spectra were also recorded as a function of temperature (range 300–360 K).

Two-dimensional correlated spectroscopy (COSY) and NOE effect spectroscopy (NOESY) experiments were carried out with data matrices of 512×2048 and 256×1024 respectively and 64 transients. Mixing time of 600 ms. was used for the NOSEY experiment. The time domain data were multiplied by appropriate window functions before Fourier transformation. J-resolved spectrum was recorded with a data matrix of 128×4096.

Results

Assignment of resonances

The resonances belonging to various spin systems from the different amino acid

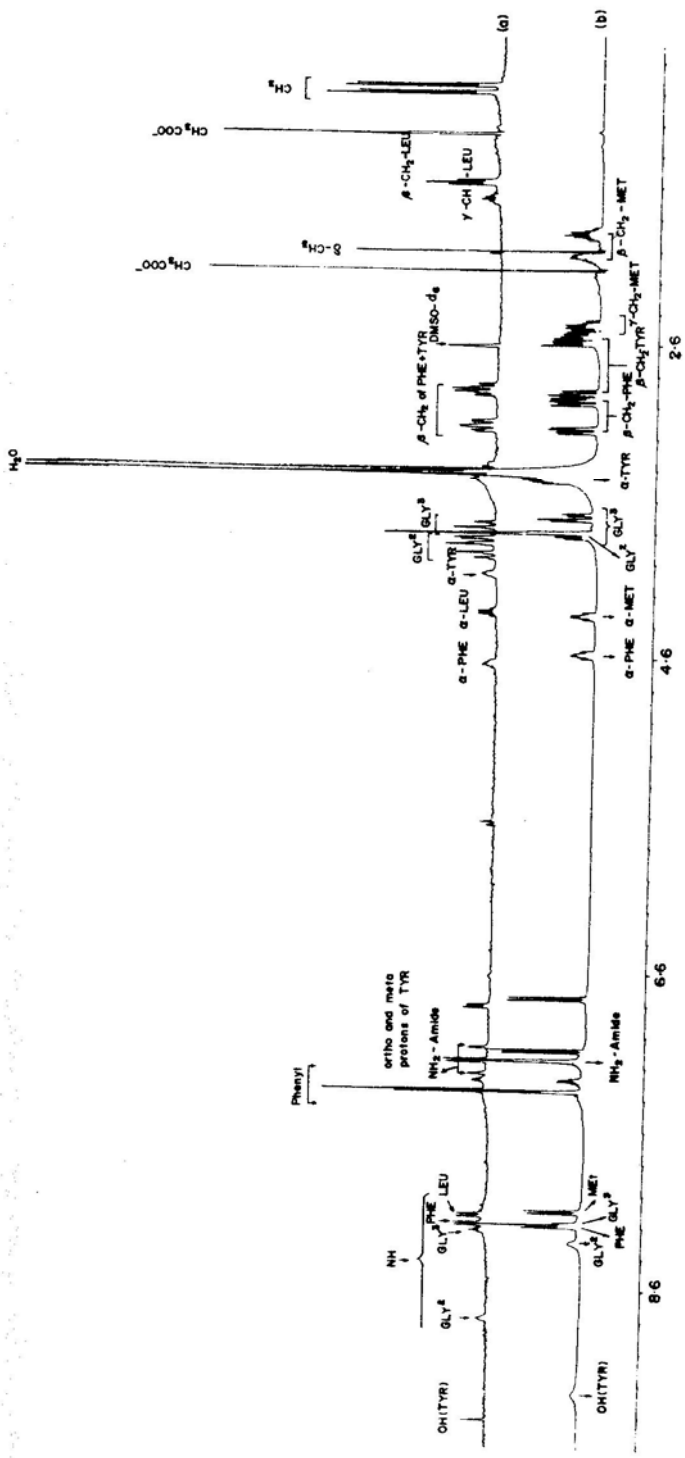

Figure 1. 500 MHz ^{1}H-NMR spectra of leucine (a) and methionine (b) enkephalinamides in DMSO-d_6 at 300 K. The assignment of resonances is indicated in the figure and the shifts are relative to TSP.

residues were identified by 2-dimensional COSY (figure 2) (Aue *et al.,* 1976). These assignments are in conformity with those reported earlier for [Met^5]- and [Leu^5]-enkephalins (Jones *et al.,* 1976; Garbay-Jaureguiberry *et al.,* 1977). The methyl resonances from CH_3COO^- and CH_3-S of [Met^5]-enkephalinamide were assigned on the basis that CH_3COO^- usually resonates around 2·2 ppm. The coupling constant data from J-resolved spectrum along with homonuclear decoupled spectra were used to estimate the proton-proton coupling constants and the chemical shifts of various protons. These NMR parameters are given in tables 1 and 2.

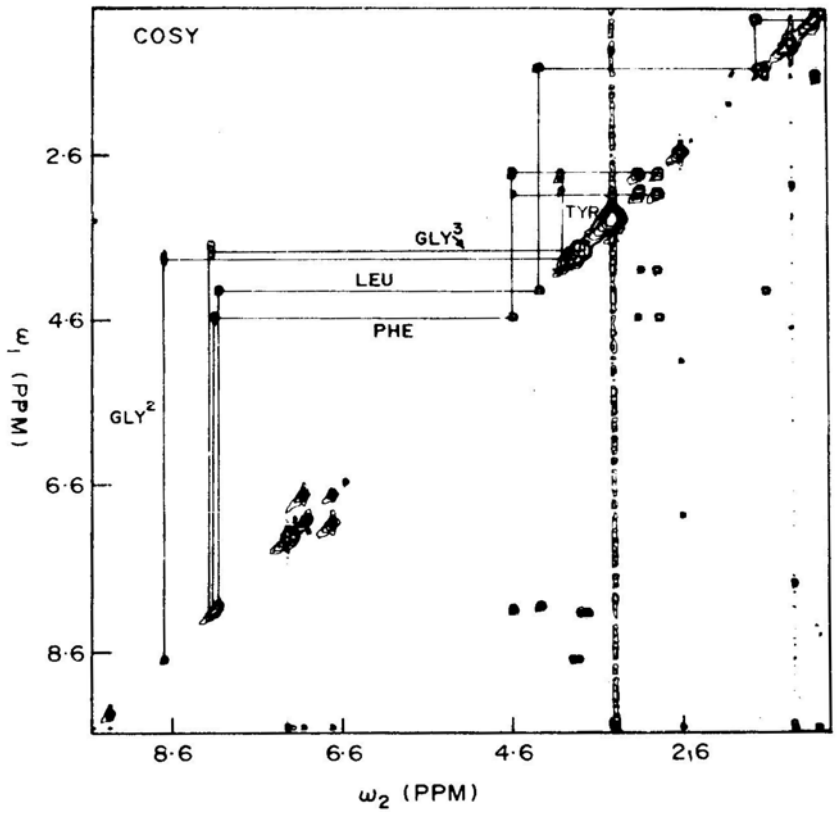

Figure 2. 2D-COSY spectrum of leucine enkephalinamide recorded with 512 × 2048 data matrix size. The digital resolution is 15·6 Hz.

The hydroxyl proton of tyrosine was identified as a sharp singlet at 9·55 ppm in [Leu^5]-enkephalinamide and as a relatively broad peak at 9·37 ppm in [Met^5]-enkephalinamide. The resonances from the acetate group CH_3COO^- were identified at 1·25 and 2·12 ppm in the spectra of [Leu^5]- and [Met^5]-enkephalinamides, respectively. The two protons of the carboxyamide group were found to be equivalent in [Met^5]-enkephalinamide and non-equivalent in [Leu^5]-enkephalinamide; their chemical shifts are given in table 1. The strongest resonance at 3·42 ppm is due to the trace of H_2O in DMSO-d_6.

Table 1. Proton chemical shifts* in leucine and methionine enkephalinamide (acetate salt) in DMSO-d_6.

Residue	CH_3COO^-	OH	O ‖ C-NH_2	NH	$C^\alpha H$	$C^\beta H_I$	$C^\beta H_{II}$	$C^\gamma H_I$	$C^\gamma H_{II}$	$C^\delta H_3$	$C^\delta H_3$	o	m	p
Leucine enkephalinamide														
Tyr^1	1·25	9·55	—	—	4·05	3·28	3·08	—	—	—	—	6·81	7·16	—
Gly^2		—	—	8·80	3·93	—	—	—	—	—	—	—	—	—
					3·82									
Gly^3		—	—	8·24	3·82	—	—	—	—	—	—	—	—	—
					3·72									
Phe^4		—	—	8·21	4·63	3·30	3·05	—	—	—	—	7·28	7·28	7·25
Leu^5		—	7·23	8·15	4·30	1·55	1·55	1·63	—	0·99	—	—	—	—
			7·04							0·93				
Methionine enkephalinamide														
Tyr^1	2·12	9·37	—	—	3·48	2·98	2·59	—	—	—	—	6·76	7·06	—
Gly^2	—	—	—	8·30	3·76	—	—	—	—	—	—	—	—	—
Gly^3	—	—	—	8·18	3·78	—	—	—	—	—	—	—	—	—
					3·70									
Phe^4	—	—	—	8·20	4·58	3·15	2·92	—	—	—	—	7·29	7·29	7·26
Met^5	—	—	7·15	8·12	4·33	1·94	2·06	2·55	2·48	—	2·0	—	—	—

*Shifts are downfield relative to TSP.

Table 2. Proton-proton coupling constants in leucine and methionine enkephalinamide (acetate salt).

Residue	$^3J_{NH\text{-}C^\alpha H}$	$^2J_{\alpha\alpha}$	$^2J_{\beta\beta}$	$^3J_{C^\alpha H\text{-}C^\beta H_I}$	$^3J_{C^\alpha H\text{-}C^\beta H_{II}}$	$^3J_{C^\beta H_I\text{-}C^\gamma H}$	$^3J_{C^\beta H_{II}\text{-}C^\gamma H}$	$^3J_{C^\gamma H\text{-}C^\delta H_3}$	$^3J_{om}$
Leucine enkephalinamide									
Tyr[1]	—	—	−14·3	8·3	4·8	—	—	—	8·6
Gly[2]	5·4	−17·0	—	—	—	—	—	—	—
Gly[3]	6·0	−17·0	—	—	—	—	—	—	—
Phe[4]	8·6	—	−13·7	9·7	4·6	—	—	—	—
Leu[5]	8·3	—		7·3	7·3	7·0	7·0	6·8	—
Methionine enkephalinamide									
Tyr[1]	—	—	−13·8	9·0	4·5	—	—	—	8·5
Gly[2]	—	—	—	—	—	—	—	—	—
Gly[3]	5·8	−16·5	—	—	—	—	—	—	—
Phe[4]	8·0	—	−14·0	9·8	4·6	—	—	—	—
Met[5]	8·1	—	−13·3	8·9	5·0	*	*	—	—

*Not obtained.

Temperature variation of the chemical shift of exchangeable protons

The chemical shifts of the amide, carboxyamide and hydroxyl protons in [Leu^5]- and [Met^5]-enkephalinamides were monitored in the temperature range of 300–360 K. All these protons are observed to shifts upfield with increasing temperature. The temperature coefficients dδ/dt observed for the amide, hydroxyl and carboxyamide protons are given in table 3. The amide proton assigned to the Gly^2 starts exchanging with H_2O beyond 340 K in [Met^5]-enkephalinamide while it does not exchange in [Leu^5]-enkephalinamide in the same temperature range. The carboxyamide protons in [Leu^5]-enkephalinamide are non-equivalent and their resonances probably collapse to a single resonance near 360 K. The carboxyamide protons of [Met^5]-enkephalinamide are equivalent at room temperature and exhibit a sharp resonance which becomes relatively broader at higher temperatures. The tyrosine hydroxyl protons in [Leu^5]- and [Met^5]-enkephalinamides have similar temperature coefficients (table 3).

Table 3. Temperature coefficient of amide, hydroxy and carboxyamide protons and side chain rotameter populations in [Leu^5]- and [Met5]-enkephalinamides.

	Temperature coefficient $\frac{d\delta}{dt}$ ($\times 10^{-3}$ ppm/°C)				Rotamer populations		
Residue	NH	OH	NH^{cis}	NH^{Trans}	g^-g^+	tg^+	tg^-
Leucine enkephalinamide							
Tyr^1	—	−5·1	—	—	0·28	0·52	0·20
Gly^2	−4·7	—	—	—			
Gly^3	−4·6	—	—	—			
Phe^4	−4·6	—	—	—	0·17	0·65	0·18
Leu^5	−5·5	—	−5·6	−5·6			
Methionine enkephalinamide							
Tyr^1	—	−6·3	—	—	0·25	0·58	0·17
Gly^2	−5·0	—	—	—			
Gly^3	−5·6	—	—	—			
Phe^4	−5·6	—	—	—	0·17	0·65	0·18
Met^5	−5·7	—	−5·8	−5·8			

Discussion

The temperature coefficient values of the amide protons of various amino acid residues in [Leu^5]- and [Met^5]-enkephalinamides suggest that none of the amide protons is involved in intramolecular hydrogen bonding. This means that all the amide protons are exposed to the solvent and the observed temperature variation is due to the breaking of intermolecular hydrogen bonds with solvent molecules on increasing temperature. In the absence of any evidence for intramolecular hydrogen bonding, it becomes quite evident that there is no folding of the peptide chain in [Leu^5]- and [Met^5]-enkephalinamides and hence their solution conformations are different from those of the native pentapeptides: [Leu^5]- and [Met5]-enkephalins which have been demonstrated to adopt a folded *β*-bend conformation under similar

conditions of solvent and temperature (Jones *et al.,* 1977; Higashijima *et al.,* 1979). The folded conformation of the native peptides is due to the electrostatic interaction between the positively charged — and negatively charged – COO^- groups at the terminii of the molecule (Higashijima *et al.,* 1979). This interaction is, however, absent in the carboxyamide derivatives which have been investigated here.

The observation of NOESY cross peaks reflects the spatial disposition of various hydrogen atoms and thus provides insight into the 3-dimensional structure of the molecule in solution (Jenner *et al.,* 1979; Kumar *et al.,* 1980). The observation of NOESY cross peaks between N_iH and $N_{i+1}H$ and $C_i^{\beta}H$ and $N_{i+1}H$ reflects long and short range ordering in the molecule. From the NOESY spectra shown in figures 3 and 4, it is quite clear that there is no long or short range ordering in these molecules. NOESY cross peaks between $C^{\alpha}H$ and $N_{i+1}H$ for some of the amino acid residues have been observed (figures 3 and 4) and these indicate the preference for extended backbone in both molecules. This deduction is in agreement with X-ray crystallographic data: [Leu^5] -enkephalin has 4 molecules per unit cell and all the 4 molecules have extended backbone conformation (Karle *et al.,* 1983).

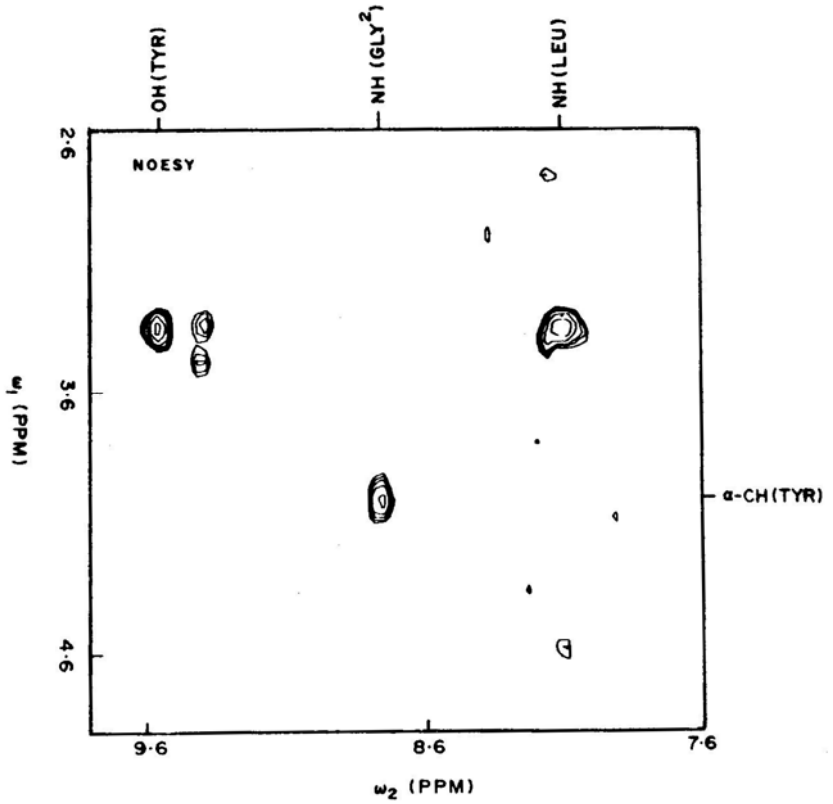

Figure 3. 2D-NOESY spectrum of leucine enkephalinamide with mixing time of 600 ms and data matrix of 256×1024.

Even though both [Leu^5]- and [Met^5]-enkephalinamides prefer extended backbone conformations, there are subtle differences in their ^{1}H-spectra. These are listed below.

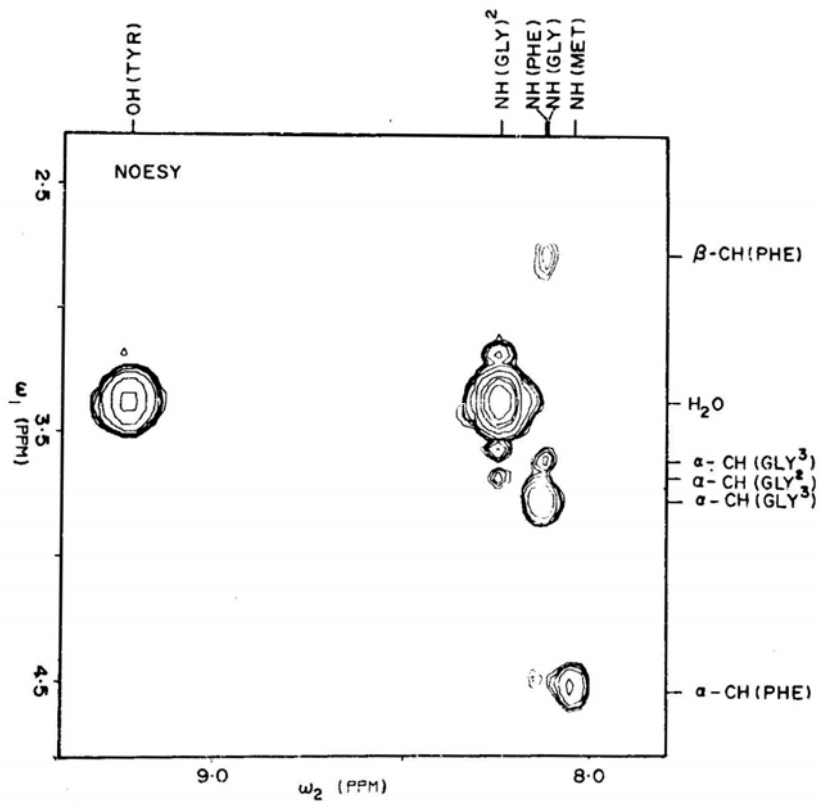

Figure 4. 2D-NOESY spectrum of methionine enkephalinamide with mixing time of 600 ms and data matrix of 256 × 1024.

(i) The most significant difference between the two spectra is the down field shift of $C^{\alpha}H$ of tyrosine in [Leu^5]-enkephalinamide (0·5 ppm) relative to the corresponding proton in [Met5]-enkephalinamide.
(ii) Methylene protons of Gly^2 in [Met^5]-enkephalinamide are equivalent while the corresponding protons in [Leu^5]-enkephalinamide are non-equivalent and have substantial shift differences.
(iii) Methyl protons of $CH3COO^-$ ion in [Leu^5]-enkephalinamide are shifted very much upfield relative to the corresponding protons in [Met^5]-enkephalinamide. The magnitude of the shift difference will increase further if the assignment of CH_3 resonances from CH_3COO^- and CH_3-S of methionine are interchanged.
(iv) The –NH proton of Gly^2 in [Met^5]-enkephalinamide starts exchanging with H_2O at 340 K while the corresponding proton in [Leu^5]-enkephalinamide is observable even upto 360 K. The former resonates at a higher field than the latter.
(v) The carboxyamide protons in [Met^5]-enkephalinamide are equivalent while those in [Leu^5] -enkephalinamide are non-equivalent and the shift difference between them is about 100 Hz.
(vi) The tyrosyl —OH group in [Leu^5]-enkephalinamide is relatively sharp and resonates at slightly lower field than the corresponding group in [Met^5] –enkephalinamide. Both these hydroxyl groups shift upfield with increasing temperature and have similar temperature coefficients.

(vii) The shift difference between *β* -CH2 protons of tyrosine in [Met^5]-enkephalinamide is much larger than that in [Leu^5]-enkephalinamide.

The shift variation of ≃ 0·5 ppm in amino acid residues is usually observed for $C^{\alpha}H$ proton whenever there is a protonation/deprotonation taking place at the $-NH_2$ group (James, 1975). The observed difference of ≃ 0·5 ppm between the $C^{\alpha}H$ protons of [Leu^5]- and [Met^5]-enkephalinamide indicates that the terminal — NH_2 group is protonated in [Leu^5]-enkephalinamide while it is not in [Met^5]-enkephalinamide. However, a comparison of the intensity of methyl resonance from CH_3COO^- in both analogues with the intensities of CH_3S (1:1) and δ–CH_3 (1:2) resonances in [Met^5]- and [Leu^5]-enkephalinamides, respectively, clearly establishes the 1:1 stoichiometry and confirms the nature of these amides as acetate salts. Such a large shift differential is difficult to explain unless there are drastic local conformational differences between the two analogues. The existence of conformational differences is supported by the following observations also.

The non-equivalence of methylene protons of both Gly_2 and Gly_3 in [Leu_5]-enkephalinamide clearly indicates some kind of order in this region of the molecule. The same region is however relatively more flexible in [Met^5] -enkephalinamide, where the protons of the CH_2 group of Gly^2 are equivalent while those of Gly^3 are non-equivalent.

The unusually large upfield shift of CH_3 resonance of CH_3COO^- in [Leu_5]-enkephalinamide can be explained only if it is assumed that the time average geometry of the molecule is such that CH_3COO^- is sandwiched between the two aromatic rings of the molecule and the ring current effects will shift CH_3 resonance to high field. This conformation is similar to one of the 4 conformations of [Leu^5]-enkephalin observed in X-ray diffraction studies (Karle *et al.,* 1983).

The differences in the NOESY spectra (figures 3 and 4) obtained under similar experimental conditions (temperature, mixing time, etc.) also indicate conformational differences: the larger number of NOESY cross peaks observed between $N_{i+1}H$ and $C^{\alpha}H$ in [Met^5] -enkephalinamide than in [Leu^5] -enkephalinamide indicates that the backbone is relatively more rigid in the former than in the latter. However, the upfield shift observed for the methyl peak of CH_3COO^- in [Leu_5]–enkephalinamide reflects a concerted motion of the rigid side chains of Tyr and Phe and the ordered backbone. This motion is relatively less concerted in the [Met5]-analogue.

At neutral pH in DMSO-d_6 the native peptides prefer a folded conformation (Garbay-Jaureguiberry *et al.,* 1976, Jones *et al.,* 1976) while the amides prefer an extended backbone geometry. This change is due to the absence of electrostatic interaction in the amides; the same interaction, due to positive and negative charges on the terminii, is responsible for the folding of the chain in the native peptides (Higashijima *et al.,* 1979). Since the amides are relatively more potent than the native peptides, it can be concluded from the present studies that the requirement of a folded conformation may not be essential and it is probably the local conformation in the backbone and the nature of functional groups that are more critical for interaction of the enkephalins with their receptor than the overall conformation of the molecule.

Conclusions

The results of the proton magnetic resonance study on [Leu_5]- and [Met_5]-enkephalinamides have revealed that the molecules prefer extended backbone in

Solution. Secondly, the backbone in [Met^5]-enkephalinamide is relatively more rigid than in [Leu^5]-enkephalinamide. Thirdly, the side chains particularly of Tyr and Phe in both the amides have definite preferences. Finally, the characteristic folded β-bend conformation of the endogenous peptides, *i.e.*, [Leu^5]- and [Met^5]-enkephalins is not retained when the C-terminal end is altered. The replacement of — COOH group by $-CONH_2$ group leads to an extended backbone structure in these pentapeptides. Since the amides are relatively more potent than the native peptides, the characteristic β-bend conformation of the native peptide is not essential for their activity.

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