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NITROGEN-15 NUCLEAR MAGNETIC RESONANCE OF [MET^5]-ENKEPHALIN: SIGNAL ASSIGNMENTS AND THE EFFECT OF pH TO THE CHEMICAL SHIFTS

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Natural abundance ^{15}N nmr spectra of [Met^5]-enkephalin and its constituent peptides were obtained. By comparison of these spectra five ^{15}N resonance signals could be assigned to each amino acid residue. In the pH titration curves of the chemical shifts the effect of the protonation at the C-terminal Met carboxyl group was observed.

[Met^5]-enkephalin (Tyr^1-Gly^2-Gly^3-Phe^4-Met^5) is one of the endogenous pentapeptides in mammalian brain with morphine-like activities, and operates at the same receptor as natural opiates.[1] In order to clarify the relationship between its conformation and activities, numerous studies have been carried out by means of proton and carbon-13 nuclear magnetic resonance (nmr).[2] Not only the conformation but the mode of interaction of the molecule is also important to understand the mechanism of the interaction with the receptor sites. Nmr is useful to get such an information about it. Particularly the ^{15}N nmr is expected to give us the most direct information of the molecular interaction, since the nitrogen atoms generally are located at the interaction sites.[3] In this communication, we will report the natural abundance ^{15}N-nmr of [Met^5]-enkephalin for the first time. We could give spectral assignments and observe the effect of pH to the chemical shifts. The present data would be quite useful for the next step of ^{15}N nmr work to detect the interaction sites.

[Met^5]-enkephalin and other six constituent peptides given below were prepared with normal liquid-phase synthesis,[4] and all the peptides were dissolved in aqueous solutions at about 500 mM in the pH region below 2. The diameter of the sample tube is 10 mmø. A JEOL-PFT-100 pulse Fourier transform nmr spectrometer at 10.05 MHz was used for ^{15}N nmr measurements. All spectra were 70,000-100,000 times accumulated at 45° pulse with 2.2 sec interval in proton noise decoupled with nuclear Overhauser effect (NOE) mode. Chemical shifts were calculated downfield from the ammonium ion nitrogen signal of external $^{15}NH_4NO_3$ in aqueous solution at pH 2.

In figure 1 five signals with negative NOE are clearly observed. They originate in four peptide-amide nitrogens and the N-terminal tyrosine's amino nitrogen. Signal assignments were performed by comparison of the chemical shifts of five constituent peptides, Phe-Met, Gly-Gly-Phe-Met, Gly-Gly, Tyr-Gly, and Tyr-Gly-Gly (Table 1) and by pH titration (Fig. 2). We also attempted to measure the spectrum of Gly-Phe-Met, but it did not dissolve in aqueous solution

at our experimental concentration at any pH. The chemical shifts of the peptide-amide nitrogens do not vary much among the peptides, but the signals of the N-terminal amino nitrogens appear about 60-90 ppm upper field from those of the peptide-amide nitrogens and the signal position of the C-terminal amide nitrogen is sensitive to the pH change in the acidic region. Obtained chemical shift values are very similar to those of previously reported ^{15}N spectra of the peptides.[5,6] As are shown in Table 1, the chemical shifts of the amide nitrogens become larger as the amide groups are located closer to the C-terminal residue. However in the cases of Gly^2 and Gly^3 their situation is reversed. The assignments of the Gly residues were confirmed by irradiating selectively at the NH proton resonance frequencies of Gly^2 (8.6 ppm) and Gly^3 (8.0 ppm).[21] The nuclei of the Gly residue next to the Tyr residue seem to be fairly deshielded. In fact the NH proton resonance of Gly^2 appears by 0.6 ppm downfield than that of Gly^3, and the ^{13}C resonance of Tyr carbonyl group is located at the lowest field among the four amide carbonyl groups.[21]

The pH titration curves are given in Figure 2. The absense of the chemical shift values between pH 4.5 and 7.0 is due to lower solubility of the sample in this pH range. The signal of the N-terminal tyrosine residue became broad and was not observable in alkaline solutions. It may be partly due to the exchange broadening between the protonated and non-protonated species and partly due to the loss of negative NOE.[7] The C-terminal methionine's amide nitrogen shifted to lower field by about 5 ppm. The obtained pK_a value from the titration curve is 2.8, which corresponds to the pK_a for the deprotonation of the C-terminal carboxylic acid group, but is smaller by 0.7 pK unit than that determined by proton nmr.[21] The discrepancy between the both experiments seems to originate in the different concentrations employed for the ^{15}N and ^{1}H-nmr measurements. There is no shift of the signal of the Phe residue by changing pH. However the signals of Gly^2 and

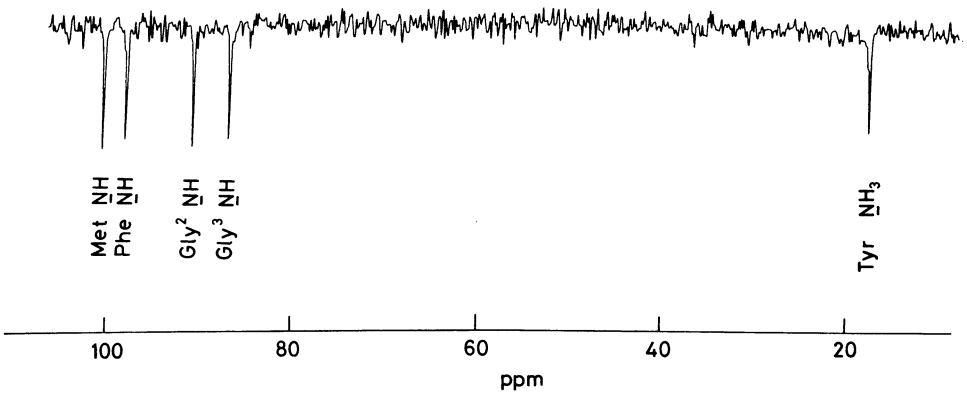

Figure 1. Proton noise decoupled, natural abundance ^{15}N nmr spectrum of [Met^5]-enkephalin in aqueous solution at pH 1.5. Concentration was 520 mM and the spectrum was accumulated 90,000 times.

Table 1. ^{15}N chemical shifts (ppm) of [Met^5]-enkephalin and its constituent peptides in aqueous solutions at around pH 1.5.

Tyr	Gly^2	Gly^3	Phe	Met
17.54	90.98	86.99	98.24	100.66
			Phe	Met
			(17.42)	101.99
	Gly^1	Gly^2	Phe	Met
	(23.11)	85.73	98.51	101.38
	Gly^1	Gly^2		
	(6.65)	88.92		
Tyr	Gly^2	Gly^3		
17.66	91.46	88.32		
Tyr	Gly^2			
17.54	91.46			

Chemical shifts are given relative to external $^{15}NH_4NO_3$. The figures in parentheses are not used for the comparison of the chemical shifts.

Gly^3 are located slightly downfield in the neutral pH region than in both the acidic and alkaline pH regions. The upfield shift around pH 7 may be attributed to the deprotonation at the amino group of the N-terminal Tyr residue, but the upfield. shifts around pH 3 are not due to the direct effect to the shifts through bonds by the protonation at the C-terminal carboxylic acid residue, since the signal of the Phe residue locating closer to the C-terminal residue does not change in this pH region. It is very probable that the Gly residues are affected by the protonation at the C-terminal group through intermolecular association in such a high concentration as 500 mM.[21] The possibility that conformational changes occur around the Gly residues accompanying the protonation at the C-terminal residue cannot be denied.

The ^{15}N chemical shifts of the peptide with morphine like activity were obtained. As the next step of the work the effect of the concentration to the ^{15}N chemical shifts and to signal NOE should be analyzed. However for such purpose natural abundance ^{15}N nmr has a limit due to low sensitivity in the spectral measurements. ^{15}N enriched enkephalin is necessary for such purpose and the enriched sample would make also possible to elucidate the interaction sites of the biologically active peptide.

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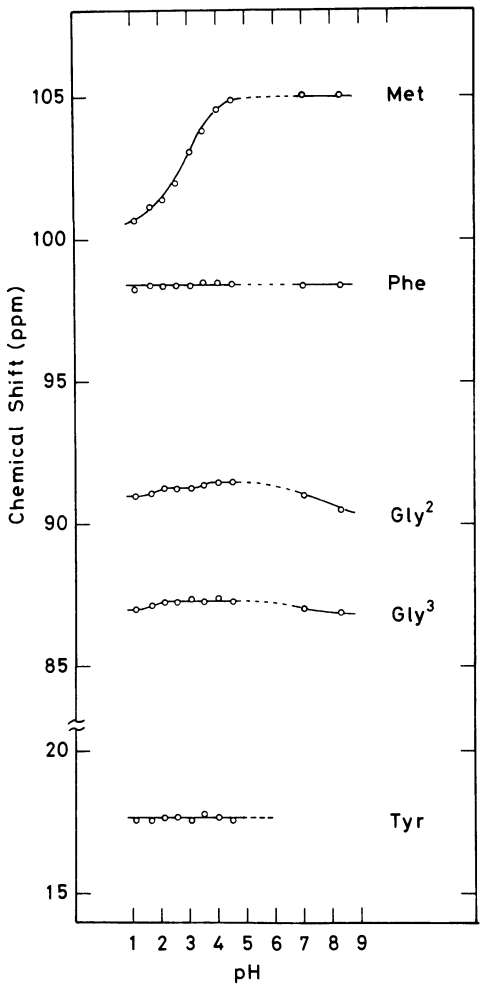

Figure 2. pH Dependence of ^{15}N chemical shifts of [Met^5]-enkephalin.

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