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A 500 MHz ^{1}H NMR SPECTROSCOPIC STUDY OF MET^5-ENKEPHALINAMIDE IN AQUEOUS SOLUTION: ETHANOL INDUCED CONFORMATIONAL CHANGES

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Summary: An analysis of spin coupling constants, $\langle^3J\rangle_{C\alpha H-NH}$, from a high resolution 500 MHz ^{1}H NMR study of [Met^5]-enkephalinamide in aqueous solution, suggested that ß-sheet structure is the likely conformer. The effect of ethanol on the conformation of [Met^5]-enkephalinamide in aqueous solution was investigated. From the upfield drift of observed chemical shifts and changes in coupling constants, especially of the amide NH resonances, it is concluded that ethanol disrupts the conformation possibly by influencing the hydrogen bonding. The above observation is consistent with a recent study of the ethanol induced conformational changes occurring in [Met^5]enkephalinamide [Rapaka, R.S. et al. (1986) Life Sciences 39,837-842].

Even before the discovery of the endogenous ligands for the opioid receptors, i.e. the enkephalins, by Hughes et al (1), several opioid receptor types were demonstrated from binding and pharmacological studies (2-5). Hiller et al (6) proposed from their binding studies that μ- and δ-receptors differ in their ligand-binding characteristics in the presence of ethanol. This difference was attributed to the differences in fluidity changes in the membrane caused by ethanol and it was proposed that binding to δ-receptors is more influenced than to μ-receptors. Conformational studies and receptor binding studies from our laboratories indicated that effects on

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ligand conformation are as important as on receptor protein. In our earlier studies, conformational changes in [Met^5]-enkephalinamide, a δ-receptor ligand, and H-Tyr-D-Ala-Gly-N-Me-Phe-Gly-ol (DAGO), a μ-receptor specific ligand were investigated using FT-IR spectroscopy (7,8). The present study was undertaken to unequivocally demonstrate the ethanol-induced hydrogen bond disruption and the resultant loss of conformation of [Met^5]-enkephalinamide. In the present report, preliminary results on the effects of ethanol on the conformations of [Met^5]-enkephalinamide in aqueous solution are presented from high resolution 500 MHz ^{1}H NMR spectroscopic study, which affords suppression of water peaks in ^{1}H NMR spectra, and offers a powerful "window" on secondary structures of polypeptides and proteins.

Materials and Methods

[Met^5]-enkephalinamide and 100% D_2O were purchased from Bachem, Torrance, CA and Sigma Chemical company, St. Louis, MO., respectively. A 15 mM solution of [Met^5]-enkephalinamide in 4:1 H_2O:D_2O mixture, pH(D) 4.47 (at 25°C) was prepared. NMR spectra were recorded on a Bruker AM500 spectrometer at Harvard University NMR Facility at the Department of Chemistry. Water resonance was suppressed by using 1331 pulse sequence. Chemical shifts, δ, were referenced to water signal (4.8 ppm). 15 μl and 120 μl of ethanol was added to a 0.5 ml aqueous solution of [Met^5]-enkephalinamide. This addition results in 3 and 24% aqueous ethanolic solutions respectively. In the earlier studies, Hiller et al (6) utilized ethanolic solutions of upto 5% (v/v). However, in this present study, 24% ethanol (v/v) in water was also used as higher concentrations of [Met^5]-enkephalinamide were required in the NMR study to obtain satisfactory signals. Spectroscopic grade ethanol was used in the preparation of the solution.

Results and Discussion

Assignment of amide NH Resonances

The amide region of the 500 MHz ^{1}H NMR spectrum of [Met^5]-enkephalinamide, 15 mM solution in 4:1 H_2O-D_2O mixture, pH(D) 4.47, at room temperature is shown in Fig. 1A. The spectral assignments were accomplished by spin decoupling and by comparison with previous studies of [Met^5]-enkephalin in $(CD_3)_2SO$ and D_2O by Higashijima et al. (9), Zetta et al. (10), and Levine et al. (11). The non-equivalent largest upfield and downfield NH resonances occurring as a broad peak at 8.60 ppm and as a triplet at 8.02 ppm were assigned to the Gly_2NH and Gly_3NH respectively. The doublets at 8.37

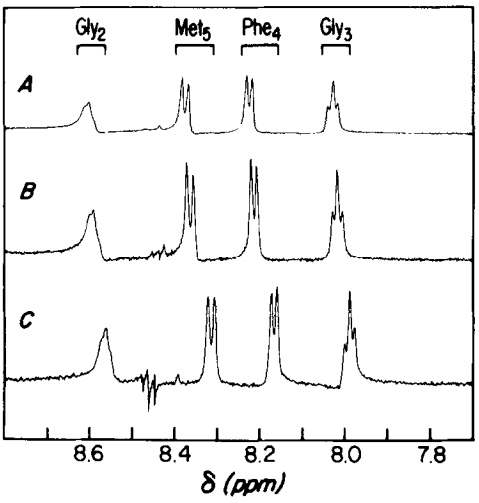

Fig. 1. A. Peptide NH region of 500 NHz ^1H NMR spectrum of 15 mM solution of [Met^5]-enkephalinamide in 4:1 H_2O-D_2O mixture, pH(D) 4.47 at room temperature; B. In the presence of 3% (v/v) ethanol; C. In the presence of 24% (v/v) ethanol.

ppm and 8.22 ppm were assigned to Met_5 NH and Phe_4 NH based on the different spin coupling constants, $\langle {}^3J \rangle_{C^{\alpha}H\text{-}NH}$, and spin decoupling experiments. In aqueous solution, NH_3^+ of Tyr_1 experiences rapid exchange with D_2O in 4:1 H_2O-D_2O mixture whereas in $(CD_3)_2SO$, Tyr_1 NH_3^+ proton resonance can be observed distinctively. Chemical shifts, δ, and spin coupling constants, J, of [Met^5]-enkephalinamide and [Met^5]-enkephalinamide in the presence of ethanol are listed in Table I.

Conformation of [Met^5]-enkephalinamide in Water

The observed spin coupling constants, $\langle {}^3J \rangle_{C^{\alpha}H\text{-}NH}$, were correlated with with Ramachandran angle,ϕ, using Karplus-like equation (12): ${}^3J_{C^{\alpha}H\text{-}NH} = A\,Cos^2\theta - B\,Cos\theta + C\,Sin^2\theta$ with the values of the coefficient A,B,C for glycyl and non-glycyl residues derived by Bystrov et al. (13). In aqueous solution of [Met^5]-enkephalinamide, Phe_4 and Met_5 NH protons show coupling constants of 6.55 Hz and 7.60 Hz, respectively. The above observed values for Phe_4 and Met_5 residues correspond to four possible values of Ramachandran angle,ϕ (IUPAC Convention): (i) 80 to 90°; (ii)30°to 40°;(iii)-160 to -170°; and (iv)-70° to -80°. The Gly_3 NH manifests an average coupling constant of 5.65 Hz which give rise

TABLE I

Peptide NH Chemical Shifts, δ (ppm ± 0.01 ppm), and Spin Coupling Constants, $^3J_{C\alpha H-NH}$(Hz ± 0.05 Hz), for [Met^5]-enkephalinamide in 4:1 H_2O-D_2O Mixture as a Function of Alcohol Concentration

Ethanol	Gly_2NH	Gly_3NH	Phe_4NH	Met_5NH
0%	8.60	8.02	8.22	8.37
$<^3J>_{C\alpha H-NH}$	a	5.65	6.55	7.60
3% (v/v)	8.59	8.01	8.21	8.36
$<^3J>_{C\alpha H-NH}$	a	5.80	6.50	7.55
24% (v/v)	8.56	7.99	8.17	8.31
$<^3J>_{C\alpha H-NH}$	a	5.65	6.40	7.65

For Tyr_1 NH_3, see text for explanation.

[a]unresolved

to φ values between ±20° to ±30°. The third possibility, φ = -160° -170° lies close to the β-sheet region and is in agreement with the observed φ angle for Phe_4 and Met_5 residues in [Met^5]-enkephalin crystal structure recently reported by Griffin et al.(14). The first possible solution for φ = 80 to 100° and the fourth possible value of φ = 70 to 80° are generally compatible with type I'/II/III' β-turn structures (15) with either Phe_4 or Met_5 as i+2 residue. But if β-turn should occur involving Gly_3-Phe_4 which has been suggested from numerous spectroscopic studies (15), then the ϕ_3 for Gly_3 of either -20° to -30° or 20° to 30° is somewhat out-of-range for the occurrence of Gly_3 in the Gly_3-Phe_4 β-turn (16). Therefore, from the present 500 MHz ^{1}H NMR study of [Met^5]-enkephalinamide in aqueous solution, at 15mM concentration, pH(D) 4.47, an extended β-sheet structure seems more likely than a β-turn structure. For a discussion of numerous spectroscopic studies of enkephalins, see Schiller et al. (15). The above conclusion is consistent with our previous FT-IR and Raman studies of [Met^5]-enkephalin (17) and the recent

x-ray studies of Griffin et al. (14) and Doi et al. (18). Nevertheless from the limited NMR study reported here where the temperature and concentration effects are not discussed, it will be premature to assume the total absence of the occurrence of ß-turn structure.

Effect of Ethanol on 500 MHz ^{1}H NMR Spectrum of [Met^5]-enkephalinamide in water.

500 MHz ^{1}H NMR spectra of [Met^5]-enkephalinamide in 4:1 H_2O-D_2O mixture in the presence of 3% and 24% ethanol are shown in Fig. 1B and 1C and the observed changes in the coupling constants, δ, and spin coupling constants, $\langle^3J\rangle_{C^\alpha H-NH}$, are listed in Table I. At 3% (v/v) ethanol, the change in the chemical shifts, δ, observed for Gly_2, Gly_3, Phe_4, and Met_5 NH resonances are less than 0.03 ppm. Nevertheless, all the four NH resonances shift upfield slightly. The change in $\langle^3J\rangle_{C^\alpha H-NH}$ are less than 0.15 Hz with the largest changes occurring in the Gly_3 $C^\alpha HNH$ coupling constant. When the concentration of ethanol is increased further, Gly_3 NH proton experiences the least chemical shift change whereas Gly_2, Phe_4, and Met_5 NH protons undergo large changes in their chemical shifts. The spin coupling constants, $\langle^3J\rangle_{C^\alpha H-NH}$, for Phe_4 and Met_5 residues also increase slightly.

The changes observed in the chemical shifts in presence of ethanol are generally indicative of the perturbation of secondary structure of [Met^5]-enkephalinamide in aqueous solution. The very slight change in Gly_2NH chemical shifts suggests that this proton does not participate in the disruption of the secondary structure induced by ethanol. On the other hand, Gly_3 NH, Phe_4 NH and Met_5 NH chemical shifts undergoing changes in δ in the presence of ethanol would implicate them in a disruption of the secondary structure of [Met^5]-enkephalinamide triggered by ethanol. The pattern of changes observed in chemical shifts is paralleled by the variation

in spin coupling constants $\langle {}^3J \rangle_{C^\alpha HNH}$ induced by ethanol. Various modes of hydrogen bonding between [Met^5]-enkephalins have been proposed by Griffin et al. (14) and others (19, and for a review see 15) and are consistent with crystal structure determinations (15). A plausible explanation of the upfield shift of the Gly_3, Phe_4, Met_5 NH protons (20) and the change in the spin coupling constants, $\langle {}^3J \rangle_{C^\alpha H-NH}$, are consistent with the general concept of disruption of the hydrogen bonding between [Met^5]-enkephalin molecules and consequent structural alterations (21-23).

In conclusion, this study provides evidence for the presence of ß-sheet structure for [Met^5]-enkephalinamide. The ß-sheet structure is destabilized by ethanol due to the disruption of H-bonds. This observation points out that ethnol-induced effect on ligand-receptor binding may be due to alteration of the ligand conformation and the importance of conformational features of the ligand in ligand-receptor interaction has been already pointed out (see 23 for review).

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References

1. Hughes, J., Smith, T.W., Kosterlitz, H.W., Fothergill, L.A., Morgan, B.A., and Morris, H.R. (1975) Nature 258, 577-580.
2. Martin, W.R., Eades, C.G., Thompson, J.A., Huppler, R.E. and Gilbert, P.E. (1976) J. Pharmacol. Exp. Ther. 197, 515-532.
3. Lord, J.A.H.,Waterfield, A.A., Hughes J.and Kosterlitz H. W.(1977) Nature 267 495-499.
4. Paterson, S.J.,Robson, L.E. and Kosterlitz H.W. (1984) The Peptides: Analysis, Synthesis, Biology, Vol. 6, Opioid Peptides: Biology, Chemistry, and Genetics, S. Udenfriend and J. Meienhofer eds. pp. 147-189, Academic Press, Inc., New York.
5. Simon, E. J. Molecular Pharmacology, Biosynthesis, and Analysis, National Institute on Drug Abuse Research Monograph, Vol. 70, R.S. Rapaka and R.L. Hawks eds, U.S. Government Printing Press, Rockville, MD, In Press.
6. Hiller, J.M., Angel, L.M., and Simon, E.J. (1981) Science, 214, 468-469.

7. Rapaka, R.S., Renugopalakrishnan, V., Goehl, T.J., and Collins, B.J. (1986) Life Sci. 39, 837-842.
8. Rapaka, R.S., Bhargava, H.N., Renugopalakrishnan, V., and Collins, B.J., Submitted.
9. Higashijima, R., Kobayashi, J., Nagai, U., and Miyazawa, T. (1979) European J. Biochem. 97,43-57.
10. Zetta, L., Cabassi, F., Tomatis, R., and Guarheri, M. (1979) European J. Biochem 95, 367-376.
11. Levine, B.A., Rabenstein, D.L., Smith, D.M, and Williams, R.J.P. (1979) Biochem, Biophys. Acta 579, 279-290
12. Karplus, M. (1979) J. Chem. Phy. 30, 11-15.
13. Bystrov, V.F., Portnova, S.L., Balashova, T.A., Kozmin, S A., Gavrilov, Yu,D., and Afanas'ev, V.A. (1973) Pure and Applied Chem. 36, 19-34.
14. Griffin, J.F., Langs, D.A., Smith, G.D., Blundell, T.L. Tickle, I.J. and Bedarkar, S. (1986) Proc. Natl Acad, Sci USA 83, 3272-3276.
15. Schiller, P.W. (1984) In THE PEPTIDES, Vol. 6: Opioid Peptides: Biology, Chemistry and Genetics, PP. 239-240, S. Udenfriend and J. Meienhofer, eds, Academic Press, New York.
16. Smith, P.A. and Pease, L.G. (1980) CRC Critical Rev. Biochem. 8, 315-399.
17. Renugoplalakrishnan, V., Rapaka, R.S., Collette, T.W., Carreira, L.A., and Bhatnagar, R.S. (1985) Biochem. Biophys. Res. Commun. 126, 1029-1035.
18. Doi, M., Ishida, T., Inoue, M., Fujiwara, T., Tomita, K.I., Kimura, T.m and Sakakibara, S (1984) FEBS Lett. 170, 229-231.
19. Khaled, M.A., Long, M.M., Thompson, W.D., Bradley, R.J., Brown, G.B., and Urry, D.W. (1977) BBRC 76, 224-231.
20. Pimentel, G.C. and McClellan, A.L. (1960) The hydrogen Bond, W.H. Freeman and Company, San Francisco, Ca., USA.
21. Mishra A.K., and Ahluwalia, J. C. (1983) Int. J. Peptide Protein Res. 21, 322-330.
22. Conio, G., Patrone, E., and Brighetti, S. (1970) J. Biol. Chem. 245, 335-3360.
23. Rapaka, R.S. (1986) Opioid Peptides: Molecular Pharmacology, Biosynthesis, and Analysis, National Institute on Drug Abuse Research Monograph, Vol. 70, R.S. Rapaka and R.L. Hawks eds, U.S. Government Printing Press, Rockville, MD.