360 MHz PROTON NMR SPECTRA OF ACTIVE AND INACTIVE DERIVATIVES OF METHIONINE-ENKEPHALIN. ASSIGNMENTS, DERIVED PARAMETERS, CONFORMATIONAL IMPLICATIONS.

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SUMMARY: The proton NMR spectra in aqueous and dimethylsulfoxide solutions have been obtained at 360 MHz for: methionine-enkephalin, leucine-enkephalin, (Phe[1])-methionine-enkephalin, norleucine-enkephalin, and des-amino-methionine-enkephalin. Resonance assignments, derived chemical shifts, spin coupling constants, and amide proton temperature dependencies (in dimethylsulfoxide) are presented.

INTRODUCTION: Considerable interest has arisen in a group of small peptides with powerful central nervous system actions. In particular, the pentapeptides with the trivial name "enkephalins"[2] represent an interesting system to study by physical methods with the aim of clarifying the role, if any, their solution conformations play in their pharmacological activity. We have entered into a systematic study of this sort and two previous reports from our laboratory have presented results[3,4]. These concerned proton NMR spectroscopy at 100 MHz where details of second order spin multiplets were too complex to analyze. Furthermore, recent investigations at 300 MHz[5,6] (references 5 and 6 appear identical) and 270 MHz[7] appear to disagree with our resonance assignments. We are therefore reporting in the present paper confirmation of our initial assignment work along with additional derived data available from 360 MHz spectra which shed some new light on the conformations of some analogs of these peptides. The pharmacological activities of the derivatives examined may be expressed as the dose of compound required to inhibit contraction of stimulated

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guinea pig ileum to 50% of control given as a relative potency (morphine = 1.0). In this unit the relative potencies of the compounds under discussion are: methionine-enkephalin (Tyr-Gly-Gly-Phe-Met): 4.0, leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu): 0.7, norleucine-enkephalin (Tyr-Gly-Gly-Phe-Nleu): 1.8, (Phe^1)-methionine-enkephalin (Phe-Gly-Gly-Phe-Met): no detectable activity[8].

We have previously proposed[4] (in summary) that the molecule of methionine-enkephalin is characterized by relative main-chain and tyrosyl side-chain rigidity coupled with phenylalanyl ring and methionine methyl intramolecular motion. On the basis of ^1H and ^{13}C spin-lattice relaxation measurements we further detected a conformational transition between dimethylsulfoxide (DMSO-d_6) and aqueous D_2O solutions.

MATERIALS AND METHODS: The peptides were synthesized by standard solid phase methods[8], subjected to countercurrent purification and isolated as the hydrochloride salts. These were dried at 40-50°C over P_2O_5 in a vacuum desiccator for 20 hours. The solutions discussed here were made up in peptide concentrations from 0.08 to 0.02 M in precision 5 mm NMR tubes under nitrogen, after vacuum drying. 100% isotopically enriched DMSO-d_6 (stored over molecular sieve) and D_2O were used but no attempt was made to exclude oxygen or paramagnetic impurities. All samples showed as undegraded material upon standard TLC analysis before and after NMR work[4]. 360 MHz spectra were obtained at a 35°C sample temperature in the Fourier Transform mode with 16K data points and 16 accumulations for each spectrum. Data on temperature dependence of amide protons were taken at 100 MHz on the New England Area Instrument in a manner previously described[4].

RESULTS: Tables I and II present results derived from the 360 MHz spectra of the various enkephalin analogs. Comparison of the α proton region of spectra from met-, leu- and nleu-enkephalins sufficed by itself to confirm our previous assignments[4] of $Met^{(5)}$ alpha and amide protons. However, we further confirmed these assignments by 360 MHz homonuclear decoupling. The constants derived for spin multiplets were on the basis of ABX analysis with theoretical fits to observed frequencies and intensities. By observation of very weak lines at 360, 270 and 100 MHz in the aromatic region of $Tyr^{(1)}$ of methionine-enkephalin in D_2O we are able to analyze the multiplet as an AA'BB' system and to determine that the satellite lines did not derive from non-equivalence in the AA' and BB' systems. All amide peaks were resolved enough to display their coupling patterns

TABLE I

Proton Chemical Shifts for methionine-enkephalin (met-Enk), leucine-enkephalin (leu-Enk), des-amino-enkephalin (dNH_2-Enk), Phe^1-methionine-enkephalin (Phe^1-Enk), and nor-leucine-enkephalin (Nleu-Enk) in DMSO-d_6 and D_2O. PPM from external 3(trimethylsilyl) tetradeutero sodium propionate (D_2O) and tetramethylsilane (DMSO) at 35°C.

		DMSO					D_2O				
Residue/Proton		Met-Enk	Leu-Enk	d-NH_2 Enk	Phe^1-Enk	N-leu-Enk	Met-Enk	Leu-Enk	d-NH_2 Enk	Phe^1-Enk	N-leu-Enk
Tyr^1	α	4.05	3.95	2.70(2.71)	-	3.98	4.27	4.28	-	-	4.26
	β_1	3.07	2.98	2.38	-	3.01	3.16	3.17	-	-	3.13
	β_2	2.19	2.83	2.39	-	2.85	3.16	3.17	-	-	3.13
	δ	7.12	7.02	6.98	-	7.06	7.19	7.19	-	-	7.15
	ϵ	6.77	6.66	6.65	-	6.70	6.89	6.90	-	-	6.86
Phe^1	α	-	-	-	4.11	-	-	-	-	4.31	-
	β_1	-	-	-	3.14	-	-	-	-	3.22	-
	β_2	-	-	-	3.03	-	-	-	-	3.22	-
	δ,ϵ,ζ	-	-	-	7.27	-	-	-	-	7.32	-
Gly^2	α_1	3.89	3.78	3.67	3.81	3.82	3.94	3.95	-	3.91	3.91
	α_2	3.77	3.66	3.67	3.70	3.71	3.89	3.90	-	3.86	3.85
	amide	8.86	8.77	8.08	8.90	8.80	-	-	-	-	-
	$d\delta$(amide)/dt	4.6*	3.6	5.0	4.1	3.6	-	-	-	-	-
Gly^3	α_1	3.79	3.68	3.69	3.73	3.72	3.89	3.89	-	3.87	3.85
	α_2	3.69	3.58	3.59	3.62	3.62	3.85	3.86	-	3.81	3.82
	amide	8.18	8.08	7.96	8.13	8.11	-	-	-	-	-
	$d\delta$(amide)/dt	3.5	3.2	4.7	3.2	2.8	-	-	-	-	-
Phe^4	α	4.61	4.52	4.54	4.56	4.58	4.65	4.67	-	4.63	4.63
	β_1	3.10	3.00	3.06	3.05	3.03	3.16	3.18	-	3.15	3.14
	β_2	2.86	2.75	2.80	2.81	2.77	3.01	3.01	-	2.90	2.98
	δ,ϵ,ζ	7.32	7.24	7.24	7.27	7.26	7.31	7.3	-	7.32	7.30
	amide	8.09	7.94	7.98	8.04	7.99	-	-	-	-	-
	$d\delta$(amide)/dt	5.4	4.7	5.6	5.3	4.9	-	-	-	-	-

TABLE I continued

Residue/Proton		DMSO					D_2O				
		Met-Enk	Leu-Enk	d-NH_2 Enk	Phe^1-Enk	N-leu-Enk	Met-Enk	Leu-Enk	d-NH_2 Enk†	Phe^1-Enk	N-leu-Enk
Met^5	α	4.53	-	4.33	4.33	-	4.47	-	-	4.47	-
	β	2.00	-	1.94	1.95	-	2.04	-	-	2.03	-
	γ	2.53	-	2.49	2.49	-	2.48	-	-	2.47	-
	δ	2.10	-	2.04	2.04	-	2.07	-	-	2.04	-
	amide	8.38	-	8.19	8.35	-	-	-	-	-	-
dδ(amide)/dt		6.2	-	-	6.4	-	-	-	-	-	-
Leu^5	α	-	4.18	-	-	-	-	4.38	-	-	-
	$β_1$	-	1.56	-	-	-	-	1.61	-	-	-
	$β_2$	-	1.51	-	-	-	-	1.63	-	-	-
	$δ_1$	-	0.86	-	-	-	-	0.92	-	-	-
	$δ_2$	-	0.80	-	-	-	-	0.86	-	-	-
	amide	-	8.24	-	-	-	-	-	-	-	-
dδ(amide)/dt		-	6.2	-	-	-	-	-	-	-	-
N-leu^5	α	-	-	-	-	4.16	-	-	-	-	4.24
	β	-	-	-	-	1.29	-	-	-	-	1.24
	γ	-	-	-	-	1.29	-	-	-	-	1.24
	δ	-	-	-	-	1.66	-	-	-	-	1.72
	ε	-	-	-	-	0.86	-	-	-	-	0.83
	amide	-	-	-	-	8.26	-	-	-	-	-
dδ(amide)/dt		-	-	-	-	6.5	-	-	-	-	-

*In units of ppm/deg Centigrade multiplied by 10^3.

†Poor quality data due to relative insolubility in aqueous solution.

TABLE II

Proton Spin Coupling Constants for: Met-Enk, Leu-Enk, dNH_2-Enk, Phe^1-Enk and N-Leu-Enk (see Table I for abbreviations) in DMSO and D_2O. A, B = β_1, β_2; proton X = α proton; J_{NH} = amide-α proton coupling constant.

Residue/J(Hz)		DMSO					D_2O				
		Met-Enk	Leu-Enk	d-NH_2-Enk	Phe^1-Enk	N-leu-Enk	Met-Enk	Leu-Enk	d-NH_2-Enk	Phe^1-Enk	N-leu-Enk
Tyr	J_{AX}	5.6	5.8	6*	-	6.3	7.5	7.6	-	-	7.4
	J_{BX}	7.7	7.8	8*	-	8.0	7.5	7.6	-	-	7.4
	$\lvert J_{AB}\rvert$	14.1	14.2	14.5*	-	14.2	-	-	-	-	-
Phe^1	J_{AX}	-	-	-	6.3	-	-	-	-	7.4	-
	J_{BX}	-	-	-	7.3	-	-	-	-	7.4	-
	$\lvert JAB\rvert$	-	-	-	14.2	-	-	-	-	-	-
Gly^2	$\lvert J_{AB}\rvert$	17.0	17.0	-	17.2	17.2	17.4	17.4	-	17.5	17.7
	J_{NH_1}	5.4	5.6	5.5	5.6	5.7	-	-	-	-	-
	J_{NH_2}	5.9	5.7	-	5.5	5.9	-	-	-	-	-
Gly^3	$\lvert J_{AB}\rvert$	16.7	16.6	16.5	16.9	16.7	16.7	16.4	-	16.7	16.6
	J_{NH}	6.2	5.9	5.9	5.8	6.1	-	-	-	-	-
	J_{NH}	5.5	5.7	5.5	5.6	5.8	-	-	-	-	-
Phe^4	J_{AX}	4.2	4.2	4.3	4.0	4.5	7.0	7.3	-	6.7	6.9
	J_{BX}	9.9	10.1	9.8	10.0	9.8	8.5	8.4	-	8.5	8.5
	$\lvert J_{AB}\rvert$	14.2	14.0	14.0	13.9	13.9	14.0	14.3	-	14.3	14.0
	J_{NH}	8.7	8.7	8.6	8.9	8.8	-	-	-	-	-

*Analyzed as a 4 spin system with the following parameters: δ_{12} = 2 Hz, δ_{34} = 2 Hz, δ = 115 Hz, $J_{12} = J_{34}$ = 14.5 Hz, $J_{13} = J_{24}$ = 8 Hz, $J_{14} = J_{23}$ = 6 Hz. J_{NH}'s were determined as follows for other residues; met^5 in met-enkephalin: 8.1 Hz, leu^5 in leu-enkephalin: 8.2 Hz, met^5 in des-amino-met-enkephalin: 8.1 Hz, met^5 in (Phe^1)-met-enkephalin: 8 Hz, N-leu in N-leu-enkephalin: 7.9 Hz.

In addition we observe the tyrosine hydroxyl proton in the appropriate cases.

DISCUSSION AND CONCLUSIONS: A striking observation is that the α protons of glycines (2) and (3) are non-equivalent in both DMSO and D_2O solutions, that the β protons of $Phe^{(4)}$ are non-equivalent in both DMSO and D_2O solutions and that the β protons of $Tyr^{(1)}$ while non-equivalent in DMSO solution become

equivalent in D_2O. Furthermore, chemical shifts and coupling constants show strong similarities through the series of peptides. The implication of the latter observation is that, whatever the functional dependence of the J_{AX}, J_{BX} upon the $\chi^{(1)}$ angles for $Tyr^{(1)}$ and $Phe^{(4)}$, there is little if any conformational determination of these angles through interactions with nearest neighbors. Thus, the $Phe^{(4)}$ pattern of coupling constants in the active met-enkephalin and the completely inactive $Phe^{(1)}$-met-enkephalin are identical while the α-β proton coupling constants at the 1 position do not depend upon whether the distal group is a phenylalanyl ring (inactive) or a tyrosyl ring (active). The temperature dependencies for amide proton shifts, independently determined in the present work, agree with our previous result for met-enkephalin. Those for the other derivatives, both active and inactive, are similarly unexceptional and therefore lend no weight to the stabilization of conformation by intramolecular hydrogen bonding in DMSO.

Our present results do not definitely answer the problem of whether or not the molecules under study are conformationally rigid over a well defined time interval. It is tempting to conclude that the non-equivalence of individual α and β proton chemical shifts, mentioned above, show that the molecules are inflexible on a chemical shift time scale. However, while this interpretation is consistent with the results it is physically possible that a complex of intramolecular motions could bring about a pattern of non-equivalence. This seems to us to be highly unlikely since, for example, the shift difference between the α protons of $Gly^{(2)}$ and $Gly^{(3)}$ remains essentially constant through the series of compounds (with the exception of $Gly^{(2)}$ in des-amino-met-enkephalin) in a given solvent system. The alternative explanation to rigidity would have to be that intramolecular rotamer populations are not a function of side-chain substitutions in these peptides.

Our results are consistent with the previous suggestion[4] that there is a conformational difference between met-enkephalin in DMSO and in D_2O, and furthermore this is true for the analogs examined here as well. Finally, com-

bined with our earlier results a model consistent with both relaxation and high resolution analysis of met-enkephalin is one in which the $Tyr^{(1)}$ aromatic ring is relatively immobile while the $Phe^{(4)}$ aromatic ring undergoes intramolecular flip-flops at a relatively rapid rate. Despite substitutions at other residues in the molecule the coupling constant analysis indicates that in DMSO the γ-ζ axes for both $Tyr^{(1)}$ and $Phe^{(4)}$ rings retain the same average directions with respect to the backbone of the peptide. The same applies for the $Phe^{(4)}$ ring in D_2O.

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