Experimental Attempt To Simulate Receptor Site Environment. A 500-MHz ^1H Nuclear Magnetic Resonance Study of Enkephalin Amides

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ABSTRACT: The amides of Leu^5-enkephalin, Met^5-enkephalin, and three analogues, D-Ala^2,Leu^5-enkephalin, (AcO)Tyr^1,Met^5-enkephalin, and (AcO)Tyr^1,D-Ala^2,Met^5-enkephalin, have been studied by means of ^1H NMR spectroscopy in two different solvent systems: Me_2SO-d_6 and $CDCl_3$. In the latter solvent the peptides were dissolved as complexes with 18-crown-6-ether, a coronand that binds strongly to the NH_3^+ groups. The crown ether complexation and the apolar solvent were used to simulate the anionic subsite of the receptor and the hydrophobic environment of the receptor cavity, respectively. The very unusual amide proton chemical shifts and their temperature coefficients suggest the presence of folded conformations in $CDCl_3$ for all peptides, consistent with several models of opioid receptors and with the crystal structure of Leu^5-enkephalin. The differences among the proposed cyclic conformations of the five peptides may be correlated, in part, with their different biological activity. All peptides in Me_2SO-d_6 are characterized by complex mixtures of extended fully solvated conformations.

Endogenous opioids have been the subject of many conformational studies in solution, particularly by means of NMR[1] spectroscopy (Beretta et al., 1984; Bleich et al., 1976; Combrisson et al., 1976; Garbay-Jaureguiberry et al., 1976, 1977; Higashijima et al., 1979; Khaled et al., 1977; Jones et al., 1976; Spirtes et al., 1978; Stimson et al., 1979). The main goal of these studies is the search for a relationship between the biological activity of the peptides and their conformation in solution.

Attempts of this nature are hampered by two fundamental difficulties: first, a flexible molecule like a linear peptide is seldom characterized, in solution, by a single (or a few) stable conformation of minimum energy but rather by a complex mixture of quasi-isoenergetic conformations effectively averaged in the NMR time scale. Second, the actual environmental in which the biological interaction takes place can have a profound influence on the conformational equilibrium. It is generally believed (Erne et al., 1985; Gremlich et al., 1983, 1984; Gremlich & Schwyzer, 1983a,b, 1984) that even before the peptide binds to a protein receptor, the change from the aqueous environment of the transport fluid to the apolar one of the membrane lipids induces a transition from more or less disordered "random coils" to fairly ordered folded conformations. Schwyzer, in fact, has recently shown (1986a,b) that the complex interactions of dynorphin (a natural tridecapeptide with κ opioid activity) with neutral lipid membranes can be interpreted in terms of the transfer of the first nine N-terminal residues to an α-helical conformation inside the hydrophobic phase. It is significant for us that the first five residues of dynorphin coincide with those of Leu-enkephalin, one of the agonists studied in the present work. Thus, it is even more important to take into account the influence of the environment inside the active site of the receptor.

Although the structure of opioid receptors is not known from direct studies, all models, based essentially on the properties of rigid nonpeptide agonists (Beckett & Casy, 1954; Portoghese, 1965; Galt, 1977; Portoghese et al., 1981; Pastore et al., 1985), point to a highly hydrophobic pocket containing a specific anionic subsite (that interacts with the positive nitrogen present in all opioids).

Accordingly, it is not surprising that so many authors, mainly on the basis of energy calculations, have proposed, as biologically active conformations for enkephalins, folded conformations in which all hydrophobic side chains are exposed (Isogai et al., 1977; DeCoen et al., 1977; Momany, 1977; Balodis et al., 1978; Loew & Burt, 1978; Manavalan & Momany, 1981; Paine & Scheraga, 1985, 1986).

On the contrary, nearly all experimental studies on the conformation of enkephalins in solution have been performed in very polar media, usually water or dimethyl sulfoxide. Water, of course, is close enough to the physiological transport medium (even if no cosolute is added) and as such can be quite useful for studies on the conformational state of the enkephalins prior to the interaction with the receptor. Me_2SO-d_6, on the other hand, is a good substitute neither for the natural transport medium nor for the receptor environment, being not only polar but also a good denaturing agent. In fact, it has been routinely used for many years to put polypeptides into the so-called random coil conformation (Bradbury et al., 1973).

In spite of many claims [e.g., see Stimson et al. (1979), Roques et al. (1976), and Jones et al. (1976)] in favor of the presence of folded structures in Me_2SO-d_6 solutions of enkephalins, Higashijima et al. (1979) have demonstrated that Met^5-enkephalin amide assumes, in Me_2SO-d_6, an essentially extended conformation, whereas the dipolar form of Met^5-enkephalin in the same solvent contains only a small fraction of a disordered folded structure, owing to the strong electrostatic interaction between the charged ends of the peptide. It seems likely that also the folded forms reported in studies of other opioid peptides in polar solvents [including very recent

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[1] Abbreviations: NMR, nuclear magnetic resonance; Me_2SO-d_6, hexadeuteriodimethyl sulfoxide; LEA, Leu^5-enkephalin amide; MEA, Met^5-enkephalin amide; DEA, D-Ala^2,Leu^5-enkephalin amide; AMEA, (AcO)Tyr^1,Met^5-enkephalin amide; ADEA, (AcO)Tyr^1,D-Ala^2,Met^5-enkephalin amide; 2D, two dimensional; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect 2D spectroscopy; COSY, correlated 2D spectroscopy; ROESY, rotating frame NOE 2D spectroscopy; DQ-COSY, double-quantum COSY; GPI, guinea pig ileum in vitro test; MVD, mouse vas deferens in vitro test; EDTA, ethylenediaminetetraacetic acid. Standard IUPAC three-letter and one-letter symbols for amino acid residues were used throughout.

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work, e.g., Gupta et al. (1986)] are mainly due to the electrostatic interaction of the zwitterion. Thus, it is difficult to evalute the intrinsic tendency, if any, of the peptide to fold as a consequence of the other intramolecular forces. On the other hand, direct dissolution of enkephalins in solvents less polar than Me_2SO-d_6 is not possible, at least for concentrations compatible with the signal to noise ratio of NMR spectrometers. Besides, charged peptides have a strong tendency, in apolar media, to form large aggregates with little or no biological significance.

Since we are interested in the conformation that the peptide can assume inside the receptor, it is necessary to devise an "organized" solvent system that can mimic the minimum requirements of the receptor. We have found a simple way of doing this and of circumventing solubility problems via complexation of the NH_3^+ group of the peptides with 18-crown-6-ether (Beretta et al., 1984; Temussi et al., 1984), a coronand that is able to solubilize potassium or ammonium salts in apolar solvents. This complexation in our view constitutes an effective simulation of the physicochemical environment of the active site of the receptor, with the crown ether acting as the anionic subsite (together with the counterion), whereas the apolar solvent provides the hydrophobic environment of the pocket. Figure 1 shows a schematic comparison of the hypothetical environment of the peptide in the active site of the receptor and of the environment in the organized medium.

We have studied the crown ether complexes of Met^5-enkephalin amide (MEA), Leu^5-enkephalin amide (LEA), D-Ala^2,Leu^5-enkephalin amide (DEA), (AcO)Tyr^1,Met^5-enkephalin amide (AMEA), and (AcO)Tyr^1,D-Ala^2,Met^5-enkephalin amide (ADEA) in $CDCl_3$ by means of 500-MHz ^{1}H NMR spectroscopy.

The amide form of these agonists does not lower their opioid activity and provides, at the same time, a cationic species that can interact with the crown ether only via the charged N-terminal. The three analogues of the natural enkephalins (i.e., DEA, AMEA, and ADEA) were chosen to investigate the conformational effect induced by small constitutional changes in a situation comparable to that of the receptor site. Besides, acetylation of the Tyr^1 OH bears a direct relationship with many theoretical investigations since it is often involved in intramolecular hydrogen bonding in energy calculations in vacuo. However, it is generally believed that this group is important in the binding process and thus it cannot possibly be involved in intramolecular bonds. Accordingly, an analogue whose OH cannot bind intramolecularly may be even more useful to study the conformational state of the typical opioid agonist inside the receptor. The results were compared in all cases with the corresponding data for the same peptides in Me_2SO-d_6.

Experimental Procedures

Materials. Leu^5-enkephalin amide acetate salt (LEA), Met^5-enkephalin amide acetate salt (MEA), and D-Ala^2,Leu^5-enkephalin amide acetate salt (DEA) were purchased from Sigma Chemical Co., St. Louis, MO. (AcO)-Tyr^1,D-Ala^2,Met^5-enkephalin amide trifluoroacetate salt (ADEA) and (AcO)Tyr^1,Met^5-enkephalin amide trifluoroacetate salt (AMEA) were generous gifts of Professor Severo Salvadori, University of Ferrara, who also checked their pharmacological profile with standard in vitro tests (GPI and MVD). Both peptides, as expected, had μ and δ activities of the order of 20% of the corresponding unacetylated enkephalin amides.

All peptides were of sufficient purity for our spectroscopic studies, as revealed by their NMR spectra.

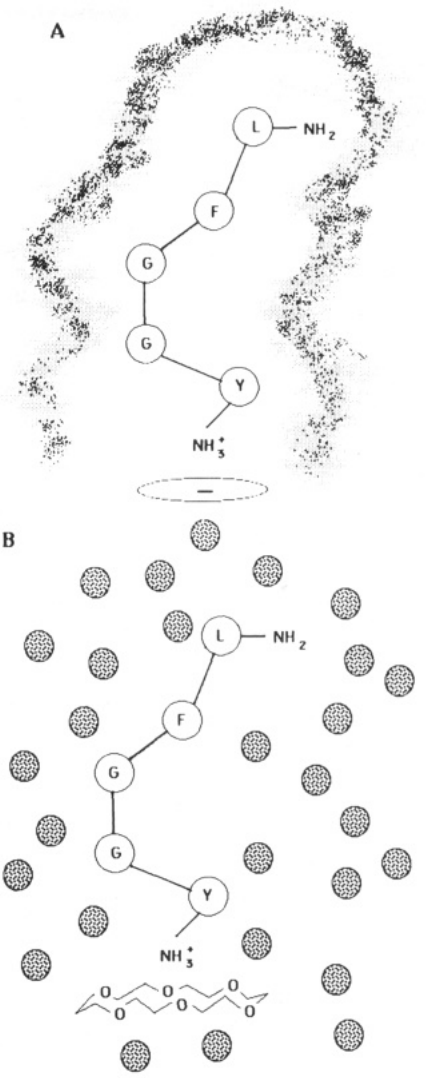

FIGURE 1: Schematic comparison between the likely environments experienced by an opioid peptide in vivo and in our experimental conditions. (A) LEA in the active site of the receptor; (B) LEA in an organized medium such as that provided by the crown ether complex in $CDCl_3$. The solvent molecules are represented as small balls.

18-Crown-6-ether and deuteriated solvents (100% $CDCl_3$, 99.8% Me_2SO-d_6) were purchased from C. Erba, Milan, Italy.

Methods. $CDCl_3$ was treated with activated molecular sieves to decrease the water impurity. The crown ether was distilled (160 °C at 0.1–0.2 Torr) in glassware previously washed with an EDTA solution (0.1 M) to eliminate possible traces of heavy metals that can interfere in NOE measurements and lyophilized before use. The complexes were prepared by dissolving equimolar amounts of the peptide and of the crown ether, lyophilizing to eliminate traces of water, and redissolving the complex (in a drybox) in 0.4 mL of $CDCl_3$ to yield (1–5) $\times$ 10^{-3} M solutions.

NMR. All spectra were recorded on a Bruker WM-500 spectrometer. 1D spectra were typically acquired in quadrature with a sweep width of 5500 Hz, a flip angle of 45°, and a memory block size of 16K, with zero filling for processing.

For measuring the DQ spectrum, we have used the pulse sequence (Mareci et al., 1983)

$$t_0\text{–}90\text{–}t\text{–}180\text{–}t\text{–}90\text{–}t_1\text{–}\alpha\text{-FID}(t_2) + t_0\text{–}90\text{–}t\text{–}180\text{–}t\text{–}45\text{–}90\text{–}t_1\text{-}\alpha\text{-FID}(t_2)$$

where the detection pulse α was chosen as 135°, $t = 1/8J$, and $t_0 = 1.5$ s. A 32-step phase cycling was used to select spe-

cifically DQ coherences. A total of 32 transients was collected for each t_1 increment by using a sweep width of 6024 Hz in the ω_2 dimension. A time domain data matrix of size 2048 × 512 was used, and the t_1 dimension was zero-filled once to improve resolution. Sine-bell windows were applied in both dimensions before the Fourier transformation was performed. The transmitter was placed in the center of the spectrum.

The COSY spectra were acquired with the original Jeener pulse sequence (Jeener, 1971; Aue et al., 1976). Appropriate phase cycling was applied to select N-type peaks. Data block sizes were 2048 addresses in t_2 and 256 or 512 equidistant t_1 values zero-filled to 1K. Before Fourier transformation, both time domains were multiplied by a sine-bell function. *J*-resolved spectra (Nagayama et al., 1981) were acquired with the following spectral parameters: t_0 = 2 s, sweep width in f_1 = 23 Hz and in f_2 = 5000 Hz, 64 t_1 values, each with 192 transients, and 8K in t_2. Data in each time domain were multiplied by a sine-bell function and then zero-filled at 128 addresses in t_1 before Fourier transformation. Quadrature detection in both dimensions was used for all the 2D experiments.

NOE experiments were recorded at 270 and 500 MHz by using standard sequences of the Bruker software package. 1D NOE experiments were performed at several different temperatures from 233 to 303 K in the difference mode. The decoupler power was the minimum needed to saturate the spins of interest. 2D experiments were performed with several mixing times at 297 K.

Results

Assignments. Assignments for Me_2SO-d_6 solutions were made by means of simple double-resonance 1D experiments, using the literature data for natural enkephalins as a guideline (Higashijima et al., 1979). Standard 2D experiments (*J*-resolved, COSY) confirmed the 1D results. The sequential assignment of the resonances of Gly^2 and Gly^3 was based mainly on a comparison with literature data (Jones et al., 1976; Higashijima et al., 1979) that show the resonance of the NH of Gly^3 consistently at high field with respect to that of Gly^2. The method of choice in this case would be the measurement of NOE's between NH's and adjacent CH's, but the enhancements observed for our peptides were too small to be reliable, an observation that is consistent with the results of a detailed study on D-Ala^2,Leu^5-enkephalin (Niccolai et al., 1980).

The spectra of the crown ether complexes in $CDCl_3$ bear no resemblance with those in Me_2SO-d_6 or in D_2O, and thus the knowledge of chemical shifts either from the literature or from our own experiments in Me_2SO-d_6 could furnish no guideline for assignments.

Figure 2 shows a comparison of the 500-MHz spectra of MEA in Me_2SO-d_6 and of its complex with 18-crown-6-ether in $CDCl_3$. Although the aliphatic regions are similar, the chemical shifts of the amide protons are dramatically different. The resonance of the methylene groups of the crown ether obscures a small part of the α-CH region, but this circumstance did not prevent a full assignment of all resonances.

2D methods proved a necessity in the case of the complexes both because of accidental superpositions and because of the unusual chemical shifts of the amide protons. Figure 3 shows a DQ-COSY spectrum of the complex of LEA in $CDCl_3$. It is possible to see that also the resonances partly obscured by the large peak of the crown ether can be correlated to the peaks of their residue. Systematic application of several 2D techniques (*J*-resolved, COSY, and less conventional techniques like the mentioned DQ-COSY) led to full assignments for all

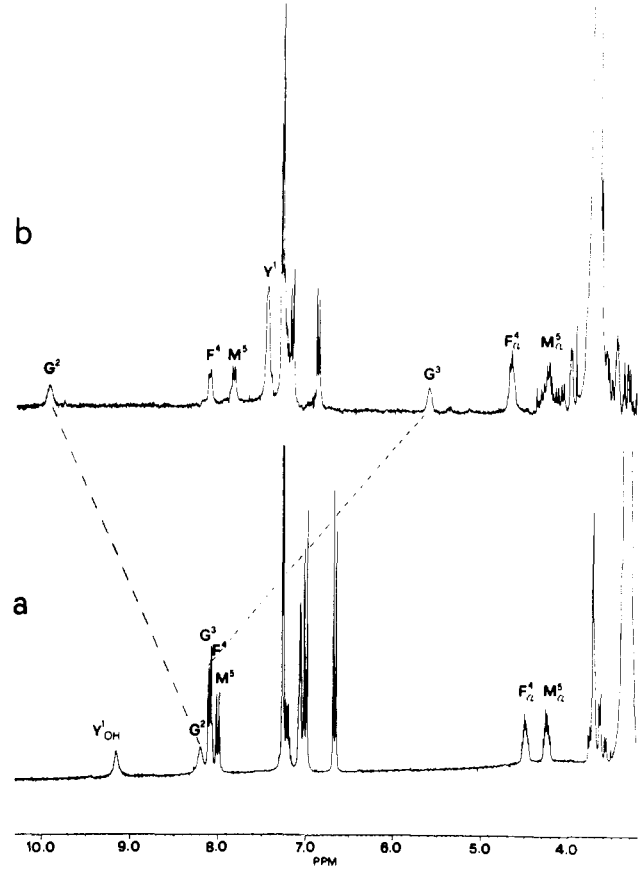

FIGURE 2: Comparison of the 500-MHz ^1H NMR spectra (region from 10.3 to 3.2 ppm) of MEA in Me_2SO-d_6 (a) and in $CDCl_3$ (b) measured at 300 K. NH resonances are indicated with the amino acid one-letter code. The dashed lines connect the resonance positions of the NH's of G^2 and G^3 in the two different solvent systems.

complexes. The sequential assignment of the resonances of Gly^2 and Gly^3 was based on a comparison of the NH chemical shifts of LEA, MEA, and AMEA with those of DEA and ADEA. It was assumed that the extremely low-field values of the NH's of D-Ala^2 are typical of the second position, and thus the corresponding low shifts of one of the Gly NH's are those of Gly^2.

Tables I and II summarize all chemical shifts, in $CDCl_3$ and in Me_2SO-d_6, for all peptides. The chemical shifts of most nonexchangeable protons are comparable to those of enkephalins reported in the literature, both for Me_2SO-d_6 and for $CDCl_3$ solutions. The chemical shifts of the α-CH groups of Tyr^1 and $CDCl_3$ differ considerably from the corresponding shifts in Me_2SO-d_6. The changes can be ascribed to the complexation of the adjacent NH_3^+ groups with the crown ether that stabilizes the ionic pair between the NH_3^+ and its counterion. The net effect is an increase of positive charge that accounts for a downfield shift similar to that observed upon acidification of water solutions for the resonances of terminal α-CH's. It is worth mentioning that these changes are paralleled by an upfield shift of the resonances of the NH_3^+ groups themselves. These resonances are often too broad to be observed in Me_2SO-d_6 but are easily identified in the $CDCl_3$ solutions of the complexes.

The chemical shift difference between the prochiral protons of the methylene groups of the glycines is greatly enhanced by complexation, at least for LEA and MEA. This parameter has often been used as an indicator of the "rigidity" of the conformation (Nagaraj & Balaram, 1978).

Although it is not fully warranted to deduct conformational rigidity from a single chemical shift parameter, it is fair to

Table I: Chemical Shifts (ppm, Referred to Internal TMS) of the Crown Ether Complexes of Enkephalin Amide in $CDCl_3$

	LEA	MEA	DEA	AMEA	ADEA
Tyr[1]					
NH_3^+	7.29	7.36	7.37	7.37	7.27
α	3.99	4.15	4.36	4.51	4.29
β_1	3.02	3.07	3.17	3.19	3.26
β_2	2.73	2.74	2.81	3.13	2.82
Ar	6.88	7.04	7.06	7.13	7.16
Ar	6.69	6.77	6.84	6.95	6.99
Gly[2]/Ala[2]					
NH	9.72	9.95	9.25	10.09	9.71
α_1	3.90	4.15	4.30	3.70	4.14
α_2	3.43	3.46		3.59	
β			0.93		0.64
Gly[3]					
NH	6.09	5.61	5.54	6.83	8.58
α_1	3.90	3.95	3.95	3.50	3.69
α_2	3.31	3.36		3.45	3.56
Phe[4]					
NH	8.05	8.03	7.97	7.22	6.87
α	4.43	4.52	4.64	4.59	4.57
β_1	3.11	3.27	3.25	3.12	3.12
β_2	3.02	3.17	3.20	2.89	3.04
Ar	7.17	7.20	7.27	7.22	7.23
Ar	7.15	7.19		7.21	7.22
Ar				7.17	
Leu[5]/Met[5]					
NH	7.43	7.75	7.45	7.51	7.27
α_1	4.35	4.46	4.46	4.47	4.52
β_1	1.50	2.16	1.50	2.18	1.87
β_2	1.56	2.06		2.06	
γ_1		2.49		2.43	2.27
γ_2		2.40		2.36	
δ_1	0.80		0.83		
δ_2	0.78		0.80		

Table II: Chemical Shifts (ppm, Referred to Internal TMS) of Enkephalin Amides in Me_2SO-d_6

	LEA	MEA	DEA	AMEA	ADEA
Tyr[1]					
NH_3^+	8.06			8.06	
α	3.99	3.92	3.36	4.06	3.97
β_1	3.00	2.81	2.82	3.11	2.99
β_2	2.82	2.77	2.48	2.92	2.91
Ar	7.05	7.28	6.98	7.30	7.27
Ar	6.70	6.69	6.65	7.09	7.09
Gly[2]/Ala[2]					
NH	8.72	8.24	8.10	8.77	8.54
α_1	3.84		4.20	3.86	4.31
α_2	3.75			3.76	
β			1.14		1.05
Gly[3]					
NH	8.12	8.13	8.23	8.19	8.26
α_1	3.75		3.72	3.76	3.70
α_2	3.65		3.57	3.66	3.61
Phe[4]					
NH	8.08	8.15	8.03	8.16	8.07
α	4.53	4.51	4.47	4.52	4.51
β_1	3.03	3.08	3.04	3.04	3.03
β_2	2.78	2.99	2.83	2.78	2.78
Ar	7.25	7.23	7.26	7.26	7.24
Ar	7.24	7.17			7.19
Ar	7.18				
Leu[5]/Met[5]					
NH	8.03	8.05	7.92	8.14	8.17
α	4.20	4.27	4.18	4.24	4.24
β_1	1.46	1.96	1.57	1.93	1.93
β_2	1.56	1.85	1.48	1.78	1.78
γ	2.50	2.44	2.50	2.55	2.40
δ_1	0.88		0.88		
δ_2	0.83		0.83		

say that very large chemical shift differences between prochiral protons monitor the existence of a marked local dissymmetry that is not consistent with extensive conformational averaging. That is, the differences observed for the methylenes of Gly[2]

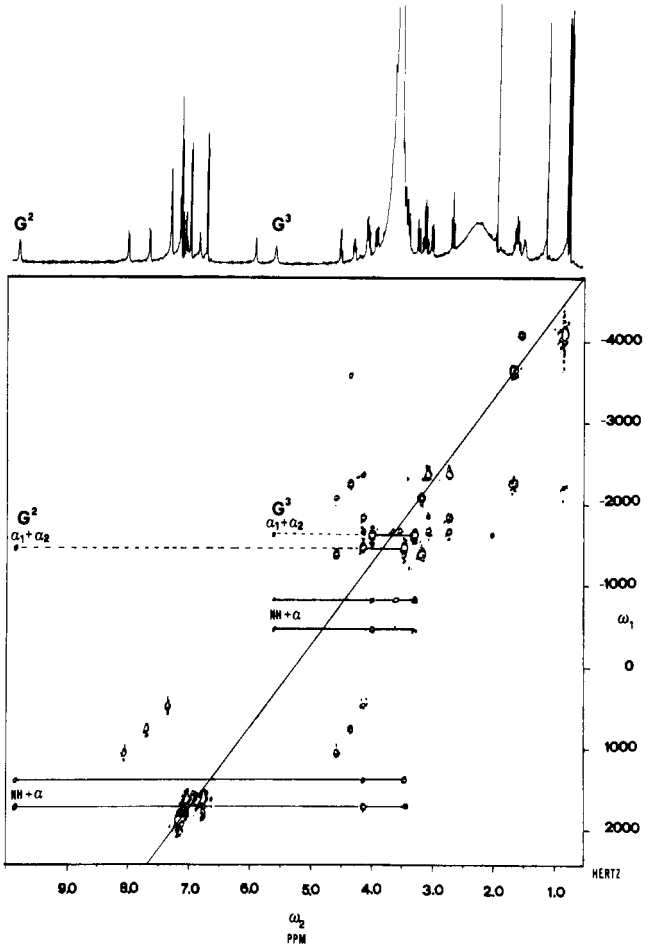

FIGURE 3: 500-MHz DQ spectrum of the LEA complex in $CDCl_3$ obtained with the delay τ = 16 ms ($1/8J$). Data are presented as absolute value contour plot. The solid line indicates the DQ diagonal ($\omega_1 = 2\omega_2$). Direct connectivities (—), at $\omega_2 = \omega_{NH}$ and $\omega_1 = \omega_{\alpha 1}$ or ω_α, and remote connectivities (---), at $\omega_2 = \omega_{NH}$ and $\omega_1 = \omega_{\alpha 1} + \omega_{\alpha 2}$, are identified for the spin systems of G^2 and G^3. On the top is shown a 1D resolution enhanced spectrum where the NH's of G^2 and G^3 are indicated. Chemical shifts are relative to the crown ether resonance arbitrarily located at 3.69 ppm.

and Gly[3] are a good indication that these residues are part of a well-defined conformation in the complexes of LEA and MEA. The chemical shifts of greatest diagnostic value for conformational analysis are, however, those of the amide protons. Deviations from average literature values for linear peptides can point to the presence of hydrogen bonds, to shielding from the solvent, or to recognizable conformational features; very low field values in particular are usually regarded as typical of protons involved in strong hydrogen bonds. It is easy to see from Table II that all peptides, in Me_2SO-d_6, show a narrow spread of chemical shifts, with values close to literature values, corresponding to the nature of the residue and to the sequence in which it is embedded. Such a behavior is typical of the presence in solution of solvated molecules in a "disordered structure" or rather in the form of a complicated mixture of essentially extended conformations in which all NH's are uniformly bound to solvent molecules (Pastore et al., 1984).

On the contrary, all complexes in $CDCl_3$ have NH chemical shifts ranging over several parts per million, with some very low field values that are generally regarded as typical of hydrogen-bonded NH's and some very high field values that are probably due to unusual conformational features.

Such a behavior is never observed in solutions of linear peptides, in polar solvents, but is typical of some cyclic peptides

Table III: Temperature Coefficients (ppb/K) of the α-CH Protons for the Enkephalin Complexes in $CDCl_3$

	LEA	MEA	DEA	AMEA	ADEA
Tyr[1]	0.83	0.64	3.87	0.21	1.57
		1.32		0.00	
Gly[2]/D-Ala[2]	0.60		1.62		0.86
		0.15		−1.29	
	1.40	0.24		0.00	−1.14
Gly[3]			3.10		
	1.41	−1.44		0.00	−1.71
Phe[4]	1.29	0.28	0.89	−0.21	−0.64
Leu[5]/Met[5]	0.30	0.21	0.55	−0.07	−0.79

in which the conformational constraints force the molecules to adopt a well-defined structure.

Temperature Studies. All systems were studied in the temperature range 290–330 K. The changes observed in the chemical shifts of nonexchangeable protons are generally rather small, as expected from the behavior in Me_2SO-d_6 (Higashijima et al., 1979), but there are a few outstanding exceptions, notably for the complex of DEA. Table III reports the temperature coefficients of the α-CH chemical shifts for the complexes in $CDCl_3$. The large coefficient of the α-CH of Tyr[1] of DEA may indicate a partial instability of the complex, whereas other nonzero coefficients may be the result of conformational transitions. These data can be used to evaluate the significance of the temperature coefficients of the NH's.

Once again the most valuable information comes from the amide protons, whose temperature coefficients have often been used to determine the accessibility to the protons of solvent molecules and/or the presence of hydrogen bonds (Kopple et al., 1969; Ohnishi & Urry, 1969). It has been shown that if an amide proton is hydrogen bonded and remains such throughout the whole temperature range examined, its chemical shift will stay approximately constant. Accordingly, very small temperature coefficients are usually taken as a strong indication that the proton is bound, in any solvent. If, however, the increase in temperature promotes a breakdown of the hydrogen bond, there are different behaviors in different solvents, depending mainly on the hydrogen-bonding ability of the solvent itself. In fact, solvents like water or dimethyl sulfoxide that are good hydrogen-bond acceptors will have a strong tendency to replace the amide carbonyl in a competitive fashion as the hydrogen bond breaks down. As a consequence, the amide proton may experience a wide range of chemical environments, not easily predictable since they are a complicated function of solvation and conformation.

In particular it is even possible for an NH to change from a weak hydrogen bond (although intramolecular) to a stronger hydrogen bond (intermolecular, with solvent molecules), in part because of the favorable concentration ratio of solvent to solute molecules.

In a relatively apolar solvent, on the other hand, it can be assumed that the amide protons that become free from an intramolecular hydrogen bond will invariably change from a bound to a nonbound state, since the interaction with the solvent is generally weak. In fact, the only solvent for which the relationship between temperature coefficients and hydrogen bonds has been established with fair accuracy is $CDCl_3$.

Stevens et al. (1980), on the basis of a careful work on model compounds, have shown that amide protons freely exposed to this solvent have chemical shift temperature coefficients of the order of −2.4 (±0.5) ppb K^{-1}; coefficients with absolute values smaller than 2.4 can be associated with hydrogens that remain bound throughout the temperature range examined, and coefficients with absolute values larger than 2.4 can be associated with hydrogens that are bound at the lower temperatures examined but become free as the temperature is raised. It is understood that these considerations are valid if and only if the temperature dependence of the chemical shift is linear and with a negative coefficient, i.e., if the chemical change monitored by the chemical shift can be described by a simple two-state equilibrium

$$-N-H\cdots OC- \;=\; -N-H \text{ (solvated)} + \; -CO$$

in which the solvated state of the NH consistently leads to a lower value of the chemical shift (i.e., to high field). Nonlinear trends and/or positive temperature coefficients may reveal the superposition of conformational equilibria that change the chemical environment of the amide proton in a manner different from the breakdown of the hydrogen bond.

Table IV: Temperature Coefficients (ppb/K) of the NH Protons of the Enkephalins in Me_2SO-d_6

	Gly/D-Ala	Gly	Phe	Met/Leu
LEA	−5.6	−5.5	−5.5	−7.0
MEA	−3.9	−4.6	−4.9	−5.6
DEA	−5.3	−5.6	−4.6	−4.7
AMEA	−6.5	−6.5	−7.1	−7.5
ADEA	−6.0	−7.1	−7.7	−7.5

Table V: Temperature Coefficients (ppb/K) of the NH Protons of the Enkephalin Complexes in $CDCl_3$

	Tyr	Gly/D-Ala	Gly	Phe	Met/Leu
LEA	−0.2	−0.7	nl[a]	−11.2	−2.8
MEA	−0.5	+1.5	nl[a]	−7.2	−4.0[b]
DEA	0.0	+0.3	−5.8	+7.9	−2.3
AMEA	−0.5	−3.0	+4.7	+1.6	−1.3
ADEA		−2.3	+0.9	−2.3	−0.9

[a] Nonlinear. [b] Sensitive to dilution.

The preceding considerations were of decisive importance in the choice of $CDCl_3$ as the apolar medium in our simulated receptor site environment. Table IV summarizes the temperature coefficients of the amide protons in Me_2SO-d_6. All values are fairly large and close to an average value of −5.9 ppb K^{-1}. As already observed for enkephalins (Higashijima et al., 1978) and dermorphins (Pastore et al., 1984), these values can be interpreted as an indication that all NH's are exposed to the solvent in approximately the same way, the main effect of the temperature increase being the destabilization of intermolecular hydrogen bonds with solvent molecules.

Accordingly, the conformational state of the peptides in Me_2SO-d_6 is probably a mixture of several extended conformations whose precise nature is difficult to establish but that are only distantly related to the "biologically active conformation". The small differences among the temperature coefficients of the NH's of different residues in the peptides examined can be ascribed to slightly different accessibilities. In principle, it might be possible to gain some conformational information from this modulation of the coefficients, at least on the different constraints imposed by the different constitutions, but the biological information is limited by the fact that the conformational state is dominated by the strong solvation by Me_2SO-d_6 molecules, a situation akin to that found in one crystal of Leu-enkephalin (Karle, 1984) where the main forces stabilizing the crystal lattice are the intermolecular hydrogen bonds of extended β-pleated sheets.

Table V summarizes the temperature coefficients of the amide protons and of the NH_3^+ groups of the crown ether complexes in $CDCl_3$. The main difference with respect to the data of Table IV is that each complex is now characterized by at least one intramolecular hydrogen bond. A preliminary

investigation (Beretta et al., 1984) on the complexes of LEA and MEA showed that at concentrations higher than 3.0×10^{-3} M moderate aggregation phenomena are present, as evidenced by the fact that some NH chemical shifts were sensitive to dilution. Care was taken to run all spectra with solutions of low enough concentration to avoid this problem, typically concentrations smaller or equal to 1.0×10^{-3} M.

Another feature common to all complexes is a very low temperature coefficient for the NH_3^+ group. This finding is consistent with the stability of the complex between the NH_3^+ and the crown ether in the whole temperature range examined.

The presence, in our systems, of positive coefficients or of nonlinear temperature dependences indicates that even in $CDCl_3$ the interpretation of temperture coefficients may not be as straightforward as for the simple model compounds studied by Stevens et al. (1980), at least whenever the peptide can undergo conformational transitions in the temperature range examined. We assumed that large positive coefficients are mainly due to conformational transitions that reorient the relative positions of bonds with large anisotropy of the magnetic susceptibility (such as the C=O bonds). It cannot be excluded, however, that even small positive coefficients may be the result of the compensation of a negative trend, due to the breakdown of a hydrogen bond, and a large positive variation. In dubious cases it is advisable to have independent indications on the existence of the hydrogen bond. A good internal check is provided by the values of the NH chemical shift themselves, at least in cases of very high (i.e., downfield) values.

Other independent measurements that have often been used are NOE enhancements, which can give information on the whole peptide conformation, and the influence of relaxation reagents or of cosolvents that can compete for hydrogen bonds.

The use of cosolvents or of relaxation agents is not generally accepted since they can cause additional conformational changes. In our systems, in fact, addition of polar solvents invariably leads to destabilization of the complexes, and also the possible conformational changes induced by relaxation reagents are not desirable since we are already dealing with a rather elaborate model system. NOE enhancements, on the other hand, were very low (essentially intraresidue, only sufficient to confirm some assignments) either because of residual flexibility of the folded conformations or, more likely, because of the unfavorable correlation times of molecules of this size at high field. Thus, we had to resort to chemical shift indications, and indeed some of the NH chemical shifts are so extreme that they can be used with some confidence as indicators of hydrogen bond formation. In particular, the NH chemical shifts of the second residue (Gly^2 or D-Ala^2) in all complexes have outstanding low-field values. They are shifted by more than 1 ppm with respect to the corresponding values in Me_2SO-d_6, in which they were already hydrogen bonded to solvent molecules. These values thus support the existence of strong hydrogen bonds even in the cases of AMEA and ADEA, for which the temperature coefficients alone would point to exposed NH's.

Another NH whose chemical shift is downfield with respect to the value in Me_2SO-d_6 is that of Gly^3 of ADEA; however, neither the absolute value of the chemical shift (8.69 ppm) nor the value of the temperature coefficients (+0.86 ppb K^{-1}) is decisive in this case. Thus, it is safer to assume, as a first approximation, that only the NH of Met^5 is hydrogen bonded in this peptide.

Conformations. The lack of long-range NOE's prevents detailed structure determinations. It is possible, however, to propose likely cyclic structures on the basis of the knowledge that some NH's are hydrogen bonded, with the aid of simple topological and energetic considerations.

Table VI: Experimental Coupling Constants $J_{NHC\alpha H}$[a] (Hz) Measured for the Peptides in $CDCl_3$

	Gly^2[b]/D-Ala	Gly^3[b]	Phe^4	Met^5/Leu^5
LEA	9.2	7.7	8.0	10.4
MEA	10.7	*c*	6.4	8.0
DEA	9.5	*c*	*c*	8.6
AMEA	13.8	9.2	8.0	8.0
ADEA	9.5	8.4	6.6	6.6

[a] Values are corrected for the electronegativity effect (Bystrov, 1976). [b] The J's of the Gly residues are the sum of J_{AX} and J_{BX}. [c] Not measurable because the peak is too broad.

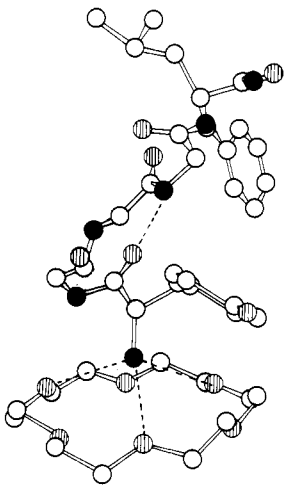

FIGURE 4: Schematic model of the complex of LEA with 18-crown-6-ether. The C_{10} ring of the peptide is arbitrarily depicted as a type I' β-turn. Filled and open circles indicate nitrogen and carbon atoms, respectively; oxygens are indicated by circles with vertical bars. Dashed lines represent hydrogen bonds.

Let us first examine the two "natural" enkephalin amides MEA and LEA. Both are characterized by two intramolecular hydrogen-bonded NH's: that of Gly^2 and that of Phe^4. If one excludes crossings, only two types of rings are possible: a C_{10} defined by the bond between the NH of Phe^4 and the CO group of Tyr^1 and a C_{11} defined by the bond between the NH of Gly^2 and the CO of Phe^4. In either case an inner (i.e., without crossing) hydrogen bond is possible, in principle, to yield a C_8 (between Gly^2 NH and Gly^3 CO) and a C_7 (between Phe^4 NH and Gly^2 CO), respectively. A simple inspection of molecular models, however, shows that C_{10} and C_8 or C_{11} and C_7 are mutually exclusive on the basis of internal energy. This observation does not contradict the experimental finding of the involvement of both Phe^4 and Gly^2 NH's in intramolecular hydrogen bonds since one of these can well be linked *intramolecularly* with one of the oxygens of the crown ether. We favor involvement of the Gly^2 NH in this bond, with the concomitant involvement of Phe^4 NH in a $1 \leftarrow 4$ β-turn with the carbonyl of Tyr^1, because of the well-known stability of C_{10} rings with respect to C_{11} rings and owing to the similarity of the temperature coefficients of the NH of Gly^2 and of the NH_3^+ of Tyr^1 that might indicate a contribution of the Gly^2 hydrogen bond to the stability of the complex.

The values of the J_{NHCH} coupling constants shown in Table VI are consistent with the family of conformations corresponding to a C_{10} ring (Bystrov, 1976). Thus, it is possible to propose a C_{10} conformation, although it is difficult to favor a given β-turn. Figure 4 shows a schematic model for the complex of LEA; the C_{10} ring is arbitrarily depicted as a type

I' β-turn, the conformation assumed by Leu-enkephalin in the solid state (Smith & Griffin, 1978; Schiller, 1984).

Another crystal structure (Karle et al., 1983) showed extended conformations linked by intermolecular hydrogen bonds in antiparallel β-pleated sheets. The forces that stabilize the two lattices can be likened to the environments of our solution study. In the crystal structure of Smith and Griffin (1978) the molecules are held essentially by van der Waals forces that do not pose severe constraints on the conformation of the independent unit, like the chloroform molecules that solvate the complexes; in the crystal structure of Karle et al. (1983), on the other hand, the intermolecular hydrogen bonds are essential to the stability of the crystal and force the enkephalin molecules to adopt fully extended conformations, in a way similar to the hydrogen bonds formed by solvating Me_2SO-d_6 molecules to favor extended conformations in solution.

Similar considerations would lead to the proposal for DEA of a C_7 conformation involving Gly^3 NH and Tyr^1 CO if the NH of D-Ala^2 is linked to one of the oxygens of the crown ether. However, the very high field value (5.54 ppm) of the chemical shift of Gly^3 NH is in conflict with the value of its temperature coefficient (−5.8 ppb K^{-1}). It is likely that this amide proton may be simply shielded by the complexation of the adjacent Ala^2 NH with the crown ether.

The conformations of the two acetylated peptides are more difficult to ascertain because, in both complexes, the NH's that can be reliably considered as hydrogen bonded are those of the last residue. The systematic change of Gly^3 and Phe^4 NH resonances with respect to the corresponding ones of MEA and DEA indicates that the (average) hydrogen-bonding state of these protons is affected by acetylation. The topological constraints are thus minimal, and it is possible to link the NH to any of the CO groups of the first three residues, although energy considerations favor once again the second residue and the corresponding 2 ← 5 β-turn. It is interesting to note that the existence of different β-turns, with respect to those of LEA and MEA, is also hinted by the spectral parameters of the Gly residues. The chemical shift differences between the prochiral protons that were as large as 0.7 ppm for LEA and MEA are now of the order of 0.1 ppm.

Conclusions

All NMR data indicate that in Me_2SO-d_6 the peptides are in a random conformation, while in $CDCl_3$ they form well-defined (ordered) folded structures. The ring size and its location along the sequence vary with the constitution of the peptide, with sharp differences for seemingly minor constitutional modifications. In particular, it is surprising to find different conformations for MEA and AMEA that differ only for the acetylation of the Tyr OH. This experimental finding can be relevant with respect to the fact that nearly all models of opiate receptors foresee a direct involvement of this OH in binding.

Modifications in the first two residues are apparently very effective in influencing the global conformation: this behavior is probably linked to the fact that the atoms of the first two residues, which are closer to the crown ether, are not free to occupy all conformational space otherwise accessible to an isolated molecule. Thus the conformational variation observed may be viewed, in part, as an artifact imposed by complexation, but it is also indicative of the importance of induced fit in the interaction of the peptides with the surface of the receptor. It can be easily surmised that the surface surrounding the anionic binding site in the receptor is far more "specialized" than that provided by the crown ether molecule. It is possible that even the differences in activity among the peptides examined may be linked, in part, to this kind of conformational effect.

The most significant result of our study, however, is that it proves for the first time that enkephalin amides can adopt a folded conformation in solution, provided the medium is made close enough to the environment inside the receptor. Previous evidence in favor of the existence of folded conformations in enkephalin solutions reflected only the tendency of a fraction of *zwitterionic* enkephalins to exist in a bent form, due to the contribution of the electrostatic (head to tail) interaction, whereas the absence of this interaction preserves full conformational flexibility to the corresponding amides.

The conformation we propose for the complexes of LEA and MEA is the 1 ← 4 turn, similar to that found in the solid state for Leu-enkephalin by Smith and Griffin [see also Schiller (1984)]. AMEA and ADEA adopt instead a conformation that is compatible with the other category of turns often proposed for enkephalins: the 2 ← 5 (Rose et al., 1985).

The conformational versatility shown by enkephalin amides is consistent with their ability to interact with at least two opioid receptors (δ and μ). It is significant that the small constitutional changes responsible for the observed conformational changes could never account for a change in specificity based on membrane catalysis (Schwyzer, 1986b) since they do not vary the net charge on the peptide.

All experimental findings presented in this paper lend strong support to the belief that folded conformations play a major role in the interaction of opioid peptides with their receptors (Pastore et al., 1985; Castiglione-Morelli et al., 1987).

Registry No. Leu^5-enkephalin amide, 60117-24-0; Met^5-enkephalin amide, 60117-17-1; D-Ala^2,Leu^5-enkephalin amide, 65189-64-2; (AcO)Tyr^1,Met^5-enkephalin amide, 110773-32-5; (AcO)Tyr^1,D-Ala^2,Met^5-enkephalin amide, 110773-33-6.

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