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LOW TEMPERATURE NMR STUDIES OF LEU-ENKEPHALINS IN CRYOPROTECTIVE SOLVENTS.

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Abstract - Leu-enkephalin (LE) and its amide (LEA) were studied by means of ^2H NMR spectroscopy, at low temperatures, in one of the so-called cryoprotective mixtures (water/DMSO in particular), i.e. mixtures that resemble water in many properties and that reach high values of the viscosity. The temperature coefficients of amide protons were measured in an unusually wide temperature range (+54 to -30 °C). All values for both LE and LEA are larger than those expected for protons involved in hydrogen bonds. Owing to the increased viscosity of the medium, it was possible, for the first time, to detect several intra- and inter-residue nuclear Overhauser effects. This implies that the structure of these peptides is not completely random, but a detailed analysis of both temperature coefficients and NOESY spectra does not support the existence of a single folded conformation either for LE and LEA. On the other hand, it proved possible to detect, for LE, a fraction of a conformer characterized by a short Gly^2 NH - Gly^3 NH distance. This finding is consistent with a type I (or type I') β-turn but not with type II β-turns.

Introduction

The structure-activity relationship of endogenous opioid peptides is dominated by conformational effects, owing to the flexibility of linear peptides. Structural studies in the solid state are of limited usefulness since the conformation of the asymmetric unit is greatly influenced by lattice forces. Solution studies can be more useful, particularly those based on highly regiospecific techniques such as NMR spectroscopy. In principle, this technique allows one to monitor intrinsic conformational tendencies as well as the influences of the environment. There are however two fundamental difficulties. In solution, a flexible molecule is seldom characterized by a single (or a few stable) conformation(s) of minimum

energy but rather by a complex mixture of quasi isoenergetic conformations averaged in the NMR time-scale. Secondly, the environment in which the biological interaction takes place influences the conformational equilibrium, but it is very difficult to reproduce even some features of this environment in solution studies[1].

Although the structure of opioid receptors is not known from direct studies, all models, based essentially on the properties of rigid nonpeptide agonists[2-6], point to a highly hydrophobic pocket containing a specific anionic subsite (that interacts with the positive nitrogen present in all opioids). Accordingly, on the basis of energy calculations, many authors have proposed, for the biologically active conformations of enkephalins, folded conformations in which all hydrophobic side-chains are exposed[7-14].

The problem of simulating the physico-chemical environment inside the receptor has been addressed specifically in our laboratory[1]. We have shown that complexation of the $-NH_3^+$ group of enkephalin amides with a crown ether can be likened to the binding of the same group to the anionic subsite of the receptor, whereas a relatively apolar solvent, like $CDCl_3$, can play the role of the hydrophobic cavity. These experimental conditions favor folded conformations of the family of β-turns[1,6].

It is equally important to study the conformational state of these peptides in the transport fluid and at the interface between water and cellular membrane. It has been pointed out[15-20] that the state of linear peptide hormones in water is probably random but that, even before a peptide binds to a protein receptor, the change from the bulk of the aqueous environment to the water-membrane interface, and finally to the apolar environment of the membrane lipids, can induce a transition from more or less disordered "random coils" to fairly ordered folded conformations. Indeed most NMR studies on opioid peptides have been performed in very polar media typical of the conditions of the transport fluids[21-29], but there is no general agreement on the conformation adopted in these media. For instance, in spite of many claims [27,29-31] in favor of the presence of folded structures in solutions of enkephalins, Higashijima et al.[25] have demonstrated that Met^5-enkephalin amide assumes, in $DMSO_{d6}$, 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.

The uncertainty on the solution conformation of these molecules is linked in part to the intrinsic difficulty of extracting structural data

from the complicated averages measured by NMR parameters[32], but also to the fact that in these studies the structural determination is often based only on the temperature coefficients of the chemical shifts of amide protons. The interpretation of these coefficients in terms of hydrogen bonds is fairly straightforward when they are close to zero, but it is very difficult in all other cases, at least in polar solvents[33]. Besides, they are usually measured in temperature ranges of only 30 °C, owing to the high melting temperature of DMSO (18 °C) and to the danger of damaging delicate peptides at temperatures higher than 60 °C.

The best way to overcome these difficulties would be to detect long-range nuclear Overhauser effects (NOE). Cyclic peptides[31-34-36] and small proteins[37-40] give strong NOEs that can be used as powerful constraints in molecular mechanics and molecular dynamics simulations of the conformation. On the contrary, in medium-sized linear peptides NOEs are often very small or quite undetectable. This behavior can be attributed to a combination of several causes; the two most important ones are the conformational flexibility and the unfavorable values of the correlation times τ_c. At the high fields necessary to fully analyze their ^1H spectra, these peptides may have values of the product $\omega\tau_c$ close to unity. This condition, in turn, corresponds to a minimum of spin-lattice relaxation time and to NOEs close to zero[41]. It is possible, in principle, to approximate the extreme narrowing condition ($\omega^2 \tau_c^2 \ll 1$) by using spectrometers that operate at lower fields, but for complex spectra like those of many peptides with biological activity, this solution is not practical because of the loss of resolution. Another way of affecting $\omega\tau_c$ is to vary the viscosity via a temperature change since, according to the microviscosity theory[42], the correlation time is directly proportional to the viscosity of the solvent. However, the changes of viscosity in the temperature range useful for NMR measurements of bioactive peptides are quite small for the pure solvents usually employed in these studies, i.e. water and DMSO. Owing to the possibility of lenghtening τ_c by increasing the viscosity some unusual solvents have been employed, like glacial acetic acid[43] or phosphoric acid[44] and, for peptides in particular, sulfolane instead of DMSO[45]. However, all the quoted solvents provide environments with properties drastically different from those of biological systems.

A satisfactory answer to many of the problems outlined above can be furnished by the use of the so-called cryoprotective mixtures. Douzou and Petsko[46] have shown that mixtures of water and alcohols, water and dimethylformamide or water and DMSO, at low temperatures, have properties close to those of water at room temperature. These mixtures have in fact been

used to investigate several enzyme-catalyzed reactions at low temperatures[46] and, more recently, to study the carbon-13 NMR relaxation of [1-^{13}C]acetyl-chymotrypsin[47]. By using such mixtures it is possible to reach unusually low temperatures, for a polar environment, that may possibly favor certain families of conformations. Furthermore, the lowering of temperature increases dramatically the viscosity[48] thus making the NOEs observable also in medium-sized peptides[49]. Finally, in these conditions (low temperature and high viscosity), it may be assumed that water is partly ordered, i.e. in a state resembling that at the interface[50] between the membrane and the transport fluid, at which the peptide molecules start to assume some preferential conformations.

We have studied Leu^5-enkephalin, Tyr-Gly-Gly-Phe-Leu (LE), and its amide (LEA) in a cryoprotective mixture by means of 500 MHz ^{1}H NMR spectroscopy with the aim of measuring temperature coefficients in a temperature range much wider than in all previous studies and of detecting structurally diagnostic NOEs.

RESULTS

The room temperature spectra of LE and LEA in a 80:10:10 $DMSO_{d6}$:^1H_2O:^2H_2O (v/v) mixture resemble the literature spectra of the same compounds and of related opioid peptides in pure polar solvents, a circumstance that greatly facilitates the identification of the resonances.

TABLE I

Chemical shifts (ppm, referred to internal TSP) of Leu-enkephalin and Leu-enkephalin amide in $DMSO_{d6}$: ^1H_2O: ^2H_2O 80:10:10 mixture at 297 K.

Residue		Leu-enkephalin	Leu-enkephalin-NH_2
	α	3.90	3.90
	β_1	3.00	2.90
Tyr^1	β_2	2.90	2.80
	$Ar_{2,6}$	7.07	7.04
	$Ar_{3,5}$	6.74	6.71
	NH	8.76	8.13
Gly^2	α_1	3.83	3.80
	α_2	3.70	3.70
	NH	8.09	≈7.2
Gly^3	α_1	3.70	3.70
	α_2	3.64	3.60
	NH	8.00	8.11
	α	4.50	4.50
Phe^4	β_1	3.00	3.10
	β_2	2.80	2.80
	Ar	7.27	7.27
	NH	8.47	8.05
	α	4.50	4.50
	β_1	1.59	1.53
Leu^5	β2	1.55	1.47
	γ	2.78	2.69
	δ_1	0.91	0.88
	δ_2	0.84	0.82

Most assignments were performed by means of 2D COSY and NOESY experiments. In fact, to the best of our knowledge, our sequential assignment of Gly^2 and Gly^3 resonances was performed for the first time from NOESY experiments (vide infra) without any recourse to analogs with specific substitutions.

Table I summarizes all relevant chemical shift information. Most chemical shifts differ only slightly from the corresponding ones in water or DMSO[6,21-30].

Figure 1 and figure 2 show the temperature dependence of the chemical shifts of the amide protons for LE and LEA respectively between 243 and 327 K. It can be appreciated that the temperature range is much wider (84°) than those usually found in the literature (ca. 30°) up to now. This is a consequence of the use of a cryoprotective mixture that allows the measurement of good spectra at unusually low temperatures, at least for very polar solvents. All Leu-enkephalin NH protons share a linear behavior (see Figure 1), with fairly large temperature coefficients. This behavior is closer to that observed in water rather than in DMSO, both for Leu-enkephalin[24] and Met-enkephalin[25]. In fact, a detailed study of the latter peptide as a function of both solvent and concentration has shown that the temperature coefficient of Met^5 NH, which is large and negative in H_2O, becomes smaller in DMSO at concentrations between 6 and 25 mM, indicating a partial shielding from the solvent.

TABLE II
Temperature coefficients of the NH resonances of Leu-enkephalin and Leu-enkephalin-NH_2 (10^3 $d\delta/dT$)

Residue	Leu-enkephalin	Leu-enkephalin-NH_2
Gly^2	-5.5	-4.9
Gly^3	-4.9	/
Phe^4	-5.8	-4.8
Leu^5	-7.1	-7.1

The data of Table II show that none of the amide protons of LE and LEA in the cryoprotective mixture is effectively shielded from the solvent and/or involved in a stable intramolecular hydrogen bonding. The only possible exception is the G^3 NH of LEA, but it is not possible to draw reliable conclusions from the three observable points at high temperatures (Figure 2). At all other temperatures this resonance was hopelessly obscured by other peaks.

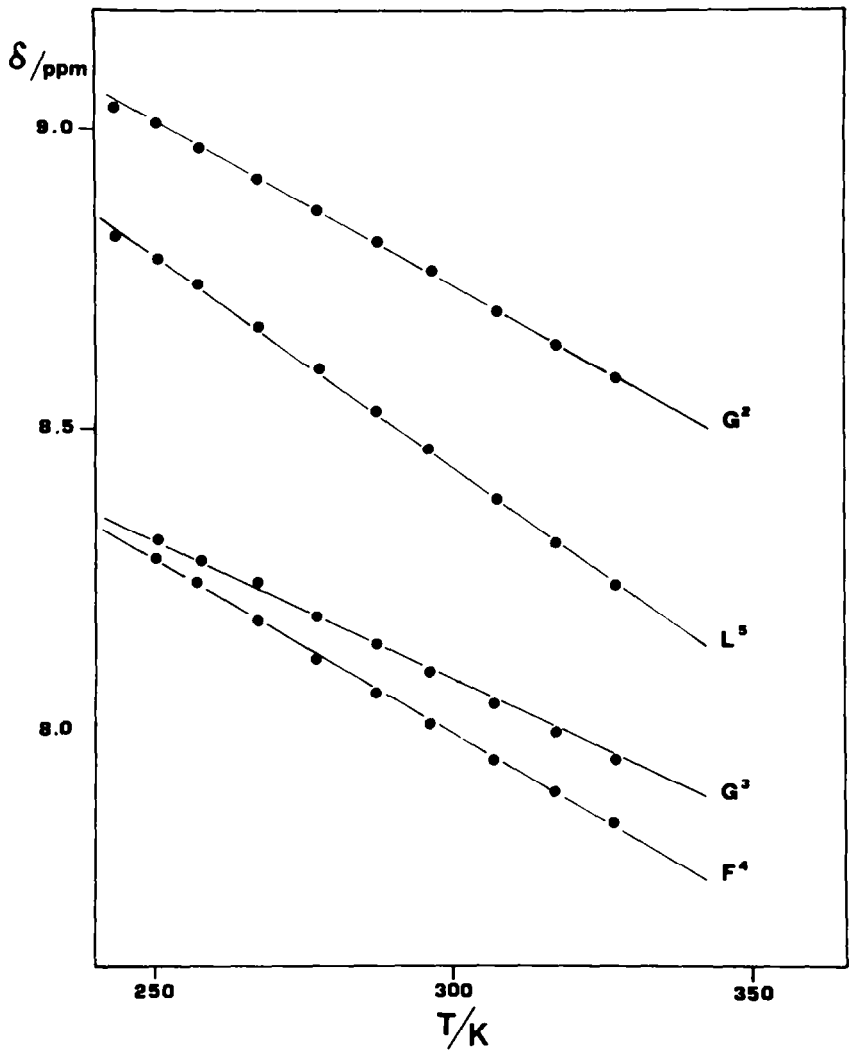

Figure 1. Temperature dependence of the chemical shifts of the amide protons of Leu-enkephalin (LE) in a 80:10:10 $DMSO_{ds}$:^2H_2O:^1H_2O mixture. The peptide concentration was 8 mM. The chemical shifts are in ppm, referred to internal TSP. The lines are labeled with the one-letter code of the corresponding residue.

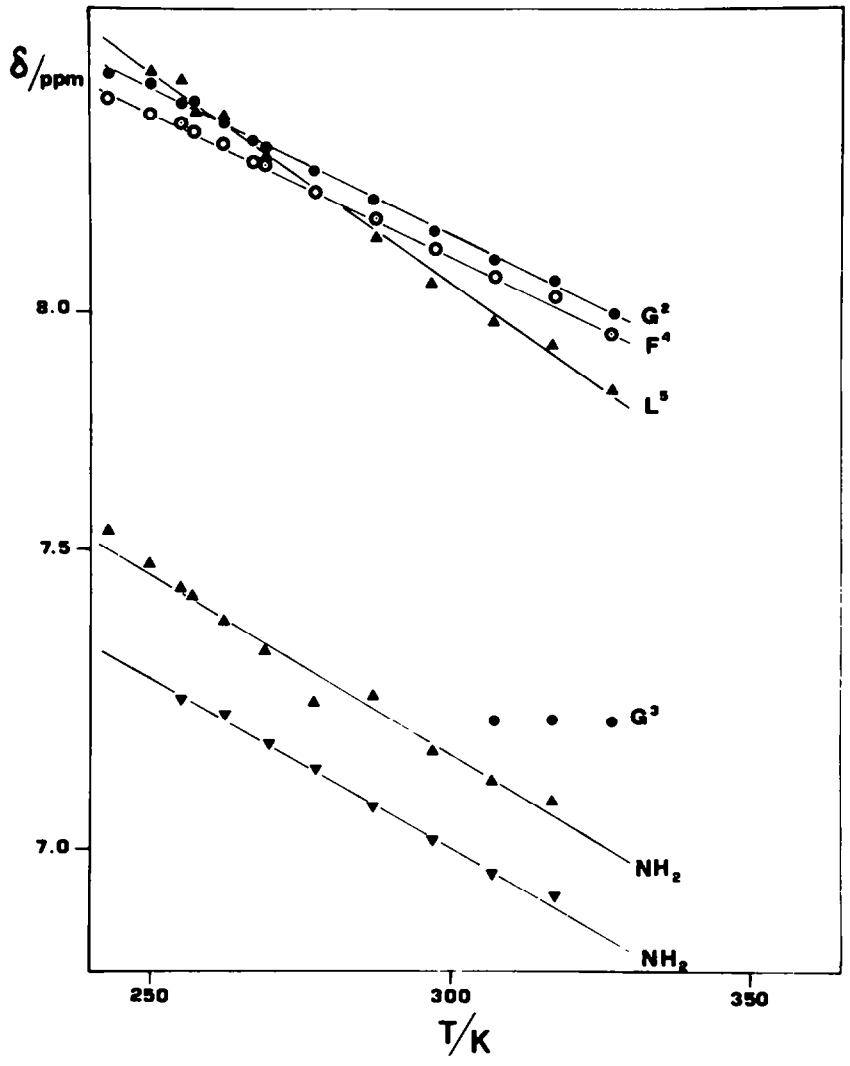

Figure 2. Temperature dependence of the chemical shifts of the amide protons of Leu-enkephalin amide (LEA) in a 80:10:10 $DMSO_{d6}$:^2H_2O:^1H_2O mixture. The peptide concentration was 8 mM. The chemical shifts are in ppm, referred to internal TSP. The lines are labeled with the one-letter code of the corresponding residue. The two lines corresponding to the two protons of the terminal amide are both labeled NH_2.

It is appropriate to recall at this point that since the chemical shifts are simple linear expansions of the contribution of individual conformers, it may be very difficult to detect small populations of folded conformers from chemical shifts or from their temperature coefficients. Besides, in a polar solvent, the change from the "bound" (i.e. intramolecularly hydrogen bonded) to the "free" state (i.e. hydrogen bonded to solvent molecules) may imply only a small chemical shift change. Thus it is imperative to be able to measure NOEs since their strongly non linear dependence on interatomic distance can be suited to detect even small populations of well defined conformers containing short proton-proton distances.

Figure 3 shows the low field portion of a phase sensitive NOESY spectrum of LE in the mentioned mixture at 277 K, with a mixing time of 500 ms. As it is well known, in such spectra cross peaks indicate dipolar coupling, and thus spatial proximity between two protons. Intrestingly, our exprimental conditions favor the observation of many negative effects. In particular, sequential NOE connectivities from the NH resonances (labeled along the diagonal in the lower part of Figure 3) to C_α H's are of importance since they are diagnostic for sequence specific assignments. LE contains two Gly residues at sites 2 and 3, whose NH's resonate at 8.19 and 8.76 ppm. The lowest field resonance shows, among others, a cross peak with C_α H of Tyr^1 (labeled $Y^1{}_\alpha$) at 3.96 ppm, while no such peak is observed for the Gly NH at 8.19 ppm. This unambigously ascribes the NH at 8.76 ppm to Gly^2 (G^2) and, consequently, the one at 8.19 ppm to Gly^3 (G^3). The other NH's also give rise to cross peaks with C_α H of the preceding residue ($L^3{}_{NH}$ with $P^4{}_\alpha$; $P^4{}_{NH}$ with $G^3{}_\alpha$; $G^3{}_{NH}$ and $G^2{}_\alpha$), as well as some intraresidue effects, all marked in Figure 3. Furthermore, the cross-peak (circled in Figure 3) that links the NH's of the two Gly's, is the only clue for the identification of a well defined conformational feature of the molecule (*vide infra*). Along with these effects due to the increased stiffness of the backbone, brought about by the increased viscosity of the medium, equally important are those relating backbone and side-chains. This type of NOEs can give information about the orientation of the side-chains. For

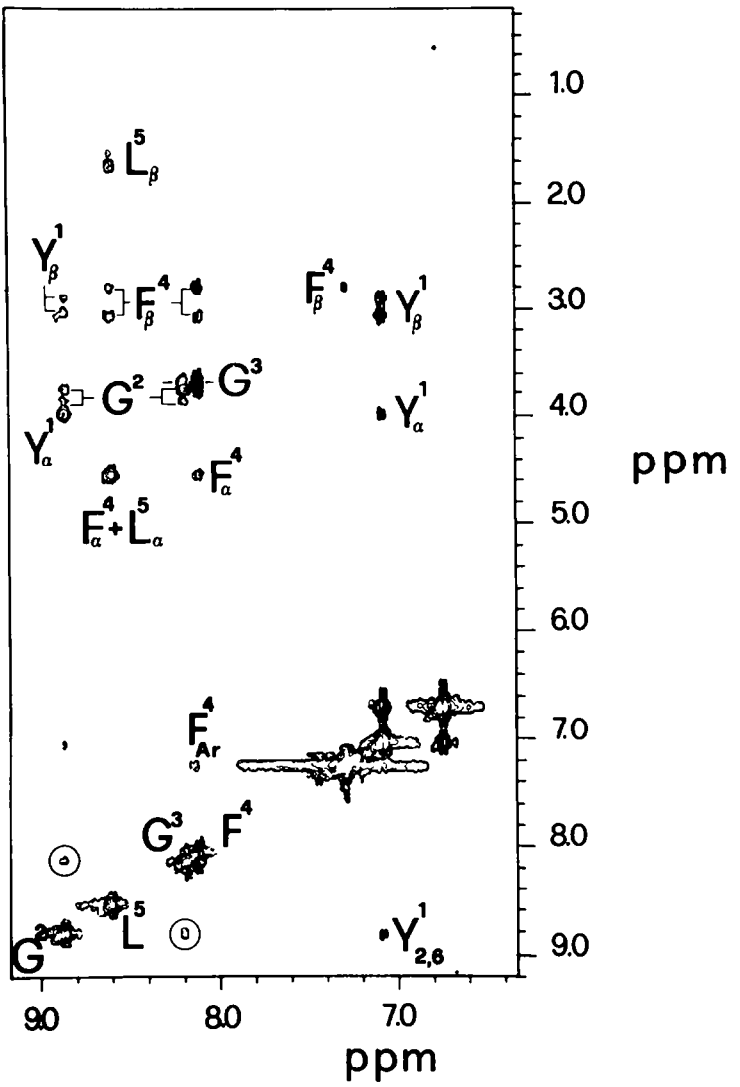

Figure 3. Low field region of a phase sensitive NOESY spectrum of 8 mM Leu-enkephalin in a 80:10:10 $DMSO_{as}$:^2H_2O:^1H_2O mixture at 277 K with a mixing time of 500 ms. The shown spectral region (ω_1 = 0.2 to 9.2 ppm, ω_2 = 6.4 to 9.2 ppm) contains cross peaks which manifest NOEs from amide and aromatic protons. All the effects are labeled using the one-letter code for the residues. The circled cross-peak connecting the Gly^2 and Gly^3 NH's is of diagnostic value in discriminating within type I and type II β-turns.

example, the presence of cross-peaks between the Gly^2 NH and the $H_{2,6}$ and the $C_\beta H_2$ protons of Tyr^1 (labeled $Y^1_{2,6}$ and Y^1_β, respectively) indicates that the amide proton feels the presence of the aromatic ring, so that its spectral appearence at 8.76 ppm could be due, in part, to the deshielding effect of the ring[31]. On the other hand, the absence of effects relating the ring protons of Phe^4 with both Gly^3 and/or Leu^5 indicates that the ring does not interact with the backbone protons.

No long range effects have been detected that could support the proposed head to tail electrostatic interaction[28] for the zwitterionic enkephalin. It is likely that this interaction is not sufficient to impose very short distances between hydrogens of the first and last residues, although it favors all folded conformations.

More difficult is the interpretation of the corresponding data for LEA. Figure 4 shows the aromatic and amide region of the LEA NOESY spectrum with 500 ms mixing time. As it can be seen in the lower left corner, the NH's of Gly^2, Phe^4 and Leu^5 (G^2, F^4 and L^5, respectively) all resonate at 8.19 ppm, while the Gly^3 NH is buried under the signals of Phe^4 aromatics at 7.22 ppm. In these conditions, specific assignments of the effects are precluded, but a qualitative interpretation of the data is possible if we assume that no big differences exist in solution between the two studied enkephalins.

Except for the effect between the Gly^2 NH and the $C_\beta H_2$ of Tyr^1, present in LE (Figure 3) but absent in LEA (Figure 4), all others are observed and labeled in Figure 4. Some ambiguities could be removed by inspecting the 2.8 - 4.0 ppm region of LEA (Figure 5). For example, the position of the Gly^3 NH is identified by the cross peak at 7.22 ppm (G^3_{NH}),which is aligned with those linking the $C_\beta H_2$ and the ring protons of Phe^4 (F^4_{Ar}). The $C_\alpha H$ of Tyr^1 is here better recognized by the well resolved pair of cross peaks with the $C_\beta H_2$ at 3.91 ppm (Y^1_α), while it is very small in Figure 4 (Y^1_α crosspeak) being bleached out by water irradiation. A comparison with the corresponding region of LE (not shown) helped in the identification of the cross peaks (Figure 5).

It seems fair to conclude that the dramatic increase of the Overhauser

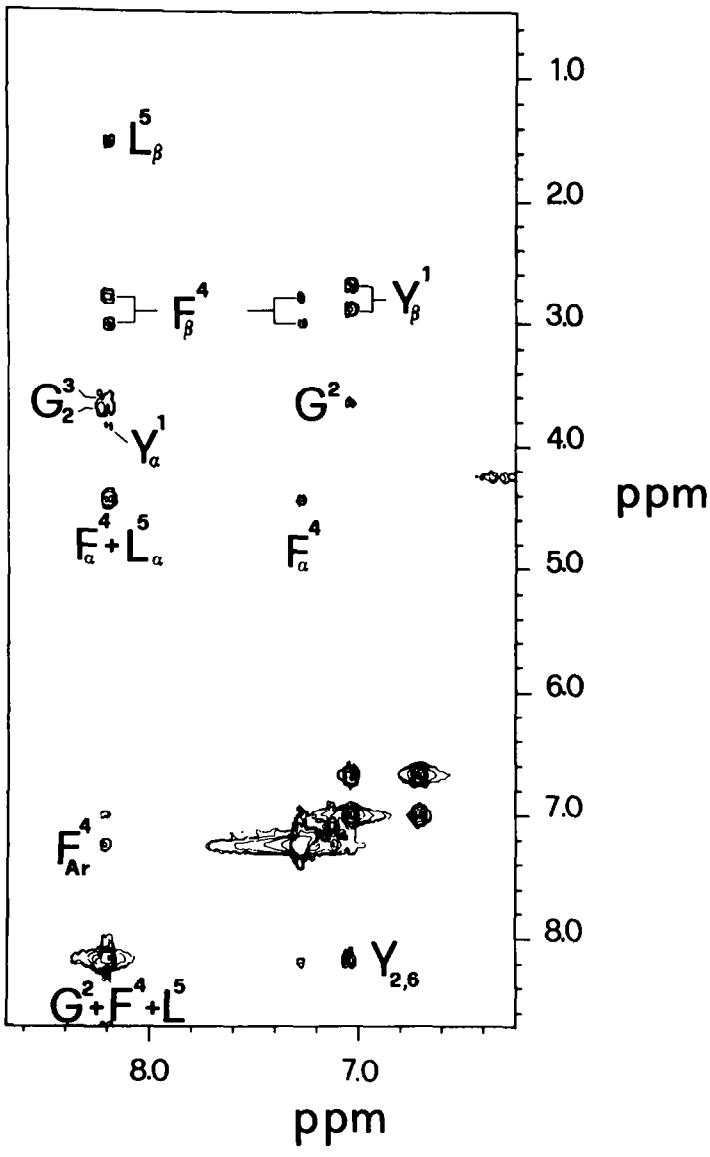

Figure 4. Aromatic and amide region of the LEA phase sensitive NOESY spectrum with 500 ms mixing time. Experimental conditions as those of Figure 3.

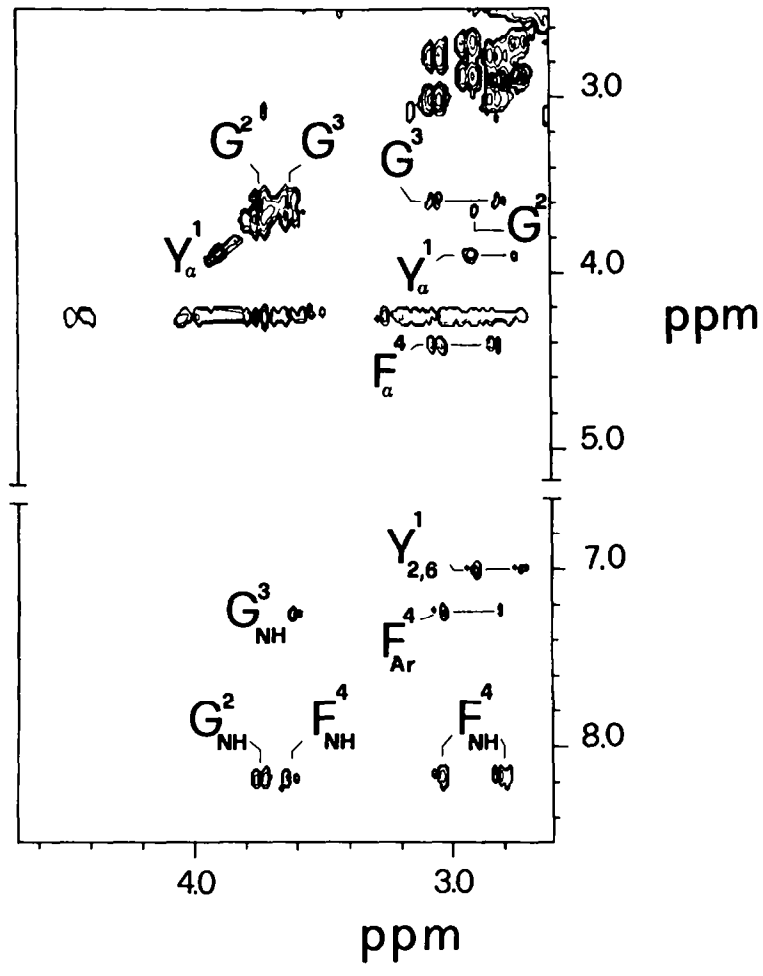

Figure 5. —Phase sensitive NOESY spectrum of LEA showing cross peaks from α and β protons. All conditions are the same as those described in Figure 3.

effects in the cryoprotective solutions of enkephalins can not be attibuted solely to the increase of rotational τ_c, brought about by the increase of viscosity. We believe that the change in the "structure" of the solvent reflected by the high viscosity favors a narrower conformer distribution. Figures 3 - 5 show that our experimental conditions favor both intra- and inter-residue NOEs; this implies that the structure of the two enkephalins is not completely random. In fact, the very detection of strong intraresidue effects points to a collection of conformers with similar backbone constraints since a truly random distribution of main chain conformations would induce a parallel random distribution of side-chain conformers.

An NOE with great diagnostic potential is that between the NH's of Gly^2 and Gly^3. It points to a local folded form and, if we limit our analysis to β-turns, it can be used to discriminate among various types. For turns of type I, the shortest distances are that between NH^2 and NH^3 and that between NH^3 and NH^4, which are of the order of < 2.5 Å [52]. Also diagnostic for turns of type I are the effects between β-proton(s) and NH of following residues, whose distances can be as low as 2.9 Å [52]. For turns of type II the shortest distances are those between $C_\alpha H^i$ and NH^{i+1} (of the order of 2.2 Å) and that between NH^3 and NH^4 (2.4 Å). The distinction between the two types relies on the presence (type I) or absence (type II) of the effect between NH^2 and NH^3, since in the type II the distance between these protons increases to 4.5 Å.

The presence of an effect between the NH's of Gly^2 and Gly^3 discriminates between the two types, indicating that LE in our solvent system contains a conformer with either a type I or a type I' β-turn.

The absence of effects for larger interatomic distances indicates that their (smaller) NOEs are nullified by the small value of the population. However, it is interesting to point out that this conformer is similar to the folded conformer we found for the crown ether complex of LEA in chloroform[6]. It is conceivable that the small population of the β-turn that shows up when moving from bulk water to the partially ordered water of the cryoprotective mixture may increase dramatically when LE enters the lipid phase of the membrane to approach the receptor. This view is consistent with the proposal of a catalytic role of the membrane[15-20] but it also hints that the "seed" of the bioactive conformation can exist in the transport fluid already.

EXPERIMENTAL

[Leu^5]-enkephalin was a gift from prof.Fred Naider (The City University

of New York, St.George Campus), and the corresponding amide was purchased from Sigma (St.Louis Mo, USA). Both were used without further purification. ^2H_2O (99.9 % atom ^2H), $DMSO_{d6}$ (99.8 % atom ^2H) and sodium 3trimethysylyl-propionate-2,2,3,3_{d4}, TSP (98 % atom ^2H) were purchased from C.Erba (Milano, Italy).

1-D NMR spectra were recorded at 500 MHz in the Fourier mode, with quadrature detection on a Bruker WM-500 spectrometer. Peptide solutions were 8 mM in 80:10:10 $DMSO_{d6}$: ^1H_2O: ^2H_2O (v/v). For the variable temperature experiments the range spun was from 243 to 327 K. Chemical shifts are reported as δ's in ppm from internal TSP. The water signal was suppressed by a low power selective irradiation in the homogated mode.

2-D NOESY experiments[53,54] were run in the phase-sensitive mode using quadrature detection in ω_1 by time proportional phase incrementation of the initial pulse[55]. Data block sizes were 2048 addresses in t_2 and 512 equidistant t_1 values, adding 80 transients for each t_1 value, with a relaxation period of 1.5 s between them, and a spectral width of 6000 Hz, yielding a resolution of 5.6 Hz/point. Before Fourier transformation, the time domain data matrix was multiplied by a Lorentz Gauss function in both dimensions.

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