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Transferred Nuclear Overhauser Effect Analyses of Membrane-Bound Enkephalin Analogues by ^{1}H Nuclear Magnetic Resonance: Correlation between Activities and Membrane-Bound Conformations†

Alain Milon,‡ Tatsuo Miyazawa,§ and Tsutomu Higashijima*
Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan
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ABSTRACT: Leu-enkephalin, [D-Ala^2]Leu-enkephalin, and [D-Ala^2]Leu-enkephalinamide (agonists) and [L-Ala^2]Leu-enkephalin (inactive analogue) bind to lipid bilayer consisting of phosphatidylcholine and phosphatidylserine. The conformations that these compounds assume, once bound to perdeuterated phospholipid bilayer, have been shown to be unique, as shown by the transferred nuclear Overhauser effect (TRNOE) of ^{1}H NMR spectroscopy. In addition, their location in the bilayer was analyzed by TRNOE in the presence of spin-labeled phospholipids. These analyses showed a clear relationship between the activity and the peptide–membrane interaction. The three active peptides, when bound to membranes, adopt the same conformation, characterized by a type II′ β-turn around Gly^3-Phe^4 and a γ-turn around Gly^2 (or D-Ala^2). The inactive analogue, [L-Ala^2]Leu-enkephalin, displayed a completely different TRNOE pattern corresponding to a different conformation in the membrane-bound state. The tyrosine residue of the active compounds is not inserted into the interior of membrane, but it is inserted into the bilayer for the L-Ala^2 analogue. According to these results, [L-Ala^2]Leu-enkephalin may be explained to be inactive because the mode of binding to the membranes is different from that of active compounds.

Enkephalins are peptides with morphine-like activity that have a primary structure Tyr-Gly-Gly-Phe-Leu (Leu-enkephalin) or Tyr-Gly-Gly-Phe-Met (Met-enkephalin) (Hughes et al., 1975). Many studies have been performed to determine their conformation–activity relationships. At first, enkephalins were assumed to take a conformation analogous to that of morphine, which has a rigid structure, because they bind to the morphine receptor (μ receptor) (Horn & Rodgers, 1976). At present, though biochemical characteristics of enkephalin receptor(s) have not yet been clarified, at least three subtypes of receptors (μ, δ, and κ) have been reported (Paterson et al., 1983). Leu-enkephalin binds to the δ receptor with the highest affinity of those three subtypes, but it also binds to the μ receptor with high affinity (Paterson et al., 1983; Kosterlitz & Paterson, 1985). These receptors have been postulated to couple with one of the inhibitory GTP-binding regulatory proteins (G_i proteins)[1] and to lower the concentration of cyclic

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* Address correspondence to this author at the Department of Pharmacology, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75235.
‡ Present address: Laboratoire de Chimie Organique des Substances Naturelles, associé au CNRS, Centre de Neurochimie, 67084 Strasbourg, France.
§ Present address: Faculty of Engineering, Yokohama National University, Hodogaya-ku, Yokohama 240, Japan.

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AMP (Klee & Nirenberg, 1976). Receptors that interact with G proteins are homologous with each other, especially in the putative seven membrane-spanning regions [see Lefkowitz and Caron (1988) and Dohlman et al. (1987) for review]. The ligand binding segment of the β-adrenergic receptor, which couples with G_s, has been identified to be in those membrane-spanning regions (Wong et al., 1988). In addition, the β-adrenergic affinity label [*N*-(bromoacetyl)amino]-cyanopindolol is reported to block the antagonist binding and to inhibit the activation of adenylate cyclase by a β-adrenergic agonist, while the labeling occurred at a nonprotein component (probably lipid) tightly associated with the β-adrenergic receptor (Chorev et al., 1985). These observations support the involvement of phospholipid membranes for their ligand binding.

Recently, many hormones or neurotransmitters, whose receptors are coupled with G proteins, have been shown to interact with phospholipid membranes. These include opioid peptides (Gysin & Schwyzer, 1983), substance P (Wu et al., 1982), glucagon (Brown et al., 1981; Wu et al., 1982), luteinizing hormone-releasing hormone (Wakamatsu et al., 1986a), and α-mating factor of yeast *Saccharomyces cerevisiae* (Higashijima et al., 1983; Wakamatsu et al., 1986b). In addition, the biological activities of these peptides have been found to be related to their affinities for phospholipid membranes and to their conformations in the membrane-bound state (Higashijima et al., 1983; Sargent & Schwyzer, 1986; Wakamatsu et al., 1987), rather than their conformations in aqueous solution, where such linear oligopeptides are not in any specific conformations. Therefore, more recent conformational studies of those oligopeptides, including enkephalins, have focused on the conformations in "membrane-mimetic" environments. However, conformational analyses of peptides as bound to phospholipid membranes are much more difficult than those for free peptides in solution. The incorporation of peptides into membranes causes drastic broadening of their NMR signals, which makes it difficult to analyze the NMR spectra with high resolution. Most ^{1}H NMR studies for peptide–membrane interaction are performed by using micelles instead of bilayer in order to avoid such drastic broadening (Brown et al., 1981; Deber & Behnam, 1985; Zetta et al., 1986). The molecular mass of dodecylphosphocholine micelles, for example, is less than 20 kDa, while that of small unilamellar vesicles of egg yolk phosphatidylcholine is larger than 2000 kDa (Huang et al., 1969). However, the packing of phospholipids in micelles is remarkably different from bilayer. Indeed, the conformation in the micelle-bound state of melittin has been found to be significantly different from that of the bilayer-bound state (Wakamatsu et al., 1986a). No doubt, bilayer membranes consisting of neutral and anionic phospholipids are more accurate models of cell membranes than are micelles.

We have demonstrated recently that the method of transferred nuclear Overhauser effects (TRNOE) with perdeuterated phospholipid vesicles is a very powerful method for determining the conformation of membrane-bound peptides. By use of this technique, membrane-bound conformations for α-mating factor (Wakamatsu et al., 1986b, 1987), melittin (Wakamatsu et al., 1986a), and mastoparan (Wakamatsu et al., 1983, 1986a) were determined in D_2O solution. However, experiments in D_2O prevent the observations of NH proton signals, which are sometimes essential for the analyses of main-chain conformation such as a β-turn. We present herein an expansion of the method for active and inactive enkephalin analogues in H_2O solution as well as D_2O solution.

In the present study, we report the membrane-bound conformations of the active enkephalin analogues Leu-enkephalin, [D-Ala^2]Leu-enkephalin, and [D-Ala^2]Leu-enkephalinamide and the inactive [L-Ala^2]Leu-enkephalin. These peptides were chosen for the following reasons. (1) If the second residue (Gly^2) is replaced by a D-amino acid, the activity is preserved or enhanced (Morley, 1980), but if it is replaced by an L-amino acid, there is a complete loss of activity, almost independent of the amino acid used. This strongly suggests a conformational explanation, although in aqueous solution no specific conformations are formed by enkephalins (Higashijima et al., 1979). It is interesting to compare the conformations of [D-Ala^2]Leu-enkephalin and [L-Ala^2]Leu-enkephalin with that of natural [Gly^2]Leu-enkephalin in order to see the effect of this second residue on the membrane-bound conformation. (2) The role of the charged C-terminal group for the formation of folded conformations is of interest because folded conformations for dipolar forms of enkephalin have been found in DMSO-d_6 solution (Roques et al., 1976; Jones et al., 1976; Stimson et al., 1979; Higashijima et al., 1979). Several groups have proposed that the main driving force for the folded conformation is the hydrogen bond between the Met^5(or Leu^5) NH and the Gly^2 CO groups, although no direct evidence to support a β-turn conformation, such as NOE data, was reported. We have suggested that the major driving force for the folded conformation of enkephalin is the electrostatic interaction between the charged N- and C-terminal groups. In fact, enkephalinamide and nondipolar forms (cationic and anionic forms) of enkephalin show no evidence for the formation of folded conformations, even in DMSO-d_6 solution (Stimson et al., 1979; Higashijima et al., 1979). Since enkephalinamide analogues are known to be biologically active, it is interesting to compare the conformation of enkephalinamide with those of other active or inactive enkephalin analogues in the membrane-bound state.

We report here that active enkephalin analogues take a common folded conformation when bound to membranes, while inactive analogue showed a clearly different interaction with membranes.

Experimental Procedures

Materials

Peptides. Leu-enkephalin and [D-Ala^2]Leu-enkephalinamide were purchased from Peptide Institute (Osaka, Japan) and Bachem Chemical (Budendorf, Switzerland), respectively. [D-Ala^2]- and [L-Ala^2]Leu-enkephalin were synthesized by the solid-phase method as described previously (Munekata et al., 1987), using Boc-amino acids and Boc-Tyr(*O*-benzyl) (Peptide Institute). The crude material obtained after HF reaction was purified by gel filtration over a Sephadex G-10 column (Pharmacia Chemicals Co., Uppsala, Sweden), and by preparative HPLC on a C-18 column (YMC-Pack S-343, 2.0 × 25 cm), eluting isocratically with 0.1% trifluoroacetic acid–20% acetonitrile in H_2O at a flow rate of 10 mL/min. The purity

[1] Abbreviations: NMR, nuclear magnetic resonance; TRNOE, transferred nuclear Overhauser effect; G proteins, guanine nucleotide binding regulatory proteins; G_s, G protein that stimulates adenylate cyclase; G_i, G protein that inhibits adenylate cyclase; HPLC, high-performance liquid chromatography; Boc, butyloxycarbonyl; DPPC, L-α-dipalmitoylphosphatidylcholine; DLPS, dilauroylphosphatidylserine; 12-SLPC, 1-acyl-2-(12-doxylstearoyl)glycerophosphocholine; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; TPPI, time-proportional phase increment; TRNOESY, two-dimensional TRNOE spectroscopy; *h*, signal at higher field; *l*, signal at lower field; $d_{AB}(i,j)$, NOE between proton A of *i*th residue and proton B of *j*th residue.

of these peptides was checked by analytical HPLC (YMC-Pack A-312, 0.6 × 15 cm) by gradient elution from 20% to 40% acetonitrile in the presence of 0.1% trifluoroacetic acid in H_2O at a flow rate of 1 mL/min, and by NMR. Impurities accounted for less than 1%, both by UV detection (220 nm) and by NMR. The activities of these synthetic peptides on the twitch responses to nervous stimulations (0.5-ms pulse width, 10 Hz, train of 10 pulses, 1-min interval) of mouse vas deferens were determined by a conventional method (Osakada et al., 1986). EC_{50}'s are 80 nM for Leu-enkephalin, 9 nM for [D-Ala^2]Leu-enkephalin, and more than 0.1 mM for [L-Ala^2]Leu-enkephalin. Those EC_{50}'s are consistent with the literature (Morley, 1980). [D-Ala^2]Leu-enkephalinamide is shown to be as active as [D-Ala^2]Leu-enkephalin (Stoineva & Petkov, 1985; Morley, 1980). In agreement with the literature, substitution of the Gly^2 residue by an L-amino acid resulted in complete loss of activity, whereas substitution by a D-amino acid resulted in retention of activity.

Liposomes. Perdeuterated L-α-dipalmitoylphosphatidylcholine (DPPC-d_{80}) was synthesized as previously described (Wakamatsu et al., 1983). Perdeuterated dilauroylphosphatidyl-*d,l*-serine (DLPS-d_{54}) and 1-acyl-2-(12-doxylstearoyl)glycerophosphocholine (12-SLPC) were generously given by Dr. Kaori Wakamatsu and Akihiko Okada in our laboratory (Wakamatsu et al., 1986a, 1987). For the liposome preparation, the phospholipids were mixed in the desired proportion in chloroform solution. This solution was evaporated in a round flask, so as to form a thin film on the flask surface. This film was exposed to high vacuum overnight to remove the remaining chloroform. D_2O was then added to make the concentration of phospholipid to 50 mM, and the film was allowed to swell overnight to form a liposome suspension. pH was adjusted to 5.0. Freezing and thawing were then repeated three times to produce liposomes that are smaller and of higher homogeneity in size (Westman et al., 1982).

Methods

NMR Spectroscopy. Proton NMR spectra were recorded on a Bruker AM-400 spectrometer equipped with an Aspect 3000 computer. The chemical shifts were measured (±0.01 ppm) relative to an internal standard of sodium 3-(trimethylsilyl)propanesulfonate. pH was measured in the NMR tubes directly before data acquisition. No buffer was added in order to keep the ionic strength to a minimum (at pH ~4 the peptide and phosphatidylserine themselves act as a buffer). Each sample was studied in D_2O at first, for easy assignment of the α,β, and side-chain protons. A pH titration was then performed in H_2O (+10% D_2O), between pH 3.0 and 6.0, by adding minute amounts of DCl and NaOD (solutions 0.3 N in D_2O). A series of decoupling experiments were performed in D_2O and H_2O solution at several pH values to confirm assignments.

For TRNOE analyses, small volumes (~10 μL) of the liposome solution described above were added to the peptide solution in H_2O (~10 mM) until the desired line broadening and TRNOE could be obtained. Finally, the sample was frozen (by liquid nitrogen) and thawed three times and then sonicated for 15 min with a bath sonicator. Liposomes at this experimental condition did not flocculate during the course of the experiment.

For NMR measurements in H_2O (including 10% D_2O) solution, the H_2O resonance was suppressed either by selective irradiation during the relaxation delay (1–2 s) or by the 1–1 hard pulse 45_x–τ–45_x (Clore et al., 1983). The latter method was chosen for the TRNOE experiments (sweep width 6000 Hz; carrier position 1000 Hz downfield from the water resonance position; interpulse delay τ 500 μs; digital resolution 1.47 Hz/point; acquisition time 0.68 s; no relaxation delay). NOE difference spectra were obtained as follows. A series of ^{1}H NMR spectra were recorded with irradiation of each proton resonance of the free peptide by homogated decoupling (0.3 s just before the 1–1 pulse) as well as two off-resonance frequencies on each extremity of the spectrum. Thirty-two transients were collected for each frequency, and this cycle was repeated 75 times, thus giving 2400 transients for each spectrum. The two off-resonance spectra were essentially same. A series of difference spectra were then calculated by subtracting the off-resonance spectrum from each on-resonance spectrum. The NOE's described were calculated as the ratio of the peak heights in the difference spectrum and the off-resonance spectrum (expressed in percent). These data have been corrected when necessary for incomplete saturation of the irradiated peak and partial saturation by the decoupler (decoupler spillover). All experiments were performed at 25 °C, if not noted otherwise.

Two-dimensional NOE (phase-sensitive NOESY) experiments were also performed for [D-Ala^2]Leu-enkephalin (20 mM) in H_2O solution at pH 3.8, in the presence of 5 mM DPPC-d_{80} and 5 mM DLPS-d_{54}. NOESY spectra were measured with a mixing time of 0.3 s in the pure-absorption mode using the TPPI method (Bodenhausen et al., 1984). Except during the acquisition period, the water proton resonance was continuously suppressed by low-power irradiation.

Table I: Proton Chemical Shift (ppm) of Enkephalin Derivatives in H_2O Solution at 25 °C

	NH	CHα	CH$_2\beta$	others
		Leu-enkephalin[a]		
Tyr^1		4.25	3.13	ar: 6.87, 7.16
Gly^2	8.60	3.84, 3.92		
Gly^3	8.00	3.80, 3.85		
Phe^4	8.05	4.63	2.99, 3.17	ar: 7.25–7.35
Leu^5	8.12	4.25	1.57	γ: 1.57; CH$_3$: 0.85, 0.89
		[D-Ala^2]Leu-enkephalin[b]		
Tyr^1		4.16	3.07, 3.19	ar: 6.90, 7.15
D-Ala^2	8.50	4.13		CH$_3$: 1.15
Gly^3	8.35	3.79, 3.82		
Phe^4	7.90	4.64	3.00, 3.18	ar: 7.26–7.37
Leu^5	8.10	4.20	1.58	γ: 1.58; CH$_3$: 0.85, 0.89
		[L-Ala^2]Leu-enkephalin[c]		
Tyr^1		4.19	3.13	ar: 6.85, 7.14
L-Ala^2	8.58	4.32		CH$_3$: 1.31
Gly^3	7.83	3.79, 3.87		
Phe^4	8.01	4.66	2.99, 3.18	ar: 7.25–7.38
Leu^5	7.92	4.19	1.56	γ: 1.56; CH$_3$: 0.85, 0.88
		[D-Ala^2]Leu-enkephalinamide[d]		
Tyr^1		4.15	3.06, 3.18	ar: 6.89, 7.14
D-Ala^2	8.47	4.15		CH$_3$: 1.15
Gly^3	8.38	3.85		
Phe^4	8.08	4.59	3.07	ar: 7.25–7.38
Leu^5	8.30	4.23	1.53	γ: 1.53; CH$_3$: 0.82, 0.89

[a] pH = 3.6. [b] pH = 3.9. [c] pH = 4.65. [d] pH = 4.13.

Results

Assignment of the ^{1}H NMR Spectra. Assignments of all proton signals are given in Table I for Leu-enkephalin, [D-Ala^2]Leu-enkephalin, [D-Ala^2]Leu-enkephalinamide, and [L-Ala^2]Leu-enkephalin at the indicated pH. The pH was selected to minimize the overlapping among NH and α resonances. In the case of Leu-enkephalin, for example, pH 3.6 had to be selected because from pH 3.8 to 4.5 there is complete overlap of the NH protons of Gly^3, Phe^4, and Leu^5; from pH 4.5 to 5.5 there is overlap between Leu α and Tyr α; and above

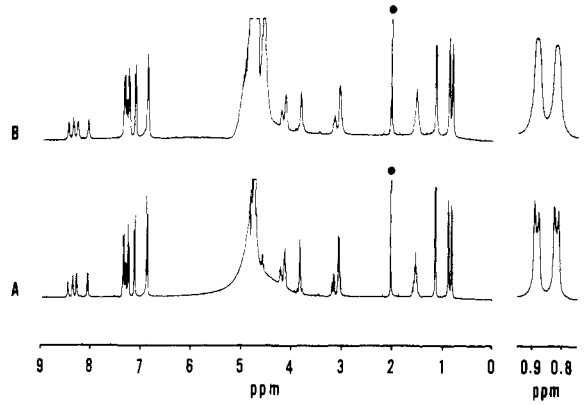

FIGURE 1: Spectra of [D-Ala^2]Leu-enkephalinamide (9.6 mM) in H_2O (10% D_2O), at pH 4.13. (A) Before the addition of liposomes. (B) After the addition: [DPPC-*d*] = [DLPS-*d*] = 3.5 mM. (●) indicates the resonance of acetate, which is the counterion of enkephalinamide. On the right side, an enlarged view of the leucine methyl signal was plotted to indicate the extent of line broadening in the conditions of the TRNOE experiments.

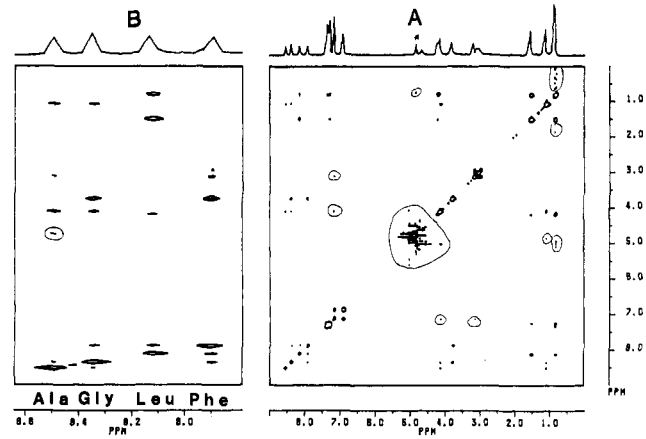

FIGURE 2: Contour plot of the symmetrized phase-sensitive NOESY spectrum (at 400 MHz) of [D-Ala^2]Leu-enkephalin (20 mM) in the presence of mixed vesicles of perdeuterated DPPC (5 mM) and DLPS (5 mM) in aqueous solution (90% H_2O and 10% D_2O) at pH 3.8 and 25 °C. The entire plot is shown in panel A (the water peak is marked with an asterisk), and an enlarged plot of the NH proton region is shown in panel B. Circled peaks are mostly due to T_1 noise. Uncircled cross peaks correspond to negative NOE's, which were confirmed by one-dimensional TRNOE experiments. The spectral width was 4000 Hz, and the carrier frequency was set at the water proton resonance. A total of 80 transients were accumulated, for each of the 512 increments, with a relaxation delay of 1 s between successive transients. The digital resolution was 15.6 Hz/point in both dimensions (no zero filling). The best spectrum was obtained without applying a window function.

pH 5.5, the NH protons of Gly^2 and Gly^3 broaden and disappear. All the assignments were confirmed by analysis of the strong intraresidue TRNOE signals in the presence of liposomes, which allow easy identification of the spin systems.

Establishment of Optimum Conditions for TRNOE Experiments. In the presence of liposomes, enkephalins exchange between a free state in solution and a membrane-bound state. This exchange caused a line broadening of the spectrum, without changing chemical shifts. As shown in Figure 1 for [D-Ala^2]Leu-enkephalinamide, the aromatic protons of Tyr^1, methyl protons of Leu^5 and Ala^2, and all the NH protons show a similar line broadening. We observed similar broadening of the entire spectra for the other three enkephalin analogues. The broadening is not specific, suggesting that no specific part of enkephalin molecules has different mobility in the membrane-bound state, unlike the case of α-mating factor (Wakamatsu et al., 1986b). The line broadening is concomitant with the appearance of negative TRNOE signals. Indeed, in the absence of liposomes, enkephalins showed only weak and positive intraresidue NOE signals under the present experimental condition. This is expected for molecules of this size and proved that the negative NOE signals observed upon the addition of membranes were not due to aggregates of peptide and arose entirely from magnetization transfer in the membrane-bound state. The extent of TRNOE observed is determined by the combinations of the conformation and correlation time at membrane-bound form, the exchange rates of enkephalin between the free and bound state, and the ratio of bound to free peptide (Clore & Gronenborn, 1982, 1983). These in turn depend on experimental conditions such as temperature, pH, ionic strength, and the [peptide]/[lipid] ratio.

(*a*) *pH Dependence of TRNOE.* We have measured the intraresidue TRNOE of Leu^5 (irradiation of Leu CH_3 signals) between pH 3 and pH 6. For all the four peptides, the NOE was maximal around pH ~4–5 and decreased both below pH 3.5 and above pH 5.5. This probably reflects weaker interaction of enkephalins with bilayer in agreement with the observation of Jarrell et al. (1980).

(*b*) *[Lipid]/[Peptide] Ratio.* The percentage of TRNOE increased with the [lipid]/[peptide] ratio (and therefore the proportion of bound peptide). However, increasing the amount of lipids had two adverse effects on the detection of TRNOE. First, it decreased the signal-to-noise ratio of TRNOE signals. Also, as the spectrum gets broader, it becomes more difficult to saturate each resonance selectively. The optimum ratio determined for each peptide was ca. 0.5.

(*c*) *Salt Concentration and Temperature.* Increased salt concentration resulted in weaker binding of enkephalins to the bilayer and therefore smaller TRNOE signals. It also caused rapid flocculation of the vesicles, thus making data acquisition for several hours impossible. Raising the temperature to 50 °C caused little change in TRNOE but increased the line broadening of NH protons through chemical exchange.

(*d*) *Time Dependence of TRNOE.* Leu CH_3 protons were irradiated during 0.05, 0.1, 0.2, 0.3, 0.5, and 1 s, and the TRNOE signals were observed on Leu α, Leu $\beta\gamma$, Leu NH, and Phe aromatic protons for the four peptides. These TRNOE signals were established in a single phase, and no lag time was observed, indicating that spin diffusion was negligible. This was also apparent from the high selectivity of the TRNOE signals. For instance, the two methyl groups of leucine gave very different TRNOE signals with Leu α and Leu $\beta\gamma$ protons. The low spin diffusion observed is due to the perdeuteration of phospholipids. Complete TRNOE experiments (irradiation of all protons in the molecule) have been performed for [D-Ala^2]Leu-enkephalin with two irradiation times (0.1 and 0.3 s). Most of the TRNOE signals were 3 times higher with 0.3 s of irradiation than 0.1 s, except the larger NOE signals which were between 2 and 3 times higher. Thus, at 0.3 s of irradiation time, the TRNOE signals were nearly proportional to the buildup rates of TRNOE and therefore to the cross relaxation rates between the bound protons (Clore & Gronenborn, 1983).

(*e*) *Decoupler Spillover.* The NH–NH NOE signals are very important for the determination of the conformation of peptides (Wüthrich et al., 1984). In order to determine NOE effects of a few percent between resonances separated by 40–80 Hz, it was necessary to check carefully for partial saturation by the decoupler. For each experiment, we have determined the saturation of a resonance as a function of its distance from the irradiated frequency. Typically, it was 4% for 40 Hz, 1% (80 Hz), 0.5% (120 Hz), and 0.15% (200 Hz). NOESY

Table II: Percentage of NOE's for [D-Ala²]Leu-enkephalin in the Presence of Bilayers[a]

	NH Ala	NH Gly	NH Leu	NH Phe	Ar Phe	Ar_l Tyr	Ar_h Tyr	α Leu	α Tyr	α Ala	α Gly	β_l Tyr	β_l Phe	β_h Tyr	β_h Phe	βγ Leu	Met Ala	Met_l Leu	Met_h Leu
NH Ala		3.1							*c*	6.0		1.4		1.4			1.0		
NH Gly	3.5		*c*	2.0	0.2					3.6	4.8						1.0		
NH Leu		*c*		8.8	0.7			3.7			0.7					6.7		1.4	1.8
NH Phe		2.1	7.7		1.2						5.9		2.8		2.8	1.0	0.6	0.4	0.4
Ar Phe			0.6	1.0												0.9	0.3	0.6	0.6
Ar_l Tyr							13.5					0.9		0.9			0.2		
Ar_h Tyr						9.2													
α Leu			8.0		0.9											4.1		2.3	10.0
α Tyr[b]	4.5	1.6	*c*					*d*				*d*		*d*		0.8	2.9		1.2
α Ala[b]																			
α Gly		7.5	1.8	11.3	1.1								1.1		1.1	0.4	0.4	0.4	
β_l Tyr[b]	0.9		1.5	4.3		1.1	1.4		3.1					22.0	52.0				
β_l Phe[b]																			
β_h Tyr	1.0								3.2			14.0							
β_h Phe			1.2	3.8	0.7								41.0			0.5		0.3	
βγ Leu			20.0	2.0	2.3			10.0					1.0		1.0		0.5	14.0	7.0
Me Ala	3.2	2.8			0.8	0.6	0.5			12	0.6					0.9		0.6	0.6
Me_l Leu			4.5	1.0	2.0			7.5								14.0	0.8		
Me_h Leu			7.2	1.0	2.1			38								8.3	0.8		

[a] [Peptide] = 9 mM, [DPPC] = [DLPS] = 2.5 mM, pH = 3.86, 25 °C. The protons are arranged in chemical shift order. No entry corresponds to no observable NOE (NOE < 0.5%). Each proton was irradiated during 0.3 s, with a decoupler power such that the proton signal was saturated to about 75%. The values indicated were corrected for incomplete saturation and for artifacts arising from partial overlapping (see text for more details). Shown vertically are irradiated resonances; horizontally, protons for which NOE was observed. [b] In this experiment, Tyr α and Ala α (underlined) could not be irradiated selectively as well as Tyr β_l and Phe β_l. [c] Due to overlapping, the possibility of the existence of a small NOE (<1%) cannot be discarded. [d] The observation was prevented by a spin-tickling effect between coupled protons.

Table III: Percentage of NOE's for [D-Ala²]Leu-enkephalinamide in the Presence of Bilayers[a]

	NH Ala	NH Gly	NH Leu	NH Phe	Ar Phe	Ar_l Tyr	Ar_h Tyr	α Leu	α Tyr[b]	α Ala[b]	α Gly	β_l Tyr	β_h Tyr	β Phe	βγ Leu	Met Ala	Met_l Leu	Met_h Leu
NH Ala		*c*		0.5	0.1	0.5			12.0			1.1	1.1			1.9		
NH Gly	*c*		*c*	1.9	0.2				2.9		6.1			0.4		1.0		
NH Leu		*c*		4.4	0.5			5.3			0.5			1.0	6.6	0.4	1.3	1.6
NH Phe	0.3	1.3	4.5		2.9						4.7			3.2	0.4	0.5	0.3	0.3
Ar Phe	0.5	0.5	0.7	1.8							0.7			1.1	0.5	0.4	0.7	0.9
Ar_l Tyr	0.6	0.2		0.4			11.4					1.8	1.8			0.5	0.2	0.2
Ar_h Tyr						13.0												
α Leu			7.6	1.1	0.8											5.1		3.2	15.0
α Tyr[b]	8.8	3.0		0.8	0.4	1.9	0.7	*d*			1.4	*d*	*d*		*c*	*d*			
α Ala[b]																			
α Gly	0.7	10.8	1.2	10.2	1.1	0.3				*d*				1.2	0.4	0.6	0.4	0.4	
β_l Tyr	1.6	0.6				2.6	0.8			3.0			37.0			0.4	0.2	0.2	
β_h Tyr[b]	1.3	0.7	1.7	6.8	2.0	1.3				3.1		1.0	20			0.7	0.4	0.4	0.4
β Phe[b]																			
βγ Leu	0.4		19.0	1.4	1.3			9.0			0.6			1.1		1.1	11.6	8.1	
Me Ala	4.4	3.3	0.7	0.8	0.8	0.7	0.6			11.1	0.7	0.1		0.7	1.2		1.1	0.8	
Me_l Leu	0.3	0.3	4.7	0.8	1.4			8.0			0.3			0.7	12.1				
Me_h Leu			5.6	0.9	2.0			30.0			0.5			0.7	9.1				

[a] [Peptide] = 9.6 mM, [DPPC] = [DLPS] = 3.5 mM, pH = 4.13, 25 °C; established in the same conditions as in Figure 1. [b] In this experiment, Tyr α and Ala α (underlined) could not be distinguished, and Tyr β_h and Phe β could not be irradiated selectively. [c] Due to overlapping, the possibility of the existence of a small NOE (<1%) cannot be discarded. [d] The observation was prevented by a spin-tickling effect between coupled protons.

spectra were measured for [D-Ala²]Leu-enkephalin in H_2O solution (Figure 2) and gave much better separation, but it was much more time consuming to get a spectrum with good signal-to-noise ratio. TRNOE patterns are essentially the same between one-dimensional TRNOE spectra and two-dimensional TRNOESY for the peptide (see below). Accordingly, only one-dimensional NOE experiments were performed for the other three peptides.

TRNOE Patterns. Figure 2 shows a TRNOESY spectrum for [D-Ala²]Leu-enkephalin, and Figure 3 shows a normal spectrum and NOE difference spectra for [D-Ala²]Leu-enkephalin by the irradiation of NH resonances. Figure 3b [irradiating the 0.15 ppm (60-Hz) lower field of Ala NH that is symmetric to Gly NH] demonstrates the spillover of the irradiation to Ala NH, but the spillover is small compared to the TRNOE on Gly NH from Ala NH (Figure 3c), or on Ala NH from Gly NH (Figure 3d). In fact, a TRNOESY spectrum (Figure 2) clearly shows the cross peak between Ala NH and Gly NH. In this manner the effect of decoupler spillover could be easily distinguished from the NH–NH NOE. From these difference spectra, the percentage of NOE was calculated (Table II; note that the data of the first four lines in Table II were calculated from the spectra of Figure 3). Strong interresidue NOE signals were observed between Ala^2 NH–Gly^3 NH (3.3%), Leu^5 NH–Phe^4 NH (8.3%), and Phe^4 NH–Gly^3 α (8.6%).

Table IV: Percentage of NOE's for [L-Ala^2]Leu-enkephalin in the Presence of Bilayers[a]

	NH Ala	NH Phe	NH Leu	NH Gly	Ar Phe	Ar$_l$ Tyr	Ar$_h$ Tyr	α Ala	α Leu[b]	α Tyr[b]	α Gly	β_l Phe	β Tyr	β_h Phe	$\beta\gamma$ Leu	Met Ala	Met$_l$ Leu	Met$_h$ Leu
NH Ala				3.4		0.7	0.6	4.6	12.8				1.5			1.5		
NH Phe			*c*	5.1	2.6	0.4	0.7	1.5			10.6	4.8		4.8	2.8	0.3	1.0	1.0
NH Leu		*c*		*c*	1.9	0.6	0.9		6.7		3.0	3.0		3.0	12.0		3.8	3.8
NH Gly	1.8	6.1	*c*		0.9	0.8	0.5	3.9			5.4		0.6		1.3	1.8		
Ar Phe		4.0	1.9	1.4		2.6	1.2		1.7		1.0	1.7		2.1	2.1	0.8	1.9	1.9
Ar$_l$ Tyr		1.2	0.5	2.1			17.0		1.8				2.5		0.4	0.3	0.4	0.4
Ar$_h$ Tyr		1.1				12.2							0.5					
α Ala	6.1	1.7	1.7	9.0	0.7	0.6	0.7		*c*				0.8			5.4	*c*	*c*
α Leu[b]	10.7	1.8	12.0	1.8	1.3	2.8	1.4	*c*				1.4	6.7	1.4	7.4	*c*	8.6	19.0
α Tyr[b]																		
α Gly		9.4	1.2	6.8	1.1	0.6	0.9								0.6	0.5	0.5	0.5
β_l Phe		7.6			2.1	*c*	*c*		*d*				*c*	59.0	1.5		0.9	0.9
β Tyr	2.9	*c*		1.8		5.9	1.8	*c*	11.8						0.7	0.6	0.9	0.9
β_h Phe		5.7	2.5		1.5	*c*	*c*					38.0	*c*		1.0		0.7	0.7
$\beta\gamma$ Leu		4.0	29.0		3.7	0.7	0.8		11.0			2.3		2.3			18.0	23.5
Me Ala	2.9	1.2	1.5	6.7	0.7	0.6	0.7	6.7							1.8		1.0	
Me$_l$ Leu		2.3	14.0	0.8	4.2	0.6	0.8	*c*	20.0			1.8		1.8	28.0	0.9		
Me$_h$ Leu		2.7	18.6		5.8	0.7	0.8	*c*	40.0			1.8		1.8	25.0	0.7		

[a] [Peptide] = 10.0 mM, [DPPC] = [DLPS] = 5.0 mM, pH = 4.64, 25 °C. [b] In this experiment, Leu α and Tyr α (underlined) could not be distinguished. [c] Due to overlapping, the possibility of the existence of a small NOE (<1%) cannot be discarded. [d] The observation was prevented by a spin-tickling effect between coupled protons.

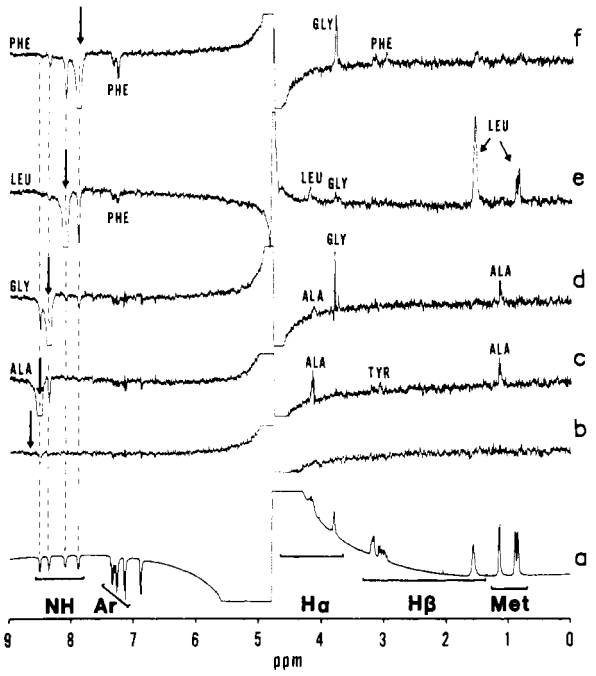

FIGURE 3: Spectrum of [D-Ala^2]Leu-enkephalin (a) and TRNOE difference spectra (c–f) obtained after irradiation (0.3 s) of the NH resonances marked by arrow with assignment on left edge. Irradiation of a frequency symmetric to the Gly NH against Ala NH resonance (b) shows the extent of saturation due to decoupling spillover. Intra- and interresidue TRNOE including NH–NH TRNOE could be observed and are shown by their assignments.

Complete NOE experiments were performed also for other two Ala-containing enkephalin analogues. Tables III and IV show TRNOE data for [D-Ala^2]Leu-enkephalinamide and [L-Ala^2]Leu-enkephalin, respectively. In preparing these tables, we have tried to distinguish clearly between those cases when no NOE could be observed because of technical reasons (as in the case of Phe α that is completely overlapped with the water peak) and when there is certainly no NOE. In the case of overlapping resonances, we have not attributed the NOE in Tables II–IV even when it was obvious for structural reasons. This will be done, when possible, later in the discussion. In the case of Leu-enkephalin (Table I), there are overlaps between many protons (NH protons of Gly^3, Phe^4, and Leu^5, α protons of Tyr^1 and Leu^5, and α protons of Gly^2 and Gly^3) so that only the following data (average value after irradiation of each proton of the pair) could be determined unambiguously (experimental conditions: [Leu-enk] = 10 mM, [DPPC] = [DLPS] = 5 mM, pH 3.6): (intraresidue TRNOE signals) Leu NH–Leu α = 3.8%, Leu NH–Leu $\beta\gamma$ = 4.9%, Leu NH–Leu $(CH_3)_h$ = 1.8%, Leu NH–Leu $(CH_3)_l$ = 1.3%, Leu α–Leu $\beta\gamma$ = 3.8%, Leu α–Leu $(CH_3)_l$ = 2.0%, Leu α–Leu $(CH_3)_h$ = 11%, Leu $\beta\gamma$–Leu $(CH_3)_l$ = 7.1%, Leu $\beta\gamma$–Leu $(CH_3)_h$ = 3.0%, Tyr ar$_h$–Tyr ar$_l$ = 5.2%, Tyr α–Tyr β = 1%, Phe NH–Phe β = 2.1%, Phe β_l–Phe β_h = 17%; (interresidue TRNOE signals) Gly^2 NH–Gly^3 NH = 0.6%, Gly^2 NH–Tyr α = 2.8%, Phe NH–Gly^3 α = 4.0%, Phe ar–Leu $\beta\gamma$ = 0.8%, Phe ar–Leu $(CH_3)_{h,l}$ = 1.0%. Here, *h* and *l* show high-field and low-field peaks of two Leu CH_3, Phe β CH_2, or Tyr aromatic protons, respectively.

The interpretation of these TRNOE patterns in terms of conformation will be detailed under Discussion, but simple comparison of the data immediately reveals that the patterns of [D-Ala^2]- and [L-Ala^2]enkephalin are clearly different. In order to make this comparison easier, we reproduced the data in a different form in Table V. On the other hand, there is a remarkable similarity between [D-Ala^2]Leu-enkephalin and [D-Ala^2]Leu-enkephalinamide. Tables II and III contain the result of two completely independent experiments, performed on compounds of different origin. However, they can be superposed almost completely. The only significant differences are that the NOE signals for Leu NH–Phe NH (4.5% compared to 8.1%) and Gly NH–Ala NH (<1% compared to 3.3%) are smaller in the case of [D-Ala^2]Leu-enkephalinamide than that of [D-Ala^2]Leu-enkephalin. For Leu-enkephalin, the NOE signals that could be determined were about half of those for the D-Ala derivatives. This is probably due to weaker interaction of Leu-enkephalin with the bilayer than D-Ala derivatives. Nevertheless, relative TRNOE values for Leu-enkephalin are similar to those for D-Ala derivatives, but not to those for [L-Ala^2]Leu-enkephalin. For example, strong TRNOE signals were observed between Phe NH–Gly^3 α, and Phe NH–Phe β, while weak TRNOE was observed between Tyr α–Tyr β, as in the cases of D-Ala derivatives but distinct from the case of [L-Ala^2]Leu-enkephalin (Tables II–IV).

Table V: Comparison of Some Important NOE Data for [D-Ala²]Leu-enkephalin and [L-Ala²]Leu-enkephalin[a]

	[D-Ala²]Leu-enkephalin					predicted turn II′			[L-Ala²]Leu-enkephalin				
	Tyr 1	D-Ala 2	Gly 3	Phe 4	Leu 5	2	3	4	Tyr 1	L-Ala 2	Gly 3	Phe 4	Leu 5
$d_{\alpha\beta}(i,i+3)$	~0	~0							~0	~0			
$d_{\alpha N}(i,i+3)$	~0	1[c]				w			*c*	1.7			
$d_{NN}(i,i+2)$	*b*	~0	~0			vw	vw		*b*	~0	~0		
$d_{\alpha N}(i,i+2)$	~0	~0	1.0			vw	w[e]		*c*	1.7	2.0		
$d_{NN}(i,i+1)$	*b*	3.3	2.0	8.2			w	s	*b*	2.6	5.6	<2	
$d_{\alpha N}(i,i+1)$	<4[c]	2.6	8.6	*b*			s[e]	w	11.8[d]	6.5	10.0	*b*	
$d_{\alpha N}(i,i)$	*b*	5.2	6.1	*b*	5.8		s	m	*b*	5.3	6.1	*b*	9.3
$d_{\beta N}(i,i)$	*b*	2.1		3.6	13.3			m	*b*	2.2		6.2	20

[a] $d_{AB}(i,i)$ is the NOE for the pair of proton A of the *i*th residue and proton B of the *j*th residue. The patterns are clearly different; note in particular $d_{\alpha N}$(Tyr,Ala), $d_{\alpha N}$(Ala,Gly), d_{NN}(Gly,Phe), and d_{NN}(Phe,Leu). w = weak; vw = very weak; m = medium; s = strong. [b] Resonances of Phe α and Tyr NH were not clearly observed. [c] Problem of overlapping resonances. [d] Assuming that $d_{\alpha N}$(Leu,Ala) is weak. [e] Only in the case of a residue Gly in position 3.

Table VI: Comparison of the NOE's Obtained with and without Spin-Labeled Phospholipid[a]

	[L-Ala²]Leu-enkephalin													
NOE between and	Tyr Ar Tyr Ar	Tyr β Tyr α	Tyr β Tyr Ar	Tyr α Ala NH	Ala NH Ala α	Leu NH Leu α	Leu NH Leu βγ	Phe NH Gly α	Leu α Leu βγ	Leu α Leu Me	Phe β phe β	Leu βγ Phe NH	Leu βγ Leu Me	Ala α Ala Me
without SLPC[b]	14.6	9	4	12	5	9	20	10	9	14; 30	49	3.5	23; 24	6
10% C12-SLPC[c]	3.5	1	~0	2	1	2	3	1	2	8; 12	15	~0	6; 4	~0

	[D-Ala²]Leu-enkephalin													
NOE between and	Tyr Ar Tyr Ar	Tyr Ar Tyr β	Ala NH Gly NH	Gly NH Phe NH	Gly NH Ala α	Gly NH Gly α	Leu NH Phe NH	Leu NH Leu βγ	Phe NH Phe α	Phe NH Phe β	Phe β Phe β	Leu βγ Phe Ar	Leu βγ Leu Me	Ala Me Ala NH
without SLPC[d]	11	1.2	3,3	2	3	6	8	13	9	3; 5	46	2	14; 8	2
10% C12-SLPC[e]	11	1.3	~0	2	1	4	3	9	8	2; 5	37	0.5	13; 8	1

[a] Note that, for [D-Ala²]Leu-enkephalin, the NOE's involving the Tyr residue are not affected by the presence of the spin-labeled phospholipid, whereas in the case of [L-Ala²]Leu-enkephalin, they are affected as much as the other residues. [b] Data taken from Table IV. [c] [Peptide] = 15 mM, [DLPS] = 2.5 mM, [DPPC] = 2.0 mM, [SLPC] = 0.5 mM, pH = 4.65. [d] Data taken from Table II. [e] [Peptide] = 8 mM, [DLPS] = 1.35 mM, [DPPC] = 1.08 mM, [SLPC] = 0.27 mM, pH = 3.84.

These results demonstrate, in the enkephalin series, a clear correlation between the activity and the membrane-bound conformation.

Transferred NOE in the Presence of Spin-Labeled Phospholipids. In an attempt to elucidate the location of the bound peptide in the bilayer, we have performed experiments in the presence of spin-labeled phospholipids (12-SLPC). The underlying principle of this technique is that, near the probe, there should be rapid relaxation of magnetization through dipolar interaction with the free radical and, therefore, the intramolecular TRNOE should be reduced (Wakamatsu et al., 1986a). Because the spin label (12-SLPC) is deep inside the bilayer, the protons that are embedded in the bilayer should be affected more than protons that are exposed to the aqueous phase. We have measured the TRNOE of [D-Ala²]Leu-enkephalin and [L-Ala²]Leu-enkephalin in the presence of bilayer containing 10% of 12-SLPC. Since we observed excess line broadening in the presence of 12-SLPC that is not perdeuterated, the [lipid]/[peptide] ratio in the presence of 12-SLPC was different than in its absence. These results are presented in Table VI. The absolute values of the TRNOE signals may not be compared directly with and without 12-SLPC because they depend on the experimental conditions (concentrations of peptide and phospholipid vesicles), but the relative change of TRNOE gives the distance between the probe and the observed protons. Suppression of TRNOE signals by the addition of 12-SLPC was observed clearly for NOE signals involving the N-terminal Tyr residue in the case of [L-Ala²]Leu-enkephalin, while those of [D-Ala²]Leu-enkephalin were not affected by 12-SLPC. These data indicate that the tyrosine residue of [L-Ala²]Leu-enkephalin is embedded in the bilayer but that the same residue of [D-Ala²]Leu-enkephalin is not.

Discussion

Interaction of Opioid Peptides with Phospholipid Vesicles. In order to observe sufficient interactions of enkephalin peptides with phospholipid vesicles (detected by broadening or by negative TRNOE's for peptide proton signals), it was necessary to add negatively charged phospholipids (phosphatidylserine) in vesicles and to keep the ionic strength low (less than 50 mM). These data suggest that enkephalins interact only weakly with phospholipid membranes. In addition, the interaction was found to decrease both below pH 3.5 and above pH 6, in agreement with Jarrell et al. (1980). The protonation of the phosphatidylserine is expected below pH 3.5, and the deprotonation of the N-terminal amino group of Tyr is expected above pH 6. Accordingly, these data show that the electrostatic force between negatively charged vesicles and positively charged enkephalin peptides is important for their interaction. Effects of the C-terminal carboxyl group on the interaction with vesicles are evidently not as important, because [D-Ala²]Leu-enkephalinamide and [D-Ala²]Leu-enkephalin showed similar interaction, as detected by TRNOE (Tables II and III). However, because the surface pH near the negatively charged bilayer is known to be lower than the pH in solution (by up to 3 pH units; MacDonald et al., 1976), the free acid peptide such as [D-Ala²]Leu-enkephalin may bind to the bilayer in the protonated form.

Membrane-Bound Conformation of the Active Analogues. The percentages of NOE between two protons described in Tables II–IV are related to the inverse sixth power of the interproton distance, so that conformations can be determined

Table VII: Comparison of Some Important Distances for [D-Ala²]Leu-enkephalin and [L-Ala²]Leu-enkephalin[a]

	[D-Ala²]Leu-enkephalin					[L-Ala²]Leu-enkephalin				
	Tyr 1	D-Ala 2	Gly 3	Phe 4	Leu 5	Tyr 1	L-Ala 2	Gly 3	Phe 4	Leu 5
$d_{\alpha\beta}(i,i+3)$	>4 (10.0)	>4 (5.0)				>4 (6.2)	>4 (6.5)			
$d_{\alpha N}(i,i+3)$	>4 (8.0)	3.7 (3.7)					3.6 (4.7)			
$d_{NN}(i,i+2)$		>4 (6.5)	>4 (4.7)				>4 (4.2)	>4 (4.7)		
$d_{\alpha N}(i,i+2)$	>4 (4.2)	>4 (4.5)	3.7 (4.0)				3.6 (4.0)	3.5 (3.9)		
$d_{NN}(i,i+1)$		3.0 (3.1)	3.3 (3.5)	2.6 (2.5)			3.3 (3.2)	2.9 (2.9)	>3.5 (4.0)	
$d_{\alpha N}(i,i+1)$	>3 (3.6)	3.1 (3.3)	2.6 (2.6)			2.6 (2.5)	2.9 (2.8)	2.7 (2.9)		
$d_{\alpha N}(i,i)$		2.8 (2.6)	2.7 (2.5)		2.8 (2.8)		3.0 (2.9)	2.9 (2.8)		2.7 (2.6)
$d_{\beta N}(i,i)$		3.3 (3.1)		3.0 (3.0)	2.4 (2.7)		3.4 (3.5)		2.9 (2.8)	2.4 (3.0)

[a] In order to compare more easily the data and the proposed models, the interproton distances (in angstroms) have been calculated by assuming a single correlation time for the whole molecule [NOE (%) = kR^{-6}]. k was calculated by using the tyrosine H_2–H_3 distance as an internal reference (k = 2600 and 3200 for [D-Ala²]- and [L-Ala²]enkephalin, respectively). The first number is the distance calculated from NOE; the second number (in parentheses) is the distance in the proposed model.

by the analyses of NOE (Jones et al., 1978; Clore & Gronenborn, 1983; Wüthrich et al., 1984). As shown in Table V, its NOE pattern of [D-Ala²]Leu-enkephalin is in quite good agreement with that expected for a type II′ β-turn structure around the Gly³-Phe⁴ residues. These NOE's exclude the possibility that membrane-bound [D-Ala²]Leu-enkephalin takes other well-characterized conformations, such as other types of β-turn structure [type I, I′, or II; see Venkatachalam (1968) for a description], extended conformation, β-sheet, α-helix, or 3_{10}-helix (Jones et al., 1978; Clore & Gronenborn, 1983; Wüthrich et al., 1984). Type I and I′ β-turn structures are excluded because the NOE between Gly NH and Phe NH [d_{NN}(Gly,Phe)] must be larger than $d_{\alpha N}$(Gly,Phe) for those β-turns. In fact, the former is 2%, while the latter is 8%, which holds for type II and II′ β-turn structures. Type II β-turn is excluded by the observation of a large 4% TRNOE of $d_{N\beta}$(Phe,Phe) and by an energy calculation that an L-amino acid in position 3 of a β-turn does not favor type I′ or type II (Venkatachalam, 1968). NOE signals of $d_{\alpha N}$(Tyr,Ala) and $d_{\alpha N}$(Ala,Gly) should be comparable with the NOE of $d_{\alpha N}$(Gly,Phe) for an extended conformation or a β-sheet structure. These were not observed in the case of [D-Ala²]Leu-enkephalin, however. In addition, the large NOE of d_{NN}(Phe,Leu) cannot be explained by an extended form or by a β-sheet structure. The helix conformations (α- and 3_{10}-helix) should give large NOE signals for proton pairs of two amino acids which are three to four residues or three residues apart, respectively, but such large NOE signals were not observed at all. On the other hand, all the observed NOE signals are explained by type II′ β-turn structure for D-Ala²-Gly³-Phe⁴-Leu⁵ residues (Table V).

Table III also suggests a γ-turn structure for Tyr¹-D-Ala²-Gly³ residues, because NOE for the proton pairs of Ala NH–Ala αH (6%) is stronger than for any other proton pairs that include an Ala residue [Ala CH_3–Ala NH (2%), Ala CH_3–Gly NH (2%), Ala NH–Gly NH (3%), and Ala αH–Gly NH (3%), except Ala αH–Ala CH_3 (7%)]. The NOE patterns support the proximity of Tyr CO and Gly NH groups (γ-turn), although direct evidence of the H bonding between the two groups should be demonstrated to confirm the γ-turn. The conformation proposed for the membrane-bound [D-Ala²]-Leu-enkephalin is presented in Figure 4. The Tyr side chain is remote from other residues, which is supported by very weak or no NOE signals between Tyr and the rest of the molecule. The Leu and Phe side chains are parallel to each other, according to the many NOE signals between these side-chain protons (Table II). This presumably constitutes the hydrophobic anchor of the peptide in the bilayer. The weak effects of spin-labeled PC on the Tyr side chain (Table VI) show that the phenol ring of Tyr is exposed to the aqueous phase. Table

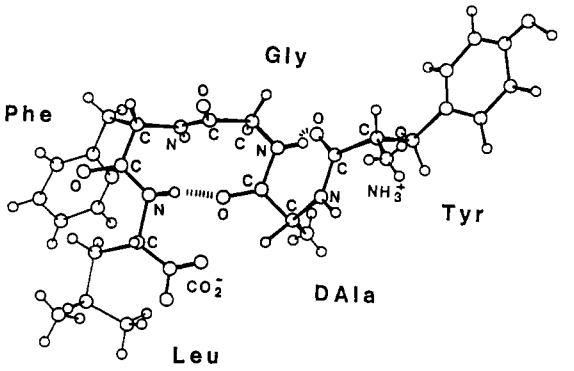

FIGURE 4: Conformation of [D-Ala²]Leu-enkephalin deduced from the TRNOE data (Table II). Side-chain conformations (χ_1, χ_2) of Phe⁴ and Leu⁵ are not defined from our NOE data, except that the Leu and the Phe side chains are close to each other. See text for the side-chain conformation of Tyr¹. The conformations of Leu-enkephalin and [D-Ala²]Leu-enkephalinamide are similar.

VII shows the comparison of the distances calculated from the NOE's and the final model we propose (Figure 4). It is clear that these two sets of distances agree very well, even if the calculation assumes a single correlation time for the whole molecule.

These arguments also show the uniqueness of the conformation for [D-Ala²]Leu-enkephalin at the membrane-bound state. This does not necessarily exclude the possibility of some different minor conformations in its membrane-bound form, or other major conformations that might exchange very slowly with free peptide in solution. (TRNOE works only in the case of fast exchange.) It is unlikely, however, that there are other major conformers that exchange very slowly with free peptides because enkephalins interact only weakly with phospholipid membranes, as discussed above.

The TRNOE patterns of [D-Ala²]Leu-enkephalinamide and [D-Ala²]Leu-enkephalin in the presence of perdeuterated phospholipid vesicles are quite similar (Tables II and III). These include large NOE's between Phe NH–Leu NH, Gly αH–Phe NH, and Gly αH–Gly NH and a small NOE between Gly NH–Phe NH. These suggest that the membrane-bound conformation of [D-Ala²]Leu-enkephalinamide is very similar to that of [D-Ala²]Leu-enkephalin.

Because of overlap of many signals of Leu-enkephalin, we could not construct its membrane-bound conformation from their NOE data. Nevertheless, we could conclude that the conformation is similar to that of [D-Ala²]Leu-enkephalin but not that of [L-Ala²]Leu-enkephalin. Two interresidue TRNOE's of $d_{\alpha N}$(1,2) and $d_{\alpha N}$(3,4) were observed without any ambiguities coming from overlaps, and the former (2.8%) is smaller than the latter (4%). This holds for [D-Ala²]Leu-

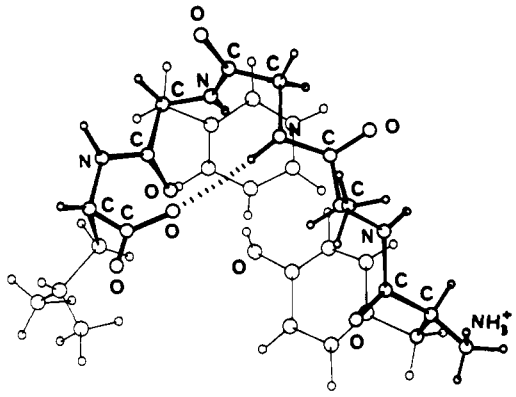

FIGURE 5: Conformation of [L-Ala^2]Leu-enkephalin deduced from the TRNOE data of Table IV. Side-chain conformations (χ_1, χ_2) of Tyr^1, Phe^4, and Leu^5 are not defined exactly, except that all the three side chains oriented to same direction (see text).

enkephalin (<4% and 8.6%, respectively) and [D-Ala^2]Leu-enkephalinamide (~3% and 7%, respectively), but not for [L-Ala^2]Leu-enkephalin (11.8% and 10%, respectively). Intraresidue TRNOE signals of the Leu residue are very different between [D-Ala]enkephalins and [L-Ala]enkephalin: for [D-Ala^2]Leu-enkephalins, the Leu $(CH_3)_h$ had an ~4–5 times larger NOE to the Leu α proton than the Leu $(CH_3)_l$, while the Leu $\beta\gamma$ protons had a 2 times larger NOE to the Leu $(CH_3)_l$ than to the Leu $(CH_3)_h$. [Gly^2]Leu-enkephalin gave almost the same relative NOE's as did [D-Ala^2]Leu-enkephalin. On the other hand, the Leu $(CH_3)_h$ of [L-Ala^2]-Leu-enkephalin showed only a 2 times larger NOE to the Leu α proton than the Leu $(CH_3)_l$, and the Leu $\beta\gamma$ had a smaller NOE to the Leu $(CH_3)_l$ than to the Leu $(CH_3)_h$. These suggest that natural [Gly^2]enkephalin and [D-Ala^2]Leu-enkephalins take similar conformations in the membrane-bound state.

Conformation of [L-*Ala*^{2}]*Leu-enkephalin.* The TRNOE pattern shown in Table IV of [L-Ala^2]Leu-enkephalin is characterized by continuous strong NOE signals of $d_{\alpha N}(i,i+1)$ (for i = 1–3), showing extended conformation for Tyr^1-L-Ala^2-Gly^3 residues. On the other hand, Gly^3-Phe^4-Leu^5 residues showed NOE patterns corresponding to none of the well-characterized classical conformations [α-helix, 3_{10}-helix, or β-turn; see Wagner et al. (1986)]. Since TRNOE signals relating the Phe αH could not be measured because of overlapping with the water peak, there are ambiguities in the conformation around the Phe residue. Nevertheless, a large NOE between Gly NH and Phe NH (5.6%) shows a turn at Gly-Phe. In addition, there were many NOE signals observed, including those between Phe aromatic and Tyr aromatic, Phe aromatic and Leu side chain, Tyr aromatic and Leu side chain (Table IV). Figure 5 shows a reasonable conformation for membrane-bound [L-Ala^2]Leu-enkephalin derived from those TRNOE data. Here we postulate a hydrogen bonding between Gly NH and C-terminal carboxyl groups. The side chains of Tyr, Phe, and Leu are oriented to same direction (downward in Figure 5), reflecting NOE signals among those side chains. These three side chains will be inserted into the bilayer according to the large effects of spin-labeled PC on entire molecule of [L-Ala^2]Leu-enkephalin. Table VII shows the distances between protons calculated from the NOE's, and the corresponding proton–proton distances of the model for [L-Ala^2]Leu-enkephalin in Figure 5. These two sets of distances agree very well, as in the case of [D-Ala^2]Leu-enkephalin, suggesting the validity of this conformation.

The differences in the membrane-bound conformations of [L-Ala^2]Leu-enkephalin and [D-Ala^2]Leu-enkephalin may be explained as follows. The D-Ala^2 α proton of the latter peptide is pointing toward the carboxy terminus (Figure 2). If the [L-Ala^2] analogue takes the same conformation as does the [D-Ala^2]Leu-enkephalin, Ala^2 CH_3 will be in contact with the C-terminal carboxyl group. Steric hindrance between the methyl group of Ala^2 and the carboxy terminus could explain why the [L-Ala^2]Leu-enkephalin should take another conformation and probably why it is inactive.

Comparison with Solution Conformations or Crystal Structure. The type II′ β-turn conformation of active enkephalins ([D-Ala^2]enkephalin, [D-Ala^2]enkephalinamide, and enkephalin) when bound to phospholipid membranes apparently corresponds well with the solution conformation proposed for enkephalin in DMSO-d_6 solution (Garbay-Jaureguiberry et al., 1983; Marion et al., 1983). In this conformation, a H bond between the Gly^2 CO and the Met^5 (or Leu^5) NH is proposed to play an important role in the structure. However, enkephalinamide does not take such a folded conformation (Higashijima et al., 1979) even in DMSO-d_6 solution. This apparent contradiction is explained as follows. As we reported previously, such a folded conformation for a dipolar form of enkephalin is stabilized by electrostatic interaction between N-terminal and C-terminal charges in DMSO-d_6 solution. On the other hand, such a head–tail electrostatic interaction is not important for the membrane-bound conformation of enkephalin analogues, because both [D-Ala^2]Leu-enkephalinamide and [D-Ala^2]Leu-enkephalin take a common β-turn for D-Ala-Gly-Phe-Leu residues, as well as a γ-turn for Tyr-D-Ala-Gly residues, when bound to phospholipid bilayer. These data show that the interaction of active enkephalins with phospholipid membranes (including acidic phospholipid) stabilizes the specific conformation in a way different from the case of the free peptide in solution.

Four different crystal structures have been solved for Leu-enkephalin. They have revealed the possible existence of a type I′ β-turn around Gly^2-Gly^3 (Smith & Griffin, 1978; Blundell et al., 1979), an extended conformation (Camerman et al., 1983; Karle et al., 1983; Griffin et al., 1986), and a partial 3_{10}-helix structure (Aubry et al., 1989). Such a diverse polymorphism illustrates the conformational mobility of enkephalins and argues that packing forces or intermolecular interactions are the major factors that determine the crystal structure.

Comparison with Active Cyclic Analogues. The conformation of the active analogues found in the membrane-bound state is very similar to the conformation of the cyclic compounds recently synthesized (DiMaio & Schiller, 1980; Mosberg et al., 1983; Mammi & Goodman, 1986). It was shown that in order to preserve the activity it is essential to leave the tyrosine residue out of the cycle, and this is achieved in the membrane-bound conformation. In the conformation presented in Figure 4, the C-terminal carboxyl group and the methyl group of Ala^2 can be connected by adding one methylene group without affecting the conformation, and then that can be the compound synthesized by DiMaio and Schiller (1980). It may be significant in this respect that a D-configuration at the second residue is also required for the activity of this cyclic compound.

Comparison with Energy Calculations. It is also interesting to compare the membrane-bound conformation with the conformations of minimum energy determined by calculations. Isogai et al. (1977) have shown that the lower energy conformation of Met-enkephalin is a type II′ β-turn centered around Gly^3-Phe^4. In more recent work from the same group, other conformations were shown to be possible (Paine &

Scheraga, 1985). The main differences between the low-energy type II′ β-turn for Gly-Gly-Phe-Leu and the membrane-bound type II′ β-turn (Figure 4) are the orientation of side chains, especially for the Tyr phenol ring. In the low-energy conformation, an intramolecular hydrogen bond between phenol OH and Gly^3 CO groups contributes to stabilization, while our data do not indicate such a hydrogen bond. If the conformation found by Isogai et al. held for [D-Ala^2]-Leu-enkephalin, large NOE's for Ala CH_3–Gly NH, Tyr aromatic–Gly NH, and Tyr aromatic–Gly NH would be observed. Such NOE signals are not found, and the hydrogen bonding between Tyr side chain and Gly^3 CO clearly plays a very minor role in the membrane-bound state. Such a very compact conformation may be found in a totally hydrophobic environment or in the isolated molecule, but not at a bilayer interface.

Location and Conformation of Tyrosine Residue of [D-*Ala*^{2}]*Leu-enkephalin and* [L-*Ala*^{2}]*Leu-enkephalin As Bound to Phospholipid Membranes.* The Tyr residue plays a crucial role for the enkephalin activity. Almost any modification at this position results in a loss of activity (Morley, 1980). Most surprisingly, it was found that tyrosine itself linked to a hydrophobic chain has morphine-like activity (Khaled et al., 1987), and it was hypothesized that tyrosine itself, if transported to the receptor site, would have opiate-like activity. In view of these results, the location of tyrosine relative to the bilayer is of extreme importance. In the case of the active compounds but not the inactive [L-Ala^2]Leu-enkephalin, the tyrosine is not embedded deeply into the bilayer (the TRNOE's were not affected by the presence of spin-labeled phospholipids). Because tyrosine is of intermediate hydrophobicity (Kyte & Doolittle, 1982), it is not surprising that tyrosine may be found both inside and outside of the membranes, depending on the conformation of the Tyr-containing peptides. Thus the presence of the tyrosine residue at the bilayer interface may be required for the activity of this peptide.

It may be interesting to point out here that the α,β dihedral angle (χ_1) of the Tyr^1 residue is very different between [D-Ala^2]- and [L-Ala^2]Leu-enkephalin: $d_{\alpha\beta}(1,1)$ is weak for the former (3.1 and 3.2% for β_l and β_h, respectively, Table II) but strong for the latter (12% for overlapped β methylene, Table IV). This supports the dihedral angle χ_1 of ca. −120° for [D-Ala^2]Leu-enkephalin. It happens to provide a distance between NH_3^+ and the OH of Tyr residue of 7.3 Å, i.e., a distance similar to the corresponding distance of morphine (7 Å).

Relation of the Membrane-Bound Conformation with the Receptor-Bound Conformation. The conformation of active enkephalins bound to the enkephalin receptor will not be exactly the same as the conformation in the membrane-bound form, since at least three types of receptors have been found (Paterson et al., 1983). Recently, a "two-stage capture model" has been proposed for the process of the recognition of peptide hormones by their receptors (Gremlich et al., 1983; Epand, 1983; Deber & Behnam, 1984; Higashijima et al., 1983; Wakamatsu et al., 1987). According to the model, peptide hormones in aqueous media are first trapped by anionic phospholipids in the outer layer of the target cell membranes, with a concomitant conformational change (first stage), and the membrane-bound peptides are then bound to the receptor with a slight conformational change (second stage).

Recently, conformation of acetylcholine bound to the nicotinic acetylcholine receptor was determined by using TRNOE (Behling, et al., 1988). Receptor-bound acetylcholine has a different conformation from that free in solution or in a crystal. Our present study shows a common conformation for all the active enkephalin analogues when bound to phospholipid membranes. Analyses of the receptor-bound conformation, which are expected to become feasible in near future, will show whether this membrane-bound conformation really corresponds well to the receptor-bound conformation of enkephalins.

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Registry No. Leu-enkephalin, 58822-25-6; [D-Ala^2]Leu-enkephalin, 64963-01-5; [D-Ala^2]Leu-enkephalinamide, 65189-64-2; [L-Ala^2]-Leu-enkephalinamide, 60284-47-1.

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