A Multidimensional ^{1}H NMR Investigation of the Conformation of Methionine-Enkephalin in Fast-Tumbling Bicelles

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ABSTRACT Enkephalins are pentapeptides found in the central nervous system. It is believed that these neuropeptides interact with the nerve cell membrane to adopt a conformation suitable for their binding to an opiate receptor. In this work, we have determined the three-dimensional structure of methionine-enkephalin (Menk) in fast-tumbling bicelles using multidimensional ^{1}H NMR. Bicelles were selected as model membranes because both their bilayer organization and composition resemble those of natural biomembranes. The effect of the membrane composition on the peptide conformation was explored using both zwitterionic (PC bicelles) and negatively charged bicelles (Bic/PG). Pulsed field gradient experiments allowed the determination of the proportion of Menk bound to the model membranes. Approximately 60% of the water-soluble enkephalin was found to associate to the bicellar systems. Structure calculations from torsion angle and NOE-based distance constraints suggest the presence of both μ- and δ-selective conformers of Menk in each system and slightly different conformers in PC bicelles and Bic/PG. As opposed to previous studies of enkephalins in membrane mimetic systems, our results show that these opiate peptides could adopt several conformations in a membrane environment, which is consistent with the flexibility and poor selectivity of enkephalins.

INTRODUCTION

Enkephalins are endogenous opioids found in the central nervous system and gastrointestinal tract where they bind preferentially to δ-opiate receptors with a significant affinity for μ-receptors (Kruk and Pycock, 1991; Schiller, 1984). These neuropeptides are composed of five residues with the following sequence: YGGF(M or L). In addition to their analgesic activity, enkephalins are also involved in the control of respiratory, cardiovascular, and gastrointestinal functions, and in neuroendocrine regulation (Cesselin, 1997; Fuxe et al., 1988). Since their identification in 1975 (Hughes et al., 1975), numerous studies have been performed to investigate the mechanism of action of enkephalins, which is still not well understood. It is believed that these flexible opioids accumulate at the neuron surface where they first interact with the membrane, and then migrate to the receptor with a bioactive conformation suitable for receptor binding (Behnam and Deber, 1984; Deber and Behnam, 1984; Gysin and Schwyzer, 1983; Sargent and Schwyzer, 1986). Hence, interaction of the opiates with the membrane lipid phase may be essential to confer a bioactive conformation on the peptide and be involved in the selection of the receptor subtype (μ, δ, or κ) (Schwyzer, 1986).

The interaction of enkephalins with model membranes has been investigated using diverse techniques including hydrophobic photolabeling (Gysin and Schwyzer, 1983), ultraviolet-visible (Young et al., 1992), and NMR (Deber and Behnam, 1984; Jarrell et al., 1980; Milon et al., 1990) spectroscopies, and revealed hydrophobic and electrostatic interactions of the neuropeptides with neutral and negatively charged lipids, respectively. In addition, a recent solid-state NMR study revealed that the insertion depth of enkephalins is modulated by the nature of the phospholipid headgroup (Marcotte et al., 2003).

To determine the role of the peptide-membrane interaction on the biological activity and receptor selection of enkephalins, comparisons have been made of the conformation of enkephalins and their analogs in water and model membranes. NMR experiments carried out in water show a random distribution of conformers for enkephalins and bioactive enkephalin derivatives (D'Alagni et al., 1996; Graham et al., 1992; Higashijima et al., 1979). These results are supported by infrared (Surewicz and Mantsch, 1988) and Raman (Takeuchi et al., 1992) spectroscopy, molecular dynamics simulations (Shen and Freed, 2002; van der Spoel and Berendsen, 1997), and by conformational analysis (Kinoshita et al., 1997). Conversely, NMR studies in membrane mimetics showed that enkephalins adopt a well-defined structure but different model systems give different structural results. Nevertheless, it is generally agreed that leu- (Lenk) and met- (Menk) enkephalins fold into a β-turn conformation when interacting with membrane-like environments such as phosphatidylcholine/phosphatidylserine (PC/PS) vesicles (Milon et al., 1990), and lyso-PC (Behnam and Deber, 1984), sodium dodecylsulfate (SDS) (Graham et al., 1992; Picone et al., 1990), or reverse bis(2-ethylhexyl) sulfosuccinate micelles (Rudolph-Böhner et al., 1997). Interestingly, studies of enkephalins and bioactive analogs

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The coordinates for the final structures have been deposited with the Protein Data Bank. The access codes are: 1PLW and 1PLX.

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do not converge to common structural elements responsible for the biological activity although it is believed that the tyrosine amide and phenolic groups, as well as the phenylalanine aromatic ring, are necessary for pharmalogical activity (Schiller, 1984; and references therein). The crystal structure of enkephalins and analogous opiates have been determined by x-ray diffraction studies and are reviewed by Deschamps et al. (1996). Extended forms are reported for both leu- and met-enkephalins (Doi et al., 1987; Griffin et al., 1986; Mastropaolo et al., 1987; Saitô et al., 1998) in addition to folded conformations for Lenk (Aubry et al., 1989; Blundell et al., 1979), depending on the crystallization solvent and degree of hydration.

To better understand the structure-activity relationship of these neuropeptides, the conformation of enkephalins needs to be determined in a model membrane system with structure and composition similar to natural biomembranes. We have, therefore, studied the conformation of Menk in binary bilayered mixed micelles or ''bicelles.'' Bicelles are discoidal bilayers typically composed of long-chain dimyristoylphosphatidylcholine (DMPC) localized in the planar section, and short-chain dicaproylphosphatidylcholine (DCPC) stabilizing the torus of the discs (Arnold et al., 2002; Picard et al., 1999; Sanders and Landis, 1994; Sanders and Schwonek, 1992; Vold and Prosser, 1996). Due to the diamagnetic susceptibility of their phospholipid components, bicelles spontaneously align in the magnetic field (B_0) with the bilayer normal perpendicular to the direction of B_0. However, this alignment depends on the temperature, concentration, and long-to-short chain lipid ratio. In this study, we have used bicelles with a small molar ratio of DMPC to DCPC (small q ratio). The resulting model membranes have a small diameter and undergo fast tumbling motion in the magnetic field, allowing the structure determination of bicelle-associated peptides by solution-state NMR techniques (Andersson and Mäler, 2002; Glover et al., 2001a; Whiles et al., 2001). These membrane systems are composed of PCs that are natural constituents of biological membranes (Cullis et al., 1996). Although their structure is mainly spherical (Glover et al., 2001b), fast tumbling bicelles are organized into a bilayer-like arrangement similar to natural membranes.

Multidimensional ^{1}H NMR was used in this study to determine the three-dimensional structure of methionine-enkephalin in fast-tumbling bicelles. To investigate the effect of the phospholipid headgroup on the conformation of this opioid peptide, negatively charged bicelles were prepared by replacing 10 mol% of DMPC with dimyristoylphosphatidylglycerol (DMPG). Because membranes of the central nervous system can be composed of up to 24% of anionic phospholipids (Hucho, 1986), DMPG-doped bicelles are suitable model membranes. First, we have determined the proportion of neuropeptide associated to the bilayers using pulsed field gradients. Then, two-dimensional NMR experiments were carried out to determine the conformation of Menk associated with the bicellar systems. Finally, the biological relevance of the conformers in terms of their opiate receptor subtype selectivity is discussed.

MATERIALS AND METHODS

Materials

Deuterated and protonated DCPC, DMPC, and DMPG were obtained from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Methionine-enkephalin was purchased from Sigma-Aldrich (Oakville, Canada) and insoluble impurities removed by washing 30 mg of peptide three times with 2 mL of deionized water, followed by centrifugation of the solution and subsequent freeze-drying of the supernatant.

Sample preparation

Bicelles were made from DMPC and DCPC at a long-chain to short-chain lipid molar ratio of 1:2. DMPC (10 mol%) was substituted for DMPG to prepare Bic/PG. A lipid-to-peptide molar ratio of 25:1 was used for all experiments. Therefore, 3.7 mg of Menk and a total of 75 mg of the different lipids were weighed and mixed in 600 μL of H_2O-D_2O (9:1), then submitted to a series of at least three freeze (liquid N_2)/thaw (37°C)/vortex shaking cycles until a transparent nonviscous solution was obtained. Deionized water was used because salt interference was observed on the binding of enkephalins to PS and PC/PS vesicles (Jarrell et al., 1980; Milon et al., 1990), and because the association of opioids to the receptor recognition sites is affected by the composition of the medium in in vitro assays of homogenates and tissue slices (Paterson et al., 1984). Lipids with deuterated acyl chains were used to limit the contribution of the lipid resonances to the ^{1}H NMR spectra. The pH of Bic/Menk and Bic/PG/Menk samples was 4.5 and 4.8 $\pm$ 0.1, respectively. The samples were stored at -20°C before their NMR analysis.

NMR experiments

NMR experiments were performed at 22°C on a Varian Inova 600-MHz NMR spectrometer using a triple-resonance XYZ-gradients probe (Varian, Palo Alto, CA). Spectra were recorded in 90% H_2O/10% D_2O. The chemical shifts were referenced relative to an external solution of 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) set to 0.0 ppm.

Diffusion measurements

The diffusion measurements were performed using the water-sLED pulse sequence (Altieri et al., 1995) in which the pulsed field gradients (PFG) also suppress the water signal. A 6-μs 90° pulse length was applied and 32 scans were recorded for each selected gradient strength (from 6.3 G/cm to 42 G/cm, with increments of 2.1 G/cm). Gradients were applied for 8.5 ms and 8-K complex data points were acquired. The recycling delay was 5.0 s and the diffusion delay 100.5 ms. The diffusion coefficients (D_S) were calculated using the equation (Altieri et al., 1995):

$$A(G) = A(0)\exp\left[-(\gamma\delta G)^2\,(\Delta - \delta/3)D_S\right], \qquad (1)$$

where $A(G)$ is the echo amplitude, γ is the ^{1}H gyromagnetic ratio, δ is the duration (s) of the PFG, G is the gradient strength (G/cm), and Δ is the time (s) between the PFG pulses. The gradient strength was calibrated using back-calculation of the coil constant from a D_2O diffusion experiment using $D_S = 1.9 \times 10^{-5}$ cm^2/s at 25°C (Longsworth, 1960).

Two-dimensional ^{1}H NMR

The two-dimensional (2D) ^{1}H NMR gradient correlation spectroscopy (gCOSY) and nuclear Overhauser enhancement spectroscopy (NOESY)

spectra were recorded with presaturation of the water peak. The 2D-gCOSY (Cavanagh et al., 1996; Hurd, 1990) spectra were obtained using a pulse length of 6 μs and a spectral width of 6 kHz in both the direct (F2) and indirect (F1) dimensions, with 2048 complex data points in F2 and 1536 complex data points in F1. Typically, four to eight scans were acquired per increment. The 2D-NOESY experiments (Jeener et al., 1979; Macura and Ernst, 1980) were carried out with a pulse length of 6 μs at different mixing times (τ_m). A τ_m of 300 ms was selected for maximum signal-to-noise and minimum spin diffusion. A spectral width of 6 kHz was used in both dimensions, with 1024 and 512 complex data points in F2 and F1, respectively. A total of 32–48 transients were recorded.

The 2D NMR spectra were processed using the NMRPipe package (Delaglio et al., 1995). A sine bell function was applied to the gCOSY spectra in both dimensions and the free induction decay size was doubled in F1 and F2 by zero filling. A cosine function was used for the NOESY spectra in both dimensions and zero filling was used to double the number of points in the free induction decay. The spectra were analyzed with the NMRView (5.0.4) software (Johnson and Blevins, 1994) for chemical shift and NOE attributions. Because the amide protons of the peptide were sensitive to water suppression, we have used the $Phe^4_{HN\text{-}H\alpha}$ NOE, corresponding to a distance of 3.05 Å, to calibrate the NOEs of the nonaromatic protons, and a distance of 2.48 Å for the $Tyr^1_{H\delta\text{-}H\varepsilon}$ NOE for calibration of the aromatic resonances (Gagné et al., 1997). A 60% error was applied to the peak integration for all distance constraint calculations.

Structure calculations

Structures were generated by simulated annealing carried out using Crystallography and NMR System (CNS) version 1.1 (Brünger et al., 1998). Two-hundred structures were calculated from an extended structure using a torsion mode, 57 and 58 NOE-based distance constraints for Menk in PC bicelles and Bic/PG, respectively, and the Phe^4 and Met^5 ϕ-angles obtained from the $^3J_{HN-H\alpha}$ coupling constants measured in the one-dimensional (1D) spectra. One-thousand high-temperature steps were used (15 ps) up to a final temperature of 50,000 K then 1000 cooling steps (250 K, 15 ps) were applied. Finally, 10 cycles of 200 minimization steps were performed. A repel constant value of 0.8 was used. The structures presented in this work include the 80 lowest total energy structures selected from the 200 calculated according to the NOE and overall energies. The software package Molmol (Koradi et al., 1996) was used to visualize the calculated structures and compute the RMSD data.

RESULTS

Chemical shift assignment

Initially, gCOSY spectra (not shown) were acquired to assign the ^{1}H chemical shifts of the phospholipids and the methionine-enkephalin in the different bicellar systems, and the latter are listed in Table 1. The peptide proton chemical shifts are similar (within 0.01 ppm) in both the zwitterionic and anionic bicellar systems.

The 1D NMR spectra of the amide and aromatic region for Menk in both the zwitterionic and anionic bicellar systems are presented in Fig. 1, *B* and *C*, and compared to the spectrum obtained in water (Fig. 1 *A*). The spectra for the bicellar systems are well resolved and demonstrate that the low-viscosity bicellar solution is a suitable medium for high-resolution NMR experiments (Vold et al., 1997). The comparison of Fig. 1 *A* with Fig. 1, *B* and *C*, reveals a small change in the chemical shifts upon binding of the peptide to both types of bicelles. Presaturation of the water signal showed an effect on the Gly^2 amide proton resonance, as observed by Deber and Behnam (1985) for the same peptide in lyso-PC micelles. Specifically, a smaller intensity was seen for this amide resonance compared to that of Gly^3, Phe^4, and Met^5 when Menk is bound to both bicellar systems. Moreover, the Gly^2 NH resonance was absent in the ^{1}H spectrum of Menk in water, indicating a rapid exchange of the free peptide form with the solvent.

TABLE 1 ^{1}H chemical shifts (±0.01 ppm) of methionine-enkephalin in zwitterionic bicelles at 22°C

Residue	NH	$C^\alpha H$	$C^\beta H$	$C^\gamma H$	$C^\delta H$	$C^\varepsilon H$	$C^\zeta H$
Tyr^1	–	4.25	3.14, 3.14		7.16, 7.16	6.86, 6.86	–
Gly^2	8.71	3.86, 3.92	–	–	–	–	–
Gly^3	8.01	3.82, 3.91	–	–	–	–	–
Phe^4	8.08	4.65	3.06, 3.17	–	7.30, 7.30	7.34, 7.34	7.25
Met^5	7.94	4.26	2.08, 1.95	2.39, 2.45	–	2.07	–

Association of met-enkephalin with bicellar systems

Before studying Menk conformation in bicellar systems, the proportion of the peptide bound to these membrane mimetics was assessed under the same conditions used for structural determination. This was performed using ^{1}H NMR with pulsed field gradients. The diffusion coefficient (D_{obs}) was obtained from the amplitude of selected resonances using Eq. 1. The value of D_{obs} used in the calculations was the average of the diffusion coefficients determined using 13–17 peptide/lipid resonances. All the plots of the normalized natural log signal intensity versus G^2 are linear, which confirms that there is no influence of intermolecular NOE on the diffusion results (Chen and Shapiro, 1999; Lucas et al., 2003).

Because the neuropeptides are in rapid exchange on the NMR timescale between the free and the bound states (Whitehead et al., 2001), the diffusion coefficient (D_{obs}) of bicelle-associated Menk thus represents an average of diffusion coefficients for the free (D_{free}) and bound (D_{bound}) species consistent with the two-site model proposed by Stilbs (1982), where D_{bound} corresponds to the bicelle diffusion coefficient. Therefore, as detailed in other related studies on neuropeptides (Gao and Wong, 1998; Whitehead et al., 2001):

$$D_{obs} = \chi_{free} D_{free} + \chi_{bound} D_{bound} \quad (2)$$

$$\text{or} \quad D_{obs} = (1 - \chi_{bound}) D_{free} + \chi_{bound} D_{bound} \quad (3)$$

where χ_{free} and χ_{bound} are the mol fractions of free and bound enkephalin, respectively. D_{free} can be obtained by measuring the diffusion coefficient of the peptide in water, whereas D_{bound} corresponds to the diffusion of the bicellar systems

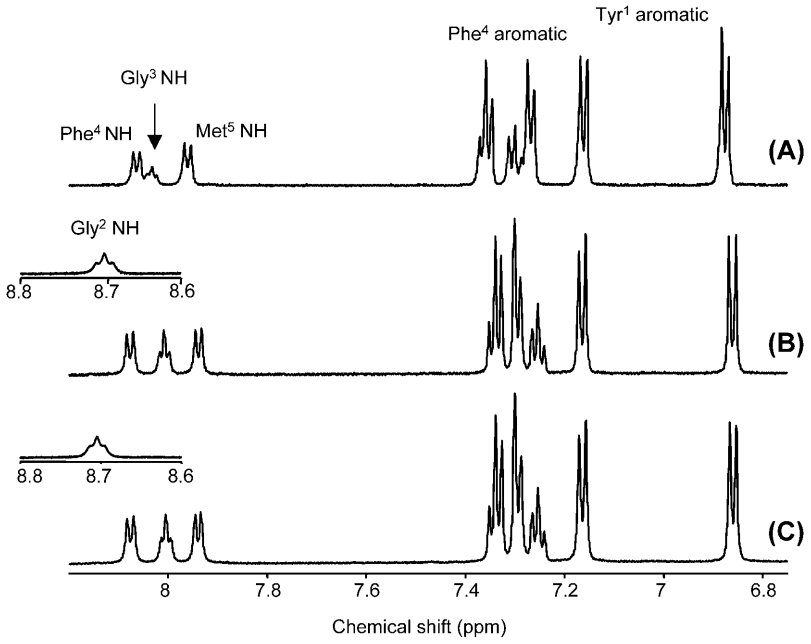

FIGURE 1 Amide and aromatic regions of the ^{1}H NMR spectra of methionine-enkephalin (*A*) in water, (*B*) in PC bicelles, and (*C*) in Bic/PG. The spectra were acquired at 22°C with a 600-MHz NMR spectrometer. The bicellar samples were prepared in H_2O/D_2O (9:1) and contain 10% (w/w) phospholipids with $q = 0.5$ and a lipid-to-peptide molar ratio of 25:1. The insert spectra are displayed with the same vertical scale.

measured using the lipid resonances. However, in a bicelle environment, the diffusion of free Menk in the aqueous solution is hindered by the presence of the bicelles themselves. It is thus essential to correct D_{free} by introducing an obstruction factor such as:

$$\langle A\rangle = 1/(1 + 0.5\phi) \tag{4}$$

$$\text{and} \quad D^{\phi}_{\text{free}} = D_{\text{free}}\langle A\rangle, \tag{5}$$

where ϕ is the volume fraction of the obstructing particles and $\langle A\rangle$ is a correction factor for spherical objects. This factor has been calculated by Gaemers and Bax (2001) for a 10% w/w bicelle solution at 20°C assuming a spherical shape and gave $\langle A\rangle = 0.95$. Because a radius of 43 Å is predicted for bicelles with $q = 0.5$ and a bilayer thickness of ~40 Å (Luchette et al., 2001), we have approximated the bicelles as spherical objects and using $\langle A\rangle = 0.95$, a D^{ϕ}_{free} of $2.86 \times 10^{-6} \pm 0.03$ cm^2/s was calculated.

Table 2 presents the diffusion coefficients measured for the bicellar systems and membrane-associated enkephalins, along with the fraction of bound peptide χ_{bound} obtained using D^{ϕ}_{free} in Eq. 3. In the case of the zwitterionic bicelles, 65% of the Menk peptides were bound whereas 59% were associated with Bic/PG10%. As shown in Table 2, increasing the proportion of anionic DMPG to 20% in bicelles has only a minor effect on the bound fraction of Menk.

TABLE 2 Diffusion coefficients and fraction of bound peptide determined from pulsed field gradient experiments at 22°C

	D_{obs} ($\times 10^{-6}$ cm^2/s)	χ_{bound} (±4%)
PC bicelles	0.53 ± 0.05	
Bic/PG20%	0.59 ± 0.06	
Menk in water	3.01 ± 0.03	
Menk in PC bicelles	1.35 ± 0.01	65%
Menk in Bic/PG10%	1.52 ± 0.01	59%
Menk in Bic/PG20%	1.55 ± 0.02	58%

Conformation of met-enkephalin in bicellar systems

The conformation of Menk associated with zwitterionic and negatively charged bicelles was determined from ^{1}H NOESY spectra. The amide/aromatic region of the NOESY spectrum is presented in Fig. 2 for Menk in zwitterionic bicelles. These results clearly show the high resolution of the 2D NMR spectra obtained for this system. Spectra with the same resolution and quality were acquired for Bic/PG. Despite the overlap of some lipid and/or peptide resonances, unambiguous NOEs could be assigned, leading to a total of 57 and 58 distance constraints for the structure calculation of Menk in PC bicelles and Bic/PG, respectively. Phe^4 and Met^5 ϕ-angle constraints of $-90°$ and $-88° \pm 20°$ were also introduced in the structure calculation and were calculated from the $^3J_{\text{NH-H}\alpha}$ couplings values of 7.8 and 7.6 Hz measured in the 1D spectra.

A NOESY experiment of Menk in water (not shown) showed no NOEs. This can be explained by the lack of a dominant dipolar relaxation pathway for the peptide at the temperature and proton frequency (600 MHz) used in this study. The peptide was, therefore, in a regime where the product of the molecular correlation time (t_c) and the Larmor frequency (ω) is close to unity, i.e., $\omega t_c \sim 1$ (Derome, 1987). Because the viscosity of the bicellar medium is close to that of water under the conditions used (Struppe and Vold, 1998), it is unlikely that conformers of free Menk in solution

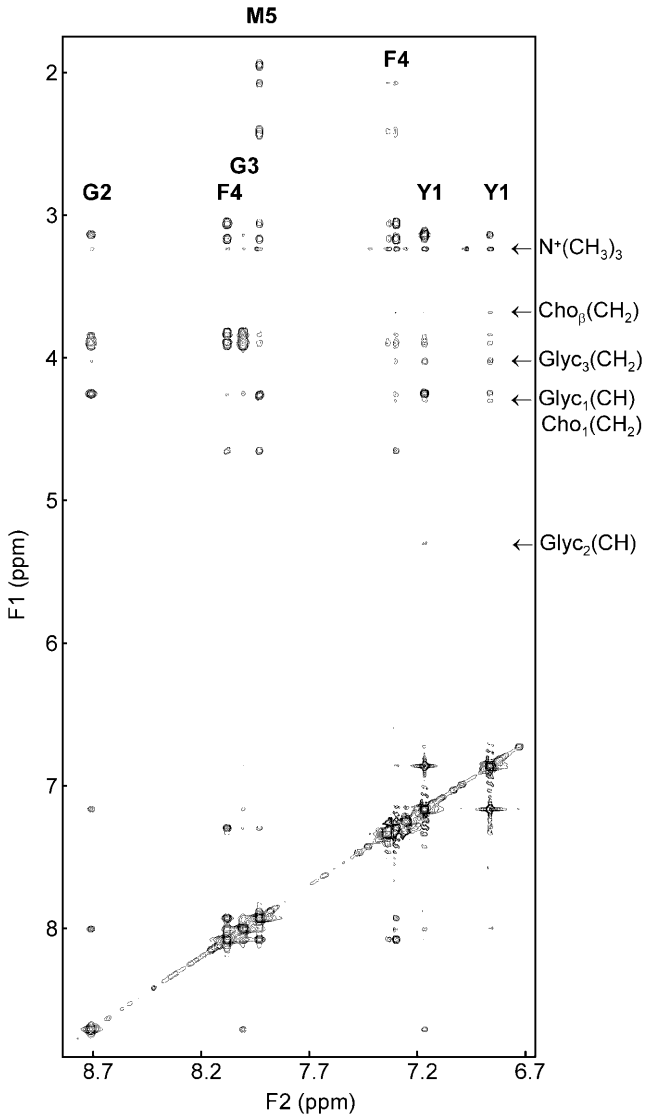

FIGURE 2 Selected region of the NOESY spectrum of methionine-enkephalin in PC bicelles. The spectrum was acquired at 22°C with a 600-MHz NMR spectrometer. The bicellar sample was prepared in H_2O/D_2O (9:1) and contain 10% (w/w) phospholipids with $q = 0.5$ and a lipid-to-peptide molar ratio of 25:1. NOEs between the peptide and the lipid glycerol (Glyc), choline (Cho), and trimethylammonium moieties are indicated.

contribute to the observed NOEs for the bicellar systems. In addition, as the experiments were performed in dilute bicellar media (10% w/w), the contribution of free peptides affected by bicelle surface effects would not be significant.

Structure of Menk in zwitterionic bicelles

Fig. 3 *A* shows the 80 structures with the lowest energy out of the 200 structures calculated for Menk in zwitterionic bicelles. These structures can be divided into three groups of conformers according to the peptide backbone, and Group I can be further separated into two subgroups according to the orientation of the Phe^4 ring with respect to the Tyr^1 side chain. Group I represents 54% of all the conformers (with 14% in Subgroup Ia and 40% in Subgroup Ib), whereas Groups II and III include 41% and 5% of the structures, respectively.

The statistics obtained for the different groups are presented in Table 3 and show low conformational energies for all conformers and no distance violation >0.12 Å. The ϕ- and ψ-angles of the peptide residues are found in the most favored regions of the Ramachandran plot, except for Group II where the dihedral angles are mainly located in additional allowed regions. The structural superposition for Groups I and II in Fig. 3 *A* is substantiated in Table 3 by a low backbone RMSD of 0.28 Å for Group Ia, 0.31 Å for Ib, and 0.22 Å for Group II. The higher RMSD calculated for the heavy atoms (ranging from 0.84 to 0.99 Å) can be explained by the higher degree of freedom of the side chains. Although the four backbone structures included in Group III are well superimposed from residues 2–5, the backbone (0.70 Å) and heavy atom (1.78 Å) RMSD values for this group are higher than those measured for Groups Ia, Ib, and II. This is mainly attributed to the considerable variation of the peptide side-chain positions observable in Fig. 3 *A*.

Table 3 shows that the Tyr^1, Phe^4, and Met^5 dihedral angles are similar in the three groups of structures. The differences between the two main families of conformers mainly arise from the ϕ-angle of Gly^2 residue, glycines being normally less constrained. In the structures of Group I, the aromatic Tyr^1 and Phe^4 rings are on opposite sides of the backbone and point toward different directions, with an average distance of 7 Å between the center of the rings. A different conformation in which the aromatic side chains are located on the same side of the backbone and point toward each other is observed for Group II where the aromatic rings are ~6 Å apart.

Hydrogen bonds are absent from the structures comprising Groups I and III, whereas H bonds are found between Tyr^1 and Gly^3 for 69% of the structures in Group II. All the structures of Menk obtained in PC bicelles are bent, forming a hydrophobic patch composed of the Tyr^1, Phe^4, and Met^5 side chains. The $^3J_{NH\text{-}H\alpha}$ coupling constant values of 7.8 and 7.6 Hz measured for Phe^4 and Met^5, respectively, in the 1D spectrum do not correspond to expected values indicative of β-turns (Hutchinson and Thornton, 1994). Therefore, the structures of Menk obtained in zwitterionic bicelles do not resemble type I, II, III, or IV β-turns previously proposed for enkephalins in different model membranes (Deber and Behnam, 1984; Graham et al., 1992; Milon et al., 1990; Rudolph-Böhner et al., 1997).

Differences and similarities between the groups of conformers are evident in Fig. 4 *A* where the minimized average structures of Groups Ia, Ib, and II are superimposed. In this figure, the backbone structures are overlaid from residues 2–5. As expected, the backbones of Subgroups Ia and Ib are superimposable. Table 5 reveals low backbone RMSD values between Groups Ia and Ib either when fit from

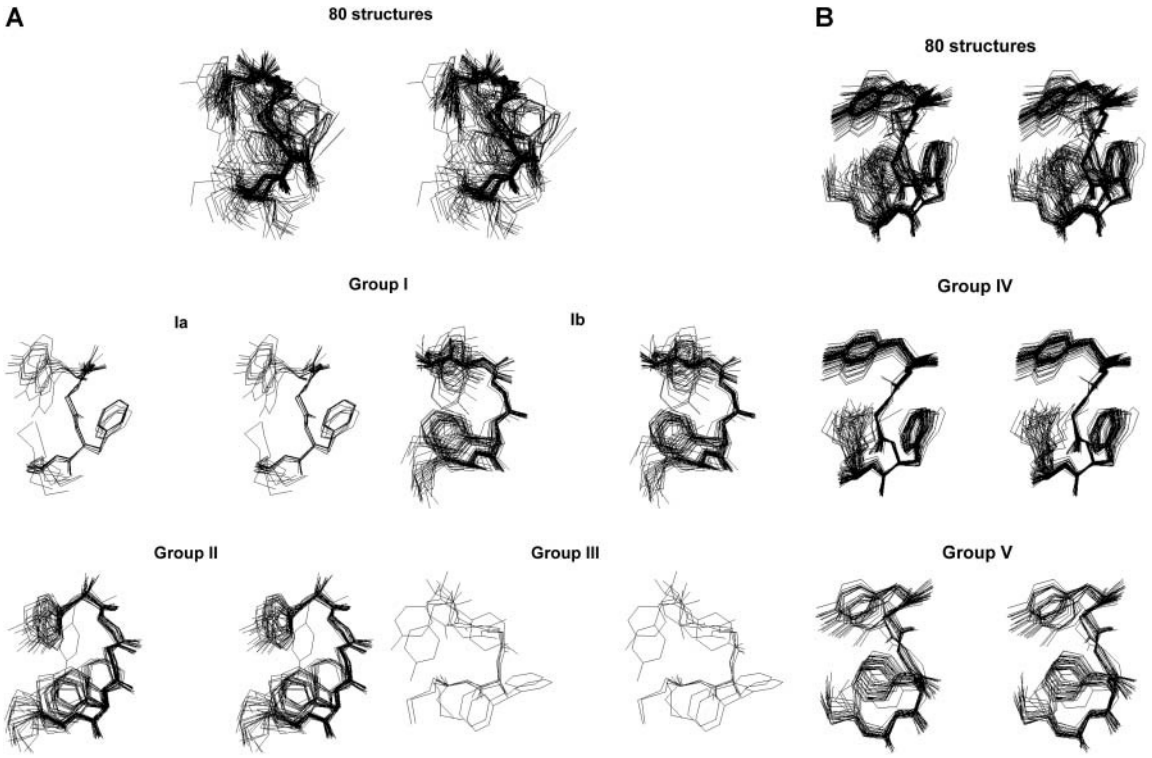

FIGURE 3 Stereoview of the structure of methionine-enkephalin in (*A*) PC bicelles, divided into three groups of conformers, and (*B*) Bic/PG, divided into two groups of conformers. All the structure figures were generated by the program Molmol (Koradi et al., 1996).

residues 1–5 (0.33 Å) or from residues 2–5 (0.25 Å). From Table 6, the Phe^4 χ_1-angle is the main difference between the two subgroups whereas the backbone torsion angles (Table 3) are similar for all the peptide residues. This further confirms that Menk structures in Subgroups Ia and Ib principally differ by the position of the phenylalanine side chain, as readily seen in Fig. 4 *A*.

The backbone superposition of Subgroups Ia and Ib to Group II (Fig. 4 *A*) gave a higher RMSD value (0.35 Å), Table 5. The heavy atom RMSD between Groups Ib and II (0.97 Å) is better than for Groups Ia/II, and is explained by the similar positioning of Phe^4 side chains. Differences between the Menk structures in Group Ia and II can be attributed to different ϕ-angle values for both glycine residues (Table 3), in addition to different Phe^4 χ_1-angles (Table 6). Further, the differences observed between the Groups Ib and II structures can be explained by the significant difference between the Gly^2 ϕ-angles.

Structure of Menk in negatively charged bicelles

Eighty structures for Menk in Bic/PG are shown in Fig. 3 *B*. They are divided into two groups according to the backbone conformation and the position of the phenylalanine side chain with respect to the tyrosine ring. These groups are called IV and V to avoid confusion with the structures found in zwitterionic bicelles. Group IV includes 68% of the structures whereas the remaining 32% make up Group V. The conformational energies of these structures are low and similar to those obtained in zwitterionic bicelles, with no distance violation >0.11 Å. The dihedral angles for the majority of residues are located in the most favored region of the Ramachandran plot, with those from other residues in additionally allowed regions. Low backbone RMSD of 0.18 Å was obtained for Group IV and 0.31 Å for Group V as shown in Table 4. The heavy atoms were also well superimposed.

Groups IV and V structures show a bent conformation with a hydrophobic patch formed by the peptide side chains. As for Menk in zwitterionic bicelles, the $^3J_{NH\text{-}H\alpha}$ coupling constant values for Phe^4 and Met^5 (7.8 and 7.6 Hz, respectively) measured from the 1D spectra do not correspond to values expected for a β-turn conformation (Hutchinson and Thornton, 1994). The Tyr^1 and Phe^4 ring centers, separated by ~7 Å in Group IV, point in opposite directions and are on opposite sides of the backbone. The contrary is observed for Group V structures where the aromatic rings are in closer proximity and ~5 Å apart. No hydrogen bonds are found in Group IV whereas H bonds are observed between the carbonyl group of Tyr^1 and the Gly^3 amide proton in some Group V structures (Table 4).

Fig. 4 *B* shows a comparison of the minimized average structures of Menk in Groups IV and V fitted from residues 2 to 5. The main structural difference around the N-terminal

TABLE 3 Statistics for the structures of methionine-enkephalin in PC bicelles

Group (%)*†	F_{noe}‡	F_{tot}‡	RMSD (Å)§ backbone heavy atoms	% Residues favored region¶	% Residues additional allowed region¶	H-bonds‖** (distance ±0.1 Å)††
Ia (14%)	0.47–1.55	10.1–13.4	0.28 ± 0.09 0.84 ± 0.18	85%	15%	No
Ib (40%)	0.39–1.79	9.2–14.0	0.31 ± 0.18 0.92 ± 0.23	60%	40%	No
II (41%)	0.23–1.76	8.9–13.0	0.22 ± 0.09 0.99 ± 0.22	33%	67%	69% Y^1-G^3 (2.8)
III (5%)	0.91–1.62	11.3–13.2	0.70 ± 0.08 1.78 ± 0.20	100%	0%	No

Residue		ϕ-angle	ψ-angle		ϕ-angle	ψ-angle
Tyr	Ia	—	169° ± 5°	II	—	169° ± 8°
Gly		−180° ± 29°	−26° ± 9°		−52° ± 7°	−34° ± 9°
Gly		114° ± 7°	53° ± 6°		70° ± 15°	53° ± 6°
Phe		−108° ± 3°	−35° ± 4°		−109° ± 2°	−40° ± 4°
Met		−74° ± 5°	—		−74° ± 12°	—
Tyr	Ib	—	165° ± 4°	III	—	165° ± 6°
Gly		−178° ± 28°	7° ± 32°		−138° ± 102°	−15° ± 45°
Gly		89° ± 39°	54° ± 5°		−140° ± 98°	−46° ± 5°
Phe		−110° ± 2°	−41° ± 2°		−98° ± 4°	−29° ± 5°
Met		−72° ± 10°	—		−70° ± 4°	—
Energies‡ ‡‡		$F_{bond} = 0.15$	$F_{cdih} = 0.012$	$F_{repel} = 1.3$	$F_{noe} = 0.95$	$F_{tot} = 10.9$

*No distance violations >0.12 Å.
†Percentage of the structures belonging to this group.
‡Energies in kcal/mol.
§Root mean square deviations to the average structure, from residues 1 to 5.
¶Values obtained from the Ramachandran plot generated by PROCHECK 3.4 (Laskowski et al., 1993).
‖Statistics on hydrogen bonds obtained with Vadar 1.3 (D. Wishart, L. Willard, and B. D. Sykes, University of Alberta Protein Engineering Network of Centres of Excellence), plus percentage of structures containing these bonds.
**C=O-NH bonds.
††Average distance between the heavy atoms (O and N) obtained with Vadar 1.3.
‡‡Average values.

explains the rather higher backbone RMSD (0.49 Å) between the two families of structures (Table 5) as compared to the groups of conformers in neutral bicelles. This can be attributed to the striking difference between the ϕ-angle values for both glycines shown in Fig. 3 *B*.

Comparison of Menk structures in PC bicelles and Bic/PG

Interestingly, the average structures of Menk in both Subgroups Ia and Ib in PC bicelles are similar to that of Group IV in Bic/PG with the aromatic rings almost superimposable and pointing in opposite directions (Fig. 5). Although already easily superimposable from residues 1 to 5 (backbone RMSD of 0.37 Å for Ia/IV and 0.43 Å for Ib/IV), the backbone RMSD between these groups is especially low when a fit is performed using residues 2–5 (0.21 Å for Ia/IV and 0.29 Å for Ib/IV). Tables 3 and 4 show only a small difference between Gly^2 ϕ-angles of Groups Ia and Ib compared to Group IV. The main difference between the conformers of Groups I and IV originates from deviations in the Phe^4 $\chi_{1,2}$-angles as can be seen in Table 6.

Although the Menk conformers comprising Group II in zwitterionic bicelles and Group V in Bic/PG contain similar H-bonds and analogous orientations of the aromatic rings, these groups include important structural differences as seen at the N-terminus (Fig. 5) and by the high RMSD values presented in Table 5. These differences cannot be attributed to the Tyr^1 and Phe^4 χ-angles (Table 6), but to differences in the Gly^2 and Gly^3 torsion angle values evident when comparing results in Tables 3 and 4.

DISCUSSION

The goal of this study was to investigate the conformation of enkephalins in fast-tumbling bicelles. The bilayer organization and composition of fast-tumbling bicelles make them relevant mimetics, and the high-resolution 1D and 2D ^{1}H spectra confirm that these bicellar systems can be used for NMR structural work. As a first step, we assessed the binding of methionine-enkephalin to these model membranes. Then, the conformation of the neuropeptide in bicellar systems with different phospholipid composition was examined and the structures compared.

Primary information on the interaction of met-enkephalin with the lipid bilayers and the peptide location was obtained from 1D NMR spectra (Fig. 1). The binding of the opiate is

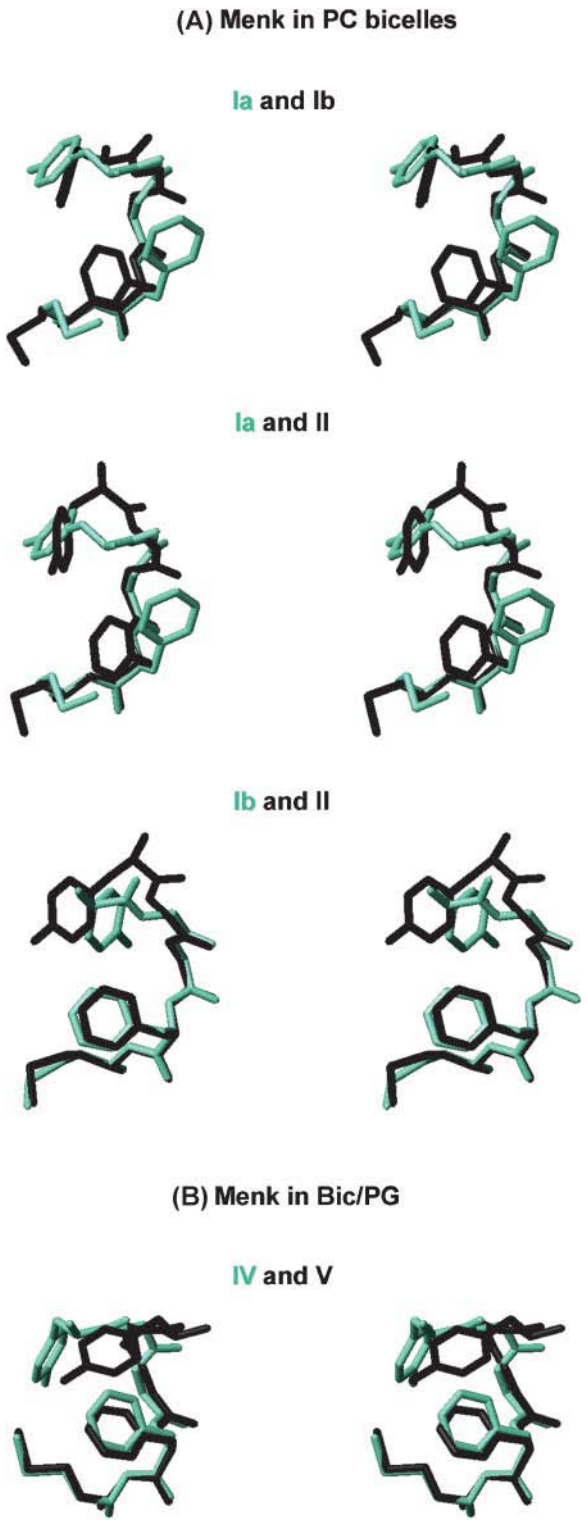

FIGURE 4 Comparison of the average structures of the different groups of conformers for methionine-enkephalin in (*A*) PC bicelles, and (*B*) Bic/PG. The superposition was performed from residues 2 to 5. The minimized average structures were calculated using the Crystallography and NMR System (CNS) (Brünger et al., 1998).

evidenced by the changes in the chemical shifts in an aqueous solution compared to the bicellar milieu. Also, the Phe^4, Met^5, and more specifically Gly^3 amide resonances are more intense in Fig. 1, *B* and *C*, compared to Fig. 1 *A*, suggesting that these protons exchange less with water when Menk is bound to the bilayers, in which they may be embedded. These findings are supported by intermolecular NOEs between the lipid headgroup region and the peptide (Fig. 2). However, despite peptide association to the bicellar systems, the Gly^2 NH is still affected by the water suppression, indicating a location of this residue closer to the membrane surface, as previously reported for Menk in lyso-PC micelles (Deber and Behnam, 1985).

Association

We have determined the proportion of met-enkephalin associated with the zwitterionic and anionic bicellar systems. From PFG experiments, 65% of the peptide molecules are associated with the neutral bicelles, and, although the results suggest decreased binding (by 5%) when negatively charged DMPG is added to the model membranes, the difference is within the error of the measurements. This proportion compares with that of another opiate, the δ-selective D-penicillamine^{2,5}-enkephalin (DPDPE), for which 40% of the peptide associate with bicelles (Rinaldi et al., 1997). Considering the lipid concentration of ~10% w/w in water used in this study, a partition coefficient of 17 (13) is calculated for Menk between PC bicelles (Bic/PG) and water. Therefore, there is a favorable partition coefficient for the lipids but the peptide is exchanging rapidly between the bicelle surface and the water. This could result from the chemical nature of the opiate peptides, which generally contain both hydrophilic and hydrophobic moieties and are also water-soluble.

Conformation

The structure of met-enkephalin was studied in both zwitterionic and negatively charged bicelles to verify the effect of the phospholipid headgroup on the peptide conformation. Calculated three-dimensional structures for Menk in phospholipid model membranes were obtained using 57 distance constraints for the peptide in PC bicelles and 58 in Bic/PG. Many NOEs were similar in both membrane systems, and correspondingly the structures obtained show important similarities. The differences between the structures can be attributed to different NOEs found between the side chains of Tyr^1 and Met^5, Phe^4 and Met^5, and to additional Tyr^1-Phe^4 NOEs in Bic/PG. More specifically, NOEs are observed for Menk between $Tyr^1C_\delta H$ and Met^5NH and between $Phe^4C_\delta H$ and $Met^5C_\alpha H$ in zwitterionic bicelles; these NOEs are absent in Bic/PG. In addition, NOEs between Phe^4 aromatic ring protons and both Tyr^1 $C_\alpha H$ and Met^5 side chains for Menk were only observed in Bic/PG.

TABLE 4 Statistics for the structures of methionine-enkephalin in Bic/PG

Group (%)*†	F_{noe}‡	F_{tot}‡	RMSD (Å)§ backbone heavy atoms	% Residues favored region¶	% Residues additional allowed region¶	H-bonds‖ ** (distance ±0.01 Å)††
IV (68%)	0.20–0.82	8.8–11.5	0.18 ± 0.10 0.72 ± 0.11	100%	0%	No
V (32%)	0.20–1.18	9.1–12.4	0.31 ± 0.13 0.80 ± 0.12	85%	15%	8% Y^1-G^3 (3.01)
Residue		ϕ-angle	ψ-angle		ϕ-angle	ψ-angle
Tyr	IV	—	176° ± 1°	V	—	174° ± 3°
Gly		149° ± 4°	−24° ± 6°		78° ± 11°	40° ± 26°
Gly		119° ± 11°	28° ± 14°		152° ± 40°	25° ± 21°
Phe		−85° ± 7°	−38° ± 2°		−85° ± 17°	−40° ± 2°
Met		−78° ± 13°	—		−83° ± 12°	—
Energies‡‡‡		$F_{bond} = 0.08$	$F_{cdih} = 0.005$	$F_{repel} = 0.1$	$F_{noe} = 0.51$	$F_{tot} = 9.8$

*No distance violations >0.11 Å.
†Percentage of the structures belonging to this group.
‡Energies in kcal/mol.
§Root mean square deviations to the average structure, from residues 1 to 5.
¶Values obtained from the Ramachandran plot generated by PROCHECK 3.4 (Laskowski et al., 1993).
‖Statistics on hydrogen bonds obtained with Vadar 1.3 (D. Wishart, L. Willard, and B. D. Sykes, University of Alberta Protein Engineering Network of Centres of Excellence), plus percentage of structures containing these bonds.
**C=O-NH bonds.
††Average distance between the heavy atoms (O and N) obtained with Vadar 1.3.
‡‡Average values.

As opposed to previous work on enkephalins in phospholipid membranes (Behnam and Deber, 1984; Milon et al., 1990), cesium perfluorooctanoate liquid crystals (Kimura et al., 1996) and surfactant micelles (Graham et al., 1992; Rudolph-Böhner et al., 1997), our structural study of Menk demonstrates that different conformers are possible for these neuropeptides in a membrane environment, which is consistent with their natural high flexibility (Paterson et al., 1984). These conformers were obtained in a mimetic system similar to biomembranes, and they could not be compared to crystal structures of Menk that exist only in extended forms unrepresentative of those obtained in lipid membranes (Deschamps et al., 1996; Doi et al., 1987; Griffin et al., 1986; Mastropaolo et al., 1987). Three families of structures were found for Menk in zwitterionic bicelles whereas two were observed in Bic/PG. In each system, these groups show different relative positions of the Tyr^1 and Phe^4 aromatic rings with respect to each other, and display different backbone characteristics particularly at the N-terminal region of the peptide. All groups of structures show low conformational energies and the backbone and heavy atom RMSDs are indicative of structural similarity within each group.

TABLE 5 Comparison of the different groups of conformers for Menk in PC bicelles and Bic/PG

Groups compared	RMSD (Å)* (residues 1–5) backbone	heavy	RMSD (Å)* (residues 2–5) backbone	heavy
Ia and Ib	0.33 ± 0.18	1.41 ± 0.23	0.25 ± 0.12	1.40 ± 0.13
Ia and II	1.09 ± 0.12	2.57 ± 0.28	0.35 ± 0.07	1.29 ± 0.32
Ib and II	1.11 ± 0.11	2.31 ± 0.26	0.35 ± 0.05	0.97 ± 0.30
IV and V	1.12 ± 0.19	2.33 ± 0.26	0.49 ± 0.13	0.93 ± 0.15
Ia and IV	0.37 ± 0.24	1.39 ± 0.11	0.21 ± 0.07	1.30 ± 0.08
Ib and IV	0.43 ± 0.16	1.29 ± 0.19	0.29 ± 0.08	1.14 ± 0.14
II and V	1.92 ± 0.06	2.19 ± 0.17	0.69 ± 0.03	1.42 ± 0.25

*Root mean square deviations of one group of conformers to the geometric mean structure of the other group determined with the software Molmol (Koradi et al., 1996).

The conformers presented in this study are not mere artifacts of the energy minimization method used for the structure calculation as we have applied a simulated annealing procedure in which the calculations are driven by experimental data (NOE and $^3J_{HN-H\alpha}$) and not by the force field. Only bond, angle, and repulsive van der Waals energy terms were used in the calculations. Attractive van der Waals and electrostatics terms were not used as they would certainly have biased the results. Hence, the force field is not favoring particular conformers, and this has been demonstrated by calculating the structure of met-enkephalin without using experimental restraints. One-hundred structures were calculated, which revealed similarly high RMSDs as compared to the average structures of Groups Ia, Ib, II, III, IV, and V. More specifically, values of 1.64 ± 0.37 Å and 3.83 ± 0.59 Å were found for the backbone and heavy atom RMSDs, respectively.

There is common agreement that the orientation of the tyrosine and phenylalanine rings with respect to each other dictates the receptor subtype selectivity, and it was originally believed that the μ-selective opiates adopted a folded

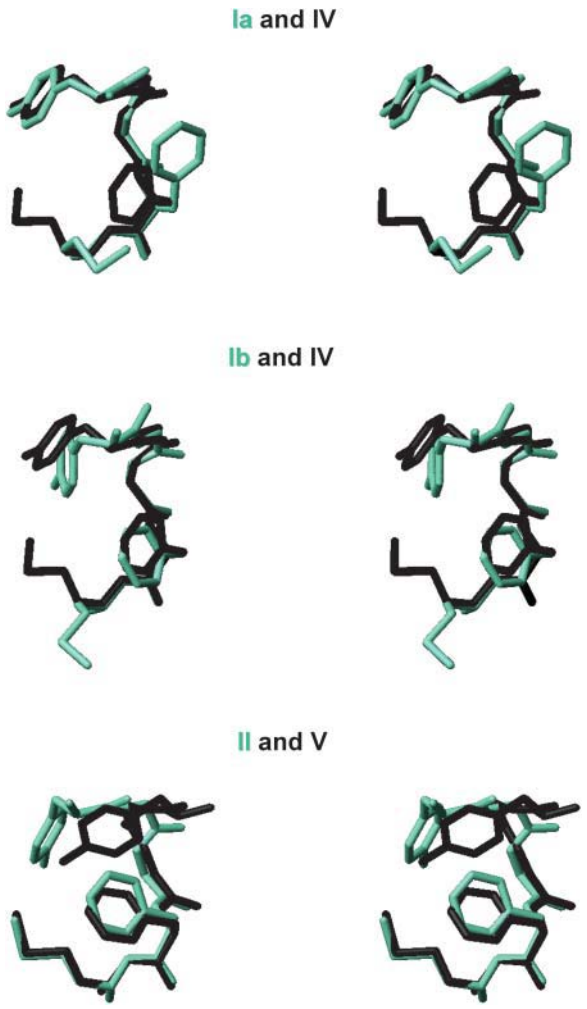

FIGURE 5 Comparison of the average structures of the different groups of conformers for methionine-enkephalin in PC bicelles to those in Bic/PG. The superposition was performed from residues 2 to 5. The minimized average structures were calculated using the Crystallography and NMR System (CNS) (Brünger et al., 1998).

conformation whereas the δ-opiates preferred an extended form (Hansen and Morgan, 1984; and references therein). Although ambiguities on the definition of μ- and δ-active conformations still remain (Deschamps et al., 1996; Hruby and Agnes, 1999), several recent studies on opioid peptides tend to suggest a folded conformation with the Tyr and Phe aromatic rings in proximity for the δ-selective opiates, whereas the aromatic rings would point in different directions in the μ-type peptides (Belleney et al., 1989; Groth et al., 1999; Hruby et al., 1988; Keys et al., 1988; Kolp et al., 1996; Lomize et al., 1996; Mosberg, 1999; Shenderovitch et al., 1991; Tourwé et al., 1995; Wang and Kuczera, 1996; Yamazaki et al., 1993).

Based on the latter structural grouping, it appears that Menk interacting with neutral and negatively charged bicellar systems would adopt conformations suitable for

TABLE 6 Statistics of the χ-angles for Tyr^1 and Phe^4 side chains of the Menk minimized average structure in each group

	Tyr^1 ($\pm 4°$)†		Phe^4 ($\pm 4°$)†	
Group*	χ_1	χ_2	χ_1	χ_2
Ia	−166°	82°	−21°	−86°
Ib	180°	74°	55°	−82°
II	−163°	74°	48°	−82°
IV	−152°	74°	48°	82°
V	−146°	82°	62°	−74°

*Minimized average structure obtained using the Crystallography and NMR System (CNS) (Brünger et al., 1998).
†χ-angle values determined by the program PROCHECK 3.4 (Laskowski et al., 1993).

binding to both μ- and δ-opiate receptors, which is consistent with the flexibility and nonspecific binding of these neuropeptides (Paterson et al., 1984). In PC bicelle systems, Groups Ia and Ib (and two structures of Group III) present similar conformations in which the tyrosine and phenylalanine rings are on opposite sides of the backbone and point in different directions. A similar conformation of Menk is also found in Bic/PG and corresponds to Group IV. This conformation would likely be compatible to μ-opiate receptors. Similar structural organizations of enkephalins with aromatic rings in opposite directions have also been found for Lenk in PC/PS vesicles (Milon et al., 1990) and cryoprotective mixtures (Picone et al., 1990).

Another Menk conformation was also obtained in which the aromatic rings are at closer distance (~5–6 Å) on the same side of the backbone and pointing toward each other. This structural arrangement would correspond to a δ-active conformation, and includes 44% of the Menk structures in PC bicelles (i.e., Group II plus two structures of Group III), and 32% of those found in Bic/PG (i.e., Group V). Despite a global structural analogy, however, Groups II and V are not identical and differ at the N-terminal portion of the peptide, as revealed by different Gly^2 and Gly^3 torsion angles. Similar conformations of enkephalins, some containing β-turns, have been reported in previous studies for Menk in lyso-PC and SDS micelles (Graham et al., 1992; Zetta et al., 1986), Lenk crystals (Aubry et al., 1989), Lenk in reverse bis (2-ethylhexyl) sulfosuccinate micelles (Rudolph-Böhner et al., 1997), and Menk in cesium perfluorooctanoate liquid crystals (Kimura et al., 1996).

Attempts have been made to rationalize the μ- and δ-receptor affinity of opioid peptides in terms of the tyrosine and phenylalanine χ_1-angle (Belleney et al., 1989; Hruby et al., 1988; Mosberg et al., 1990; Nikiforovitch et al., 1991; Shen and Freed, 2002; Tourwé et al., 1995; Wang and Kuczera, 1996; Yamazaki et al., 1993). The comparison of these angles to the values listed in Table 6 for Menk in neutral and zwitterionic bicelles is however complicated by the discrepancies observed in Tyr and Phe side-chain orientations in the different studies (Hruby and Agnes,

1999; Mosberg, 1999). Moreover, many of the opioid molecules used for the χ_1-angle investigations are small peptides with only one amino acid between the Tyr and Phe aromatic rings, and the Phe χ_1-angle value for opiate activity might depend on the Phe position in the peptide sequence. Further studies are thus necessary to shed light on the structure-activity relationships.

As detailed in the Results section, the structural differences between Groups Ia/1b and II could be attributed to small differences in Gly^2 ϕ-angles. In zwitterionic bicelles, both the μ- and δ-selective conformers appear to be probable. However, the Ramachandran plots suggest that the μ-selective conformers would be geometrically favored. More specifically, the backbone torsion angles for most of the peptide residues in Groups Ia, Ib, and III are found in favored regions of the Ramachandran plot as compared to Group II (δ-type) for which the majority (67%) of the residues are located in additionally allowed regions. It is therefore likely that the μ-binding form of Menk predominates in neutral bicelles.

The results obtained for Menk in negatively charged bicelles suggest that the μ-selective conformation could be favored over the δ-type. Sixty-eight percent of Menk structures belong to the μ-type Group IV whereas 32% belong to the δ-selective Group V. However, according to the Ramachandran plots, the dihedral angles are in the most favored regions for the majority of the residues, suggesting that both types of structures would be geometrically favored.

It is possible that additional NOEs and/or residual dipolar coupling constraints for Menk in the bicellar systems could refine the structure calculation and determine a preference for only one conformer, or that additional constraints could confirm the possibility of several conformers. Structural calculations using ambiguous NOEs (data not shown) were performed and the same number of groups of conformers were obtained for Menk in both PC bicelles and Bic/PG. The 80 structures shown in this study for met-enkephalin in bicelles and Bic/PG result from angle and distance restraints and are all energetically favorable and, thus, possible. Nevertheless, it is also conceivable that geometrically or energetically less favored structures could be stabilized in cell membranes when interacting with the lipids.

It is noteworthy that some NOE distance constraints were different for Menk in zwitterionic and negatively charged membranes, resulting in structural differences between the conformers obtained in PC bicelles and Bic/PG. This suggests that the peptide conformation could be affected by the membrane composition. The structural differences between the two types of conformers in zwitterionic and anionic bicelles could be due to the mobility of the N-terminus, which is related to the variations observed in the ϕ- and ψ-angles of the glycine residues, a naturally less constrained amino acid.

Structural differences between the conformers of Menk in PC bicelles and Bic/PG are not surprising. Enkephalins are flexible neuropeptides and their conformation is dependent on their environment. Biologically, opiate receptors are mainly found in the peripheral nervous system, the spinal cord and in several regions of the brain where the ratio of μ- and δ-binding sites has been shown to vary (Paterson et al., 1984). As different cells compose these parts of the body, it is expected that their membrane composition differs (Yorek, 1993). Our results suggest, therefore, that variations in the biological membrane composition would have an effect on the conformation adopted by enkephalins.

Interestingly, all the structures obtained in this study tend to form a hydrophobic patch by means of the residue side chains (Tyr, Phe, Met). This patch can be explained by the distance restraints resulting from the NOESY experiments. In particular, correlation peaks were observed between the peptide backbone protons and the Tyr^1, Phe^4, and Met^5 side chains. In addition, numerous NOEs were also observed between the Phe^4 and Met^5 side chains (Fig. 2). Therefore, from the ^{1}H NMR data, it appears that once in contact with an amphiphilic lipid membrane, the flexible opiate peptides could adopt different (μ- or δ-) conformations, and expose their hydrophobic patch to the apolar region of the bilayer with their amide region close to the surface. Our results are consistent with the amphiphilic character of enkephalins and their natural flexibility.

CONCLUSION

The conformation of methionine-enkephalin in fast-tumbling bicelles was investigated using neutral and negatively charged bicellar systems and multidimensional ^{1}H NMR. PFG experiments suggest favorable binding of the water-soluble neuropeptide to both the PC bicelles and Bic/PG. In addition, upon interaction with cell membranes, two main conformations of enkephalins are possible, which could correspond to μ- and δ-selective conformers. This result is consistent with the flexible nature and poor receptor selectivity of these neuropeptides. To our knowledge, this study of enkephalins is the first to evidence the possibility of both μ- and δ-active conformers for the enkephalins in membrane mimetics. The conformers of Menk in zwitterionic and negatively charged bicelle systems presented in this work may be different to those of the receptor-bound peptide in the human body. However, our results suggest that a variety of conformations are possible for the binding to each receptor subtype. Studies of enkephalins bound to μ- and δ-receptors are needed to complete our understanding of the biological activity of enkephalins and their mechanism of action.

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