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# Interaction of the Neuropeptide Met-Enkephalin with Zwitterionic and Negatively Charged Bicelles as Viewed by $^{31}\text{P}$ and $^2\text{H}$ Solid-State NMR

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**ABSTRACT** The interaction of the neuropeptide methionine-enkephalin (Menk) with bicelles was investigated by solid-state NMR. Bicelles composed of dimyristoylphosphatidylcholine (DMPC) and dicaproylphosphatidylcholine (DCPC) were modified to investigate the effect of the lipid headgroup and electrostatic charges on the association with Menk. A total of 10 mol % of DMPC was replaced by zwitterionic phosphatidylethanolamine (DMPE), anionic phosphatidylglycerol (DMPG), or phosphatidylserine (DMPS). The preparation of DMPE-doped bicelles (Bic/PE) is reported for the first time. The  $^{31}\text{P}$  and  $^2\text{H}$  NMR results revealed changes in the lipid dynamics when Menk interacts with the bicellar systems.  $^2\text{H}$  NMR experiments showed a disordering effect of Menk on the lipid chains in all the bicelles except Bic/PG, whereas the study of the choline headgroups indicated a decreased order of the lipids only in Bic/PE and Bic/PG. Our results suggest that the insertion depth of Menk into bicelles is modulated by their composition, more specifically by the balance between hydrophobic and electrostatic interactions. Menk would be buried at the lipid polar/apolar interface, the depth of penetration into the hydrophobic membrane core following the scaling Bic > Bic/PE > Bic/PS at the slightly acidic pH used in this study. The peptide would not insert into the bilayer core of Bic/PG and would rather remain at the surface.

## INTRODUCTION

Enkephalins are neurotransmitters found in the human central nervous system, especially in regions of the brain and spine associated with diffuse pain pathways (Hucho, 1986; Kruk and Pycoc, 1991). These opiate pentapeptides have the same receptors as morphine. In addition to the central control of respiration, they work to inhibit pain signals. Enkephalins are composed of five amino acids with the following sequence: Tyr-Gly-Gly-Phe-(Met or Leu) (Hughes et al., 1975). It is believed that these neuropeptides interact with the nerve cell membrane in order to adopt a bioactive conformation that will then fit onto the receptors (Gysin and Schwyzer, 1983; Behnam and Deber, 1984; Deber and Behnam, 1985; Sargent and Schwyzer, 1986). According to this mechanism called “membrane catalysis,” the polar headgroups of the lipids at the cell membrane surface interact with these amphiphilic hormones which enter into the membrane via hydrophobic interactions and undergo a conformational change. Then, the enkephalins would migrate to the receptor with the suitable structure for binding. Consequently, the study of enkephalin/membrane interactions is of great importance to obtain a better knowledge of pain relief mechanisms and, in the long term, to develop synthetic analgesics.

In order to better understand the interaction of enkephalins with neuronal membranes, numerous experiments have been conducted using circular dichroism (D’Alagni et al., 1996),

UV-visible (Young et al., 1992), UV-Raman (Takeuchi et al., 1992), and nuclear magnetic resonance (NMR) spectroscopy which have brought valuable conformational information. The NMR experiments performed in water showed no distinguishable secondary structure for enkephalins and bioactive enkephalin derivatives (Higashijima et al., 1979; Graham et al., 1992; D’Alagni et al., 1996), a result supported by molecular dynamics simulations (van der Spoel and Berendsen, 1997; Shen and Freed, 2002) and numerical analyses (Kinoshita et al., 1997). Phospholipidic membrane mimicking systems have been used to obtain relevant conformational and structural data on membrane-associated enkephalins by NMR spectroscopy. The interaction of enkephalins or enkephalin derivatives has therefore been studied in lysophosphatidylcholine (Behnam and Deber, 1984; Deber and Behnam, 1984; Zetta et al., 1986; Hicks et al., 1992) and lysophosphatidylglycerol micelles (Deber and Behnam, 1984), phosphatidylserine (PS) vesicles (Jarrell et al., 1980; D’Alagni et al., 1996), and in binary lipid mixtures such as PC/PS bilayers (Milon et al., 1990) and DMPC/DCPC bicelles (Sanders and Landis, 1994, 1995; Rinaldi et al., 1997). Sodium dodecylsulfate (SDS) micelles have also been used (Zetta et al., 1986; Picone et al., 1990; Graham et al., 1992; Hicks et al., 1992). Although structural results vary among these studies, there is a common agreement that both methionine- and leucine-enkephalin fold into a  $\beta$ -turn structure in lipid environments, stabilized by a H-bond between Gly<sup>2</sup> C=O and Met<sup>5</sup> (Leu<sup>5</sup>) N–H (Behnam and Deber, 1984; Milon et al., 1990) and that electrostatic interactions are responsible for the association of Menk with the membrane, with Tyr<sup>1</sup> located at the headgroup/acyl chain interface (Deber and Behnam, 1984; Milon et al., 1990). Although membrane catalysis is believed to play an important role in enkephalin biological activity, no extensive studies

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have been performed to compare the effects of the phospholipid headgroup on the localization of these peptides in the membrane. Therefore we have investigated the interaction of methionine-enkephalin (Menk) as viewed by the phospholipids using bicelles as model membranes.

Bicelles are composed of long- and short-chain phosphatidylcholines, typically dimyristoyl- and dicaproylphosphatidylcholine (DMPC and DCPC), although other long-chain lipids can also be used (Cho et al., 2001; Tiburu et al., 2001). These systems have the propensity to align in the magnetic field ( $B_0$ ) with the bilayer normal perpendicular to the direction of  $B_0$  at temperatures above 30°C and for DMPC/DCPC molar ratios greater than 2.5:1 (Sanders and Landis, 1994; Sanders et al., 1994; Raffard et al., 2000). It is believed that oriented bicelles are organized into a disklike morphology (Sanders and Schwonek, 1992; Sanders et al., 1994; Vold and Prosser, 1996). From  $^{31}\text{P}$ ,  $^2\text{H}$ , and 2D  $^1\text{H}$  NMR experiments and simulations (Vold and Prosser, 1996; Sternin et al., 2001), DMPC would be segregated on the bicelle rim while DCPC molecules would compose the planar section. However, recent small angle neutron scattering (SANS) studies (Nieh et al., 2001, 2002) and diffusion measurement of tracers in bicelles and water by NMR spectroscopy (Gaemers and Bax, 2001) suggest that oriented bicelles could adopt a lamellar DMPC structure perforated with toroidal defects created by DCPC domains. Results published by Sternin et al. (2001) could not distinguish between disks and perforated bilayers, but recent electron microscopy data (Arnold et al., 2002) revealed a discoidal shape for oriented bicelles. This model is supported by geometrical studies and spectral simulations which did not depend on the DMPC/DCPC molar ratio (Picard et al., 1999; Arnold et al., 2002). Nevertheless, in both perforated lamellae and discoidal models, the bicelle planar section is interesting for the study of peptide-membrane interactions since, unlike micelles which have a strong curvature, its flat surface is more similar to that of biomembranes, allowing proteins such as enzymes to retain their biological activity (Sanders and Landis, 1995; Czerski and Sanders, 2000). In addition, bicelles constitute an interesting mimicking medium for the study of membrane peptides since they are mainly composed of phosphatidylcholines which are important natural constituents of biomembranes (Cullis et al., 1996).

In order to better mimic the lipid diversity of biological membranes, attempts have been made to modify the composition of bicelles with phospholipids of different headgroups. So far, there are reports of the existence of bicelles doped with negatively charged lipids such as dimyristoylphosphatidylglycerol (DMPG) and dimyristoylphosphatidylserine (DMPS) (Struppe et al., 1998, 2000; Crowell and Macdonald, 1999). PS is found in human membranes whereas PG exists in prokaryote membranes. The aim of our work is to study the effect of the phospholipid headgroups on the interaction of Menk with biomembranes. We have therefore modified bicelles by substituting 10 mol %

of DMPC by either DMPG, DMPS, or dimyristoylphosphatidylethanolamine (DMPE). To our knowledge, the preparation of bicelles doped with DMPE (Bic/PE) is reported for the first time. Phosphatidylethanolamines are normal constituents of biomembranes, composing 15% of myelin membranes and 18% of erythrocyte membranes (Cullis et al., 1996). As a natural component of myelin membranes, PE is relevant for the study of the interaction of Menk with nerve cell membranes.

In this work, the effects of the modification of bicelle composition with DMPE, DMPG, and DMPS are first studied and emphasis is put on the new Bic/PE system. Although there are reports on the conformation of enkephalins in different lipid micelles, the perturbation created on model membranes by these neuropeptides have not been studied. We have therefore investigated the effect of the phospholipid headgroups on the interaction of methionine-enkephalin with bicelles and proposed models of insertion of enkephalins in zwitterionic and negatively charged bicelles. Information on the changes in the phospholipid headgroup dynamics is obtained by static and magic angle spinning (MAS)  $^{31}\text{P}$  NMR and  $^2\text{H}$  NMR spectroscopy is used to assess changes in the lipid acyl chain ordering and the phosphatidylcholine headgroup conformation.

## MATERIALS AND METHODS

### Materials

Dicaproylphosphatidylcholine (DCPC), dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylethanolamine (DMPE), dimyristoylphosphatidylglycerol (DMPG) and dimyristoylphosphatidylserine (DMPS), both in their protonated and deuterated forms, have been purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. Methionine-enkephalin has been obtained from Sigma-Aldrich (Oakville, Ontario, Canada) and extracted from its water insoluble impurities by washing 30 mg powder three times with 2 mL deionized water. The solution was centrifuged and the supernatant freeze dried.

### Sample preparation

Bicelles were made of DMPC and DCPC at a long chain-to-short chain lipid molar ratios of 2.70:1 for the  $^{31}\text{P}$  NMR experiments and 3.55:1 for the  $^2\text{H}$  NMR studies. In the modified bicellar systems, DMPC was replaced by 10 mol % of DMPE, DMPG, or DMPS. We have used 20% w/v of lipids in deionized water to avoid the interference of salts on the interaction of Menk with the lipid bilayers (Jarrell et al., 1980; Milon et al., 1990). The pH of all samples was  $\sim 5.5 \pm 0.1$ . A total of 25 mg of the different lipids were weighed and mixed in 100  $\mu\text{L}$  water, then submitted to at least three series of freezing (liquid  $\text{N}_2$ )/heating (40°C)/vortex shaking cycles until a viscous transparent gel was obtained. The peptide was also added to the lipid mixture before the addition of water. A lipid-to-peptide molar ratio of 25:1 was used for all experiments. The samples were stored at  $-20^\circ\text{C}$  before their analysis.

### NMR experiments

The static  $^{31}\text{P}$  NMR spectra of the bicellar systems were obtained at 162.0 MHz on a Bruker Avance 400 NB spectrometer (Bruker, Wissembourg,

France) using a phase-cycled Hahn echo pulse sequence with gated broadband proton decoupling (Rance et al., 1983). The samples were placed in a solid-state NMR 4-mm tube inserted into a 5-mm glass tube. 4096 data points were recorded, and a deuterium ( $D_2O$ ) lock was used. Typically, 256 scans were acquired with a  $90^\circ$  pulse length of  $6.0 \mu s$  and an interpulse delay of 4 s. A line broadening of 50 Hz was applied to all spectra. An 1800-s equilibration delay was allowed between each experiment at different temperatures. The chemical shifts were referenced relative to external  $H_3PO_4$  85% (0 ppm).

The  $^{31}P$  MAS spectra of the bicelles were recorded at 121.6 MHz on a Bruker ASX-300 spectrometer (Bruker Canada Ltd., Milton, Ontario, Canada). A 4-mm probe head was used for the magic angle spinning experiments. A Hahn echo sequence was used with gated broadband proton decoupling. A total of 1200 scans were acquired with a  $90^\circ$  pulse length of typically  $5.0 \mu s$ , an interpulse delay of 4 s, 4096 data points and a line broadening value of 50 Hz. A 1800-s equilibration delay was allowed between each experiment at different temperatures. The chemical shifts were referenced relative to external  $H_3PO_4$  85% (0 ppm).

All the  $^2H$  NMR spectra were carried out at 46.1 MHz on a Bruker ASX-300 spectrometer (Bruker Canada Ltd., Milton, Ontario, Canada) using a 4 mm sample tube inserted into a 5-mm coil of a homebuilt probe head. A quadrupolar echo sequence was used for the acquisition of the data (Davis et al., 1976). A  $90^\circ$  pulse length of  $4.7 \mu s$  was used with 2800 scans, 4096 data points and a line broadening value of 75 Hz. The recycle time was set to 0.5 s. Although this delay will most likely lead to a saturation of the chain methyl groups, the change in intensity will not affect the measurements of the quadrupolar splittings. The deuterium longitudinal ( $T_{1\rho}$ ) relaxation times were measured using a standard inversion-recovery pulse sequence coupled to the quadrupolar echo sequence, with a recycle time of at least  $5T_1$ . Transverse ( $T_{2\rho}$ ) relaxation data were obtained with a quadrupolar echo pulse sequence with variable delays ( $\tau$ ) and a recycle time of 0.5 s. A total of 4000 scans per spectra were acquired for both the  $T_{1\rho}$  and  $T_{2\rho}$  experiments.

## RESULTS

### Characterization of the bicellar systems

#### $^{31}P$ NMR

We have attempted in the present study to incorporate phosphatidylethanolamine into bicelles as this phospholipid is a natural constituent of myelin membranes. This would therefore allow the preparation of a mimicking system interesting for the study of neuron membrane-neurotransmitter interactions. Hence, DMPC was substituted with 10 mol % of DMPE since these lipids have the same acyl chain length. The DMPC and DCPC  $^{31}P$  characteristic resonances in well-aligned bicelles can be unambiguously attributed (Sanders and Schwonek, 1992; Picard et al., 1999; Arnold et al., 2002) and revealed that it is not possible to make bicelles with higher DMPE proportions. This was shown by the superposition of an isotropic signal and a powder pattern over the spectra typical of oriented bicelles, suggesting the coexistence of different lipid structures for Bic/PE made with more than 10 mol % of DMPE. Therefore, bicelles composed of 10 mol % of DMPG and DMPS were also prepared although it has been shown that DMPC can be replaced by up to 25 mol % of these anionic phospholipids (Struppe et al., 1998, 2000; Crowell and Macdonald, 1999; Whiles et al., 2001). The bicelle spectra show DMPC and DCPC resonances characteristic of oriented bilayers from  $33^\circ C$ , as

shown in Table 1, except for Bic/PS which orients from  $39^\circ C$  to  $50^\circ C$ . Bic/PE aligns within the narrowest range of temperatures (i.e.,  $33^\circ C$ – $36^\circ C$ ) whereas Bic and Bic/PG orient at temperatures up to  $45^\circ C$  and  $39^\circ C$ , respectively. All the systems display optical clarity within their range of orientation temperatures, likely indicating the formation of small structures such as bicelles.

Fig. 1 shows the  $^{31}P$  NMR spectra of the different bicelles at  $36^\circ C$ , except for Bic/PS which is presented at its lowest temperature of orientation ( $39^\circ C$ ). All these spectra are typical of well-aligned systems (Sanders and Schwonek, 1992; Crowell and Macdonald, 1999). At temperatures in the range of bicelle existence, two resonances are seen for normal bicelles, with an additional peak for modified bicelles. As described in previous reports, the most upfield and intense resonance is attributed to molecules (mostly DMPC) located in the planar section of the bicelles and aligned perpendicular to the direction of the magnetic field (Sanders and Schwonek, 1992; Picard et al., 1999; Nieh et al., 2002). The downfield resonance is attributed to molecules (mostly DCPC) on the highly curved region of the bicelle torus (Picard et al., 1999; Stermin et al., 2001). The third resonance in modified bicelles is attributed to DMPX (X standing for E, G, and S) as described in a previous study (Crowell and Macdonald, 1999). The resonances attributed to DMPG and DMPE in the bicelles are downfield to that of DMPC whereas the DMPS resonance appears upfield. The position of the DMPX resonances relative to that of DMPC in the bicelle spectra is consistent with the  $90^\circ$  orientation chemical shift ( $\delta_\perp$ ) observed in the  $^{31}P$  powder spectra of the different phospholipids (results not shown). This observation was also made with cardiolipin-doped bicelles (Parker et al., 2001).

As seen in Fig. 1, the DMPC and DCPC linewidths remain essentially unchanged in the presence of DMPE, whereas both phosphatidylcholine resonances are broader and more asymmetric when DMPG is added. Interestingly, the presence of DMPS does not affect the symmetry and linewidth of the DMPC and DCPC resonances. Finally, an upfield shift of the DMPC and DCPC resonances occurs when bicelles are doped with the zwitterionic and anionic lipids but this effect is more important in Bic/PE and Bic/PG.

**TABLE 1** Range of orientation temperatures ( $\pm 2^\circ C$ ) obtained from the  $^{31}P$  NMR spectra for the different bicellar systems in the absence and in the presence of Menk

System	$q = 2.70$		$q = 3.55$	
	Orientation temperatures ( $^\circ C$ )	Orientation temperatures ( $^\circ C$ ) + Menk	Orientation temperatures ( $^\circ C$ )	Orientation temperatures ( $^\circ C$ ) + Menk
Bic	33–45	33–45	30–44	35–46
Bic/PE	33–36	33–42	30–40	33–40
Bic/PG	33–39	33–42	32–44	32–49
Bic/PS	39–50	36–45	30–46	32–49

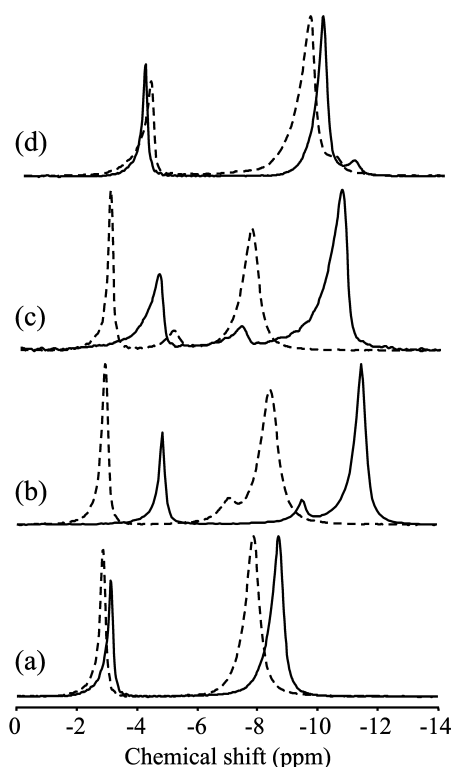


FIGURE 1  $^{31}\text{P}$  NMR spectra of the different bicellar systems in the absence (*solid*) and in the presence (*dotted*) of Menk. (a) Bic, 36°C, (b) Bic/PE, 36°C, (c) Bic/PG, 36°C, and (d) Bic/PS, 39°C. The bicellar samples were prepared in water and contain 20% (w/w) phospholipids with  $q = 2.70$  and a lipid-to-peptide molar ratio of 25:1.

#### $^2\text{H}$ NMR of deuterated acyl chains

$^2\text{H}$  NMR experiments on bicelles made of DMPC- $\text{d}_{27}$  have been performed to investigate the effect of DMPE and anionic lipids on the DMPC chain ordering. We have used a long-to-short chain molar ratio  $q$  of 3.55 since it has been shown that the diameter of discoidal bicelles increases linearly with  $q \geq 3$  (Vold and Prosser, 1996). Bigger bicelles were used to minimize the contribution of bicelle wobbling to the quadrupolar splittings in order to better assess any variation in the lipid chain ordering. As determined from the lineshape of the  $^2\text{H}$  NMR spectra, the different bicellar systems orient in a wider range of temperatures, with Bic/PE system being oriented up to 40°C, as shown in Table 1. This is consistent with the temperature-composition diagram proposed by Raffard et al. (2000) which shows that bicelles composed of 78 mol % DMPC ( $q = 3.55$ ) align on a wider range of temperatures than bicelles with 73 mol % DMPC ( $q = 2.70$ ).

The  $^2\text{H}$  NMR spectra of the different bicellar systems were compared at 37°C and are shown in Fig. 2. These spectra are typical of bicelles oriented at 90° with respect to the magnetic field, with well-defined resonances observed for most of the deuteron positions along the lipid acyl chain (Sanders and Schwonek, 1992; Struppe et al., 1998). The

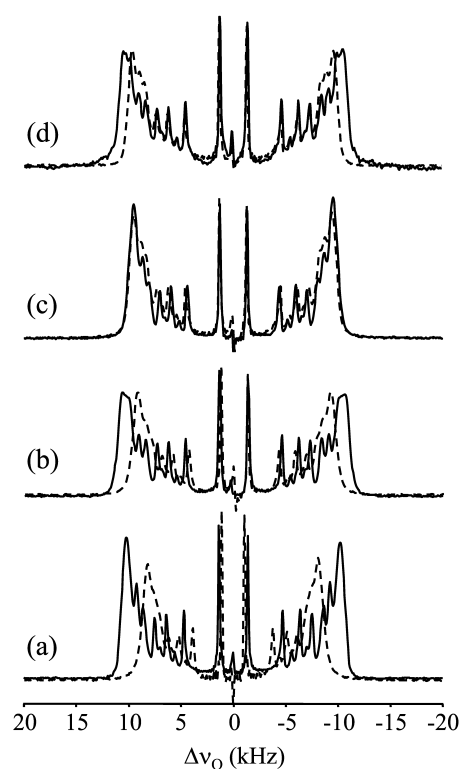


FIGURE 2  $^2\text{H}$  NMR spectra of the different bicellar systems containing DMPC- $\text{d}_{27}$  in the absence (*solid*) and in the presence (*dotted*) of Menk at 37°C. (a) Bic, (b) Bic/PE, (c) Bic/PG, and (d) Bic/PS. The bicellar samples were prepared in water and contain 20% (w/w) phospholipids with  $q = 3.55$  and a lipid-to-peptide molar ratio of 25:1.

quadrupolar splitting ( $\Delta\nu_Q$ ) values were used to determine variations in lipid chain ordering (Seelig, 1977) and the results obtained for the plateau and methyl regions of the lipid acyl chains are shown in Table 3. A decrease (increase) in the quadrupolar splittings is indicative of a disordering (ordering) effect of the peptide on the lipid acyl chains.

Fig. 2 shows that the doping of bicelles with DMPE induces a small increase in the DMPC acyl chain order whereas the presence of DMPG induces a small disordering effect, with the DMPC quadrupolar splittings reduced by  $\sim 6\%$  at both the plateau and methyl positions. No significant change is observed with the addition of DMPS. However, the spectra of DMPG- $\text{d}_{54}$  and DMPS- $\text{d}_{54}$  in Bic/PG and Bic/PS are almost superimposable to that of DMPC- $\text{d}_{27}$  (Fig. 3), which is indicative of a similar chain ordering of the two lipids in these bicellar arrangements. These findings have been verified with bicelles composed of DMPC- $\text{d}_{54}$  and gave similar results. Interestingly, when comparing the  $^2\text{H}$  NMR spectrum of DMPC- $\text{d}_{27}$  ( $\text{d}_{54}$ ) with DMPE- $\text{d}_{54}$  in Bic/PE (Fig. 3 and Table 2), it can be observed that the quadrupolar splitting is much greater ( $\sim 12\%$ ) in the plateau region of the DMPE acyl chain as compared to DMPC, which is indicative of a better ordering.

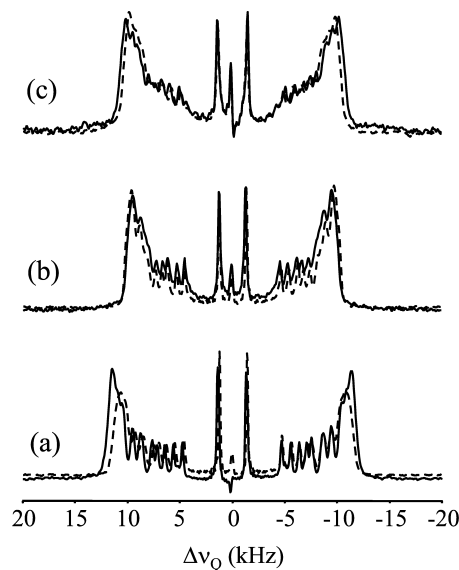


FIGURE 3  $^2\text{H}$  NMR spectra of the different bicellar systems composed of DMPX- $\text{d}_{54}$  in the absence (*solid*) and in the presence (*dotted*) of Menk at  $37^\circ\text{C}$ . (a) Bic/PE, (b) Bic/PG, and (c) Bic/PS. The bicellar samples were prepared in water and contain 20% (w/w) phospholipids with  $q = 3.55$  and a lipid-to-peptide molar ratio of 25:1.

*$^2\text{H}$  NMR of the deuterated choline headgroup*

$^2\text{H}$  NMR was also used to monitor changes in the conformation and order of the choline headgroup when modifying bicelles with a third phospholipid. To do so, DMPC deuterated on both the  $\alpha$ -methylene group (close to the phosphate group) and on the  $\beta$ -methylene group (close to the trimethylamine group) was used. The resulting NMR spectrum contains two resonances with large and small quadrupolar splittings respectively attributed to the  $\alpha\text{-CD}_2$  and  $\beta\text{-CD}_2$ . Previous studies have shown that the  $\alpha$ - and  $\beta$ -deuterons are sensitive to changes in conformation and order of the PC headgroup (Seelig et al., 1987) and this property has been extensively used to examine the electrostatic interactions between charged species and the membrane surface. In such cases, counterdirectional changes in the magnitudes of the  $\alpha\text{-CD}_2$  and  $\beta\text{-CD}_2$  quadrupolar splittings are indicative of changes in the membrane surface charge density. In this respect, phosphatidylcholine behaves like

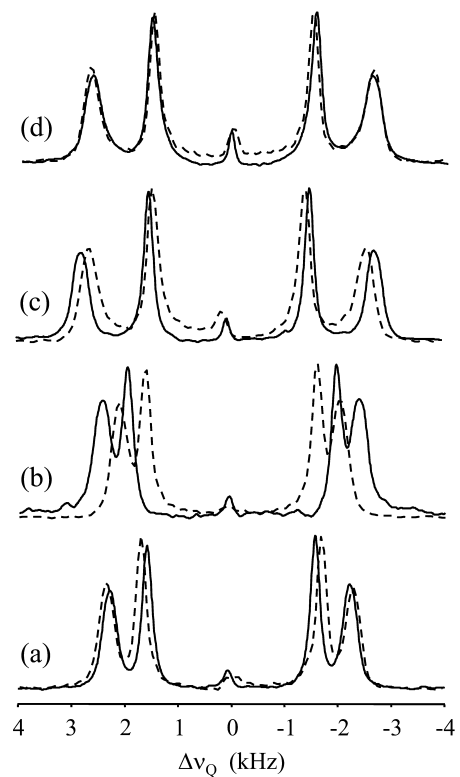


FIGURE 4  $^2\text{H}$  NMR spectra of the different bicellar systems with deuterated choline headgroups in the absence (*solid*) and in the presence (*dotted*) of Menk at  $37^\circ\text{C}$ . (a) Bic, (b) Bic/PE, (c) Bic/PG, and (d) Bic/PS. The bicellar samples were prepared in water and contain 20% (w/w) phospholipids with  $q = 3.55$  and a lipid-to-peptide molar ratio of 25:1.

a “molecular voltmeter” and in the presence of a negatively charged species at the membrane surface, an increase (decrease) in the  $\alpha\text{-CD}_2$  ( $\beta\text{-CD}_2$ ) is observed whereas a positive charge has the opposite effect.

Fig. 4 shows the  $^2\text{H}$  NMR spectra of the different bicellar systems prepared with DMPC- $\text{d}_4$ . The resonances attributed to both the  $\alpha$ - and  $\beta$ -deuterons are well resolved and the spectra are indicative of bilayers aligned with their main axis perpendicular to the magnetic field. The results presented in Table 3 indicate increases of 25% and 8% in the  $\alpha$ - and  $\beta\text{-CD}_2$  quadrupolar splittings, respectively, when DMPE is added to bicelles, suggesting a better ordering of the DMPC

**TABLE 2** Quadrupolar splittings of the plateau ( $\Delta\nu_p$ ) and methyl ( $\Delta\nu_m$ ) regions of DMPC- $\text{d}_{27}$  and DMPX- $\text{d}_{54}$  in the different bicellar systems at  $37^\circ\text{C}$  in the absence and in the presence of Menk at a  $q$  ratio of 3.55:1

System	$\Delta\nu_p$ (kHz)	$\Delta\nu_p$ (kHz) + Menk	Difference	$\Delta\nu_m$ (kHz)	$\Delta\nu_m$ (kHz) + Menk	Difference
Bic	20.4	16.6	−19%	2.75	2.33	−15%
Bic/PE	21.2	18.4	−13%	2.75	2.55	−7%
Bic/PG	19.0	18.9	−1%	2.59	2.65	+3%
Bic/PS	21.0	19.2	−8%	2.64	2.66	+1%
Bic/PE- $\text{d}_{54}$	22.8	21.5	−6%	2.69	2.67	−1%
Bic/PG- $\text{d}_{54}$	19.3	19.0	−2%	2.61	2.61	0%
Bic/PS- $\text{d}_{54}$	20.4	19.6	−4%	2.94	2.83	−4%

The error on the quadrupolar splitting values is estimated to be  $\pm 3\%$ .

**TABLE 3** Quadrupolar splittings of the  $\alpha$ - and  $\beta$ -deuterons of DMPC- $d_4$  in the different bicellar systems at 37°C in the absence and in the presence of Menk, at a  $q$  ratio of 3.55:1

System	$\Delta\nu_\alpha$ (kHz)	$\Delta\nu_\alpha$ (kHz) + Menk	Difference	$\Delta\nu_\beta$ (kHz)	$\Delta\nu_\beta$ (kHz) + Menk	Difference
Bic	4.46	4.66	+4%	3.13	3.40	+9%
Bic/PE	4.80	4.13	-14%	3.93	3.21	-18%
Bic/PG	5.49	5.19	-6%	3.03	2.88	-5%
Bic/PS	5.20	5.32	+2%	3.09	2.98	-4%

The error on the quadrupolar splitting values is estimated to be  $\pm 3\%$ .

headgroups. As the differences in  $\Delta\nu_\alpha$  and  $\Delta\nu_\beta$  values could be attributed to a change of orientation of the phospholipid headgroup, the tilt angle ( $\theta_t$ ) was calculated as described by Pinheiro et al. (1994) and no change in the choline tilt angle was observed. The difference in the choline deuteron response is therefore likely due to the lack of sensitivity of the  $\beta$ -deuterons in bicelles, as proposed by Crowell and Macdonald (1999).

The presence of anionic phospholipids in bicelles results in an increase of the DMPC  $\Delta\nu_\alpha$  and a small decrease in  $\Delta\nu_\beta$  (2% on average) as shown in Fig. 4. The effects of the anionic phospholipids on the  $\alpha$ - and  $\beta$ -splittings are not surprising as they are typical of a conformational change of the choline headgroup when interacting with a negatively charged species. Here again, the different response of the  $\alpha$ - and  $\beta$ -deuterons could be attributed to the lack of sensitivity of the  $\beta$ -position and was also observed when cardiolipin was added to bicelles (Parker et al., 2001). These results are supported by tilt angle calculations which showed a change in  $\theta_t$  from  $70^\circ \pm 2^\circ$  to  $37^\circ \pm 2^\circ$  on average when bicelles are doped with DMPG and DMPS. The results are also consistent with those obtained by Crowell and Macdonald (1999) with bicelles containing different proportions of DMPG.

### Effect of Menk on the bicellar systems

The effect of Menk on the four bicellar systems was studied by  $^{31}\text{P}$  and  $^2\text{H}$  NMR. The results are presented below and will be further analyzed in the Discussion section.

#### $^{31}\text{P}$ NMR

The effect of Menk on the different bicellar systems was first studied by static  $^{31}\text{P}$  NMR and the spectra are shown in Fig. 1. The integrity of these systems appears to be preserved when Menk is added as not only the spectra are typical of well-aligned bicelles but the samples were also optically clear. A lipid-to-peptide molar ratio of 25:1 was used as methionine-enkephalin is expected to be fully bound to the lipids at this ratio according to previous studies in similar membrane environments (Deber and Behnam, 1984; Sanders and Landis, 1994). Moreover, considering the  $\text{pK}_a$  values of the amino (7.5) and carboxyl (3.9) groups determined by Jarrell et al. (1980), Menk likely exists in a zwitterionic form at the pH ( $\sim 5.5$ ) used in the present study.

A first effect of the peptide is seen on the range of orientation temperatures of modified bicelles at the  $q$  value of 2.70. As observed in Table 1, Bic/PE aligns at higher temperatures in the presence of Menk. The degree of alignment of Bic/PE is also slightly affected by the presence of Menk as revealed by the  $^{31}\text{P}$  NMR spectra. A broadening of the phospholipid resonances occurs for Bic/PE when Menk is added while no significant changes are observed for the nonmodified bicelles (Bic). The temperature range in which anionic Bic/PG aligns is increased by the presence of Menk, whereas the opposite effect is observed for Bic/PS as shown in Table 1. The  $^{31}\text{P}$  spectra presented in Fig. 1 show narrower and more symmetric lipid resonances when the peptide is added to Bic/PG, while a slight broadening and asymmetry is seen for Bic/PS.

Another effect of Menk on the bicellar systems is a downfield shift of the phospholipid resonances. This effect is particularly important for Bic/PE and Bic/PG with a chemical shift variation of  $\sim 30\%$  on average for the different lipid resonances. These changes in the chemical shift values could be due to a modification of the phosphorus atom environment upon interaction of the phospholipid headgroups with a deshielding moiety of Menk. This would result in a change of the lipid isotropic chemical shift ( $\delta_{\text{iso}}$ ). It is also possible that Menk affects the mobility of the phospholipids in the bicelle systems and the headgroup orientation (Cullis et al., 1976; Seelig, 1977; Seelig and Seelig, 1980).

In order to verify the first hypothesis, we have performed  $^{31}\text{P}$  magic angle spinning (MAS) NMR experiments on the bicellar systems. The spectra are shown in Fig. 5 and the different lipid resonances could not be resolved under these experimental conditions, except for Bic/PG. No significant change in the  $\delta_{\text{iso}}$  chemical shift is observed when Menk is added to the different systems. The lipid resonance linewidth is also unchanged when Menk is added.

#### $^2\text{H}$ NMR of deuterated acyl chains

In order to gain information on the effect of the peptide on the acyl chain ordering, we have prepared bicellar systems with DMPC deuterated on the *sn*-2 acyl chain (DMPC- $d_{27}$ ). The  $^2\text{H}$  NMR spectra in the absence and in the presence of Menk are presented in Fig. 2. A first observation is an important decrease of the quadrupolar splittings at all the positions on the acyl chain when Menk is interacting with

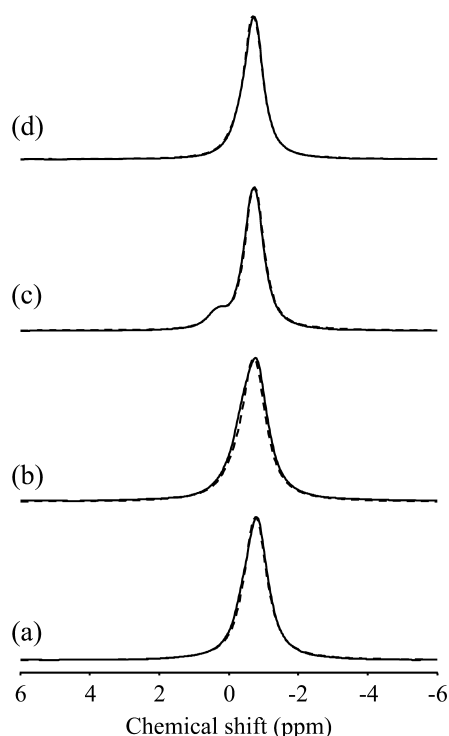


FIGURE 5  $^{31}\text{P}$  MAS NMR spectra of the different bicellar systems in the absence (solid) and in the presence (dotted) of Menk at  $37^\circ\text{C}$ . (a) Bic, (b) Bic/PE, (c) Bic/PG, and (d) Bic/PS. The bicellar samples were prepared in water and contain 20% (w/w) phospholipids with  $q = 2.70$  and a lipid-to-peptide molar ratio of 25:1.

Bic and Bic/PE. The quadrupolar splitting values at the plateau and methyl regions are reduced by 19% and 15% respectively for Bic, and by 13% and 7% for Bic/PE (Table 2). The smaller change at the phospholipid chain terminus is not surprising since greater motions exist before the addition of the peptide. A decreased ordering is also observed for the DMPE acyl chains in Bic/PE, as shown in Fig. 3. However, the effect of Menk on DMPE is smaller than that observed on DMPC as the plateau quadrupolar splitting is reduced by only 6% and  $\Delta\nu_m$  remains unchanged.

As observed in Figs. 2 and 3, the effect of Menk on Bic/PS is mainly localized at the region of the lipid chains close to the polar/apolar interface, with a decrease in  $\Delta\nu_p$  of 8% and 4% for DMPC and DMPS, respectively (Table 2). Finally, no significant change is induced by Menk on both the DMPC and DMPC acyl chains in Bic/PG, as seen in Figs. 2 and 3.

#### $^2\text{H}$ NMR of the deuterated choline headgroup

The effect of Menk on the DMPC headgroup conformation has been studied as it is sensitive to the electric surface charge and headgroup ordering (Seelig et al., 1987). Bicellar systems composed of DMPC- $d_4$  were thus used. The spectra are shown in Fig. 4 and the corresponding quadrupolar splittings are presented in Table 3. It can be observed that the

effect of Menk on Bic and Bic/PS is very small, within the error of the measurements. In the other zwitterionic system Bic/PE, both the  $\alpha$ - and  $\beta$ - $\text{CD}_2$  splittings are decreased in the presence of the peptide by 14% and 18%, respectively, whereas Fig. 4 shows a decrease of 6% and 5% in the  $\alpha$ - and  $\beta$ -splittings of Bic/PG respectively upon the interaction with Menk. Finally, no change was observed on the choline headgroup tilt angle nor on the choline headgroup spin-lattice and spin-spin relaxation times for all the systems.

## DISCUSSION

The first goal of the present study was to substitute bicelles with different phospholipids in order to investigate the effect of the lipid headgroup on the interaction of Menk with biomembranes. A new bicellar system, Bic/PE, was prepared and shown to be suitable for the study of peptide/membrane interactions. In the second part of the study, Menk was added to the bicellar systems to gain information on the interaction of enkephalins with lipid membranes. The  $^{31}\text{P}$  and  $^2\text{H}$  NMR spectra typical of well-aligned bicelles show that the bicelle integrity is preserved for all the systems when interacting with Menk, providing a good system for studying the effect of this peptide on aligned membranes. Our results suggest a different degree of insertion of Menk in the four membrane systems. The results obtained for each type of bicelle are discussed below.

#### Effect of modifying the bicelle composition

The results obtained in the present solid-state NMR study indicate that the modification of the bicelle composition leads to aligned systems with different characteristics. First, the  $^{31}\text{P}$  NMR results show that bicelles doped with 10 mol % of DMPE are magnetically alignable bilayers suitable for the study of peptide-lipid systems. The phospholipid resonances observed in Fig. 1 *b* are indicative of a great degree of alignment for this nerve membrane mimicking system. They also reveal that DMPE affects the PC headgroup motions as the DMPC and DCPC resonances are shifted upfield while the  $\delta_{\text{iso}}$  remains unchanged. The  $^{31}\text{P}$  NMR study also reveals that these new zwitterionic bicelles align within a smaller range of temperatures as compared to Bic or anionic bicelles.

The  $^2\text{H}$  NMR data show that the degree of lipid ordering is slightly increased when DMPE is added to bicelles, especially at the polar head/lipid chain interface. This result could be attributed to the small size of the phosphatidylethanolamine headgroup, allowing the phospholipids to be closer to each other with the  $\text{NH}_3^+$  groups hydrogen bonded to the  $\text{PO}_4^-$  moieties (Seelig, 1978). Interestingly, the DMPE acyl chains showed a better ordering as compared to DMPC (Fig. 3). The presence of gel-phase DMPE in fluid Bic/PE can not be excluded. Previous studies have shown the coexistence of fluid PC and gel-phase PE at temperatures between the phase transition of the isolated phospholipids



(Sackmann, 1978, Arnold et al., 1981). An increase of the  $\alpha$ -quadrupolar splitting, and to a lesser extent of  $\Delta\nu_\beta$ , was observed for the deuterated choline headgroup, indicating a better ordering when bicelles are doped with DMPE. The difference in the  $\alpha$ - and  $\beta$ -deuteron response could not be due to a change in the PC headgroup orientation as the tilt angle remained unchanged. It is thus more likely attributable to a lack of sensitivity of the  $\beta$ -deuterons in bicelles as proposed by Crowell and Macdonald (1999).

The substitution of DMPC with 10 mol % of DMPS resulted in no significant effect on the lipid chain ordering. In addition, the order of DMPS is similar to that of DMPC (Figs. 2 and 3), in agreement with the miscibility of PC and PS observed in a previous study (Silvius and Gagné, 1984). The  $^{31}\text{P}$  NMR results revealed that the Bic/PS system aligns at higher temperatures, above the DMPS fluid-to-gel phase transition of 35°C, and that the degree of alignment is not perturbed by the presence of DMPS. Moreover, the presence of DMPS in bicelles induces only a minor shift in the  $^{31}\text{P}$  resonances positions, indicating only small effects on the phospholipid dynamics. Finally, the  $^2\text{H}$  NMR investigation of the effect of DMPS on the choline headgroups in Bic/PS showed a voltmer effect of the anionic lipid, which is consistent with the increased surface charge of the bilayers.

Interestingly, the presence of DMPG has a greater effect on the phospholipid dynamics in bicelles as revealed by an upfield shift of both the DMPC and DCPC  $^{31}\text{P}$  resonances. In addition, this anionic lipid affects the symmetry and linewidth of the phosphatidylcholine  $^{31}\text{P}$  resonances, as compared to DMPS (Fig. 1). As proposed by Struppe et al. (2000), this could be explained by a decreased liquid crystalline ordering due to the electrostatic repulsion between the charged headgroups. This repulsion could be due to interactions between adjacent bicelles. The repulsive effect of the negative charges in anionic bicelles is not seen in Bic/PS. This could be explained by the physical properties of DMPS which headgroup is composed of an acidic moiety with a  $\text{pK}_a$  of 5.5 (Marsh, 1990). Therefore, only about half of the DMPS molecules would be negatively charged at the pH of 5.5 used in this study. Correspondingly, the observed effect of DMPS on the choline deuterated headgroup is smaller than that of DMPG (Fig. 4).

In addition to a smaller degree of alignment, Bic/PG aligns within a smaller range of temperatures, as compared to Bic. Moreover, a smaller ordering was observed along both the DMPC and DMPG acyl chains. Since PG and PC have a similar headgroup structures, a similar ordering was expected in both phospholipids when mixed together (Seelig and Seelig, 1980). These results are consistent with a previous study reporting only slight changes in the lipid chain order when substituting DMPC by 25 mol % of DMPG in bicelles (Struppe et al., 1998).

The overall results suggest that the bicelles become slightly more ordered in the presence of DMPE whereas DMPG tends to fluidize the system. On the other hand, the

presence of DMPS does not significantly affect the bicelles. The doping of bicelles with 10 mol % of PE, PG, and PS therefore allows the modulation of the bicelle properties which are of great importance when studying peptide-lipid interactions.

## Effect of Menk on the different bicellar systems

### *Menk in nonmodified bicelles*

A clear picture of the interaction of methionine-enkephalin with nonmodified bicelles can be deduced from the results obtained by  $^{31}\text{P}$  and  $^2\text{H}$  NMR. First, the bicelle orientation temperatures are not changed by the presence of Menk, nor the degree of alignment. The chemical shift variation of DMPC and DCPC induced by the interaction with Menk could not be attributed to a change in the phosphorus nucleus environment, as verified by  $^{31}\text{P}$  MAS NMR, but is most likely due to a small disordering of the lipid phosphate group or to a change in the headgroup orientation. Since the study of the lipid choline moiety by  $^2\text{H}$  NMR revealed no change in the tilt angle, nor in the longitudinal and transverse relaxation times, it is therefore more likely that Menk has a dynamic effect on the lipid headgroup motions with correlation times greater than  $10^{-5}$  s (Macdonald, 1997).

In the light of these results, it appears that Menk could be located at the polar/apolar interface of the bilayer, allowing a greater area per lipid and consequently a decrease in the lipid chain ordering (Koenig et al., 1999). This location is in agreement with a previous study (Deber and Behnam, 1984) which explained the association of enkephalins with lysophosphatidylcholine via hydrophobic interactions. The binding would favor a peptide structure which places the hydrophobic moieties of the peptide on a non polar face suitable for the interaction. In the model proposed by Behnam and Deber (1984), Menk would fold into a  $\beta$ -turn structure with an H-bond between  $\text{Gly}^2 \text{C}=\text{O}$  and  $\text{Met}^5 \text{NH}$ , positioning the side-chain substituents of amino acids such as Met, Phe, and likely Tyr toward the hydrophobic interior of the lipid system.

### *Menk with Bic/PE*

The results obtained by  $^{31}\text{P}$  and  $^2\text{H}$  NMR suggest that the Bic/PE order is highly affected by the presence of methionine-enkephalin. The system aligns within a broader range of temperatures when Menk is added, likely indicating that the peptide prevents these bicelles to break into different structures such as micelles or bilayers. In addition, an important disordering effect is observed on the choline headgroup by  $^2\text{H}$  NMR, as revealed by a decrease in both the  $\alpha$ - and  $\beta$ - $\text{CD}_2$  splittings upon the addition of the peptide. As described for the pure bicelles, the significant downfield shift of the  $^{31}\text{P}$  resonances in the presence of the peptide might be explained by an increased motion of the phosphate moiety

although a conformational change can not be excluded. Moreover, the peptide has a disordering effect on both the DMPC and DMPE acyl chains but the extent of this effect is slightly greater on DMPC. A preferential interaction of Menk with DMPC is possible but further experiments would be necessary to confirm this result.

Our data suggest that Menk is located at the bilayer interface, altering the hydrogen bonds between the phospholipid headgroups and resulting in an increased disorder of the headgroup. The location of Menk at the interface would also allow greater motion of the lipid acyl chains but to a lesser extent than the effect observed on Bic. Methionine-enkephalin in Bic/PE would be inserted into the membrane but most likely with a reduced penetration of the peptide in the bilayer interface as compared to bicelles. Since the PC and PE headgroup structures are similar, the same conformation would be expected for the peptide in Bic and Bic/PE.

#### *Menk with Bic/PG*

A different type of interaction of Met-enkephalin is observed when 10 mol % of DMPC is substituted by anionic DMPG. The results obtained in the present study suggest an association of the peptide at the bilayer surface with specific effects on the lipid headgroups. First, a significant downfield shift of the lipid resonances in the presence of Menk was observed by  $^{31}\text{P}$  NMR, with no change in the choline tilt angle and spin-lattice and spin-spin relaxation times. This suggests an increased motion of the lipid phosphate moiety although the possibility of a conformational change can not be excluded. A small decrease in both the methylene  $\Delta\nu_\alpha$  and  $\Delta\nu_\beta$  quadrupolar splitting values is indicative of a disordering effect of the peptide on the PC headgroups in Bic/PG. The  $^{31}\text{P}$  NMR spectra show an increase in the range of orientation temperatures of Bic/PG upon the interaction with the enkephalin. Since it has been suggested that Menk can interact electrostatically with lysoPG micelles via its  $\text{NH}_3^+$  group at a pH of 6 (Deber and Behnam, 1984), the Menk positive moiety could partially neutralize the DMPG negative charge when added to Bic/PG. This would reduce the repulsion between the Bic/PG bilayers and consequently increase the range of orientation temperatures. This is in agreement with the  $^{31}\text{P}$  spectrum which shows narrower and more symmetric lipid resonances when the peptide is added, indicative of a greater degree of alignment (Arnold et al., 2002). Finally,  $^2\text{H}$  NMR reveals no effect of the peptide on both the DMPC and DMPG acyl chains.

Our results therefore suggest that Met-enkephalin has a different interaction with Bic/PG as compared to the model proposed for zwitterionic bicelles. It is very likely that the peptide interacts electrostatically with DMPG and sits at the surface of the bilayer where it would not perturb the lipid chain ordering. According to Kyte and Doolittle's hydrophobicity indices, Menk is both hydrophilic and hydropho-

bic (Kyte and Doolittle, 1982). It is also water soluble and attempts have been made to determine the structure of enkephalins and analogs in an aqueous environment (Graham et al., 1992; Gußmann et al. 1996; Amodeo et al., 1998; Fiori et al., 1999). It is believed that an equilibrium involving different conformers would exist in solution (Schiller, 1984, and references therein), and bent structures including  $\beta$ -turns have been observed (Schiller, 1984; Gußmann et al. 1996; Fiori et al., 1999). At the slightly acidic pH used in this study, it is very likely that the Menk's positive charge at the N-terminal interacts with the DMPG negative charge in Bic/PG. It is therefore possible to imagine a bent conformation for Menk in which the contact of the hydrophobic side chains with water would be prevented by the formation of a cluster exposing the more hydrophilic amides to the aqueous environment, and the N-terminal group would interact electrostatically with the bilayer negative charge. These results slightly differ from those obtained by Deber and Behnam which proposed that methionine-enkephalin would interact both hydrophobically and electrostatically with lysoPG micelles.

#### *Menk with Bic/PS*

Surprising results are obtained when Menk is added to Bic/PS. Although a similar interaction would be expected with both anionic bicelles, Met-enkephalin seems to associate differently with Bic/PS and Bic/PG. This difference could however be due to the pH value used in this study at which only about half of the DMPS molecules are charged.

First, the  $^{31}\text{P}$  NMR data indicate that the range of orientation temperatures is slightly reduced when Menk is added to Bic/PS. In addition, the lipid resonances are broadened and more asymmetric, likely due to a greater orientation distribution of the bicellar system. It thus appears that Menk affects the bicelle orientation, which could be attributed to a destabilization of the lipid arrangement at higher temperatures. The changes observed in the lipid  $^{31}\text{P}$  chemical shifts in the presence of Menk are small and both the  $\alpha$ - and  $\beta$ -quadrupolar splittings of the deuterated choline headgroups show practically no changes for Bic/PS upon the interaction with Menk, indicating very little effect of the peptide on the lipid headgroup. Finally, the acyl chain order is only slightly decreased in the presence of Menk.

Previous studies (Jarrell et al. 1980; Milon et al., 1990) have proposed that the  $\text{NH}_3^+$  group of enkephalin is essential for binding to PS and PC/PS vesicles and, as discussed above, half of DMPS molecules in Bic/PS are negatively charged at a pH of 5.5. It is thus possible that Menk interacts with the PS carboxylate group but as a first step in the binding of the peptide to the Bic/PS membranes. The calculation of the association constants ( $K_a$ ) shows that it is most likely that Menk interacts with DMPC molecules in the Bic/PS system. The  $K_a$  of enkephalins in lysoPC ( $3.14 \times 10^1 \text{ M}^{-1}$ ,  $23^\circ\text{C}$ ) (Deber and Behnam, 1985) and the dissociation constant of  $4 \times 10^{-1} \text{ M}$

(thus  $K_a = 0.25 \times 10^1 \text{ M}^{-1}$ ,  $30^\circ\text{C}$ ) for the neuropeptides in PS vesicles (Jarrell et al., 1980) have been determined by  $^{13}\text{C}$  NMR at a pH of  $\sim 6$ . The association constant values can also explain the different effect of the peptide on anionic Bic/PG and Bic/PS. More specifically, the electrostatic interactions would be favored in the Bic/PG system as a  $K_a$  of  $5.2 \times 10^1 \text{ M}^{-1}$  ( $23^\circ\text{C}$ ) has been calculated for enkephalins interacting with lysoPG micelles (Deber and Behnam, 1984).

In general, these results suggest a different interaction of Menk with Bic/PS as compared to the other anionic bicelles. As the free energy of association of Menk is greater for PC than PS, it is possible that the peptide is first electrostatically attracted by DMPS and then interacts hydrophobically with DMPC. On average, the peptide would be localized at an intermediate position between the membrane surface and interface as the effect of Menk on the DMPC acyl chains is smaller than what is observed for Bic and Bic/PE. The DMPC headgroup conformation and acyl chain ordering (close to the interfacial region) would be slightly disrupted, with little effect on the phospholipid headgroup motion. The small effects induced by Menk on Bic/PS are in agreement with previous studies in PS/PC vesicles (Jarrell et al., 1980; Milon et al., 1990).

## CONCLUSIONS

The goal of the present study was to investigate the effect of different lipid polar headgroups on the interaction of the peptide methionine-enkephalin with lipid membranes. The preparation of Bic/PE is reported for the first time and the NMR data show that DMPE induces a better ordering of the phospholipid acyl chains and headgroup in the bicelles. Though these novel bicelles orient within a narrower range of temperatures, this system is stable and readily amenable to the study of peptide structure. Our study has shown that replacing 10 mol % of DMPC in bicelles with phospholipids of different headgroup characteristics induces modifications in the bicelle properties.

We have performed a detailed study of the effect of the phospholipid headgroups on the interaction of methionine-enkephalin with bicelles. First, it was shown that the bicelle integrity is preserved upon the addition of Met-enkephalin. The determination of the peptide conformation is thus possible in the bicellar systems studied in this work and is currently performed in our laboratory. Our results also suggest that Menk has a similar interaction with both zwitterionic Bic and Bic/PE. We suppose the insertion of Menk at the lipid chain/headgroup interface which would decrease the acyl chain order and likely affect the headgroup motion.

Though a similar interaction of Menk was expected with both anionic bicelles, the results are surprisingly different and most likely attributed to the pH of the sample at which only about half of the DMPS molecules bear a negative charge. Met-enkephalin would be located at the polar/apolar interface

in Bic/PS whereas it would sit on the bilayer surface in Bic/PG. A common feature could be electrostatic interactions between the Menk  $\text{NH}_3^+$  and the PG and PS negative charge, but hydrophobic interactions with DMPC would be more important in the interaction of Menk with Bic/PS.

In the light of this study, it appears that the depth of insertion of Menk in the bicellar systems is modulated by the composition of the bilayers such as Bic > Bic/PE > Bic/PS > Bic/PG under the slightly acidic pH conditions used in this study. More specifically, the insertion of enkephalins in the membranes would depend on the balance between electrostatic and hydrophobic interactions.

It is known that enkephalins are flexible peptides and that their conformation is thus medium-dependent. It is also believed that in addition to structural requirements, the interaction of opiate peptides with the membrane lipid phase would be involved in the receptor subtype ( $\mu$ ,  $\delta$ , or  $\kappa$ ) selection (Schwyzer, 1986). The opioid receptors are distributed in the central and peripheral nervous systems, as well as in the intestinal tract (Paterson et al., 1984), and it is very likely that the membrane composition of the numerous cells composing these body parts varies. Our results therefore suggest that variations in the pH and membrane composition at different locations in the body might not only induce preferential subtype selections for enkephalins but also favor certain conformations and locations of the peptide in the membrane.

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