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Conformational study of [Met5]enkephalin-Arg-Phe in the presence of phosphatidylserine vesicles

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The interaction of [Met5]enkephalin-Arg-Phe with phosphatidylserine (PtdSer) was studied by circular dichroism (CD), two-dimensional nuclear magnetic resonance spectroscopy, hybrid distance geometry simulated annealing (DG-SA) and molecular dynamics (MD) calculations.

The very low solubility of [Met5]enkephalin-Arg-Phe and the instability of the solution containing PtdSer vesicles at low pH values did not allow us to observe the amide proton resonances in the usual two-dimensional NMR work. NOESY cross-peaks of protons of side chains from two-dimensional NMR were converted into distances which were used as restraints for modelling with DG-SA and MD. Our results indicate that, in aqueous solutions at pH 7.68 [Met5]enkephalin-Arg-Phe exists in the absence of PtdSer as a random distribution of conformers, whereas in the presence of PtdSer it adopts conformations containing a common orientation of the bonds of Cα2, Cα3, Cα4 and Cα5, although different orientations of the peptide planes are consistent with the results.

Two of the reported conformers from MD simulations are characterized by the presence of a 2←4 γ and inverse γ turns centered on Gly3. A gradual decline of order was observed when moving from the central moiety of the peptide to both the N-terminus and C-terminus. Finally, the DG-SA and MD calculations resulted in a structure such that the orientation of the Phe4 and Met5 side chains favours hydrophobic interactions with the apolar portion of the PtdSer vesicle to form a hydrophobic cluster. These data support the hypothesis of a role of lipids to modify the conformation of [Met5]enkephalin-Arg-Phe to permit the interactions with the receptor site.

Keywords: [Met5]enkephalin-Arg-Phe; phosphatidylserine vesicle; NMR; distance geometry; molecular dynamics calculation.

The existence of opioid peptides in the mammalian brain was described in the mid 1970s as result of an explicit search for the endogenous receptors of opium alkaloids [1, 2]. The molecular structure of these receptors, however, has been clarified only from 1984 onwards [3–7]. In the intervening years, a body of data was collected indicating the existence of interactions between opioid peptides and several acidic lipids, notably phospholipids [8]. Opioid peptides bind different lipids, specifically phosphatidylserine [9, 10] and gangliosides [11]. The binding is sensitive to lipid-specific reagents such as phospolipase A [9] or aryl sulfatase [12]. In addition, the binding reduction caused by the above agents can be suppressed by the addition of the appropriate lipids [13]. In all opioid peptides studied, lipid binding appears to induce a shift towards a more ordered structure. On the basis of these data, proteolipidic models have been proposed for the opioid receptors [14]. The determination of the molecular structure of opioid receptors, however, superseded these models, at least in their original formulation: opioid receptors belong to the large and extensively studied family of the guanine-nucleotide-binding regulatory(G)-protein-coupled receptors [15]. In all of these receptors, the interactions with the ligand take place with the side chains of the amino acid residues lining the cavity formed by the seven α-helical transmembrane segments of the protein's single polypeptide chain. As noted above, it is difficult to account for an involvement of lipids in such a model.

However, the experimental evidence for lipid involvement appears too firm to be disregarded. The data could be made to agree with the accepted model for opioid receptors under a hypothesis involving two-stage recognition of the opioid peptides [16–21]. According to this hypothesis, in a polar environment the peptide exists in an extended conformation. The interaction with membrane lipids induces a partially ordered conformation (first stage). In this conformation, the peptide complies with the constraints given by the relative position of the side chains of the residues forming the receptor's binding site, and is able to bind the receptor. This process induces another

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Abbreviations. [Met5]enkephalin-Arg-Phe, Tyr-Gly-Gly-Phe-Met-Arg-Phe, (MEAP); PtdSer, phosphatidylserine; COSY-DQF, correlation spectroscopy double-quantum filtered; TPPI, time-proportional phase increments; ROESY, rotating-frame dipolar-correlated two-dimensional spectroscopy; DG-SA, hybrid distance geometry simulated annealing; MD, molecular dynamics.

conformational change (second stage), that fine-tunes the peptide conformation to the constraints imposed by the relative position of the amino acid residues involved in the binding process. Existing data on the structure of opioid peptides support this model. On the basis of both theoretical and experimental studies, the degree of ordered structure of opioid peptides such as enkephalins is generally very limited in polar environments [22, 23]. In less polar environments, however, the structure of the pentapeptides shifts towards a β-turn stabilized by intramolecular hydrogen bonds [24]. Data concerning longer polypeptides like endorphins allow similar interpretation: a reduction of the environment polarity induces an increase of ordered structure, in this case an α-helix [11]. Yet in the case of opioid peptides a two-stage interaction is not supported by direct experimental evidence, a workable model for the dynamics of transfer from the lipid to the opioid receptor is also lacking. Finally, the functional significance of the above-mentioned changes can be overestimated: simple energy considerations predict an increase of structural order upon reduction of the environment polarity. Conformational changes directed towards an increase of structural order can be shown in the case of peptides for which no proteolipidic model has been suggested [25]. The long-term goal of the present work is to study the connection between structural changes induced in opioid peptides by interactions with phospholipids and binding of these peptides to their receptors. The immediate aim is to study the interaction of the bioactive heptapeptide [Met5]enkephalin-Arg-Phe [26, 27] with phosphatidylserine (PtdSer) vesicles by circular dichroism, two-dimensional NMR spectroscopy, hybrid distance geometry simulated annealing (DG-SA) and molecular dynamics (MD) calculations.

In the present work we have used a low concentration of PtdSer and peptide and a peptide/phospholipid molar ratio of 3:1. These experimental conditions differ from those generally used for analogous studies. They were dictated by the low solubility of the peptide and the narrow pH interval of stability of the solution containing the PtdSer vesicles. However, these experimental conditions may better represent the early stage of the binding mechanism. Despite the restrictions imposed by the pH conditions, which did not allow us to observe the amide proton resonances in the two-dimensional NMR experiments, and by the peptide/phospholipid molar ratio, which limit the observable results, it was possible to detect a difference between the structure of the peptide in aqueous solution and in the presence of PtdSer vesicles. Ten interresidue NOEs were detected. These NOEs were used as restraints for a subsequent DG and MD analysis and gave a unique folding of Cα2, Cα3, Cα4 and Cα5 carbon atoms of the peptide moiety. No information was obtained for the orientation of the peptide planes, due to the lack of the amide-proton resonances. This folding determines an orientation of Phe4 and Met5 side chains on the same side of the backbone, thus forming a hydrophobic cluster that can favour the interaction with the apolar portion of the PtdSer vesicles.

MATERIALS AND METHODS

Materials. The following materials were obtained through the listed suppliers: [Met5]enkephalin-Arg-Phe and PtdSer from Serva Feinbiochemia, and deuterium oxide (minimum isotopic purity 99.96%) from Aldrich Chemical Co. All other chemicals were of reagent grade and used without further purification.

Sample preparation. The solutions were prepared by dissolving different amounts of the peptide by adding 10 mM sodium phosphate, pH 7.68. The phosphate buffer for NMR measurements was prepared in D_2O, the pH values reported were meter readings in D_2O and were not corrected for isotope effects at the glass electrode (pD $\approx$ 8.08). PtdSer vesicles in 10 mM sodium phosphate buffer were prepared as described earlier [28] and added in a 0.3 molar ratio to the peptide. The experimental conditions were dictated by the low solubility of the peptide, and by the pH interval of stability of the solution containing the PtdSer vesicles. The peptide concentration was in the range 0.39−1.03 mM. The rather high peptide/phospholipide molar ratio made it more difficult to observe the interaction effect, but was imposed by the need for collection measurable NMR signals in a reasonably short time. It should be noted that [Met5]enkephalin-Arg-Phe is poorly soluble in water, and adding PtdSer makes it even less soluble. However, these experimental conditions are likely to represent the early stage of the peptide-phospholipid interaction, where a fast exchange between the bound and free forms may occur. With these experimental conditions of fast exchange between bound and free forms of the peptide the spin-diffusion effects are reduced.

Circular dichroism. CD spectra were recorded with a Jasco J-500A spectropolarimeter equipped with a temperature-regulated cell assembly, and a slit program to obtain a wavelength accuracy of better than 0.5 nm. Quartz cuvettes of 0.01-, 0.1-, 0.2-, 1.0- and 2.0-cm pathlength were used. The instrument was flushed with dry nitrogen and calibrated with androsterone (1.69 mM in dioxane, $[\theta]_{304}$ = 11180 degree · cm^2 · dmol^{-1}). Spectra were recorded at 38 °C both in the presence and in the absence of PtdSer at pH 7.68 in 10 mM sodium phosphate. Possible concentration effects were evaluated by recording CD spectra at pH 7.68 and at a peptide concentration ranging from 0.39 mM to 1.07 mM, the concentrations at which NMR measurements were accomplished. Molar ellipticities measured under these conditions varied only 2−4%, eliminating the possibility of concentration effects within the range used.

NMR spectroscopy. All the spectra were taken at 38 °C on Varian XL-300, Bruker AM 400 and AM 500 instruments operating at 299.90, 400.13 and 500.13 MHz, respectively. Two-dimensional NMR experiments were performed in phase-sensitive mode with time-proportional phase increment (TPPI) phase cycling [29] using 2 K of memory for 512 increments. The number of scans was optimized to obtain a satisfactory signal to noise ratio. Correlation experiments were performed in double quantum filtered mode (COSY-DQF) [30, 31]. Total correlation experiments (TOCSY) used the MLEV-17 spin-lock composite pulse [32, 33] with a typical mixing time of 0.120 s for observation of remote connectivities. NOE dipolar correlated two-dimensional spectra were obtained using the NOESY pulse sequence [34] or rotating-frame dipolar correlated two-dimensional spectroscopy (ROESY) [35, 36]. The mixing times for the magnetization exchange were 0.080, 0.150, 0.200, 0.250, 0.300, 0.500 and 0.800 s. All data were processed on a microVAX II computer system using the TRITON software from multidimensional processing software package written by Boelens, R. & Vuister, G. University of Utrecht, The Netherlands and courtesy of R. Kaptein. The free induction decays (FID) were weighted in both dimensions by a sine-bell apodization function typically shifted by 90°. The final two-dimensional NMR spectra consisted of 1024×1024 data point matrices with a digital resolution of 5 Hz/point. Baseline correction was performed in both dimensions with a four-term polynomial fit. Peak volumes were measured from NOESY spectra by using the routine of the TRITON software package. The rate of build up of NOESY peaks confirmed that spin diffusion was not significant over a range of mixing times from 0.080 s to 0.800 s.

It is obvious that the intensity of NOEs cannot be considered strictly proportional to distance and, due to the low concentration, a build up computation cannot be properly made. We chose the 0.500-s mixing time NOESY spectrum for a more detailed

analysis, classifying volumes as strong, medium and weak, corresponding to interproton-distance restraints of 0.18−0.30, 0.18−0.40 and 0.18−0.50 nm, respectively [37], for subsequent DG and MD analysis. As will be reported in the following sections, flat-bottom restraints were used in the simulations; this implies that the restraint potential used in the distance restrained simulations acts as an upper limit. Distances between methylene and aromatic-ring protons were not assigned and were calculated with respect to the average position of these protons and the upper limits of the corresponding restraints were corrected as described by Wüthrich et al. [38]. Restraints involving pseudo atoms were conveniently treated.

We wish to indicate that the presence of spin diffusion, even if reduced, lowers the volume of the peaks detected. For this reason the values of the upper bounds chosen represent the maximum allowed distance compatible with the experimental data. It is important to note that the 3:1 molar ratio adopted strongly favours the fast exchange between the free and bound forms. As a consequence the residence lifetime becomes rather short, thus reducing the build up of NOEs which rise with a time constant comparable to the T_1 relaxation time. The subsequent magnetization transfer to other protons, responsible for the three-spin effect, is further decreased.

The reduction of the residence lifetime implies that the conformation derived from the experimental data may represent the early stage of the binding mechanism.

The results obtained with DG and MD calculations give interresidue proton distances shorter than the maximum allowed distances. Peaks that were observed only in the 0.800-s spectrum were classified as very weak and were not included in the calculations. Due to the lack of unequivocal assignments of Gly2 and Gly3 because of peak overlap the following combinations of NOE with Tyr1 and Phe4 were modeled and analyzed: Tyr1 to Gly3, and Gly2 to Phe4 (set I); Tyr1 to Gly3, and Gly3 to Phe4 (set II); Tyr1 to Gly2, and Gly2 to Phe4 (set III); Tyr1 to Gly2, and Gly3 to Phe4 (set IV).

Distance-geometry–simulated annealing procedure. Distance geometry, simulated annealing, energy minimization and the subsequent analysis of the structures were carried out on a Silicon Graphics IRIS 4D/220 GTX workstation using the program X-PLOR [39]. The calculations were based on the hybrid distance-geometry−dynamical-simulated-annealing protocol [40−42]. The Lennard-Jones potentials were taken by the CHARMM empirical energy function [43].

The peptide torsion angles were restrained in *trans* conformation by adding a proper dihedral potential.

Following the procedure of Nilges et al. [41], we used 100 cycles of Powell minimization [44] of van der Waals, bond and NOE terms and 100 subsequent cycles of the bond angles terms to improve the covalent geometry of the embedded structures. They were followed by a molecular-dynamics simulation stage, starting at a simulated temperature of 2000 K, to introduce the chirality and planarity. The correct handedness of the structure was established on the basis of the lowest energy of the embedded structures. The subsequent stage was the cooling of the structures to a final temperature of 100 K with increased van der Waals terms. Finally 200 steps of Powell minimization of the structures were performed. The obtained structures were then refined by a further simulated-annealing stage consisting of 1000 steps of molecular-dynamics calculation at 2000 K then of 1000 cooling steps to a final temperature of 100 K. The van der Waals interactions were softened to enable atoms to move through each other. The structures obtained were then subjected to 100 cycles of energy minimization by using the conjugated gradients Powell algorithm. The criteria of acceptance adopted for the generated structures were deviation of the actual distance, r_{ij}, from the experimental upper distance, r_{ij}^+, determined by the NOE intensity, $r_{ij} \leq r_{ij}^+ + 0.05$ nm; rms differences for the covalent bond deviation from ideality <0.001 nm; rms difference for bond angles deviation from ideality $<2°$.

Restrained molecular dynamics *in vacuo*. The calculations were based on an energy function approach: the molecule's total energy was given by the combination of an empirical and an effective energy term.

$$E_{total} = E_{empirical} + E_{effective}$$

The CHARM22 force field [43] was used in the calculations. Two different dielectric permittivities were used: ε of 80, to mimic a polar environment, and ε of 10, to embed the peptide in a membrane-like environment [45]. PtdSer vesicles were not explicitly included in the calculation, but their effects were implicitly reflected by the experimentally determined NOE restraints. The effective energy function (soft-square function) for the distance restraints, E_{dist}, used was

$$E_{dist} = k_{dist} \Sigma [r_{ij} - r_{ij}^+]^2 \quad \text{if } r_{ij} > r_{ij}^+$$

$$E_{dist} = 0 \quad \text{if } r_{ij}^- \leq r_{ij} \leq r_{ij}^+$$

$$E_{dist} = k_{dist} \Sigma [r_{ij} - r_{ij}^-]^2 \quad \text{if } r_{ij} < r_{ij}^-$$

where r_{ij}^+ and r_{ij}^- are the upper and lower bounds of the distance, reported in the NMR section. Due to the small value of r_{ij}^- ($r_{ij}^- = 0.18$ nm) the restraint potential acts as an upper bound limit. r_{ij} is the calculated distance and k_{dist} was set at 238.9 kJ · mol^{-1} · nm^{-2}. An additional effective energy function, E_{dihe}, was used, in the runs at high temperature, to forbid the *cis* conformation of the peptide torsion angles. All the bond lengths were kept fixed with the SHAKE algorithm [46]. The temperature of the system was maintained constant by weak coupling to a thermal bath [47]. The time-step of the integrator was 2 fs.

Starting from an extended conformation of the polypeptide, 5 ps of restrained MD simulations at 300 K were performed to equilibrate the molecule. The friction coefficient for the coupling with the thermal bath was 10 ps^{-1}. Subsequently the temperature was raised to 800 K in 5 ps using a friction coefficient for the coupling with the thermal bath of 0.5 ps^{-1}. For the conformational search 50-ps MD simulations at 800 K were performed, including E_{dist} and E_{dihe} with a friction coefficient of 10 ps^{-1}. Ten snapshot structures were chosen (every 5 ps) and cooled using a simulated-annealing stage from 800 K to 300 K. The conformers were equilibrated with 10-ps MD simulations at 300 K without E_{dihe}. This protocol of simulations was applied for simulations with both $\varepsilon = 80$ and $\varepsilon = 10$.

RESULTS AND DISCUSSION

CD measurements. CD spectra of [Met5]enkephalin-Arg-Phe were recorded as described in Materials and Methods, as a preliminary experiment to ascertain the presence of interaction between [Met5]enkephalin-Arg-Phe and PtdSer vesicles. At pH 7.68 the spectrum was characterized by two positive, partially resolved dichroic bands (Fig. 1). In the absence of the lipid these were localized at 216.5 nm and 211.5 nm. In the presence of PtdSer with a peptide/phospholipid molar ratio of 3:1, these bands were broader and, therefore, less resolved. The presence of the lipid induced a modest reduction of the rotational strength with respect to that measured with pure peptide solution. Although the location of the dichroic signal suggests an involvement of π-π^* and n-π^* transitions of the peptide groups, these assignments for [Met5]enkephalin-Arg-Phe are affected by the presence of three residues with an aromatic side chain. The CD spectra of several proteins have positive bands near 230 nm,

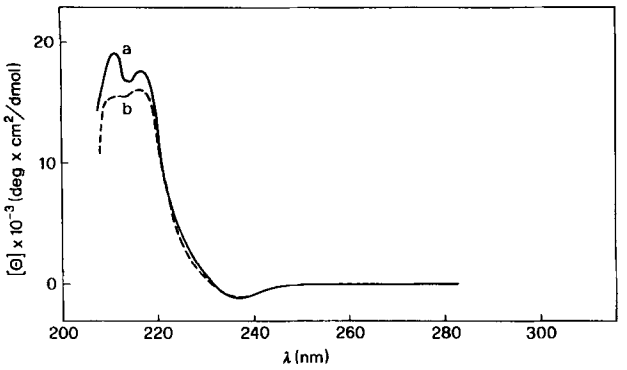

Fig. 1. CD spectra of [Met5]enkephalin-Arg-Phe at $T = 38$ °C. (a) c = 0.399 mM, in 10 mM sodium phosphate, pH 7.68; (b) as in (a) with PtdSer at c = 0.119 mM.

which have been attributed to the L_a band of tyrosine [48, 49]. The CD data can be interpreted as evidence for considerable variations of the conformation of the heptapeptide as a consequence of the interaction with PtdSer. A study of the probable and average conformations of [Met5]enkephalin [50], for which the peptide sequence is the same as that of the first five residues of [Met5]enkephalin-Arg-Phe, showed that a γ-turn is the thermodynamically preferred conformation. The calculated [51] CD spectra for γ-turns show a negative CD band at long wavelength, centered at approximately 230 nm. This theoretical prediction is supported by spectra of cyclic model peptides [52–55] in non-polar solvents [52–54] and in aqueous environments [55], and by spectra of model peptides that assume predominantly the γ-turn conformation in non hydrogen-bonding solvents [56, 57]. The CD spectrum of [Met5]enkephalin-Arg-Phe recorded in the presence of PtdSer at pH 7.68 is characterized by molar ellipticity values lower than those obtained in the absence of PtdSer. An increase of the molar ellipticity values might take place, due to a positive contribution of the aromatic groups [48, 49, 58–62]. These may have a positive CD band in the 220–230-nm region, where coupling between aromatic and amide transitions may occur.

Therefore the CD results do not appear to contrast with the hypothesis that in the presence of PtdSer [Met5]enkephalin-Arg-Phe assumes a folded conformation including a γ-turn, as supported by NMR and MD results.

NMR measurements. Proton assignments employed homonuclear decoupling experiments, two-dimensional COSY-DQF and TOCSY experiments at different mixing times. All of the resonance assignments are reported in Table 1. The assignment of the CHα of Phe4 and Phe7 residues was solved according to the data reported in the literature for random-coil polypeptides [63] and to the studies carried out on [Met5]enkephalin [64]. The assignment was confirmed by the observation of NOEs between resonances of the protons of Phe4 and those of Met5, as presented below. The only ambiguities were the individual assignment of the overlapping CH_2 resonances of Gly2 and Gly3. The fast exchange of the amide protons at pH 7.38 and 38 °C in H_2O/D_2O (9:1) solution did not allow us to observe the amide protons. In the case of Gly2 and Gly3 the individual assignment would be possible only by identifying the corresponding amide resonances, thus these resonances at this stage were considered as ambiguously assigned and their NOEs were not considered as initial restraints for the computer simulation.

The NMR study of the free peptide in solution was performed by ROESY and NOESY experiments. The presence of only intraresidue dipolar contacts in these experiments, taken at different mixing times, indicates a high degree of flexibility of the peptide in solution and the presence of random distributed conformers.

Addition of PtdSer to [Met5]enkephalin-Arg-Phe results in a broadening of the NMR resonances, thus indicating a direct interaction between the peptide and the vesicle. No significant change in the chemical-shift values indicate the presence of a fast-exchange dynamics between free and bound forms of [Met5]enkephalin-Arg-Phe.

Preliminary information on the interaction of the peptide with PtdSer vesicles was deduced from the proton T_1 relaxation times (Table 1). These values indicated a global reduction of the correlation time of the peptide when interacting with PtdSer. Because of the fast exchange between free and bound states of the peptide, effects on individual residues were only slightly visible. A major shortening of relaxation times is evident only for the Tyr1 and Met5 residues (Table 1). This result indicates the involvment of this moiety in the binding mechanism of [Met5]-enkephalin-Arg-Phe with PtdSer vesicles.

Two-dimensional NMR NOESY experiments were performed in D_2O. This has the effect of making inapplicable the usual NMR procedure for determining the peptide structure in solution. Only the few and usually weak NOEs between protons of the side chains of the residues can be observed. To validate the observed NOEs several mixing times were applied (see Materials and Methods). It is well known, however, that increase of the mixing time can give rise to ambiguities in the interpretation of NOEs in terms of interatomic distances due to the possibility that the three-spin effect (spin diffusion) occurs. Nevertheless the 3:1 molar ratio which produces a fast-exchange dynamics between the free and bound form markedly reduces the residence lifetime of the latter form, thus decreasing its extent as already mentioned in Materials and Methods.

Fig. 2A–C shows parts of the aliphatic regions and the CHα/aromatic region of the NOESY spectrum taken at 0.500 s mixing time. All of the cross-peaks observed at different mixing times are summarized in Table 2. The observed intramolecular dipolar contacts confirmed the assignments. In particular the NOE between the CHα/aromatic protons of Phe4 and the NOEs between these protons and the Met5 confirmed the tentative assignment of Phe4. The dipolar contact between the $CH_2\beta$ of Tyr1 and the two glycines was not assigned. Subsequent MD calculations showed that both the Gly2 and Gly3 are at time-averaged distances suitable to result in magnetization transfer.

The aromatic protons of the two Phe4 and Phe7, well resolved from those of Tyr1, give NOEs with the two overlapped resonances of the Gly2,3 CH_2 resonances. Because the peptide is highly flexible under these experimental conditions, long-range NOEs between Phe7 and the glycine residues are unlikely. We therefore assigned the NOEs to the dipolar interaction between the glycines and Phe4. In this case the computer calculations indicated that the candidate for the interaction observed is the contact between CHα of Phe4 and CH_2 of Gly2.

The presence of ten interresidue cross-peaks (Table 2) implies that the interaction with PtdSer vesicles induces an ordered conformation of the peptide. The weak dipolar contacts detected between Gly2,3 and one proton of $CH_2\beta$ of Tyr1 (Fig. 2A) as well as the strong cross-peak between CHα and one proton of $CH_2\beta$ of Tyr1 suggest that the N-terminus is partially involved in the lipid-headgroup-binding process. In particular, the intraresidue NOE in Tyr1 indicates that the free rotation of the aromatic ring is strongly hindered. The presence of NOE peaks between Phe4 and Met5 (Fig. 2C) suggests that the interaction with PtdSer vesicles affects the conformation of [Met5]enkephalin-Arg-Phe by restraining the orientation of the molecular moiety between Phe4 and Met5 residues. In particular, at all mixing

Table 1. Proton chemical shifts (δ) and spin-lattice relaxation times (T_1). Results obtained on a Varian XL-300 spectrometer; δ (in ppm) are referred to HDO placed at 4.75 ppm after standardization of the spectrometer to external tetramethylsilane. The T_1 standard deviations are less than 5% of the observed values.

Residue	Protons	[[Met5]Enkephalin-Arg-Phe] = 0.972 mM in 10 mM sodium phosphate, pH 7.68, at 38.0 ± 0.1°C		[[Met5]Enkephalin-Arg-Phe] = 1.07 mM [PtdSer] = 0.321 mM in 10 mM sodium phosphate, pH 7.68, at 38.0 ± 0.1°C	
		δ	T_1	δ	T_1
		ppm	s	ppm	s
Tyr1	α	3.88	1.539	3.90	0.411
	β	2.99	0.392	3.05	0.300
	β	2.98	0.356	3.05	0.300
	δ	7.25	1.338	7.25	0.632
	ε	6.96	2.285	6.97	0.834
Gly2,3	α	3.97, 3.96	0.466, 0.416	3.96, 3.95	0.378, 0.398
Phe4	α	4.56	1.228	4.59	–
	β	3.27	0.398	–	–
	β	2.96	0.364	–	–
Met5	α	4.48	1.159	4.48	0.688
	β	2.03	–	2.03	–
	γ	2.52	0.424	2.52	0.378
	δ	2.17	2.087	2.17	1.187
Arg6	α	4.38	1.042	4.38	0.612
	β	1.88	0.311	1.88	0.125
	β	1.76	0.316	1.77	0.105
	γ	1.66	0.382	1.66	0.276
	δ	3.22	0.452	3.27	0.356
Phe7	α	4.70	–	–	–
	β	3.16	0.250	–	–
	β	3.11	0.292	–	–
Phe7,4	δ, ε, ζ	7.30	1.288	7.36	0.833
		7.36	1.297	7.38	0.861
		7.38	1.640	7.41	0.983
		7.39	1.530	7.46	0.960
		7.41	1.457	7.48	0.918

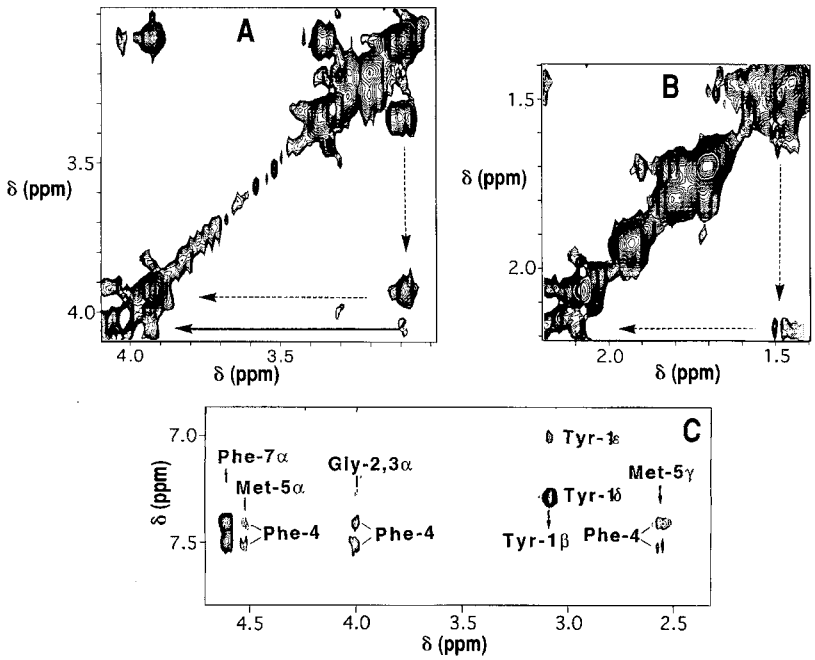

Fig. 2. The 400-MHz NOESY spectrum at 0.500 s of mixing time of [Met5]enkephalin-Arg-Phe in the presence of PtdSer vesicles. (A) Part of the alkyl region containing the cross-peaks between CHα and one of the proton of $CH_2\beta$ of Tyr1 (dashed arrow) and one of the proton of $CH_2\beta$ of Tyr1 and CH_2 of Gly2,3 (solid arrow); (B) part of the alkyl region containing the intermolecular cross-peak between the CH_2 envelope of PtdSer and the CH_3 of Met5 (dashed arrow); (C) part of the aromatic/alkyl region with dipolar contacts between Phe4 and Met5 residues.

Table 2. NOEs of [Met5]enkephalin-Arg-Phe (1.07 mM) in the presence of PtdSer (0.321 mM) in 10 mM sodium phosphate, pH 7.68, at 38.0±0.1 °C. Separate NOESY experiments were performed with mixing times of 0.080, 0.150, 0.200, 0.300, 0.500 and 0.800 s. S, strong = 0.18–0.30 nm; M, medium = 0.18–0.40 nm; W, weak = 0.18–0.50 nm.

Cross-peak	NOE type
Tyr1 CHα to Tyr1 CH$_2\beta'$	S
Tyr1 CH$_2\beta$ to Tyr1 CH$_2\beta'$	S
Tyr1 CH$_2\beta'$ to Tyr1 CHδ	S
Tyr1 CH$_2\beta'$ to Tyr1 CHε	W
Tyr1 CHδ to Tyr1 CHε	S
Tyr1 CH$_2\beta'$ to Gly2,3 CH$_2$	W
Gly2,3 CH$_2$ to Phe4 CHδ	M
Gly2,3 CH$_2$ to Phe4 CHε	W
Phe4 CHα to Phe4 CH$_2\beta$	S
Phe4 CHα to Phe4 CHδ	M
Phe4 CHα to Phe4 CHε	W
Phe4 CHδ to Met5 CHα	W
Phe4 CHε to Met5 CHα	W
Phe4 CHδ to Met5 CH$_2\gamma$	W
Phe4 CHε to Met5 CH$_2\gamma$	M
Met5 CHα to Met5 CH$_2\beta$	S
Met5 CH$_2\beta$ to Met5 CH$_2\gamma$	S
Met5 CHα to Met5 CH$_2\gamma$	M
Met5 CH$_2\beta$ to Met5 CH$_2\gamma$	S
Met5 CHα to Met5 CH$_3$	W
Met5 CHα to Arg6 CH$_2\gamma$	W
Met5 CH$_2\beta$ to Arg6 CHα	W
Met5 CH$_2\gamma$ to Arg6 CHα	W
Met5 CH$_3$ to PtdSer CH$_2$	M
Arg6 CHα to Arg6 CH$_2\gamma$	S
Arg6 CHα to Arg6 CH$_2\delta$	M
Arg6 CH$_2\gamma$ to Arg6 CH$_2\delta$	S
Arg6 CHα to Arg6 CH$_2\beta$	S
Phe7 CHα to Phe7 CH$_2\delta$	S

times, the presence of a cross-peak between the methyl group of Met5 and the methylene protons of the fatty acid chains of PtdSer vesicle (Fig. 2B) indicates that this residue is embedded in the hydrophobic region of the vesicle. The fatty acid chain protons give rise to a unique envelope of resonances in the NMR spectrum, making it impossible to deduce the exact position of [Met5]enkephalin-Arg-Phe within the vesicle. The peptide C-terminus seems to be less important for this interaction. The only dipolar contacts were detected between CH$_2\gamma$ of Met5 and CHα of the Arg6, and between the CH$_2\beta$ of Met5 and CHα of Arg6 at 0.800 s mixing time. All the NOEs detected at different mixing times are reported in Table 2.

DG-SA calculations. The limited number of restraints obtained from the NMR measurements and the ambiguities in the assignment of Gly2 and Gly3 complicated the determination of the arrangement of the molecule. To solve the ambiguities in the NOEs assignments and to analyze the conformational behavior in the bound state we modelled all the various combinations of distances between the two glycines, Tyr1 and Phe4 (see Materials and Methods). Use of a DG-SA approach [41] ensured a good screening of all backbone and side-chain conformations. 20 conformers were generated for each set of distances. After distance geometry and refinement procedures, the number of accepted conformers were 16, 17, 16 and 18 for set I, II, III and IV, respectively. The conformational energies of all the accepted conformers were analyzed and the different sets were compared. The average conformational energies values of sets I and III were 0.6 kJ/mol and 0.8 kJ/mol, respectively. In contrast, the

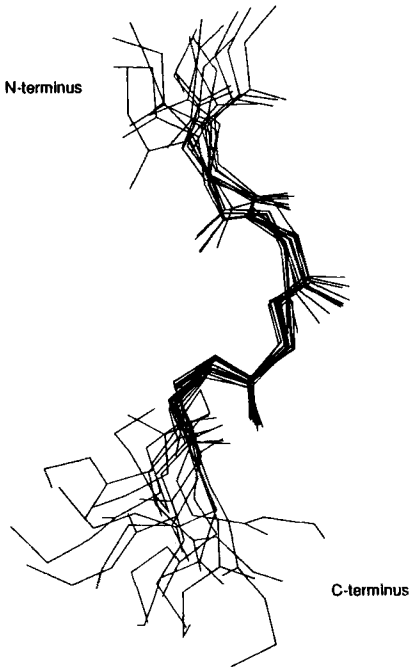

Fig. 3. Superimposition of DG-SA structures showing only the [Met5]enkephalin-Arg-Phe backbones.

conformational energies of sets II and IV were both 4.8 kJ/mol. In particular, these last structures showed values of ϕ and ψ of Phe4 in the L region of the Ramachandran plot (see BHRL-code) [65]. These values are anomalous for the [Met5]enkephalin and [Met5]enkephalin analogs, as reported in other calculations in which the ϕ and ψ Phe4 angles span only the B and H regions [66].

We therefore restricted our calculations to set I and III, consistent with an assignment of the NOEs of the Phe4 to Gly2. It was not possible, however, to assign the NOE between Tyr1 and the two glycines.

All conformers obtained from the two sets of distances showed the same folding of the Cα carbons of Gly2, Gly3, Phe4 and Met5. The rmsd among all these atoms was less than 0.03 nm (Fig. 3). These results suggest the existence of a unique folding of the polypeptide moiety in the bound state. We can expect that the same folding has been obtained with both methods (DG-SA and MD) and with two different values of the dielectric constant. It has to be noted that there are no NOEs observed between protons of the backbone, but there are six inter-residue contacts between protons belonging to side chains. The low value of the rmsd is not surprising as it is obtained by averaging the position of four atoms only. For the side chains and for the residues Tyr1, Arg6 and Phe7 several different conformations were allowed. For the side chains and for the residues Tyr1, Arg6 and Phe7 several different conformations were allowed. This unique folding of Cα2, Cα3, Cα4, Cα5, can be obtained with different orientations of the peptide planes. The different orientation of the peptide planes allowed by the simulation is due to the lack of the resonances of the amide protons. The allowed conformations for Gly3 and Phe4 (Fig. 4), agree with previously reported data on [Met5]enkephalin and low-energy conformers for [Met5]enkephalin analogs [66].

Molecular-dynamics calculations. The results obtained by DG calculations showed that only sets I and III were allowed and that all conformers obtained give the same folding of Cα2, Cα3, Cα4 and Cα5. For this reason,we performed the MD calculations

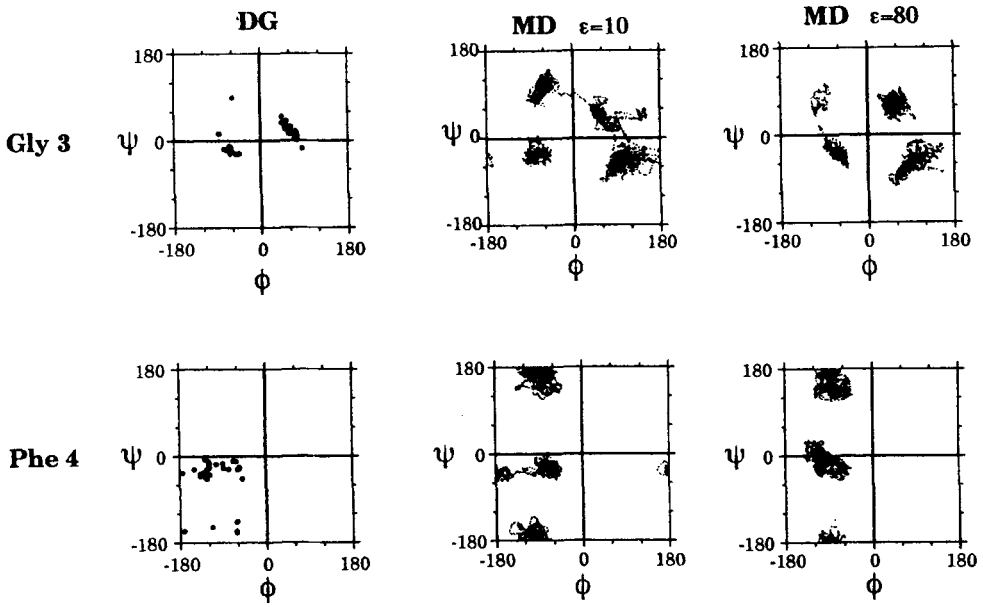

Fig. 4. Ramachandran plots showing the ϕ and ψ angles of the allowed conformational regions from DG-SA and MD ($\varepsilon = 10$ and $\varepsilon = 80$) of Gly3 and Phe4 residues.

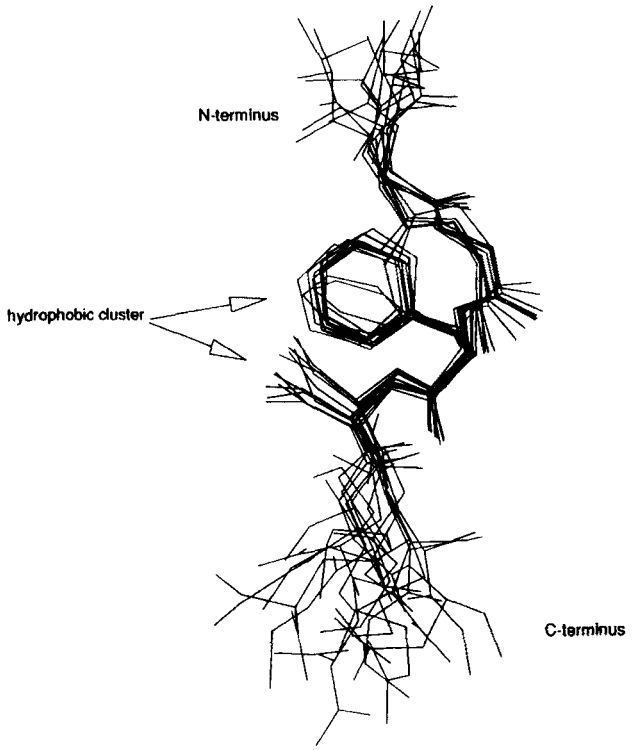

Fig. 5. Superimposition of DG-SA structures showing the Phe4 and Met5 residues side-chain orientations.

using only set I, as constraints anticipating that the MD simulations performed with set I would give distances in agreement also with set III. Two different values of the dielectric constant were used: $\varepsilon = 80$ and $\varepsilon = 10$, to mimic a polar and a membrane-like environments, respectively [45]. Embedding the peptide in a continuum dielectric model simulating a membrane environment is a better representation of the situation experienced by the N-terminus and the central segment of the peptide interacting with PtdSer vesicle. However, a more polar environment is experienced by the C-terminus and has been suggested for the formation of the bioactive conformers of [Met5]- enkephalin analogs in other theoretical studies [67]. We simulated the dynamics of the peptide in both the environments and we analyzed the trajectories versus time of all the conformers obtained after SA procedures.

The results of the MD simulations confirmed the unique folding of the polypeptide moiety in the bound state (Cα2−Cα5) obtained by DG calculations.

Note that the hydrophobic side chains of the Phe4 and Met5 are oriented on the same side with respect to the backbone (Fig. 5). This orientation favours the interaction of the hydrophobic side chains with the apolar portion of the PtdSer vesicle to form a hydrophobic cluster as reported for [Met5]enkephalin in other theoretical calculations [68].

The two dielectric constants did not change the nature of the structures found. The partial screening of the electrostatic interactions in the simulations with ε of 80 allowed only a different structural reordering of the side chains and did not influence the backbone orientations. Among the structures found, two conformers are characterized by the presence of a 2←4 γ-turn and inverse γ-turn, with a H-bond between the CO of Gly2 and NH of Phe4. These turns have already been suggested for [Met5]enkephalin [29, 50, 68]. The average potential energy of the conformation characterized by the γ-turn is 3.6 kJ/mol higher than the other conformers, consistent with the findings of Paine and Sheraga [50].

The Ramachandran plots for Gly3 and Phe4 obtained by DG-SA and MD calculations are reported in Fig. 4. The dihedral angle values of the peptide backbone and their fluctuations, for ε of 80 and ε of 10 are reported in Tables 3 and 4.

The data obtained are consistent with the existence of randomly distributed conformers of [Met5]enkephalin-Arg-Phe in aqueous solution and a process of structural reordering when present in a membrane-like environment. The experimental conditions have been chosen in order to be in the presence of a fast exchange between the free and bound form of [Met5]enkephalin-Arg-Phe. This condition strongly reduces the spin-diffusion effects which occur extensively when the lifetime of the bound form is rather long with respect to the NOESY mixing time.

The NMR results, obtained under the fast-exchange conditions, show that, in the early stage of the binding step, an extrin-

Table 3. Average values and rms fluctuation of backbone dihedral angles and energies of different conformers from the molecular dynamics calculations with $\varepsilon = 80$.

Conformer	Tyr1		Gly 2		Gly 3		Phe 4		Met 5		Arg 6		Phe 7		$E_{(tot)}$
	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	
	degrees														kJ/mol
I	−54 ± 18	−26 ± 51	100 ± 38	22 ± 25	53 ± 12	61 ± 9	−86 ± 11	48 ± 159	−16 ± 38	−39 ± 12	−128 ± 17	−57 ± 13	−116 ± 28	−26 ± 54	6.5 ± 4.1
II	3 ± 165	−105 ± 84	−87 ± 25	119 ± 20	−66 ± 14	−34 ± 15	−75 ± 17	140 ± 45	67 ± 7	133 ± 17	−114 ± 19	64 ± 28	−119 ± 26	128 ± 25	4.8 ± 2.9
III	−9 ± 96	−62 ± 9	113 ± 28	110 ± 77	−89 ± 46	75 ± 21	−112 ± 13	3 ± 15	−131 ± 13	−132 ± 19	−89 ± 23	−56 ± 11	−120 ± 20	−56 ± 19	4.1 ± 2.1
IV	−64 ± 153	−59 ± 12	91 ± 14	0 ± 38	74 ± 11	−76 ± 12	67 ± 8	−60 ± 158	−49 ± 10	−12 ± 10	−101 ± 15	115 ± 20	−77 ± 16	125 ± 10	9.6 ± 1.9
V	69 ± 14	−58 ± 13	108 ± 15	−59 ± 16	111 ± 19	−46 ± 16	−85 ± 20	−22 ± 14	−109 ± 18	135 ± 22	−95 ± 63	95 ± 117	70 ± 9	−75 ± 24	5.0 ± 3.3

Table 4. Average values and rms fluctuation of backbone dihedral angles and total energies of different conformers from molecular dynamics calculations with $\varepsilon = 10$.

Conformer	Tyr1		Gly 2		Gly 3		Phe 4		Met 5		Arg 6		Phe 7		$E_{(tot)}$
	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	
	degrees														kJ/mol
I	21 ± 70	−63 ± 10	107 ± 56	112 ± 46	−61 ± 13	102 ± 16	−103 ± 19	−49 ± 158	−42 ± 26	5 ± 30	−79 ± 26	120 ± 85	−111 ± 25	−43 ± 45	1.2 ± 5.0
II	−40 ± 67	123 ± 18	−89 ± 21	−17 ± 37	91 ± 32	16 ± 39	−77 ± 131	−37 ± 8	−83 ± 118	7 ± 151	−119 ± 28	−36 ± 78	−109 ± 28	−50 ± 46	0.5 ± 5.5
III	33 ± 96	−31 ± 57	−97 ± 73	−43 ± 27	95 ± 67	−48 ± 24	−80 ± 22	146 ± 39	60 ± 10	108 ± 28	−100 ± 29	−9 ± 28	−105 ± 30	−54 ± 35	8.1 ± 3.3
IV	71 ± 19	−59 ± 13	103 ± 58	−72 ± 25	106 ± 21	−49 ± 14	−70 ± 12	−25 ± 9	−97 ± 17	−13 ± 15	−107 ± 22	113 ± 21	−101 ± 23	−52 ± 17	1.4 ± 4.5
V	61 ± 7	115 ± 92	−78 ± 81	81 ± 27	−4 ± 54	82 ± 26	−97 ± 15	3 ± 166	−47 ± 11	−12 ± 15	−108 ± 22	−28 ± 44	−111 ± 73	60 ± 97	0.2 ± 3.8
VI	−7 ± 106	−62 ± 17	98 ± 25	106 ± 26	−73 ± 14	−32 ± 12	−85 ± 20	−22 ± 14	−109 ± 18	135 ± 22	−95 ± 63	95 ± 117	70 ± 9	−75 ± 24	2.4 ± 3.6

sic interaction occurs between the [Met5]enkephalin-Arg-Phe and the PtdSer vesicle which strongly influences the folding of the peptide. The limited number of NOESY cross-peaks and the degeneracies of the Gly2,3 resonances indicate the high flexibility of the peptide.

The conformations obtained by MD are close to those obtained by DG-SA and show a well-defined folding of the Cα2, Cα3, Cα4 and Cα5. This folding can be obtained with different orientations of the peptide planes. Among the conformers obtained by MD calculations two of them are characterized by the presence of a 2←4 γ and inverse γ-turns centered on Gly3. These allowed conformations have a higher energy and are in agreement with those reported by Paine and Sheraga [50] for [Met5]enkephalin. The unique folding of the central moiety of the peptide is characterized by the presence of the same orientation of the two hydrophobic side chains Phe4 and Met5 to form a hydrophobic cluster interacting with the apolar portion of PtdSer vesicle, in agreement with the existing data reported for the [Met5]enkephalin conformers [68].

All the data are consistent with a mechanism of interaction in which the peptide folds in the presence of PtdSer vesicle with both the hydrophilic termini near the membrane/water interface and the hydrophobic interior buried in the hydrocarbon phase of the PtdSer vesicle. The N-terminus seems to be more involved in the interaction with the polar headgroups than the C-terminus which exhibits more structural disorder.

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