

# Interaction of $\alpha$ -Melanocyte Stimulating Hormone with Binary Phospholipid Membranes: Structural Changes and Relevance of Phase Behavior

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**ABSTRACT** The interaction of  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) with negatively charged binary membrane systems composed of either 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine/1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)], (DMPC/DMPG) or DMPC/1,2-dimyristoyl-*sn*-glycero-3-phosphate (DMPC/DMPA), both at a 3:1 ratio, was studied using complementary techniques (differential scanning calorimetry, infrared and ultraviolet absorption spectroscopy, and steady-state and time-resolved fluorescence). The peptide structure in buffer, at medium to high concentrations, is a mixture of aggregated  $\beta$ -strands and random coil, and upon increasing the temperature the random coil configuration becomes predominant. At low concentrations (micromolar) there are essentially no aggregates. When in interaction with the lipidic systems this transition is prevented and the peptide is stabilized in a specific conformation different from the one in solution. The incorporation of  $\alpha$ -MSH into phosphatidic acid-containing systems produced a significant alteration of the calorimetric data. Lateral heterogeneity can be induced by the peptide in the DMPA-containing mixture, at variance with the one of DMPG. In addition, the lipid/water partition coefficient for the peptide in the presence of DMPC/DMPA is greater in the gel phase as compared to the fluid phase. From the high values of limiting anisotropies it can be concluded that the peptide presents a very reduced rotational dynamics when in interaction with the lipids, pointing out to a strong interaction. Overall, these results show that the structure and stability of  $\alpha$ -MSH in a negatively charged membrane environment are substantially different from those of the peptide in solution, being stabilized in a specific conformation that could be important to eliciting its biological activity.

## INTRODUCTION

$\alpha$ -Melanocyte stimulating hormone ( $\alpha$ -MSH) is a peptide hormone known for its role in regulating skin pigmentation in vertebrates (Eberle, 1988). The primary structure of  $\alpha$ -MSH is Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub>. It appears that the peptide has no preferred structure in water (Biaggi et al., 1997), being very flexible, whereas all its synthetic analogs with superpotent biological activity present a  $\beta$ -turn (or sometimes another kind of turn) stabilized within their central region comprising residues 6–9, i.e., His-Phe-Arg-Trp (Sawyer et al., 1980, 1982; Al-Obeidi et al., 1989). This Trp-containing region of the peptide is furthermore the minimum melanotropic message sequence, essential for ligand binding and biological function (Hruby et al., 1987).

$\alpha$ -MSH has a net +1 charge at physiological pH, and a mean hydrophobicity and hydrophobic moment that are indicative of a receptor or specific lipid-mediated membrane interaction, and not a surface-seeking characteristic peptide (González et al., 1996).  $\alpha$ -MSH should be, according to these authors, classified as a secondary amphiphilic

peptide with a low penetration into the lipid monolayer and a small effect on its thermotropic properties.

It was recently demonstrated by fluorescence spectroscopy that  $\alpha$ -MSH and analogs interact with lipid vesicles composed of one anionic phospholipid, as concluded from alterations of its photophysical parameters (Ito et al., 1993; Macêdo et al., 1996). The fluorescence parameters were dependent on the lipid used, but the interaction was greater in the liquid crystalline phase as compared to the gel phase. The interaction was mainly electrostatic, because changes in the peptide (Ito et al., 1993), bilayer (Biaggi et al., 1996), or peptide/monolayer (González et al., 1996) parameters could not be detected by a wide variety of techniques when using zwitterionic phospholipids. In a recent molecular dynamics study (Pascutti et al., 1999) the peptide was reported to acquire a stable structure in a low dielectric constant medium, as compared to the structure in water, where a hydrophilic core was surrounded by a hydrophobic surface, forming a  $\beta$ -turn structure.

All these results suggest that the lipid matrix of the cell membrane could be a key factor for the peptide biological activity, namely by stabilizing an appropriate conformation. In line with this, we have studied in detail the interaction of  $\alpha$ -MSH with two-component lipid bilayers composed of a zwitterionic phospholipid (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, DMPC) and a negatively charged one (1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)], DMPG, or 1,2-dimyristoyl-*sn*-glycero-3-phosphate, DMPA) by using high-sensitivity differential scanning calorimetry (DSC),

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Fourier-transform infrared spectroscopy (FTIR), and steady-state and time-resolved fluorescence spectroscopy. The main issues addressed were the extent of lipid/peptide interaction, the effect of the peptide on the structure and thermotropism of phospholipids, and the structural features of the peptide, both in the aqueous and lipidic phases.

## MATERIALS AND METHODS

### Chemicals

$\alpha$ -MSH and D<sub>2</sub>O were obtained from Sigma Chemical Co. (St. Louis, MO). The lipids DMPC, 1,2-dimyristoyl-d<sub>54</sub>-sn-glycero-3-phosphocholine (DMPC<sub>d54</sub>), DMPA, and DMPG were obtained from Avanti Polar Lipids (Birmingham, AL). The buffer used was 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) at pH 7.4, 20 mM NaCl, and 0.1 mM EDTA. All reagents were used as received.

### Differential scanning calorimetry

Samples containing 2.6 mmol of phospholipids (DMPC<sub>d54</sub> plus either DMPA or DMPG at a 3:1 molar ratio) dissolved in chloroform/methanol (1:1 v/v) were mixed alone or with  $\alpha$ -MSH, also in the same solvent, to give a final phospholipid to peptide molar ratio of 10:1. The mixtures were dried under vacuum for 3 h to remove all traces of the organic solvents. Then, multilamellar vesicles (MLV) were formed in 1.4 ml buffer heated at a temperature  $\sim 10^\circ\text{C}$  above the temperature of the gel-to-liquid-crystalline phase transition ( $T_m$ ) of the mixture and frozen at  $-80^\circ\text{C}$ , this process being repeated five times. Differential scanning calorimetry was performed in a high-resolution Microcal MC-2 calorimeter (Microcal Inc., Northampton, MA). Differences in the heat capacity between the sample and the reference cell were obtained by raising the temperature at a constant rate of  $45^\circ\text{C/h}$  over a temperature range of  $5$ – $60^\circ\text{C}$ . The excess heat capacity functions were obtained after baseline subtraction and correction for the instrument time response. Unless otherwise stated, the third scan was used for transition temperature and enthalpy calculations.

### Infrared spectroscopy

Samples containing the same quantity of lipids and peptide as described above were hydrated in the same deuterated buffer at  $\text{pD} = 7.4$ . In order to wash unbound peptide, vesicles were pelleted by centrifugation and washed with the same deuterated buffer, freeze-thawed, and centrifuged again. Approximately 20  $\mu\text{l}$  of the pelleted sample were placed between two CaF<sub>2</sub> windows separated by a 50- $\mu\text{m}$ -thick Teflon spacer in a liquid demountable cell (Harrick, Ossining, NY). Peptide solutions of 12.8 mM concentration obtained under bath sonication were used for obtaining the spectra in buffer. The spectra were obtained in a Nicolet 580 FTIR spectrometer using a deuterated triglycine sulfate detector. Each spectrum was obtained by collecting 200 scans with a triangular function at a resolution of  $2\text{ cm}^{-1}$ . For temperature studies, samples were scanned between  $5$  and  $70^\circ\text{C}$  with  $2^\circ\text{C}$  intervals and a 2-min delay between each consecutive scan. Data treatment and resolution enhancement methods performed according to previously published methods (Kauppinen et al., 1981) were made using Spectra Calc or Grams software (Galactic Industries, Salem, NH). The frequency of the band due to the CH<sub>2</sub> or CD<sub>2</sub> symmetric stretching vibration was calculated from the center of gravity taking the top 10 points of each specific band and fitted to a Gaussian band. The criterion used for buffer subtraction in the C=O and amide regions was the removal of the band near  $1210\text{ cm}^{-1}$ , and a flat baseline between  $1800$  and  $2100\text{ cm}^{-1}$ .

## Absorption and fluorescence studies

For fluorescence studies, large unilamellar vesicles (LUV) prepared by the extrusion technique were used (Hope et al., 1985). First, MLV were made as described for DSC, except that DMPC was used instead of DMPC<sub>d54</sub>. Extrusion cycles were then performed on MLV suspensions with Nucleopore polycarbonate filters, first with  $0.4\text{ }\mu\text{m}$  pore diameter until no resistance was offered, followed by eight cycles with  $0.1\text{ }\mu\text{m}$  pore diameter filters. The final concentration of total phospholipid in LUV suspensions was determined by phosphorus analysis (McClare, 1971).

Direct dissolution of the peptide in a small volume of buffer ( $\sim 0.1$  absorbance at  $290\text{ nm}$ ) was achieved by vortexing and bath sonication (Bandelin Sonorex RK156). Then, the samples were prepared by taking aliquots of the peptide solution and adding the same volume of lipid suspension plus buffer in different proportions. The final peptide absorbance at  $290\text{ nm}$  was  $\sim 0.05$  in all samples. This corresponds to a peptide concentration of  $\sim 30\text{ }\mu\text{M}$  ( $\epsilon \sim 3500\text{ M}^{-1}\text{ cm}^{-1}$  (Wetlaufer, 1962)).

Absorbance spectra were carried out in a Shimadzu UV-3101PC absorption spectrophotometer using spectral bandwidths of  $2.0\text{ nm}$ . The correction for light scattering was carried out subtracting the baseline from an identical lipidic suspension. Steady-state fluorescence spectra were obtained in a SLM-Aminco 8100 Series 2 spectrofluorimeter. Emission spectra were corrected using standard emission spectra of L-Tyr and L-Trp (Chen, 1967).  $5\text{ mm} \times 5\text{ mm}$  quartz cuvettes were used and temperature was controlled up to  $\pm 0.5^\circ\text{C}$  in a thermostatted cuvette holder. Spectral bandwidth was  $2\text{ nm}$  for excitation and  $4\text{ nm}$  for emission in most measurements.

The time-resolved instrumentation (correlated single-photon timing technique) was previously described (Loura et al., 1996). Excitation was at  $295\text{ nm}$  and emission (at  $350\text{ nm}$ ) was detected at the magic angle ( $54.7^\circ$ ) relative to the vertically polarized beam. The number of counts on the peak channel was 20,000 and the number of channels per curve used for analysis was 800, with 8.4 or 11 ps/channel at  $37^\circ\text{C}$  and 14 or 15.3 ps/channel at  $20^\circ\text{C}$ . Data analysis was carried out using a nonlinear, least-square iterative convolution method based on the algorithm of Marquardt (1963). The goodness-of-fit was judged from the reduced  $\chi^2$ , weighted residuals, and autocorrelation plots.

The average lifetime  $\bar{\tau}$  of a fluorophore with a complex fluorescence decay is defined as (e.g., Lakowicz, 1999),

$$\bar{\tau} = \frac{\sum_i a_i \tau_i^2}{\sum_i a_i \tau_i} \quad (1)$$

where  $a_i$  are the normalized pre-exponentials (amplitudes) and  $\tau_i$  the lifetime components. For the purpose of partition coefficients determination, the lifetime-weighted quantum yield,  $\langle\tau\rangle$ , leads to simpler formalisms, and it will be used throughout this work

$$\langle\tau\rangle = \frac{\sum_i a_i \tau_i}{\sum_i a_i} \quad (2)$$

The time-resolved fluorescence anisotropies were determined using Glan-Thompson polarizers, and calculated (e.g., Lakowicz, 1999) according to:

$$r(t) = \frac{I_{VV}(t) - GI_{VH}(t)}{I_{VV}(t) + 2GI_{VH}(t)} \quad (3)$$

where the different intensities are the vertical ( $I_{VV}$ ) and horizontal ( $I_{VH}$ ) components of the fluorescence emission with excitation vertical to the emission axis. The intensity decays of polarized light  $I_{VV}(t)$  and  $I_{VH}(t)$  were obtained with the same accumulation time. The instrumental factor  $G = I_{HV}/I_{HH}$  for the time-resolved instrumentation is  $G = 1$ , once a scrambler is used before the detector. The limiting values of anisotropy at infinite time ( $r_\infty$ ) (Best et al., 1987), were determined directly from the plot

of  $r(t)$  vs.  $t$ . For this purpose there is no need of deconvolution with the laser pulse profile.

## RESULTS

### Differential scanning calorimetry

DSC was used to study the effect of α-MSH on phospholipid mixtures composed of either DMPC<sub>d54</sub>/DMPA or DMPC<sub>d54</sub>/DMPG, 3:1. Thermograms recorded for the phospholipid mixture DMPC<sub>d54</sub>/DMPA, either in the absence or in the presence of α-MSH, are shown in Fig. 1 *A*. In the absence of α-MSH, the transition peak for the mixture DMPC<sub>d54</sub>/DMPA was broad and asymmetrical, with a maximum at ~22°C, in agreement with the literature (Garidel et al., 1997), and no pretransition was observed at variance with the pure phospholipid.  $T_m$  for the mixture is close to the one observed for the pure DMPC<sub>d54</sub>, as  $T_m$  is ~20°C for DMPC<sub>d54</sub> (Muga et al., 1991) and 50°C for DMPA (Garidel et al., 1997). The asymmetric broadening can be related to a non-ideal behavior of the phospholipid mixture. Upon incorporation of α-MSH a small shift in the onset temperature of the gel-to-liquid-crystalline phase transition ( $T_c$ ) of

~2°C is observed, as is the appearance of sharper peaks within the broad component. These peaks (at ~23°C, 27°C, and 32°C) were only apparent in the pure phospholipid mixture. Despite the significant effect on the thermogram, α-MSH did not significantly affect the enthalpy change of the gel-to-liquid-crystalline phase transition of the phospholipid mixture.

Thermograms recorded for the phospholipid mixture DMPC<sub>d54</sub>/DMPG, either in the absence or in the presence of α-MSH, are shown in Fig. 1 *B*. The mixture DMPC<sub>d54</sub>/DMPG has ideal behavior, and as it is found in the pure phospholipids, presents a pretransition at ~12°C and a highly cooperative main transition at ~22°C, in agreement with the literature (Muga et al., 1991). When α-MSH was incorporated in this mixture, the pretransition was abolished, the  $T_m$  value decreased slightly to ~21°C, and at the same time the peak broadened slightly, indicating a decrease in the cooperativity. The incorporation of α-MSH did not significantly affect the enthalpy change of the gel-to-liquid-crystalline phase transition of the phospholipid mixture.

### Determination of the lipid/water partition coefficients of the peptide

Upon incorporation of Trp-containing peptides into membranes, an increase in Trp fluorescence lifetime usually takes place, and there is a concomitant increase in its fluorescence quantum yield. Any of these parameters can be used to quantify the extent of interaction of the peptide with different membrane model systems, as previously shown (Santos et al., 1998), but time-resolved measurements have the advantage of avoiding the artifacts introduced by light-scattering of the lipid suspension. The quantification should be based on the determination of the lipid/water partition coefficient (White et al., 1998), which can be described by

$$K_p = \frac{n_L/V_L}{n_W/V_W} \quad (4)$$

where  $n_i$  stands for moles of peptide in phase  $i$  and  $V_i$  for volume of phase  $i$ . The phase is either aqueous ( $i = W$ ) or lipidic ( $i = L$ ). The relationship and assumptions used to determine  $K_p$  were previously described (Santos et al., 1998),

$$\langle \tau \rangle = \frac{\langle \tau \rangle_W + \langle \tau \rangle_L K_p \bar{V}_L [L]}{1 + K_p \bar{V}_L [L]} \quad (5)$$

where the lifetime-weighted quantum yield of the peptide  $\langle \tau \rangle$  is calculated through Eq. 2 for each lipid concentration  $[L]$ . A nonlinear fit of Eq. 5 to the data yields the values of  $\langle \tau \rangle$  in phase  $i$  ( $i = W, L$ ) and  $K_p$ . The values of  $\bar{V}_L$  used in this work (0.637 M<sup>-1</sup> for vesicles at 20°C and 0.698 M<sup>-1</sup> for vesicles at 37°C) are extrapolated from available data in the literature (Marsh, 1990). In Fig. 2 are shown the fluorescence lifetime-weighted quantum yields  $\langle \tau \rangle$  of α-MSH

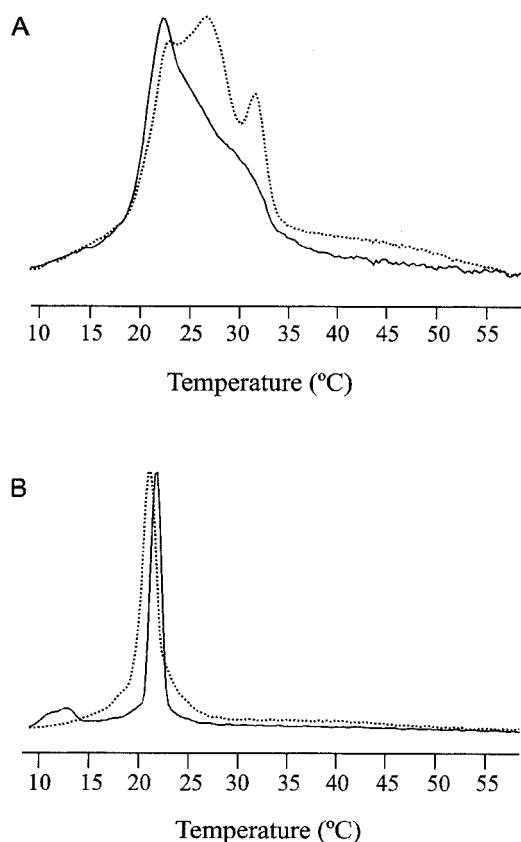


FIGURE 1 DSC heating thermograms for aqueous dispersions of mixtures at a 3:1 molar ratio of DMPC<sub>d54</sub>/DMPA (*A*) and of DMPC<sub>d54</sub>/DMPG (*B*), with (dotted lines) or without (solid lines) α-MSH at a phospholipid/peptide molar ratio of 10:1.

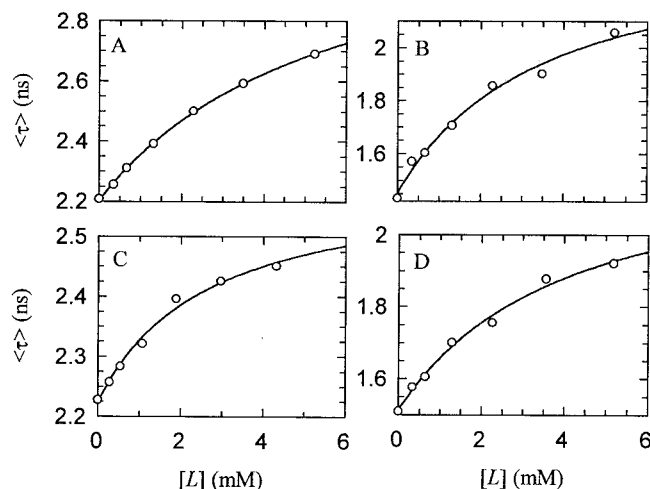


FIGURE 2 Lifetime-weighted quantum yield ( $\langle\tau\rangle$ ) of  $\alpha$ -MSH ( $\lambda_{\text{exc}} = 295$  nm) versus lipid concentration  $[L]$  of DMPC/DMPG (3:1) (A and B) and DMPC/DMPA (3:1) (C and D) in the gel phase at 20°C (A and C) and in the fluid phase at 37°C (B and D). Peptide concentration ( $\sim 30$   $\mu$ M) was kept constant during the experiments. The solid line is the fit of Eq. 5 to the data and the best-fit parameters are shown in Table 1.

versus lipid concentration (either DMPC/DMPG or DMPC/DMPA mixtures both at a 3:1 molar ratio) at 20°C and 37°C.  $\langle\tau\rangle_{\text{w}}$  and the best-fit parameters  $\langle\tau\rangle_{\text{L}}$  and  $K_{\text{p}}$  are shown in Table 1. Although the four  $K_{\text{p}}$  values have the same order of magnitude, there are clear differences depending on the lipidic phase; the values for the fluid phase (37°C) are similar for both lipidic mixtures, but for the gel phase (20°C) the partition coefficient for the DMPC/DMPA system is higher as compared to the one for DMPC/DMPG.

### Steady-state fluorescence spectra and anisotropy

The fluorescence spectra of the peptide under different experimental conditions are shown in Fig. 3. At the excitation wavelength of  $\lambda_{\text{exc}} = 290$  nm, the emission is due to the single Trp residue, and its interaction with the lipidic systems can be appreciated from the spectral shifts observed. The maximum emission wavelength in buffer ( $\lambda_{\text{em}} = 349$  nm) undergoes a blue-shift ( $\lambda_{\text{em}} = 346$  nm) in the presence of the lipidic mixture DMPC/DMPG (3:1) in the gel phase,

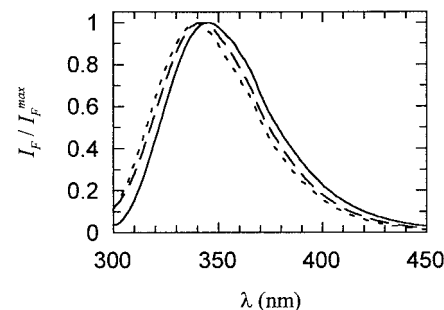


FIGURE 3 Corrected fluorescence emission spectra ( $\lambda_{\text{exc}} = 290$  nm) of  $\alpha$ -MSH at 20°C in aqueous solution (solid line), incorporated in gel phase LUV of DMPC/DMPG (3:1) (dashed line), and DMPC/DMPA (3:1) (dotted line). The spectra were corrected using Eq. 7 as described in the text.

this effect being greater ( $\lambda_{\text{em}} = 343$  nm) in the case of DMPC/DMPA (3:1), also in the gel phase. For the fluid phase, an identical spectral shift ( $\Delta\lambda$ ) is observed for both lipidic systems (Table 1).

The incorporation of the peptide in the membrane is not quantitative. If the partition coefficients  $K_{\text{p}}$  are known, the fraction of peptide in the aqueous phase and in the membrane surface can be determined. Because the samples with the highest concentration of lipid still have an appreciable amount of peptide in water (e.g., in the case of DMPC/DMPG at 20°C,  $\sim 52\%$  of the peptide), the spectra obtained do not correspond only to  $\alpha$ -MSH incorporated into the membrane. The spectra shown in Fig. 3 are corrected for this effect and were calculated from the experimental spectra in water and at the highest concentration of lipid. The molar fraction of the peptide in the aqueous phase is given by

$$x_{\text{w}} = \frac{1}{K_{\text{p}} \bar{V}_{\text{L}}[L] + 1} \quad (6)$$

and in this way the spectrum in the membrane  $F_{\lambda}^{\text{L}}$  is determined from

$$F_{\lambda}^{\text{L}} = C \cdot \left( F_{\lambda}^{\text{L+W}} - x_{\text{w}} \frac{1}{1 + \langle\tau\rangle_{\text{L}}/\langle\tau\rangle_{\text{w}}} F_{\lambda}^{\text{W}} \right) \quad (7)$$

where  $F_{\lambda}^{\text{i}}$  is the spectral distribution (unitary area) function of  $\alpha$ -MSH experimentally determined in water ( $i = \text{W}$ ), and at the highest concentration of lipid ( $i = \text{L} + \text{W}$ ).  $C$  is a

TABLE 1 Lipid/water partition coefficients ( $K_{\text{p}}$ ), and  $^9\text{Trp}$  photophysical parameters of  $\alpha$ -MSH in different systems

System	$T$ (°C)	$K_{\text{p}}^{\text{(lipid/water)}/10^2}$	$\langle\tau\rangle$ (ns)	$\Delta\lambda$ (nm)	$r_{\infty}$
Buffer	20	—	$2.21 \pm 0.02$	—	0
	37	—	$1.51 \pm 0.03$	—	0
DMPC/DMPG (3:1)	20	$2.8 \pm 0.2$	$3.22 \pm 0.05$	3.0	$0.125 \pm 0.006$
	37	$4.0 \pm 1.4$	$2.44 \pm 0.16$	5.0	$0.090 \pm 0.015$
DMPC/DMPA (3:1)	20	$6.0 \pm 1.8$	$2.60 \pm 0.05$	6.0	$0.127 \pm 0.021$
	37	$3.5 \pm 1.0$	$2.25 \pm 0.11$	5.0	$0.114 \pm 0.017$

$\langle\tau\rangle$ , Lifetime-weighted quantum yield;  $\Delta\lambda$ , membrane/water spectral-shift;  $r_{\infty}$ , limiting anisotropy.



normalization constant. The spectral shifts presented in Table 1 are obtained after this correction.

Steady-state fluorescence anisotropy values,  $\langle r \rangle$ , were determined according to Eq. 3 using the steady-state intensities of the components. An anisotropy value of  $\langle r \rangle = 0.0144 \pm 0.0003$  (mean standard error of four different experiments) was obtained at 20°C ( $\lambda_{\text{exc}} = 290$  nm), this being identical to the one obtained in the fluid phase (37°C),  $\langle r \rangle = 0.0146 \pm 0.0007$ .

### $\alpha$ -MSH structure in water and in the presence of lipids by IR

The infrared spectrum of the amide I' region of  $\alpha$ -MSH in D<sub>2</sub>O buffer, at pH 7.4 and at different temperatures, is shown in Fig. 4 A. The amide I' of  $\alpha$ -MSH in the 1700–1600 cm<sup>-1</sup> region is formed by different underlying components that give place to a broad and asymmetric band. Whereas at 15°C the most characteristic feature is a band with maximum intensity at 1618 cm<sup>-1</sup>, at higher temperatures there is a dominant band with a maximum at 1649 cm<sup>-1</sup> (Fig. 4 A). Other smaller components are also discernible in the original spectra at ~1685 and 1586 cm<sup>-1</sup>. Bands appearing at ~1618 cm<sup>-1</sup> accompanied by high-wavenumber counterparts at ~1680–1685 cm<sup>-1</sup> have been previously reported as originated from self-associated peptides forming an intermolecular network of hydrogen-bonded  $\beta$ -strands (Arrondo and Goñi, 1999; Krimm and Bandekar, 1986).

Bands located at ~1656–1650 cm<sup>-1</sup> are usually observed for  $\alpha$ -helix in D<sub>2</sub>O solution, and bands appearing at ~1644–1642 cm<sup>-1</sup> can be assigned in D<sub>2</sub>O to unordered (random) structures (Arrondo and Goñi, 1999). The frequency of the 1649 cm<sup>-1</sup> band is somewhat below the typical range for most  $\alpha$ -helical structures. In this way we obtained circular dichroism (CD) spectra (results not shown), which allows unequivocally assigning this band to a random coil structure, coexisting with the previously described structure ( $\beta$ -strands). In addition, the CD spectra were carried out at a lower concentration (millimolar range), showing that the aggregates detected by IR are not due to any solubilization problem at the higher concentrations.

It is apparent in Fig. 4 A that upon a temperature increase, the area corresponding to the bands at 1618 and 1685 cm<sup>-1</sup> (intermolecularly associated  $\beta$ -strands) decreases, whereas at the same time the area of the 1649 cm<sup>-1</sup> (random coil structure) component band increases. A small but noteworthy inflection can be observed in the temperature variation pattern at ~40°C (Fig. 4 B). Decomposition analysis of the infrared spectra at different temperatures showed that these spectra can be described as linear combinations of only two distinct basis spectra, which would correspond to the peptide either in aggregated form or with random coil structure (Fig. 4, C and D). Whereas the area of the aggregated component is about the same as the random coil structure at 15°C (Fig. 4 D), it decreases to 17% at 70°C accompanied by a concomitant increase of the random coil structure (Fig. 4 C). These data suggest that the structural transition

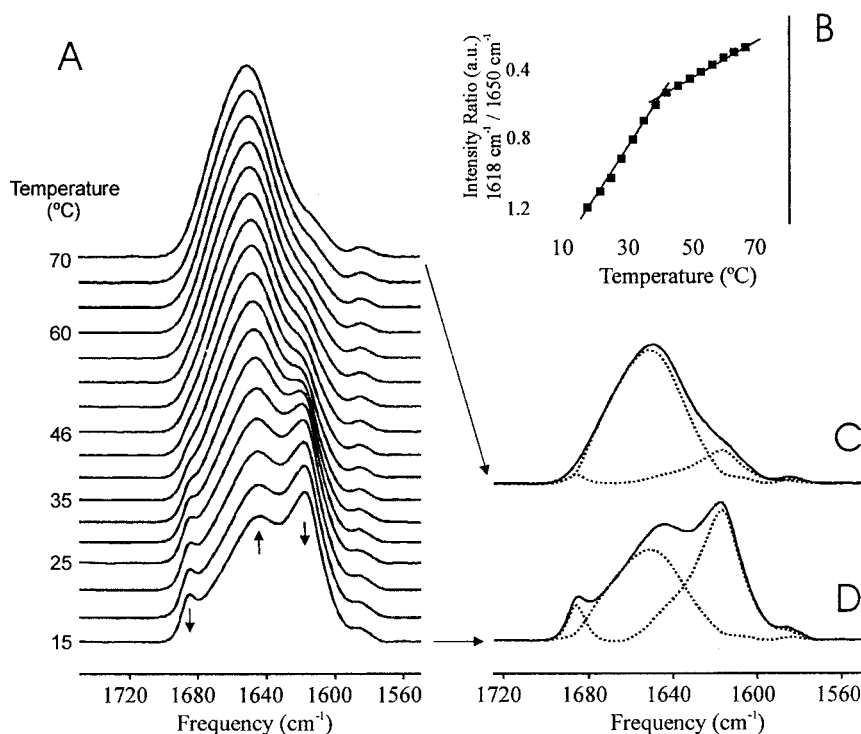


FIGURE 4 Infrared spectra in the amide I' region of  $\alpha$ -MSH in solution at different temperatures as indicated (A), plot of the 1618 cm<sup>-1</sup>/1650 cm<sup>-1</sup> band intensity ratio versus temperature (B), and two-basis spectra fitting of spectra obtained at 70°C (C) and 15°C (D). Arrows in (A) indicate the direction change of the bands upon increase in temperature.

observed upon increasing the temperature is a two-state process involving the contribution from two distinct conformations.

The infrared spectra of the amide I' band of  $\alpha$ -MSH in the presence of either DMPC<sub>d54</sub>/DMPA or DMPC<sub>d54</sub>/DMPG at 45°C are shown in Fig. 5, *A* and *B*, respectively, and as described in the Materials and Methods section, the spectra correspond essentially to the peptide in interaction with the phospholipid membrane. The amide I' band of the peptide when bound to DMPC<sub>d54</sub>/DMPA is similar to the amide I' envelope observed for the peptide at low temperature in aqueous solution (see Fig. 4 *A*), but a difference in conformation can be inferred from the spectral shifts (1618 cm<sup>-1</sup> to 1621 cm<sup>-1</sup> and 1685 cm<sup>-1</sup> to 1694 cm<sup>-1</sup>). As observed in the derivative and deconvoluted spectra shown in Fig. 5 *A*, there are additional components within the amide I' envelope, which could be assigned to different substructure components. Interestingly, the spectrum does

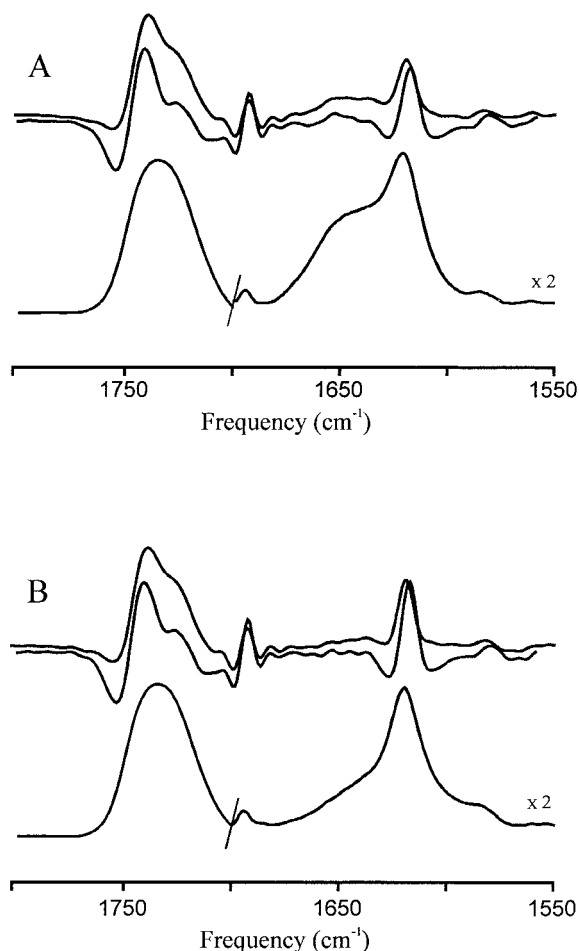


FIGURE 5 Infrared spectra in the C=O and amide I' region of  $\alpha$ -MSH in the presence of DMPC<sub>d54</sub>/DMPA (*A*) and DMPC<sub>d54</sub>/DMPG (*B*) above the gel-to-liquid-crystalline phase transition of the phospholipid mixture. The derivative and deconvoluted spectra are shown above the original spectra (see text for details).

not present a change upon temperature increase (results not shown), indicating a particularly stable entity when bound to the phospholipid. The amide I' band of  $\alpha$ -MSH when bound to DMPC<sub>d54</sub>/DMPG is different from that found for the peptide in the presence of DMPC<sub>d54</sub>/DMPA, being similar to one of the components found previously in the solution structure, that corresponding to the aggregated form of the peptide (see Fig. 4 *D*). As noted for the other lipidic system, no temperature variation was observed.

### Time-resolved fluorescence spectroscopy

The  $\alpha$ -MSH tryptophanyl fluorescence decay in buffer is complex (described by three exponentials), and from the best-fits (reduced  $\chi^2 \leq 1.2$ ), a short component ( $\tau_1 = 0.448$  ns (20°C) or 0.307 ns (37°C);  $a_1 = 0.18$ ), an intermediate component ( $\tau_2 = 2.00$  ns (20°C) or 1.36 ns (37°C);  $a_2 = 0.48$ ), and a long component ( $\tau_3 = 3.45$  ns (20°C) or 2.35 ns (37°C);  $a_3 = 0.34$ ) are obtained. In the presence of both lipidic systems at the two temperatures studied a variation of both the lifetimes and amplitudes of the components is observed. The data for the DMPC/DMPA (3:1) mixture at 20°C is depicted in Fig. 6, and the trend of variation is

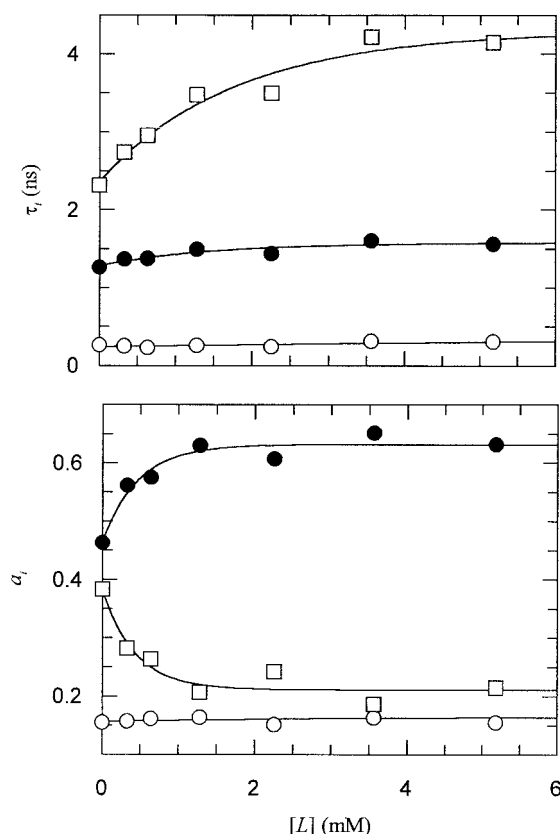


FIGURE 6 Fluorescence lifetime components  $\tau_i$  and respective normalized pre-exponentials  $a_i$  for  $\alpha$ -MSH ( $\lambda_{\text{exc}} = 295$  nm,  $\lambda_{\text{em}} = 350$  nm) versus lipid concentration  $[L]$  (DMPC/DMPA (3:1) LUV) at 20°C. The solid lines are merely guides to the eye and have no physical meaning.

similar to the one obtained for the DMPC/DMPG mixture (results not shown). The short lifetime is essentially invariant, while the intermediate one increases slightly and reaches a plateau, and a marked increase is observed for the long one, which varies from 3.45 ns in buffer up to 6.72 ns for the highest lipid concentration. Regarding the amplitudes, that of the short component shows a very slight increase; the amplitude of the intermediate component increases significantly and for the amplitude of the long one a correspondent decrease is obtained, both reaching a plateau at higher lipid concentrations. The amplitudes and the lifetimes of the components are similar to those obtained by Ito et al. (1993). From the partition coefficient study previously described (Eq. 5),  $\langle\tau\rangle_L$ , the lifetime-weighted quantum yield of the peptide interacting with the membrane, was determined and the values for the two membrane model systems are presented in Table 1. The  $\langle\tau\rangle_L$  values are longer in the DMPC/DMPG vesicles as compared to the DMPC/DMPA ones at the same temperature.

The time-resolved anisotropies of α-MSH in water and at the highest lipid concentration were obtained and the decay for the DMPC/DMPA (3:1) mixture (37°C) is presented in Fig. 7. When in water, the Trp residue fluorescence anisotropy decays to zero, as expected, and values for the rotational correlation time  $\Phi = 0.520 \pm 0.016$  ns (20°C) and  $\Phi = 0.317 \pm 0.009$  ns ( $T = 37^\circ\text{C}$ , result not shown) are obtained, considering for the fundamental anisotropy  $r_0 = 0.24$  ( $\lambda_{\text{exc}} = 295$  nm, Valeur and Weber, 1977). When in interaction with the membrane, the anisotropy of α-MSH decays to a value larger than zero ( $r_\infty$ ). Considering that even at the highest lipid concentration a considerable fraction of peptide still remains in water, the limiting anisotropy should be corrected for this effect as previously described for the fluorescence spectra. This is carried out according to the equation:

$$r_\infty^L = \left(1 + \frac{x_W \langle\tau\rangle_W}{x_L \langle\tau\rangle_L}\right) \cdot r_\infty \quad (8)$$

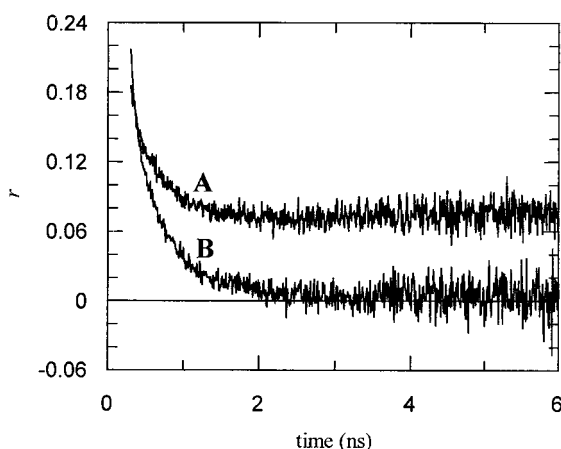


FIGURE 7 Anisotropy decays of α-MSH ( $\lambda_{\text{exc}} = 295$  nm) in buffer (A) and in the presence of 5.2 mM of DMPC/DMPA (3:1) LUV at 37°C (B).

where  $r_\infty^L$  is the limiting anisotropy of α-MSH when in the membrane and  $r_\infty$  is the experimentally determined value for the highest lipid concentration. The other parameters are described in Table 1 or obtained from Eq. 6. For both lipidic systems  $r_\infty^L$  is larger at the lower temperature, but no significant variation was observed between the two lipidic systems.

### Thermotropic behavior of the lipids from the $\text{CH}_2(\text{CD}_2)$ stretching vibration

In order to obtain further information on the transitions observed in the thermograms (Fig. 1), we have studied the effect of α-MSH on the phase transition of the phospholipid mixtures by IR (Fig. 8). Because in this study one of the phospholipids was acyl chain-perdeuterated (DMPC<sub>d54</sub>), and the other was not, it is possible to independently detect changes that might occur with each one.

The temperature dependence of the  $\text{CH}_2/\text{CD}_2$  symmetric stretching frequency is shown in Fig. 8 A for DMPA and in Fig. 8 B for DMPC<sub>d54</sub> in the DMPC<sub>d54</sub>/DMPA mixture. There, a single broad transition starting at  $\sim 22^\circ\text{C}$  and ending at  $\sim 35^\circ\text{C}$  is observed (mean values of  $T_m = 29.0^\circ\text{C}$  (DMPA) and  $T_m = 28.8^\circ\text{C}$  (DMPC<sub>d54</sub>)), quite similar to the gel-to-liquid-crystalline phase transition found by DSC for the same lipidic system (see Fig. 1 A). When α-MSH was present, a small shift of the mean  $T_m$  to higher temperatures ( $T_m = 30.2^\circ\text{C}$  (DMPA) and  $T_m = 30.4^\circ\text{C}$  (DMPC<sub>d54</sub>)) was observed, again in agreement with the DSC data. It should be emphasized that the presence of α-MSH induced a shift of the DMPA frequency to higher values, but only at temperatures above  $T_m$ , i.e., when the phospholipid mixture is in the liquid-crystalline phase (Fig. 8 A). This effect, not so apparent in DMPC<sub>d54</sub> (Fig. 8 B), might indicate the presence of lipid aggregates above  $T_m$ , enriched in DMPA bound to α-MSH.

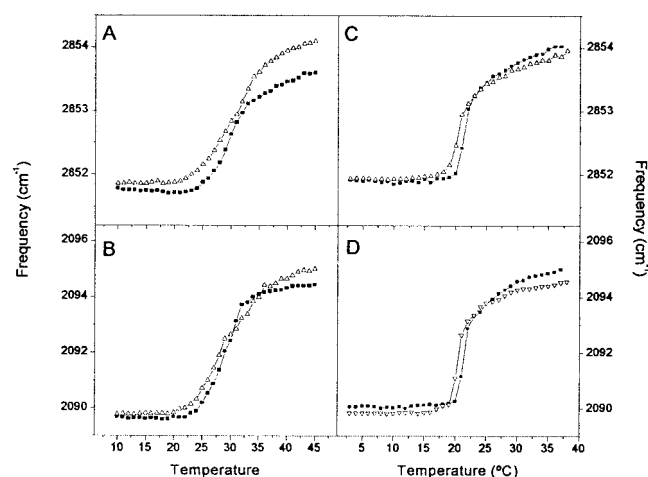


FIGURE 8 Temperature dependence of the frequency of the symmetrical  $\text{CH}_2$  stretching (A and C) and symmetrical  $\text{CD}_2$  stretching (B and D) of mixtures containing DMPC<sub>d54</sub>/DMPA (A and B) and DMPC<sub>d54</sub>/DMPG (C and D) in the absence (■) and in the presence (▽) of α-MSH.

The temperature dependence of the  $\text{CH}_2$  symmetric frequency of the phospholipid mixture  $\text{DMPC}_{\text{d54}}/\text{DMPG}$  is shown in Fig. 8 *C* for DMPG and in Fig. 8 *D* for  $\text{DMPC}_{\text{d54}}$ . Single cooperative transitions at  $\sim T_m = 21.8^\circ\text{C}$  ( $\text{DMPC}_{\text{d54}}$ ) and  $T_m = 21.5^\circ\text{C}$  (DMPG) were observed, corresponding to the gel-to-liquid-crystalline phase transition of the phospholipid mixture (see the DSC data presented in Fig. 1 *B*). When  $\alpha$ -MSH was present in the mixture, similar transitions were observed, but they showed small shifts to lower temperatures ( $T_m = 20.7^\circ\text{C}$  ( $\text{DMPC}_{\text{d54}}$ ) and  $T_m = 20.3^\circ\text{C}$  (DMPG)), again in agreement with the DSC data.

## DISCUSSION

### Thermotropic behavior of the phospholipids in the presence of $\alpha$ -MSH by calorimetry and infrared spectroscopy

As shown in Fig. 1, the  $\text{DMPC}_{\text{d54}}/\text{DMPA}$  system displayed a non-ideal behavior because at least two peaks could be observed in the DSC thermogram. Upon incorporation of  $\alpha$ -MSH, a significant change was observed. This effect was related to that observed by IR (Fig. 8), as  $\alpha$ -MSH induced a significant change in the  $\text{CH}_2$  stretching frequency of DMPA as compared to the  $\text{CD}_2$  stretching frequency of DMPC, suggesting a preferential interaction of the peptide with DMPA-enriched domains. The fact that no significant effect is apparent in the gel phase is probably due to the peptide incorporation in the line defects between the gel domains (Loura et al., 1996), and so there is not such a strong lipid perturbation in that case. It is interesting that a smooth transition is observed in all cases, and there is no evidence of, e.g., shoulders that would correspond to the peaks observed in DSC. This would mean that the different lipidic domains would have no significant variations in the stretching frequencies depending on their composition, or otherwise they would superimpose preventing the observation of inflections.

In contrast to the  $\text{DMPC}_{\text{d54}}/\text{DMPA}$  system, the  $\text{DMPC}_{\text{d54}}/\text{DMPG}$  behaves as an ideal system, where only one peak could be discerned. Upon  $\alpha$ -MSH binding to this system, a broadening of the transition peak was observed, as was a slight shift of  $T_c$  to lower temperatures, and by IR we found no significant differences in the  $\text{CH}_2$  and  $\text{CD}_2$  stretching frequencies.

In conclusion,  $\alpha$ -MSH establishes an interaction with the phospholipid molecules, probably via the intercalation of the peptide molecule between the phospholipid headgroups and perturbing, although slightly, the cooperative behavior of the phospholipid acyl chains. As it was noted before,  $\alpha$ -MSH has a net +1 charge at physiological pH and a low hydrophobicity, implying an essentially electrostatic interaction between the peptide and the anionic phospholipids. This interaction is more effective with the DMPA lipid containing system as compared to DMPG.

### Lipid/water partition coefficients of the peptide

Several studies indicate that the peptide does not interact with neutral membranes composed of only zwitterionic phospholipids (Ito et al., 1993; de Kroon et al., 1991; Biaggi et al., 1996), i.e., the hydrophobic contribution for the partition coefficient is not significant. In fact, all the partition coefficients found in this work are of the same order of magnitude because in all cases the global charge of the lipid vesicles is the same (25% anionic phospholipid molecules). However, the different values obtained for the partition coefficients in each case reveal that other characteristics of the system are important besides its global charge. In the case of DMPC/DMPG the result is not surprising, because previous studies point out to a stronger interaction of the peptide with phospholipid vesicles in the liquid-crystalline rather than in the gel phase (Ito et al., 1993), although no quantified values (i.e.,  $K_p$  or binding constants) were presented for the gel phase. The opposite behavior observed with DMPC/DMPA is unexpected. Although DMPC forms a homogeneous mixture with DMPG, DMPA presents a clear evidence of domain formation (phase separation) from the DSC thermograms (Fig. 1). It is possible that the domains enriched in the anionic phospholipid induce a stronger electrostatic interaction with the peptide, and as described before the interfaces between these domains would facilitate the peptide incorporation.

Preliminary results of both steady-state and time-resolved fluorescence on the interaction of  $\alpha$ -MSH with a mixture of DMPC/1,2-dimyristoyl-*sn*-glycero-3-phosphoserine (for which a phase diagram similar to the DMPC/DMPA mixture is reported (Silvius and Gagné, 1984; Graham et al., 1985)) also indicate a stronger interaction in the gel phase rather than in the fluid (unpublished results).

### Steady-state fluorescence spectra and anisotropy

The maximum fluorescence emission for  $\alpha$ -MSH in buffer is at 349 nm, a value very close to the maximum determined for Trp in buffer (350 nm). This result implies that the Trp residue in  $\alpha$ -MSH is strongly solvated, as expected for a small peptide with no tertiary structure. The interaction of the peptide with the lipidic system is clearly shown from its blue-shifts (3–6 nm, depending on temperature and lipidic system, see Table 1), pointing out to a more hydrophobic environment of the fluorophore (Lakowicz, 1999).

The results for the system with DMPG are in agreement with the studies of Ito et al. (1993), in which the shift was greater when the peptide was interacting with membranes in the liquid crystalline phase. However, when the anionic lipid is DMPA this is not the case. The information we can derive from this observation is that the hydrophobicity of the environment surrounding the Trp residue of  $\alpha$ -MSH is greater in DMPC/DMPA vesicles in the gel phase than in



DMPC/DMPA and DMPC/DMPG vesicles in the fluid phase. A more efficient fluorophore solvation happens for DMPC/DMPG in the gel phase. Once the hydration of the lipid bilayers is greater in the fluid phase (Cevc and Marsh, 1987), for the DMPC/DMPG system we can conclude from the relative spectral shifts that  $\alpha$ -MSH penetrates deeper into the membrane in the liquid-crystalline phase. Regarding the DMPC/DMPA model membrane, and as the shift is only 1 nm or less for the fluid phase, no information about its location can be obtained. However, IR data are indicative of a deeper penetration in the fluid phase, as concluded from the greater disordering of DMPA acyl chains.

Information about the eventual aggregation of peptide in water, at the low concentrations used in fluorescence experiments, can be obtained from the anisotropy data.

If a spherical geometry is assumed for the peptide, the steady-state anisotropy can be determined from the Perrin equation (Lakowicz, 1999),

$$\langle r \rangle = r_0 / (1 + \tau / \Phi) \quad (9)$$

where  $r_0$  is the fundamental anisotropy,  $\tau$  the fluorescence lifetime, and  $\Phi = \eta V / RT$  is the rotational correlation time,  $\eta$  and  $V$  being the solvent viscosity and the rotating unity volume, respectively.

The peptide volume can be estimated as  $V = 1821 \text{ \AA}^3$  (Zamyatin, 1972), and for water ( $\eta = 1.00 \text{ cP}$  ( $20^\circ\text{C}$ );  $\eta = 0.69 \text{ cP}$  ( $37^\circ\text{C}$ )), values of  $\phi = 0.45 \text{ ns}$  ( $20^\circ\text{C}$ ) and  $\phi = 0.29 \text{ ns}$  ( $37^\circ\text{C}$ ) are obtained.

From the average fluorescence lifetime (Eq. 1) of the peptide ( $\bar{\tau} = 2.71 \text{ ns}$  ( $20^\circ\text{C}$ ) and  $\bar{\tau} = 1.85 \text{ ns}$  ( $37^\circ\text{C}$ )) and the fundamental anisotropy  $r_0 = 0.105$  at  $\lambda_{\text{exc}} = 290 \text{ nm}$  (Valeur and Weber, 1977), values of  $\langle r \rangle = 0.015$  ( $20^\circ\text{C}$ ) and  $\langle r \rangle = 0.014$  ( $37^\circ\text{C}$ ) are obtained. These are very close to the experimental ones, and in this way we can conclude that the peptide is essentially monomeric at the physiological concentrations used in the fluorescence measurements (micromolar). In fact, for a dimer (2 V), or a larger aggregate (e.g., 5 V), values of  $\langle r \rangle = 0.026$  ( $20^\circ\text{C}$ ) and  $\langle r \rangle = 0.047$  ( $20^\circ\text{C}$ ), respectively, would be theoretically expected. The problems arising at the concentrations used in the other techniques (DSC, IR) will be discussed later.

### Peptide structure by infrared spectroscopy

We found that the amide I band of  $\alpha$ -MSH in buffer is dependent on temperature: a mixture of two structures, random coil and hydrogen-bonded  $\beta$ -strand at low temperature, and nearly all random coil at high temperatures. At variance, this alteration is not observed in the presence of the lipid vesicles under all conditions tested. This behavior is in contrast with other systems, such as gramicidin S in the presence of anionic phospholipids (Lewis et al., 1999). The conformation of  $\alpha$ -MSH does not change with the physical state of the host lipid bilayer, i.e., when the phospholipid

changes from the gel to the liquid-crystalline phase. In this way  $\alpha$ -MSH would be largely excluded from the lipid bilayer and associated with the lipid polar headgroups at the bilayer surface. Given the relatively small size of  $\alpha$ -MSH, such an observation could be attributed to the formation of some type of extended intermolecular aggregates at the phospholipid surface (Thiaudière et al., 1991). This result would be in consonance with the lipid/water partition coefficients and the small spectral shifts obtained by fluorescence spectroscopy (Table 1).

It should also be noted that there are differences in the amide I band of  $\alpha$ -MSH depending on the lipidic system because, as shown in Fig. 5, the proportion of random coil is greater in the presence of DMPC/DMPA than in the presence of DMPC/DMPG. These differences in spectroscopic behavior are also correlated with differences in calorimetric behavior and fluorescence lifetime-weighted quantum yields (see below), and all point to specific interactions with the lipid headgroup. In case that a strong interaction with the lipid acyl chains would exist, significant differences would be apparent for the peptide interaction with gel and fluid phases.

Due to its intrinsic lower sensitivity, it is well known that the concentrations used in the DSC and IR experiments are in general much higher than the physiological ones (submicromolar or lower). In the present work, we showed from the anisotropy data that in all studies involving fluorescence (e.g., partition constants), the monomeric species is the one under study. At variance, both from CD and IR, evidence for aggregate formation was obtained at lower temperatures, and in this way the physiological relevance of these data deserves detailed attention.

It should be stressed that at the highest temperature ( $70^\circ\text{C}$ ), the peptide structure is essentially a random coil in water, i.e., similar to the physiological monomeric species, so the different structures observed in the presence of the two lipids can be safely concluded at this temperature. In addition it was observed that the peptide structure in the presence of lipids is temperature-invariant, at variance with the studies in water. This means that at lower temperatures the interactions with the lipid and not peptide-peptide interactions are the controlling factor. Interestingly, this effect is greater for DMPC/DMPA as compared to DMPC/DMPG, in agreement with the information derived from the fluorescence data.

### Time-resolved fluorescence spectroscopy

The lifetime-weighted quantum yield,  $\langle \tau \rangle$ , of  $\alpha$ -MSH in water at  $20^\circ\text{C}$  is very close to the value for Trp in aqueous solution at the same temperature ( $\langle \tau \rangle = 2.25 \text{ ns}$ ) (Lakowicz, 1999) and just slightly higher than the value for  $\alpha$ -MSH reported in the literature ( $\langle \tau \rangle = 2.09 \text{ ns}$ ) (Ito et al., 1993). If we extrapolate the values at different temperatures reported in that work, the fluorescence lifetime at  $37^\circ\text{C}$  ( $\langle \tau \rangle = 1.51$

ns) is in very good agreement with our data. The complex decay obtained for peptides in aqueous solution is presently assigned to different rotational conformers of the indole ring (Willis and Szabo, 1992). Upon interaction with the lipidic system the peptide undergoes alterations of its secondary structure, and these can be appreciated from the trend of variation of both lifetime components and pre-exponentials (Fig. 6).

It is difficult to infer conclusions about the secondary structure of the peptide by the fluorescence decays in the different environments mainly because there are still very few studies made on peptides in which the Trp residue is in a position of known structure and, e.g., Dahms and Szabo (1995) point to subtleties that require caution when predicting structures. However, from the trend of variation of the time-resolved data, it can be concluded that upon membrane incorporation,  $\alpha$ -MSH undergoes strong structural changes, which is in agreement with the IR data. However, it should be stressed that this pattern of fluorescence time-resolved data only reports the peptide structure in the vicinity of the Trp residue, whereas global information about all the peptide structure is obtained from IR. Thus, there is no contradiction when the very same pattern of variation was obtained for both lipids, at variance with IR data, which allowed concluding that intermolecular aggregates are the dominant structural feature in DMPG-containing vesicles, while in DMPA ones some random structure is present.

The lifetime-weighted quantum yields of the peptide interacting with the different membranes,  $\langle\tau\rangle_L$ , are longer for DMPC/DMPG as compared to the DMPC/DMPA mixture (Table 1), probably due to the structural differences found by IR spectroscopy. The fact that  $\langle\tau\rangle_L$  is longer in DMPG-containing vesicles but the spectral shift is smaller does not represent any contradiction. In fact, there is no clear correlation between quantum yield and the wavelength of maximum emission in proteins with a single Trp residue (Lakowicz, 1999).

The values obtained for the rotational correlation times  $\Phi$ , assuming a spherical rotor, are in reasonable agreement with the ones expected from the Perrin equation (Eq. 9), once that an average lifetime is used to describe the complex decay.

Our study of time-resolved fluorescence anisotropy in membranes was restricted to the limiting anisotropies  $r_\infty$  because the correction for the fraction of peptide in the aqueous phase would be too critical to allow the determination of the dynamics of the system contained at the earlier times of the decay. The limiting anisotropies obtained are in all cases very high, considering that the fluorophore is not a typical hydrophobic molecule incorporated into the membrane core with a serious restriction to its motion. This means that in all cases the peptide is strongly adsorbed at the membrane/water interface and the wobbling motion of the Trp is very restricted, even in the fluid phase. Considering that most of the studies (e.g.,  $K_p$ ,  $\Delta\lambda$ , DSC) carried out for the DMPC/DMPA mixture in the gel phase gave pecu-

liar results due to the phase separation observed by DSC, we could also anticipate a greater value for  $r_\infty$ , which is not observed. It can then be concluded that for DMPC/DMPG less peptide incorporation is obtained (lower  $K_p$ ), but the peptide in the membrane has a hindered rotation similar to the one observed for the DMPC/DMPA mixture. Irrespective of the structure adopted by the peptide in the vesicles, not only it is thermally stabilized, it is also very rigid. The limiting anisotropies  $r_\infty$  are related to the order parameter  $S$ , and the fundamental anisotropy  $r_0$  through the following relationship (e.g., Lakowicz (1999)):

$$S^2 = \frac{r_\infty}{r_0} = \left\langle \frac{3 \cos^2 \theta - 1}{2} \right\rangle^2 \quad (10)$$

where  $\theta$  is the angular rotation of the emission transition moment and the angle brackets indicate an average over all the fluorophore population. If we consider a fundamental anisotropy for Trp excitation at 295 nm of  $r_0 = 0.3$  (Valeur and Weber, 1977), an angle  $\theta = 29^\circ$  is obtained for the gel phase, or  $\sim 30$ – $33^\circ$  for the liquid-crystalline phase. The Trp containing central 6–9 region of  $\alpha$ -MSH is the minimal melanotropic message sequence (Hruby et al., 1987). In a recent molecular dynamics study (Pascutti et al., 1999) the peptide acquired, in a low dielectric constant medium, a packed conformation that remained stabilized for  $>7.0$  ns of the simulation. A common feature of various superpotent synthetic analogs of the native hormone is the stabilization of a turn involving the Trp residue (Sawyer et al., 1980, 1982). The behavior of the fluorescence decay components indicates a similar structure in the region of the Trp residue region, and the limiting anisotropies reveal an effective immobilization of the indole ring in both lipidic systems, above and below the  $T_m$ .

## CONCLUDING REMARKS

The interaction of  $\alpha$ -MSH with (3:1) lipid mixtures containing the same net charge (DMPC/DMPG or DMPC/DMPA) was studied by complementary methods.

It was concluded that  $\alpha$ -MSH in buffer at higher concentrations undergoes a transition from an aggregated plus random coil structures to essentially random coil upon temperature increase, this transition being prevented when in interaction with negatively charged vesicles. These can stabilize specific structures different from the ones in solution, as concluded from IR and fluorescence data. A strong interaction of the peptide with both lipidic systems was also concluded from the high limiting anisotropies observed both above and below  $T_m$ .

The peptide can induce lateral heterogeneity in the DMPC/DMPA lipidic systems, as seen from the strong alterations observed in the calorimetric thermograms, and in addition a greater partition coefficient is obtained for this last system in the gel phase as compared to the fluid phase. Analogs of

$\alpha$ -MSH with superpotent biological activity possess a defined and stable structure. It is reasonable to predict that the interaction of  $\alpha$ -MSH with negatively charged membranes induces and stabilizes a specific conformation, probably involving the Trp-containing message region, which could be similar to the one found for the superpotent analogs, and therefore necessary for its biological activity.

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J.V. and M.P. contributed equally to this work.

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