

## Melanotropic peptides–lipid bilayer interaction. Comparison of the hormone $\alpha$ -MSH to a biologically more potent analog

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### Abstract

The interaction of the native peptide  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) and the biologically more active analog [Nle<sup>4</sup>, D-Phe<sup>7</sup>]- $\alpha$ -MSH(MSH-I) with lipid vesicles was studied by spin label electron spin resonance (ESR) spectroscopy and circular dichroism (CD). Using spin labels located at the membrane interface and at different depths along the acyl chain, it was shown that the binding of both peptides to the membrane induces tighter lipid packing at all the monitored positions. However, the effect of the analog on the spin label ESR parameters was much more evident, and suggested that it penetrates farthest into the lipid matrix than the native molecule. Lipid partition coefficients were calculated based on the effect the peptides cause on the ESR spectra of spin labels incorporated in the membrane. For the biologically more potent peptide, the partition coefficient was found to be about 4-times greater than that of the native hormone. For the same concentration of peptide bound to the membrane, MSH-I was found to cause a slightly greater effect on the membrane structure than  $\alpha$ -MSH, in accord with its possible deeper penetration into the bilayer. CD spectra in aqueous solution and in the  $\alpha$ -helix inducing solvent 2,2,2-trifluoroethanol showed that the two peptides have somewhat different structures in solution, though similar conformational changes occur in both peptides as a result of their interaction with negatively charged vesicles or micelles. The higher peptide–lipid association constant and the deeper penetration of the analog into lipid bilayers could be related to its greater activity and/or prolonged action. © 1997 Elsevier Science B.V.

**Keywords:** Melanotropin; Peptide–lipid interaction; Spin label; Circular dichroism

Abbreviations: ESR, electron spin resonance; CD, circular dichroism; NMR, nuclear magnetic resonance; SDS, sodium dodecyl sulfate;  $\alpha$ -MSH,  $\alpha$ -melanocyte stimulating hormone; MSH-I, [Nle<sup>4</sup>, D-Phe<sup>7</sup>]- $\alpha$ -MSH, 4-norleucine, 7-D-phenylalanine- $\alpha$ -melanocyte stimulating hormone; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 5-, 7-, 9- and 12-SASL, 5-, 7-, 9-, and 12-doxylstearic acid spin label; SSL, stearamide spin label;  $T_c$ , lipid main transition temperature;  $A_{max}$ , outer hyperfine splitting;  $S_{eff}$ , effective order parameter;  $\tau_{||}$ , rotational correlation time about the molecular symmetry axis;  $\tau_{\perp}$ , rotational correlation time perpendicular to the molecular symmetry axis; TFE, 2,2,2-trifluoroethanol

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## 1. Introduction

In most vertebrates the tridecapeptide  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH; Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH<sub>2</sub>) is the physiologically relevant hormone regulating skin pigmentation, causing darkening. In culture, both in normal and abnormal (melanoma) cells,  $\alpha$ -MSH and its derivatives have been reported to stimulate adenylate cyclase, and to increase the activity of the enzyme tyrosinase, which controls the melanin synthesis [1–4].  $\alpha$ -MSH is also involved in many other biological functions, such as fetal growth and behavior [5].

Several modifications made within the primary structure of  $\alpha$ -MSH have yielded peptides with superpotent activity [1,6]. The present work focuses on the native hormone  $\alpha$ -MSH and the analog [Nle<sup>4</sup>, D-Phe<sup>7</sup>]- $\alpha$ -MSH (hereafter referred to as MSH-I), wherein norleucine was substituted for methionine, and D-phenylalanine was substituted for the L-enantiomer. The latter was reported to exhibit an increased potency and prolonged activity in the frog skin bioassay when compared to the native molecule [1]. It was suggested that the increased potency of MSH-I could be attributed to a reverse turn within the  $\alpha$ -MSH<sub>4–10</sub> sequence which would be conformationally-stabilized by the D-Phe<sup>7</sup> substitution [7]. Though the prolonged activity of MSH-I in vivo could be ascribed to its resistance to degradative inactivation by serum enzymes, its ultra-long action in vitro remains to be understood. It was suggested [1] that MSH-I could be sequestered into some compartment of the membrane, possibly irreversibly bound to a receptor.

Although different activities of  $\alpha$ -MSH analogs may reflect different strengths of binding to protein membrane receptors, the peptide–lipid interaction could also be relevant to both the potency and the duration of the peptide action. According to the hypothesis of the lipid phase as a catalyst for the peptide biological action [8,9], the peptide–bilayer interaction could favor a particular peptide conformation appropriate for its interaction with the receptor site, and/or increase the local peptide concentration close to the receptor. Alternatively, the peptide could slightly change the membrane fluidity, triggering structural modifications in membrane proteins,

which would enhance the peptide–receptor interaction.

In a previous work we showed that both the native hormone  $\alpha$ -MSH and the analog MSH-I interact with anionic lipid bilayers, turning the membrane more rigid [10]. We used electron spin resonance (ESR) spectroscopy of spin labels incorporated in the lipid phase, and NMR of deuterated lipids. However, it remained to be shown whether the different effects the two peptides cause on the bilayer structure were due to their different lipid partition coefficients, or to a relative different penetration into the membrane hydrophobic core.

The present work extends those studies, using stearic acids labeled at four different positions along the acyl chain and at the headgroup. This approach provides information on the motional and structural alterations caused by the peptides at different depths of the lipid bilayer, making possible a comparison between the effects due to the native hormone and its analog at the different positions. To further understand the peptide–bilayer interaction, lipid partition coefficients for the two peptides were calculated, based on the effect they cause on the membrane structure. Considering those partition coefficients, it was possible to compare the alterations in the bilayer caused by the two peptides when present at the same concentration in the membrane. Structural modifications on the conformation of the peptides were monitored by their circular dichroism (CD) spectra.

## 2. Materials and methods

### 2.1. Materials

The peptides  $\alpha$ -MSH and MSH-I, and SDS (sodium dodecyl sulfate) were purchased from Sigma Chemical Co. (St. Louis, MO). Phospholipids, DMPG (1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol) and DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), were obtained from Avanti Polar Lipids (Birmingham, AL). The spin labels 5-, 7-, 9- and 12-SASL (5-, 7-, 9- and 12-doxylstearic acid spin label) were either purchased from Sigma or kindly donated by A. Watts, from the University of Oxford, UK. SSL (stearamide spin label) was a gift from S. Schreier Laboratory, at the University of São

Paulo. The buffer used for the ESR experiments was 10 mM Hepes (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid) at pH 7.4. For the CD experiments 5 mM phosphate buffer, pH 7.4, was used. All reagents were used without further purification.

## 2.2. ESR sample preparation

Stock solutions of spin labels were prepared in chloroform and stored at  $-80^{\circ}\text{C}$ . The membranes were multilamellar dispersions of lipids (10 mM) containing 1 mol% of spin label. A lipid film was formed from a chloroform solution of lipids and spin labels, dried under a stream of  $\text{N}_2$  and left under vacuum for a minimum of 5 h, to remove all traces of the organic solvent. Liposomes were prepared by the addition of the buffer solution, without or with the desired concentration of melanotropic peptides, followed by vortexing. Large unilamellar vesicles (LUV), prepared by the method of extrusion [11], were also used, yielding results similar to those obtained with liposomes.

## 2.3. CD sample preparation

Large unilamellar vesicles were prepared as described above, and sonicated until the sample was optically clear, producing no obvious light scattering. Peptide solution was added to the final desired concentration.

## 2.4. ESR spectroscopy

ESR measurements were performed in a Bruker ER 200D-SRC spectrometer interfaced with an IBM-PC like computer for spectrum digitalization. A field modulation amplitude of 0.08 mT and microwave power of 10 mW were used. The temperature was controlled to about  $0.5^{\circ}\text{C}$  with a Bruker B-ST 100/700 variable temperature device. The temperature was always varied from  $40^{\circ}\text{C}$  to  $0^{\circ}\text{C}$  and monitored with a Fluke 51 K/J thermometer. As most of the stearic acid spin labels presented a small partition in solution, the ESR spectra shown here are the result of the subtraction of free aqueous label signal from the composite spectrum, using the software EPRANALA (J. Rowntree, P. Fajer and B. Bennet, University of Oxford, UK). The LOWFIT program

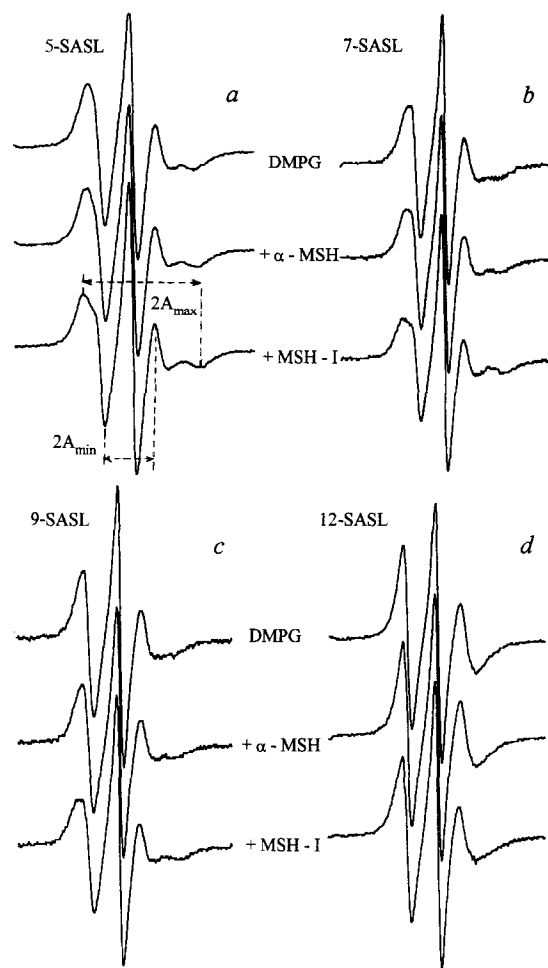


Fig. 1. ESR spectra of stearic acid spin label positional isomers, n-SASL, in DMPG dispersion (10 mM Hepes, pH 7.4) in the absence and presence of 10 mol% of  $\alpha$ -MSH or MSH-I. Total spectral width is 100 G. Temperature =  $40^{\circ}\text{C}$ .

(B. Bales) was used for the SSL spectra simulations. For the measurement of the spectrum parameters the ORIGIN software (MicroCal Software, MA, USA) was used.

Effective order parameters,  $S_{\text{eff}}$ , were calculated from the expression

$$S_{\text{eff}} = \frac{A_{\parallel} - A_{\perp}}{A_{zz} - (1/2)(A_{xx} + A_{yy})} \frac{a'_0}{a_0}$$

where  $2A_{\parallel}$  (or  $2A_{\max}$ ) is the maximum hyperfine splitting and

$$A_{\perp} = A_{\min} + 1.4 \left[ 1 - \frac{A_{\parallel} - A_{\min}}{A_{zz} - (1/2)(A_{xx} + A_{yy})} \right]$$

where  $2A_{\min}$  is the measured inner hyperfine splitting [12,13]. (For  $2A_{\max}$  and  $2A_{\min}$  see Fig. 1.) The polarity was normalized from  $a'_0/a_0$  where

$$a'_0 = (1/3)(A_{xx} + A_{yy} + A_{zz})$$

and

$$a_0 = (1/3)(A_{\parallel} + 2A_{\perp})$$

$A_{xx}$ ,  $A_{yy}$  and  $A_{zz}$  being the principal values of the hyperfine tensor for doxylpropane [12]. All the data shown here are the mean of at least three experiments. Standard deviations were calculated for all the experimental data, considering three different experiments with ten independent measurements for each ESR spectrum. In Fig. 2 and Fig. 5, they were

found to be around 2%. In the other figures, whenever the standard deviation is greater than the symbol size it is shown in the graphic.

## 2.5. Circular dichroism

Spectra were recorded at 35°C on Jasco J-720 and CD6 Jobin Yvon spectropolarimeters, with quartz cells of 1 mm path length. Spectra were baseline corrected by using the appropriated reference, buffer or vesicle suspension. Typically five spectra were recorded and averaged over the range of 185 to 260 nm. The data are expressed as mean residue ellipticity,  $[\theta]$ , in  $\text{deg}/\text{cm}^2 \text{dmol}^{-1}$ .

## 3. Results and discussion

### 3.1. ESR of spin labels at different depths of the carbon chain

Fig. 1 shows the ESR spectra of positional isomers of spin labeled stearic acids incorporated in DMPG liposomes, in the absence and in the presence of the two peptides. The fluidity of the membrane microenvironment can be quantified by measuring the outer maximum hyperfine splitting  $A_{\max}$  which contains contributions from both the amplitude and the rate of motion of the spin label chains ([14], and references therein).  $A_{\max}$  decreases as the region monitored by the spin label becomes less organized and/or more mobile. Comparing the  $A_{\max}$  values obtained with the different labels incorporated in pure lipid bilayers (filled symbols in Fig. 2a–d) it is clearly seen that, above the lipid main transition temperature,  $T_c$ , spin labels placed deeper in the acyl chain monitor a more mobile environment. Therefore, despite the known vertical fluctuation of stearic acid labels [15–17], their ESR spectra monitor, on average, the characteristic flexibility gradient toward the terminal methyl end of the chain. This result is similar to others reported in the literature [18–20], and can also be confirmed by the different  $a_0$  values measured for the different labels. The labels closer to the membrane surface yield higher  $a_0$  values (results not shown), indicating a more polar environment [14].

Above  $T_c$ , the addition of the analog MSH-I to

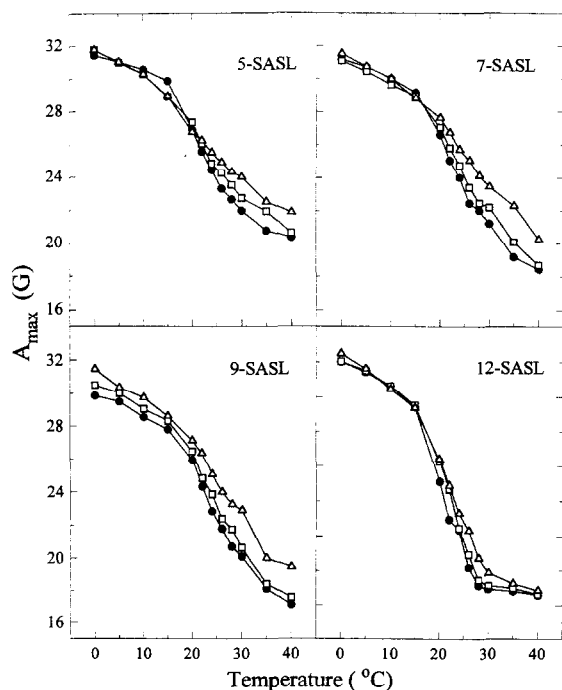


Fig. 2. Temperature dependence of the maximum hyperfine splitting constant,  $A_{\max}$ , of n-SASL stearic acid positional isomers in DMPG dispersion in the absence of peptide (●), and in the presence of 10 mol% of  $\alpha$ -MSH (□) or MSH-I (△).

DMPG liposomes significantly enhances the packing of the bilayer (Fig. 2), increasing the  $A_{\max}$  values of all labels placed along the acyl chain, with a greater effect in the C-7 and C-9 positions. The native hormone  $\alpha$ -MSH also causes an ordering effect in the membrane, though less evident.

Below  $T_c$ , apart for the 9-SASL, both peptides cause only minor changes on the  $A_{\max}$  values. The variations observed on the 9-SASL  $A_{\max}$  values in the presence of the two peptides are under investigation and will not be discussed here.

Fig. 3 shows the depth profile of the calculated effective order parameters ( $S_{\text{eff}}$ ) (see Section 2). The shown  $S_{\text{eff}}$  values were calculated at 30°C, but similar profiles were obtained for all temperatures above  $T_c$ .  $S_{\text{eff}}$  is a parameter similar to  $A_{\max}$  in the sense that it contains contributions from both order and rate of motion, although the principal contribution to  $S_{\text{eff}}$  is the amplitude of segmental motion of the acyl chain [21]. Different from  $A_{\max}$ , in the  $S_{\text{eff}}$  values the distinct polarities of the spin labels microregions were taken into account [12]. The variation of  $S_{\text{eff}}$  also indicates that the analog causes a stronger effect on the bilayer packing as compared to the native molecule. It is interesting to note that whereas for the hormone the labels placed at C-5 and C-7 on the acyl chain are more sensitive to the interaction peptide–lipid, for the analog the C-7 and C-9 are the most affected positions. Those results probably indicate

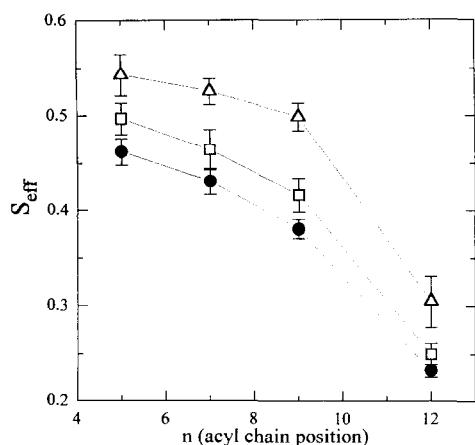


Fig. 3. Effective order parameter,  $S_{\text{eff}}$ , as a function of the nitroxide position,  $n$ -SASL stearic acid isomers in DMPG dispersion in the absence of peptide (●) and in the presence of 10 mol% of  $\alpha$ -MSH (□) or MSH-I (△). Temperature = 30°C.

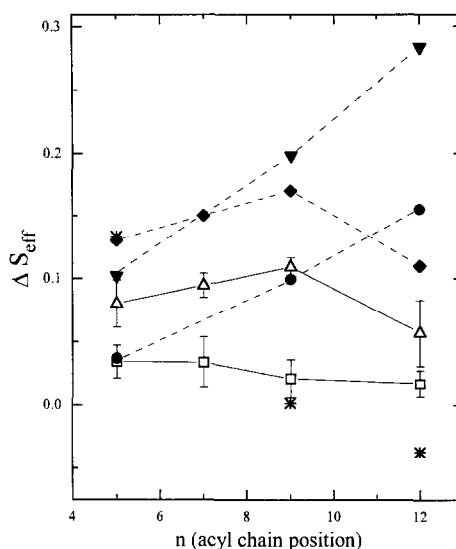


Fig. 4. Depth profiles of the variation of the effective order parameter,  $S_{\text{eff}}$ , relative to the pure lipid bilayer, of stearic acid positional isomers in DMPG dispersions, in the presence of 10 mol% of  $\alpha$ -MSH (□) and MSH-I (△), and 0.3 M NaCl (★). Temperature = 30°C. The profiles are compared to those of zeaxanthin (●) and cholesterol (▼) calculated from [23], and that of Apocytochrome  $c$  (◆), calculated from [19].

that MSH-I penetrates deeper into the bilayer than  $\alpha$ -MSH.

The deeper penetration of the analog MSH-I into the membrane core has also been suggested by the parallax method, using steady-state fluorescence spectroscopy [22], despite the controversy about the method. The authors used as quenchers the same stearic acid labels used here, and phospholipid derivatives labeled at different positions of the *sn*-2 chain. They concluded that the Trp residue of the analog goes deeper into the bilayer as compared to the native molecule.

However, there is not an obvious correlation between the depth of penetration of a molecule into the bilayer core and the variation of the ESR spectrum of a nitroxide probing a certain region of the bilayer. Depending on the order/mobility of the label, its ESR spectrum will be more or less sensitive to changes on its micro-environment. Therefore, for a better analysis of the data, the  $\Delta S_{\text{eff}}$  (difference relative to the pure lipid bilayer) depth profiles of the two melanotropic peptides in DMPG membranes (Fig. 4) were compared to those of the carotenoid zeaxanthin and cholesterol in DMPC (calculated from

[23]), and that of the extrinsic membrane protein apocytochrome *c* in bovine phosphatidylserine dispersion (calculated from [19]). The profile shown by the analog MSH-I is different from that of zeaxanthin and cholesterol, which are known to intercalate into the bilayer, and mostly disturb the C-12 chain region. However, MSH-I profile parallels that of apocytochrome *c* [19], which is supposed to partially penetrate into the apolar region of the membrane [24], mainly affecting the C-7 and C-9 positions. Although order parameters have not been calculated, the equivalent  $\Delta A_{\max}$  profile found for the extrinsic basic protein from the myelin sheath (MBP) (calculated from [25]), which is also supposed to penetrate into the bilayer, follows the same pattern as that found for apocytochrome *c* [19] and MSH-I (data not shown).

Considering that the two peptides are positively charged molecules (equally charged), Fig. 4 shows the changes of the effective order parameter obtained for DMPG in the presence and in the absence of an excess of charge, 0.3 M of NaCl. It can be clearly seen that a possible bilayer packing caused by the increase of the  $\text{PG}^-$  shielding, due to the high concentration of ions at the membrane surface, leads to a  $\Delta S_{\text{eff}}$  depth profile different from that obtained in the presence of the peptides.

All of the ESR spectra of the spin label positional isomers seem to consist of a single component only. Hence, different from the proteins mentioned above, with MSH-I there is no evidence of a second, more motionally restricted component, which would be taken as direct evidence of peptide penetration into the bilayer. However, it has been recently reported that the comparatively small lung surfactant protein SP-C, which is supposed to be a transmembrane  $\alpha$ -helix peptide, reduces the mobility of the phospholipid acyl chains, without yielding a more immobilized ESR signal [20]. There are other similar reports in the literature [26].

### 3.2. ESR of spin labels at the bilayer interface

The stearamide spin label, SSL, was used to monitor the membrane headgroup region. Different from the stearic acid probes, where the anisotropic rotation is preferentially about the nitroxide *z* axis, the SSL rotation is axially symmetric about the *x*

axis, which is reflected in the higher amplitude of the  $M_I = +1$  line (corresponding to the nitrogen quantum number  $M_I = +1$ ) as compared to the other lines (top spectrum in Fig. 5a). The narrow lines

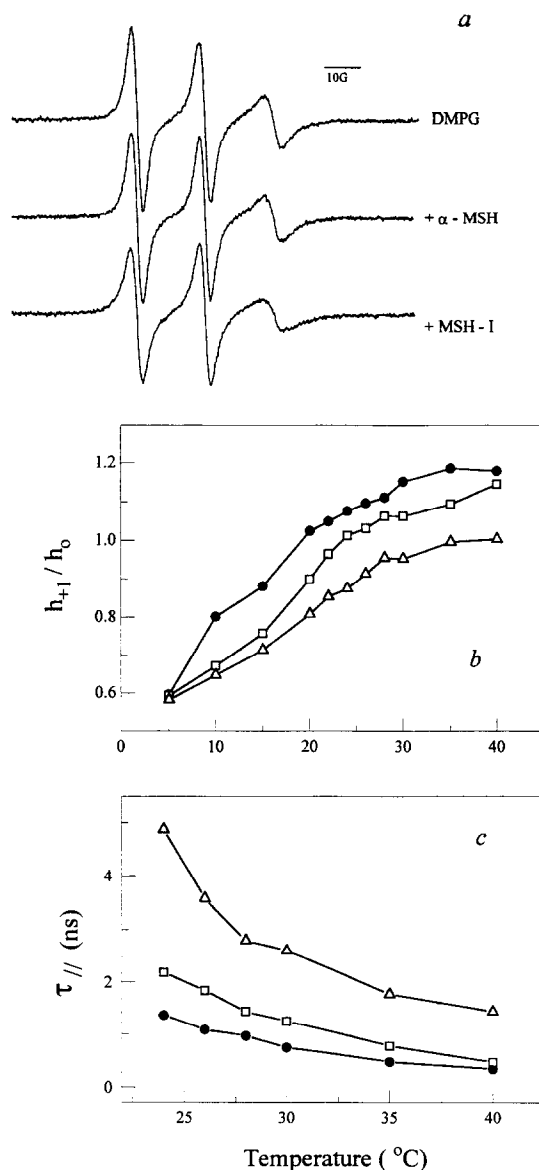


Fig. 5. Dependence of the ESR spectra of SSL in DMPG liposomes on the presence of 10 mol% of  $\alpha$ -MSH ( $\square$ ) or MSH-I ( $\triangle$ ). ( $\bullet$ ) corresponds to pure DMPG dispersions. (a) ESR spectra at a temperature of 30 $^{\circ}\text{C}$ . Temperature variation of (b) the ratio between the amplitude of the lines corresponding to  $M_I = +1$  and  $M_I = 0$ , and (c) the spin label rotation correlation time about the molecular symmetry axis.

obtained with SSL incorporated in DMPG vesicles indicate the low order and high mobility of the nitroxide moiety in the head group region. The packing effect due to the interaction peptide–bilayer is evinced by the broadening of the SSL ESR lines, mainly with MSH-I (Fig. 5a).

The alterations caused by the peptides on the bilayer interface can be evaluated by the decrease of the empirical parameter  $h_{+1}/h_0$  (ratio between the amplitude of the lines corresponding to  $M_I = +1$  and  $M_I = 0$ ) calculated from the SSL ESR spectra. Both peptides render the membrane surface more compact, as their effect in decreasing the  $h_{+1}/h_0$  ratio is in the same direction as that caused by diminishing the temperature (Fig. 5b). Again, the analog is much more effective in changing the membrane structure than the native hormone.

Rotation correlation times were calculated according to [27]. The spectra were simulated using the corrections for inhomogeneous broadening due to unresolved hyperfine structure, as suggested by [28]. Fig. 5c shows that the effect of the peptides on the  $\tau_{||}$  values (rotation correlation time about the molecular symmetry axis) is also similar to that caused by decreasing the temperature. This result is similar to that obtained with NMR of headgroup deuterated lipids [10]. It was shown that both peptides decrease the spin lattice relaxation time of the DMPG  $\alpha$ -deuterons, therefore increasing the carbon–deuterium segmental motion correlation time. In accordance with the present work it was found that the effect of the analog MSH-I was greater than that of the native peptide.

The values of  $\tau_{\perp}$  (rotation correlation time perpendicular to the molecular symmetry axis) were not very sensitive to the presence of the peptides.

Considering that there is probably a small orienting potential in the membrane interface, the calculated correlation times should be taken as comparative parameters and not as true numerical values. A more comprehensive motional model [29] would be necessary for an accurate analysis of the SSL EPR spectra.

### 3.3. Peptides–membrane partition coefficient

To further understand the binding of the peptides to DMPG vesicles, membrane partition coefficients,

$K_p$ , were calculated based on the effect the peptides cause on the structure of the bilayers [30]. It is assumed that the magnitude of the effect observed is only determined by the number of moles of peptides bound to the membrane,  $n_M$ . The above hypothesis was supported by the calculation of the membrane partition coefficient of the local anesthetic tetracaine using two different techniques, fluorescence and ESR spectroscopy [30].

$K_p$  is defined as

$$K_p = \frac{n_M/V_M}{n_{H_2O}/V_{H_2O}}$$

where  $n_{H_2O}$  is the number of moles of peptides in water, and  $V_M$  and  $V_{H_2O}$  are the volumes of membrane and water, respectively.

Fig. 6a and b show the variation of the  $A_{max}$  parameter of 5-SASL incorporated in DMPG vesicles, for different concentrations of peptides and lipids. The dashed lines correspond to constant effects obtained for different lipid and peptide total concentrations ( $n_T$  is the total number of moles of peptide), leading to the same concentration of peptide actually bound to the membrane. It was shown that a plot of the  $n_T$  and  $V_M$  values generated by the dashed lines (Fig. 6a and b) yields the peptide concentration in the membrane,  $C_M$ , and the  $K_p$  value, as follows [30]:

$$n_T = \frac{C_M \cdot V_{H_2O}}{K_p} + C_M \cdot V_M$$

Accordingly, partition coefficients of  $360 \pm 70$  and  $1330 \pm 160$  were calculated for  $\alpha$ -MSH and MSH-I, respectively. Similar values ( $290 \pm 54$  and  $1360 \pm 160$ ) were obtained using the  $h_{+1}/h_0$  parameter of SSL spin labels incorporated in DMPG vesicles (results not shown). It is important to point out that  $K_d$  values were previously calculated by fluorescence spectroscopy [22], but due to some problems with the methodology used there those values cannot be directly compared to the values obtained here (Lamy-Freund and Ito, in preparation).

To check whether the greater effect of MSH-I on DMPG bilayer structure was only due to its higher lipid association constant, the alterations caused by the same amount of the two peptides on the membrane structure were compared. Assuming 360 and

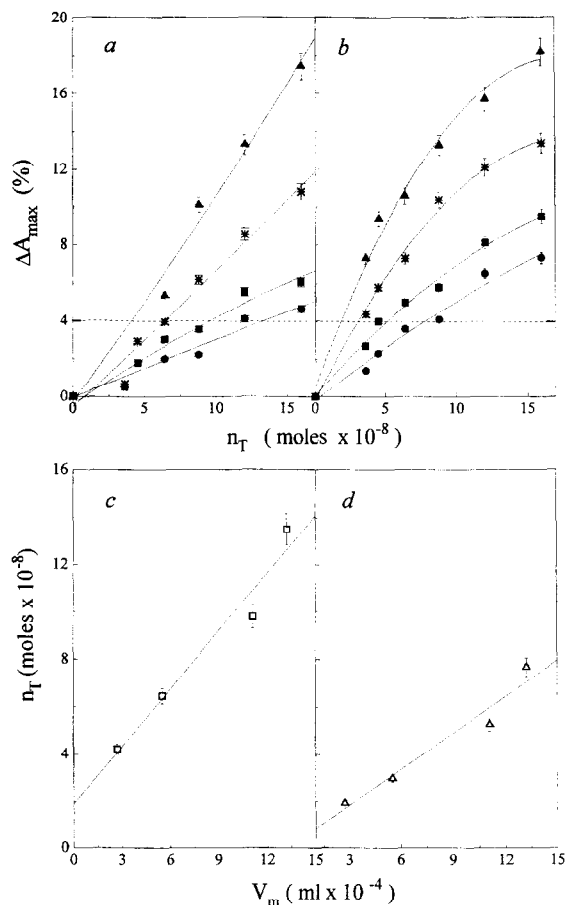


Fig. 6. Effect of (a)  $\alpha$ -MSH and (b) MSH-I on the maximum hyperfine splitting,  $A_{\max}$ , parameter of 5-SASL in DMPG dispersions lipid concentration (mg/ml): ( $\blacktriangle$ ) 3.4, ( $*$ ) 6.9, ( $\blacksquare$ ) 13.8, ( $\bullet$ ) 16.0. The dashed lines parallel to the abscissa generate sets of  $n_T$  and  $V_M$  values for which the effect of the peptide is the same. c and d show the plots of  $n_T$  as a function of  $V_M$  for the sets generated by the dashed lines in a and b, respectively.

1330 as partition coefficients for  $\alpha$ -MSH and MSH-I, respectively, the variations of  $A_{\max}$  in the 5-SASL spectra were calculated as a function of the actual peptide concentration interacting with the bilayer. Fig. 7 shows that for the same percentage of peptide bound to the membrane the packing effect is somewhat greater for the analog MSH-I.

In principle, one could use the partition coefficients calculated above and repeat the experiments shown in Figs. 2–5, using  $\alpha$ -MSH and MSH-I concentrations that would yield the same amount of peptide bound to the membrane. For instance, con-

sidering  $[DMPG] = 10 \text{ mM}$  ( $8 \times 10^{-7} \text{ mol}$  in  $80 \mu\text{l}$ ), and keeping  $[MSH-I] = 1 \text{ mM}$  ( $n_T = 8 \times 10^{-8} \text{ mol}$ ), as used before, from the definition of the partition coefficient, and using  $K_p = 1330$  for MSH-I, one can calculate the amount of bound MSH-I,  $n_M = 7.2 \times 10^{-8} \text{ mol}$ . Hence, considering  $K_p = 360$  for  $\alpha$ -MSH, it would be necessary to have  $n_T = 10.1 \times 10^{-8} \text{ mol}$  of the native peptide to get the same amount of the two peptides bound to the bilayer. However, Fig. 6a shows that the variation of  $A_{\max}$ , for 10 mM DMPG ( $*$ ), from  $n_T = 8 \times 10^{-8}$  to  $10.1 \times 10^{-8} \text{ mol}$  is rather small. Hence, the corrections to be done in Figs. 2–5 do not seem to be very relevant. It is important to note that in the calculations above (and in the data in Figs. 2–5) the molar percentage of bound peptide (relative to DMPG) is rather high, around 9%. For lower peptide/lipid ratios the difference between the effect of the two peptides is not so well detected by that method (Fig. 7).

Considering the calculated partition coefficients, and the different effects the same amount of the two peptides cause on the bilayer structure, we can infer that the analog MSH-I not only preferentially binds to the bilayer, as compared to the native molecule, but it also seems to change the bilayer structure more pronouncedly. This is in accord with the obtained depth profile (Fig. 3), which suggests that MSH-I

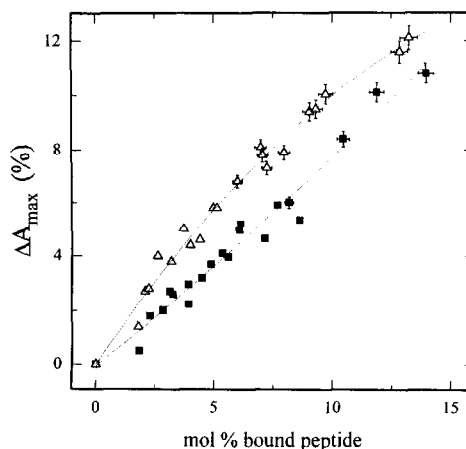


Fig. 7. Dependence of the outer hyperfine splitting of 5-SASL in DMPG dispersions (10 mM Hepes, pH 7.4), on the actual concentration of  $\alpha$ -MSH ( $\blacksquare$ ) or MSH-I ( $\triangle$ ) bound to the liposomes ((moles of bound peptide/moles of DMPG)  $\times$  100).

disturbs the membrane in a position deeper into the bilayer than that felt by the presence of  $\alpha$ -MSH.

### 3.4. Circular dichroism

Although the two peptides display similar CD spectra in aqueous medium (full lines in Fig. 8a and b), with a large negative band about 200 nm, indicative of random coil, their behavior in an  $\alpha$ -helix inducing solvent, TFE, is rather different. The peptide CD spectra in the presence of different concentrations of TFE (Fig. 8a and b) clearly show that  $\alpha$ -MSH much more easily acquires an  $\alpha$ -helix structure than the analog MSH-I. The unordered structure

found for the two peptides in aqueous solution is in accord with the NMR studies with 4–11  $\alpha$ -MSH fragment analogs [7], which suggested that their backbone may possess considerable conformational flexibility. Otherwise, it was found that in the D-Phe<sup>7</sup> analogs, such as MSH-I, the interaction between His<sup>6</sup>, D-Phe<sup>7</sup> and Arg<sup>8</sup> amino acid side chain groups might stabilize some type of  $\beta$ -like structure [7]. Hence, although no  $\beta$  structure is obviously detected in the MSH-I CD spectrum, its difficulty in acquiring an  $\alpha$ -helix conformation, as compared to the native molecule, is certainly an indication of some other preferential structure in the water medium.

Considering the difficulty in analyzing the CD spectra of small peptides, one can speculate that the large positive band around 185 nm, observed for MSH-I in aqueous solution (full line in Fig. 8b or d), could be an indication of some sort of secondary structure. Recently, structure modeling calculations, considering rotameric conformations of Trp side chain, found that the most stable conformations for both  $\alpha$ -MSH [31] and MSH-I (Jacchieri and Ito, personal communication) presented  $\beta$ -like structures. However, the energy barrier for interconversion among different conformational families was much lower for  $\alpha$ -MSH than for its analog. Hence, the similar energies for a variety of different structures could explain the  $\alpha$ -MSH's more flexible structure, favoring an  $\alpha$ -helix formation in an appropriate solvent (Fig. 8a and b).

The tendency of both peptides to form a  $\beta$ -like structure is evinced in the presence of either anionic SDS micelles or DMPG vesicles. Although rather small, Fig. 8c and d show a clear indication of a negative band around 220 nm upon the peptides interaction with micelles or vesicles. This is likely to be due to a small amount of  $\beta$ -structure present in the peptide conformation. Previously, it was reported [32] that  $\alpha$ -MSH at low pH (2–3) adopted a  $\beta$ -form in the presence of SDS, but only below the surfactant critical micellar concentration.

It is interesting to note that the two melanotropic peptides display similar CD spectra either in the presence of SDS micelles or DMPG vesicles. Moreover, although the spin label ESR results presented here, and fluorescence data [22] show that the analog MSH-I goes deeper into the bilayer than the native peptide, the conformational alterations in the struc-

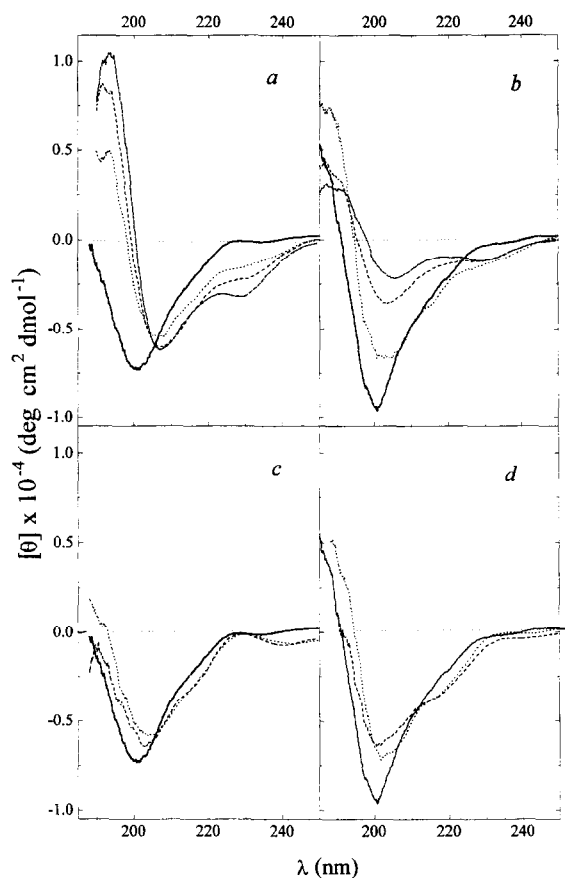


Fig. 8. CD spectra of  $\alpha$ -MSH (a and c) and MSH-I (b and d), in 5 mM phosphate buffer, pH 7.4 (—). In a and b, the spectra were recorded in the presence of 50% (· · ·), 75% (---) and 100% (— · —) in volume of TFE, and in c and d in 40 mM SDS (· · ·) and 5 mM DMPG (---). Peptide concentration was  $1.2 \times 10^{-4}$  M. Temperature = 35°C.

ture of the two peptides upon interaction with vesicles, as monitored by CD, are rather similar. One must keep in mind that the ESR data were obtained with multilamellar liposomes, whereas sonicated unilamellar vesicles were used in the CD experiment.

#### 4. Concluding remarks

It is shown here that the interaction between the melanotropic peptides and negatively charged lipids results in motional restriction of the lipids, to a certain extent resembling the interaction of surface membrane proteins. No effect was observed with zwitterionic vesicles (DMPC). As discussed before [10], this might be attributed to the low concentration of the cationic peptides on the surface of zwitterionic bilayers.

Considering that both the native hormone  $\alpha$ -MSH and the more active analog MSH-I have the same net positive charge, and that the present work focuses on the difference of their interaction with lipid bilayers, no electrostatic correction through the Gouy–Chapman potential was found necessary. It is shown here that the overall perturbation on the lipid structure on peptide binding is greater for the analog than for the native peptide. The initial interaction is primarily electrostatic, but apparently there is a subsequent penetration of the peptides, mainly the analog MSH-I, into the hydrophobic core of the bilayer.

CD results indicate that both peptides acquire a certain percentage of  $\beta$ -like structure upon interaction with anionic vesicles or micelles. However, CD spectroscopy does not allow the discrimination between peptide structural alterations caused by electrostatic interaction with the negative surface charge of DMPG vesicles (or SDS micelles), and the actual penetration of the peptide into the bilayer, or both effects.

The results presented here should be regarded only as an indication of what might happen in a biological system, as they were obtained with a very simple model system, consisting of single phospholipid vesicles. Our data suggest that the more potent peptide MSH-I is the one that presents the highest peptide–lipid association constant and the deepest penetration into the membrane, consequently being the one that most disturbs the bilayer structure.

Considering that the lipid packing has been reported to modulate the conformation and related activity of several membrane proteins (see, for instance, [33,34]), it could be speculated that the MSH-I interaction with lipid bilayers could play a role in the peptide enhanced biological activity, inducing an appropriate receptor conformation. On the other hand, the superpotent action of MSH-I could be partially explained by its higher partition coefficient in membranes, which would lead to an increased local concentration close to the receptor. Additionally, the MSH-I prolonged biological activity in cultured cells, after saline rinse [35], could be related to its greater affinity for the lipid phase, in line with the previous suggestion that the analog MSH-I could be somehow sequestered by the melanotropin receptor membrane [35].

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