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Estimation of average depth of penetration of melanotropins in dimyristoylphosphatidylglycerol vesicles

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Abstract

The interaction of α-melanocyte stimulating hormone (α-MSH) and its analogs [Nle^4,D-Phe^7]-α-MSH (MSH-I) and [Nle^4,Asp^5,D-Phe^7,Lys^{10}]-α-MSH(4–10) (MSH-II) with vesicles of dimyristoylphosphatidylglycerol (DMPG) was studied by steady-state fluorescence spectroscopy. The association constants for the interaction were obtained from binding isotherms. Electrostatic effects on the interaction were taken into account through calculation of Gouy–Chapman potentials. The quenching of fluorescence of the peptides by acrylamide and nitroxide labeled lipids demonstrated that insertion of the peptides into the lipid phase of the vesicles causes the changes in the hormone's fluorescence in the presence of DMPG. The parallax method was employed for the estimation of an average depth of penetration of the peptides in the DMPG vesicles. It was found that the Trp residue in α-MSH and in MSH-II is positioned around the carbons 6 and 8 of the aliphatic chain. The analog MSH-I goes deeper into the bilayer compared to the others peptides, and the Trp residue locates between carbons 10 and 11 of the acyl chain. The average depth of penetration shows correlation with the number of lipid molecules that interact with one molecule of peptide. There is no direct correlation between the association constants for the lipid–peptide interactions and the depth of penetration of the hormone.

Keywords: Melanotropic hormones; Fluorescence spectroscopy; Parallax method; Peptide–lipid interaction; Lipid vesicles

1. Introduction

α-Melanocyte stimulating hormone (α-MSH) is the physiologically relevant hormone regulating skin pigmentation in most vertebrates [1,2]. It is a tridecapeptide whose amino acid sequence is Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 and the central sequence His-Phe-Arg-Trp is essential for the biological activity of the hormone. A conformational analysis performed on α-MSH [3] has shown that one of the lowest-energy conformations available to the peptide contains a β-turn in the central region, in agreement with early molecular dynamics simulations [2]. Several analogs of the hormone have been synthesized and, among them, the substitution of Nle for Met^4 and the D-enantiomer of Phe^7 for the corresponding L-amino acid, which favours the β-turn, resulted in a more potent derivative. This analog, hereafter referred to as MSH-I, exhibited increased potency and prolonged activity in the frog skin bioassay, when compared to the native hormone [1]. Another analog, Ac-

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[Nle^4,Asp^5,D-Phe^7,Lys^{10}]-α-MSH(4–10), hereafter referred to as MSH-II, contains a cyclic lactam bridge between Asp^5 and Lys^{10} resulting in a compact structure. It is about 90 times more potent than α-MSH in the lizard skin bioassay, with residual activity when compared to the native molecule [4].

Recently it has been demonstrated by fluorescence spectroscopy that α-MSH and the analogs MSH-I and MSH-II interact with vesicles of palmitoyloleoylphosphatidylserine (POPS) and dimyristoylphosphatidylserine (DMPS): increase in fluorescence intensity, blue shift of the spectral position of the emission, changes in anisotropy and fluorescence decay parameters are indicative that the tryptophan residue of melanotropins moves to a less polar environment when lipid vesicles are present in the aqueous medium [5]. The experimental data were interpreted as originating from the insertion of the region of the peptide containing Trp into the lipid phase of the vesicles, and analysis of the results suggested a correlation between the biological activity of the hormones and the extent of interaction with the vesicles, supporting the hypothesis of an active role of the membrane as a catalyst for ligand–receptor interactions [6,7]. However, it has been argued that modifications in fluorescence parameters of a peptide in the water–membrane interface could also be interpreted as resulting from local alterations in the environment around the Trp residue, due to conformational changes of the peptide induced by interaction with the vesicles, and not necessarily from its insertion into the aliphatic region.

In order to obtain more information about the possible insertion of the melanotropins into lipid bilayers, we examined in more detail the interaction of α-MSH, MSH-I and MSH-II with vesicles of dimyristoylphosphatidylglycerol (DMPG), that has been subject of preliminary measurements described in a previous paper [5]. The binding constants for the interaction were determined from steady-state fluorescence measurements, using the intensity of fluorescence as relevant parameter to obtain the amount of peptide bound to the vesicles. As both the lipids and peptides are charged, the Gouy–Chapman potential was employed to take into account electrostatic effects in the interaction. To check if the peptide is really inserting into the lipid phase, we tried to determine the position of the Trp residue inside the bilayer, using information from fluorescence quenching experiments. Acrylamide, a quencher that, typically, remains in the aqueous medium, was used in a qualitative verification of the extent of its interaction with the Trp residue, either in the presence or in the absence of lipid vesicles. We applied the parallax method [8] to the quenching of Trp fluorescence by nitroxide labeled lipids, for the estimation of the penetration depth of the peptides into the DMPG bilayer.

2. Materials and methods

α-MSH and MSH-I were purchased from Sigma (St. Louis, MO), and MSH-II was kindly donated by Dr. Victor Hruby from The University of Arizona. The peptides were used without additional purification, at concentrations of $(1.0–2.5) \times 10^{-5}$ M, in 0.01 M phosphate, pH 7.0, containing 0.01 M NaCl.

The phospholipid dimyristoylphosphatidylglycerol (DMPG) and the spin-labeled phospholipids 1-palmitoyl-2-(5-doxyl)stearoylphosphatidylcholine (5-PCSL), 1-palmitoyl-2-(10-doxyl)stearoylphosphatidylcholine (10-PCSL) and 1-palmitoyl-2-(12-doxyl)stearoylphosphatidylcholine (12-PCSL) were obtained from Avanti Polar Lipids (Birmingham, AL). The spin-labeled lipids 7-doxylstearic acid (7-SASL), 9-doxylstearic acid (9-SASL) and 14-doxylstearic acid (14-SASL) were a kind gift by Dr. Anthony Watts from Oxford University. Acrylamide was obtained from Merck. The lipids and all other chemicals were used as received.

Lipid vesicles were prepared by the method of extrusion [9]. Initially, a solution of lipid (either pure DMPG or a mixture of DMPG and labeled lipid) in chloroform was dried under vacuum during 2 h. The film thus obtained was resuspended in buffer at a concentration near to 2 mM and the resulting suspension was then extruded at 30°C, a temperature above the transition from gel to liquid crystalline phase for DMPG vesicles. The final steps of the extrusion were made using 0.1 μm pore diameter polycarbonate membranes, resulting in large unilamellar vesicles (LUV), as expected [10], and checked by electron microscopy. In the experiments with labeled lipids, the films were prepared from a chloroform solution containing DMPG/*n*-PCSL at molar ratio 90:10.

Optical absorption spectra were measured using a Hewlett Packard 8452A diode array spectrophotometer. Steady-state fluorescence was measured with a Hitachi 3010 spectrofluorimeter and spectra were corrected for the instrumental sensitivity variation with wavelength.

Lipid titration was performed by adding small amounts of the concentrated lipid vesicle suspensions to the melanotropin solutions and monitoring the changes in Trp fluorescence intensity. The concentration of peptide bound to the vesicles $[P_b]$ was estimated from the titration curves, according to $[P_b] = [P_t](I - I_o)/(I_{max} - I_o)$, where $[P_t]$ is the total concentration of peptide, I is the fluorescence intensity after the addition of an aliquot of lipid, I_o is the initial intensity of the peptide in the absence of lipid, and I_{max} is the plateau value of the fluorescence intensity reached after the addition of an excess of lipid. The intensities I were measured as the amount of fluorescence (at an arbitrary scale) at the wavelength of maximum emission. When necessary, usually at DMPG concentrations above 100 μM, intensities were corrected for increase in scattering, revealed by increase in the absorbance of the samples.

As next described, titration curves were employed for the determination of binding constants through Scatchard plot analysis and corrections due to Gouy–Chapman potential. Titration curves were also obtained for interactions with vesicles containing labeled lipids and the plateau values were used for the parallax method calculations.

3. Results and discussion

Interaction of α-MSH, MSH-I and MSH-II with vesicles of DMPG resulted in increase in Trp fluorescence intensity, blue shift of the emission spectrum and increase in anisotropy of fluorescence, as previously observed for interaction of melanotropins with POPS and DMPS [5]. Titration curves as illustrated in Fig. 1 were obtained at 30°C, the temperature where the lipid system is in the liquid crystalline phase. The increase in fluorescence intensity was accompanied by a blue shift in the emission spectrum, which ranged from 12 nm (for α-MSH and MSH-I) to 15 nm (for MSH-II). This last value is higher than that reported previously [5], obtained from a set of independent measurements. It is possible that saturating conditions were not attained in that work, for the result was not obtained from a titration curve.

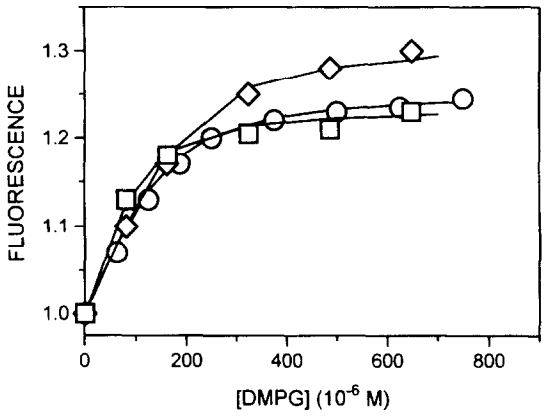

Fig. 1. Relative increase in fluorescence intensity of melanotropins in the presence of DMPG vesicles. Excitation, 290 nm; peptide concentration, 1.5×10^{-5} M, in 0.01 M phosphate buffer, pH 7.0, 0.01 M NaCl, 30°C. (I_0 is the intensity without lipid.) (○) α-MSH; (□) MSH-I; (◇) MSH-II. Solid lines were obtained from values of peptide bound $[P_b]$ calculated according to Eq. 1 as a function of total lipid concentration [L] and using the experimental value for the total peptide concentration $[P_t]$ and the adjusted parameters reported in Table 1 for K_d and n.

3.1. Binding constants

From the titration data illustrated in Fig. 1 the concentration of peptide bound $[P_b]$ was obtained as a function of the lipid concentration [L]. In the case when one molecule of peptide (P) forms a complex (PL_n) with n molecules of lipid (L) acting as independent binding sites, the equilibrium may be described by the conventional binding isotherm [11,12]

$$X = [P_f]/(K_d + n[P_f]) \quad (1)$$

where $X = [P_b]/[L_t]$ is the number of moles of peptide bound per mole of total lipid, $[P_f]$ is the concentration of free peptide, and K_d is the dissociation constant.

The values of n and K_d were determined from the conventional reciprocal plot ($1/X$ vs. $1/P_f$) or by the Scatchard plot (X/P_f vs. X), according to

$$X/[P_f] = 1/K_d - nX/K_d \quad (2)$$

A typical Scatchard plot is shown in Fig. 2. Both

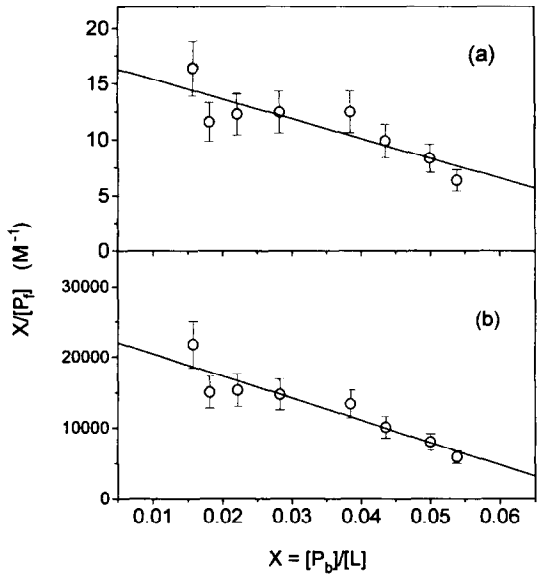

Fig. 2. Scatchard plot for the titration of α-MSH with DMPG vesicles. (a) Conventional analysis; (b) analysis with Gouy–Chapman correction. Solid lines are best linear regression fits to the data. Excitation, 290 nm; peptide concentration, 1.5×10^{-5} M, in 0.01 M phosphate buffer, pH 7.0, 0.01 M NaCl, 30°C.

reciprocal and Scatchard plots gave similar values of K_d and n for each of the peptides in interaction with vesicles of DMPG (Table 1). The differences between results from the two plots, for a given peptide, are within the reported errors in the values of K_d and n.

As discussed in a previous paper [5], the melanotropins interact with negatively charged vesicles and the electrostatic effect should be considered for these systems, similar to what was reported in the binding of small peptides such as neuropeptide substance P [13] and melittin [14,15] to acidic vesicles. The procedure, described in the references above, are based on the Gouy–Chapman theory [16]. The surface charge density (S) at the surface of a vesicle is related to the electrostatic potential ψ_0 (known as Gouy–Chapman potential) through

$$S = \{2000\varepsilon_R\varepsilon_0 RT\Sigma c_i[\exp(-z_i F_0 - \psi_0/RT) - 1]\}^{1/2} \quad (3)$$

where ε_R is the dielectric constant of water, ε_0 is the permittivity of free space, R is the gas constant, F_0 is the Faraday constant, c_i is the concentration of the ith electrolyte in the bulk aqueous phase (in mol/l) and z_i is the signed charge of the ith species.

The incorporation of positively charged peptides in the vesicles decreased the surface charge density and, consequently, the magnitude of the Gouy–Chapman potential. The value of the surface charge density (S) has been determined from the mole fraction X_L of charged lipids (of charge z_L) and the mole fraction X_b of peptide (of charge z_p) bound to the vesicles, through the relation

$$S = e_0(z_L X_L + z_p X_b)/[A_L(1 + X_b A_p/A_L)] \quad (4)$$

where A_L and A_p are the surface areas of lipid and peptide, respectively. The following values were employed for the calculation of S: the product $z_L X_L$ is -1, for we used pure DMPG vesicles; the peptide has a net positive charge $+1$ at the pH used in the experiments; X_b was obtained from the titration data as $X_b = X/0.56$, where $X = [P_b]/[L_t]$ as defined previously and the factor 0.56 accounts for the asymmetry in the lipid concentration in the outer and inner layers of the vesicles [17]; the value for A_L was assumed to be 60 Å^2, according to Cullis and Hope [17]; the value of 200 Å^2 was used for A_p, following the reports by Seelig and MacDonald [13] and Beschiachvilli and Seelig [14].

The distribution of charged molecules in the medium is dependent on the Gouy–Chapman potential, ψ_0, through a Boltzmann relation between populations in equilibrium:

$$[P_m] = [P_{eq}]\exp(-\psi_0 z_p F_0/RT) \quad (5)$$

where $[P_m]$ is the concentration of charged peptides adjacent to the membrane surface and $[P_{eq}]$ is the

Table 1
Binding parameters for interaction of melanotropins with DMPG vesicles in 10 mM phosphate buffer, pH 7.0, 10 mM NaCl, 30°C

	Uncorrected		Corrected	
	K_d (10^{-6} M)	n	K_d (10^{-3} M)	n
α-MSH	40±3	12±2	55±5	10±2
MSH-I	32±4	20±4	38±5	22±3
MSH-II	24±4	10±3	21±4	9±3

Values were calculated either with Gouy–Chapman correction for the electrostatic potential (column corrected) as without correction (column uncorrected).

concentration in the bulk solution, equivalent to $[P_f]$ in Eq. 1.

Scatchard and reciprocal plots corrected for electrostatic effects were made using the concentration of peptides calculated according to Eq. 5 and the values obtained are shown in Table 1. The shapes of these plots are not significantly different from the conventional analysis made without considering electrostatic effects (Fig. 2b), contrary to that reported for the interaction of peptides like neuropeptide P and melittin with acidic lipids [13,14]. However, it should be noticed that the charge of the melanotropins (+1) is well below the charges of neuropeptide P (+3) or melittin (+6). The calculated values of $[P_m]$ are about three orders of magnitude higher than $[P_f]$, and this increase in the concentration of peptides in the layer next to the surface of the vesicles can be attributed to the electrostatic interactions between charged peptides and lipids. The interaction of the peptides accumulated close to the surface of the bilayer then proceeds through hydrophobic effects, and the values of the dissociation constants are three orders of magnitude greater than those obtained by the conventional plots. This indicates that observation of interaction between melanotropins and neutral lipids could be verified in experiments conducted at concentrations of peptides three orders of magnitude higher than those employed in this work, that were the usual ones in fluorescence spectroscopy.

The analog MSH-II presents the lowest value of the dissociation constant K_d for the interaction with DMPG vesicles and the native hormone α-MSH gives the highest value. These results parallel those obtained for the interaction of melanotropins with POPS and DMPS vesicles [5]. However, the difference between the K_d values for MSH-II and α-MSH in the interaction with DMPG is not so pronounced as in the interaction with POPS and DMPS. This is due to the fact that α-MSH shows higher affinity to DMPG vesicles than to phosphatidylserine lipids, while the opposite behavior is verified for MSH-II. The dissociation constant value for the analog MSH-I is less sensitive to the different lipids studied.

3.2. Quenching by acrylamide

It was recently verified that α-MSH fluorescence is quenched by acrylamide with positive deviation

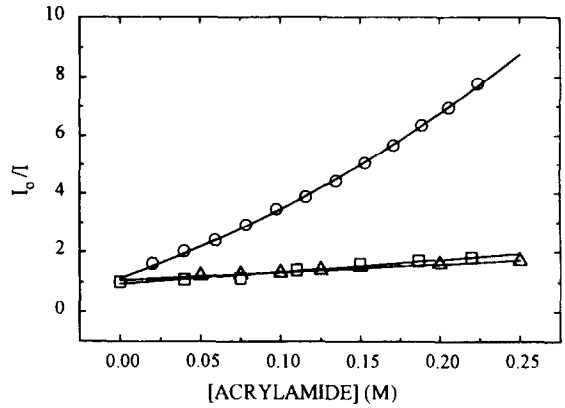

Fig. 3. Stern–Volmer plots for acrylamide quenching of melanotropins fluorescence. (○) α-MSH in 0.01 M phosphate buffer, pH 7.0, 0.01 M NaCl, 30°C; (□) MSH-I in the presence of DMPG vesicles; (△) α-MSH in the presence of DMPG vesicles. [lipid]/[peptide] > 50. Peptide concentration 1.5×10^{-5} M, excitation 290 nm.

from the normal Stern–Volmer plot [18]. We observed that MSH-I and MSH-II exhibit the same behavior in aqueous medium, without lipid vesicles (open circles in Fig. 3). In this case, the intensity of fluorescence (I) is related to the quencher concentration [Q], through $I_0/I = (1 + K_{SV}[Q])\exp(V[Q])$ (Fig. 3). Here I_0 is the intensity in the absence of quencher, K_{SV} is the dynamic Stern–Volmer constant and V is known as static quenching constant. Typical values for Stern–Volmer modified parameters are $K_{SV} = 19.5 \pm 0.7\ M^{-1}$ and $V = 1.6 \pm 0.16\ M^{-1}$, basically the same for all the peptides, showing that the Trp residue is equally exposed to the aqueous environment in the melanotropins we examined.

Bhattacharyya and Basak [18] showed that in the presence of reverse micelles of sodium dioctylsulfosuccinate (AOT), the extent of quenching is decreased, and the results were interpreted as originating from the restricted access of the Trp residue to acrylamide due to the interaction of the peptide with the micelles. In the interaction of melanotropins with DMPG, we also observed that when DMPG vesicles are present, there is a decrease in the acrylamide quenching of the fluorescence from the hormones (Fig. 3).

The quenching of Trp fluorescence by acrylamide is less efficient in the presence of lipid vesicles. As

the quenching originates from collisions between the fluorophore and acrylamide molecules, and as these are not bound to the lipid phase, the results indicate a decrease in the access of Trp to the interaction with the quencher. It would be possible that the charged lipid surface could exert some repulsion over the acrylamide molecules, impairing the Trp quenching, and the results are not conclusive about the insertion of the peptide into the lipid phase of the bilayer. However, results as shown in Fig. 3 allows the discrimination between melanotropins interacting with DMPG and peptides free in solution. We further analyzed the interaction between the hormones and DMPG, using quenchers located inside the bilayer, in previously known positions. The estimation of the distance between the Trp residue and the center of the bilayer was then performed using the so-called parallax method.

3.3. Depth of penetration

The penetration depth of Trp in lipid bilayers can be determined by comparing the efficiencies of quenching by nitroxides spin labels incorporated into the lipids at different distances from the bilayer surface. It is assumed that the suppression is static, because the lateral diffusion of the lipids in bilayers is slowly enough for the fluorophore and the quencher to maintain a fixed distance during the excited state lifetime. The evaluation of the fluorophore position inside the bilayer is made using data of quenching by quenchers located at two different positions in the hydrocarbon chain, as described by Chattopadhyay and London [8].

Shortly, from Perrin's equation for the analysis of the static quenching with fluorophores and quenchers randomly distributed in a plane [19], in the rigid sphere (of radius R_c) approximation of Birks [20], the ratio between the fluorescence intensity (I_1) measured with the quencher near to the bilayer surface and the intensity (I_2) with the quencher in a deeper position is given by

$$\frac{I_1}{I_2} = \frac{I_1/I_0}{I_2/I_0} = \left[\exp\left(-\pi R_c^2 C + \pi z_{1F}^2 C + \pi x^2 C\right)\right] / \left[\exp\left(-\pi R_c^2 C + \pi z_{2F}^2 C + \pi x^2 C\right)\right] = \exp\left\{\pi C\left[z_{1F}^2 - z_{2F}^2\right]\right\} \quad (6)$$

where C is the mole fraction of quencher divided by lipid area, z_{1F} and z_{2F} are the vertical distances between the fluorophore and the quencher 1 and 2, respectively, and x is the lateral distance between fluorophore and quencher. As the quenchers are bound to the hydrocarbon chain in known positions, the distance L_{21}, that is the difference in depth between the two quenchers, is also known. Using $z_{2F} = L_{21} + z_{1F}$, one obtains

$$z_{1F} = \frac{\left(-\frac{1}{\pi C}\ln\frac{I_1}{I_2} - L_{21}^2\right)}{2L_{21}} \quad (7)$$

Finally, having the value of z_{1F}, the distance z_{cF} from the fluorophore and the center of the bilayer is

$$z_{cF} = z_{1F} + L_{c1} \quad (8)$$

where L_{c1} is the distance from the center of the bilayer and the quencher 1.

However, a modification of the above treatment has to be applied when one of the quenchers is near to center of the bilayer, for in this case quenchers in the opposite layer of the bilayer contributes to the decrease in the intensity of fluorescence. Now the ratio between the intensities I_1 and I_2 defined as above, is

$$\frac{I_1}{I_2} = \left[\exp\left(-\pi R_c^2 C + \pi z_{1F}^2 C + \pi x^2 C\right)\right] / \left[\exp\left(-\pi R_c^2 C + \pi z_{2F}^2 C + \pi x^2 C\right) \times \exp\left(-\pi R_c^2 C + \pi z_{2tF}^2 C + \pi x_{2tF}^2 C\right)\right] \quad (9)$$

The index t refers to the values related to the quencher in the opposite layer of the membrane. The distance from the fluorophore and the quencher in the opposite layer, z_{2tF}, is equal to $2L_{c2} + z_{2F}$, where L_{c2} is the distance from the deepest quencher and the center of the bilayer. In this way, Eq. 7 can be rewritten as

$$Z_{1F} = -2L_{21} - 2L_{c2} \pm \left[\frac{-1}{\pi C}\ln\frac{I_1}{I_2} + 2L_{21}^2 + 4L_{21}L_{c2} + R_c^2\right]^{1/2} \quad (10)$$

A point that deserves attention is the extent of modifications in the lipid bilayer due to the interac-

tion with the peptides. It has been observed by analysis of electron paramagnetic resonance signals of spin labels incorporated in oriented bilayers of DMPG, that the membrane fluidity is modified in the interaction with α-MSH and MSH-I [21]. However, these effects are less important at the high lipid:peptide ratios used in the our experiments. On the other hand, an analysis considering probe and quencher movements was presented by Abrams and London [22], who showed that, typically, there are little dynamics effects on the average insertion depth of fluorophores in bilayers. We assume that in our case, average values of penetration depth are being determined, in the same way that the method has been used in studies of insertion of peptides in lipid bilayers [23,24] and discussed in a recent paper by Jones and Gierasch [25].

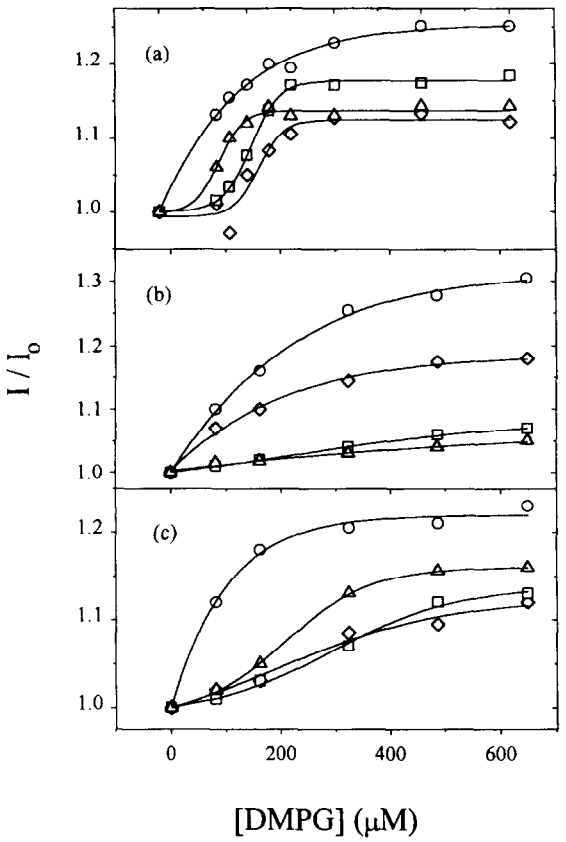

Fig. 4. Relative increase in melanotropins fluorescence in the presence of vesicles of DMPG/nitroxide labeled lipids (molar ratio 90/10). (a) α-MSH; (b) MSH-I; (c) MSH-II. 0.01 M Phosphate buffer, pH 7.0, 0.01 M NaCl, 30°C. (○) DMPG–DMPC; (□) DMPG–10-PCSL; (△) DMPG–12-PCSL; (◇) DMPG–5-PCSL.

Table 2
Distances (Å) between the tryptophan residue in melanotropins and the center of DMPG bilayers

Pair of quenchers	α-MSH	MSH-I	MSH-II
7-SASL–14-SASL [a]	8.7	–	–
7-SASL–9-SASL	7.0	–	–
5-PCSL–10-PCSL	7.7	3.7	6.8
5-PCSL–12-PCSL	5.8	3.2	6.1
5-PCSL–12-PCSL [a]	6.9	4.4	7.1

10 mM Phosphate buffer, pH 7.0, 10 mM NaCl, 30°C.
[a] Calculated using Eq. 10. Results with estimated error of ±1 Å.

The depth of penetration of α-MSH, MSH-I and MSH-II in DMPG vesicles was estimated using the nitroxide labeled phospholipid *n*-PCSL (1-palmitoyl-2-(*n*-doxyl)stearoylphosphatidylcholine), in which the doxyl group quenches the tryptophan fluorescence. The calculations for the pair 5-PCSL–12-PCSL used Eq. 10, for one of the quenchers is near to the center of the bilayer. For the pair 5-PCSL–10-PCSL, Eq. 7 was used. Values of 11 Å for R_c, and 60 Å^2 for the surface area of one lipid molecule were employed in Eqs. 7 and 10 [26]. Values for L_{21}, L_{c1} and L_{c2} used in Eqs. 7–9, were obtained taking 0.9 Å as the distance between two carbons of the aliphatic chain and assuming the separation between the last carbon and the center of the bilayer as 0.45 Å [27–30].

From the titration curves with vesicles of DMPG–*n*-PCSL (Fig. 4) it is possible to verify that the extent of quenching depends on the position of the doxyl group in the aliphatic chain. From these curves we estimated the plateau values of the fluorescence, corresponding to the extrapolated intensity at lipid/peptide ratios higher than 60. These estimated values of fluorescence intensity in the presence of vesicles (I_i), for each DMPG–*n*-PCSL mixed vesicles, were compared to the values without lipids (I_0) and the ratios (I_i/I_0) were then used for the calculation of the distances z_{1F} and z_{cF}.

The estimates of penetration depth are presented in Table 2. Each of the values is affected by an error of about 1 Å due to uncertainties in the measurements and also to the acyl chain dynamics. Acyl

labels placed deep in the membrane undergo large vertical fluctuations [31] and as the parallax method compares quenching from shallow and deep labels, the computed distances should be taken as average locations of the fluorophore inside the bilayer.

The results clearly demonstrate that melanotropins penetrate into the lipid phase of DMPG vesicles and indicate that the penetration depth is not the same for the different peptides. For α-MSH the strongest quenching is seen for vesicles containing 5-PCSL. Eq. 7 for the pair 5-PCSL–10-PCSL gave for Trp in α-MSH a position 7.7 Å distant from the center of the bilayer (calculated assuming that the dimensions in the normal direction of the bilayer are determined mainly by the 14 carbon aliphatic chain of DMPG). For the pair 5-PCSL–12-PCSL the distance was calculated as 5.8 Å (Table 2). Considering the possible effect of quenching from the 12-PCSL spin label located in the internal layer of the vesicle, the use of Eq. 10 gave 6.9 Å for the distance of Trp to the center of the bilayer. This means that the tryptophan residue of α-MSH is located in between the carbons at positions 6 and 8 of the aliphatic chain of the bilayer.

Fig. 4b shows that MSH-I is strongly quenched by 10-PCSL and by 12-PCSL. This suggests a deep penetration of the peptide inside the vesicle and calculated distances between Trp and the center of the bilayer ranged from 3.2 Å to 4.4 Å (Table 2). This corresponds to a location of the residue between carbon 10 and carbon 11 of the aliphatic chain. On the other hand a shallow location for MSH-II is suggested by the curves in Fig. 4c showing stronger quenching by 5-PCSL. The calculations for MSH-II gave distances of 6.1 Å to 7.1 Å from the center of the bilayer, corresponding to a position for the Trp residue near carbon 7 of the acyl chain.

The values obtained are representative of the average penetration of the Trp residue with estimated dispersion of 1 Å, originating from a distribution of depth distances for the nitroxide group, mainly affecting the deep labels, and the fluorophore inside the bilayer. We also conducted experiments for the interaction of α-MSH with the labeled fatty acid *n*-SASL (*n*-doxylstearic acid, $n = 7$, 9 or 14) mixed to DMPG (Table 2). In this case, we obtained results for the penetration depth of Trp from α-MSH inside DMPG vesicles comparable to those calculated with *n*-PCSL, despite of the fact that the fatty acid posses a higher mobility in the bilayer, leading to some degree of delocalization of the doxyl group.

Even taking into account the dispersion inherent to the method, and that the distances are average locations of the fluorophore, it can be said that the analog MSH-I has a deeper penetration in DMPG bilayers, compared to the other peptides. The Trp residue in α-MSH and in MSH-II have a similar location in DMPG vesicles.

4. Concluding remarks

The experiments of quenching of melanotropins fluorescence by acrylamide and labeled phospholipids demonstrated that α-MSH and the analogs MSH-I and MSH-II really penetrate into the aliphatic region of DMPG vesicles. This observation supported the supposition that the modifications of the fluorescence parameters in the presence of acidic vesicles originated from the insertion of the peptide into the lipid phase. Therefore, the alternative hypothesis can be discarded that the changes in the fluorescence parameters could be attributed to the burying of Trp residue from the aqueous media, resulting from conformational alterations in the peptides after they were anchored to the membrane surface.

Some comparison can be performed between the results of the depth of penetration and the binding constants for the interaction with DMPG vesicles. Although there is the possibility that different oligomeric states for the peptides are present, in the calculations of the binding constants it was assumed that a complex of one molecule of peptide with *n* lipid molecules was formed. The different insertions of melanotropins seems to be correlated with the number of lipid molecules in the complex: the deepest located MSH-I has a larger number of lipids involved in the interaction with DMPG vesicles, while the peptides α-MSH and MSH-II, with a more superficial location, interact with a lower number of lipids. Accordingly, electron paramagnetic resonance results (Lamy-Freund, personal communication) indicate that DMPG vesicles are more affected by interaction with MSH-I than with α-MSH. On the other hand, the deeper insertion of MSH-I does not

imply a higher association constant with the lipids: the small analog MSH-II has higher affinity to the lipid vesicles, even if not located deep inside the bilayer; the native hormone, with shallow location in the bilayer, has the lowest association constant, and the more penetrating MSH-I has an intermediate affinity to the vesicles. The association constant is related to ΔG, the free energy change for the peptides in aqueous solution and complexed to the lipids, and the results indicate that the higher change is verified for MHS-II, followed by MSH-I and α-MSH. As this change contains energetic and entropic contributions, it is not possible to correlate directly the location of the peptide inside the membrane and the amount of energy and entropy change during the interaction. Time-resolved experiments [5] show that all the melanotropins have three-exponential decay both in aqueous solution as well as in the presence of DMPG vesicles. However, while the lifetimes and the relative contributions of the components to the total fluorescence are different for the three peptides in aqueous medium, after interaction with the vesicles, one observes similar lifetimes (long component: 4.13–4.88 ns, intermediate: 1.74–1.93 ns, short: 0.46–0.49 ns) and similar relative contributions to fluorescence (long component: 29–38, intermediate: 52–57, short: 10–13). These results suggest different conformations for the peptides in aqueous medium and similar conformations after interaction with DMPG. The free energy change in the interaction, reflected by the association constants, contains also contributions due to these conformational modifications and thus are not directly correlated only to the penetration depth of the peptide in the bilayer.

It is interesting to point out that the relative binding constants between the melanotropins studied and DMPG follows the pattern observed in their interaction with POPS and DMPS [5], and the results correlate with the observed higher biological activity of the cyclic analog MSH-II in biological assays [4] compared to the analog MSH-I and the native hormone α-MSH. These bioassays were performed in natural tissues and it may be said that vesicles of a single phospholipid do not reflect the complexity of natural membranes. Despite the limitation of single lipid vesicles as model for biological membranes, the results demonstrating the insertion of melanotropins into the vesicles support the hypothesis of the lipid phase as a catalyst for the biological action of the peptides.

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