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Peptide–lipid interaction monitored by spin labeled biologically active melanocortin peptides

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Abstract

The present work comparatively analyzes the interaction of α-MSH and its more potent and long-acting analog [Nle^4, D-Phe^7]α-MSH (NDP-MSH) with lipid bilayers. The peptides were spin labeled with Toac (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) at the N-terminal, as those derivatives had been previously shown to keep their full biological activity. Due to the special rigidity of the Toac covalent binding to the peptide molecule, this spin label is highly sensitive to the peptide backbone conformation and dynamics. The peptides were investigated both by the electron spin resonance (ESR) of Toac^0 and the time resolved fluorescence of Trp^9 present in the peptides. The Toac^0 ESR of the membrane-bound peptides indicates that the two peptides are inserted into the bilayer, close to the bilayer surface, in rather similar environments. A residue titration around pK_a 7.5, possibly that of His^6, can be clearly monitored by peptide–lipid partition. Trp^9 time resolved fluorescence indicates that the peptides, and their Toac-labeled derivatives, present rather similar conformations when membrane bound, though Trp^9 in NDP-MSH, and in its Toac-labeled derivative, goes somewhat further down into the bilayer. Yet, Toac^0 ESR signal shows that the Toac-labeled N-terminal of NDP-MSH is in a shallower position in the bilayer, as compared to the hormone.

Keywords: Melanocortins; Toac; ESR; α-MSH; NDP-MSH; Bilayer-partition; pH titration; Trp; Time resolved fluorescence

1. Introduction

Melanocortin peptides are known to interact with membrane proteins belonging to the super family of receptors coupled to the G-protein, which stimulate the adenosine cyclic 3′,5′-phosphate (cAMP) signal transduction pathway [11]. However, the lipid phase of the cell membrane possibly plays an active role in the peptide–receptor interaction, not only by increasing the peptide concentration at its surface, by electrostatic effects (anionic lipids/cationic peptide), but also by changing the peptide structural conformation, making it adjustable to the receptor. Besides, the peptide–lipid partition would make the peptide more accessible to the membrane protein receptor, and/or the presence of the peptide inside the bilayer could change the bilayer structure favoring an appropriate receptor conformation.

Considering the possible biological relevance of the lipid phase, α-MSH and several active analogs have been tested for lipid affinity, membrane depth penetration and peptide/bilayer structural alterations. Using lipids spin-labeled at different chain positions, incorporated in dimyristoyl phosphatidylglycerol (DMPG) bilayers, we have shown that the two cationic peptides, the hormone α-MSH (Ac-Ser^1-Tyr^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-NH^2) and the biologically more potent analog [Nle^4, D-Phe^7]α-MSH (NDP-MSH), interact with anionic DMPG bilayers, changing the membrane

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packing and altering the amount of water molecules in the bilayer core [5,6,13]. They interact with both the gel and fluid phases of the lipid. For fluid DMPG bilayers, above 35 °C, the hormone and the analog were shown to harden the bilayer packing at all depths [6,13], and α-MSH was found to decrease the membrane hydrophobic barrier, increasing interchain hydration [13]. At low temperatures, from 5 to 15 °C, in the highly organized lipid gel phase, α-MSH effect is rather complex, being bilayer position dependent: it significantly decreases the bilayer packing at the bilayer core and close to the lipid/water interface, but seems to increase the packing around the 9th to 12th acyl chain C-atoms. Though the actual position of α-MSH or NDP-MSH in DMPG membranes is still a matter of discussion, the peptides structural bilayer alterations are certainly related to their penetration into the membrane and could not be simply explained by surface charge effects [13].

On the other hand, the peptide–lipid interaction has also been monitored through the alterations on the Trp^9 fluorescent properties in the presence of membranes. Tryptophan fluorescence, both static and time resolved, and its suppression by nitroxide moieties positioned at different bilayer depths confirm that α-MSH, and some potent analogs, including NDP-MSH, partially penetrate lipid bilayers [20,22,12]. Based on the parallax method [7] it was proposed that, in α-MSH, the average depth of Trp penetration into DMPG bilayers was around the sixth to eighth carbon of the lipid acyl chain [22]. By the same methodology, the analog NDP-MSH was found to go deeper into the bilayer, with the Trp residue located between carbons 10 and 11.

The present work focuses on a comparative study of peptide–lipid bilayer interaction, with the hormone α-MSH and its biologically more active analog NDP-MSH. Here, the peptide–lipid interaction was monitored by electron spin resonance (ESR), with peptides labeled with the paramagnetic amino acid Toac (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid) at the N-terminal. The use of Toac for peptide labeling was first successfully carried out almost two decades ago by the synthesis of the dipeptide Toac-Gly [27] and of the octapeptide angiotensin II (AngII) analogs $Toac^0$- and $Toac^1$-AngII [28]. Later, an alternative approach enabled insertion of this spin probe at any position of the peptide backbone [23]. The special tetra substituted cyclic structure of Toac renders high sensitivity to this probe to monitor conformational features and dynamics of the molecule or system where it is linked. Owing to this unique characteristic, a great amount of different Toac application has been witnessed in last years encompassing peptides [4,25,29,36,38,40] or even peptide-polymers studies [8,9,30]. Among the peptides studied, acetyl-$Toac^0$-α-MSH [3] and acetyl-$Toac^0$-[Nle^4, D-Phe^7]α-MSH [26] were shown to retain entirely their native biological potency.

Toac-labeled and unlabeled peptides, incorporated in DMPG bilayers, were also studied via Trp^9 time resolved fluorescence. The tryptophan fluorescence study allows a comparison between the peptides and their Toac-labeled derivatives. Moreover, the ESR of $Toac^0$ and the fluorescence of Trp^9 give complementary information about the whole structure of the peptides.

DMPG, used here as a lipid model system of acidic domains in natural cells, increases the cationic peptide local concentration at the membrane surface by electrostatic effects. Due to the 14 C-atoms acyl chain, this lipid presents a gel-fluid transition around 20 °C, allowing the study of lipid bilayers in the gel and fluid phases, in a quite convenient range of temperature. The two phases would mimic acidic micro-regions of different fluidity present in biological membranes at physiological temperatures. Low ionic strength DMPG dispersions are used to increase the magnitude of the membrane surface potential, therefore increasing the local peptide concentration. Those dispersions are known to present an “intermediate phase”, over the temperature interval 17–35 °C, between the gel and fluid phases, with rather complex characteristics [21,34]. Therefore, most of the peptide–lipid interaction studies presented here were done with DMPG in the gel, intermediate and fluid phases, between 5 and 45 °C.

2. Materials and methods

2.1. Materials

Melanocortin peptides were synthesized as mentioned in previous papers [3,26]. HEPES (4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The sodium salt of the phospholipid DMPG (1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol) was obtained from Avanti Polar Lipids (Birmingham, AL, USA).

2.2. Peptide preparation

Stock solutions of peptides were prepared in water. Fluorescence and electron spin resonance (ESR) measurements were made diluting the stock solution with 10 mM HEPES buffer pH 7.4 to the desired final peptide concentration.

2.3. Lipid dispersion preparation

A film was formed from a chloroform solution of DMPG, dried under a stream of N_2 and left under reduced pressure for a minimum of 2 h, to remove all traces of the organic solvent. Liposomes were prepared by the addition of peptide HEPES buffer solution, followed by vortexing, with the lipids in the fluid phase. When necessary, the pH of the dispersion was adjusted to the desired value by addition of small aliquots of concentrated NaOH or HCl stock solutions. The DMPG final concentrations were 50 or 1 mM for electron spin resonance (ESR), and 1 mM for fluorescence spectroscopy.

2.4. ESR spectroscopy

ESR measurements were performed with a Bruker EMX spectrometer. Field modulation amplitude of 0.5 G and microwave power of 10.13 mW were used. The temperature was controlled to about 0.2 °C with a Bruker BVT-2000 variable temperature device. The temperature was monitored with a Fluke 51 K/J thermometer with the probe placed just above the cavity. The magnetic field was measured with a Bruker ER 035 NMR Gaussmeter, and, when necessary, the WINEPR software (Bruker) was used.

For the highly anisotropic spectra yielded by peptides incorporated in DMPG vesicles, the isotropic hyperfine splitting (a_0) was calculated from the expression [15,17]

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

where $A_{\parallel}$ (=A_{max}) is the maximum hyperfine splitting directly measured in the spectrum, and

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

where $2A_{min}$ is the measured inner hyperfine splitting, and A_{xx} = 5.9 G, A_{yy} = 5.4 G and A_{zz} = 32.9 G are the principal values of the hyperfine tensor for doxylpropane [19].

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

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

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

2.5. Fluorescence spectroscopy

Time-resolved experiments were performed using an apparatus based on the time-correlated single photon counting method. The excitation source was a Tsunami 3950 Spectra Physics titanium-sapphire laser, pumped by a 2060 Spectra Physics argon laser. The repetition rate of the 5 ps pulses was set to 400 kHz using the pulse picker 3980 Spectra Physics. The laser was tuned to give output at 888 nm, and a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) gave the 296 nm excitation pulses that were directed to an Edinburgh FL900 spectrometer. The spectrometer was set in L-format configuration, the emission wavelength was selected by a monochromator, and emitted photons were detected by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. The FWHM of the instrument response function was typically 50 ps, determined with a time resolution of 12 ps per channel. Measurements of the peptides decays were made using time resolution of 12 ps per channel.

The software provided by Edinburgh Instruments was used to analyze the individual decay curves. The adequacy of the multi-exponential decay fitting was judged by inspection of the plots of weighted residuals and by statistical parameters such as reduced χ^2.

2.6. pH titration

The Henderson–Hasselbalch equation describes pH titration of individual sites. For a molecule with more than one titratable site, as the peptides used herein, the total protonation curve can be described by a weighted sum of Henderson–Hasselbalch curves. As discussed by Onufriev et al. [31], the total average protonation of a molecule containing N sites, can be expressed by

$$\gamma = \sum_{i}^{N} c_i \frac{10^{pK_i - pH}}{1 + 10^{pK_i - pH}} \quad (4)$$

where γ is a parameter representative of the molecule pH titration, like the lipid-partition in Fig. 5, where c_i is the contribution of the individual Henderson–Hasselbalch curve corresponding to the titratable site i characterized by its pK_i value.

3. Results and discussion

3.1. Toac ESR studies

The ESR spectra yielded by 0.1 mM Ac-Toac^0-α-MSH and Ac-Toac^0-[Nle^4, D-Phe^7]α-MSH in the presence of 50 mM DMPG vesicles are shown in Fig. 1. The spectra, obtained with the lipids in the gel (15 °C) and fluid phases (45 °C), are certainly very different from those yielded by the peptides in aqueous solution (Fig. 1c). They seem to be composite signals: a large amount of a rather anisotropic signal, typical of spin labels incorporated in membranes, coexisting with a small percentage of a very narrow signal, mainly evident at the high field feature (see arrows in Fig. 1). The latter looking very much similar to the signal yielded by the peptides in aqueous solution (Fig. 1c). Accordingly, the Toac-labeled peptides spectra in DMPG were subtracted from the "free" peptide signal, at the appropriate temperature. Fig. 2 shows the resultant spectra, after the subtractions. The subtraction was done by subtracting a weighted free spectrum. The weight of the free signal was varied until the resulting spectrum looked like a one component signal, as shown in Fig. 2. This subtraction proceeds by trial and error, judging by eye the quality of the resultant spectrum. Up to 30 °C the subtraction was rather straightforward. For higher temperatures, the free signal obtained at a somewhat lower temperature had to be used in the subtraction, for a good match of the free component in the composite spectra. That could possibly be attributed to an increase in the rate of exchange between the peptides in aqueous medium and in the membrane, with the temperature rise, slightly increasing the line width of the free signal [2]. Hence, a low temperature signal would somehow mimic the line width broadening.

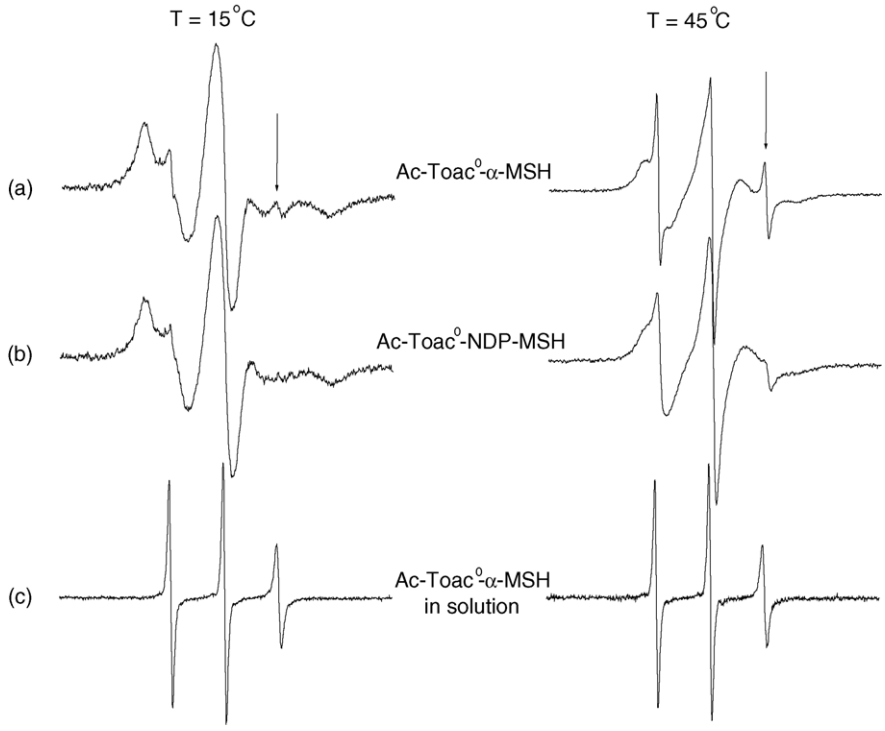

Fig. 1. ESR spectra of 0.1 mM Toac-peptides in 50 mM DMPG dispersion (a and b), and in aqueous solution (c), at 15 and 45 °C. Total spectra width 100 G. HEPES buffer, pH 7.4.

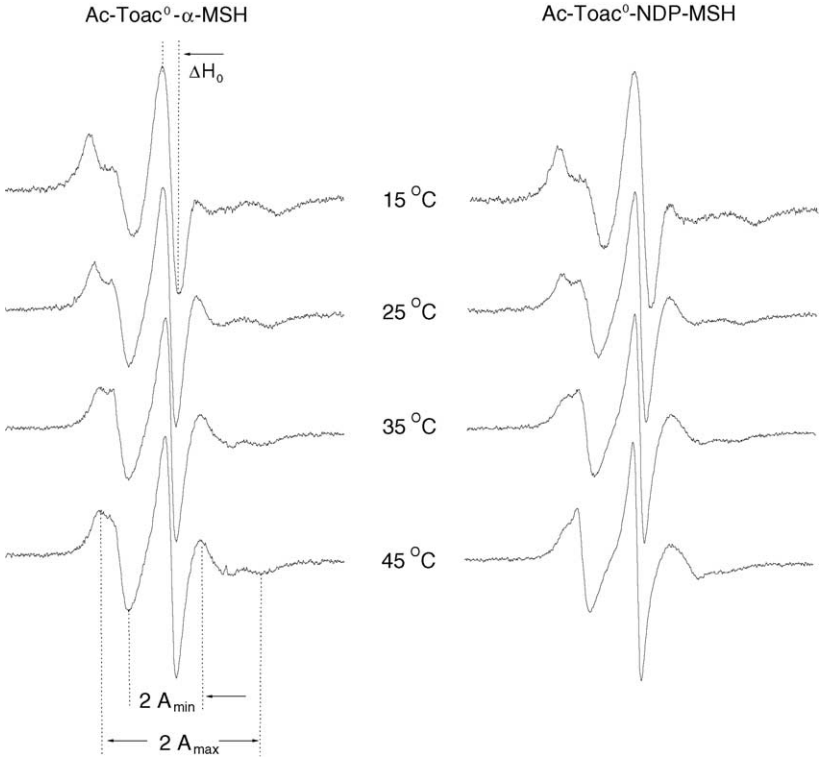

Fig. 2. ESR spectra of 0.1 mM Ac-Toac^0-α-MSH and Ac-Toac^0-NDP-MSH in 50 mM DMPG dispersions at 15, 25, 35 and 45 °C, subtracted from the ESR signal yielded by the peptides in aqueous solution. Total spectra width 100 G. The spectral parameters A_{max}, A_{min} and ΔH_0 are indicated. HEPES buffer, pH 7.4.

The relatively good subtracted spectra, shown in Fig. 2, allow us to conclude that the spin-labeled peptides partition between the water and lipid media. Double integration gives the fractions of the components present in the composite spectrum. At the lipid concentration used (50 mM), the peptides are mostly in the bilayer, the amount in solution varying from 2 to 6%, as the temperature increases (see further discussion on peptide–lipid–water partition below). The spectra of Toac in both peptides are rather anisotropic, indicating that the peptides are not placed at the bilayer surface, but are going into the bilayer. It is important to have in mind that the rotational rate of the Toac spin label is tied to the rotational rate of the peptide backbone, therefore reflecting the peptide as a whole. This conclusion is supported by the work of Victor and Cafiso [39], who showed that, when membrane-bound, Toac-peptides containing only the cationic residue Lys display a relatively isotropic ESR spectra, which can be attributed to the peptide in the aqueous double layer, at the membrane surface. The substitution of two or more Lys residues by Phe drastically changes the Toac-peptide ESR spectrum, making it rather anisotropic, similar to the spectra of Toac-melanocortins showed here. It is suggested [39] that the backbone of the peptides containing phenylalanine lays several angstroms below the level of the lipid phosphate. In the presence of DMPG bilayers, Toac bound at the N-terminus of both α-MSH and NDP-MSH has its movement strongly restricted, indicating its insertion into the membrane, at all temperatures investigated, with the lipid in fluid, intermediate and gel phases.

The Toac ESR signal was analyzed via three parameters, directly measured in the spectra, sensitive to the spin-label movement/order: the maximum hyperfine splitting A_{max} and the width of the central line, shown in Fig. 2, and the effective order parameter, discussed in Section 2. The three parameters decrease as the Toac environment gets more fluid [19]. While the Toac central line width for the peptides in solution is nearly insensitive to the temperature variation,[1] the Toac-peptide incorporated in the membrane strongly feel the bilayer temperature dependence (Fig. 3). However, it is interesting to note that the Toac-bound peptide does not monitor the DMPG temperature transition, as monitored by a phospholipid spin labeled at the acyl chain down in the bilayer core [34], but feels the lipid temperature packing variations as a lipid spin-labeled at the head group [6]. Still noteworthy is the similarity between the Toac microenvironment in the two labeled peptides: the three measured parameters are rather similar for the two peptides.

The Toac location in the membrane can also be monitored via its nitrogen isotropic hyperfine splitting, a_0. It has been shown that the magnitude of the nitrogen isotropic hyperfine splitting (a_0, one-third of the trace of the hyperfine tensor) depends on several factors which increase the unpaired electron spin density at the nitrogen nucleus, like solvent polarity,

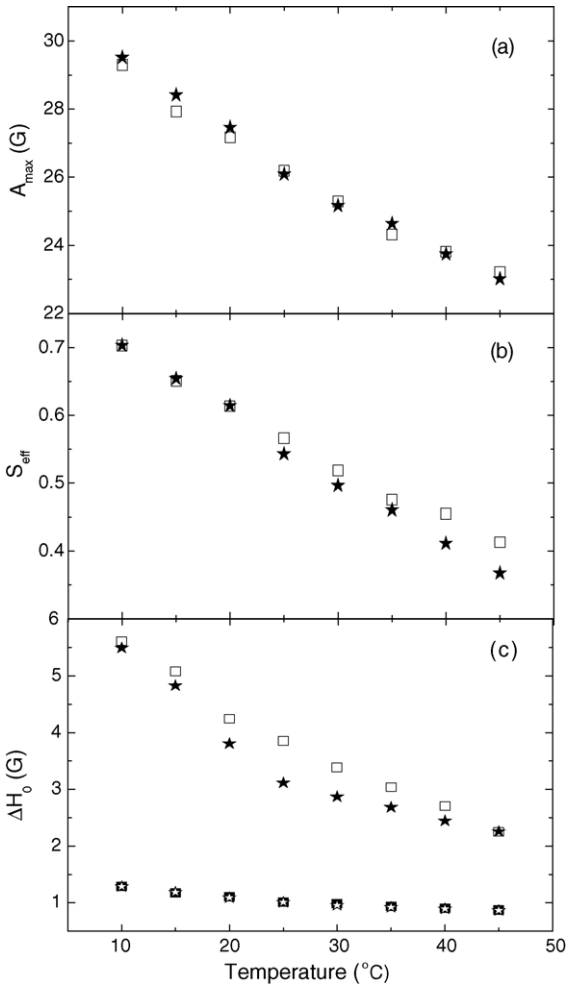

Fig. 3. Temperature dependence of the maximum hyperfine splitting A_{max} (a), effective order parameter S_{eff} (b), and central peak line width ΔH_0 (c), measured on the ESR spectra of 0.1 mM (□) Ac-Toac^0-α-MSH and (⋆) Ac-Toac^0-NDP-MSH, in 50 mM DMPG. The central peak line width ΔH_0 (c) was also measured on the ESR spectra of (■) Ac-Toac^0-α-MSH and (☆) Ac-Toac^0-NDP-MSH in aqueous medium. HEPES buffer, pH 7.4.

the presence of electric fields or electron transfer complex [16,35]. For labels inside a lipid bilayer, there are strong indications that an increase in a_0 is mainly related to the increase in the amount of nitroxide–water hydrogen bonding [16,24]. As expected, for the Toac-peptides in aqueous medium the hyperfine splitting is rather high, around 16.25 G, and does not vary much with temperature (Fig. 4). The relatively low a_0 values yielded by the Toac-peptides in the presence of DMPG membranes, about 15.2 and 15.5 G, for Ac-Toac^0-α-MSH and Ac-Toac^0-[Nle^4, D-Phe^7]α-MSH, respectively (Fig. 4), indicate that the Toac group is incorporated into the DMPG membrane, but not down into the bilayer core, where the expected a_0 value would be even lower. The relatively higher value found for the analog suggests that the Toac at the N-terminal of NDP-MSH is located at a shallower position. It is interesting to have in mind that Toac-peptides in micelle structures present a rather isotropic ESR signal, with high a_0 value, around 16.2 G [40].

[1] This parameter is the only one that can be compared with those of the Toac membrane-bound spectra.

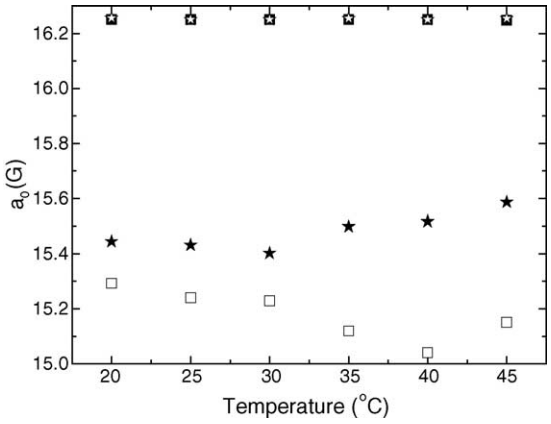

Fig. 4. Temperature dependence of the isotropic hyperfine splitting a_0, measured on the ESR spectra of 0.1 mM (□) Ac-Toac^0-α-MSH and (⋆) Ac-Toac^0-NDP-MSH, in 50 mM DMPG, and on the ESR spectra of (■) Ac-Toac^0-α-MSH and (☆) Ac-Toac^0-NDP-MSH in aqueous medium. HEPES buffer, pH 7.4.

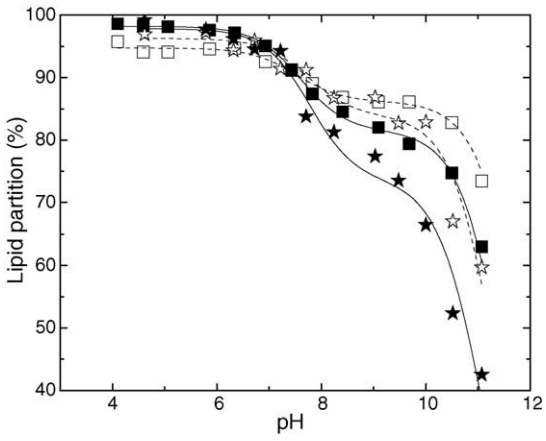

Fig. 5. pH dependence of the percentage of lipid-partition of 5×10^{-5} M of Ac-Toac^0-α-MSH (squares) and Ac-Toac^0-NDP-MSH (stars), at 10 °C (opened) and 40 °C (closed), in 1 mM DMPG. The fitting curves (Eq. (1)) for data at 10 °C (- - -) and 40 °C (—) are shown.

Peptide–lipid interaction studies with trichogin GA IV, a short lipopeptaibol antibiotic (10 amino acid residues), Toac-labeled at the first, fourth and eighth backbone position, found rather anisotropic ESR spectra for the peptides labeled at the three positions, in the presence of egg-lecithin membranes [25]. The measured A_{max} values for these peptides are fairly higher than those found for the Toac^0-labeled melanocortins in fluid DMPG bilayers, indicating a more restricted movement for Toac at the middle of a peptide backbone, as expected. However, the calculated nitrogen hyperfine splittings are rather similar to those of melanocortins, between 15.1 and 15.4 G (see Fig. 4). By the similar values presented by Toac bound at the three positions in trichogin, first, fourth and eighth, it was concluded that the peptide lays parallel to the membrane surface, not very deep into the bilayer [25]. That also seems to be the melanocortins location in a bilayer, allowing their charged residues to be at the polar interface.

The peptides lipid–water partitioning was carefully studied with 5×10^{-5} M peptides in the presence of 10^{-3} M DMPG, where the water partition is sufficiently high to be properly calculated by spectra subtraction. As discussed above, the peptides lipid–water partitioning can be well evaluated by the subtraction of the free peptide signal from the composite spectrum yielded by Toac-labeled peptides in the presence of DMPG lipid vesicles. The peptide–lipid partition was studied at two temperatures, 10 and 40 °C, with the lipid in the gel and fluid phases, respectively, at different pH values (Fig. 5).[2] Below pH 7, the peptide–lipid partitioning is rather high, both the hormone and the more active analog NDP-MSH presenting similar lipid partitioning values, at the lipid gel and fluid phases. Interestingly, the Toac-peptide–lipid partition can well monitor the titration of a group, around pH 7–8. The obtained pK_a values, yielded by the fittings shown in Fig. 5, with the Henderson–Hasselbalch equation (Eq. (1)), are shown in Table 1. The pK_a values are rather similar for the two peptides, at the two temperatures. The titratable group could possibly be His^6, whose de-protonation would make the peptides less positive, therefore, would decrease their local concentration at the negatively charged DMPG bilayer surface, leading to a lesser amount of peptide inside the membrane. The higher pK_a values measured for His, as compared to the pK_a found in aqueous solution (about 6.1) [14], is possibly related to the local low pH value at the negative DMPG bilayer surface.

Though less probable, the monitored residue titration could be that of Glu^5, increased by a few pH units due to the close proximity to the negatively charged DMPG phosphate group. As the pH increases, both labeled peptides go out of the membrane. That is possibly related to the loss of one more positive charge, as indicated by the Henderson–Hasselbalch fitting (Table 1), possibly that of Lys^{11} or Tyr^2, making the peptides neutral, therefore, drastically diminishing their concentration at the DMPG bilayer surface. Curiously, the peptides membrane-partition is somewhat higher in the bilayer highly packed DMPG gel phase, at 10 °C, than in the lipid fluid phase, at 40 °C (Fig. 5). That could possibly be attributed to the tighter lipid packing at the gel phase, increasing the bilayer surface density charge (magnitude), hence increasing the peptide local concentration at the bilayer interface. Also interesting is the higher lipid partitioning of the hormone α-MSH as compared to the analog NDP-MSH, for high pH values.

Table 1
pK_a values determined from the fitting (Eq. (1)) of the pH dependence of the peptide–lipid partition (Fig. 5)

	T (°C)	pK_1	pK_2
Ac-Toac^0-α-MSH	10	7.5 ± 0.1	11.9 ± 0.1
	40	7.6 ± 0.1	11.5 ± 0.1
Ac-Toac^0-NDP-MSH	10	7.7 ± 0.4	11.4 ± 0.1
	40	7.8 ± 0.2	11.1 ± 0.1

[2] The data should be analyzed for pH values higher than 5, to be sure DMPG is fully deprotonated [33].

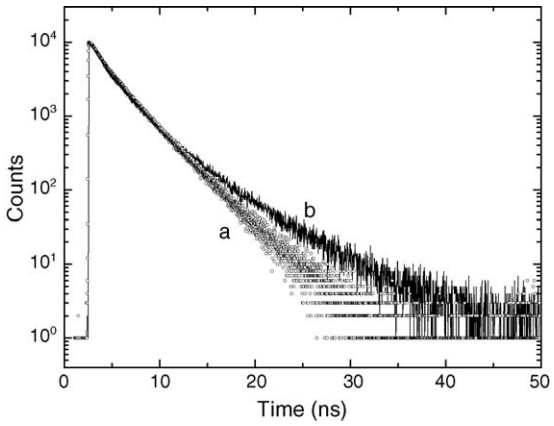

Fig. 6. Excited state decay of 5×10^{-5} M α-MSH in (a, ◯) aqueous medium and in (b, –) 1 mM DMPG. HEPES buffer, pH 7.4, 25 °C. Excitation wavelength 296 nm, emission wavelength 355 nm.

3.2. Trp time resolved fluorescence studies

Fig. 6 shows the fluorescence decay profiles of Trp^9 in α-MSH, in the absence and presence of DMPG bilayers. The experimental data, measured in emission wavelength 355 nm, could be described by a multi-exponential decay function $I(t) = \sum_i \alpha_i \, e^{-t/\tau_i}$, where α_i and τ_i are, respectively, the pre-exponential factor and the lifetime of the component i of the decay. At 25 °C, the data obtained for the peptides in DMPG (Table 2) can be compared with those in aqueous medium, previously published [14]. As shown before, in the pH interval between 5.0 and 7.0 the peptide–lipid partitioning is high, above 95% (Fig. 5) and fluorescence data are representative of the peptides in the bilayer. Comparison was made with fluorescence data in aqueous medium obtained at pH 5.0 in order to have the same ionization state for the His side chain residue, both in absence and in presence of DMPG vesicles. Mean values $\langle\tau\rangle$ can be calculated from intensity weighted lifetimes according to $\langle\tau\rangle = \sum \alpha_i \tau_i^2 / \sum \alpha_i \tau_i$. The longer excited state mean value lifetime for the peptide in the presence of lipid membrane (Table 2), evident in the slower decay curve, indicates the Trp^9 penetration into the lipid hydrophobic region, as discussed before [20]. The penetration of Trp^9 into DMPG bilayers is mainly evident by the great increase of the long lifetime component τ_1, for the four peptides, though there is also a significant increase in τ_2 for NDP-MSH and Ac-$Toac^0$-NDP-MSH (Table 2). The extent of increase in the mean lifetime is not the same as the increase in the lifetime components because the distribution of pre-exponential factors is also affected in the interaction with the bilayer.

It is interesting to examine the Trp lifetime pre-exponential factors. As observed in Table 3, complementing the information from Table 2, for the peptides inserted into the bilayer, the pre-exponential factors of intermediate and short lifetime component are approximately the same, and the contribution of the long lifetime component amounts to values near 10–15% of the total population. The presence of three lifetime components for Trp in peptides has been attributed to the occurrence of different rotational conformers of the indole ring around the C_α–C_β bond of the alanyl side chain [37,41] and several recent studies support the model [10,18], allowing a correlation between lifetimes and Trp side-chain χ_1 rotamer distributions. Several cyclic hexapeptides containing a single Trp residue were examined by fluorescence and ^{1}H NMR [32], and lifetime components ranging from 2.7 to 5.5 ns were assigned to g^- rotamer. In most cases, the rotamer g^+ is less populated, with a short lifetime, having the smaller pre-exponential factor.

The occurrence of Trp rotamers in α-MSH and analogs in solution was observed by NMR studies, and it was reported that the g^+ conformer is less populated [1]. Cyclic melanocortin peptides were the subject of a recent NMR study reported by Ying et al. [42] showing that in aque-

Table 2
Trp excited state lifetimes (τ_i), mean values $\langle\tau\rangle$, and normalized pre-exponential factors (α_i) in melanocortin peptides (5×10^{-5} M) in aqueous solution and DMPG dispersion

		τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	$\langle\tau\rangle$ (ns)	α_1	α_2	α_3
α-MSH	H_2O[a]	3.68 ± 0.05	2.28 ± 0.05	0.54 ± 0.01	2.80	0.27 ± 0.03	0.62 ± 0.04	0.11 ± 0.01
	DMPG[b]	5.28 ± 0.05	2.36 ± 0.04	0.59 ± 0.02	3.21	0.14 ± 0.01	0.43 ± 0.01	0.43 ± 0.02
	%[c]	+43	+4	+9	+15			
Ac-$Toac^0$-	H_2O[a]	3.22 ± 0.04	2.03 ± 0.03	0.50 ± 0.01	2.45	0.26 ± 0.02	0.62 ± 0.03	0.12 ± 0.01
α-MSH	DMPG[b]	4.64 ± 0.04	2.05 ± 0.03	0.37 ± 0.02	2.86	0.13 ± 0.01	0.37 ± 0.30	0.50 ± 0.04
	%[c]	+44	+1	−26	+17			
NDP-MSH	H_2O[a]	3.40 ± 0.10	1.95 ± 0.05	0.48 ± 0.03	2.73	0.39 ± 0.03	0.52 ± 0.04	0.09 ± 0.01
	DMPG[b]	5.94 ± 0.11	2.46 ± 0.03	0.58 ± 0.03	3.12	0.09 ± 0.01	0.42 ± 0.05	0.49 ± 0.05
	%[c]	+75	+26	+21	+14			
Ac-$Toac^0$-	H_2O[a]	3.10 ± 0.10	1.70 ± 0.06	0.42 ± 0.02	2.43	0.36 ± 0.04	0.52 ± 0.05	0.12 ± 0.01
NDP-MSH	DMPG[b]	6.39 ± 0.47	2.16 ± 0.15	0.38 ± 0.03	3.38	0.08 ± 0.01	0.33 ± 0.03	0.59 ± 0.05
	%[c]	+106	+27	−10	+39			

Excitation and emission wavelengths 296 and 355 nm, respectively. Hepes buffer, 25 °C. [DMPG] = 1 mM.

[a] Lifetimes (from Global analysis) and pre-exponential factors (at pH 5, see text) from [14].

[b] Values are averages calculated between pH 5 to 7 (see text).

[c] $[(\tau_{lipid} - \tau_{aqueous})/\tau_{aqueous}] \times 100$.

Table 3
Trp excited state lifetimes (τ_i), and normalized pre-exponential factors (α_i) in melanocortin peptides (5×10^{-5} M) in DMPG gel (10 °C) and fluid (40 °C) bilayers

	T (°C)	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	α_1	α_2	α_3
α-MSH	10	6.02 ± 0.10	2.68 ± 0.16	0.71 ± 0.03	0.16 ± 0.01	0.45 ± 0.06	0.39 ± 0.01
	40	4.00 ± 0.11	1.80 ± 0.10	0.53 ± 0.04	0.13 ± 0.02	0.44 ± 0.07	0.43 ± 0.01
Ac-Toac^0-	10	5.37 ± 0.21	2.36 ± 0.13	0.58 ± 0.06	0.14 ± 0.02	0.40 ± 0.05	0.46 ± 0.05
α-MSH	40	3.74 ± 0.15	1.64 ± 0.05	0.47 ± 0.04	0.08 ± 0.02	0.43 ± 0.01	0.49 ± 0.02
NDP-MSH	10	6.63 ± 0.16	2.86 ± 0.08	0.71 ± 0.02	0.16 ± 0.02	0.43 ± 0.03	0.41 ± 0.04
	40	5.07 ± 0.17	2.09 ± 0.07	0.59 ± 0.05	0.07 ± 0.01	0.43 ± 0.03	0.50 ± 0.07
Ac-Toac^0-	10	5.81 ± 0.20	2.36 ± 0.16	0.56 ± 0.04	0.12 ± 0.01	0.38 ± 0.01	0.50 ± 0.02
NDP-MSH	40	4.23 ± 0.15	1.71 ± 0.07	0.47 ± 0.02	0.08 ± 0.01	0.39 ± 0.04	0.53 ± 0.04

Excitation and emission wavelengths 296 and 355 nm, respectively. Excited state lifetimes, and normalized pre-exponential factors are averages calculated between pH 5–7 (see text). Hepes buffer. [DMPG] = 1 mM.

ous medium trans/g^- rotamers predominate and g^+ rotamers account for less than 20% of the total population. The variation of the pre-exponential factors with the partition of the peptide in DMPG suggests that the g^- rotamer, which predominates in aqueous medium [14], is less present in the lipid bilayer. The opposite trend was observed for the g^+ rotamer, associated to the short lifetime component, which becomes more populated with the insertion of the peptides into the vesicles. It is suggested that the change in rotamers population has its origin in the conformational changes driven by the different environment around the peptides interacting with DMPG bilayers.

While in aqueous medium lifetimes from NDP-MSH were smaller than those from α-MSH [14], insertion of peptides into the bilayers leads to the opposite situation, and the lifetimes of the more potent analog are higher than those of the natural hormone (Table 3). Thus, despite the similarities in the rotamer population of the two peptides, the above result suggests that the environment around Trp^9 in NDP-MSH is more hydrophobic compared to the fluorophore in α-MSH, in agreement with the observation of its deeper penetration in DMPG bilayers [22]. As Trp^9 is in the critical message region of α-MSH comprising the sequence His-Phe-Arg-Trp, the results stress the importance of the hydrophobic environment around the aromatic residues for the biological activity of melanocortic peptides. The lifetimes of Toac-labeled peptides are smaller than the lifetimes of non-labeled peptides, due to energy transfer between Trp and the Toac group [14]. However it is to be noticed that comparison of the results from Toac-α-MSH and Toac-NDP-MSH follows the same behavior as the non labeled peptides, indicating the more hydrophobic environment around Trp in the analog peptide, which maintain its biological activity even with binding to the spin label group. It is also interesting to observe that ESR results indicate that the Toac group in NDP-MSH is in a more polar environment compared to Toac in α-MSH, in contradiction with fluorescence results. However the information from the paramagnetic group is related to the N-terminal region, indicating that the peptide is structured so that the N-terminal and the central core occupy different positions in the bilayer.

4. Conclusions

Due to the special rigidity of the Toac covalent binding to the peptide molecule, the Toac spin label is highly sensitive to the peptide backbone conformation and dynamics. Despite the insertion of this non-natural probe to the structure of the melanocortin peptides α-MSH and the more potent analog NDP-MSH, those peptides, labeled at the N-terminal, were shown to retain their entire biological activity [3,26]. In the present work, Ac-Toac^0-α-MSH and Ac-Toac^0-[Nle^4, D-Phe^7]α-MSH were comparatively investigated concerning their lipid partition, their lipid position inside the bilayer, and the structural alterations felt by those peptides when inserted into the less polar bilayer environment. The peptides are studied via two complementary approaches: the Toac^0 ESR and the Trp^9 fluorescent properties.

Trp time resolved fluorescence data indicate that the peptides, and their Toac-labeled derivatives, present rather similar conformations when membrane bound. This result parallels those found for the peptides in aqueous medium [14]. The clear distinction between the ESR spectra of Toac-bound peptide in solution and in DMPG bilayer allows a very reliable calculation of the lipid-partition of the peptides. It also permits a good analysis of the peptide-bound ESR signal, after spectral subtractions. It also permits the analysis of the peptide-bound ESR signal, after spectral subtractions. A residue titration around pH 7.5, possibly that of His^6, can be clearly monitored by peptide–lipid partition. This is an interesting result, in the sense that all the data obtained with the peptides around neutral pH, in the presence of anionic aggregates, should take into consideration that the peptides are partially positively double-charged and partially monocharged.

It is clearly shown that the two peptides incorporate into DMPG bilayers, both in the more rigid gel lipid phase, and in the fluid bilayer. They seem to be localized close to the bilayer surface, at not very different positions. However, Trp^9 in NDP-MSH is in a somewhat deeper position inside the bilayer, as indicated by the longer excited state lifetimes, though Toac^0 points to a shallower position for the N-terminal in this peptide, as compared to the hormone. The equilibrium

among the three Trp rotamers, g^-, g^+ and trans, changes when the peptides are inserted into the DMPG bilayer, indicating that they undergo structural modifications upon bilayer penetration. The four peptides studied, α-MSH, NDP-α-MSH, and their Toac-labeled derivatives, seem to present similar average conformation inside the membrane, as they present similar equilibrium Among the three Trp rotamers, when inserted into the DMPG bilayer, indicated by the similar Trp lifetime pre-exponential factors.

Toac-labeled peptide can detect conformational changes on peptide backbone due to membrane, or receptor, interaction, as well as monitor peptide partition between two different environments. The Toac-labeling of melanotropins at different backbone positions will further increase the possibilities of monitoring peptide structural alterations.

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