Eur Biophys J (2000) 29: 38–47

ARTICLE

Ana Lucia Carneiro Fernandes Souto · Amando Siuiti Ito

Tryptophan fluorescence studies of melanotropins in the amphiphile-water interface of reversed micelles

Received: 4 August 1999 / Revised version: 17 December 1999 / Accepted: 4 January 2000

Abstract We report studies on the interaction of α-melanocyte stimulating hormone (α-MSH) and a synthetic analogue (MSH-I) with reverse micelles prepared from the amphiphilic sodium bis(2-ethylhexyl)sulfosuccinate in isooctane. The tripeptide lysyl-tryptophyl-lysine and the isolated amino acid tryptophan were also investigated as simpler compounds interacting with the micelles. Tryptophan fluorescence parameters (spectral position of emission band, anisotropy, and lifetime decay) demonstrated that in the presence of reverse micelles the environment around the fluorophore is less polar and more rigid than bulk water. Those parameters are sensitive to the changes induced in the micelles by the presence of water. In large micelles having a water/amphiphile molar ratio above 10, the modifications detected by fluorescence are small and the location of the fluorophore is not affected by a further increase in the concentration of the bulk water. The results, with additional support from quenching experiments, indicated that the different compounds occupy different positions in the large reverse micelles, but in any case they are in the interface region, without dispersing into the bulk water. From decay associated spectra, conformations were identified showing different degrees of tryptophan exposition to polar and nonpolar local environments. The conformation related to the long lifetime has its tryptophan more exposed to water while that associated to the intermediate lifetime has that residue stabilized in nonpolar media. The native hormone α-MSH and the analogue MSH-I present similar conformations in dry micelles. However, in buffer and in the large hydrated micelles, differences in conformations are evident, and could be related to the different physiological activity of the peptides.

A.L.C.F. Souto · A.S. Ito (✉)
Instituto de Física, Universidade de São Paulo,
Caixa Postal 66318,
05315-970 São Paulo, SP, Brazil
e-mail: amando@if.usp.br

Key words Time-resolved fluorescence · AOT reverse micelles · Melanotropins conformation · Heterogeneous fluorescence decay

Introduction

The hormone peptide α-melanocyte stimulating hormone (α-MSH) is secreted by the intermediate lobe of vertebrate pituitaries and is related to several physiological and neurological processes. It is a linear tridecapeptide having the amino acid sequence Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, and it has long been known as the relevant hormone regulating skin pigmentation (Sawyer et al. 1980). More recently, evidence was found of its action also as a neurotransmitter or neuromodulator in learning, memory, and attention (Jegou et al. 1993).

From studies relating chemical structure to biological activity of the hormone, it was established that the central 6–9 tetrapeptide, His-Phe-Arg-Trp, is essential for the action of the hormone as a pigmenting agent (Hruby et al. 1984). Exploration of the characteristics of that central region led to the development of the agonist [Nle4, D-Phe7] α-MSH (hereafter referred to as MSH-I), which in frog skin bioassay exhibits increased potency and prolonged activity, as compared to the native hormone (Sawyer et al. 1980; Al-Obeidi et al. 1989a). The superpotency of MSH-I was attributed to a β-turn conformation stabilized by a D-Phe7 residue and a possible Glu5-Arg8 or Glu5-Lys11 salt bridge (Hruby et al. 1988; Sugg et al. 1988; Al-Obeidi et al. 1989b). It was suggested that structural features of the lateral chains of the central 6–9 sequence also would be of importance, and enhanced biological activity could be observed in those analogues having, in one surface of the peptide, the residues His, Phe, and Trp and, in the other surface, the residue Arg (Sugg et al. 1988; Hruby et al. 1993).

The melanotropins interact with receptors that have been cloned and identified as pertaining to the

superfamily of G-protein receptors and some important amino acids for the receptor-ligant interaction were identified in the extracellular loops of the receptor in human melanoma (Chhajlani and Wikberg 1992; Mountjoy et al. 1992; Grantz et al. 1994; Chhajlani et al. 1996). On the other hand, several reports indicate that the native hormone and some potent analogues show affinity for lipid membranes (Ito et al. 1993; Biaggi et al. 1996; Macêdo et al. 1996). A correlation was observed between an increase in pigmenting activity and the strength of the interaction with model membranes; in particular, the results of fluorescence (Ito et al. 1993) and circular dichroism (Biaggi et al. 1997) suggest structural differences for the peptides in water and in the lipid phase of vesicles, with a possible stabilization of the β-turn in the central region of the peptide. Those results emphasize the importance of the lipid phase in the interaction of the hormone with its receptor in the biological membrane.

A model for the interface between lipid membranes and water is the self-organized aggregation of amphiphiles stable in organic solvents and in the presence of limited amounts of water (Lenz et al. 1995). The reverse micelles formed in this ternary system can mimic the hydrophobic regions around and inside receptors in biological membranes, permeated by the presence of water molecules structured near to charged and hydrophilic groups. Inside the controlled volume of the reverse micelles, the water molecules, if present in sufficient amount, can have the properties of bulk water, modeling the aqueous environment far from the membrane surface.

In order to investigate the behavior of the melanotropins in such a controlled environment, we studied the fluorescent properties of the Trp9 residue of the amino acid sequence of α-MSH, in the presence of reverse micelles formed in the system made by the nonpolar solvent isooctane, the amphiphile sodium bis(2-ethylhexyl)sulfosuccinate (AOT), and various and controlled concentrations of water. Previous work by Bhattacharyya and Basak (1993) suggested different locations for α-MSH and the heptapeptide δ-MSH inside AOT reverse micelles, possibly influenced by electrostatic interactions between charged side-chain residues in the peptides and the surfactant. We studied also the behavior of the potent analogue MSH-I, compared to that of the native hormone α-MSH. The tripeptide lysyl-tryptophyl-lysine (LTL) was examined as a model for a positively charged peptide and we used the isolated amino acid tryptophan as a reference for the properties of the fluorophore interacting with reverse micelles. Steady-state and time-resolved parameters of the compounds were determined at various contents of water in the reverse micelles, characterized by the water/amphiphile molar ratio, w_o. Experiments of fluorescence quenching by acrylamide were also performed, giving additional information about the behavior of the melanotropins in the presence of the reverse micelles. Special attention was devoted to the decay associated spectra (DAS) of the compounds obtained from a global analysis of decay curves measured at different emission wavelengths, and to the correlation between these spectra and structural features of the peptides.

Materials and methods

α-MSH, MSH-I, LTL, and tryptophan were purchased from Sigma (St. Louis, Mo.); isooctane, AOT, and acrylamide were obtained from Sigma-Aldrich. All chemicals were utilized without additional purification.

Reversed micellar suspensions were prepared by a simple solubilization of AOT on a required amount of isooctane to have a final concentration of 50 mM, and vortex mixing the suspension. The peptides were incorporated in the reverse micelles by adding small aliquots (a few microliters) of concentrated stock solutions in buffer (10^{-3} M in 50 mM phosphate buffer, pH 7.4) to 1 ml of the 50 mM suspension of AOT in isooctane, followed by shaking until clear. The amount of stock added was controlled to give the desired values of the parameter w_o that is the ratio between the number of water molecules and the number of AOT molecules. During each series of experiments, w_o was increased by further addition of small aliquots of the stock to the same AOT-isooctane-water suspension. For quenching experiments, a concentrated stock solution of acrylamide (2 M) was prepared in 50 mM phosphate buffer, pH 7.4. Small aliquots (a few microliters) were serially added to the samples either in buffer (peptide concentration 2×10^{-5} M) on in the reverse micelles with $w_o = 16$ (peptide concentration 1.5×10^{-5} M).

Optical absorption measurements were performed using an HP 8452 A diode array spectrophotometer. For steady-state fluorescence experiments we employed a Fluorolog 3 Jobin Yvon-Spex spectrometer. Excitation and emission slits of 1 nm or 2 nm bandpass were used, depending on the fluorescence intensity of the sample. Fluorescence quantum yields were determined using tryptophan in pH 7 buffer as a reference, taking the value 0.14 as standard. The excitation wavelength was 295 nm to avoid emission from the tyrosine residue in melanotropins. In the acrylamide quenching experiments the excitation wavelength was set to 300 nm and corrections were made for the absorbance of the quencher, although its value was always lower than 0.10 at that wavelength. The steady-state fluorescence anisotropy was measured at a wavelength emission of 350 nm, using Glan Taylor polarizers in a L-format configuration. The temperature was controlled using a Forma Scientific 2006 thermal bath.

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 3980

Spectra Physics pulse picker. The laser was tuned to give an output at 890 nm, and a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) gave the 297 nm excitation pulse that was directed to an Edinburgh FL900 spectrometer. The L-format configuration of the spectrometer allowed the detection of the emission at a right angle to the excitation. The emission wavelength was selected by a monochromator, and emitted photons were detected by a Hamamatsu R3809U microchannel plate photomultiplier. The FWHM of the instrument response function was typically 45 ps and a time resolution of 12 ps per channel was employed. Software provided by Edinburgh Instruments was used to analyze the decay curves. The adequacy of the exponential decay fitting was judged by inspection of the plots of weighted residuals and by statistical parameters such as reduced chi-square. Decay associated spectra (DAS) were generated by a global analysis of data sets obtained at different emission wavelengths, using the analysis software from Edinburgh Instruments.

Results

Spectral position

In phosphate buffer, pH 7.4, the peptides presented maximum fluorescence emission at wavelengths near to 355 nm, characteristic of the exposition of the tryptophan residue to the polar aqueous environment, in agreement with previous results of fluorescence of melanotropins (Ito et al. 1993). In reverse micelles having the lowest water content, with w_o = 2, the emission spectra of all samples were blue shifted, showing maxima near to 334 nm (Fig. 1). This means that in those small and dry micelles the tryptophan, either isolated or within the peptides, is in a medium of low polarity, far from the small amount of the structural water present around the polar heads of AOT. Similar blue-shifted emission spectra in reverse micelles were observed in α-MSH by Bhattacharyya and Basak (1993) and in tryptophan by Singh and Aruna (1995).

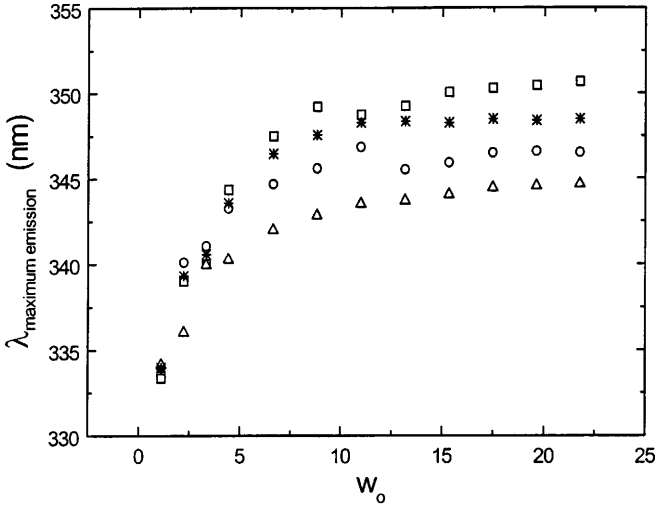

Fig. 1 Dependence of maximum emission wavelength on water content (w_o) of the micelles. △ α-MSH; ✱ MSH-I; ○ LTL □ tryptophan. Excitation wavelength 295 nm

Titration experiments showed that the maximum of the fluorescence emission spectra was shifted to longer wavelengths with increase in the water content of the micelles (Fig. 1). Above w_o = 10, the changes were very small and, although the emission spectra in the large micelles were red shifted compared to those in the small dry micelles, they were blue shifted compared to the spectra in phosphate buffer (Fig. 1). This means that the polarity of the environment around the fluorophore in the large reverse micelles is intermediate between the high value of the bulk aqueous phase and the low value of the AOT hydrocarbon chains.

Quantum yield

Quantum yields of the peptides in phosphate buffer, pH 7.4, are typical of tryptophan in peptides: 0.09 for α-MSH, 0.095 for MSH-I, and 0.08 for LTL (Ito et al. 1993). In the nonpolar medium of the dry micelles (w_o 2), absence of water around the tryptophan residue decreased nonradiative deactivation processes, and the blue shift of the emission spectrum was accompanied by an increase in quantum yield. The quantum yields in dry micelles were higher for α-MSH and MSH-I (0.13 and 0.15, respectively) compared to LTL and tryptophan (0.08 and 0.09, respectively). Values for α-MSH are comparable to those reported by Bhattacharyya and Basak (1993).

Increase in the water content of the micelles in the w_o range between 2 and 10 resulted in a decrease of the quantum yields, which remained practically constant above w_o = 10 (Fig. 2). The final equilibrium values of the quantum yield in large micelles were lower than those measured in the buffer solution, being equal to 0.075 for α-MSH and MSH-I and 0.04 and 0.03 for LTL and tryptophan, respectively. This is another indication that in the large micelles the local environment around the fluorophore is different from the bulk water.

Anisotropy

The anisotropy of the zwitterionic tryptophan measured in buffer was low, as expected for such a small molecule. Larger values were found for LTL (0.014) and melanotropins (around 0.030), with only small changes in temperatures between 15 and 30 °C. The anisotropy of the compounds increased to values around 0.13 in the small micelles (w_o 2). The fluorophore seems to be immobilized in the small micelles and the anisotropy values reflect the depolarization due to the movement of

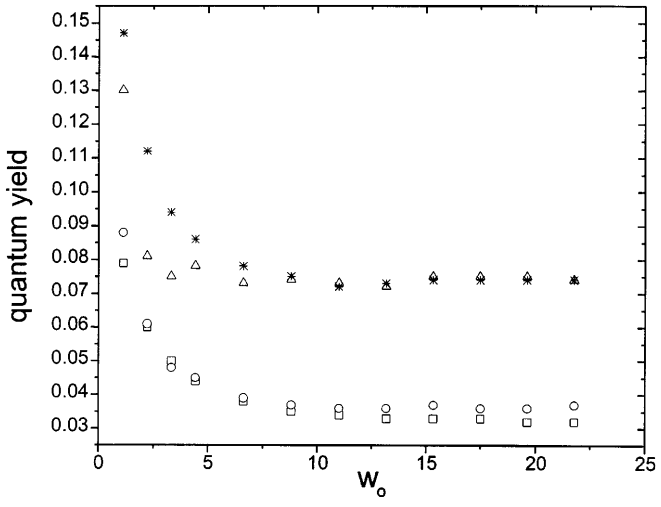

Fig. 2 Dependence of quantum yield on water content (w_o) of the micelles. △ α-MSH; ✱ MSH-I ○ LTL; □ tryptophan. Excitation wavelength 295 nm

the whole micelles. Titration of the reverse micelles, increasing w_o, continually lowered the anisotropy values until about 0.10 for the peptides and 0.06 for free tryptophan (Fig. 3). Diminution of the anisotropy with increasing water content in the micelles could be the result of two opposite trends: an increase in depolarization due to a more fluid environment caused by the influx of water in the interface region of AOT, and a decrease in depolarization due to the larger size of the micelles. The final values of the anisotropy in the titration experiments were higher than those measured in buffer, indicating again that in the large micelles, with a high water content, the peptides were not free in the bulk aqueous phase.

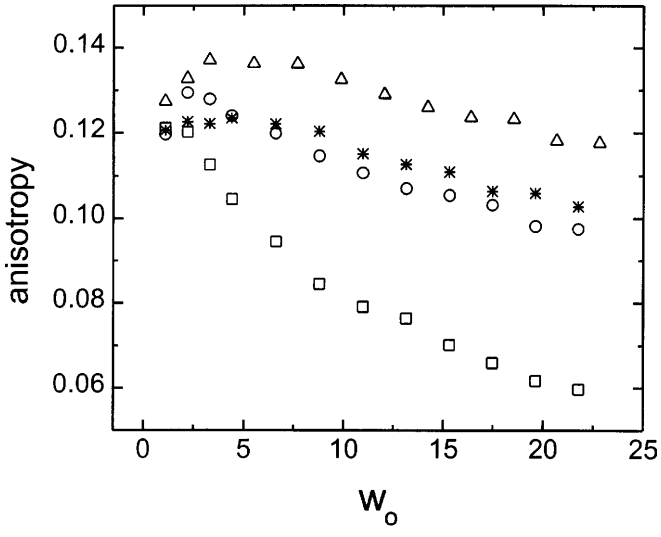

Fig. 3 Variation of anisotropy with water content (w_o) of the micelles. △α-MSH; ✱ MSH-I; ○ LTL; □ tryptophan. Excitation wavelength 295 nm

Lifetime of fluorescence

A very common observation is the multi-exponential fluorescence decay of tryptophan in proteins and peptides, and previous reports showed that the same applied to the melanotropins in aqueous medium and in the presence of vesicles and micelles (Bhattacharyya and Basak 1993; Ito et al. 1993). In this work we observed that, in reverse micelles, three exponential decay components were required to fit the experimental decay data, as judged by the statistical criteria reported in Materials and methods.

Visual inspection of the decay profile of the tryptophan residue in the peptides indicated that the decay kinetics was dependent on the amount of water present in the reverse micelles in the w_o interval between 2 and 10. Also here, only small changes were detected above w_o 10, indicating that for this water/amphiphile ratio a limiting value was already attained for the partition of the peptides in the water/AOT/isooctane system.

A detailed analysis of the time-resolved fluorescence results will be presented in a following section. We examine here the overall characteristics of the fluorescence decay of the peptides under different conditions of the reverse micelles, through the inspection of mean lifetimes for the decay kinetics measured at the wavelength emission of 350 nm. The mean lifetime $\langle\tau\rangle$ is obtained from a weighted mean value calculated from the individual lifetimes (τ_i) and the corresponding pre-exponential factors (a_i) according to

$$\langle\tau\rangle = \frac{\sum a_i\tau_i^2}{\sum a_i\tau_i}$$

Mean lifetimes in buffer were higher for tryptophan (3.0 ns) compared to the peptides (2.31 ns for LTL, 2.35 ns for α-MSH and 2.38 ns, for MSH-I respectively), in agreement with the quantum yield results. Additional routes for the deactivation of tryptophan are present in peptides, involving quenching by either the peptide bonds or other residues in the macromolecule (Chen and Barkley 1998). In the small and drier micelles (w_o 2), longer mean lifetimes were measured for the peptides (2.7, 3.2, and 3.4 ns for LTL, α-MSH, and MSH-I, respectively), while the mean lifetime for the zwitterionic tryptophan decreased to 2.6 ns. In this environment, interaction of free tryptophan with the AOT polar head groups may introduce new deactivation routes for the excited state. Increase in the water content of the micelles resulted in a fall of the mean lifetime values and limiting values were attained for the micelles having w_o above 10 (Fig. 4). The final calculated mean lifetimes were smaller than those obtained in buffer solution for tryptophan, LTL, and α-MSH (1.7, 1.6, and 2.2 ns, respectively), a result that is parallel to the titration observed through the quantum yield values. An exception is the analogue MSH-I, whose mean lifetime in buffer (2.4 ns) is slightly smaller than its value in the large micelles (2.67 ns). The results reported in this section

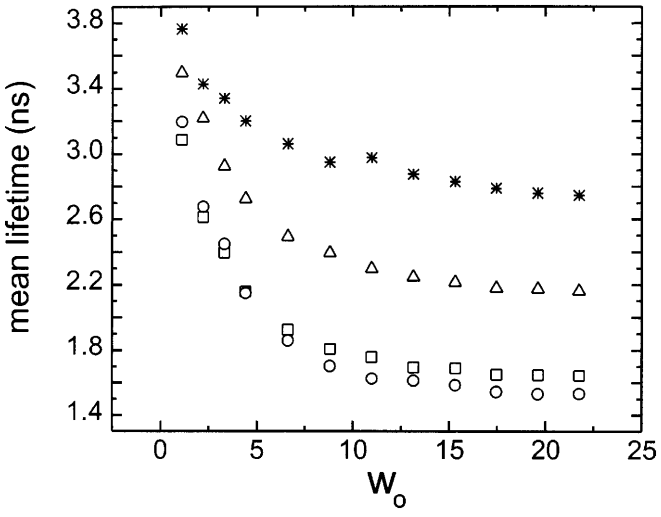

Fig. 4 Variation of mean lifetime with water content (w_o) of the micelles. △α-MSH; ✱ MSH-I; ○ LTL; □ tryptophan. Excitation wavelength 298 nm; emission at 350 nm

refer to emission wavelengths of 350 nm. Decay curves were obtained at several emission wavelengths and the data are presented in the last section of these results.

Quenching

The accessibility of the fluorophores to acrylamide was observed through quenching experiments performed either in phosphate buffer or in the large reverse micelles having $w_o = 16$. Stern-Volmer plots using fluorescence intensity data presented a small extent of positive deviation from linearity (Fig. 5) and a best fit to the experimental data was obtained using the modified Stern-Volmer expression:

$$\frac{I_0}{I} = (1 + K_{SV}[Q])\exp(V[Q]).$$

that includes a sphere of action, characterized by the parameter V, within which occurs the supression of fluorescence. In the expression, K_{SV} is the Stern-Volmer constant and [Q] is the concentration of the quencher.

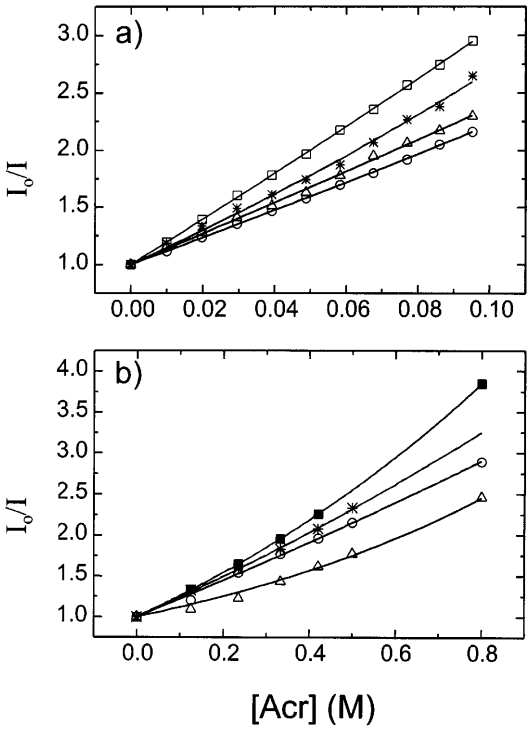

Fig. 5 Stern-Volmer plots for quenching of fluorescence by acrylamide, from intensity measurements: **a** In phosphate buffer, pH 7.4; **b** in reverse micelles, $w_o = 16$. △ α-MSH; ✱ MSH-I; ○ LTL; □ tryptophan. Excitation wavelength 300 nm

Results for α-MSH and MSH-I in buffer were similar to those reported previously (Bhattacharyya and Basak 1993; Macêdo et al. 1996) and we observed here that the fluorescence of zwitterionic tryptophan was more supressed by acrylamide than the fluorescence of the peptides (Fig. 5a and Table 1). This is in agreement with data from the peak position of fluorescence emission, indicating that the Trp residue in the peptides is less exposed to the aqueous solvent.

The Stern-Volmer plots obtained from the mean lifetimes were linear and the constants resulting from the fit of the data to the normal Stern-Volmer equation were

Table 1 Quenching parameters: Stern-Volmer constant (K_{SV}), volume of the sphere of action (V), and bimolecular constant (k_q) for samples in aqueous buffer solution and in reverse micelles with $w_o = 16$

	K_{SV} (M^{-1})[a]	V (M^{-1})[a]	K_{SV} (M^{-1})[b]	K_q (10^9 M^{-1} s^{-1})
α-MSH				
Buffer 7.4	16.0 ± 1.0	1.1 ± 0.5	13.4 ± 0.2	5.7 ± 0.4
Micelles $w_o = 16$	0.81 ± 0.05	0.47 ± 0.035	0.93 ± 0.03	0.42 ± 0.02
MSH-I				
Buffer pH 7.4	14.5 ± 0.8	0.9 ± 0.4	13.0 ± 0.2	5.5 ± 0.3
Micelles $w_o = 16$	2.1 ± 0.2	0.23 ± 0.10	3.5 ± 0.2	1.3 ± 0.1
LTL				
Buffer pH 7.4	13.5 ± 0.2	1.0 ± 0.1	13.4 ± 0.3	5.8 ± 0.1
Micelles $w_o = 16$	2.1 ± 0.2	0.11 ± 0.07	1.07 ± 0.07	0.67 ± 0.04
Trp				
Buffer pH 7.4	20.5 ± 0.1	1.0 ± 0.1	23.1 ± 0.3	7.7 ± 0.1
Micelles $w_o = 16$	1.97 ± 0.02	0.50 ± 0.01	1.7 ± 0.1	1.0 ± 0.1

[a] From intensity plots
[b] From lifetime plots

slightly smaller than those calculated from intensity plots (Table 1), suggesting the occurrence of a contribution from static quenching to the steady-state data. A bimolecular rate constant, k_q, proportional to the diffusion coefficients of the interacting molecules, can be derived according to $k_q = K_{SV}/\langle\tau_0\rangle$, from the mean lifetime $\langle\tau_0\rangle$ measured in absence of quencher and the dynamic Stern-Volmer constants. The values obtained for tryptophan and α-MSH ($7.7 \times 10^9\,M^{-1}\,s^{-1}$ and $5.7 \times 10^9\,M^{-1}s^{-1}$, respectively) in buffer solution were comparable to those reported in the literature (Lakowicz 1983; Bhattacharyya and Basak 1993). Bimolecular rate constants of similar magnitude were obtained for MSH-I and LTL (Table 1).

Quenching by acrylamide was also examined in reverse micelles of AOT having $w_o = 16$, an experimental condition that should be representative of the equilibrium attained by the peptides in the large reverse micelles. Compared to the measurements in buffer, there was a large decrease in the extent of quenching (Fig. 5b), meaning that in the reverse micelles the tryptophan residue in the peptides is less accessible to the quencher. This qualitative observation was evident from both steady-state data and time-resolved experiments. Stern-Volmer plots showed a drastic decrease in the quenching constants in the presence of reverse micelles, compared to those obtained from measurements performed in buffer solution. The bimolecular constant k_q decreases 5–10 times, reflecting the increased restrictions to the collisions between fluorophore and quencher in the reverse micelles (Table 1). Our values for α-MSH ($0.42 \times 10^9\ M^{-1}\ s^{-1}$) are comparable to those previously reported (Bhattacharyya and Basak 1993).

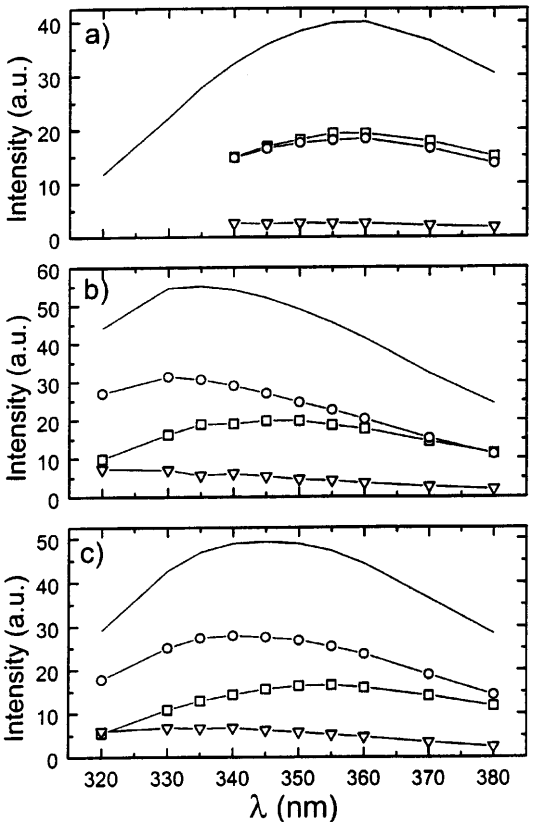

Fig. 6 DAS of α-MSH: **a** in phosphate buffer, pH 7.4, 3.73 ns component (□), 1.60 ns component (○), 0.28 ns component (▽), and steady-state spectrum (—); **b** in reverse micelles, w_o = 2, 5.07 ns component (□), 2.07 ns component (○) 0.34 component (▽), and steady-state spectrum (—); **c** in reverse micelles, w_o = 20, 3.73 ns component (□), 1.51 ns component (○), 0.29 component (△), and steady-state spectrum (—)

Decay associated spectra (DAS)

For a given compound, decay curves were obtained at different emission wavelengths and the decay times were nearly constant across the fluorescence spectrum. Then a global analysis of the data sets measured at different wavelengths was performed to obtain the fluorescence spectrum of each decay component, generating the DAS (Knutson et al. 1983). We obtained the DAS for the compounds in buffer and in the extremes of small (w_o 2) and large reverse micelles (w_o 20). In all compounds, data were best fitted to three exponential decays and the DAS are illustrated in Fig. 6 for α-MSH. Results for the peptides and tryptophan are summarized in Table 2, which also presents the fractional contribution to the total fluorescence emission, obtained from the integration of the spectral curve corresponding to each lifetime. Wavelengths indicated in Table 2 correspond to the position of the maximum from each spectral curve present in the DAS.

Lifetimes resulting from global analysis of α-MSH and MSH-I data in phosphate buffer (Table 2) were comparable to those previously reported, approximately 3.2 ns for a long component, 1.5 ns for an intermediate component, and 0.30 ns for a short component (Ito et al. 1993). From the relative contributions of each component to the total fluorescence (Table 2), it is observed that the long lifetime component predominates in the spectra of MSH-I, the long and intermediate lifetimes have almost equal contributions in α-MSH, and the intermediate lifetime component predominates in LTL and tryptophan. We also noted the presence of a small amount of a long lifetime component of 7.23 ns in tryptophan, which contributed to increase the mean lifetime. In all cases the maximum emission associated with each of the three lifetimes was located around 355 nm, a spectral position corresponding to the fluorophore exposed to the aqueous environment.

Quite distinct DAS were obtained in reverse micelles with w_o = 2. Similarities between α-MSH and MSH-I are evident, like increased lifetimes and similar distribution among the components of the decay, with 50% contribution from the intermediate lifetime (2.07 ns in α-MSH, 2.24 ns in MSH-I, Table 2). Furthermore, that component has the maximum contribution to fluorescence at 330 nm, being mainly responsible for the blue shift observed in the steady-state emission spectra. The short lifetime (0.34 ns in α-MSH and 0.42 ns in MSH-I)

Table 2 Lifetime parameters from global analysis of decay datasets measured at different wavelengths, in buffer solution and in reverse micelles: τ_i lifetime; p_i percentile contribution of lifetime component i obtained from integration of the corresponding spectral component; λ_i peak position (in nm) of the spectrum of component i

	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	p_1	p_2	p_3	λ_1	λ_2	λ_3
α-MSH									
Buffer pH 7.4	3.33	1.6	0.28	48.1	45.5	6.4	355	355	350
$w_o = 2$	5.07	2.07	0.34	37	52.5	10.5	350	330	320
$w_o = 20$	3.73	1.51	0.29	32	55.4	12.5	355	340	330
MSH-1									
Buffer pH 7.4	3.07	1.57	0.25	58.5	37.9	3.6	355	355	350
$w_o = 2$	5.36	2.24	0.42	40.4	49.5	10.5	350	335	320
$w_o = 20$	4.33	1.67	0.29	42.6	46.4	11	355	345	330
LTL									
Buffer pH 7.4	3.38	1.95	0.53	18.8	72.4	8.8	355	355	350
$w_o = 2$	6.0	2.43	0.83	16.7	62.9	20.4	350	345	335
$w_o = 20$	2.47	1.3	0.36	30.6	54.5	15.0	350	345	330
Tryptophan									
Buffer pH 7.4	7.32	2.79	0.51	6.6	88.5	4.8	355	360	350
$w_o = 2$	4.34	1.69	0.35	38	46	15.4	350	335	335
$w_o = 20$	2.30	1.04	0.21	50.3	37.0	12.7	355	345	340

component that contributes 10% to the total emission was also strongly blue shifted, while the spectrum of the long lifetime component (around 5 ns) maintained its maximum near to 350 nm. In the tripeptide LTL the intermediate and the short lifetime (2.43 ns and 0.83 ns) components were less blue shifted compared to those in the melanotropins. However, their relative contributions account for about 83% of the total fluorescence, resulting in the same blue shift of the steady-state spectra observed in the melanotropins. On the other hand, free tryptophan presented the greatest changes both in the lifetimes and in their relative contributions to fluorescence, but maintaining the same trend observed in the peptides that associates the intermediate and short lifetimes mainly to a nonpolar environment.

In reverse micelles with $w_o = 20$, the DAS were different from those obtained either in aqueous medium or in the small, drier reverse micelles. The long lifetime and its relative contribution in the analogue MSH-I were slightly higher than those of the native hormone α-MSH, leading to the higher mean lifetime. The results suggest that, although α-MSH and MSH-I have similar structures in the low polarity environment of dry reverse micelles, they can have different conformations in the large reverse micelles. The differences in conformations could be the same as those existing in the membrane–water interface in the cells, accounting for the different biological activity of the hormones.

In the large micelles, the small tripeptide LTL showed lifetimes smaller than those presented by the hormones, indicating the occurrence of nonradiative processes of decay that are not present in the melanotropins. Despite this fact, the relative distribution of the lifetimes is similar to that observed in α-MSH, with the main contribution to the steady-state emission of fluorescence coming from the intermediate lifetime, whose maximum emission occurred in wavelengths typical of a nonpolar environment. Distinct behavior was observed for free tryptophan: in the large micelles, compared to the buffer, the relative contribution of the intermediate lifetime was strongly decreased, and blue shifted; the long lifetime population, which is not wavelength shifted, increases its contribution to total fluorescence, as does the short lifetime component whose maximum is blue shifted. Besides these changes in the populations, the lifetimes are significantly smaller than those in buffer. Even if tryptophan perceives a polar environment, its DAS is very different from that of the buffer, confirming that the water around the molecule in the large reverse micelles is not the bulk water.

The analysis of the DAS gives additional information related to the blue shift of the emission observed in the experiments of fluorescence quenching by acrylamide. From the decay curves we observed a systematic decrease in the contribution from the long lifetime component when the acrylamide concentration increased, and a corresponding rise in the contribution of the short lifetime component. Examination of the relative contribution of the components of the decay revealed that the population having a longer lifetime, and associated with a polar environment, is more quenched than those presenting shorter lifetimes and associated with the nonpolar medium. The decrease in the contribution from the conformations related to the polar component, more accessible to the quencher, resulted in the observed blue shift of the steady-state spectra of the peptides with increasing concentration of acrylamide.

Discussion

Steady-state fluorescence parameters for α-MSH, MSH-I, and LTL in buffer were similar to those usually found for other tryptophan containing peptides. The residue is probing an aqueous environment, even if it is slightly less polar in the melanotropins than in the tripeptide LTL. As could be expected, the quenching by acrylamide was more pronounced in the free amino acid than in the peptides, reflecting the need for the quencher to

match with a specific part of the peptides, and resulting in the smallest bimolecular constants for the largest molecules. Furthermore, differences in anisotropies reflect the different sizes of the molecules probed by the tryptophan residue.

The large micelles probably best reproduce the overall environment sensed by the peptides in the presence of the lipid phase of a biological membrane. Electron paramagnetic resonance measurements of spin-labeled fatty acids have shown that, below $w_o = 10$, the addition of water to the reverse micelles changes the solvent polarity in the interface region of the micelles, and the water molecules remain structured around the polar head group of the amphiphile (Kommareddi et al. 1993; Ikushima et al. 1997). Above $w_o = 10$, the modifications in the order parameter and rotational correlation times of the spin labels could be explained by the increase in the amount of bulk water inside the micelles. Thus, the large micelles to which we refer in this work (w_o 20) contain structured water around the head group of the amphiphiles, extending to its hydrocarbon chain, and also an amount of water that is enough to reproduce the properties of an aqueous bulk medium inside the micelles. We observed that increase of the water concentration above $w_o = 10$ does not alter the peptide fluorescence parameters, indicating that the interaction of the compounds with the reverse micelle is not affected by further addition of bulk water. Thus, steady-state fluorescence parameters pointed to a location for the tryptophan residue in the peptides intermediate between the bulk phase and the hydrophobic region of the large reverse micelles.

In the experiments with reverse micelles, the concentration of amphiphiles was always in large excess compared to that of the peptides, the minimum ratio being 2250:1 when w_o was set equal to 20. As observed before (Ito et al. 1993), melanotropins interact with negatively charged vesicles and direct micelles, and a limiting distribution is effectively attained at lipid/peptide ratios above, typically, 50. Thus, at the AOT/peptide concentrations used here, it is expected that the peptides are bound to the nonpolar phase of the micelles and this binding distribution, comprising electrostatic interactions, is probably present in the plateau region of Figs. 1–4 corresponding to w_o values above 10. The results presented may indicate that, below $w_o = 10$, additional binding to the lipid surface could be promoted by the decrease in the volume of the aqueous phase, or, alternatively, that the changes in the fluorescence parameters reflect modifications in the polarity of the medium promoted by the decrease in the volume of the aqueous phase. A definite answer cannot be given without additional analysis of the interaction of the peptides with the reverse micelles.

An interesting point needing further elucidation is the effect of the electrostatic interactions between the negatively charged micelle surface and the positively charged peptides. Electric potential energy can be calculated from the Poisson–Boltzmann equation, under spherical symmetry, but it is necessary to have a better knowledge of surface charge densities. The use of lipophilic fluorescent pH indicators could be helpful to investigate the electric properties of the reverse micelle surface, as well as in the determination of the local pH, which could be influencing the fluorescence parameters. Such investigation is currently under study (Fornés et al. 1999) and may provide relevant information about the interaction between peptides and reverse micelles.

Nevertheless, the time-resolved data and global analysis of the decay curves provide some additional information about the conformation of the peptides in interaction with the reverse micelles. In isolated tryptophan, the heterogeneous decay is related to the occurrence of different rotameric conformations (Szabo and Rayner 1980), leading to distinct rates for nonradiative decay processes like electron transfer (Goldman et al. 1995). Studies in protein crystals that present three exponential decay kinetics (Dahms et al. 1995) have shown the existence of different rotamers of the tryptophan residue side chain. Conformational characteristics of the macromolecules can be related to the different rotamers of the tryptophan residue (Jacchieri and Ito 1995) and this assumption leads to the view that different distribution of the lifetimes could be associated with different conformations of the peptide, consistent with recent studies of model indole fluorophores (Chen and Barkley 1998).

It was recently shown by molecular dynamics simulation performed for tenths of nanoseconds that the native hormone α-MSH has a flexible structure in water, without stabilization of any preferential conformation (Pascutti et al. 1999). The results from the DAS of α-MSH in buffer indicated the occurrence of conformations associated with different nonradiative decay rates and their relative contributions to the total fluorescence were not the same as measured for the analogue MSH-I. Even assuming that the modifications promoted in the amino acid sequence of the native hormone did not modify its characteristic flexibility in aqueous solution at room temperature, the differences observed in the DAS suggest that the conformations of α-MSH and MSH-I are significantly different in water.

In the small reverse micelles, a totally different environment is probed by the tryptophan residue, for the several fluorescence parameters were characteristics of a nonpolar medium. Similarities between α-MSH and its analogue MSH-I are evident from the time-resolved results, and DAS for both peptides show a similar distribution of contributions from the long, intermediate, and short lifetimes. Furthermore, in both cases the emission of the long lifetime is centered at 350 nm, while the intermediate and short lifetimes present maxima around 330 and 320 nm, respectively. According to MD simulations (Pascutti et al. 1999), α-MSH in a medium with low dielectric constant presents the stabilization of a compact conformation showing a β-turn involving the residues His6, Phe7, and Arg8. The results from DAS indicate that both peptides are, in fact, constrained to

assume similar conformations in the nonpolar environment of the dry reverse micelles, and these conformations, stabilizing the β-turn, are different from those presented by the peptides in the buffer solution.

In the large micelles there is apparently a contribution to the total fluorescence coming from the long lifetime component, which has maximum emission at 355 nm. This component can be associated with a peptide conformation corresponding to a rotameric state where the tryptophan residue is probing a polar environment. However, the main contribution to the fluorescence of melanotropins comes from the intermediate lifetime component, with a significant contribution also from the short lifetime component. Both lifetimes present maximum spectral contribution at short wavelengths, indicating that in the melanotropins the tryptophan residue is sensing preferentially the less polar medium of the hydrocarbon region of the amphiphile. We emphasize that, in these micelles, the DAS from the peptides were not equal, with different contributions from the lifetime components, indicating differences in the conformations of the peptide despite their location in an environment of similar polarity.

From the time-resolved data we deduce that similar conformations are presented by the native and the analogue hormones in the low polar environment of small and dry reverse micelles. However, the changes promoted in the amino acid sequence of the analogue MSH-I lead to conformations distinct from those attained by the native hormone in buffer and in the large reverse micelles. This stresses the importance of the mixed environment of the micelle lipid phase, affected by the presence of water molecules, and modulating the conformational arrangement of melanotropins. We conclude that the difference in the peptide conformations in an environment having physicochemical properties intermediate between the polar water and the nonpolar lipid phase, and presumed to be similar to that present near to a membrane receptor, could be related to the observed significantly different biological activities of the native hormone α-MSH and the synthetic analogue MSH-I.

Acknowledgements The authors thank Fundação de Amparo a Pesquisa no Estado de São Paulo (FAPESP) for financial support. A.L.C.S.F. also thanks Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) for a fellowship.

References

Al-Obeidi F, Hruby VJ, Castrucci AML, Hadley ME (1989a) Design of potent linear α-melanotropin 4–10 analogue modified in positions 5 and 10. J Med Chem 32: 174–179

Al-Obeidi F, Castrucci AML, Hadley ME, Hruby VJ (1989b) Potent and prolonged acting cyclic lactam analogues of α-melanotroin: design based on molecular dynamics. J Med Chem 32: 2554–2561

Bhattacharyya K, Basak S (1993) Fluorescence study of melanocyte stimulating hormones in AOT reverse micelles. Biophys Chem 47: 21–31

Biaggi MH, Pinheiro TJT, Watts A, Lamy-Freund MT (1996) Spin label and ^{2}H-NMR studies on the interaction of melanotropic peptides with lipid bilayers. Eur Biophys J 24: 251–259

Biaggi MH, Riske KA, Lamy-Freund MT (1997) Melanotropic peptides-lipid bilayer interaction. Comparison of the hormone α-MSH to a biologically more potent analogue. Biophys Chem 67: 139–149

Chen Y, Barkley MD (1998) Toward understanding tryptophan fluorescence in proteins. Biochemistry 37: 9976–9982

Chhajlani V, Wikberg JE (1992) Molecular cloning and expression of the human melanocyte stimulating hormone receptor cDNA. FEBS Lett 309: 417–420

Chhajlani V, Xu X, Blauw J, Sudarshi S (1996) Identification of ligand binding residues in extracellular loops of melanocortin 1 receptor. Biochem Biophys Res Commun 21: 9521–9525

Dahms TES, Willis KJ, Szabo AG (1995) Conformational hetrogeneity of tryptophan in a protein crystal. J Am Chem Soc 117: 2321–2336

Fornés J, Ito AS, Curi R, Procopio J (1999) pH fluctuations in unilamellar vesicles. Phys Chem Chem Phys 1: 5133–5139

Goldman C, Pascutti PG, Piquini P, Ito AS (1995) On the contribution of electron transfer reactions to the quenching of tryptophan fluorescence. J Chem Phys 103: 10614–10620

Grantz I, Shimoto Y, Konda Y, Miwa H, Dickinson CJ, Yamada TY (1994) Molecular cloning, expression and characterisation of a fifth melanocortin receptor. Biochem Biophys Res Commun 200: 1214–1220

Hruby VJ, Wilkes BC, Cody WL, Sawyer TK, Hadley ME (1984) Melanotropins: structural, conformational, and biological considerations in the development of superpotent and superprolonged analogues. Pept Protein Res 3: 1–64

Hruby VJ, Cody WL, Castrucci AML, Hadley ME (1988) Conformational and biological analysis of α-MSH fragment analogues with sterically constrained amino acid residues. Collect Czech Chem Commun 53: 2549–2566

Hruby VJ, Sharma SD, Toth K, Jaw JY, Al-Obeidi F, Sawyer TK, Hadley ME (1993) Design, synthesis, and conformation of superpotent and prolonged acting melanotropins. Ann NY Acad Sci 680: 51–63

Ikushima Y, Saito N, Arai M (1997) The nature and structure of water/AOT/ethane (w/o) microemulsion under supercritical conditions studied by high-pressure FT-IR spectroscopy. J Colloid Interface Sci 186: 254–263

Ito AS, Castrucci AML, Hruby VJ, Hadley ME, Krajcarski DT, Szabo AG (1993) Structure-activity correlation of melanotropin peptides in model lipids by tryptophan fluorescence studies. Biochemistry 32: 12264–12272

Jacchieri S, Ito AS (1995) Characterization of structural transitions from aqueous solution to a lipid phase for α-MSH. Int J Quantum Chem 53: 335–341

Jegou S, Blazques C, Delbende C, Bunel DT, Vaudry H (1993) Regulation of α-melanocyte stimulating hormone release from hypothalamic neurones. Ann NY Acad Sci 680: 260–278

Knutson JR, Beechem JM, Brand L (1983) Simultaneous analysis of multiple decay curves – a global approach. Chem Phys Lett 102: 501–507

Kommareddi NS, John VT, Waguespack YY, McPherson GL (1993) Temperature and gas pressure induced microstructural changes in AOT water-in-oil microemulsions: characterization through electron paramagnetic resonance spectroscopy. J Phys Chem 97: 5752–5761

Lakowicz JR (1983) Principles of fluorescence spectroscopy. Plenum Press, New York

Lenz VJ, Federwisch M, Gattner HG, Bradenburg D, Höcker H, Hassiepen U, Wollmer A (1995) Structure and rotational dynamics of fluorescently labeled insulin in aqueous solution and at the amphiphile–water interface of reversed micelles. Biochemistry 34: 6130–6141

Macêdo ZS, Furquim TA, Ito AS (1996) Estimation of average depth of penetration of melanotropins in dimyristoylphosphatidylglycerol vesicles. Biophys Chem 59: 193–202

Mountjoy KG, Robbins LS, Mortrud MT, Cone RD (1992) The cloning of a family of genes that encode the melanocortin receptors. Science 257: 1248–1251

Pascutti PG, Jackoud LE-J, Mundim KC, Bisch PM, Ito AS (1999) Molecular dynamics simulation of α-melanocyte stimulating hormone in a water-membrane model interface. Eur Biophys J 28: 499–509

Sawyer TK, Sanfilipo PJ, Hruby VJ, Engel MH, Heward CB, Burnett JB, Hadley ME (1980) 4-Norleucine, 7-D-phenylalanine-α-melanocyte-stimulating hormone: a highly potent α-melanotropin with ultralong biological activity. Proc Natl Acad Sci USA 77: 5754–5758

Singh AK, Aruna RV (1995) Fluorescence studies of tryptophan and tryptophan-retinal Schiff base in reverse micellar matrix. J Photochem Photobiol A 89: 247–250

Sugg EE, Castrucci AML, Hadley ME, Binst G, Hruby VJ (1988) Cyclic lactam analogues of Ac-[Nle^4]α-MSH_{4-11}-NH_2. Biochemistry 27: 8181–8188

Szabo AG, Rayner DM (1980) Fluorescence decay of tryptophan conformers in aqueous solution. J Am Chem Soc 102: 554–563