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Interaction of adrenocorticotropin peptides with microheterogeneous systems — A fluorescence study

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ABSTRACT

Adrenocorticotropin (ACTH) and α-melanocyte stimulating hormone (α-MSH) are peptides which present many physiological effects related to pigmentation, motor and sexual behavior, learning and memory, analgesia, anti-inflammatory and antipyretic processes. The 13 amino acid residues of α-MSH are the same initial sequence of ACTH and due to the presence of a tryptophan residue in position 9 of the peptide chain, fluorescence techniques could be used to investigate the conformational properties of the hormones in different environments and the mechanisms of interaction with biomimetic systems like sodium dodecyl sulphate (SDS) micelles, sodium dodecyl sulphate-poly(ethylene oxide) (SDS-PEO) aggregates and neutral polymeric micelles. In buffer solution, fluorescence parameters were typical of peptides containing tryptophan exposed to the aqueous medium and upon addition of surfactant and polymer molecules, the gradual change of those parameters demonstrated the interaction of the peptides with the microheterogeneous systems. From time-resolved experiments it was shown that the interaction proceeded with conformational changes in both peptides, and further information was obtained from quenching of Trp fluorescence by a family of N-alkylpyridinium ions, which possess affinity to the microheterogeneous systems dependent on the length of the alkyl chain. The quenching of Trp fluorescence was enhanced in the presence of charged micelles, compared to the buffer solution and the accessibility of the fluorophore to the quencher was dependent on the peptide and the alkylpyridinium: in ACTH(1–21) highest collisional constants were obtained using ethylpyridinium as quencher, indicating a location of the residue in the surface of the micelle, while in α-MSH the best quencher was hexylpyridinium, indicating insertion of the residue into the non-polar region of the micelles. The results had shown that the interaction between the peptides and the biomimetic systems where driven by combined electrostatic and hydrophobic effects: in ACTH(1–24) the electrostatic interaction between highly positively charged C-terminal and negatively charged surface of micelles and aggregates predominates over hydrophobic interactions involving residues in the central region of the peptide; in α-MSH, which presents one residual positive charge, the hydrophobic interactions are relevant to position the Trp residue in the non-polar region of the microheterogeneous systems.

1. Introduction

Melanocortins, which include adrenocorticotropic hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH) are derived from a 36-kDa precursor protein, proopiomelanocortin. There are many evidences indicating that melanocortins and melanocortin receptors are implicated in a variety of important functions, beside their effect on melanocytes [1]. Melanocortins possess a large number of physiological activity, inducing stimulatory action on learning, attention and memory, alterations in motor and sexual behavior, inhibition of food intake, and there is a strong evidence that melanocortins facilitate regeneration of injured nerves. Beyond this, they can promote central effects such as analgesia and antipyretic effects and peripheral effects such as powerful anti-inflammatory and lipolytic action [1]. Recent studies examining melanocortins and their receptors, have shown their relevance to health, for they have crucial significance in inflammatory and autoimmune diseases that upset the balance of the body's systems [2].

The linear tridecapeptide α-melanocyte stimulating hormone (α-MSH) has the amino acid sequence Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2 which is the same initial NH_2-terminal sequence of the longer peptide ACTH. It is recognized that α-MSH presents several potent biological activities such as stimulation of cutaneous pigmentation, bodyweight regulation and induction of grooming behavior, as well as anti-inflammatory and antipyretic effects [3]. The anti-inflammatory effect occurs through the action of the peptide on peripheral inflammatory cells, glial inflammatory cells,

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and on central nervous system (CNS) receptors that activate descending anti-inflammatory neural pathways [4]. These properties are also related to inhibition of nitric oxide synthase induction and nitric oxide production in liver and microglia and cyclooxygenase (COX-2) expression in hypothalamus [5]. α-MSH is also a natural antimicrobial peptide with a broad spectrum of activity [6] and presents action on the cardiovascular and renal systems [7]. The amino acid sequence His-Phe-Arg-Trp is sufficient for receptor recognition and analogues peptides containing this core sequence can be used in the treatment of malignant melanoma [8].

ACTH is a longer peptide containing 39 amino acids, from which the initial 13 residues are the same as α-MSH sequence. It stimulates adrenal and accelerates cortisol secretion, and is secreted from pituitary, in a process controlled by circadian rhythm, negative feedback from plasma cortisol and neurogenic stimulation [9]. Several N-terminal segments of ACTH were found to have hormonal activities and specific receptor binding properties: ACTH(1–24) binds to steroidogenic receptors, while ACTH(1–10) was found to bind preferentially to central nervous receptors. The fragment ACTH(11–24) is an antagonist for steroidogenesis without any agonist properties [10]. ACTH may enhance memory cytotoxic responses through a combination of mechanisms such as direct cell alterations or synergy with regulatory cytokines [11]. The hormone increases steroid production in adrenal cells and, by increasing NO synthesis, could exert a negative control of steroidogenesis [12].

Beyond specific interactions of melanotropin peptides with receptors, their activity can be modulated by interactions with biological membranes [13]. The dynamics of peptides in the presence of amphiphilic aggregates like micro-heterogeneous systems made of water-soluble polymers and surfactants in aqueous solution are of great interest to understand such process, and also have important technological implications [14]. Sodium dodecyl sulphate (SDS) micelles, which are able to solubilize proteins and other organic compounds, are considered simplified model for cell membranes, and several works report studies about the interaction between peptides and SDS micelles [14–16]. The aggregates of SDS with neutral polymers, such as poly(ethyleneoxide) (PEO), are also subject of interest in processes involving peptides. The interaction of the detergent with the polymer occurs in such way that the micelle surface will be protected from the contact with water molecules, and the structure of polymer-surfactant aggregates consists of spherical micelle beads, surrounded by polymer chains and connected by polymer strands [17].

A third kind of microheterogeneous system of interest consists of hydrophobic and hydrophilic block copolymers, which at low concentration can form micelles, where the hydrophobic groups are in the nucleus and the hydrophilic groups are in the outer part of the micelles, forming a corona which acts as steric stabilizer for the hydrophobic region in the aqueous environment [17,18]. The polymeric micelle nucleus shows structural and functional similarities to the plasma lipoproteins, and presents the capacity to transport lipophilic substances, acting as a long time circulation delivery system of hydrophobic molecules [19]. There are particular interest in self-assembled micelles with poly(ethyleneoxide) (PEO) as the corona-forming block because of its excellent biocompatibility, non-toxicity [20] and prevention against opsonization, avoiding clearance by reticuloendothelial system and increasing the circulation time. The hydrophobic block is formed by a large variety of polymers, and among them the polypropileneoxide (PPO) has shown interesting results [20]. Triblock copolymers of PEO–PPO–PEO can form micelles in aqueous solution at certain conditions, i.e. polymer concentration and temperature. The commercial copolymer known as F127, where the molar weight of the PPO fraction is of approximately 4000, has been widely used for the preparation of thermally reversible gels for the transdermical, injectable and controllable delivery of hydrophilic and hydrophobic drugs [21].

In this paper we report a fluorescence study of the interaction between the hormone peptides α-MSH and ACTH(1–24) and biomimetic systems. We investigated the properties of the interaction analyzing steady-state and time-resolved fluorescence properties of the tryptophan residue present in position 9 of the amino acid sequence of the peptides. Contributions from electrostatic and hydrophobic with microheterogeneous systems where examined, taking into account that hydrophobic residues present in α-MSH are also present in ACTH(1–24) which, in addition, possess 5 positively charged residues in the C-terminal region. The biomimetic systems were the negatively charged SDS micelles and SDS-PEO aggregates, and the neutral LUTROL® F127 polymeric micelles, in aqueous phosphate buffer solution (PBS). As a tool to obtain information about the location of the Trp residue in the presence of the biomimetic systems, we explored the quenching promoted by alkylpyridinium halides, a family of cationic surfactants containing the pyridinium moiety which is an efficient quencher for tryptophan fluorescence, as verified by the decrease in the fluorescence emission of that residue in BSA and small peptides [22]. The convenience of using alkylpyridinium halides as quencher comes also from their high affinity to amphiphilic aggregates with negatively charged surface like SDS [23] and the dependence of its hydrophobicity on the size of the alkyl chain, whose increase causes reduction of the intramicellar mobility and the exchange rate with the aqueous phase [24]. Results from quenching experiments with alkylpyridinium quenchers are thus informative about the location of the peptides in the microheterogeneous systems.

2. Materials and methods

ACTH(1–24) (sequence Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro) and α-melanocyte-stimulating hormone (α-MSH) were purchased from Sigma and used as received. Sodium dodecyl sulphate (99%, Sigma, St. Louis) was purified by recrystallization from ethanol. Poly(ethylene oxide) (PEO, average molecular weight 8000 g/mol — Aldrich) and LUTROL® F127 (BASF) were used as received. The fluorescence quenchers N-Ethylpyridinium (NEP^+) and N-Hexylpyridinium (NHP^+) chlorides were prepared as described previously [24]. N-Dodecylpyridinium chloride (NDP^+) was purchased from Aldrich and recrystallized from acetone.

The absorption spectra were registered on a Hitachi U-3000 spectrophotometer. Tryptophan fluorescence measurements and titration curves were performed on a Hitachi F4500 spectrofluorimeter. The anisotropy experiments in the steady state were made in a Hitachi F-3010 spectrofluorimeter equipped with polarizer filters and the fluorescence anisotropy was calculated taking into account the grating correction factor G. The anisotropy values reported in the paper are average of four measurements.

Fluorescence intensity decay and time-resolved anisotropy were measured 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 the solid state laser Millenia X Spectra Physics. The repetition rate of the pulses was set to 4.0 MHz using 3980 Spectra Physics pulse picker. The laser was tuned so that 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 the emitted photons were detected by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. Soleil–Babinet compensator in the excitation beam and Glann–Thomson polarizer in the emission beam were used in anisotropy experiments. The FWHM of the instrument response function was typically 100 ps and the time resolution was 12 ps/channel. A software provided by Edinburgh Instruments was used to

analyze the individual decays, which were fitted to multi-exponential curves:

$$I(t) = \sum_i \alpha_i e^{(-t/\tau_i)} \quad (1)$$

where α_i are the pre-exponential factors and τ_i are the lifetimes. The quality of the fit was judged by the analysis of the statistical parameters reduced-χ^2 and Durbin–Watson, and by the inspection of the residuals distribution. Mean lifetimes were calculated from intensity weighted lifetimes according to:

$$\langle \tau \rangle = \frac{\sum \alpha_i \tau_i^2}{\sum \alpha_i \tau_i} \quad (2)$$

where τ_i and α_i are lifetime and pre-exponential factor of the component i of the decay.

In the fluorescence measurements, the following concentrations were used: SDS (0.050 M), SDS (0.050 M)-PEO (2%) and LUTROL® F127 (2%). In each system, aliquots of hormone stock solution (1×10^{-3} M) were added to volumetric flasks to give a hormone final concentration of 1×10^{-5} M. In titration experiments, SDS and polymers were gradually added to the suspension. All aqueous solutions were stirred for 40 min using a magnetic stirr barr. The concentration of N-alkylpyridinium ions was calculated based on the absorption measurements (λ_{max} 258 nm). Aliquots of concentrated solutions of the quenchers (0.15 M) were added directly to the cuvette via a calibrated Hamilton microsyringe, and the emission intensities or probe fluorescence decay was registered after each addition. All measurements were performed at 293 K.

3. Results and discussion

3.1. Peptide fluorescence in PBS

The steady state emission spectra of ACTH(1–24) and α-MSH in buffer solution, pH 7.4, under excitation at 295 nm, are typical of peptides with tryptophan side chain exposed to water, showing a broad band emission peaked in the region of 350 nm. Relatively low steady state anisotropy values, around 0.010, were obtained for the peptides in aqueous medium, and the time-resolved anisotropy decay (Fig. 1) was fitted to a two-component exponential curve (Table 1). Similar to other tryptophan containing peptides observed, the short rotational correlation time around 0.100 ns could be ascribed to the local Trp movement and the long correlation time near to 1.0 ns should describe the overall motion of the whole peptide. Despite of the greater number of residues in ACTH(1–24) compared to α-MSH, their steady state and time resolved anisotropy values are smaller, indicating differences in the spatial arrangement of the two peptides in PBS aqueous solution.

Table 1
Fluorescence parameters maximum emission wavelength (λ_{em}), anisotropy (R), rotational correlation time (Φ_i) and initial anisotropies (B_i) for α-MSH and ACTH(1–24) (1.0×10^{-5} M) in PBS and in the presence of microheterogeneous systems

	λ_{em}	R	Φ_1 (ns)	Φ_2 (ns)	B_1	B_2
α-MSH						
PBS (0.01 M)	350	0.015	0.93	0.16	0.12	0.09
SDS (0.05 M)	341	0.066	7.53	0.32	0.10	0.08
SDS (0.05 M)-PEO (2%)	338	0.057	5.95	0.48	0.13	0.05
LUTROL® F127 (2%)	351	0.026	2.39	0.11	0.15	0.07
ACTH(1–24)						
PBS (0.01 M)	352	0.010	0.47	0.05	0.12	0.10
SDS (0.05 M)	336	0.059	3.76	0.52	0.15	0.05
SDS (0.05 M)-PEO (2%)	337	0.049	2.74	0.28	0.13	0.07
LUTROL® F127 (2%)	351	0.023	1.34	0.14	0.12	0.09

The fluorescence decay profiles in PBS (Fig. 2) were fitted to a tri-exponential curve (Table 2), and the three lifetime components are attributed to the occurrence of different rotational conformers of the indole ring from Trp around the C_α–C_β bond of the alanyl side chain. Mean intensity weighted lifetimes of 2.77 and 2.56 ns were calculated for ACTH(1–24) and α-MSH respectively, values in agreement with previous reports for melanotropins. The set of values of anisotropy and intensity decay are in agreement with previous report for Trp in melanocyte stimulating hormones and derivatives [13,25] or in melittin [26] and bee venon extract in aqueous medium at neutral pH [27].

3.2. Fluorescence in microheterogeneous systems

In the presence of amphyphylic aggregates the fluorescence parameters changed, indicating modifications in the environment around the Trp residue, due to interaction between the hormone peptides and the microheterogeneous systems. In the presence of SDS micelles and SDS-PEO aggregates, the emission bands were blue-shifted by about 10 nm (Table 1), suggesting the location of Trp residue in a less

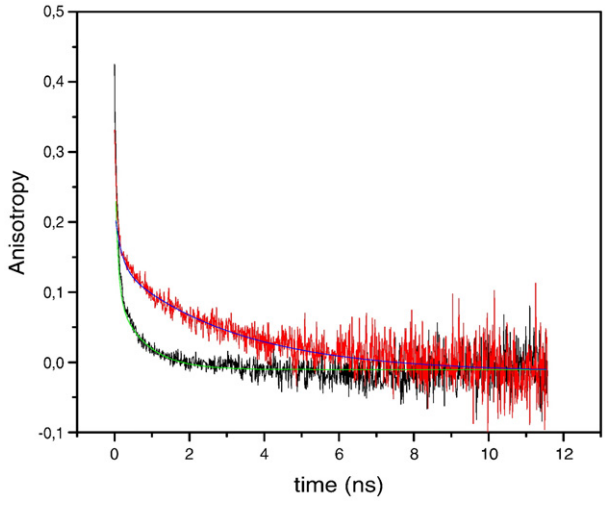

Fig. 1. Fluorescence anisotropy decay of ACTH(1–24) in the presence of SDS 0.05 M (red line) and of polymeric LUTROL® F127 (2%) micelles (black line). Hormone concentration was 1×10^{-5} M, excitation wavelength 295 nm, temperature 20 °C. Also shown are the curves representing the best fit to the anisotropy decay data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

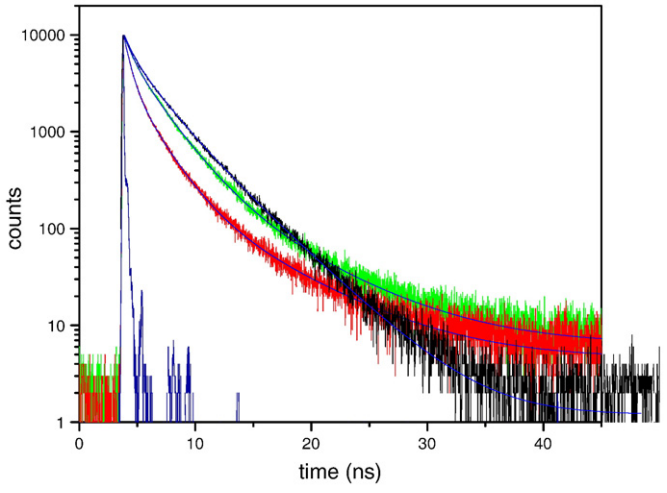

Fig. 2. Fluorescence decay for ACTH(1–24), concentration 1.0×10^{-5} M, excitation wavelength 295 nm. Black line represents decay in buffer, green line in the presence of polymeric micelles LUTROL® F127 (2%) and red line in SDS 0.05 M micelles. Also shown are the instrument response profile (blue line) and the curves (blue lines) representing the best fit to multiexponential decay data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Lifetime (τ_i) and pre-exponential factor (α_i) and weighted mean lifetime $<\tau>$, obtained from multiexponential fit decay profiles

Medium	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	α_1	α_2	α_3	$<\tau>$ (ns)
α-MSH							
PBS	3.49	1.64	0.15	0.23	0.38	0.40	2.57
SDS	4.53	1.29	0.20	0.06	0.40	0.54	2.16
SDS-PEO	5.60	1.73	0.30	0.08	0.53	0.39	2.81
LUTROL	4.03	1.78	0.29	0.21	0.55	0.24	2.77
ACTH(1–24)							
PBS	3.92	1.64	0.15	0.18	0.40	0.42	2.71
SDS	6.50	1.54	0.31	0.05	0.45	0.50	2.61
SDS-PEO	5.71	1.69	0.34	0.05	0.47	0.48	2.44
LUTROL	7.30	2.07	0.36	0.04	0.60	0.36	2.93

Excitation and emission wavelengths were 296 nm and 350 nm respectively, peptide concentration was 1.0×10^{-5} M. PBS 0.01 M, SDS 0.050 M, PEO and LUTROL, 2%.

polar environment. The blue shift was not observed in the presence of copolymer micelles, indicating that the Trp residue is exposed to water in the hydrophilic region of the copolymers.

Another evidence of interaction came from the steady state anisotropy (R), which increased by a factor of 4 to 6 in the presence of SDS and SDS-PEO aggregates, and by a factor of 2 in the copolymer micelles (Table 1). The association of hormones peptides with the aggregates reduces their mobility, as reflected by the increase in steady state anisotropy. The time-resolved anisotropy decay curves (Fig. 1) reveal a slowing down in the rotational motion of the fluorophore in the aggregates, and the curves were fitted to bi-exponential functions. The rotational correlation time of the overall tumbling of the peptide (Φ_1) and of Trp residue in the peptide chain (Φ_2) increased compared to the values obtained in phosphate buffer (Table 1), indicating restricted motion due to association of the probe with the aggregates, the effect being more pronounced in SDS micelles and less evident in the polymeric micelles. Despite the same general trend presented by both hormone peptides in interaction with microheterogeneous systems, some differences arose from the anisotropy experiments: the Trp residue in α-MSH, which has one residual positive charge, presents lower rotational diffusion, or longer rotational correlation time, compared to Trp in the fragment ACTH(1–24) which has 24 amino acids and a +6 residual positive charge, located in the 11 to 24 region. The result is in agreement with the suggestion that ACTH(1–24) locates in the surface region of negatively charged micelles due to the strong electrostatic interactions, and the hydrophobic residues like Trp have shallow insertion into the non-polar region [10,26]. On the other hand, the hydrophobic residues of α-MSH, which lacks the positive residues of ACTH(1–24) can penetrate the non-polar region of micelles, resulting in increased restriction for the rotational diffusion of Trp side chain.

The fluorescence decay was also affected by the presence of the aggregates (Fig. 2). When the polarity of the Trp environment is reduced it is expected an increase in lifetime due to the elimination of fast deactivating process present in polar environments. As seen in Table 2, the values of all the three lifetimes increased in the microheterogeneous systems, the main enhancement being observed in long and short lifetimes. However, due to the relative increase of the pre-exponential factor of the short component and corresponding decrease in the contribution from the long component, in some cases the average fluorescence lifetime decreased.

A correlation between Trp lifetimes and rotamers in proteins and peptides came from comparison of fluorescence, crystallographic and ^{1}H NMR data [28–30]. For many peptides the long lifetime component (2.7 to 5.5 ns) can be assigned to Trp g^- rotamer, while the less populated rotamer g^+ is related to the short lifetime component [30]. Theoretical calculations [31] indicated that in g^- rotamers of Trp the intramolecular electron transfer process competes with fluorescence as a deactivation route for the excited state while in the g^+ rotamer the electron transfer rate predominates over the fluorescence. It was shown that in Trp there is a pH dependent interconversion between long and intermediate lifetimes reflecting interconversion between g^- and *trans* rotamers [32], and in melanocyte stimulating hormone the changes in parameters of fluorescence decay came from the conformational changes driven by modifications in the ionization stated of titratable residues in the peptides [33].

The fluorescence decays of α-MSH and ACTH(1–24) in buffer indicate predominance of trans and g^+ rotamers, associated to the intermediate and short lifetimes. In SDS micelles, for both peptides, there is a significant decrease in the relative contribution of the long lifetime component (α_1 in Table 2) associated to g^- rotamer, leading to the decrease in the mean lifetimes, as previously observed for melittin [26] and bee venon extract [27]. In SDS-PEO aggregates, the decay of α-MSH and ACTH(1–24) presented decrease in the long lifetime pre-exponential factor (α_1) and increase in the contribution from the intermediate lifetime associated to *trans* rotamer. In LUTROL polymeric micelles, the peptides behaved differently: while the same pattern of decrease in the long lifetime population was observed in ACTH(1–24), in α-MSH there was a decrease in the short lifetime population, keeping unchanged the amount of g^- population associated to the long lifetime. Thus, the time resolved data show that insertion of both peptides into the microheterogeneous systems is accompanied by changes in the equilibrium between rotameric conformations of Trp.

3.3. Steady state fluorescence quenching

The fluorescence from α-MSH and ACTH(1–24) was quenched by alkylpyridinium ions in PBS aqueous solution, as well as in the presence of the microheterogeneous systems. The Stern–Volmer constant (K_{SV}) values were obtained as usual, from the plots of F_0/F as a function of quencher concentration [Q], where F_0 and F are the fluorescence intensities in the absence and in the presence of the quencher. The plots were linear in the concentration range of quencher used, as shown in Fig. 4, and the quenching constants in the various systems are listed in Table 3. In PBS aqueous solution, for both peptides, the short chain quencher ethylpyridinium (NEP^+) was the most efficient, as expected for a process of diffusional nature, because higher diffusion coefficient is presented by smaller quencher molecule. We noticed also that quenching of emission from ACTH(1–24) in PBS was higher than for α-MSH, showing that structural differences between the peptides may an play important role in the accommodation of the alkylpyridinium near to the Trp residue. Smaller values of K_{SV} for dodecyl-pyridinium can be originated from effects promoted by the long acyl chain: decrease in the diffusion coefficient and steric restrictions imposed to the approximation between Trp and the quencher group.

The alkylpyridinium ions have high affinity for negatively charged aggregates [23,24] and the probability of contact between fluorophore and quencher in the presence of SDS and SDS-PEO micelles is increased, enhancing the quenching compared to PBS medium (Table 3). In those micelles the highest K_{SV} values for ACTH(1–24) fluorescence quenching was observed for N-ethylpyridinium (NEP^+), indicating that its Trp

Table 3
Stern–Volmer constants (K_{SV}) for the fluorescence quenching of α-MSH and ACTH(1–24) (1.0×10^{-5} M) by alkylpyridinium ions

	NEP^+	NHP^+	NDP^+
α-MSH K_{SV} (M^{-1})			
PBS (0.01 M)	174±2	149±1	143±8
SDS (0.05 M)	271±4	448±19	365±12
SDS (0.05 M)-PEG (2%)	286±9	459±14	436±14
LUTROL® F127 (2%)	108±1	223±3	115±4
ACTH(1–24) K_{SV} (M^{-1})			
PBS (0.01 M)	371±5	228±11	199±15
SDS (0.05 M)	498±16	416±13	321±10
SDS (0.05 M)-PEG (2%)	584±28	452±17	174±5
LUTROL® F127 (2%)	235±9	211±8	144±7

residue is located near to the surface of the aggregates, the preferential location of NEP^+. The results are in agreement with those reported for the quenching of ACTH(1–24) by acrilamide in the presence of negative charged small unilamellar vesicles, where it was concluded that the Trp residue is located near the membrane interface [34]. The results are also consistent with the observation by NMR that the peptide backbone, for the most part, was located in the SDS–water interfacial area: electrostatic interactions originated in the positive charges of Lys and Arg residues present in the segment 11–24 of ACTH(1–24), and hydrophobic effects driven by Trp, Tyr, Phe, and Met side chains present in segment 1–10, combine to the surface-binding mode of interaction between ACTH(1–24) and negatively charged SDS micelles [10,26]. The quenching process was more efficient in SDS-PEO aggregates, which has an average aggregation number smaller than SDS micelles [27]. This fact leads to an enhancement in the average occupation number (number of quencher molecules by micelles), increasing the rate of encounter between probe and quencher.

On the other hand, the α-MSH peptide presents higher quenching by N-hexylpyridinium (NHP^+) ion, indicating a deeper insertion of Trp residue in the interface region of the micelles, compared to ACTH(1–24). The result agrees with those obtained from studies of location of Trp from MSH in unilamelar dimiristoylphosphatidylglycerol vesicles using the parallax method, where it was shown that the fluorescent residue is near to the carbon 6 of the aliphatic chain [35]. The fluorescent properties of the Trp residue in α-MSH were also studied in the presence of reverse micelles of sodium bis(2-ethyl-hexyl)sulfosuccinate (AOT), and the results showed its location in the hydrophobic chains region of AOT interface [36,37].

In the LUTROL polymeric micelles the K_{SV} values were smaller, a result related to the lower affinity of the peptide to that microheterogeneous system, as well as to the absence of Coulombic interaction among quencher ions and micellar surface. Also to be noticed is that the extent of quenching promoted by the long chain dodecylpyridinium in LUTROL decreased compared to the value in PBS, due to the restrictions imposed from the micellar environment to the motion of the long chain quencher.

3.4. Time-resolved fluorescence quenching

Time-resolved fluorescence measurements showed that the emission decay curves of the hormones were fitted to three-exponential curves, the multiplicity of tryptophan lifetime related to the presence

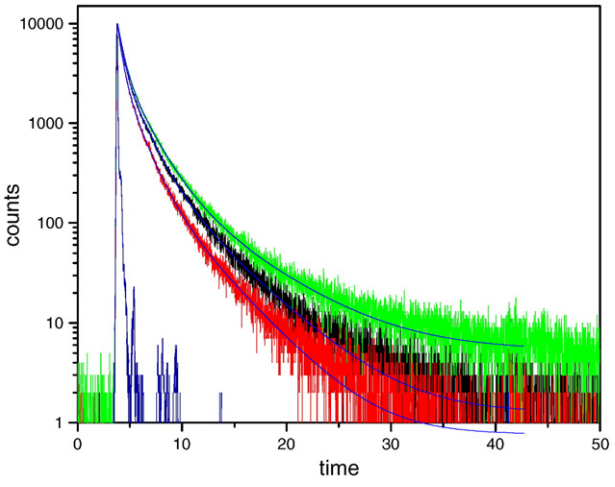

Fig. 3. Fluorescence decay of ACTH(1–24) and quenching of by N-dodecylpyridinium ion in SDS micelles (0.05 M). Green line represents the decay without quencher, black and red lines are the decay with addition of quencher (concentrations of 1.0×10^{-3} and 4.5×10^{-3} M respectively). Also shown are the instrument response profile (blue line) and the curves representing the best fit to multiexponential decay data. Hormone concentration 1.0×10^{-5} M and excitation wavelength 295 nm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

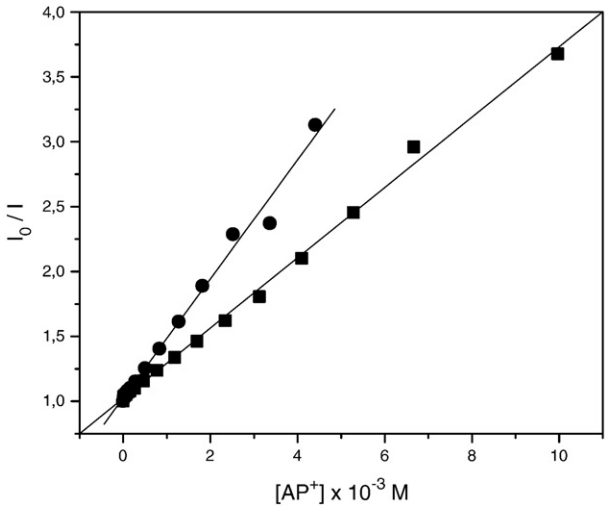

Fig. 4. Stern–Volmer plot from intensity data for the quenching of α-MSH (1.0×10^{-5} M) by N-alkylpyridinium ions (■ NEP^+, ● NHP^+) in the presence of SDS (0.05 M)-PEO (2%) aggregates. Excitation wavelength 295 nm, temperature 20 °C, intensities obtained from integration of emission spectra.

of tryptophan in different rotamers, which can be quenched with different extents [28,29]. In proteins with hydrophobic and hydrophilic regions, charged alkylpyridinium can quench the surface exposed tryptophan residues, but depending on the size of the alkyl chain, they can penetrate into the interior of the protein matrix and can also quench partially buried tryptophan residues [28]. Our results showed that the addition of alkylpyridinium ions increased the rate of non-radiative deactivation of ACTH(1–24) and α-MSH, either in phosphate buffer as in the presence of microheterogeneous systems (Fig. 3), making faster the excited state decay.

The degree of accessibility of the Trp to the quencher in PBS and in the micelles can be estimated from the collisional quenching constant (k_q) which is calculated from the equation $K_D = k_q \cdot \tau_o$, where the dynamic quenching constant (K_D) was obtained from Stern–Volmer plots of the average lifetimes and τ_o is the average lifetime without quencher (Fig. 5). We noticed that the three lifetimes are equally affected by the interaction with alkylpyridinium, indicating that even during the lifetime of the shortest component (around 0.2 ns), the molecules are close enough to quench the fluorescence. For the

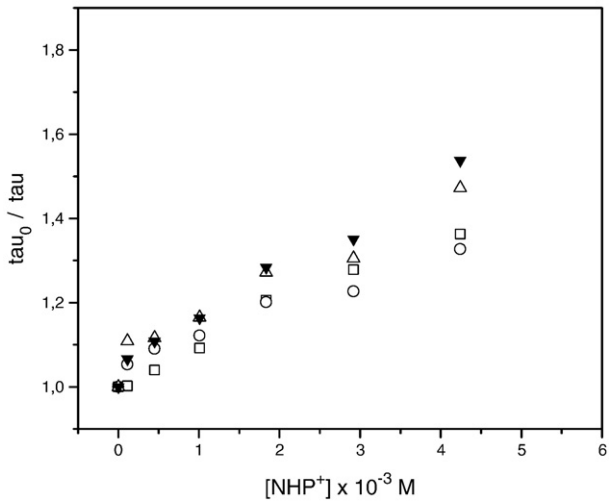

Fig. 5. Stern–Volmer plot from time resolved decay data for fluorescence quenching of ACTH(1–24) by N-hexylpyridinium ions, in the presence of SDS (0.05 M)-PEO (2%) micelles. Hormone concentration 1.0×10^{-5} M, excitation wavelength 295 nm, temperature 20 °C. Lifetimes obtained from best fit to multiexponential decay profiles: □ long lifetime; ○ intermediate lifetime; Δ short lifetime; ▼average lifetime.

Table 4
Bimolecular collisional rate constants (k_q) for tryptophan in MSH and ACTH(1–24) and alkylpyridinium ions

	NEP^+	NHP^+	NDP^+
α-MSH k_q (10^8 M^{-1} s^{-1})			
PBS (0.01 M)	117	88	50
SDS (0.05 M)	7.5	8.3	5.1
SDS (0.05 M)-PEO (2%)	4.1	8.4	4.6
LUTROL® F127 (2%)	1.3	1.7	1.2
ACTH(1–24) k_q (10^8 M^{-1} s^{-1})			
PBS (0.01 M)	56	37	20
SDS (0.05 M)	8.7	7.6	6.6
SDS (0.05 M)-PEO (2%)	5.4	4.7	4.1
LUTROL® F127 (2%)	2.2	2.1	1.3

calculations of K_D it was assumed that alkylpyridinium ions incorporate into the micelles and, instead of the bulk quencher concentration, we used the local concentration in the volume of the aggregates, obtained as the ratio between total concentration of the quencher ($[Q_{total}]$) and the product of surfactant concentration ($[D]$) by the its molar volume (determined from the molecular weight and density).

In PBS solution the values of k_q for the quenching of MSH by ethylpyridinium and hexylpyridinium (near to 1.0×10^{10} M^{-1} s^{-1}, Table 4) are relatively large compared to usual values reported for collisional quenching involving smaller molecules like Trp and O_2. Considering the dependence of collisional quenching on diffusion process, and the molecular dimensions of the peptides and alkylpyridinium, one would expect smaller values for k_q. The experimental results suggest that other interactions between MSH and quencher are present, p.ex. electrostatic, favoring encounters of the molecules which are not driven by purely diffusive mechanisms, and enhancing the quenching of fluorescence. As ACTH(1–24) has positive residues in the segment 11–24, its electrostatic attraction with alkylpyridinium ions is smaller than that presented by α-MSH, resulting in decrease of k_q values compared to the melanotropic hormone.

In the presence of the microheterogeneous systems the values obtained for the bimolecular collisional constant decreased by one or two orders of magnitude. In the charged SDS and SDS-PEO micelles, electrostatic interactions and hydrophobic effects combine to promote binding of both fluorophore and quencher to the aggregates, but also reduce the mobility, or the diffusion coefficient of both quencher and peptide. In this picture, if the quencher and the fluorophore are far apart in the aggregates, the reduced mobility will prevent encounters during the lifetime of the excited state. The results for k_q in LUTROL where there are no electrostatic effects are representative of the low diffusion of the molecules inside the aggregates.

In SDS micelles and SDS-PEO aggregates, the quenchers with short chain (NEP^+ and NHP^+) may dynamically interact with Trp residues, leading to the highest values of k_q (Table 4). For ACTH(1–24) the highest collisional constant was observed with the short chain ethylpyridinium quencher, and, among these, the quenching process was more efficient in SDS micelles. For α-MSH, the quenching with the intermediate chain NHP^+ was the most efficient, a result consistent with a deeper location in the micelle of the Trp residue in α-MSH compared to ACTH(1–24). As expected, the less mobile quencher (NDP^+) gave the lowest values of collisional constant.

4. Conclusions

In buffer solution the same rotamer distribution for Trp side chain residue is present either in the longer peptide ACTH(1–24) as in the shorter α-MSH, showing that the increase in peptide chain length has no effect in the peptide structure around the Trp residue. The interaction of the hormone peptides with the microheterogeneous systems proceeded with conformational changes in the peptides, assessed by the modifications in the pre-exponential factors of the lifetime components. Similar rotamer distributions for Trp side chain in α-MSH and ACTH(1–24) are seen in the presence of SDS micelles and SDS-PEO aggregates, where the contribution of the long lifetime, related to g^- rotamer, markedly decreased. In the presence of the neutral triblock copolymers micelles of PEO–PPO–PEO, the conformational changes are more pronounced for α-MSH, indicating that the charged residues in the C-terminal of ACTH (1–24) decrease its affinity to the hydrophobic core of the polymeric micelles.

In both peptides the extent of modifications in the polarity around the Trp side chain, observed from the blue-shift of the emission, and the decrease in rotational diffusion, verified from anisotropy results, were more pronounced when the surface of the micelles was negatively charged (SDS and SDS-PEO), demonstrating the relevance of the electrostatic contribution to the interaction. However, despite of the stronger electrostatic attractive potential between ACTH(1–24) and the charged SDS and SDS-PEO micelles, the variations in their fluorescence anisotropy parameters are slightly smaller than those presented by α-MSH, showing that the restrictions to the rotational movement of Trp promoted by the micelles are higher in the shorter peptide.

Differences in the behavior of the peptides are valid also for the translational diffusion. The quenching promoted by alkylpyridinium ions was dependent on the length of the acyl chain and in the micellar environment, which imposes restrictions to the translational diffusion of both fluorophore and quencher, the highest collisional constants were observed in the presence of charged micelles. The ethylpyridinium was the most efficient quencher of Trp fluorescence in ACTH (1–24), indicating that in the long peptide, the Trp residue has better contact with the quencher which has higher exchange rate with the aqueous phase. On the other hand, in α-MSH the best quencher was hexylpyridinium, showing that in the less charged and smaller peptide, the Trp residue has higher penetration in the non-polar region of the micelles, and better contact with a more hydrophobic quencher, compared with ACTH(1–24). In the polymeric micelles the electrostatic effects are not present and the Trp residue in both peptides is subjected to similar constraints, concerning its rotational and translational diffusion properties.

The results stress the fact that the positively charged residues in C-terminal region of ACTH(1–24) favor interaction with negatively charged micelles, and make more difficult the insertion of the hydrophobic moiety containing the Trp residue into the non-polar region of micelles. One may expect thus that despite similarities in the initial N-terminal sequence, ACTH(1–24) behaves differently in the presence of cell membranes, compared to the shorter α-MSH.

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