Comparative EPR and fluorescence conformational studies of fully active spin-labeled melanotropic peptides

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Abstract Similar to melanocyte stimulating hormone (α-MSH), its potent and long-acting analogue, [Nle^4, D-Phe^7]α-MSH, when labeled with the paramagnetic amino acid probe 2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid (Toac), maintains its full biological potency, thus validating any comparative structural investigations between the two labeled peptides. Correlation times, calculated from the electron paramagnetic resonance signal of Toac bound to the peptides, and Toac–Trp distances, estimated from the Toac fluorescence quenching of the Trp residue present in the peptides, indicate a more rigid and folded structure for the potent analogue as compared to the hormone, in aqueous medium. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Peptide; α-Melanocyte stimulating hormone; Electron paramagnetic resonance; 2,2,6,6-Tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid; Fluorescence; Spin label

1. Introduction

In most vertebrates, the linear tridecapeptide α-melanocyte stimulating hormone (α-MSH; acetyl-SYSMEHFRWGKPV-amide) is known as the most relevant physiological hormone regulating skin darkening [1]. This peptide is also involved in a variety of other physiological and neurological processes, such as fetal growth, thermoregulation, food intake, learning, memory and attention [2]. The minimal sequence required to stimulate melanocytes was determined to be the core 6-9 fragment, generally referred to as the primary active site or 'message sequence' [3], due to its conservation in several species. Among the several α-MSH analogues, [Nle^4, D-Phe^7]α-MSH (hereafter referred to as NDP-MSH) has been identified as very potent and long-acting analogue in various vertebrate species [4,5]. Due to its high activity and enzymatic resistance, this analogue has been used in the characterization of melanocortin receptors, mainly by radioligand binding analysis [5,6].

Although the important question about the hormone structure–activity relationship has been largely addressed in the melanotropic peptides literature, there is not a consensus about the conformations acquired by both α-MSH and NDP-MSH, either in solution or during the membrane–receptor binding. Several studies have indicated that the peptides assume a folded conformation in aqueous medium (a β-structure or a hairpin loop), comprising the critical 6-9 fragment, and it was postulated that the increased potency of the NDP-MSH derivative was due to a reverse turn-type structure stabilized by the D-Phe^7 substitution [7–9]. Otherwise, there are investigations pointing to a rather flexible backbone for α-MSH in solution, whereas the presence of the D-Phe^7 residue favors a more folded structure for the NDP-MSH analogue [10–12]. It is noteworthy that in the presence of either anionic sodium dodecylsulfate micelles or dimyristoyl phosphatidylglycerol vesicles, a small percentage of the proposed biological relevant structure (a β-like conformation) could be observed in the circular dichroism spectra of both peptides [11]. That result should be regarded in the light of the possible catalytic role of the membrane bilayer phase, inducing a particular peptide conformation appropriate for its interaction with the receptor [13].

To initiate an alternative conformational approach for melanotropic peptides studies, we have recently reported [14] the synthesis of a paramagnetic α-MSH analogue labeled with Toac, an amino acid-type spin probe (2,2,6,6-tetramethylpiperidine-*N*-oxyl-4-amino-4-carboxylic acid) earlier introduced [15,16] in the peptide chemistry field. As emphasized in those works, due to the special rigidity of its 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 in its structure, the synthesized Ac-Toac^0-α-MSH analogue maintained its original potency in the frog *Rana catesbeiana* skin bioassay thus becoming [14] the first example of fully active spin-labeled peptide hormone.

By pursuing the synthesis of alternative fully active paramagnetic melanotropic derivatives for further comparative physiological studies, the present work describes the successful

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Abbreviations: Abbreviations for amino acids and nomenclature of peptide structure follow the recommendations of the IUPAC-IUB, (1989) J. Biol. Chem. 264, 668–673; EPR, electron paramagnetic resonance; Toac, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid

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Toac-labeling of the more potent and long-acting NDP-MSH derivative. The findings here detailed for the synthesized Ac-Toac^0-NDP-MSH analogue demonstrate that the NDP-MSH original activity was entirely maintained after this structural modification, thus validating any further conformational studies of this class of peptides. In addition to the electron paramagnetic resonance (EPR) analysis of the Toac signal of the labeled melanotropic peptides, the presence of the naturally fluorescent Trp residue in their core sequences has also allowed structural comparisons between labeled α-MSH and NDP-MSH analogues via the Trp fluorescence quenching due to energy transfer to the Toac molecule.

2. Materials and methods

2.1. Peptide synthesis

The NDP-MSH and Ac-Toac^0-NDP-MSH analogues were synthesized in the same manner as already detailed for the native α-MSH and its Ac-Toac^0-α-MSH derivative [14]. The base labile 9-(fluorenylmethyloxycarbonyl) group (Fmoc) was used for temporary protection of the Toac α-amine group which allows its insertion at any position in the peptide sequence without the danger in decomposing its nitroxide function during the synthesis cycle. After HF cleavage, the extracted spin-labeled analogue was submitted to alkaline treatment for complete reversion (monitored by analytical high performance liquid chromatography (HPLC)) of the N–O protonation that occurs during this anhydrous acid treatment. After purification in preparative HPLC (C_{18} (octadecyl) column) using aqueous 0.02 M ammonium acetate (pH 5.0) and 60% acetonitrile solutions as solvents A and B, the purified NDP-MSH and Ac-Toac^0-NDP-MSH peptides were characterized by analytical HPLC, mass spectrometry and amino acid analysis.

2.2. Biological assay [17,18]

The thigh and dorsal skin of the frog, *R. catesbeiana*, was excised, cut in square (2×2 cm) pieces, which were placed between two PVC rings and kept for 1 h in Ringer's solution. After this period, the melanin granules were aggregated in the perinuclear region of the melanocytes, which assumed a punctate state, leading to skin lightening. Upon addition of Ac-NDP-MSH or Toac^0-NDP-MSH to the medium, the pigment disperses out into the dendritic processes of the cell, resulting in skin darkening. The changes in skin color (decrease in skin reflectance) were monitored with a Photovolt reflectometer and expressed as percent change of the initial value. Dose–response curves and the EC_{50}s (the concentration eliciting 50% of the maximal darkening, confidence interval (CI) of 95%) were determined for the peptides. The data were compared employing the SNK test ($P \leq 0.05$).

2.3. Sample preparation

For the spectroscopic measurements, stock solutions of the peptides (10^{-3} M) were prepared in water and diluted to the final desired concentrations. The values of pH were adjusted with HCl or NaOH.

2.4. EPR spectroscopy

EPR measurements were performed at 25°C with a Bruker EMX spectrometer. A field-modulation amplitude of 0.5 G and microwave power of 5 mW were used. The temperature was controlled to about 0.2°C with a Bruker BVT-2000 variable temperature device. The spectral parameters were found by fitting each line to a Gaussian–Lorentzian sum function taking advantage of the fact that the sum function is an accurate representation of a Voigt function [19]. The corrected B and C parameters were calculated as described by Bales [19], as a function of the three line heights, making the corrections for the contribution of non-resolved hyperfine splittings. Rotational correlation times were calculated with both parameters, and called τ_B and τ_C, using the principal components of the g and hyperfine tensors of doxyl-propane, and 3300 G for the magnetic field [20]. Considering that those two correlation times should be identical for rapid, isotropic movement, and due to the difficulty in postulating a preferential rotational axis, the movement asymmetry will be estimated by the ratio between τ_C and τ_B. The hyperfine splitting, a_o, was taken to be one-half the difference in the resonance fields of the high- and low-field lines (fit by the computer program).

2.5. Optical and fluorescence spectroscopies

Optical absorption measurements were performed with a HP 8452 A diode array spectrophotometer. For steady state fluorescence experiments a Fluorolog 3 Jobin Yvon-Spex spectrometer was used, with excitation and emission slits of 1 mm or 2 mm, depending on the fluorescence intensity of the sample. The excitation wavelength was set to 295 nm and the quantum yields were estimated using the Trp value 0.14 as a reference. 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 with the pulse picker 3980 Spectra Physics. The laser was tuned to give an output at 897 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 emission wavelength was selected by a monochromator, and emitted photons were detected at right angle from excitation by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. The FWHM (full width at half maximum) of the instrument response function was typically 45 ps and the time resolution 12 ps per channel. A software provided by the Edinburgh Instruments was used to analyze the decay curves and the adequacy of the fitting was judged by the inspection of the weighted residual plots and by the analysis of statistical parameters such as reduced chi-square.

3. Results and discussion

The in vitro frog (*R. catesbeiana*) skin bioassay was performed as previously described [17,18]. NDP-MSH from Sigma, NDP-MSH and Ac-Toac^0-NDP-MSH (this paper) are full agonists on the frog melanocyte, promoting a dose-dependent skin darkening (Fig. 1), and are about five to eight times more potent than α-MSH in this bioassay (1.96×10^{-10} M [14]). The EC_{50}s and the CIs of 95% were 2.4×10^{-11} M (0.38–8.57), 3.8×10^{-11} M (0.59–13.56) and 3.7×10^{-11} M (0.58–13.21), respectively, and were not statistically different. After the removal of these compounds, and several rinses in Ringer's solution, the reversal of the maximal responses was not achieved, at least until 90 min (data not shown). It had

Table 1
EPR parameters for free Toac and Toac bound to the peptides: nitrogen isotropic hyperfine splitting, a_o, and rotational correlation times, τ_B and τ_C

	System	a_o (G)	τ_B (ns)	τ_C (ns)	τ_C/τ_B
	Toac (pH 5.0)	16.330	0.034	0.036	1.06
pH 3.0	Ac-Toac^0-α-MSH	16.238	0.314	0.370	1.18
	Ac-Toac^0-NDP-MSH	16.231	0.333	0.408	1.22
pH 5.0	Ac-Toac^0-α-MSH	16.249	0.306	0.359	1.17
	Ac-Toac^0-NDP-MSH	16.241	0.321	0.391	1.22
pH 9.0	Ac-Toac^0-α-MSH	16.252	0.301	0.353	1.17
	Ac-Toac^0-NDP-MSH	16.242	0.335	0.407	1.22

The estimated errors in the values of a_o and τ_B or τ_C are 0.005 G and 0.005 ns, respectively.

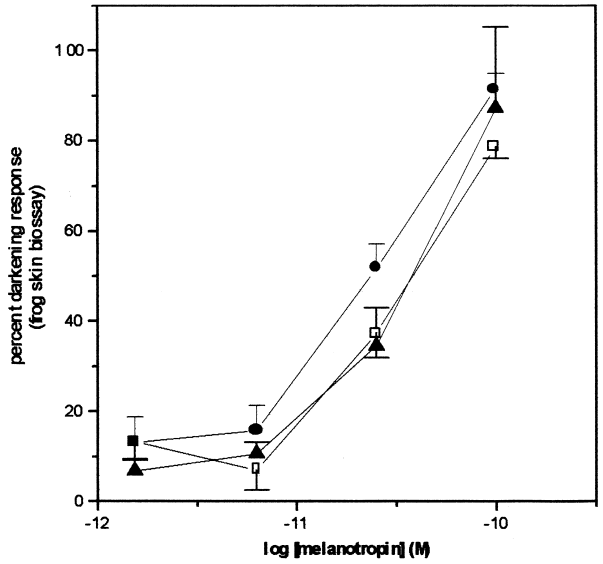

Fig. 1. Dose–response curves to Ac-Toac^0-NDP-MSH (▲) as compared to NDP-MSH (□, from Sigma; ●, this paper) in the frog *R. catesbeiana* skin bioassay. Each point represents the mean ($n=8$) ± S.E.M. darkening response at the concentration noted.

already been demonstrated that NDP-MSH is a potent and long-acting agonist [4], and these properties were not changed with the insertion of the spin label, Toac.

In aqueous solution, the paramagnetic Ac-Toac^0-NDP-MSH analogue displays an EPR spectrum similar to that obtained with Ac-Toac^0-α-MSH already reported [14]. The parameters yielded by the EPR spectra of the two Toac-bound peptides at different pH values are comparatively summarized in Table 1. The EPR parameters (rotational correlation times, τ_B and τ_C, and nitrogen isotropic hyperfine splitting, a_0) are also compared to those of the free Toac molecule. Considering that the free and the peptide-bound Toac EPR spectra are typical of the motional narrowing region (between 10^{-11} and 10^{-9} s), the τ_B and τ_C values were calculated according to Schreier et al. [20] using the corrections for non-resolved hyperfine splittings proposed by Bales [19] (see Section 2). As expected, the τ values of both Toac-bound melanotropic peptides are one order of magnitude higher than those of the free Toac molecule in solution, indicating that the Toac peptide EPR spectrum reflects the mobility of the peptide backbone. This is in close accordance with data already reported for other small paramagnetic labeled active peptides [14–16]. Moreover, considering that the ratio between the two correlation times, τ_C/τ_B, can be used as an estimation of the anisotropy of the spin-labeled systems [20], it is evident that the binding to the peptides also turns the Toac movement less isotropic (higher τ_C/τ_B ratio). It is interesting to note that the correlation times are slightly higher for the Toac-containing NDP-MSH as compared to Ac-Toac^0-α-MSH: τ_C values are around 10%, 9% and 15% higher at pH 3, 5 and 9, respectively. Besides displaying a slower rate of movement, the former peptide also shows a more anisotropic rotation than when bound to the latter ($(\tau_C/\tau_B)=1.22$ and 1.17, respectively), at the three pH values. Those results suggest that the two peptides are characterized by different conformers.

The analysis of Toac nitrogen isotropic hyperfine splitting (a_o) values, which are sensitive to the polarity of the nitroxide group micro-environment [21], also reveals a significant difference between the free and the peptide-bound spin label: in the EPR spectra of the Toac-bound peptide, a_o is 0.08–0.10 G lower than that of the free paramagnetic molecule. That difference could be attributed to a less polar environment neighboring the Toac nitroxide function, when inserted in the peptide structure, and/or to a differentiated unpaired electron distribution over the N-O moiety of the spin probe, when in the free or peptide-bound states. Otherwise, the a_o values of the Ac-Toac^0-α-MSH are somehow higher than those of Ac-Toac^0-NDP-MSH, in all pH values. Considering that the covalent binding of Toac in the two peptides is identical, that difference could only be assigned to slightly different environments experienced by the paramagnetic molecule in the two peptides. Taking together, the higher correlation times, the higher anisotropy of movement, and the lower hyperfine splitting indicate a slightly more folded structure for the Toac-labeled potent analogue as compared to Ac-Toac^0-α-MSH.

Optical absorption spectra of Toac, α-MSH and Ac-Toac^0-α-MSH in aqueous solution, pH 7.0, are illustrated in Fig. 2.

Table 2
Mean lifetime of Trp fluorescence decay, $\langle\tau\rangle$, and estimated Trp–Toac distances in Toac-bound peptides, r

	System	$\langle\tau\rangle$ (ns)	r (Å)
pH 3.0	α-MSH	1.95	
	Ac-Toac^0-α-MSH	1.89	16.7
	NDP-MSH	2.13	
	Ac-Toac^0-NDP-MSH	1.75	12.1
pH 5.0	α-MSH	2.14	
	Ac-Toac^0-α-MSH	2.03	15.3
	NDP-MSH	2.30	
	Ac-Toac^0-NDP-MSH	1.95	12.6
pH 9.0	α-MSH	2.62	
	Ac-Toac^0-α-MSH	2.32	13.1
	NDP-MSH	2.70	
	Ac-Toac^0-NDP-MSH	2.17	12.1

The estimated error in the values of $\langle\tau\rangle$ is 0.05 ns.

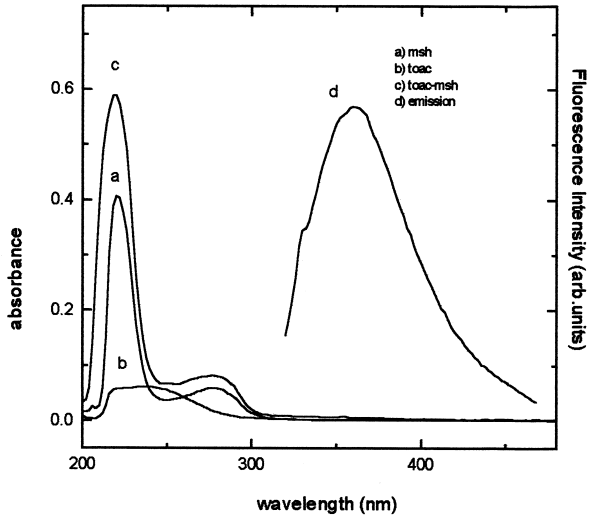

Fig. 2. Optical absorption spectra (optical path length 1.0 cm) of α-MSH (a), Toac (b) and Ac-Toac^0-α-MSH (c), and the fluorescence emission spectra of Ac-Toac^0-α-MSH excited at 295 nm (d). Peptides and Toac concentration 2.5×10^{-5} M, in aqueous solution, pH 7.0, 22°C.

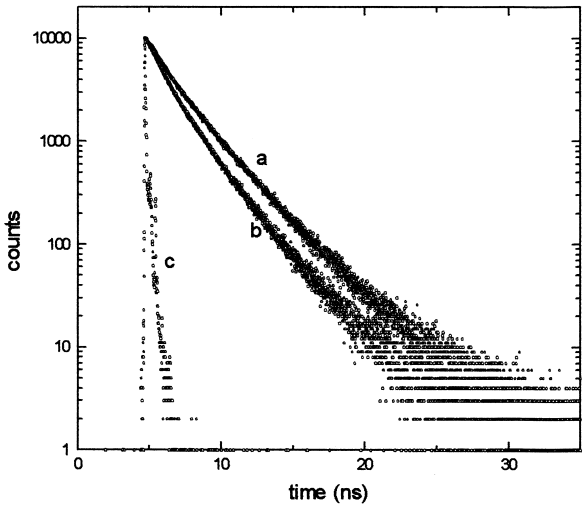

Fig. 3. Fluorescence decay of α-MSH (a), and Ac-Toac^0-α-MSH (b) in aqueous solution, pH 7.0. Concentration 2.5×10^{-5} M, 22°C. The instrument response function at 297 nm excitation is also shown (c).

In the near-UV region, around 280 nm (extinction coefficient $\approx 5.7\times10^3$ M^{-1} cm^{-1}), the spectrum of the labeled peptide is dominated by the electronic transitions characteristic of the Trp residue, superimposed to a small contribution from the Toac molecule. The Ac-Toac^0-α-MSH also presents a very weak absorption band centered around 410 nm, with an extinction coefficient about 20 M^{-1} cm^{-1}, as observed recently [22] in Toac-bound peptides (not evident in Fig. 2, due to the scale used). The same features are present in the spectrum of the analogue Ac-Toac^0-NDP-MSH (data not shown). Pure Toac samples, excited at 295 nm, do not show any fluorescence emission. However, under excitation at that wavelength, both labeled and unlabeled peptides present an emission band centered around 355 nm, typical of Trp in aqueous environment (Fig. 1d). The values of the quantum yield of α-MSH and NDP-MSH are about 0.090, comparable to previous reports for the peptides [23]. Compared to non-labeled peptides, the fluorescence intensity of Trp in the Toac-bound peptides decreases, due to the quenching by the Toac group. In acidic pH values the quantum yields of unbound and Toac-bound peptides decrease with respect to the values at higher pH.

The excited state decay of all the compounds, labeled or not, was found to be heterogeneous, as observed before for the non-labeled peptides in neutral pH [23], and could be properly fit with three lifetime components. The α-MSH and NDP-MSH lifetime values for the high pH samples were also comparable to those previously reported for the peptides in buffer, pH 7.4. However, the Toac-labeled peptides presented faster fluorescence decay (Fig. 3), compatible with Trp fluorescence quenching by the spin label. The decrease in lifetime can be examined by calculating a mean lifetime $\langle\tau\rangle$ for the decay, according to $\langle\tau\rangle = \Sigma a_i\tau_i^2/\Sigma a_i\tau_i$, where τ_i and a_i are the lifetime and the pre-exponential factor of the i-th component, respectively. As observed in Table 2, lifetimes of Toac-labeled peptides are systematically lower than those of original peptides, independent of the solution pH value.

Mechanisms like charge transfer or collisional quenching can contribute to the decrease of Trp fluorescence, and processes requiring close proximity between the fluorophore and nitroxide spin labels were assumed, for example, in the parallax method employed to estimate the penetration depth of melanotropins into the lipid bilayer of phospholipid vesicles [24]. In the present case, as mentioned, the Toac peptides present a very weak absorption band centered around 410 nm, overlapping the red tail of the Trp emission band, allowing for fluorescence resonant energy transfer between the side chain residue as donor and the spin probe as acceptor. The Trp residue and the Toac group are far apart in the peptide chain and the Förster model was recently applied to the study of the Toac-labeled peptides [22], and a Förster distance (R_o) of 9.3 Å was calculated for the pair Trp–Toac. Likewise, an estimation of the intramolecular Toac–Trp distances in the labeled melanotropins can be done, based on the calculated mean lifetimes (Table 2). A donor–acceptor distance, $r = R_o(1/E-1)^{1/6}$, can be estimated from the efficiency of energy transfer, given by $E = 1-\langle\tau_{da}\rangle/\langle\tau_d\rangle$, where $\langle\tau_{da}\rangle$ and $\langle\tau_d\rangle$ are the mean lifetimes for the Toac-labeled and unlabeled peptides, respectively, and using the R_o value calculated by Pispisa et al. [22]. The estimated Toac–Trp distances given in Table 2 were obtained under the assumption of fast movement for donor and acceptor during the fluorescence lifetime. As usually reported in the literature, donor–acceptor distances are estimated within 20% error.

Our results demonstrate that, in all pH values, the Toac–Trp distance is shorter in NDP-MSH, as compared to the native peptide α-MSH. That finding taken together with the results from EPR spectroscopy as discussed above indicate a more compact conformation for the potent analogue and fit with the proposed turn in the NDP-MSH conformation [10–12]. It should also be noted that the analogue conformation does not seem to be dependent on the sample pH value, whereas the Toac–Trp distance in α-MSH increases when the pH decreases. Changes in the pH of the medium affect the protonation state of several side chain groups in the peptides. Therefore, the consequences of those modifications on the conformation of the peptides could be evaluated from a detailed study of EPR and fluorescence parameters of labeled and non-labeled melanotropins as a function of the medium pH value. We stress that the Toac–Trp distances presented here are estimates of mean distances. More detailed information about the conformation of the peptides will depend, for example, on the analysis of the decay associated spectra, effects of the protonable groups, and analysis of resonant energy transfer in the framework of donor–acceptor distance distribution models [25].

The Toac-labeling approach has been considered unique and with great potential for application in a variety of biochemical applications [26]. In this context, the relevance of the present work refers to the introduction of a second fully active Toac-labeled peptide hormone in the literature and its usefulness for structural investigation of melanotropic peptides, in aqueous solution. Otherwise, when membrane-mimetic systems are taken into account, it is already known that this class of peptides strongly interacts with anionic micelles and vesicles, partially penetrating their hydrophobic core [11,27]. We can therefore envisage that, as an expected extension of the present work, conformational changes upon insertion of Toac-containing α-MSH and/or NDP-MSH peptides into the lipid phase could be deeper investigated by simultaneous analysis of the fluorescence properties of Trp residue and the paramagnetic properties of Toac, possibly inserted in different positions of the melanotropic peptide sequence.

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