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# Acid–Base Titration of Melanocortin Peptides: Evidence of Trp Rotational Conformers Interconversion

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***Abstract:*** *Tryptophan time-resolved fluorescence was used to monitor acid–base titration properties of α-melanocyte stimulating hormone (α-MSH) and the biologically more potent analog [Nle[4], D-Phe[7]]α -MSH (NDP-MSH), labeled or not with the paramagnetic amino acid probe 2,2,6,6-tetramthylpiperidine-N-oxyl-4-amino-4-carboxylic acid (Toac). Global analysis of fluorescence decay profiles measured in the pH range between 2.0 and 11.0 showed that, for each peptide, the data could be well fitted to three lifetimes whose values remained constant. The less populated short lifetime component changed little with pH and was ascribed to Trp* g+ $\chi_1$ *rotamer, in which electron transfer deactivation predominates over fluorescence. The long and intermediate lifetime preexponential factors interconverted along that pH interval and the result was interpreted as due to interconversion between Trp* g- *and* trans $\chi_1$ *rotamers, driven by conformational changes promoted by modifications in the ionization state of side-chain residues. The differences in the extent of interconversion in α-MSH and NDP-MSH are indicative of structural differences between the peptides, while titration curves suggest structural similarities between each peptide and its Toac-labeled species, in aqueous solution. Though less sensitive than fluorescence, the Toac electron spin resonance (ESR) isotropic hyperfine splitting parameter can also monitor the titration of side-chain*

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*residues located relatively far from the probe.*  Biopolymers (Pept Sci) 80: 643–650, 2005

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## INTRODUCTION

The linear tridecapeptide (Ac–Ser[1]–Tyr[2]–Ser[3]–Met[4]–Glu[5]–His[6]–Phe[7]–Arg[8]–Trp[9]–Gly[10]–Lys[11]–Pro[12]–Val[13]–$NH_2$), α-melanocyte stimulating hormone (α-MSH), is well known for the relevant physiological role regulating skin darkening, and its involvement in a variety of other physiological processes, like energy homeostasis, feeding behavior, sexual function, and exocrine gland function, etc.[1,2] 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 in various vertebrate species.[3] It has been suggested that the increased potency of the NDP-MSH derivative is due to a reverse turn-type structure stabilized by the D-Phe[7] substitution in the critical His[6]–Phe[7]–Arg[8]–Trp[9] fragment.[4] The presence of Trp residue in position 9 allowed the use of fluorescence spectroscopy in several studies about the hormone and analogues, like the investigation of conformational properties of the peptides in aqueous solution and in interaction with phospholipid bilayers,[5] estimation of depth of penetration in dimyristol phosphatidylglycerol (DMPG) vesicles by parallax methods,[6] conformation induction by trifluoroethanol,[7] verification of the positioning of the peptides in the amphiphile–water interface of reversed micelles,[8] determination of intramolecular distance distributions in α-MSH and NDP-MSH labeled with *o*-aminobenzoic acid by Förster resonance energy transfer,[9] and examination of the structure of analogues containing β-(2-naphthyl)-D-alanine in aqueous solution and in the presence of DMPG vesicles.[10]

It is known that Trp fluorescence properties are strongly affected by interaction with neighboring groups and by physicochemical factors that induces quenching of the indole emission. Besides processes involving collisions between tryptophan and quencher, many other mechanisms may contribute to nonradiative decay of the fluorophore, including temperature-dependent solvent interactions, intersystem crossing, excited-state proton transfer, and intramolecular electron transfer.[11–14] Carbonyl compounds can quench indol fluorescence, due to the formation of an excited state charge transfer complex between indole and a carbonyl group[12]; recent calculations by Callis and Vivian put in evidence the importance of a Trp ring-to-amide backbone charge transfer state as a promoter of quenching of the indole emission.[15] The changes in the pH of the medium may also influence the Trp decay in proteins and peptides, and it was recently shown that Trp is an useful fluorescent probe to study acid–base equilibria of Trp derivatives and Trp-containing peptides, applying the global analysis technique to a set of decay profiles measured in several pH values.[16] The pH changes affect the decay by modifications in the rates of nonradiative decay processes competing with fluorescence either through direct interaction of titratable residues with the indol ring or by modifications in the local environment affecting the mechanisms of excited state deactivation.

In the present work, the Trp[9] time-resolved fluorescence of two peptides, A-MSH and [Nle[4], D-Phe[7]]α-MSH (NDP-MSH), was examined in the pH range between 2.0 and 11.0. As the peptides contain several titratable residues, a complex pattern of decays could be present in that range of pH values. We analyzed the heterogeneous fluorescence decay profiles using the global analysis technique,[17] as applied by Marquezin et al.[16] in the study of Trp-containing derivatives. The experimental decay profiles could be well fitted to a three-exponential decay function and the pH-dependent modifications in the preexponential factors were correlated to the occurrence of different peptide conformations associated with different Trp rotamers. The results could be interpreted based on the induction of conformational changes on the peptides by alterations in the protonation state of side-chain residues.

In addition, we studied by fluorescence and by electron spin resonance (ESR) spectroscopy the peptides α-MSH and NDP-MSH containing the amino acid spin probe Toac (2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid). The probe has been used for peptide labeling to monitor conformational features and dynamics of the macromolecule[18–23] and attention has been paid recently to the first two examples of Toac-bearing peptides that retained entirely the native biological potency: the derivatives of the α-melanocyte stimulating hormone (acetyl-Toac[0]-α-MSH)[24] and of its more potent analog NDP-MSH (acetyl-Toac[0]-[Nle[4], D-Phe[7]]α -MSH).[25] The experiments with the peptides labeled with Toac allowed

comparison of the fluorescence results with data from ESR measurements of the Toac group, examining the pH dependence of isotropic hyperfine splitting and rotational correlation times of the paramagnetic probe. The combined analysis of fluorescence and ESR data is a useful approach, allowing us to obtain complementary information about the peptides.

## MATERIAL AND METHODS

The peptides were synthesized as mentioned in previous articles.[13,14] Stock solutions of peptides were prepared in water. Fluorescence and ESR measurements were made diluting the stock solution with HEPES [4-(2-hydroxyethyl)-1-piperizineethanesulfonic acid] buffer pH 7.4 to final concentration of $5 \times 10^{-5} M$. The pH of the solution was adjusted to the desired value by addition of small aliquots of concentrated NaOH or HCl stock solutions.

### Fluorescence Spectroscopy

Time-resolved experiments were performed using an apparatus based on the time-correlated single photon counting method. The excitation source was a Tsunami 3950 Spectra Physics titanium-sapphire laser, pumped by a 2060 Spectra Physics argon laser. The repetition rate of the 5-ps pulses was set to 400 kHz using the pulse picker 3980 Spectra Physics. The laser was tuned to give output at 888 nm, and a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) gave the 296-nm excitation pulses that were directed to an Edinburgh FL900 spectrometer. The spectrometer was set in L-format configuration, the emission wavelength (355 nm) was selected by a monochromator, and emitted photons were detected by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. The full width of half maximum (FWHM) of the instrument response function was typically 50 ps, determined with a time resolution of 12 ps per channel. Measurements of the peptides decays were made using time resolution of 12 ps per channel.

The software provided by Edinburgh Instruments was used to analyze the individual decay curves, and to perform global analysis of a set of decay profiles. The adequacy of the multiexponential decay fitting was judged by inspection of the plots of weighted residuals and by statistical parameters such as reduced chi-square.

### ESR Spectroscopy

ESR measurements were performed with a Bruker EMX spectrometer. Field-modulation amplitude of 0.5 G and microwave power of 10.13 mW were used. The temperature was controlled to about 0.2°C with a Bruker BVT-2000 variable temperature device. The temperature was monitored with a Fluke 51 K/J thermometer with the probe placed just above the cavity. The magnetic field was measured with a Bruker ER 035 NMR Gaussmeter. Spectral parameters were measured using the Win-EPR Software. The Toac spectral parameters were found by fitting each line to a Gaussian–Lorentzian sum function[26] taking advantage of the fact that the sum function is an accurate representation of a Gaussian–Lorentzian convolution, the Voigt function.[27] The corrected $B$ and $C$ parameters were calculated as described by Bales,[27] as a function of the three line heights, making the corrections for the contribution of nonresolved hyperfine splitting. Rotational correlation times $\tau_B$ and $\tau_C$ were calculated with both parameters and using the principal components of the $g$ and hyperfine tensors of doxil-propane. 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).

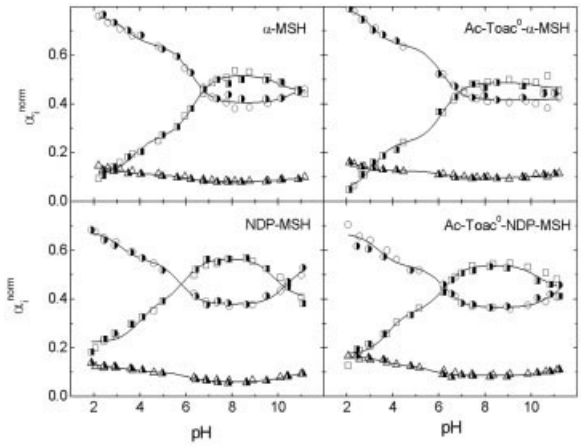


**FIGURE 1** Normalized preexponential factors $\alpha_1^{norm}$ (□ and ■), $\alpha_2^{norm}$ (○ and ◑), and $\alpha_3^{norm}$ (△ and ▲), from global analysis of Trp fluorescence decay in aqueous medium at 22°C, for two different samples (open and half full symbols) in the peptides α-MSH, NDP-MSH, Ac-Toac⁰-α-MSH and Ac-Toac⁰-NDP-MSH. $\alpha_1^{norm}$, $\alpha_2^{norm}$, and $A_3^{norm}$ are related to the Trp excited state lifetimes $\tau_1$, $\tau_2$, and $\tau_3$, respectively. The fitting parameters (solid line) are shown in table.

### pH Titration

The Henderson–Hasselbalch equation describes pH titration of individual sites. For a molecule with more than one titratable site, as the peptides used herein, the total protonation curve can be described by a weighted sum of Henderson–Hasselbalch curves. As discussed by Onufriev et al.,[28] the total average protonation of a molecule containing $N$ sites, can be expressed by

$$\gamma = \sum_{i}^{n} c_i \frac{10^{pK_i - pH}}{1 + 10^{pK_i - pH}} \qquad (1)$$

where $\gamma$ is a parameter representative of the molecule pH titration, like the normalized preexponential factor $\alpha_i^{norm}$ in Figure 1 or the isotropic hyperfine splitting ($a_0$) in Figure 3, and $c_i$ is the contribution of the individual Henderson–

**Table I Trp Mean Lifetimes (ns) in Melanocortin Peptides in Aqueous Solution, 22°C, Calculated at Different pH Values**

| | pH 3.0 | pH 5.0 | pH 8.0 |
|---|---|---|---|
| $\alpha$-MSH | 2.30 | 2,48 | 2.86 |
| Toac-$\alpha$-MSH | 1.98 | 2.14 | 2.45 |
| NDP-MSH | 2.20 | 2.40 | 2.72 |
| Toac-NDP-MSH | 1.85 | 2.06 | 2.37 |

Hasselbalch curve corresponding to the titratable site *i* characterized by its $pK_i$ value.

## RESULTS AND DISCUSSION

### Trp Time-Resolved Fluorescence

Fluorescence decay profile of the peptides, measured in emission wavelength 355 nm and at a given pH value, could be described by a multiexponential decay function $I(t) = \Sigma_i \alpha_i e^{-t/\tau_i}$ where $\alpha_i$ and $\tau_i$ are, respectively, the preexponential factor and the lifetime of the component *i* of the decay. Several groups in the peptides contribute to the deactivation of indole-excited state, as reviewed by Engelborghs,[29] and the presence of ionizable groups in $\alpha$-MSH and analogs renders the fluorescence emission of Trp[9] highly sensitive to the pH of the medium. Mean values $\langle\tau\rangle$ calculated from intensity weighted lifetimes according to $\langle\tau\rangle = \Sigma\alpha_i\tau_i^2/\Sigma\alpha_i\tau_I$, at different pH values, demonstrated that the fluorescence quenching of the peptides is progressively higher with increase in acidity of the medium (Table I).

For a deeper understanding of the pH effects on the emission from the peptides, the global analysis technique was applied to the set of decay profiles measured at several pH values. Despite the presence, in the peptides, of several titratable residues in the pH range 2.0–11.0, the data were well fitted to three lifetimes ($\tau_1$, $\tau_2$, $\tau_3$) (Table II) whose values could be made constant across the pH range studied, and with the corresponding preexponential factors ($\alpha_1$, $\alpha_2$, $\alpha_3$), markedly dependent on the pH of the medium (Figure 1). In all four peptides, there is a short lifetime component, corresponding to 10% of the fluorescent species that is insensitive to pH changes (Figure 1). Opposite to that, there is an interconversion between the long lifetime and the intermediate lifetime preexponential factors in the whole range of pH studied, clearly visualized in pH values near to 4.0, 6.0, and 10.0. A direct correlation of the tryptophan lifetime component interconversion with the exchange between protonated/deprotonated side-chain residues species within a given peptide conformation seems improbable because it would be expected that one of the lifetimes should disappear when the corresponding species disappear. Additionally, one could also expect a larger number of lifetime components if there was a one-to-one correspondence between lifetime and ionization state of the tritratable residues Glu, His, Lys, and Tyr.

The relatively less complex picture emerging from the global analysis suggests an alternative explanation, based on the hypothesis that in the entire pH range, the lifetime components interconversion reflects the equilibrium between different peptide conformations. The presence of three lifetime components for Trp in peptides has been attributed to the occurrence of different $\chi_1$ rotamers of the indole ring around the $C_\alpha$–$C_\beta$ bond of the alanyl side chain[30,31] and despite of the controversy about the origin of Trp heterogeneous decay, several recent studies support the rotamer model. Hellings et al.[32] reported a method to link Trp rotamers with fluorescence lifetime and it was stated that for 10 lifetimes in eight proteins there is an objective identification of the corresponding rotamer. Amphipathic peptides containing a single tryptophan were studied by Clayton and Sawyer,[33] who showed that comparison with crystallographic data of Trp in unordered regions of proteins and in $\alpha$-helical regions supported a correlation of the lifetimes with rotamer distributions. Pan and Barkley[34] studied the fluorescence of a single Trp residue present in seven cyclic hexapeptides possessing a

**Table II Trp Lifetimes (ns) in Melanocortin Peptides, Obtained from Global Analysis of Decay Profiles Measured at Different pH Values in Aqueous Solution, 22°C**

| | $\tau_1$ | $\tau_2$ | $\tau_3$ | $\chi^2_G$ [a] |
|---|---|---|---|---|
| $\alpha$-MSH | 3.68 ± 0.05 | 2.28 ± 0.05 | 0.54 ± 0.01 | 1.17 |
| Toac-$\alpha$-MSH | 3.22 ± 0.04 | 2.03 ± 0.03 | 0.50 ± 0.01 | 1.20 |
| NDP-MSH | 3.40 ± 0.10 | 1.95 ± 0.05 | 0.48 ± 0.03 | 1.27 |
| Toac-NDP-MSH | 3.10 ± 0.10 | 1.70 ± 0.06 | 0.42 ± 0.02 | 1.49 |

[a] $\chi^2_G$ is the global chi-square value, for all the decay profiles analyzed. For each decay, the chi-square was found to be less than 1.1.

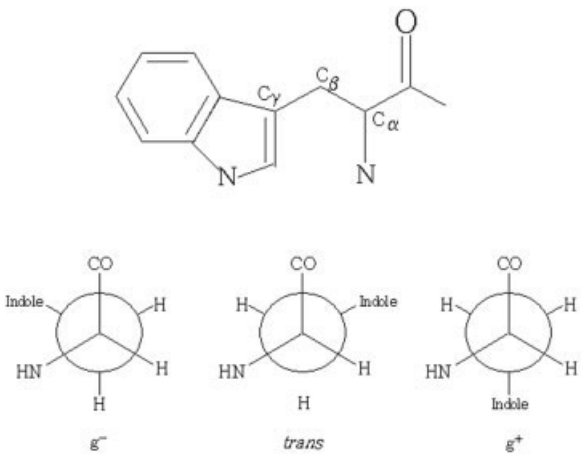


**FIGURE 2** Schematic drawing showing the definition of tryptophan rotamers. The $\chi_1$ is the rotation angle around the central bond between N–$C_\alpha$–$C_\beta$–$C_\gamma$ (upper part of figure). *Gauche minus* ($g^-$), *trans* ($t$), and *gauche plus* ($g+$) rotamers correspond to $\chi_1$ angles equal to $-60°$, 180°, and $+60°$, respectively. Lower part of figure shows the Newman projections indicating the rotation of the indole group about the $C_\alpha$—$C_\beta$ bond.

single backbone conformation. The triple exponential fluorescence emission was analyzed in view of the solution structure and Trp side-chain $\chi_1$ rotamer populations determined by $^1$H-NMR. For all peptides except one, the preexponential value of the major lifetime component is in reasonable agreement with the population of the major $\chi_1$ rotamer. In five peptides the major lifetime component is assigned to $g^+$ rotamer (see Figure 2 for illustration of Trp rotamers), with lifetimes ranging from 2.7 to 5.5 ns; in one peptide the major lifetime component (lifetime 0.5 ns) is assigned to a *trans* rotamer. In most cases, the rotamer $g^+$ is less populated and the short lifetime has the smaller preexponential factor.

The occurrence of Trp rotamers in $\alpha$-MSH and analogues in solution was observed by NMR studies, and it was reported that the $g^+$ conformer is less populated.[35] Predictions from computer simulations performed using different approaches agreed with the experimental data, and it could be expected distinct conformational families associated with Trp rotamers, from which *trans* and $g^-$ states were most populated.[43] Cyclic melanocortin peptides were the subject of a recent NMR study reported by Ying et al.[2] showing that *trans*/$g^-$ rotamers predominate and $g^+$ rotamers account for less than 20% of the total population. The experimental observations also supported the hypothesis of a reverse turn structure in the message region of $\alpha$-MSH comprising the sequence Glu–His–Phe–Arg, as observed earlier in NMR studies of noncyclic analogues.[36,37]

Interpretation of Trp heterogeneous decay in terms of lifetime distributions is also possible when multiple isoenergetic structures can be assigned to the macromolecule.[38] However, this is not always the case, as verified for example in amphipathic peptides, where interpretation of the results in terms of a lifetime distribution model was not consistent with experimental data.[33] In our study good fittings were obtained with single sets of three lifetimes quite distinct among themselves and presenting a standard deviation much smaller than the differences from each other (Table II).

Considering that each lifetime is associated with a Trp rotational conformer, the results from global analysis strongly suggest interconversion among rotameric conformations of Trp induced by the titration of ionizable side-chain groups. That is explicit in the variation of the preexponential factors with the medium pH, as seen in Figure 1. A general trend is evident in the titration curves: when the pH is raised from 2.0 to 9.0, the population of the long lifetime component increases with a corresponding decrease in the concentration of the intermediate one. Above pH 9.0, there is an inversion in the progress of the curves related to those populations. Theoretical calculations reported by Goldmann et al.[39] showed that in the Trp $g^-$ rotamers the electron transfer process competes with fluorescence as a deactivation route for the excited state and the $g^+$ rotamers present very high electron transfer rate, predominating over the fluorescence. We are then supposing that the interconversion between long and intermediate lifetimes reflects interconversion between $g^-$ and *trans* rotamers. The change in rotamers population has its origin in the conformational changes driven by modifications in the ionization stated of titratable residues in the peptides.

For the Toac-labeled peptides, the three lifetimes are significantly shorter than for the unlabeled peptides (Table II), indicating a quenching of the Trp fluorescence by the Toac group, as discussed before.[25] Distinct from that previous publication, NDP-MSH presents somewhat shorter lifetimes than the hormone, suggesting that the Trp residue is more exposed to the polar solvent in the analogue as compared to $\alpha$-MSH (see Table II), based on the shortening of the Trp excited state lifetime due to solvent collisional nonradiative deactivation. The global analysis indicated that the difference between the peptides extended to the large range of pH values over which the study was performed.

## pH Titration

To better understand the pH effects on the peptides, the dependence on the medium pH of the three pre-

**Table III Average Apparent pK Values Determined from the Fitting [Eq. (1)] of the pH Dependence of the Normalized Preexponential Factors, $\alpha_i^{norm}$, Associated with Long, Intermediate, and Short Trp Lifetime Components (Figure 1)**

| | $pK_1$ | $pK_2$ | $pK_3$ |
|---|---|---|---|
| $\alpha$-MSH | 3.46 ± 0.23 | 6.18 ± 0.13 | 10.27 ± 0.26 |
| Toac-$\alpha$-MSH | 2.91 ± 0.33 | 6.14 ± 0.03 | 10.61 ± 0.39 |
| NDP-MSH | 3.48 ± 0.44 | 5.91 ± 0.17 | 10.13 ± 0.27 |
| Toac-NDP-MSH | 3.57 ± 0.20 | 6.04 ± 0.34 | 10.59 ± 0.30 |

exponential factors, $\alpha_1$, $\alpha_2$, and $\alpha_3$, corresponding, respectively, to the long lifetime ($\tau_1$), the intermediate lifetime ($\tau_2$), and the short lifetime ($\tau_3$) components, were fitted by a sum of Henderson–Hasselbalch equations (see Material and Methods), yielding the apparent pK values for the titratable sites of the peptides. As shown in Figure 1 and Table III, the curves corresponding to the preexponential values, for the four peptides, were well fitted with three pK values, which find correspondence to titration of the residues $Glu^5$, $His^6$, and $Lys^{11}$ or $Tyr^2$.

It is noteworthy that, in all peptides, the residue Glu titrates with a lower apparent pK value (around 3.4) as compared to the free amino acid, which presents a pK = 4.07.[40] This same residue, in a dipeptide (Ac–Glu–Trp-$NH_2$) presented an apparent pK of 4.5,[16] also measured by the Trp lifetime decay profiles. Simulation studies on $\alpha$-MSH and analogues and solution structures obtained from NMR experiments emphasized the reverse turn in the peptide, stabilized by interaction between $Glu^5$ and $Lys^{11}$ residues.[2,41] Therefore, the lower apparent pK value found for $Glu^5$ in melanotropins can be attributed to the interaction with the positively charged group, decreasing the local proton concentration. It is interesting to note that, though the $Glu^5$ residue is far from $Trp^9$ in the melanotropins, its ionization causes an increase in the long lifetime component ($\tau_1$), and a decrease in the intermediate lifetime ($\tau_2$) conformer (Figure 1), a result similar to that observed in Ac–Glu–Trp–$NH_2$,[24] where the two residues are close together.

In melanotropins, deprotonation of His increases the population of $\tau_1$ lifetime and decreases the one corresponding to $\tau_2$, almost without affecting that related to $\tau_3$ (Figure 1). A distinct behavior was found in the dipeptide Ac–His–Trp–$NH_2$,[16] where both $\tau_1$ and $\tau_2$ species increase with His deprotonation, and that corresponding to $\tau_3$ markedly decreases. In melanotropins, His titration yields apparent pK around 6, similar to the value obtained for the free amino acid,[39] whereas in the dipeptide Ac–His–Trp–$NH_2$ the found pK was rather higher, around 6.43.[16] It is noteworthy that the result suggests the interaction of $His^6$ with a positively charged group, which is in agreement with structural studies proposing the stacking of $Phe^7$ and $Trp^9$ and segregation of $His^6$ and $Arg^8$ residues in the external side of the peptide chain.[2,4]

The highest apparent pK values obtained from the curves fitting, around 10.5 (Table II), are rather similar for the four peptides and are close to the values corresponding to the titration of side chain groups like $\epsilon$-amine from free $Lys^{11}$ or phenol from free $Tyr^2$, 10.54 and 10.46, respectively.[40] Despite of that similarity, NDP-MSH is significantly more sensitive to the titration at high pH value, presenting a greater variation in the preexponential factors and hence in the rotameric conformations (Figure 1), indicating a different average conformation for this peptide in aqueous solution, as compared to the native hormone. On the other hand, the presence of Toac poses some restriction to conformational changes at high pH values, decreasing the extent of interconversion between the long and the intermediate lifetimes (Figure 1).

## Toac ESR

As discussed before,[24] the ESR spectra of Toac-labeled melanotropins in aqueous solution give rotational correlation times values ($\tau_B$ and $\tau_C$) indicative of Toac anisotropic movement, and one order of magnitude higher than those obtained for free Toac. Figure 3 indicates that the titration of the peptide side chains does not affect the rotational correlation times, $\tau_B$ being found around 0.30 ns and $\tau_C$ around 0.37 ns, for both peptides, showing that the Toac movement in the two peptides is similar. However, the isotropic hyperfine splitting ($a_o$), which is sensitive to the microenvironment polarity[42] felt by the Toac group, well monitors the residues titration (Figure 4). That is rather interesting, considering the Toac position at the beginning of the peptide chain, and the position of those groups in the peptide backbone so that the sensitivity could be related to an average conformational structure for the peptides in aqueous solution such that the ionizable groups affect the polarity around the Toac group. Although some difference in

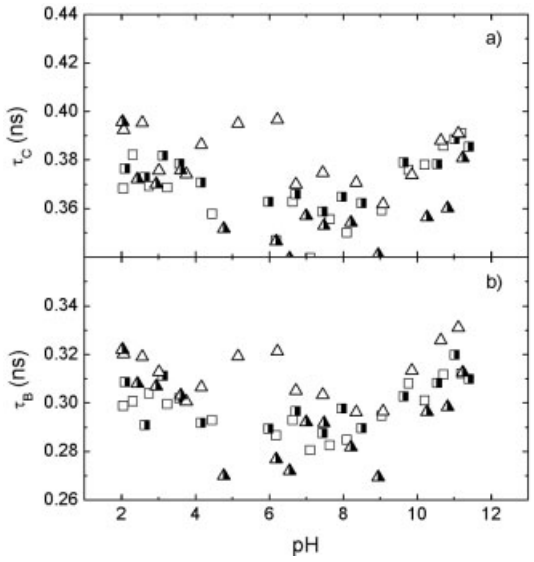


**FIGURE 3** The pH dependence of the rotational correlation times $\tau_C$ (a) and $\tau_B$ (b) of Toac in Ac-Toac$^0$-$\alpha$-MSH (□ and ◨) and Ac-Toac$^0$-NDP-MSH (△ and ◭), for two different samples (open and half full symbols), in aqueous solution at 22°C.

$a_o$ values can be observed between the hormone and its more potent analogue (Figure 4), the latter presenting in average higher $a_o$ values, the structure of the two peptides in aqueous medium cannot be clearly differentiated by the ESR spectra of the Toac-bound group. Like the Trp residue in NDP-MSH, the Toac group in the analogue also feels a slightly more polar environment than that probe in $\alpha$-MSH (somewhat higher $a_o$ values, as seen in Figure 3). Though the $a_o$ values are sensitive to the titration of peptide residues, the uncertainty in the values is too large to allow for a good fitting of the curves, based on the Henderson–Hasselbalch equation. However, the dependence of the isotropic hyperfine splitting $a_o$ with the medium pH could be well fitted with a curve using the pK values obtained from the fluorescence decay global analysis (Figure 4).

## CONCLUSIONS

Even if mean lifetimes can be employed to follow an overall dependence, with pH, of fluorescence changes promoted by protonation/deprotonation of residues like Glu, His, Lys, or Tyr, the global analysis technique applied to a set of decay profiles allows the interpretation of experimental data based on conformational changes imposed to the peptides by alterations in the medium pH. In neutral pH, conformational families in A-MSH and NDP-MSH are stabilized so that Glu$^5$–Lys$^{11}$ and His$^6$–Arg$^8$ interactions are relevant, and the sizeable Trp $g^-$ and *trans* rotamers are associated to the predominant long and intermediate lifetimes. Decrease in the medium pH, starting from neutrality, causes His$^6$ protonation and the subsequent conformational changes in the peptides promotes the increase in *trans* rotamer population reflected in the raising of the intermediate lifetime preexponential factor. In more acidic pH, protonation of Glu$^5$ disrupts interaction with Lys$^{11}$, changing peptide conformation and further raising *trans* rotamer and intermediate lifetime population. In the other extreme, of high pH values, conformational changes promoted by deprotonation of Lys$^{11}$ causes similar effects in the interaction with Glu$^5$, leaning again to the predominance of *trans* rotamers.

The higher amount of $g^-$ rotamers in the neutral region is more evident in NDP-MSH and Toac-NDP-MSH, reflecting structural differences between the more potent analog and the native hormone. Apart from the small quenching of fluorescence induced by Toac, it does not greatly affect the structural arrangements of A-MSH and NDP-MSH, a result consistent with the observed conservation of biological activity of the labeled hormones.

Although considering that in a biological assay a multiplicity of factors may be involved when the medium pH changes, it would be interesting to verify how the alterations in pH affect the activity of $\alpha$-MSH and NDP-MSH. Concern must be paid to the fact that local pH near to the membrane surface and in the

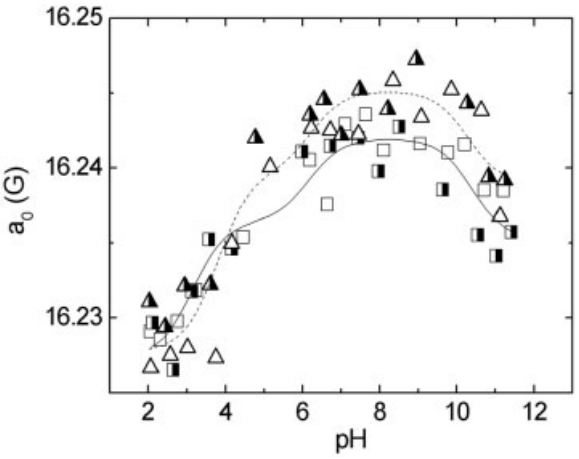


**FIGURE 4** The pH dependence of the isotropic hyperfine splitting ($a_o$) of Toac in Ac-Toac$^0$-A-MSH (□ and ◨) and Ac-Toac$^0$-NDP-MSH (△ and ◭), for two different samples (open and half full symbols), in aqueous solution at 22°C. The solid and dashed lines corresponding to Ac-Toac$^0$-$\alpha$-MSH and Ac-Toac$^0$-NDP-MSH respectively, were drawn using the fitting parameters obtained from FIGURE 1 and listed in Table III.

environment around the sites of interaction of the hormones and its receptors may be different from the bulk pH. As we observed, conformational changes are experienced by the hormones with the variation of pH, and an active conformation may be the one associated with the local pH in the interacting site. The observation that Toac may also be informative about structural changes in the peptides makes it an useful tool to investigate system without a fluorescent group or in situations where the fluorescence data are not enough to describe the system under study.

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