Fluorescence Study of Conformational Properties of Melanotropins Labeled with Aminobenzoic Acid

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ABSTRACT The native hormone α-melanocyte-stimulating hormone (α-MSH) and its more potent analog [Nle^4,D-Phe^7]α-MSH (NDP-αMSH), labeled at the amino terminal with the fluorescent aminobenzoic acid (Abz) isomers, were examined by fluorescence methods. We observed energy transfer between the tryptophan^9 residue acting as donor and Abz as acceptor, the transfer being more pronounced to the *ortho*-form of the acceptor. Within the hypothesis that different peptide conformations coexist in equilibrium during the fluorescence decay, we supposed that the intensity decay was modulated by an acceptor-donor distance distribution function $f(r)$. From the time-resolved fluorescence experimental data, we recovered the distance distribution between Abz and Trp^9, using the CONTIN program, within the framework of the Förster resonance energy transfer model. The methodology proved to be useful to provide quantitative information about conformational dynamics of melanotropins and its dependency on the solvent. In aqueous medium, α-MSH has a broad Abz-Trp^9 distance distribution, reflecting the structural flexibility of the peptide. Three different distance populations could be identified in the labeled analog NDP-αMSH in water, indicating distinct conformational states for the synthetic peptide, compared with the native hormone. Measurements in trifluoroethanol resulted in the recovery of two Abz-Trp^9 distance populations, both for the native and the analog hormones, reflecting the decrease, induced by the solvent, of the conformational states available to the peptides.

INTRODUCTION

The hormone peptide α-MSH is a linear tridecapeptide having the amino acid sequence Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, and it has been known for years as the relevant hormone regulating skin pigmentation (Sawyer et al., 1980). Evidence was found of its action also as neurotransmitter or neuromodulator in learning, memory, and attention (Jegou et al., 1993). From studies relating chemical structure and biological activity of the hormone as a pigmenting agent, it was established that the central 6–9 tetrapeptide, His-Phe-Arg-Trp, is essential for its action (Hruby et al., 1984). Exploration of the characteristics of that central region was conducted for the development of the agonist [Nle^4,D-Phe^7]α-MSH (hereafter referred to as NDP-αMSH), which in the frog skin bioassay exhibits increased potency and prolonged activity, as compared with the native hormone (Sawyer et al., 1980; Al-Obeidi et al., 1989a). The superpotency of NDP-αMSH was attributed to a β-turn conformation stabilized by a D-Phe^7 residue and a possible Glu^5-Arg^8 or Glu^5-Lys^{11} salt bridge (Sugg et al., 1988; Hruby et al., 1988; Al-Obeidi et al., 1989b).

The melanotropins interact with receptors that have been cloned and identified as pertaining to the superfamily of receptor coupled to the G-protein, and some important amino acids for the receptor-ligand interaction were identified in the extracellular loops of the receptor in human melanoma (Mountjoy et al., 1992; Chhajlani and Wikberg 1992; Gantz et al., 1994; Chhajlani et al., 1996). On the other hand, several reports indicated that the native hormone and some potent analogs show affinity for the lipid phase of model membranes (Ito et al., 1993; Macêdo et al., 1996; Souto and Ito, 2000). By fluorescence spectroscopy a correlation was observed between an increase in pigmenting activity and the strength of interaction with model membranes, and those results emphasized the importance of the lipid phase in the interaction of the hormone with its receptor in the biological membrane.

Aiming to unravel some of the molecular factors associated with melanotropin peptides, the α-MSH molecule was recently labeled (Barbosa et al., 1999) with a paramagnetic amino acid inserted between its acetyl group and the Ser^1 residue for further investigation through the electron spin resonance method. In that case, the spectroscopic probe used was the small cyclic TOAC marker introduced earlier for peptide labeling (Nakaie et al., 1981, 1983; Marchetto et al., 1993). The synthesized acetyl-TOAC^0-α-MSH was fully active in the frog skin bioassay, hence becoming the first biologically active spin-labeled peptide reported that still maintained entirely its native potency.

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Abbreviations for amino acids and nomenclature of peptide structure follow the recommendations of the IUPAC-IUB (1989, *J. Biol. Chem.* 264:668–673). Other abbreviations used: Abz, amino benzoic acid; Ac, acetyl; Boc, tert-butyloxycarbonyl; C_{18}, octadecyl; FWHM, full width at half-maximum; HPLC, high-performance liquid chromatography; MSH, melanocyte-stimulating hormone; NATA, *N*-acetyl tryptophanamide; TFE, trifluoroethanol; TOAC, 2,2,6,6-tetramethylpiperidine-1-oxyl-4-amino-4-carboxylic acid.

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Fluorescence spectroscopy also has been used in the study of melanotropins examining the emission properties of the tryptophan residue located at position 9. On the other hand, Abz is a small fluorescent molecule with size and structure comparable to TOAC and natural amino acids and has been used as an extrinsic probe for peptides. Its spectroscopic characteristics when bound to amino acids and small peptides were studied in aqueous medium (Ito et al., 1998) as well as in interaction with amphiphilic aggregates made of SDS micelles (Turchiello et al., 1998). Abz was used as a convenient donor group in peptides that are substrates for several proteolytic enzymes, forming a donor-acceptor pair with *N*-[2,4-dinitrophenyl]-ethylenediamine. That pair was employed, for example, in the investigation of proteases such as human tissue kallikrein (Chagas et al., 1995; Del Nery et al., 1995). In those works, peptides with the sequence of human kininogen that spanned the region that contains bradykinin were synthesized with Abz and *N*-[2,4-dinitrophenyl]-ethylenediamine as donor-acceptor pair and assayed as kallikrein substrates.

A novel approach is presented here for the study of α-MSH and its potent NDP-αMSH analog, both labeled at position zero of their sequences with the fluorescent Abz probe. To deeper evaluate the structural requirements for fluorescence investigation, the *ortho*-, *meta*-, and *para*-derivatives of the Abz molecule were used for labeling. We checked the occurrence of energy transfer between the Trp^9 residue acting as a donor and Abz molecule as an acceptor. In the Förster's model for a single fixed distance r between donor and acceptor, the energy transfer rate (k_T) is given by $k_T = R_o^6/\tau_d r^6$ where R_o is the Förster distance, which is dependent on the spectral overlap between donor's emission and acceptor's absorption, and τ_d is the donor's lifetime. It results from the model that the additional route for de-excitation affects the fluorescence decay of the donor, and the time evolution of the fluorescence intensity turns out to be given by

$$I(t) = I_0\, exp\left[\left(-\frac{1}{\tau_d} - \frac{R_0^6}{\tau_d r^6}\right)t\right] \qquad (1)$$

However, when the donor-acceptor distance is not unique, the decay becomes more complex, preventing the use of a single exponential decay function, as provided by the equation above, for fitting to the experimental decay data. If this is the case for labeled MSH we cannot use, for example, the steady-state intensity data to obtain a single donor-acceptor distance in the peptide. Within the hypothesis of the occurrence of different peptide conformations that coexist in equilibrium during fluorescence decay, we suppose that the intensity decay is modulated by a donor-acceptor distance distribution function $f(r)$. From the experimental decay data, it is possible to recover that distance distribution, which would reflect the flexibility of the peptide.

In the present work we report measurements of steady-state and time-resolved fluorescence spectroscopy of α-MSH and NDP-αMSH labeled with the Abz group, including the absorption and emission properties of its *o*-, *m*- and *p*-forms. We investigated the conformational flexibility of the native hormone α-MSH and its analog NDP-αMSH, through the analysis of the complex decay kinetics of the labeled peptides, using the program CONTIN to obtain the Trp^9-Abz distance distribution function $f(r)$. Such a procedure has been applied, for example, in conformational studies of peptides such as galanin (Kulinski et al., 1997) and brakykinin (Souza et al., 2000). The conformational constraints imposed by the solvent were also investigated, studying the peptides in water and in TFE.

MATERIALS AND METHODS

Peptide synthesis

The α-MSH, NDP-αMSH, and their Abz-labeled analogs were synthesized according to the Boc solid-phase method (Barany and Merrifield, 1980; Stewart and Young, 1984). The peptides were synthesized on a 0.2-mmol scale using methylbenzhydrylamine-resin as the solid support, and the following amino acid side-chain protecting groups were employed: benzyl for Ser, cyclohexyl for Glu, 2-bromobenzyloxycarbonyl for Lys, and *p*-toluenesulfonyl for His and Arg. The fluorescent *o*-, *m*-, and *p*-Abz derivatives were also protected with the N^{α}-Boc group. Optimized coupling conditions were applied for the synthesis based upon our peptidyl-resin solvation approach (Cilli et al., 1996) and using Boc-amino acid/2-(^{1}H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/1-hydroxybenzotriazole (1:1:1) components in the presence of diisopropylethylamine in *N*-methylpirrolidinone. The acetylation of the peptide N-terminal amino group was done with acetic anhydride/*N*,*N*-dimethylformamide (1:4, v/v) solution containing 0.1 ml of pyridine for 30 min. The peptides were cleaved from the resin with HF:*o*-cresol:dimethyl sulfide:ethanedithiol (8.5:0.5:0.5:0.5, v/v) solution at 0°C for 90 min. After HF evaporation, the resin was washed with ethyl acetate and dried, and the peptide was extracted into 5% acetic acid in water and lyophilized.

All peptides were purified by preparative HPLC (C_{18} column) using aqueous 0.02 M ammonium acetate (pH 5.0) and 60% acetonitrile solutions as solvents A and B, respectively (linear gradient of 30–70% B in 2 h; flow rate of 10 ml/min). After the purification, the peptides were characterized by analytical HPLC, matrix-assisted laser desorption ionization mass spectrometry and amino acid analysis.

Measurements

Stock solutions of peptides were prepared in water. Optical absorption and fluorescence measurements were made by diluting the stock solution with HEPES buffer, pH 7.4, or TFE to final peptide concentrations between 2×10^{-5} M and 4×10^{-5} M. In TFE experiments, pH was adjusted to 7.0 with addition of NaOH.

Optical absorption measurements were performed using an HP 8452 A diode array spectrophotometer. For steady-state fluorescence experiments we employed a Fluorolog 3 Jobin Yvon-Spex spectrometer. Excitation and emission slits of 1- or 2-nm bandpass were used, depending on the fluorescence intensity of the sample. Fluorescence quantum yields for tryptophan emission were determined at 23°C using NATA in pH 7.0 as a reference, taking the value 0.14 as standard. Excitation wavelength was 295 nm to avoid emission from the tyrosine residue in melanotropins. Abz emission quantum yield was determined with excitation 310 nm, using 0.60 in ethanol as standard (Melhuish, 1961). Steady-state fluorescence anisotropy was measured at wavelength emission 350 or 420 nm, with excitation 295 or 310 nm, respectively, using Glan Thompson polarizers in an

L-format configuration. The temperature was controlled using a Forma Scientific 2006 thermal bath.

Time-resolved experiments were performed using an apparatus based on the time-correlated single photon counting method. The excitation source was a Tsunami 3950 Spectra Physics titanium-sapphire laser, pumped by a 2060 Spectra Physics argon laser. The repetition rate of the 5-ps pulses was set to 400 or 800 kHz using the pulse picker 3980 Spectra Physics. The laser was tuned to give output at 930 or 888 nm, and a third harmonic generator BBO crystal (GWN-23PL Spectra Physics) gave, respectively, the 310- or 296-nm excitation pulses that were directed to an Edinburgh FL900 spectrometer. The L-format configuration of the spectrometer allowed detection of the emission at a right angle from the excitation, and for anisotropy measurements it was employed a Glan Thompson polarizer in the emission beam and a Soleil Babinet compensator in the excitation beam. The emission wavelength was selected by a monochromator, and emitted photons were detected by a refrigerated Hamamatsu R3809U microchannel plate photomultiplier. The FWHM of the instrument response function was typically 45 ps, determined with a time resolution of 6.0 ps per channel. Measurements of the peptide decays were made using a time resolution of 12 ps per channel or 6 ps per channel, depending on the presence of the acceptor. Software provided by Edinburgh Instruments was used to analyze the decay curves, and the adequacy of the multi-exponential decay fitting was judged by inspection of the plots of weighted residuals and by statistical parameters such as reduced χ^2.

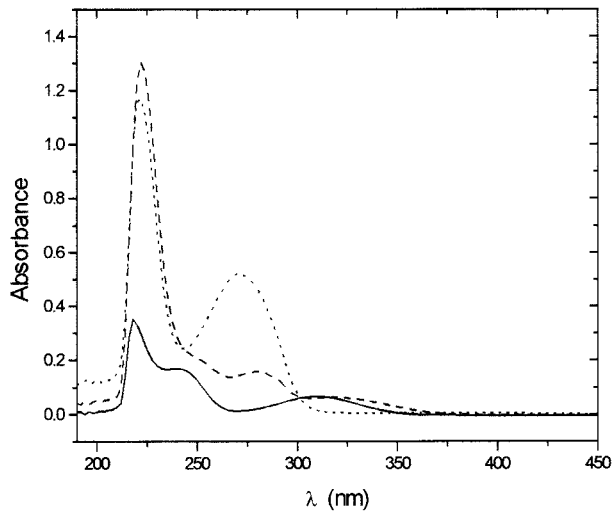

FIGURE 1 Optical absorption spectra of *o*-Abz (—) and *o*-Abz-MSH (- - -), concentration 3×10^{-5} M in HEPES 10 mM, pH 7.4, 23°C. Also shown is the spectrum of the acetylated compound Ac-*p*-Abz-MSH (· · ·) measured under the same conditions.

Computation

The fluorescence decay of the donor, characterized by its lifetime τ_d, becomes faster in the presence of the acceptor bound to the peptide. Equation 1, describing the intensity decay in the case of a fixed distance between donor and acceptor, fails to fit to the experimental decay when more than one distance is present during the time decay of the fluorophore.

If the distance distribution can be represented by a function $f(r)$, it can be recovered from the experimental data by the use of the CONTIN program (Provencher, 1982) that inverts general systems of linear algebraic equations of the type:

$$y(t_k) = \int_a^b f(r)K(r, t_k)dr + \sum_{j=1}^{N_L} L_{kj}\beta_j, \quad k = 1, \ldots N_y \qquad (2)$$

In this work, $y(t_k)$ corresponds to the experimentally observed intensity of fluorescence at the instant t_k. The distance distribution function $f(r)$ is recovered within a chosen interval for r, initially set between 5 and 45 Å, divided in N equally spaced intervals, and the integral is converted, by numerical integration, to a summation in r_j. The function inside the integral is then written as $K(r_j, t_k)$ and corresponds to Eq. 1, written for the instant t_k and deconvoluted from the instrument response function, and assumed to be valid for each distance r_j within the chosen interval.

The second term in Eq. 2 accounts for impurities that may be present contributing to the decay profile. The program recovers the parameter β_j corresponding to the contribution L_{kj} of the jth impurity to the fluorescence intensity at the instant t_k. In the analysis we also imposed the constraint of non-negativity for the distribution function. The best solution was found using the weighted least-squares method with the employment of a regularizer based on the principles of parsimony (Provencher, 1982).

The distance R_o as used in equation for the function $K(r_j, t_k)$ comes from the expression $k_T = R_o^6/\tau_d r^6$ for the energy transfer rate and was obtained from the spectral data of the donor's emission $F_d(\lambda)$ and acceptor's extinction coefficient for absorption $\epsilon_a(\lambda)$, through

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\Phi_d}{128\pi^5 n^4 N_0}\int F_d(\lambda)\varepsilon_a(\lambda)\lambda^4 d\lambda, \qquad (3)$$

where n is the refractive index of the medium, Φ_d is the quantum yield of the donor, and κ is the orientational term dependent on the relative angles between dipole moments from donor and acceptor.

RESULTS

Optical absorption

Representative optical absorption spectra of the native α-MSH labeled with Abz are presented in Fig. 1. In the near-UV region, the absorption spectrum of *o*-Abz-MSH is dominated by two main bands, around 315 nm and 280 nm. The characteristic band due to the $^1A \rightarrow L_b$ transition in *o*-Abz free in aqueous solution, centered at 310 nm, is displaced to 315 nm in *o*-Abz-MSH. That red shift was typically observed in amidated derivatives of *o*-Abz, such as *o*-Abz-NH_2, *o*-Abz-amino acids, and *o*-Abz-peptides (Ito et al., 1998; Turchiello et al., 1998). We can see in Fig. 1 that acetylation of Abz bound to α-MSH caused a decrease in the intensity of the band at 310 nm, the larger effect being observed in Ac-*p*-Abz-MSH. We obtained similar spectra for compounds having the Abz group bound to the analog NDP-αMSH, indicating that the fluorescence properties of the probe are independent of the modifications in the amino acid sequence of the native hormone that produced the more potent analog.

Optical absorption spectra were obtained in the concentration range between 1.0×10^{-5} M and 4.0×10^{-5} M, and results for the extinction coefficients at 310 nm and 280 nm are presented in Table 1. We can see that, despite the spectral displacement of the $^1A \rightarrow L_b$ transition of *o*-Abz, the extinction coefficient of *o*-Abz-MSH is almost the same as that of free *o*-Abz. Acetylation of the probe modified its electronic structure, as observed by the significant decrease of ϵ_{310} in the acetyl-Abz-MSH compounds. Major alter-

TABLE 1 Optical absorption and fluorescence parameters for labeled and non-labeled melanotropins in 10 mM HEPES, pH 7.4, at 23° C

	ε_{310} (M cm)$^{-1}$	ε_{280} (M cm)$^{-1}$	QY
o-Abz	$(2.4 \pm 0.1) \times 10^3$	$(0.7 \pm 0.1) \times 10^3$	0.6
o-Abz-MSH	$(2.3 \pm 0.1) \times 10^3$	$(5.76 \pm 0.01) \times 10^3$	0.35
Ac-*o*-Abz-MSH	$(1.0 \pm 0.1) \times 10^3$	$(5.69 \pm 0.01) \times 10^3$	0.076
Ac-*m*-Abz-MSH	$(0.44 \pm 0.04) \times 10^3$	$(5.31 \pm 0.01) \times 10^3$	0.004
Ac-*p*-Abz-MSH	$(0.41 \pm 0.02) \times 10^3$	$(15.7 \pm 0.01) \times 10^3$	0.003
o-Abz-NDP-MSH	$(2.3 \pm 0.1) \times 10^3$	$(5.57 \pm 0.02) \times 10^3$	0.41
Ac-*o*-Abz-NDP-MSH	$(0.7 \pm 0.3) \times 10^3$	$(5.70 \pm 0.02) \times 10^3$	0.012
α-MSH	$(0.10 \pm 0.01) \times 10^3$	$(5.70 \pm 0.02) \times 10^3$	0.09*
NDP-MSH	$(0.13 \pm 0.02) \times 10^3$	$(5.90 \pm 0.03) \times 10^3$	0.095*

Extinction coefficients at 310 and 280 nm were obtained from absorbance measurements in the concentration range between 1.0×10^{-5} M to 4.0×10^{-5} M. Results in the last column are for Abz fluorescence quantum yield, with excitation at 310 nm.

*Results for Trp^9 fluorescence quantum yield, with excitation at 290 nm.

ations occurred in acetyl-*p*-Abz-MSH, which presented a very low extinction coefficient at 310 nm and the masking of the 280-nm band by a more intense band located at 268 nm, revealing that the *p*-isomer has an electronic structure very different from the other Abz isomers.

The extinction coefficient of free *o*-Abz at 280 nm was significantly smaller than at 310 nm (Table 1). The same was observed in several Abz-amino acids and in Abz-peptides without Tyr or Trp (Ito et al., 1998). In the Abz-labeled melanotropins ϵ_{280} ranged from 5.6×10^3 to 5.8×10^3 (M cm)$^{-1}$, close to the values measured for non-labeled α-MSH and NDP-MSH (Table 1). Thus we can assume that the extinction coefficient measured at that wavelength in the Abz-labeled melanotropins is due to absorption by Tyr^2 and Trp^9 residues. Similar values of ϵ_{280} were obtained for non-acetylated and acetylated Abz-melanotropins (with exception of the above mentioned Ac-*p*-Abz-MSH), indicating that the binding of the acetyl group that affects the electronic structure of the Abz probe does not modify the electronic properties of the aromatic residues in the native hormone and in the analog NDP-αMSH.

Steady-state fluorescence

A broad and intense fluorescence emission band centered at 396 nm was obtained, exciting *o*-Abz in aqueous solution at 310 nm. The non-acetylated *o*-Abz-labeled melanotropins presented the emission band red-shifted to ~420 nm (Fig. 2), and decreased quantum yield (Table 1), similar to previous observations in Abz-amino acids and Abz-bradykinin (Ito et al., 1998; Souza et al., 2000). The acetylated *o*-Abz compounds also presented red-shifted emission, and the intensity was strongly reduced. The *m*- and *p*-forms of acetylated Abz bound to the melanotropins were not fluorescent with excitation at 310 nm (Fig. 2 and Table 1).

In Fig. 3 the fluorescence emission of melanotropins can be

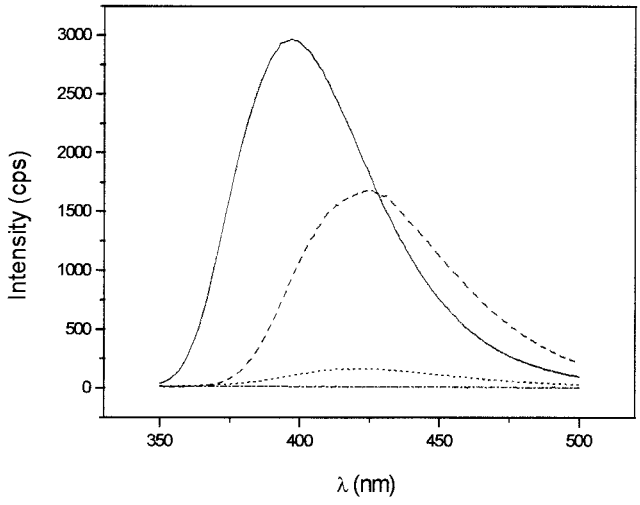

FIGURE 2 Emission fluorescence spectra of *o*-Abz (—) and *o*-Abz-MSH (— — —) concentration 3×10^{-5} M in HEPES 10 mM, pH 7.4, 23°C; excitation wavelength, 310 nm. Also shown are spectra of the acetylated compounds Ac-*o*-Abz-MSH (· · ·) and Ac-*p*-Abz-MSH (– · –), measured under the same conditions.

compared with that of *o*-Abz-labeled melanotropins excited at 290 nm. The non-labeled melanotropins in aqueous medium presented an emission maximum around 355 nm, with quantum yield around 0.10, determined using the quantum yield of 0.14 for NATA as reference. The labeled melanotropins excited at 290 nm presented an emission band at 355 nm and also a strong emission centered at 420 nm, which is absent in the spectra of non-labeled melanotropins. Excitation spectra of

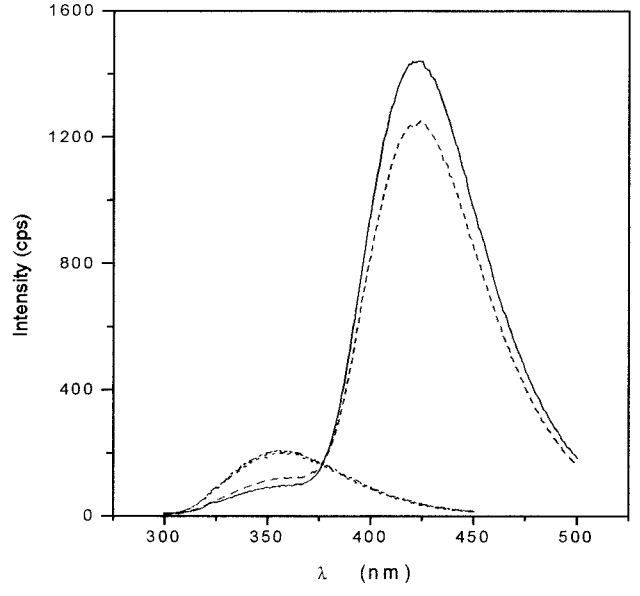

FIGURE 3 Emission fluorescence spectra of *o*-Abz-MSH (——) and *o*-Abz-NDP-MSH (— — —), concentration 3×10^{-5} M in HEPES 10 mM, pH 7.4, 23°C; excitation wavelength, 290 nm. Also shown are spectra of αMSH (· · ·) and NDP-MSH (– · –), measured under the same conditions.

TABLE 2 Lifetime (τ_i) and normalized pre-exponential factor (a_i) for fluorescence decay of *o*-Abz bound to melanotropins

	τ_1 (ns)	a_1	τ_2 (ns)	a_2	τ_3 (ns)	a_3	τ_{med} (ns)
o-Abz	8.58	1.0					8.58
o-Abz-MSH	8.32	0.684	3.58	0.316			6.81
o-Abz-NDP-MSH	8.97	0.415	4.95	0.397	0.54	0.188	5.78
Ac-*o*-Abz-MSH	7.26	0.148	1.88	0.169	0.37	0.723	4.69

Excitation was at 310 nm and emission at 420 nm. Concentration was 4.0×10^{-5} M, in HEPES buffer 10 mM, pH 7.4, at 23° C.

o-Abz free in aqueous solution indicated that there is no emission in the region around 355 nm that could be attributed to that fluorophore. Thus we can assume that in *o*-Abz-melanotropins, the fluorescence centered at 355 nm was originated from the excitation of the Trp^9 residue of the peptide, which is exposed to the aqueous environment. Fig. 3 illustrates also the most relevant result of the binding of *o*-Abz to α-MSH or NDP-αMSH: a noticeably large decrease in the emission of tryptophan fluorescence and a concomitant increase in the emission band centered at 420 nm. Part of the emission at 420 nm is due to direct excitation of *o*-Abz, but the intensity of that band in *o*-Abz-MSH and *o*-Abz-NDP-αMSH is by far higher than would be obtained if Abz fluorescence were the sole origin of the emission. The simultaneous decrease in the emission at 355 nm and the increase in the emission at 420 nm can be attributed to the energy transfer between Trp^9 acting as a donor and *o*-Abz acting as an acceptor.

Time-resolved fluorescence

The fluorescence decay kinetics of *o*-Abz in HEPES buffer, pH 7.4, examined at 400 nm with excitation at 310 nm, was adequately described by a monoexponential function, and we measured a lifetime decay of 8.58 ns (Table 2), very close to the value of 8.54 ns previously reported for *o*-Abz in water (Souza et al., 2000). The emission decay at 420 nm of *o*-Abz-MSH and *o*-Abz-NDP-αMSH excited at 310 nm presented shorter decay components in addition to the long component of free *o*-Abz (Table 2). Although some contribution from tryptophan emission can be present at 420 nm, the occurrence of multi-exponential decay in *o*-Abz peptides had already been reported, and the values obtained here are comparable to those reported for *o*-Abz-bradykinin, a labeled peptide without tryptophan (Souza et al., 2000). Acetylation of *o*-Abz-MSH decreased the mean decay lifetime, although the extent of decrease is not so pronounced as the diminution in quantum yield.

As usually found in Trp-containing peptides, the melanotropins α-MSH and NDP-αMSH in buffer, pH 7.4, presented heterogeneous decay under excitation at 296 nm, which fitted to a three-exponential function (Table 3). Excitation of Trp^9 in the *o*-Abz-labeled peptides also resulted in heterogeneous decay kinetics, and a fit to a three-exponential function for emission at 350 nm was possible, although the dispersion in the lifetimes was larger than that obtained in the fit for non-labeled peptides. Mean lifetimes $\langle\tau\rangle$ were calculated as a simple weighted average value from the individual lifetimes and the corresponding normalized pre-exponential factors, a procedure that properly accounts for the contributions from the short lifetimes (Kulinski et al., 1997). It is clearly seen in Table 3 that the mean lifetimes for peptides bound to *o*-Abz decreased, a result that can be visualized in Fig. 4, which illustrates the faster decay of *o*-Abz-MSH compared with the native hormone. These results also indicate the occurrence of energy transfer between Trp^9 and *o*-Abz in the labeled peptides.

Decay curves were obtained at different emission wavelengths. With excitation at 310 nm, the decay profiles corresponding to *o*-Abz emission were practically insensitive to changes in the emission wavelength. On the other hand, excitation of Trp^9 resulted in longer decay profiles for longer wavelengths. The decay times were nearly constant across the Trp^9 fluorescence spectrum, and a long lifetime component appeared in the spectral region corresponding to the *o*-Abz emission. The relative contribution of the three lifetimes in different wavelengths were determined by performing a global analysis of data sets of decays measured at different emission wavelengths in the range from 340 to 400

TABLE 3 Lifetime (τ_i) and normalized pre-exponential factor (a_i) for Trp^9 fluorescence decay in labeled and non-labeled melanotropins, with excitation at 296 nm and emission at 360 nm Concentration was 4.0×10^{-5} M, in HEPES buffer 10 mM, pH 7.4, 23° C. Results in the last two rows are for measurements in TFE

	τ_1 (ns)	a_1	τ_2 (ns)	a_2	τ_3 (ns)	a_3	$\langle\tau\rangle$ (ns)
MSH	3.41	0.307	2.02	0.349	0.472	0.344	1.91
NDP-MSH	3.97	0.178	2.33	0.538	0.570	0.284	2.12
o-Abz-MSH	2.62	0.074	1.22	0.475	0.307	0.451	0.91
o-Abz-NDP-MSH	2.86	0.029	1.15	0.451	0.300	0.520	0.76
Ac-*o*-Abz-MSH	3.63	0.165	2.11	0.351	0.421	0.484	1.54
MSH/TFE	4.51	0.033	1.50	0.364	0.59	0.603	1.05
MSH1/TFE	5.8	0.045	1.85	0.357	0.58	0.598	1.27

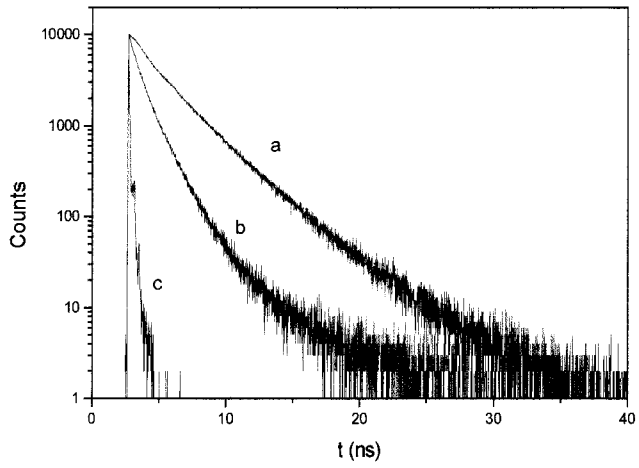

FIGURE 4 Fluorescence decay curves for α-MSH (*a*) and *o*-Abz-MSH (*b*) in HEPES, pH 7.4. Excitation wavelength, 296 nm; emission wavelength, 350 nm. Curve *c* is the instrument response decay curve.

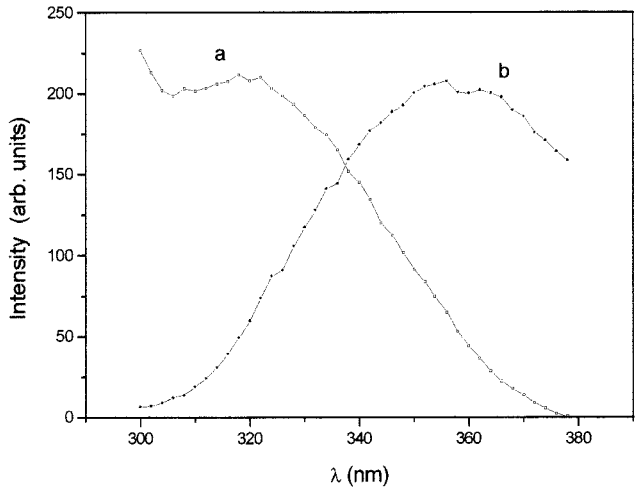

FIGURE 5 Spectral superposition of o-Abz optical absorption (*a*) and tryptophan fluorescence emission (*b*) in HEPES, pH 7.4.

nm. The decay-associated spectra for α-MSH and some analogs have been extensively examined in water and in the presence of model membranes (Ito et al., 1993; Souto and Ito, 2000), and the relative contributions of each decay component were dependent on the environment around the Trp residue. In the present work it becomes evident that in *o*-Abz-MSH and *o*-Abz-NDP-αMSH there is, in addition to the three lifetimes from tryptophan, a contribution from a long-lifetime component of ~7.5 ns, which predominates in longer wavelengths and is characteristic of the *o*-Abz emission (Table 4). The presence of this component due to *o*-Abz emission, coming from the excitation of Trp^9 at 296 nm, provides additional evidence for the energy transfer between that residue and the extrinsic fluorophore.

Energy transfer

Donor-acceptor pair

As shown above, in *o*-Abz-labeled melanotropins there is energy transfer between the Trp^9 residue and the *o*-Abz group. In the Förster model, the rate of transfer depends on the overlap integral *J* calculated in the spectral range where the fluorescence emission of the donor superimposes to the optical absorption of the acceptor (Fig. 5). Values of *J* obtained from Trp^9 fluorescence in non-labeled α-MSH and NDP-αMSH and *o*-Abz absorption in labeled peptides were used in Eq. 3 for the calculation of the Förster distance R_o, and the results are presented in Table 5. Calculations were performed from data obtained in buffer pH 7.4 and TFE.

In the calculation of R_o we employed the values of 1.334 for the index of refraction in water and 1.291 in TFE. Quantum yields for the donor in the absence of acceptor were obtained from experiments with non-labeled MSH and NDP-αMSH, and the values were 0.090 and 0.095, respectively, in water and 0.060 and 0.057, respectively, in TFE. We used the value 2/3 for the orientational factor κ^2, assuming fast movement of the donor and acceptor (Dale and Eisinger, 1974) based on anisotropy decay data as described in the following section.

Rotational correlation times

The anisotropy decays of tryptophan in labeled and non-labeled melanotropins were measured, as illustrated in Fig. 6, and two rotational correlation times were necessary to fit the experimental data (Table 6). The short component, on

TABLE 4 Results from global analysis of fluorescence decays measured at several emission wavelengths, with excitation at 296 nm

	o-Abz-MSH				*o*-Abz-NDP-MSH			
	7.17 ± 0.70	2.6 ns	1.20 ns	0.30 ns	7.62 ± 0.30	2.86 ns	1.15 ns	0.30 ns
345	0.000 (0.3)	0.018 (21.5)	0.113 (63.4)	0.103 (14.4)	0.000 (0.2)	0.007 (10.9)	0.104 (69.0)	0.115 (19.9)
350	0.000 (0.9)	0.019 (22.4)	0.110 (62.0)	0.101 (14.5)	0.001 (4.2)	0.009 (13.1)	0.106 (65.0)	0.111 (14.5)
360	0.001 (3.6)	0.019 (22.3)	0.113 (60.6)	0.101 (13.5)	0.045 (65.5)	0.026 (14.0)	0.073 (16.2)	0.076 (4.4)
380	0.043 (61.5)	0.027 (14.0)	0.082 (19.8)	0.076 (4.6)	0.101 (83.3)	0.037 (17.4)	0.033 (4.1)	0.036 (1.2)
400	0.115 (87.9)	0.016 (12.1)			0.111 (88.1)	0.015 (11.9)		

Numbers in the columns are normalized pre-exponential factors and in parentheses are percentile contribution to total fluorescence.

TABLE 5 Parameters for energy transfer between the Trp[9]-*o*-Abz pair in melanotropins in HEPES buffer, pH 7.4, and in TFE, pH 7.0

	n	ϕ	J ($M^{-1}cm^3$)	R_o (Å)
o-Abz-MSH in buffer	1.334	0.090	1.25×10^{-15}	16.6
o-Abz-NDP-MSH in buffer	1.334	0.095	1.06×10^{-15}	16.3
o-Abz-MSH in TFE	1.291	0.060	1.80×10^{-15}	16.8
o-Abz-NDP-MSH in TFE	1.291	0.057	1.06×10^{-15}	17.1

the order of 100 ps, can be ascribed to local tryptophan motion, and the long correlation time originates from the overall tumbling of the whole peptide. The higher viscosity of TFE compared with the buffer leads to the increase in the long rotational correlation time whereas the short correlation time is decreased. Using the anisotropy decay parameters it is possible to estimate the order parameter (S) and the related wobbling cone (θ_o) (Kulinski et al., 1997), and the values obtained indicated that the restriction to the motion of the Trp[9] is comparable to that reported for Trp[2] in the galanin peptide (Kulinski et al., 1997). In the last row of Table 6 we present results for the anisotropy decay of the probe *o*-Abz in NDP-αMSH. A very short component, within the limit of detection of the instrument, can be identified, together with a longer rotational correlation time. The result is comparable to that obtained previously for bradykinin labeled with *o*-Abz, indicating a fast movement of the group bound to the peptide. An estimate for the error in the energy transfer rate due to the use of the value 2/3 for the orientational factor was made by Kulinski et al. (1997). From anisotropy decay data the authors have shown that in the galanin peptide labeled with dansyl or dinitrophenyl groups as acceptors of the energy transferred from the tryptophan residue, the characteristic deviations in the results of R_o amounted to ~20%. Based on our results we also estimate the same deviations in the calculated R_o values.

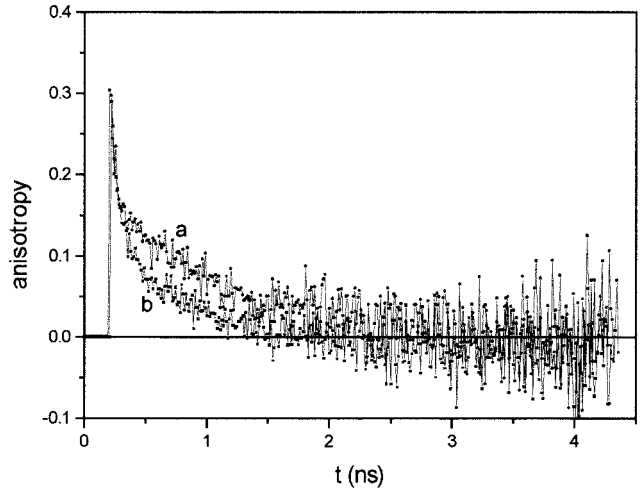

FIGURE 6 Anisotropy decay of o-Abz-MSH in TFE solution pH 7.0 (*a*) and in HEPES pH 7.4 (*b*). Excitation, 296 nm; emission, 350 nm.

Distance distribution function in buffer

In the time-resolved experiments we observed that the fluorescence decay of the peptides containing the Trp[9]-*o*-Abz pair was faster than that presented by the non-labeled peptide, as illustrated in Fig. 4, and the mean lifetimes decreased from the nanosecond to the sub-nanosecond range. No attempts were made to find donor-acceptor distance from steady-state intensity data, for the time-resolved results evinced the inadequacy of the assumption of a single distance between the donor-acceptor pair.

Equation 2 was then used, trying to fit the experimental decay curves to the distance distribution model, to get a distribution function $f(r)$ without any a priori assumption concerning the distances r or the shape of the distribution curves. We employed the CONTIN program (Provencher, 1982) for the analysis of the experimental data, including fluorescence decay parameters needed to account for the possible presence of contaminants such as free fluorescent probe. Quality of the fit was judged by the statistical parameter χ^2 and by plots of residues. Typical decay data, plotted with the corresponding fitted curve, are illustrated in Fig. 7 for *o*-Abz-MSH in buffer, pH 7.4. The function $f(r)$ resulting from the fit is presented in Fig. 8 *a*, showing that the distances between *o*-Abz and Trp[9] within the labeled peptide were distributed among a broad interval with maximum at 15.8 Å and FWHM of 5.7 Å. The procedure was repeated for the potent analog NDP-αMSH labeled with *o*-Abz. In that homolog, recovered distance distributions presented three populations in buffer, with the peak positions in 8.0, 13.0, and 16.0 Å and FWHM for each peak of ~1.5 Å.

Distance distribution in TFE

We also examined the peptides in TFE, observing the same general trend: strong quenching of fluorescence of the donor due to the presence of the acceptor group *o*-Abz bound to the N-terminal, with corresponding fastening of the fluorescence emission decay. The resulting distance distributions recovered using the program CONTIN are illustrated in Fig. 8 *b*. We can observe that in TFE the labeled native hormone presented two distance populations between donor and acceptor, centered at 11 and 17 Å, indicating equilibrium stabilization between only two main conformations. In the labeled analog *o*-Abz-NDP-αMSH, the three peaks for the distance distribution in buffer merged to two peaks in TFE, located at 13 and 15.5 Å.

DISCUSSION AND CONCLUSION

o-Abz bound to melanotropins maintains its main electronic properties, particularly a high fluorescence quantum yield.

TABLE 6 Rotational correlation times (ϕ_i), partial anisotropies (b_i), order parameter (S), and wobbling cone angles (θ_o) for Trp[9] in non-labeled and labeled melanotropins in HEPES, pH 7.4, at 23° C and in TFE

	ϕ_1 (ps)	ϕ_2 (ns)	b_1	b_2	S	θ_o
α-MSH	69 ± 24	0.670 ± 0.030	0.048	0.101	0.82	28.7
NDP-MSH	89 ± 25	0.780 ± 0.030	0.069	0.130	0.81	30.0
o-Abz-MSH	120 ± 20	0.700 ± 0.050	0.116	0.127	0.72	36.7
o-Abz-NDP-MSH	95 ± 18	0.828 ± 0.072	0.089	0.107	0.74	35.5
α-MSH/TFE	110 ± 30	1.75 ± 0.18	0.122	0.078	0.62	43.6
o-Abz-MSH/TFE	38 ± 12	1.13 ± 0.09	0.124	0.165	0.76	34.2
o-Abz-NDP-MSH/TFE	47 ± 17	1.12 ± 0.10	0.085	0.154	0.80	30.5
o-Abz-NDP-MSH*	10 ± 12	0.51 ± 0.20	0.2	0.01	0.22	70.8

Excitation, 297 nm; emission, 355 nm.
*Last row of data is for *o*-Abz excitation (305 nm), with emission at 400 nm.

Excitation can be done above 320 nm without exciting Trp[9] or Tyr[2] residues, giving strong emission in the spectral blue region. This result raises the possibility that labeling the peptide with *o*-Abz could be useful to visualize interactions between the hormone and cell components, because few natural chromophores absorb in that spectral region. For example, interaction between labeled melanotropins and membrane receptors in cells could be directly observed by selective excitation of *o*-Abz and using fluorescence microscopy methods to detect the emission above 420 nm in the blue region of the spectrum. We also verified that acetylated *o*-Abz, *m*-Abz, and *p*-Abz have low fluorescence and are not adequate to that purpose.

Spectral superposition between *o*-Abz absorption and Trp[9] fluorescence emission leads to Förster resonance energy transfer. In labeled melanotropins the emission from excited Trp[9] is significantly reduced compared with non-labeled peptides. We examined the conformational dynamics of the melanotropins from the time-resolved data for fluorescence intensity and anisotropy decay, assuming that the donor-acceptor distance is not constant during the lifetime of the excited state of tryptophan. Using the CONTIN program we recovered the distance distribution between *o*-Abz and Trp[9], assuming the validity of the Förster model for any given fixed distance and estimating donor acceptor distances within 20% uncertainty for the Förster distance R_o. The number of peaks in the recovered distributions is the minimum possible for a good fit of the data, evaluated through the χ^2 parameter and the residual distribution. The short distances obtained for Abz-NDP-αMSH are near to the lowest possible that can be recovered by Förster resonance energy transfer in the present case, where R_o is ~16 Å. Even if larger uncertainties should be affecting that short distance, the fit of the experimental data, which were obtained with a time resolution of 6 or 12 ps/channel, required the presence of a population with distance below 10 nm.

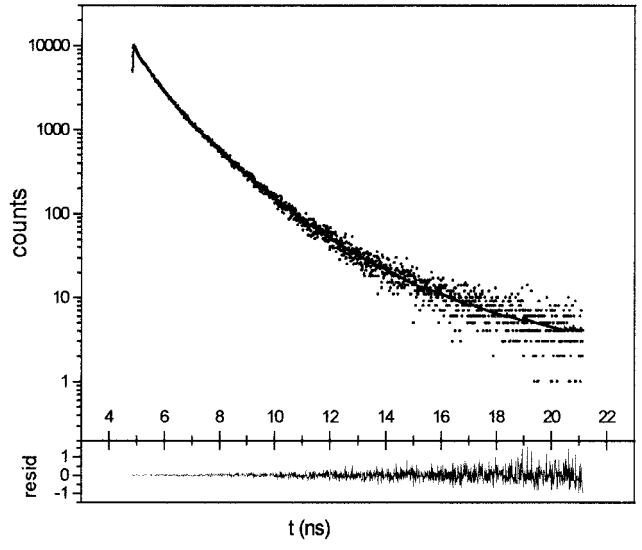

FIGURE 7 Typical decay curve (— — —) for o-Abz-MSH in HEPES pH 7.4 and fit (——) to the distance distribution model using the CONTIN program. Also shown is the plot of residuals for the fit.

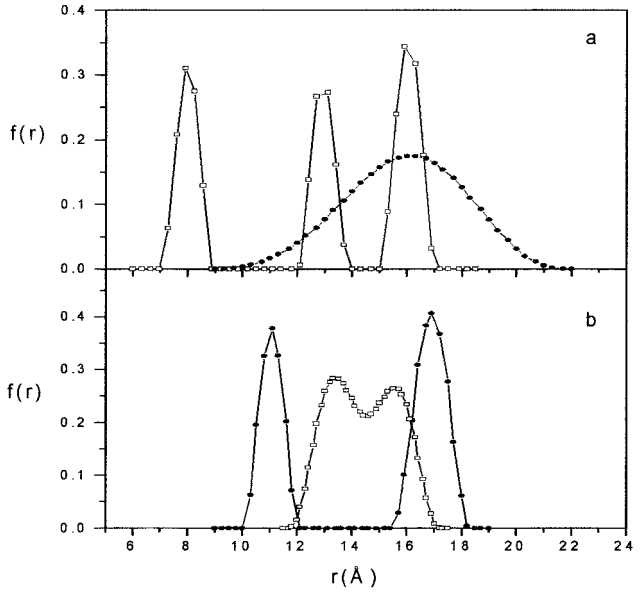

FIGURE 8 (*a*) Distance distribution recovered from the fluorescence decay profile for *o*-Abz-MSH (●) and *o*-Abz-NDP-MSH (□) in HEPES pH 7.4; (*b*) Distance distribution for *o*-Abz-MSH (●) and *o*-Abz-NDP-MSH (□) in TFE.

Recently, Pascutti et al. (1999) reported results of molecular dynamics simulation in a medium of high dielectric constant, showing that over more than a 10-ns simulation, the native hormone α-MSH presented a flexible structure, without stabilization of any preferential conformation. It is possible that there are interconversions among several available conformational states with small energy separation, as suggested by energy minimization calculations performed in the gas phase (Jacchieri and Ito, 1995). A conformational search for α-MSH structures in aqueous solution using a potential of mean force to model the effects of solvation was reported by Prabhu et al. (1999a). Lowest-energy structures presented a reverse turn in the central region of the peptide containing the message sequence His^6, Phe^7, Arg^8, Trp^9. In those families, varying degrees of interaction between side chains are present, resulting in different distances between the residues.

The distance distribution function recovered from the time-resolved fluorescence data of *o*-Abz-MSH in buffer revealed a broad distance distribution between *o*-Abz and Trp^9, which could reflect both the structural flexibility of the peptide as the distinct inter-residual distances corresponding to different conformational families. In another paper, Prabhu et al. (1999b) examined the dynamics of α-MSH over a time scale of 100 ns in aqueous environment at room temperature, using Langevin dynamics on a potential of mean force, treated with the integral equation theory. An initial end-to-end distance of 41 Å corresponding to extended structure decreased and remained between 19 and 33 Å. The message region presented a reverse turn that was conformationally more rigid than the region near the N-terminal. The larger flexibility of the N-terminal region resulted in the relatively large end-to-end distances and in distances between 15 and 20 Å for the side chains Tyr^2 to Trp^9 separation. The results that we obtained for the Abz-Trp^9 distances in α-MSH are thus consistent with those structural simulations for the peptide in aqueous medium.

The modified analog NDP-αMSH has increased potency, and NMR results suggested that D-Phe^7 substitution could stabilize some type of β-like structure (Hruby et al., 1988), although no β structure could be obviously detected in CD experiments. The lowest-energy structure from the simulation reported by Prabhu et al. (1999a) is well folded, with stacking of the Phe^7 phenyl ring and the Trp^9 indole ring, and the folded structure is reflected in short distances between Tyr^2 and Trp^9 side chains. Our results for the distance distribution between Abz and Trp^9 in Abz-NDP-αMSH indicated the occurrence of relatively compact conformational states in the modified hormone, compared with the native one. Instead of the broad distribution obtained for α-MSH, three different distance populations could be identified, with shorter Abz-Trp^9 distances (Fig. 8 *a*). The proposed turned conformation induced by D-Phe^7 could account for the occurrence of a short Abz-Trp^9 distance population, in dynamic equilibrium with longer distance populations.

Peptide secondary structure is sensitive to solvent effects. In TFE, peptides such as galanin and α-MSH have increased content of α-helical structure (Kulinski et al., 1997; Biaggi et al., 1997) and bradykinin present stabilization of β-turn conformations, either in the C-terminal region or in the N-terminal region (Cann et al., 1994; Kotovych et al., 1998). Mukhopadhyay and Basak (1998) observed by CD spectroscopy changes in α-MSH secondary structure at varying percentages of TFE. The spectral modifications were interpreted as resulting from a decreasing proportion of peptides in the random coil state and increasing proportion in the folded state. Our measurements performed in *o*-Abz-MSH in TFE resulted in the recovery of two Abz-Trp^9 distance populations, reflecting the conformational restrictions imposed by the solvent: instead of the broad distribution recovered for *o*-Abz-MSH in aqueous medium, we obtained two populations in TFE, and the increasing proportion of the folded state is represented by the recovery of a short distance population. On the other hand, the three distance populations obtained for the labeled analog *o*-Abz-NDP-αMSH in water merged to two in TFE, indicating a decrease in the conformational states available to the peptide and the stabilization of two conformations centered in distances separated by ~3 Å only. In the CD work on melanotropins by Biaggi et al. (1997), it was reported that changing the solvent from water to TFE produced modifications in both α-MSH and NDP-αMSH spectra, but the secondary structure of the hormones were not the same. In this work, we observed that, although different, the Abz-Trp^9 distance distributions recovered for *o*-Abz-MSH and *o*-Abz-NDP-αMSH in TFE have similar features.

Previous time-resolved fluorescence results (Ito et al., 1993; Souto and Ito, 2000) suggested conformational changes when both native and synthetic hormones move from the aqueous medium to the lipid phase of membrane models. Such changes were also observed in molecular dynamics simulations of the native hormone in the presence of a model lipid-water interface (Pascutti et al., 1999). The present results indicated that the more potent analog has folded conformations stabilized in aqueous medium, instead of the characteristic flexibility of the native hormone. Furthermore, it was also observed that in the less polar solvent TFE, both native and analog hormones present two distance populations, a result that gives support to the qualitative indications of similarity in the conformation of the peptides in nonpolar environment, as mentioned above. The folded conformation induced by the less polar solvent, and already present in the analog NDP-αMSH, can be important for the biological activity regulated by the interaction with the membrane receptor that has local regions of different polarity. The methodology for the recovery of distribution distances from time-resolved fluorescence data, in the framework of the Förster resonance energy transfer model, was proved useful to provide quantitative information about conformational dynamics of melanotropins and its dependency on the solvent. The determination of intramolecular

donor-acceptor distances within peptides gives important information complementary to other spectroscopic techniques, providing distance constraints that can be used for structure determination and correlation with hormone biological activity.

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