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INTERACTION OF THE PEPTIDE HORMONE ADRENOCORTICOTROPIN, ACTH(1-24), WITH A MEMBRANE MODEL SYSTEM: A FLUORESCENCE STUDY*

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Abstract—The peptide hormone adrenocorticotropin and a related peptide were studied in solution and in interaction with a model system of membranes (small unilamellar vesicles of dipalmitoylphosphatidylcholine and 17% dimyristoylphosphatidylglycerol) *via* fluorescence spectroscopy. In aqueous solution, intramolecular distances between the fluorescent residues R(Tyr^2–Trp^9) = 9.2 Å and R(Trp^9–Tyr^{23}) ≥ 18 Å were obtained, in agreement with molecular models.

Interaction of the peptide with the negatively charged membrane is evident from the alteration of the Trp photophysical parameters (quantum yield, fluorescence spectra and anisotropy), with a partition constant between the lipidic and aqueous phase of $K_p = 1–2 \times 10^3$.

The existence of two populations of Trp in the membrane, which are distinctly accessed by acrylamide, was concluded from the tryptophan fluorescence quenching study; the two fractions are located near the membrane interface as inferred from its fluorescence quenching by the 5-doxylstearate and 16-doxylstearate lipophilic quenchers. This result is further supported by energy transfer experiments to the 3-(9-anthroyloxyl)stearic acid and 12-(9-anthroyloxyl)stearic acid probes.

INTRODUCTION

Adrenocorticotropin tetracosapeptide (ACTH[1–24])‡ is a very potent synthetic fragment of the native hormone, ACTH(1–39),[1] and it mainly affects protein phosphorylation and adenylcyclase activity.[2]

In order to elucidate its structure–function relationship, studies of this peptide were carried out in aqueous solutions using photophysical techniques,[3–6] but information on its interaction with membranes received less attention; an infrared spectroscopy study (infrared attenuated total reflectance [IR-ATR]) produced evidence of its incorporation in dry or wet membranes,[7] and its binding to negatively charged lipids was also reported.[2]

ACTH(1–24) has three fluorescent amino acids, a single tryptophan (Trp^9) and two tyrosine residues (Tyr^2 and Tyr^{23}). It is the purpose of this work to report information on ACTH(1–24) structure (distances) in aqueous solution and when interacting with a model system of membranes (small unilamellar vesicles [SUV] of dipalmitoylphosphatidylcholine [DPPC] + 17% dimyristoylphosphatidylglycerol [DMPG]), namely its incorporation, location and dynamics, using fluorescence techniques. A concomitant study of the related peptide [Phe^2][Nle^4]ACTH(1–24), in which the Tyr^2 is replaced by a phenylalanine residue, was also carried out.

MATERIALS AND METHODS

Chemicals. The ACTH(1–24) (91% pure) was from Sigma Chemical Co. (St. Louis, MO) and was not purified further; the related peptide [Phe^2][Nle^4]ACTH(1–24) (95% pure) was obtained from Neosystem (Strasbourg, France). The lipids DPPC and DMPG were from Sigma and Aldrich Chemical Co. (Milwaukee, WI), respectively, and used as received. The fluorescent probes 3-(9-anthroyloxyl)stearic acid (3-AS) and 12-AS and the spin probes 5-doxylstearate (5-NS) and 16-NS were from Molecular Probes Inc. (Eugene, OR). Spectroscopic grade solvents, tryptophan and tyrosine were from Merck (Darmstadt, Germany) and the buffer 2-(*N*-morpholino)ethanesulfonic acid (MES) was obtained from Fluka (Buchs, Switzerland).

Preparation of phospholipid vesicles. A film of lipid obtained from evaporation of a chloroform solution (30 m*M*, DPPC + 17% DMPG) was kept under vacuum for more than 6 h and then solubilized in buffer, by vortexing and warming the solution above the phase transition temperature. These multilamellar vesicles (MLV) were disrupted to SUV by sonication (Branson, 40 W), until no significant decrease in the scatter intensity of the suspension was observed (30 min). The vesicles were annealed for 10 min at 50°C, to decrease structural defects in the bilayer, which would induce its fusion, and then submitted to low-speed centrifugation to separate titanium particles arising from the sonication.

The peptide hormone ACTH, the related peptide, and/or membrane probes *n*-NS and *n*-AS were added to the lipid solutions from a stock solution in MES buffer or in ethanol. For the highest concentration of ethanol used, 2% (vol/vol), no alteration in the bilayer structure is reported,[8] and no change in the intensity of light scattered by the vesicles was found. The incubation time in the dark for incorporation was 2 h. A steady intensity of fluorescence was observed in all cases.

Absorption and fluorescence measurements. Absorption spectra were recorded on a Perkin-Elmer Lambda 15 spectrophotometer, using 0.5 cm cuvettes.

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‡*Abbreviations:* ACTH, adrenocorticotropin; CD, circular dichroism; DMPG, dimyristoylphosphatidylglycerol; DPPC, dipalmitoylphosphatidylcholine; IR-ATR, infrared attenuated total reflectance; MES, 2-(*N*-morpholino)ethanesulfonic acid; MLV, multilamellar vesicles; *n*-AS, *n*-(9-anthroyloxyl)stearic acid; *n*-NS, *n*-doxylstearate; Nle, norleucine; SUV, small unilamellar vesicles.

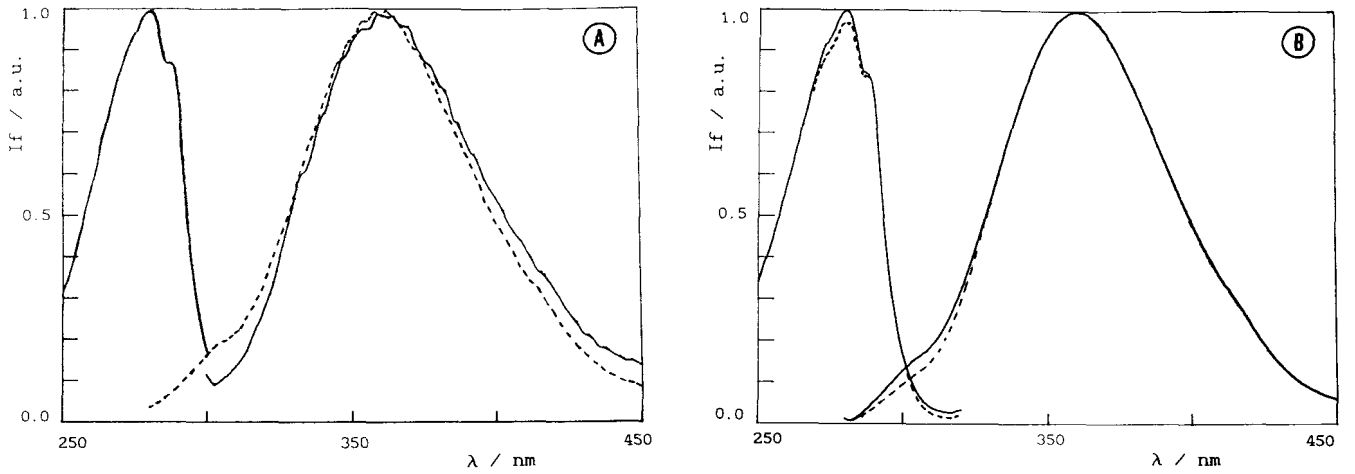

Figure 1. (A) Corrected excitation (λ_{em} = 360 nm) and emission (– – –, λ_{exc} = 275 nm; ——, λ_{exc} = 297 nm) fluorescence spectra of ACTH in aqueous solution. (B) Corrected excitation (λ_{em} = 360 nm) and emission (λ_{exc} = 275 nm) fluorescence spectra of ACTH (——) and related peptide (– – –).

Steady-state excitation and emission spectra were carried out using 0.5 cm × 0.5 cm cuvettes in a Spex F112 A Fluorolog spectrofluorometer with a double emission monochromator and a thermostatting unit. Excitation and emission bandwidths were 4.5 nm in most measurements. Correction of excitation and emission spectra were performed using a rhodamine-B quantum counter solution and a standard lamp, respectively.[9]

All the fluorescence measurements were carried out in a right-angle geometry, and the eventual artifacts arising in a steady-state experimental approach were taken into account.

Quantum yields of Trp in ACTH and in the related peptide were determined *vs* Trp in water (Φ_F = 0.16, 20°C,[10] this value being independent of the excitation wavelength[11]). In aqueous solution the emission spectra of the amino acid tryptophan and of the Trp residue in the peptide are very similar, and quantum yield was measured at the maximum fluorescence emission intensity, λ = 360 nm. Fluorescence measurements in lipid solutions were obtained in a magic angle arrangement.

Intramolecular energy transfer efficiency E_{ET}, from Tyr to Trp residues was determined from the dependence of peptide quantum yield (measured at the Trp maximum emission wavelength) on the excitation wavelength[3] (Eq. 1); $\Phi_F(\lambda_{exc})$ is the peptide quantum yield at excitation wavelength λ_{exc}, and $f_{Tyr}(\lambda_{exc})$ and f_{Trp} (λ_{exc}) are the fractions of light absorbed at λ_{exc} by Trp and Tyr residues, respectively.

$$\Phi_F(\lambda_{exc}) = \Phi_F(297 \text{ nm})[f_{Trp}(\lambda_{exc}) + f_{Tyr}(\lambda_{exc})E_{ET}] \quad (1)$$

The value of Φ_{ACTH}(297 nm) is identical to Φ_{Trp}, as at this wavelength the Tyr residues do not absorb (f_{Tyr}[297 nm] = 0). The light fraction absorbed by the Tyr residues in the two peptides was determined from the absorption spectra of the two amino acids in buffer, considering f_{Tyr}(275 nm) = 0.347.[3] In this way it was assumed that the absorption spectra of these chromophores in aqueous solution and in the peptide structure are identical.

The peptide concentration in the energy transfer experiments was 2×10^{-5} *M*, as determined from the absorption spectrum at 280 nm, assuming that ε is the addition of the amino acids' absorption, (ε[Trp] = 5309 M^{-1} cm^{-1} and ε[Tyr] = 1122 M^{-1} cm^{-1}).

The critical radius of energy transfer, R_o, was evaluated from Förster's formula, rewritten as[12]

$$R_o = 0.02108[k^2\Phi_D n^{-4} \int_o^\infty I_f(\lambda)\epsilon(\lambda)\lambda^4 \, d\lambda]^{1/6} \quad (2)$$

with R_o in nm, where k^2 is the orientational factor, Φ_D is the donor quantum yield in the absence of the acceptor, n is the refractive index of the medium, $I_f(\lambda)$ is the normalized fluorescence spectrum, $\epsilon(\lambda)$ is the molar absorption coefficient in M^{-1} cm^{-1} and λ is in nm. In the above expression the orientational factor corresponding to a dynamic isotropic (or pseudoisotropic) regime of transfer, k^2 = ⅔, was assumed.

Fluorescence anisotropies were determined from Eq. (3), where I_{vv} and I_{vh} are fluorescence intensities the two subscripts indicating the orientation of the excitation and emission polarizers, respectively, and $G = I_{hv}/I_{hh}$ is the instrumental factor. Glan-Thompson polarizers were used, the excitation and emission wavelengths were 290 nm and 350 nm, respectively, and "blank" intensities were taken into account.

$$r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh}) \quad (3)$$

The pH of the solutions was measured in a CRISON microph 2001.

RESULTS

The fluorescence emission spectrum (λ_{exc} = 297 nm) of ACTH in aqueous solution is dominated by the single Trp residue (Fig. 1A); when excited at 275 nm the emission spectrum shows an inflection near 300 nm, and the emission maximum underwent a small blue shift of 2 nm. The related peptide excitation and emission spectra (Fig. 1B) are very similar to that of ACTH, although the inflection near 300 nm is now less intense.

Upon addition of ACTH or related peptide to the lipidic solution (DPPC + 17% DMPG) the light scatter largely increases and the emission spectrum of the peptides undergoes a significant blue shift as shown in Fig. 2 for ACTH. No such hypsochromic effect was observed in the absence of DMPG.

The quantum yield of Trp increases upon interaction of the peptides with the membrane, as shown in Table 1. It should be stressed that the values of Φ in the presence of lipid can be biased due to the contribution of light scatter.

The dependence of the peptide's quantum yield on excitation wavelength (measured at the Trp maximum emission wavelength) was used to determine the energy transfer efficiency E_{ET}, from the Tyr residues to Trp from Eq. (1), and the results are shown in Fig. 3. The intramolecular energy transfer in ACTH is quite efficient: E_{ET} = 0.3, this being a mean value of the two tyrosine residues that can participate as donors; for the related peptide where Tyr[2] is absent, E_{ET} ≃ 0, *i.e.* no energy transfer from Tyr[23] is operative.

The interaction of both peptides with the lipid is also shown from the variation of fluorescence anisotropy as depicted in Fig. 4; for both systems an increase from its value in aqueous

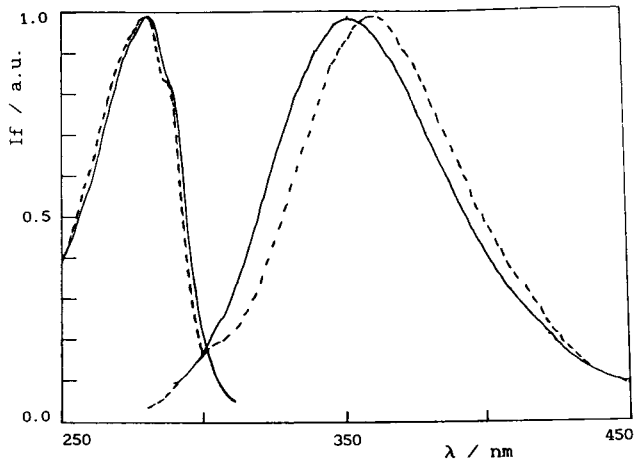

Figure 2. Corrected excitation (λ_{em} = 360 nm) and emission (λ_{exc} = 275 nm) fluorescence spectra of ACTH in aqueous solution (– – –) and in interaction with the membrane model system (——) (SUV of DPPC + 17% DMPG, lipid concentration [L] = 1.1×10^{-3} *M*).

solution $r_w \simeq 0.02$ is observed upon increasing the lipid concentration.

A study of ACTH and the related peptide fluorescence quenching by acrylamide was carried out in the presence and absence of lipid. In aqueous solutions linear Stern-Volmer relationships were obtained (Fig. 5A), the slope (Stern-Volmer constant, K_{sv}) for ACTH, $K_{sv} = 11.2\ M^{-1}$, being similar to the one for the related peptide $K_{sv} = 9.0\ M^{-1}$. In the presence of lipid, the Stern-Volmer plots are nonlinear, presenting a downward curvature (Fig. 5B). This is an indication that a fraction of fluorophores is not accessible to the quencher. This effect is more pronounced for ACTH than for the related peptide.

The Stern-Volmer plot of ACTH fluorescence quenching by 5-NS and 16-NS shows an upward curvature compatible with the existence of both dynamic and static quenching (Fig. 6), and in addition fluorescence quenching by 5-NS is more efficient than by 16-NS. In these plots the fluorescence intensity was corrected for the fraction of peptide in aqueous solution, $f_w = 0.1$ (see Discussion), as these fluorophores are not accessible to the *n*-NS hydrophobic quenchers. The concentration of *n*-NS in the membrane was determined assuming a partition constant $K_p = 1.257 \times 10^4$ for 5-NS and $K_p = 3.340 \times 10^3$ for 16-NS.[13]

The fluorescence quenching of ACTH by energy transfer

Table 1. Quantum yield, Φ, of ACTH and related peptide in aqueous solution (pH = 7, 10 m*M* MES buffer) and in interaction with a membrane model system (SUV of DPPC + 17% DMPG) (lipid concentration 1.1×10^{-3} *M*)

	Φ (275 nm)	Φ (297 nm)
ACTH		
Aqueous solution	0.11	0.16
Membrane model system	0.16	0.30
Related peptide		
Aqueous solution	0.12	0.16
Membrane model system	0.17	0.32

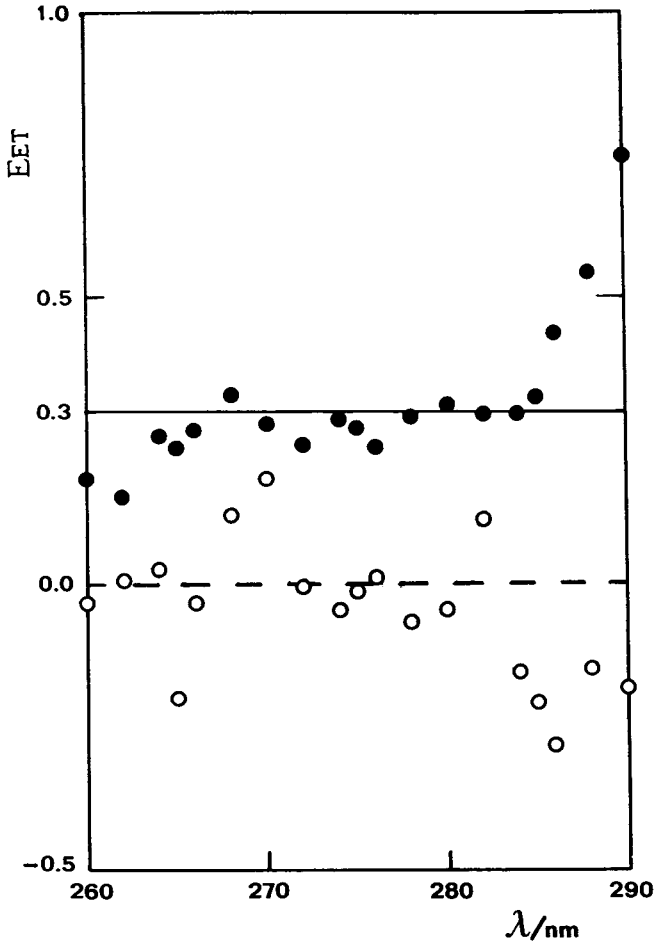

Figure 3. Energy transfer efficiency, E_{ET}, from Tyr to Trp residue in ACTH (●) and in the related peptide (○).

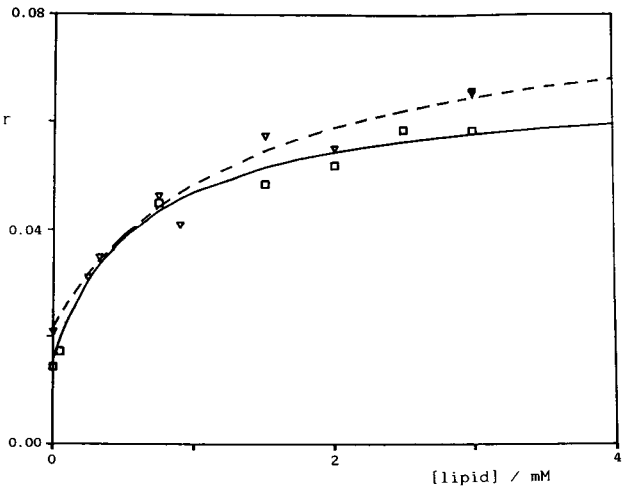

Figure 4. Fluorescence anisotropy, r, *vs* lipid concentration, for ACTH (□) and for the related peptide (▽); fit of Eq. (6) to experimental data for ACTH (——, $K_p = 2.3 \times 10^3$ and $r_l = 0.07$) and for the related peptide (– – –, $K_p = 1.1 \times 10^3$ and $r_l = 0.08$).

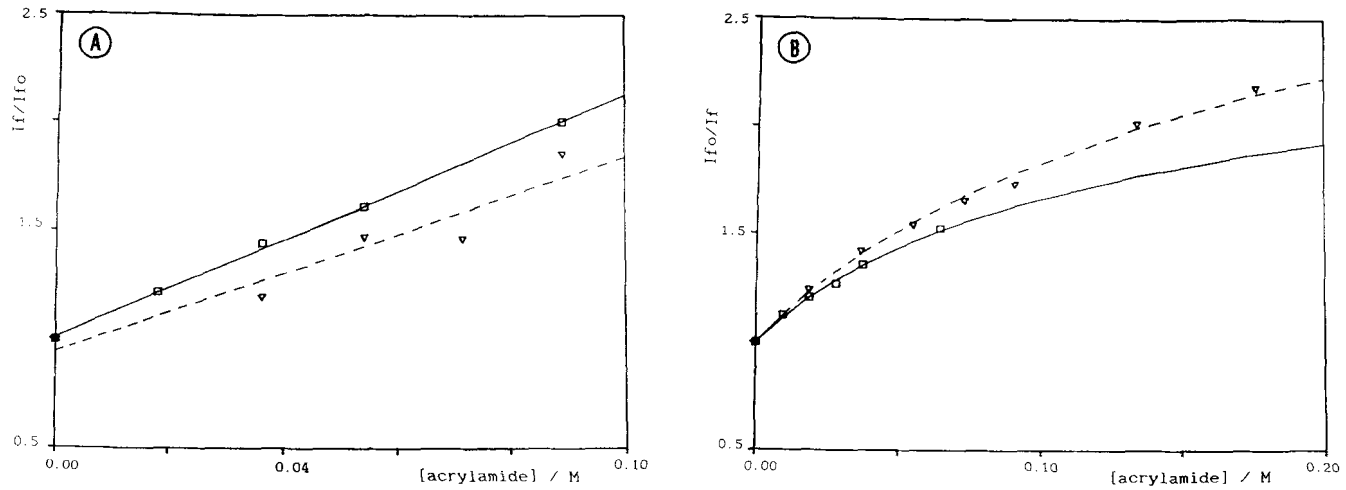

Figure 5. (A) ACTH (□) and related peptide (▽) fluorescence quenching by acrylamide, in aqueous solution. (B) ACTH (□) and related peptide (▽) fluorescence quenching, by acrylamide in the presence of the membrane model system ([L] = 2 × 10^{-3} *M*); fit of Eq. (7) to experimental data for ACTH (——) and related peptide (– – –) with parameters described in Table 2.

to the membrane probes 3-AS and 12-AS is shown in Fig. 7. The fluorescence intensities were again corrected to take into account the peptide concentration in the aqueous phase, as its energy transfer to acceptors in the membrane is negligible. As shown in Fig. 7, the energy transfer efficiency is similar to both 3-AS and 12-AS probes.

DISCUSSION

The emission spectrum of ACTH (λ_{exc} = 297 nm) in aqueous solution (λ_{max} = 360 nm), shown in Fig. 1A, is very similar to that of Trp (λ_{max} = 362 nm) (result not shown), pointing out that the Trp residue in ACTH is in a very polar environment. The inflection observed at λ_{em} = 300 nm when exciting at shorter wavelength (λ_{exc} = 275 nm) arises from the emission of Tyr residues. Accordingly, its contribution is smaller for the related peptide (Fig. 1B), where only one Tyr is present. The red shift observed in ACTH upon increasing the excitation wavelength (Fig. 1A) is due to a red-edge effect,[14,15] and is not related to the spectral composition of the emission of the two chromophores.

The blue shift of the emission spectrum of ACTH in the presence of SUV (DPPC + 17% DMPG), shown in Fig. 2, is clear evidence of the interaction of the peptide with the membrane model system. In addition the hypsochromic effect indicates that Trp is now in a much less polar environment than in the aqueous solution. The existence of a net negative charge for the membrane is essential to promote this interaction, no spectral alterations being noticed with DPPC in the absence of DMPG. This is compatible with the total positive charge of the peptide (+6). The electrostatic interaction induces SUV fusion and increases light scattering.

The energy transfer experiment, namely the study of a related peptide with a single donor, Tyr^{23}, where no transfer was observed, implies that in ACTH the only operative pro-

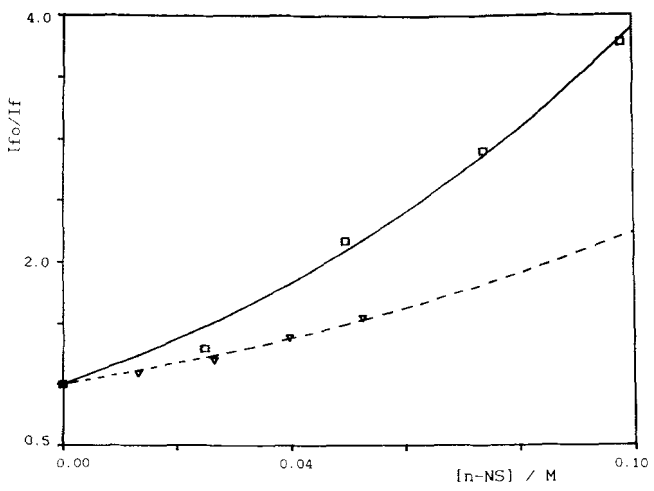

Figure 6. ACTH fluorescence quenching by 5-NS (□) and by 16-NS (▽) ([L] = 2 × 10^{-3} *M*); fit of Eq. (8) to experimental data for 5-NS (——, k_q = 2.2 × 10^9 M^{-1} s^{-1} and R = 13.7 Å) and 16-NS (– – –, k_q = 0.4 × 10^9 M^{-1} s^{-1} and R = 13.7 Å).

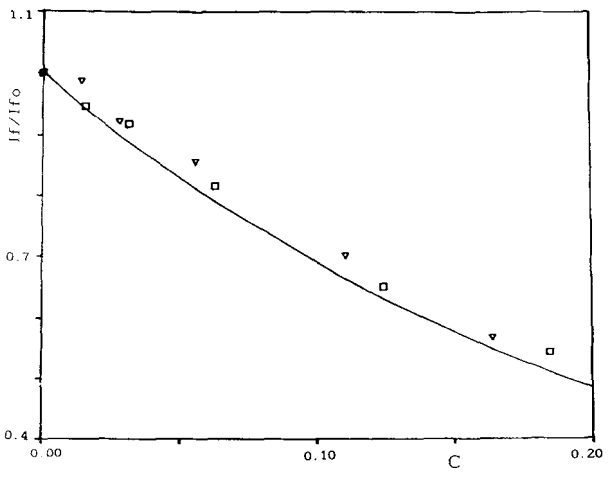

Figure 7. Relative fluorescence intensity, I_f/I_{fo}, for ACTH as a function of surface concentration of acceptor C (number of acceptor molecules in a surface area of R_o^2 Å^2) for 3-AS (□) and 12-AS (▽), incorporated in lipid ([L] = 2 × 10^{-3} *M*); Theoretical expectation for energy transfer in two dimensions[29] (——).

cess involves Tyr^2 (donor) and Trp^9 (acceptor) with an efficiency $E_{ET} = 0.6$. Eisinger[3] obtained for ACTH(1–24) a similar value of $E_{ET} = 0.33$; to discriminate between the two possible donors, two other peptide fragments with single Tyr donors were used, with values of $E_{ET}(Tyr^2) = 0.5$ and $E_{ET}(Tyr^{23}) = 0.15$ reported. The experimental conditions described in that work are very critical, namely optical densities were very high (OD $\simeq$ 10), *i.e.* solutions were very concentrated and intermolecular energy transfer may have occurred as recognized. This would explain why a contribution from Tyr^{23} ($E_{ET}[Tyr^{23}] = 0.15$) was observed to be significant, at variance with our result $E_{ET}(Tyr^{23}) \simeq 0$.

Interchromophoric distances R can be determined from Eq. (4), once the Förster critical radius R_0 for the donor–acceptor pair is known.[9]

$$R = (E_{ET}^{-1} - 1)^{1/6} R_0 \quad (4)$$

For the determination of R_0 the most critical assumption in this case is the quantum yield of tyrosine (donor), which ranges from 0.02 to 0.07.[16] As a similar peptide with no acceptor (Trp^9) is not available, a literature value[3] $R_0 = 9.8$ Å was considered. In this way a distance $R(Tyr^2–Trp^9) = 9.2$ Å is obtained from Eq. (4). This value is in agreement with the theoretical expectations[17] for both an α-helix (10.5 Å) and a random coil structure (10.1 Å). For a small peptide there is no significant distance variation for the two structures, although the existence of a random coil seems more likely in aqueous solution, as reported from circular dichroism (CD) experiments.[18]

The experimental evidence showing negligible energy transfer between Tyr^{23} and Trp^9 also contains information on distances. Assuming an experimental error of $E_{ET} = 0.05$, this would imply a distance of $R(Trp^9–Tyr^{23}) \geq 18$ Å, considering $R_0 = 11$ Å for this pair.[3] Between these two residues, a distance of 21 Å is expected for an α-helix,[17] in agreement with the determined lower bound of 18 Å. For a random coil the expected distance would be of 14.2 Å,[17] incompatible with the conclusion of the energy transfer experiment. For very long peptides Brant and Flory introduced a correction factor in the random coil model, taking into account rotational restrictions of the peptide bonds.[19,20] On this basis a distance of R = 21.6 Å would be obtained, this one being again identical to the expected one for an α-helix and compatible with the lower limit of R = 18 Å, obtained in this work.

We have also tried to carry out the energy transfer study (*i.e.* determination of distances) in the presence of lipid. To rationalize these results it would be necessary to have access to the absorption spectra of the chromophores when in interaction with the membrane, in order to obtain the fractions of absorbed light (Eq. 1). This cannot be overcome due to the strong scatter of the solutions, and the spectra of the peptide in buffer cannot be used because the spectral shifts are significant.

The increase of anisotropy in the presence of lipid is further evidence for the lipid–peptide interaction. Although fluorescence lifetimes were not determined in these conditions, the increase of Trp anisotropy is in agreement with the more restricted depolarization dynamics of the chromophore when in the membrane. In addition these anisotropy data allow the determination of the partition constant K_p of the peptide between the two phases, defined as the concentration ratio of the compound in the lipidic and aqueous phase, Eq. (5)

Table 2. Stern-Volmer rate constants (K_{sv}) and bimolecular quenching rate constants (k_q) for ACTH and related peptide fluorescence quenching by acrylamide (Eq. 7) in aqueous phase and in the presence of lipid

	Aqueous phase		Membrane phase			
	K_{sv}^w (M^{-1})	$k_q^w \times 10^{-9}$ (M^{-1} s^{-1})	K_{sv}^l (M^{-1})	$k_q^l \times 10^{-9}$ (M^{-1} s^{-1})	f_{la}	f_{ln}
ACTH	11.2	4.4	23.2	4.9	0.5	0.4
Parent peptide	9.0	3.6	21.5	4.6	0.6	0.3

$$K_p = \frac{n_l/V_l}{n_w/V_w} \quad (5)$$

where n_l and n_w are the moles of the partitioning compound in the lipid and the aqueous phase, respectively, and V_l and V_w are the volume of each phase.

The anisotropy is an additive parameter,[9] and Eq. (6) is derived based on the following grounds: (1) the quantum yield and the absorption coefficient are identical in the two phases, (2) the lipid and water density are identical, (3) the volume of the aqueous phase is the total volume and (4) all the lipidic volume is accessible for the peptide interaction

$$r = \frac{(r_l - r_w)[L]}{\dfrac{1000}{M_l K_p} + [L]} + r_w \quad (6)$$

where r, r_w and r_l are the total anisotropy and the anisotropy in the aqueous and in the lipidic phase, respectively; M_l is the lipid molar mass (720 g mol^{-1}); and [L] is the lipid concentration.

The best fit of Eq. (6) to the experimental results is shown in Fig. 4; values of $K_p = 2.3 \times 10^3$ and $r_l = 0.07$ for ACTH and $K_p = 1.1 \times 10^3$ and $r_l = 0.08$ for the related peptide were obtained. The recovered values are very similar for both peptides, *i.e.* substitution of Tyr^2 in ACTH for Nle has introduced no alterations in the peptide affinity for the membrane, and in addition the rotational dynamics of Trp^9 in both compounds, as concluded for the r_l values, are also very similar.

Linear Stern-Volmer relationships were obtained for the quenching of ACTH and the related peptide by acrylamide (Fig. 5A). The mean lifetime of Trp in ACTH is $\bar{\tau} = 2.53$ ns,[6] and in this way the bimolecular quenching rate constants $k_q = 4.4 \times 10^9$ M^{-1} s^{-1} (ACTH) and $k_q = 3.6 \times 10^9$ M^{-1} s^{-1} (related peptide) are obtained, assuming invariance of Trp lifetime in both peptides (Table 2). These rate constants are very close to the ones obtained from the Smoluchovski equation ($k_q = 6.2 \times 10^9$ M^{-1} s^{-1}), where diffusion constants were determined from the modified Stokes-Einstein equation and the molecular radii were obtained from addition of molecular volumes;[21] this observation is in agreement with the reported diffusion-controlled Trp quenching by acrylamide.[22,23]

The fluorescence quenching kinetics of Trp when the peptides are interacting with the lipid must take into account that a fraction of the peptide fluorescence (f_w) arises from the peptide remaining in the aqueous phase. The modified Stern-

Volmer relationship, considering the different Trp locations, is Eq. (7).

$$I_{fo}/I_f = 1 \Big/ \left(\frac{f_w}{1 + K_{sv}^w[Q]} + \frac{f_{la}}{1 + K_{sv}^l[Q]} + f_{ln} \right) \quad (7)$$

Once the value of K_{sv}^w is known from the fluorescence quenching study in aqueous solution, the values of f_{la} and f_{ln}, the fractions of peptide fluorescence in the lipid-accessible and nonaccessible to the quencher, respectively, and K_{sv}^l, the Stern-Volmer rate constant, can be determined from the best fit of Eq. (7) to experimental data, and they are presented in Table 2. It should be stressed that a model with just two accessible Trp populations (in water and in the membrane) could not describe the experimental data, *i.e.* it was necessary to invoke a nonaccessible fraction of fluorophore incorporated in the membrane. In the fitting process it was assumed that the quenching rate constant of the accessible peptide fluorescence in the membrane cannot be larger than the quenching rate constant of the peptide fluorescence in the aqueous phase and, in this way, $f_w = 0.1$ was considered. Due to the uncertainty associated with the determination of K_p values, this value seems a reasonable one.

The bimolecular rate constants presented in Table 2 were determined assuming fluorescence lifetimes of $\bar{\tau} = 2.53$ ns for ACTH in aqueous solution[6] and $\bar{\tau} = 4.7$ ns when in the membrane. This last value was estimated from the reported increase of fluorescence lifetime of bombesin (a single Trp peptide) in the presence of membranes of lysolecithin.[24]

It cannot be inferred from these experiments that the population of Trp nonaccessible to the quencher is deeply buried in the membrane. As will be shown later, both the energy transfer experiments (to the *n*-AS probes), and the Trp quenching by lipophilic probes (*n*-NS), point to an essentially superficial location of Trp.

The upward curvature verified in the ACTH fluorescence quenching by both 5-NS or 16-NS (Fig. 6) can be rationalized considering the contributions of static (active sphere model) and dynamic quenching mechanisms,[9] Eq. (8)

$$I_{fo}/I_f = (1 + K_{sv}[Q])e^{0.0025R^3[Q]} \quad (8)$$

where I_{fo}/I_f is the ratio of the fluorescence intensity of the peptide fraction in the membrane in the absence and in the presence of a concentration [Q] of quencher in the membrane, R is the active sphere radius in Å, and K_{sv} is the Stern-Volmer constant. From a fit of Eq. (8) to experimental data (Fig. 6), dynamic quenching by 5-NS was found to be much more efficient ($K_{sv} = 10.5\ M^{-1}$) than the one observed for 16-NS ($K_{sv} = 1.8\ M^{-1}$), whereas the active sphere radius is R = 13.7 Å for both quenchers, this radius value being close to the sum of molecular radii of ACTH and quencher. Within the same approximation considered in the Trp quenching by acrylamide, values of the quenching rate constants ($k_q = 2.2 \times 10^9\ M^{-1}\ s^{-1}$ for 5-NS and $k_q = 0.4 \times 10^9\ M^{-1}\ s^{-1}$ for 16-NS) are obtained. The higher quenching efficiency by 5-NS means that the Trp residue is closer to the carbon 5 position of the fatty acid than to the carbon 16 position. Considering that these probes are located at a graded series of depths in the membrane,[25] this result is evidence for the location of the Trp near the membrane interface.

As shown in Fig. 7, identical energy transfer efficiencies were obtained from Trp to the membrane probes 3-AS and 12-AS. In order to determine R_o (Eq. 2), the fluorescence emission spectrum of ACTH in interaction with the membrane, $I_f(\lambda)$, was obtained at a lipid concentration of $3.3 \times 10^{-3}\ M$, as in this case the fraction of peptide in the aqueous phase ($f_w < 0.05$) is negligible; the refractive index of the membrane was assumed to be 1.44.[26] A Förster critical radius $R_o = 24$ Å was obtained, this value being the same for both probes (3-AS and 12-AS) as the absorption spectra of these two acceptors is identical. Various studies using different techniques[27,28] point out that these probes are located at a graded series of depths in the membrane. In this way direct inspection of Fig. 7 showing that the energy transfer efficiency is probe invariant allows us to conclude that the Trp residue of ACTH is not deeply buried in the membrane.

We have further rationalized the energy transfer efficiency *via* the model for Förster energy transfer in two dimensions.[29] For this purpose the phospholipid head-group area for our system was considered to be 50 Å^2.[30] As depicted in Fig. 7, there is qualitative agreement; the experimental energy transfer efficiency is slightly lower than the theoretical expectation, the latter considering a random distribution of both donor and acceptor on the same plane. These assumptions are not verified in this experiment, *i.e.* donor and acceptor are located at different depths in the membrane, and the acceptor is known to self-aggregate within the membrane;[31] these facts imply the observed deviation. It should be stressed that information on the Trp location as discussed earlier (*i.e.* between the 3-AS and 12-AS probes) is not hampered by this fact.

CONCLUSIONS

A fluorescence study of ACTH(1–24), a peptide hormone with three fluorescent residues (Tyr^2, Trp^9 and Tyr^{23}), was carried out in aqueous solution and in interaction with a model system of membranes (SUV of DPPC + 17% DMPG), and a concomitant study of a related peptide in which Tyr^2 is absent was also made.

Intramolecular distances of $R(Tyr^2–Trp^9) = 9.8$ Å and $R(Tyr^{23}–Trp^9) \geq 18$ Å were obtained from energy transfer experiments, these values being compatible with molecular models for an α-helix or a random coil structure in which rotational restrictions were considered.

Interactions of the peptide with the model system of membranes was evident from the blue shift of Trp fluorescence and from the increase in its fluorescence quantum yield and anisotropy. This last parameter allows determination of the partition constant of the peptide between the lipidic and aqueous phase, values of $K_p = 2.3 \times 10^3$ (ACTH) and $K_p = 1.1 \times 10^3$ (related peptide) being obtained. In the absence of a negative charge in the membrane, no interaction with the peptides was noticed, pointing to the relevance of electrostatic interactions.

Fluorescence quenching of Trp emission by acrylamide was studied both in the absence and presence of lipid and, in the latter case, nonlinear Stern-Volmer plots were obtained. Its rationalization was made invoking the existence of a nonaccessible fraction of Trp to the quencher.

The lipophilic 5-NS and 16-NS probes were used to obtain information on Trp location. The fluorescence quenching of the Trp has both a static and a dynamic contribution, this

last one being greater for 5-NS ($k_q = 2.2 \times 10^9 M^{-1} s^{-1}$) than for 16-NS ($k_q = 0.4 \times 10^9 M^{-1} s^{-1}$). This result is also evidence for a Trp location near the interface. In addition, identical energy transfer efficiencies were obtained from Trp to the membrane probes 3-AS and 12-AS, showing that Trp is not deeply buried in the membrane. The energy transfer process was qualitatively described by a model for energy transfer in two dimensions.

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