Biochimica et Biophysica Acta, 1068 (1991) 81–86

ADONIS 000527369100332A

BBAMEM 75345

Fluctuation and rotation of human growth hormone-releasing factor in the presence and the absence of phospholipid bilayer analyzed by time-resolved fluorescence depolarization

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(Received 18 March 1991)

Key words: Growth hormone-releasing factor; Fluctuation; Rotation; Fluorescence depolarization; Phospholipid

Time-resolved fluorescence depolarization measurements were carried out for human growth hormone-releasing factor analog ([Trp^{10}]-hGRF(1–29)NH_2), where the Trp^{10} residue was incorporated as a fluorescent probe, in the presence and the absence of 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol(DMPG) liposome and in aqueous 2,2,2-trifluoroethanol (TFE) solution. The fluorescence lifetimes and the rotatory correlation times of the peptide in each medium were determined. The apparent volumes of the rotatory Brownian motion unit calculated from these fluorescent parameters indicate the different mode of the fluctuation and/or the rotation of the peptide in each medium, such as: (i) In the aqueous solution, several segments of the peptide fluctuate individually. (ii) In the DMPG bilayer, both the local fluctuation of Trp residue alone and the rotation of the whole molecule exist. (iii) In the aqueous TFE solution, the monomeric peptide rotates as a rigid ellipsoid.

Introduction

Most of linear bioactive peptides such as hormones and neurotransmitters do not exist in an ordered structure in aqueous solutions [1]. The conformation of the peptide changes from a flexible one into an inherent one, only when the peptide reaches biomembrane [2,3]. Therefore, in order to understand the action mechanism of bioactive peptides, it is important to investigate the static and dynamic structures of the peptide interacting with biomembrane as well as the structures of the peptide alone in solution. However, since the intact biomembrane is constructed from many complicated components, phospholipid liposome is generally used for an environmental model of biomembrane.

Growth hormone-releasing factor (GRF) is an amidated peptide consisting 44 amino acid residues [4,5]. It is one of hypothalamic hormone, which regulates the secretion of pituitary hormone. GRF probably binds to a certain receptor located on plasma membrane of the pituitary cell and activates the adenylate cyclase-cAMP system [6]. However, the receptor has not been isolated so far. By the physiological studies of a number of synthetic fragments and analogs, the active core of GRF, called 'message' segment [7], has been proved to reside in the N-terminal region. In particular, the amidated fragment between Tyr^1 and Arg^{29} of human GRF (hGRF(1–29)NH_2) possesses almost the same activity as intact human GRF [8].

In our previous work, we revealed that hGRF(1–29)NH_2 does not exist in an ordered structure in aqueous buffer solution and that its flexible conformation is almost independent of pH, temperature and ionic strength [9]. The peptide binds to acidic phospholipids, but not to neutral ones [10]. The conformation of the peptide changes to a helix-rich one with binding to DMPG bilayer above the gel-liquid crystal phase transition temperature (T_c) [11]. In addition, the peptide also turns to a helix on addition of TFE to aqueous buffer solution and almost the whole molecule forms a complete helix in 50 vol% TFE [9].

On the basis of the two-stage capture model [2] or the multiple sequential steps model [3], a bioactive peptide reaches the lipid membrane prior to binding to

Abbreviations: GRF, growth hormone-releasing factor; hGRF, human growth hormone-releasing factor; DMPG, 1,2-dimyristoyl-*sn*-glycero-3-phospho-*rac*-glycerol; TFE, 2,2,2-trifluoroethanol; T_c, the temperature of gel-liquid crystal phase transition.

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a target receptor. Thus, the dynamics of a peptide in a membrane is an important factor for the action of a peptide as well as the static conformation of a peptide in the membrane.

In the present study, we carried out the fluorescence depolarization measurements of [Trp^{10}]-hGRF(1–29)NH_2, in which the Tyr^{10} residue of hGRF(1–29)NH_2 is replaced with a Trp residue as a fluorescent probe, in the presence and the absence of DMPG liposome as well as in aqueous TFE solution, in order to investigate the fluctuation and the rotation of the peptide in each environment.

Materials and Methods

Chemicals

TFE was obtained from Wako Pure Chemicals and the other chemicals were of the best available commercial grade. Ultra-pure water provided by Mill-Q (Millpore) was used.

Peptide

[Trp^{10}]-hGRF(1–29)NH_2 (Fig. 1) was synthesized by the solid-phase method using an Applied Biosystems 430A peptide synthesizer. The deprotection and the purification were the same as described previously [12]. The purified peptide showed a single peak in isocratic reverse-phase HPLC and was identified by amino acid analysis after acid hydrolysis [13]. The concentration of the peptide was determined by the absorption spectra in water based on the molar absorption coefficients of *N*-acetyltyrosine ethyl ester and *N*-acetyltryptophan ethyl ester [14].

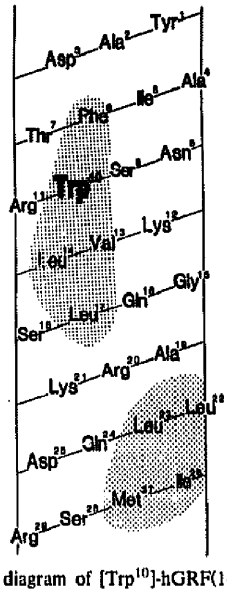

Fig. 1. Helical net diagram of [Trp^{10}]-hGRF(1–29)NH_2. Incorporated Trp residue is located in hydrophobic area.

Lipid

Semi-synthetic DMPG was purchased from Nippon Oil & Fats. In order to avoid light-scattering effect and absorption-flattening effect of lipid assembly [15], size-ordered liposome was prepared using an Extruder (The Liposome Company) after the treatment of freeze-thaw cycles [16]. The small unilamellar vesicle was confirmed by differential scanning calorimetry [17] and its diameter was found to be below 50 nm by a dynamic light-scattering measurement. The concentration of lipid was determined by colorimetry of molybdenum blue at 660 nm, which was obtained by reducing the mixture of ammonium molybdate and the lipid hydrolyzate [18].

Circular dichroism

Circular dichroism (CD) spectra were recorded from 200 to 250 nm on a Jasco J-600 spectropolarimeter equipped with a thermostated cell holder. The instrument was calibrated with ammonium *d*-10-camphorsulfonate [19]. The detailed conditions of the measurement were described previously [9]. The CD data were expressed as the mean residue molar ellipticity [θ].

Time-resolved fluorescence

Time-resolved fluorescence depolarization measurements were performed on Horiba NAES-1100 time-resolved spectrofluorometer by time-correlated photon counting technique. The light source was self-excited discharge lamp filled with 0.6–1.0 MPa H_2 and emitted stable pulses (1.8 ns half-width and 7 kHz frequency). Light at 280 nm was selected by a monochromator in order to excite the Trp^{10} residue, polarized vertically with a Glan-Taylor prism, and focused on the sample in a thermostated cell holder. Both vertically and horizontally polarized components, $I_{\parallel}(t)$ and $I_{\perp}(t)$, of fluorescence emission were detected by a Polaroid HNP'B film set in vertical position and in horizontal position, respectively, followed by the filtration with a Hoya UV-32 filter, which effectively blocked off the excitation light and the Raman scattering light from solvent. The fluorescence intensity of the solution without peptide was also measured separately as a blank fluorescence and subtracted from the data of the corresponding peptide-containing sample. The absorbance of the samples at 280 nm was usually kept below 0.06 to avoid inner filter effect. The apparatus response function of exciting light, $g(t)$, was measured simultaneously by splitting exciting light. The instrumental time-lags between $I(t)$ and $g(t)$ were adjusted with colloidal silica suspension as a light scatter before every set of the measurements.

Evaluation of fluorescence data

From the blank-subtracted fluorescence intensities, $I_{\parallel}(t)$ and $I_{\perp}(t)$, the pseudo-total fluorescence intensity, $I_T^*(t)$, and the pseudo-difference intensity, $I_D^*(t)$, are defined as

$$I_T^*(t) = I_{\parallel}(t) + 2SGI_{\perp}(t) \quad (1)$$

$$I_D^*(t) = (I_{\parallel}(t) - SGI_{\perp}(t))/D \quad (2)$$

where S is the ratio of the sensitivity of the detection system for vertically polarized light to horizontal one. G is the ratio of the integrated $g(t)$ as the measurement for $I_{\parallel}(t)$ so far for $I_{\perp}((t)$. D is the correction factor due to scattering effect of a turbid sample. In this study, S was determined to be 1.083 by the measurement of colloidal silica suspension and D was taken as 1.0 because of the negligible scattering effect of the prepared size-order liposome. The value of G ranged between 1.03 and 1.36.

The total fluorescence intensity, $I_T(t)$, and the difference intensity, $I_D(t)$, are calculated by the deconvolution of following equations

$$I_T^*(t) = \int_0^t g(t') I_T(t - t')\, dt' \quad (3)$$

$$I_D^*(t) = \int_0^t g(t') I_D(t - t')\, dt' \quad (4)$$

where $I_T(t)$ and $I_D(t)$ are regarded as the multiple exponential approximation, such as

$$I_T(t) = a_0 \sum_i \alpha_i \exp(-t/\tau_{T,i}) \left[\sum_i \alpha_i = 1\right] \quad (5)$$

$$I_D(t) = b_0 \sum_j \beta_j \exp(-t/\tau_{D,j}) \left[\sum_j \beta_j = 1\right] \quad (6)$$

The fluorescence anisotropy, $r(t)$, is analyzed by fitting $r(t)I_T(t)$ to $I_D(t)$ by the least-squares method, where $r(t)$ is assumed as the following models [20,21]:

$$r(t) = r_0 \sum_k \gamma_k \exp(-t/\tau_{c,k}) \left[\sum_k \gamma_k = 1\right] \quad (7)$$

$$r(t) = r_0 \left(\sum_k \gamma_k \exp(-t/\tau_{c,k}) + \gamma_\infty\right) \left[\sum_k \gamma_k + \gamma_\infty = 1\right] \quad (8)$$

where r_0 and τ_c denote the intrinsic depolarization factor and the rotatory correlation time, respectively.

Results and Discussion

CD measurements

The CD spectra of [Trp[10]]-hGRF(1–29)NH_2 and hGRF(1–29)NH_2 in Fig. 2 indicate that the replacement of Tyr residue by Trp residue does not affect the conformation of the whole molecule in the aqueous solution, in DMPG bilayer, and in 50 vol% TFE.

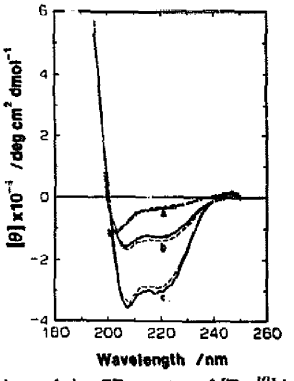

Fig. 2. Comparison of the CD spectra of [Trp[10]]-hGRF(1–29)NH_2 (solid lines) with that of hGRF(1–29)NH_2 (broken lines) at 30° C. (a) In 5 mM potassium phosphate buffer with 50 mM KCl (pH 7.0), [peptide] = 5.5 and 5.3 μM. (b) In the above aqueous solution containing DMPG vesicles, [peptide] = 9.1 and 2.7 μM, [DMPG] = 149 and 72 μM. (c) In the above aqueous solution containing TFE, [peptide] = 2.7 and 2.7 μM, [TFE] = 50 and 50 vol%.

Time-resolved fluorescence measurements

The fluorescence decays of [Trp[10]]-hGRF(1–29)NH_2 in the aqueous solution, in the DMPG solution, and in 50 vol% TFE were measured at 30° C. (Tc for DMPG bilayer is 23° C [22].) The amount of DMPG was considered to make almost all of the peptide bind in the lipid bilayer based on the above results in CD measurements and our previous investigation [9–11]. Observed $I_T^*(t)$ and $I_D^*(t)$ were deconvoluted into multiple exponential approximation by Eqns. 3 and 4. Then, the fluorescence life times, τ_T, and the time constants of difference intensity, τ_D, are determined as a function of the number of exponential components, i and j, by Eqns. 5 and 6, respectively. The results are listed in Tables I and II with other related parameters.

The average fluorescence lifetime, $\bar{\tau}_T$, in the DMPG solution is longer than that in the aqueous solution at all values of i (Table I), demonstrating that the Trp residue is protected by the DMPG bilayer against the contact with water. However, the values of $\bar{\tau}_T$ in the TFE solution were surprising at first glance, because the wavelength at maximum fluorescence of the peptide in 50 vol% TFE (343 nm) is shorter than that in the aqueous solution (352 nm) and is longer than that in the DMPG solution (328 nm) as reported previously [11]. Although the question is still open, it is most likely that the Trp residue is quenched by some approaching carbonyl groups [23,24] and/or basic groups [23,25] with the formation of helix structure in the TFE solution.

TABLE I

Fluorescence lifetimes and related parameters of [Trp[10]]-hGRF(1–29)NH$_2$ at 30° C

Medium	i	$\tau_{T,i}$(ns)	α_i	$\bar{\tau}_T$ [d](ns)	χ^2
Aqueous [a]	1	2.43	1.0	2.43	6.23
	2	1.82	0.844	2.49	1.84
		4.11	0.156		
	3	0.672	0.311	2.58	1.66
		2.44	0.678		
		8.98	0.011		
DMPG [b]	1	2.88	1.0	2.88	29.3
	2	2.05	0.882	3.18	4.89
		6.04	0.118		
	3	1.68	0.672	3.23	4.54
		3.68	0.308		
		9.63	0.020		
TFE [c]	1	1.70	1.0	1.70	11.2
	2	1.42	0.952	1.87	2.11
		4.62	0.048		
	3	1.05	0.644	1.89	1.27
		2.25	0.353		
		10.8	0.003		

[a] 10.1 μM peptide in 5 mM potassium phosphate buffer containing 50 mM KCl.

[b] 10.2 μM peptide and 170 μM DMPG in the above buffer.

[c] 9.7 μM peptide and 50 vol% TFE in the above buffer.

[d] $\bar{\tau} = \sum_i \alpha_i \tau_{T,i}^2 / \sum_i \alpha_i \tau_{T,i}$

TABLE II

Time constants of difference intensity and relating parameters of [Trp[10]]-hGRF(1–29)NH$_2$ at 30° C

Medium	j	$\tau_{D,j}$(ns)	β_j	$\bar{\tau}_D$ [a](ns)	χ^2
Aqueous	1	0.394	1.0	0.394	0.46
	2	0.393	0.328	0.394	0.52
		0.394	0.672		
	3	0.386	0.263	0.394	0.59
		0.396	0.266		
		0.397	0.471		
DMPG	1	2.95	1.0	2.95	5.25
	2	1.24	0.765	3.26	3.11
		4.92	0.235		
	3	0.749	0.525	3.29	3.06
		2.36	0.345		
		5.67	0.130		
TFE	1	0.977	1.0	0.977	0.87
	2	0.977	0.609	0.977	0.89
		0.977	0.391		
	3	0.974	0.258	0.977	0.91
		0.977	0.408		
		0.979	0.334		

[a] $\bar{\tau}_D = \sum_j \beta_j \tau_{D,j}^2 / \sum_j \beta_j \tau_{D,j}$

The reliability showed by χ^2 in Table I improves obviously with increasing the number of i from 1 to 2. On the other hand, the improvements at 'aqueous' and 'TFE' in Table II do not occur like those in Table I in spite of the increase of number j. In general, increasing the number of parameters in regression analysis increases apparent reliability; however, too many parameters bring confusion to the analysis. To simplify further calculation, therefore, double exponential approximation ($i = 2$) was adopted for τ_T in every medium, whereas single one ($j = 1$) for τ_D in the aqueous solution and in the aqueous TFE, and double one ($j = 2$) for τ_D in the DMPG solution were chosen. Their original data and fitted curves are shown in Fig. 3 as normalized intensities.

The fluorescence anisotropy parameters were calculated by Eqn. 7 ($k = 1,2$) and Eqn. 8 ($k = 1$) as shown in Table III. Judging from the values of variance, σ^2, in the fitting, the single exponential model is suitable for the fluctuating mode of the peptide in the aqueous

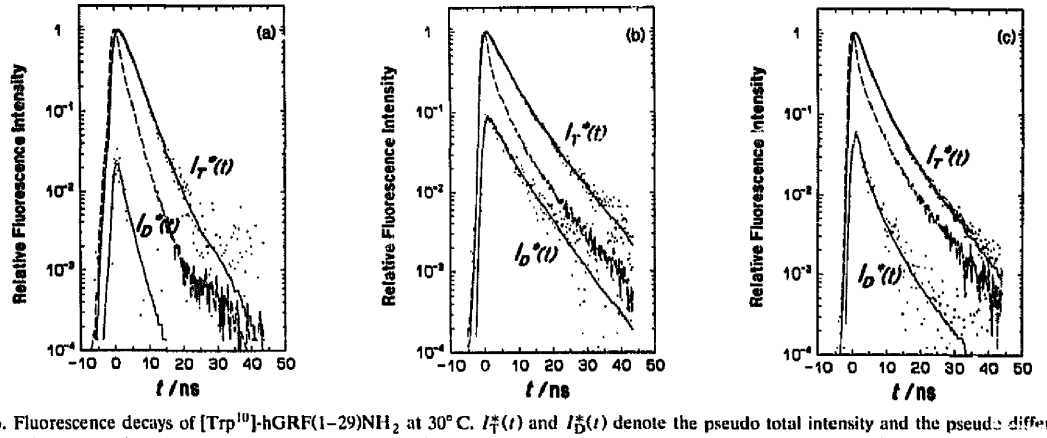

Fig. 3. Fluorescence decays of [Trp[10]]-hGRF(1–29)NH$_2$ at 30° C. $I_T^*(t)$ and $I_D^*(t)$ denote the pseudo total intensity and the pseudo difference intensity (dots; experimental data, solid lines; fitted curves). Broken lines show the apparatus response function. (a) In the aqueous solution, [peptide] = 10.1 μM, $i = 2$, $j = 1$. (b) In the DMPG solution, [peptide] = 10.2 μM, [DMPG] = 170 μM, $i = 2$, $j = 2$. (c) In the TFE solution, [peptide] = 9.7 μM, [TFE] = 50 vol%, $i = 2$, $j = 1$.

TABLE III

Fluorescence anisotropy parameters of [Trp[10]]-hGRF(1–29)NH$_2$ at 30° C

Medium	i	j	k	$\tau_{C,k}$(ns)	γ_k	γ_∞	r_0	σ^2	Remark
Aqueous	2	1	1	0.488	1.0	–	0.0623	$0.22 \cdot 10^{-9}$	a
			2	0.438	0.496	–	0.0623	$1.5 \cdot 10^{-9}$	a
				0.547	0.504				
			1	0.485	0.996	0.004	0.0623	$3.2 \cdot 10^{-9}$	b
DMPG	2	2	1	12.7	1.0	–	0.0996	$5.5 \cdot 10^{-6}$	a
			2	0.892	0.253	–	0.107	$1.1 \cdot 10^{-6}$	a
				97.2	0.747				
			1	0.994	0.285	0.715	0.107	$0.89 \cdot 10^{-6}$	b
TFE	2	1	1	2.79	1.0	–	0.0670	$1.2 \cdot 10^{-8}$	a
			2	2.65	0.852	–	0.0670	$1.4 \cdot 10^{-8}$	a
				3.79	0.148				
			1	2.73	0.979	0.021	0.0672	$1.9 \cdot 10^{-8}$	b

[a] Calculated by Eqn. 7.
[b] Calculated by Eqn. 8.

solution and in the aqueous TFE, while both double exponential and single exponential with plateau models are adequate for that in the DMPG solution.

The values of the intrinsic depolarization factors, r_0, listed in Table III are approximately constant. However, these values are apparently smaller than those in the literature [20], where the fluctuations of several Trp-containing artificial peptides in lipid were reported. These differences seem mainly due to the resolution of the instrument at initial short time.

The apparent volume of the Brownian motion unit, fv_e, in each medium was estimated from the corresponding rotatory correlation time, τ_c (Table IV).

In the aqueous solution, fv_e was estimated by the Stokes-Einstein law as

$$fv_e = \tau_c kT/\eta \qquad (9)$$

where v_e and f denote the effective volume and the shape factor, respectively, k is the Boltzmann constant, T the temperature, the η the viscosity of the medium [21]. When the shape of the moving body is approximated to a sphere, $f = 1$, and in other cases, $f > 1$. The value of fv_e in the aqueous solution (2.56 nm^3) corresponds to about 10-times volume of a hydrate amino acid [26], indicating that several segments in [Trp[10]]-hGRF(1–29)NH$_2$ fluctuate individually as a unit of Brownian motion in the aqueous solution. The result agrees with the presence of flexible random coil conformation of hGRF(1–29)NH$_2$ in the aqueous solution analyzed by CD spectra [9].

TABLE IV

Apparent volume of the rotatory Brownian motion unit of [Trp[10]]-hGRF(1–29)NH$_2$ at 30° C

Medium	i	j	k	η_{30}($\times 10^{-3}$ Ns/m^{-2})	fv_e (nm^3)	Remark
Aqueous	2	1	1	0.797 [a]	2.56	d
DMPG	2	2	2	30 [b]	0.124, 13.6	d
			1	30 [b]	0.139	e
TFE	2	1	1	1.75 [c]	6.67	d

[a] The value corresponds to that of pure water at 30° C.
[b] The value is cited from the literature [21].
[c] The value is determined by the authors using an Ubbelohde meter corrected with pure water.
[d] Calculated by Eqns. 7 and 9.
[e] Calculated by Eqns. 8 and 9.

In the DMPG solution, both the double exponential model and the single exponential with plateau model are suitable as mentioned above. Both the volumes of fv_e (0.124 and 0.139 nm^3) calculated from $\tau_{c,1}$(0.892 ns) in the former model and $\tau_{c,1}$(0.994 ns) in the latter model are no more than one amino acid-volume, indicating the local fluctuation of the Trp residue alone. On the other hand, both the large values of $\tau_{c,2}$ (97.2 ns) in the former model and of γ_∞ (0.715) in the latter model prove that the mobility of the whole peptide is reduced by the interaction with DMPG bilayer. For membrane proteins, the relaxation time for the rotation around an axis normal to the membrane plane, $\phi_\parallel$ is given by [27]

$$\phi_\parallel = kT/4\pi a^2 h\eta \qquad (10)$$

where a and h are the radius of membrane proteins and the length immersed in membrane, respectively. If the peptide rotates individually as a monomer in the membrane (i.e., self-association does not occur), roughly calculated $\phi_\parallel$ has the order of 10^2 ns. The order is comparable with the value of $\tau_{c,2}$, hinting the individual monomeric rotation of the peptide in membrane. However, the fluorescence lifetime of the Trp residue is too short to describe the rotation of the

whole peptide in the membrane minutely. Therefore, it is necessary to observe its rotation using longer range method such as phosphorescence or EPR for further discussion.

In the TFE solution, fv_e is 6.67 nm^3. Since whole of hGRF(1–29)NH_2 folds into a complete helix in 50 vol% TFE [9], it is reasonable to approximate the shape of [Trp^{10}]-hGRF(1–29)NH_2 to the ellipsoid having 0.6-nm equatorial radius and 2.1-nm longitudinal radius. (These values were determined from the average radius and pitch of α-helix [28,29].) Although the theoretical fluorescence anisotropy of a ellipsoid is represented with three exponential forms [30], it is possible to describe the anisotropy with a single exponential form and parameter f for an experimental procedure [31]. In the case of such the ellipsoid in homogeneous medium, the value of the shape factor f is led to the range from 1.8 to 2.8 under the condition of the axial ratio, $\rho = 2.1/0.6 = 3.5$, and the angle between the longitudinal axis of the ellipsoid and the emission moments of the fluorophore, $\theta = 0 \sim 45$ degree [31]. (ρf in this paper corresponds to $1/f^3$ in Ref. 31). Thus, the observed apparent volume (6.67) belongs within the theoretical range (5.7 ~ 8.9), which was calculated from f and the volume of the above ellipsoid as follows: $5.7 \sim 8.9 = (1.8 \sim 2.8) \cdot ((4/3) \cdot \pi \cdot 0.6^3 \cdot 3.5)$. These results indicate that the helical peptide exists as a monomer and rotates as one rigid body in the TFE solution.

In conclusion, the time-resolved fluorescence depolarization study has revealed that the mode of the fluctuation and the rotation of [Trp^{10}]-hGRF(1–29)NH_2 depends on the surrounding medium as follows; (i) several segments of the peptide fluctuate individually in the aqueous solution, (ii) the local fluctuation of the Trp residue alone and the rotation of the whole peptide exist in the DMPG bilayer above its T_c, and (iii) the whole peptide rotates as a rigid ellipsoid in the aqueous TFE solution.

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