The Tendency of Magainin To Associate upon Binding to Phospholipid Bilayers

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ABSTRACT: Fluorescence energy transfer (FET) from [Trp[16]]-magainin-2-amide (Trp-Mag) and [D-Ala[15],D-Trp[16]]magainin-2-amide (DD-Trp-Mag) to N^{α}-dansyl-magainin-2-amide (DNS-Mag) was used to study the association of magainin 2 analogs bound to phosphatidylglycerol vesicles. As shown by circular dichroism and fluorescence spectroscopy, the all-L-analogs exist in a helical conformation and are completely bound to the lipid membrane. The observed FET between Trp-Mag and DNS-Mag is rather small and increases with the DNS-Mag surface concentration. The experimentally determined transfer efficiency is lower than predicted for monomeric magainin analogs randomly distributed exclusively at the outer leaflet of lipid vesicles. These observations can be explained by two different models of spatial distribution for the monomeric magainin analogs. The first model takes into account translocation of magainin which might result in a uniform distribution of magainin at the inner and outer vesicle leaflets. The second model assumes that at least one shell of lipids exists between two magainin molecules, thus reducing the probability of direct contact. Both models explain the measured FET without any contribution of stable associates of magainin analogs. Furthermore, for Trp-Mag and DD-Trp-Mag, an identical energy transfer efficiency was observed, although the nonhelical double-D substituted analog should have a significantly reduced association tendency resulting in decreased FET. Our conclusion that the observed FET is not the result of magainin association is confirmed by the equivalence of the measured energy transfer efficiencies.

Magainin 2 is a linear peptide consisting of 23 amino acid residues (Zasloff, 1987). It exhibits a broad spectrum of antibacterial and antifungal activity but is not hemolytic and therefore is of pharmaceutical interest (Zasloff et al., 1988; Soravia et al., 1988). Being positively charged, it shows high affinity to negatively charged lipid bilayers (Matsuzaki et al., 1991; Wieprecht et al., 1996). Solid state NMR (Bechinger et al., 1993) and CD[1] spectroscopy (Wieprecht et al., 1996) confirm the high probability of magainin to form an amphipathic α-helical structure.

On the basis of investigations of both biological and model membrane systems, it has been suggested that magainin exerts its activity by permeabilizing the lipid bilayer. (Westerhoff et al., 1989; Matsuzaki et al., 1991; Bessalle et al., 1990; Juretic et al., 1994). However, the exact mechanism of membrane disturbance is still controversial. Grant et al. (1992) proposed that magainin bound at the outer surface of a lipid vesicle causes a temporary packing imbalance between the two leaflets of the bilayer, thus increasing its permeability for hydrophilic substances. The formation of ion-gating transmembranal pores was suggested by the conductivity experiments of Duclohier et al. (1989) and Cruciani et al. (1992). The formation of active associated structures is indicated by the positive cooperativity of membrane permeabilization and cytotoxic activity (Juretic et al., 1994; Haimovich et al., 1995). Positive cooperativity was also observed for magainin binding at lipid bilayers derived from calcein leakage from negatively charged lipid vesicles and the dependence of fluorescence quantum yield of bound [Trp[12]]magainin on peptide surface concentration (Matsuzaki et al., 1991, 1994). Such results imply the existence of preformed associates which are expected to facilitate pore formation (Duclohier et al., 1989; Cruciani et al., 1992; Vaz Gomes et al., 1993). The pore model suggested by Matsuzaki et al. (1994, 1995a) assumes a side-by-side dimerization of magainin helices. However, association of lipid-bound magainin has not yet been proved by any direct method.

The method of choice for investigating the association of lipid-bound peptides is measurement of the resulting intermolecular FET (Chung et al., 1992). The aim of the present work is the application of this technique to study the association of lipid-bound magainin. Mag, having phenylalanine in position 16 replaced by tryptophan, was used as the fluorescence donor and DNS-Mag as the acceptor. To compare the behavior of the helical Trp-Mag with that of a nearly nonhelical analog, we employed a Trp-Mag analog with alanine 15 and tryptophan 16 substituted by their D-enantiomers (DD-Trp-Mag). Double D-amino acid substitution has recently been shown to significantly disturb the helical conformation of lipid-bound magainin and amphipathic model peptides (Wieprecht et al., 1996; Dathe et al., 1996a) and is expected to perturb peptide association as reported for D-substituted pardaxin (Pouny & Shai, 1992).

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[1] Abbreviations: CD, circular dichroism; DNS, 5-(dimethylamino)-naphthalene-1-sulfonamide (dansyl amide); EDTA, ethylenediaminetetraacetic acid; FET, fluorescence energy transfer; LUVs, large unilamellar vesicles; MALDI-MS, matrix assisted laser desorption/ionization mass spectrometry; Mag, magainin-2-amide (Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-NH_2); DD-Mag, [D-Ala[15],D-Phe[16]]magainin-2-amide; Trp-Mag, [Trp[16]]magainin-2-amide; DD-Trp-Mag, [D-Ala[15],D-Trp[16]]-magainin-2-amide; DNS-Mag, N^{α}-dansylmagainin-2-amide; NMR, nuclear magnetic resonance spectroscopy; POPG, 1-palmitoyl-2-oleoylphosphatidyl-DL-glycerol; SUVs, small unilamellar vesicles; Tris, tris(hydroxymethyl)aminomethane.

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MATERIALS AND METHODS

Materials. Mag, Trp-Mag, DD-Mag, DD-Trp-Mag, and DNS-Mag were synthesized by the solid-phase method using the Fmoc strategy (Beyermann et al., 1992). After cleavage of the N-terminal Fmoc group from the fully protected peptide on the resin, the dansyl residue was incorporated by reaction of the N-terminal amino group with dansyl chloride. Final cleavage resulted in peptide amides which were purified by preparative HPLC on PolyEncap A300 to give final products >98% pure by RP-HPLC analysis. Peptide identity was established by mass spectrometry (MALDI II, Kratos, Manchester, U.K.) and the peptide content by quantitative amino acid analysis.

POPG was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The amino acid standard from Sigma Chemie (Dreisenhofen, Germany) was used as tryptophan standard for the quantum yield determination.

Vesicle Preparation. Dried POPG was suspended in Tris buffered solution (10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, pH = 7.4) and suspension used for the preparation of LUVs by freeze-thawing in liquid nitrogen for seven cycles and then extruding through polycarbonate filters (six times through two stacked 0.4 μm pore size filters followed by eight times through two stacked 0.1 μm pore size filters) using a thermobarrel extruder (Lipex Biomembranes Inc., Vancouver, Canada). The mean diameter of the LUVs was determined by dynamic light scattering to be 98 nm (N4 Plus, Coulter Corp., Miami, FL). SUVs for CD spectroscopy were prepared by sonication of the lipid suspension (25 min under nitrogen in ice water) using a titanium-tipped ultrasonicator. The lipid concentration was determined by phosphorus analysis (Böttcher et al., 1961).

Fluorescence Measurements. FET was characterized by the ratio I_{DA}/I_D, where I_{DA} represents the fluorescence intensity of the donor (Trp-Mag, DD-Trp-Mag) in the presence and I_D in the absence of the acceptor (DNS-Mag). The FET and hence the ratio I_{DA}/I_D depends on the distance between the two fluorophores (r)

$$I_{DA}/I_D = 1/(1 + (R_0/r)^6) \quad (1)$$

The characteristic distance R_0 is determined by the spectroscopic properties of the investigated system (Förster, 1948)

$$R_0^6 = (8.79 \times 10^{-28}\ \text{mol})(n^{-4} q_D k^2 J) \quad (2)$$

where n is the refractive index of the medium ($n = 1.4$; Fung & Stryer, 1978; Rapaport & Shai, 1992), k is the orientation factor ($k^2 = 2/3$), q_D is the quantum yield of the donor in the absence of acceptor, and J is the spectral overlap integral, $J = \int I_D(\lambda)\epsilon_A(\lambda)\lambda^4\, d\lambda$, which is estimated from the corrected and area normalized emission spectrum of the donor $I_D(\lambda)$ and the molar absorption coefficient of the acceptor $\epsilon_A(\lambda)$.

The FET was measured on a Perkin-Elmer LS 50B fluorimeter equipped with a custom-made thermostated cell holder adapted to quadratic cells with an inner length of 4 mm. The fluorescence was excited at 280 nm (slit = 5 nm) using an additional interference filter IF280 (Carl Zeiss, Jena, Germany). Emission spectra were measured using the 5 nm slit of the emission monochromator. The inner filter effect caused by the presence of peptide chromophores and light scattering by the lipid vesicles was corrected by the method of Lakowicz (1983). Background intensity measured for samples containing the same amount of nonlabeled magainin was subtracted from the observed intensities.

Tryptophan fluorescence intensities $I_{DA}$and I_D were measured at 337 nm with a bandpass of 10 nm and a red edge filter WG320 (Schott, Mainz, Germany) using an integration time of 5 s.

Fluorescence due to the dansyl residue (I_A and I_{AD} in the absence and the presence of a donor, respectively) excited at 280 nm (see above) was observed at 502 nm with a bandpass of 5 nm.

The corrected emission spectra for determination of quantum yields and spectral overlap were measured on a photon-counting fluorimeter FS900CDT (Edinburgh Analytical Instruments, Edinburgh, U.K.) without a filter (slits = 1.8 nm). Spectral sensitivity was determined using a calibrated deuterium lamp and a freshly prepared magnesium oxide scatterer. Quantum yields were determined comparing the area of the corrected fluorescence spectra of a 10 μM Trp-Mag or DD-Trp-Mag solution in 1 mM POPG-LUVs excited at 280 nm with that of a tryptophan standard in 3 mM phosphate buffer of pH = 7.0. A quantum yield of 0.14 was used for the tryptophan standard (Wiget et al., 1978).

Absorbance measurements were made with a Lambda 9 spectrometer (Perkin-Elmer, Überlingen, Germany) using the accessory for turbid samples. If necessary, an UG11 filter (Schott, Mainz, Germany) was set in front of the multiplier to block dansyl fluorescence.

Time-resolved fluorescence was measured at 330 nm (bandpass = 12.6 nm) with the photon-counting fluorimeter (Edinburgh Analytical Instruments) using a hydrogen-filled coaxial nanosecond flashlamp for excitation (280 nm, slit = 6 nm) and a time-correlated single-photon-counting system FL900CDT (time per channel = 0.0244 ns). Fluorescence decay of Trp-Mag in the presence of POPG-LUVs, background fluorescence of the POPG-LUVs in the presence of Mag, and the lamp impulse were measured interleaved. The lamp impulse was registered using the light scattered at 340 nm from a Ludox suspension (Edinburgh Analytical Instruments). Data analysis was done using the Edinburgh Instrument software. The results are given as lifetimes and fractional intensities in percent of total intensity.

All measurements were performed in buffer (10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, pH = 7.4) at a temperature of 25.0 ± 0.2 °C. Samples were prepared from lipid stock solutions by adding aliquots of peptide stock solutions so as to give various surface concentrations of the acceptor. For measuring I_D and I_A, unmodified magainin was added to the samples to guarantee equal peptide concentration in the solutions used for the determination of I_{DA}, I_D, and I_{AD}, I_A, respectively. The given ratios I_{DA}/I_D and standard deviations were obtained from the ratios of three pairs of samples calculated from the mean of five measurements of I_D and four interleaved measurements of I_{DA}. The measured fluorescence intensities did not show time dependency after an equilibration time of 1 min at lipid-to-peptide ratios $C_L/C_P \geq 100$ (mol/mol). Since I_{DA}/I_D measured at $C_L/C_P = 20$ decreased slightly with time, data measured between 1 and 5 min after peptide lipid mixing were used.

CD spectroscopy. CD measurements of magainin peptides (50 μM) in the presence of POPG-SUVs (1 mM in 10 mM Tris, 154 mM NaCl, 0.1 mM EDTA, pH = 7.4) were

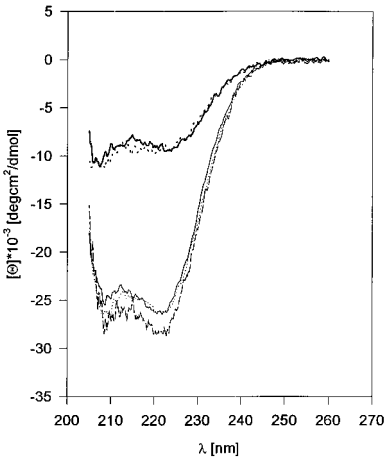

FIGURE 1: CD spectra of magainin peptides (peptide concentration 50 μM) in the presence of POPG-SUVs (lipid concentration = 10 mM) lower curves: —, Trp-Mag; ···, Mag; — — —, DNS-Mag, upper curves: —, DD-Trp-Mag; •••, DD-Mag.

performed on a J 720 spectrometer (Jasco) in a cell of 1 mm path length at room temperature. For each spectrum, six scans were accumulated and the baseline (POPG-SUVs without peptide) was subtracted. Helicity was calculated from the mean residue ellipticity [Θ] at 222 nm (Chen et al., 1972).

RESULTS

Characterization of the Fluorescent Magainin Analogs. Since the CD spectra of Trp-Mag and Mag in POPG vesicle suspension superimpose (Figure 1), substitution of phenylalanine in position 16 by tryptophan does not influence the peptide conformation. Both spectra are characteristic for a helical conformation and correspond to a helicity of 75%. The helicity of the DNS-Mag was found to be somewhat higher (86%). This increase has to be associated with sulfonation of the N-terminal amino group. Protection of the N-terminus reduces the helical dipole and is expected to stabilize a helical conformation (Shoemaker et al., 1987).

Double D-amino acid substitution at positions 15 and 16 causes a similar disturbance of the helical structure in DD-Mag and DD-Trp-Mag as shown by the reduced CD intensity (Figure 1). A helicity of 26% was determined for the two double D-substituted magainin analogs. More than 99% of the peptides are bound under the used experimental conditions as shown by binding studies of magainin and double D-isomers (Wieprecht et al., 1996). Thus, the spectra presented reflect the conformations of the completely bound magainins.

The fluorescence spectra of the tryptophan-containing analogs bound to POPG-LUVs are blue-shifted in comparison with the emission spectra of the same peptides in buffer solution (Figure 2). The quantum yields are 0.197 ± 0.01 for Trp-Mag and 0.174 ± 0.01 for DD-Trp-Mag. The blue-shift and the increased quantum yield (Table 1) point to a more hydrophobic environment of the tryptophan side chain in the lipid-bound peptides (Burstein et al., 1973; Chung et al., 1992). Reduction of the molar lipid-to-peptide ratio from 125 to 20 changes neither the wavelength of the emission maximum nor the shape of the fluorescence spectrum nor the quantum yield, confirming that Trp-Mag and DD-Trp-Mag are completely bound at negatively charged vesicles under these conditions.

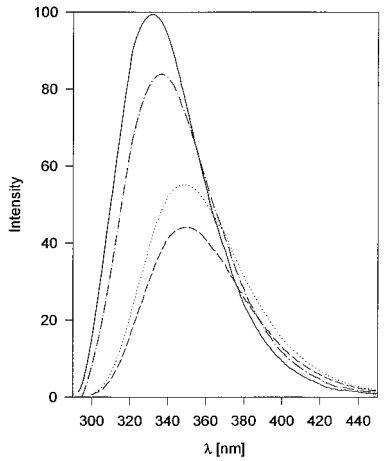

FIGURE 2: Corrected fluorescence emission spectra of Trp-Mag and DD-Trp-Mag in the presence and absence of POPG-LUVs: —, Trp-Mag in POPG; —·—, DD-Trp-Mag in POPG; ···, DD-Trp-Mag in buffer; - - -, Trp-Mag in buffer; (concentration of each peptide = 10 μM; lipid concentration = 1 mM).

Table 1: Fluorescence of Trp^{16}-Magainin Analogs in Buffer and in the Presence of POPG-LUV[a]

	C_L. (mM)	λ_{max} (nm)	q_D
Trp-Mag	0	350	0.103 ± 0.01
Trp-Mag	1	331.5	0.197 ± 0.01
DD-Trp-Mag	0	349	0.126 ± 0.01
DD-Trp-Mag	1	336	0.174 ± 0.01

[a] Maxima of the corrected emission spectra (λ_{max}) and quantum yields (q_D) were measured at lipid concentration (C_L) and peptide concentration = 10 μM.

Table 2: Fluorescence of DNS-Mag in the Presence of POPG-LUV[a]

C_L	C_L/C_{DNS}	λ_{max} (nm)	I_{502nm}/C_{DNS}
1.8 mM	625	499	69 ± 2
1.0 mM	200	498	69 ± 1
1.0 mM	20	498	73 ± 2
buffer	0	543	3.9 ± 0.1

[a] Dansyl fluorescence emission maxima without spectral correction (λ_{max}) and relative intensities at 502 nm (I_{502nm}) excited at 340 nm were measured at different lipid concentrations C_L and DNS-Mag concentrations C_{DNS}.

The fluorescence spectrum of DNS-Mag in the presence of POPG-LUVs is blue-shifted by 42 nm, the intensity being enhanced by a factor of about 17 compared to the buffer (Table 2). These fluorescence changes also indicate a nonpolar environment of the N-terminal dansyl group in the lipid-bound state (Li et al., 1975). Since no changes are observed upon decreasing the molar lipid-to-peptide ratio from 625 to 20, it can be concluded that under these conditions DNS-Mag is also completely lipid-bound.

The functional properties of Mag, Trp-Mag, DNS-Mag, and DD-Trp-Mag were characterized by their growth inhibiting effect on *Escherichia coli* DH 5α bacteria. Under the used conditions [see Dathe et al. (1996a)], the bacterial growth after 1 h of incubation at 20 μM peptide concentration was 59, 70, 26, and 100%, respectively, compared to 100% growth of the control. While the all L-peptides were biologically active, DD-Trp-Mag did not influence the bacterial growth. The result with DD-Trp-Mag is in accordance with observation of an other D-amino acid substituted magainin (Chen et al., 1988).

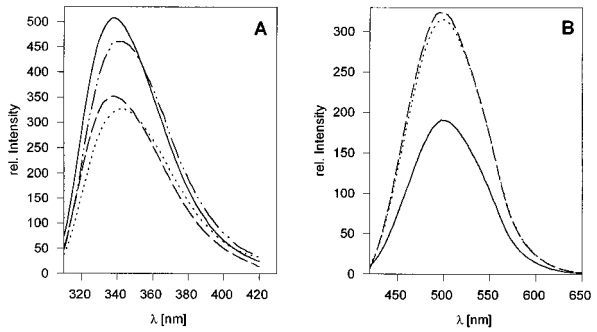

FIGURE 3: (A) Fluorescence emission spectra of Trp-Mag and DD-Trp-Mag in equimolar mixtures with Mag and DNS-Mag: —, Trp-Mag + Mag; – –, Trp-Mag + DNS-Mag; ••—••, DD-Trp-Mag + Mag; •••, DD-Trp-Mag + DNS-Mag. (B) Fluorescence emission spectra of DNS-Mag in equimolar mixtures with —, Mag; – –, Trp-Mag; •••, DD-Trp-Mag; (peptide concentration = 25 μM; lipid concentration = 1 mM).

Table 3: Comparison of FET of Trp-Mag and DD-Trp-Mag to DNS-Mag[a]

			I_{DA}/I_D measured at 337 nm	
C_{DNS} (μM)	C_{DNS}/C_P	C_L/C_P	Trp-Mag	DD-Trp-Mag
7.5	0.75	125	0.900 ± 0.013	0.898 ± 0.03
7.5	0.75	100	0.855 ± 0.01	0.860±0.015
9.0	0.9	100	0.800 ± 0.03	nd
25.0	0.5	20	0.693 ± 0.02	0.689 ± 0.03

[a] Ratio I_{DA}/I_D was determined using Trp-Mag or DD-Trp-Mag at different lipid-to-peptide ratios; C_{DNS} = molar concentration of DNS-Mag, C_P = total peptide concentration of all magainin analogs, C_L = lipid concentration; nd = not determined.

Intermolecular FET. To detect the FET from Trp-Mag to DNS-Mag the fluorescence spectrum of Trp-Mag was compared with the corresponding spectrum of a mixture of Trp-Mag and DNS-Mag in the presence of POPG-LUVs at a molar lipid:peptide ratio of 20 (Figure 3A). The maxima and shape of the emission spectra are identical, although the intensity of the Trp emission is reduced in the presence of the acceptor; I_{DA}/I_A was determined to be 0.693 (Table 3). If the intensity change is caused by fluorescence energy transfer from tryptophan to the dansyl chromophore, the fluorescence intensity emitted by DNS-Mag should increase by the factor

$$I_{AD}/I_A = 1 + (\epsilon_{Trp-Mag}C_{Trp}/\epsilon_{DNS-Mag}C_{DNS})e_{FET} \quad (3)$$

I_{AD}/I_A is the ratio of fluorescence intensities emitted at 502 nm by the acceptor in the presence (I_{AD}) and absence (I_A) of the donor. C_{Trp} and C_{DNS} are the molar concentrations of Trp-Mag and DNS-Mag, $\epsilon_{Trp-Mag}/\epsilon_{DNS-Mag} = 2.3$ is the estimated ratio of the absorption coefficients of Trp-Mag and DNS-Mag at the excitation wavelength 280 nm, and $e_{FET} = 1 - I_{DA}/I_D$ is the transfer efficiency determined from the tryptophan fluorescence intensity. The calculated value of the ratio I_{AD}/I_A corresponds to the observed increase in the fluorescence intensity of DNS-Mag (Table 4, Figure 3B), demonstrating that changes in fluorescence intensities are caused exclusively by FET. For the FET of Trp-Mag and DD-Trp-Mag to DNS-Mag, R_0 values of 2.27 and 2.22 nm, respectively, were obtained according to eq 2 using the corrected spectra of tryptophan emission (Figure 2), the quantum yields given in Table 1, and the absorption spectrum of DNS-Mag bound to POPG-LUVs (not shown).

Table 4: FET Measured by DNS-Mag Emission and Predicted by Trp-Mag Quenching[a]

			I_{AD}/I_A measured at 502 nm		I_{AD}/I_A predicted by eq 3	
C_{DNS} (μM)	C_{DNS}/C_{Trp}	C_L/C_P	Trp-Mag	DD-Trp-Mag	Trp-Mag	DD-Trp-Mag
7.5	3	125	1.09 ± 0.03	1.06 ± 0.03	1.08	1.08
25.0	1	20	1.70 ± 0.03	1.66 ± 0.04	1.71	1.71

[a] I_{AD}, I_A are the fluorescence intensities emitted at 502 nm by DNS-Mag in the presence or absence of Trp-Mag, respectively; I_{AD}/I_A predicted by eq 3 as described in the text using the experimentally determined ratios I_{DA}/I_D given in Table 3; C_{Trp} = molar concentration of Trp-Mag or DD-Trp-Mag, C_{DNS} = molar concentration of DNS-Mag, C_P = total peptide concentration of all magainin analogs, and C_L = lipid concentration.

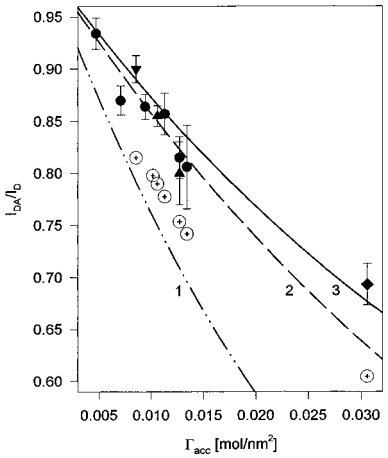

FIGURE 4: Fluorescence intensity of Trp-Mag as a function of DNS-Mag surface concentration. I_{DA}/I_D = ratio of tryptophan fluorescence intensities at 337 nm in the presence (I_{DA}) and absence of the acceptor (I_D); Γ_{acc} = number of bound DNS-Mag molecules per total vesicle surface (eq 6) ●, C_L = 2 mM, C_L/C_P = 100; ▼, C_L = 1 mM, C_L/C_P = 125; ▲, C_L = 1 mM, C_L/C_P = 100; ◆, C_L = 1 mM, C_L/C_P = 20. Curves calculated for C_L/C_P = 100: curve 1 calculated according to eqs 4, 5, and 7, assuming that magainin is bound exclusively at the outer leaflet with R_c = 1 nm; curve 2 calculated according to eqs. 4−6 with R_c = 1 nm assuming a distribution of magainin on the outer and inner leaflets; curve 3 calculated as curve 1 but using R_c = 2 nm. ⊕, I_{DA}/I_D ratios predicted for random dimerization of magainin peptides including 10% of the molecules by $I_{DA}/I_D = 0.9\ M + 0.1[M(1 - C_{DNS}/C_P) + DC_{DNS}C_P]$. C_{DNS}/C_P is the fraction of DNS-Mag in relation to the total amount of magainin, D = 0.05 is the relative fluorescence intensity of the donor in a hypothetical dimer (see text), and M is the intensity ratio I_{DA}/I_D of monomeric magainin taking into account the FET between magainin molecules randomly distributed at outer and inner leaflets (curve 2); peptide concentrations as given in Table 3. It is assumed that the FET between randomly distributed Trp-Mag and homodimers of DNS-Mag is nearly equivalent to the FET between randomly distributed Trp-Mag and an equal number of monomeric DNS-Mag molecules. FET of monomeric Trp-Mag and homodimers of Trp-Mag to randomly distributed DNS-Mag is assumed to be identical.

The change of the tryptophan fluorescence intensity at 337 nm was used to quantify the FET according to eq 1. As shown in Table 3, the ratio I_{DA}/I_D decreases with a decreasing lipid-to-peptide ratio, thus, corresponding to the increasing surface concentration of magainin (Figure 4). However, the observed changes are rather small and cannot be explained exclusively by FET within oligomers. In two-dimensional systems such as lipid bilayers, the probability of finding an acceptor molecule in the environment of an excited Trp-Mag molecule cannot be neglected. Thus, FET between

monomeric magainin analogs has to be taken into consideration. This transfer is a function of the surface concentration of the lipid-bound acceptor (Γ_{DNS}) and the distance of closest approach between donor and acceptor (R_C). An approximation for $\Gamma_{DNS} \leq 0.5/R_0^2$ and $R_C < 3.5$ nm was derived by Wolber and Hudson (1979):

$$I_{DA}/I_D = a \exp(-b\Gamma_{DNS}R_0^2) + c \exp(-d\,\Gamma_{DNS}\,R_0^2) \quad (4)$$

The parameters *a*, *b*, *c*, and *d* were given as functions of R_C/R_0. For a model of two α-helical magainin molecules, an R_C value of about 1 nm was estimated (Molecular visualization software Sybyl, Tripos Inc., St. Louis, MO). The surface concentration of DNS-Mag (Γ_{DNS}) was estimated from the number of DNS-Mag molecules and the surface area (*A*) of all vesicles in the observed volume (area per volume unit in nanometers squared per liter).

$$\Gamma_{DNS} = C_{DNS}N/A \quad (5)$$

C_{DNS} is the molar concentration of DNS-Mag and *N* is the Avogadro number.

The total (inner plus outer) surface area of the lipid vesicles per unit volume (A_t) was estimated from the molar lipid concentration (C_L) and the total molar concentration of all types of magainin (C_P) assuming an area per lipid molecule $A_L = 0.68$ nm^2 (Evans et al., 1987) and an increase of surface area per adsorbed magainin molecule $A_P = 2.75$ nm^2 (Dathe et al., 1996b).

$$A_t = N(C_LA_L + C_PA_P) \quad (6)$$

Assuming that magainin is bound exclusively at the outer surface of the vesicles and that only 54% of the lipid molecules are in the outer leaflet of a vesicle with a diameter of 98 nm, the following expression has been derived for the surface of the outer leaflet per unit of volume:

$$A = 0.54N(C_LA_L + C_PA_P) \quad (7)$$

Curve 1 in Figure 4 shows the calculated ratio I_{DA}/I_D for FET of monomeric magainin molecules randomly distributed at the outer surface of the vesicles (eqs 4, 5, and 7). If the magainin molecules are associated, an additional FET within the associates is expected along with lower I_{DA}/I_D ratios than those given by curve 1 (Figure 4). However, the experimentally determined values are significantly higher than those determined from curve 1. Consequently, the observed FET provides no evidence for peptide association.

The experimentally observed FET of Trp-Mag and DD-Trp-Mag are identical (Table 3), showing that both α-helical Trp-Mag and the nearly nonhelical DD-Trp-Mag exist in nearly identical states with respect to association.

In order to examine the homogeneity of the state of bound Trp-Mag in the presence and absence of DNS-Mag, the fluorescence decay of Trp-Mag bound to POPG-LUVs was investigated. The fluorescence decay of Trp-Mag in the absence of DNS-Mag can be described by three fluorescence lifetimes ($\tau_1 = 4.30$ ns, $\tau_2 = 1.97$ ns, $\tau_3 = 0.49$ ns, with 60.9%, 30.2%, and 8.9% fractional intensity, respectively; mean lifetime $\tau_D = 3.25$ ns). The measurement in the presence of DNS-Mag is documented in Figure 5. To test whether the decay curve contains contributions of short lifetimes which could be expected for associated molecules,

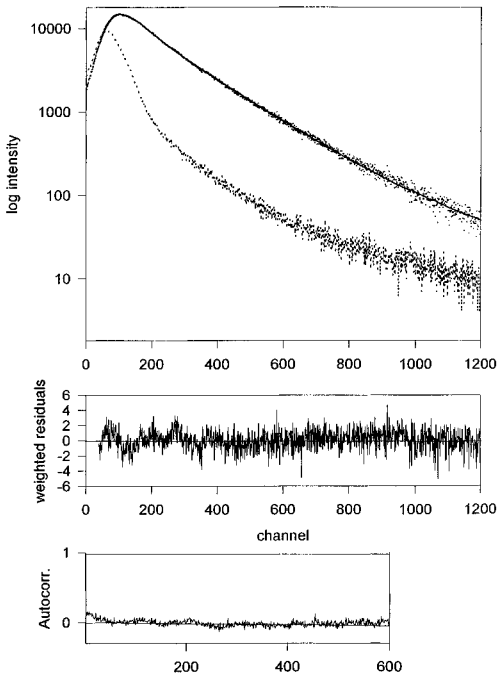

FIGURE 5: Fluorescence decay of Trp-Mag bound to POPG-SUVs in the presence of DNS-Mag (upper curve): peptide concentration = 50 μM, lipid concentration 2 = mM ($\Gamma_{acc} = 0.007\,07$ mol/nm^2). The decay times were determined to be 3.66 (68.5%) and 1.1 ns (31.5%). The mean lifetime is 2.86 ns. The lower curve shows the lamp impulse. The time per channel is 0.0244 ns.

a fixed value of 0.1, 0.2, or 0.4 ns was used in fitting procedures with three exponents. The data cannot be reasonably fitted by a three-exponential function. The fractional intensity of the short lifetimes was found to be below 2%. The determined lifetimes were $\tau_1 = 3.66$ ns (68.5%) and $\tau_2 = 1.10$ ns (31.5%) with a mean lifetime of $\tau_{DA} = 2.86$ ns. The ratio of mean lifetimes ($\tau_{DA}/\tau_D = 0.88$) and intensities ($I_{DA}/I_D = 0.87 \pm 0.014$) determined under identical conditions were found to be equal.

DISCUSSION

The positive cooperativity of membrane permeabilization by magainin observed in different systems (Matsuzaki et al., 1991; Juretic et al., 1994; Haimovich et al., 1995) implies that the association of lipid-bound magainin is important for its activity. Spectroscopic investigations showed that magainin forms a helix in the lipid-bound state which is oriented parallel to the bilayer surface (Bechinger et al., 1993; Matsuzaki et al., 1994; Wieprecht et al., 1996). On the basis of the slight positive cooperativity observed in peptide binding at model bilayers and the dependence of fluorescence quantum yield of [Trp^{12}]magainin 2 on the peptide surface concentration, the formation of magainin dimers was suggested (Matsuzaki et al., 1994). Side-by-side dimerization was suggested as the initial step in the formation of transmembranal pores (Matsuzaki et al., 1994, 1995a,b). The aim of our work was to examine the associational state of magainin analogs in the lipid-bound state by FET.

Our experiments show that in the presence of a 10-fold excess of DNS-Mag the Trp-Mag fluorescence intensity is reduced by about 20% compared to that of Trp-Mag in the absence of acceptor. The observed reduction in Trp-Mag fluorescence intensity is exclusively caused by FET to DNS-Mag (Table 4).

At the surface of vesicles energy transfer may be observed between monomeric magainin molecules and also within stable associates of Trp-Mag and DNS-Mag. From the fact that all determined ratios I_{DA}/I_D lie above curve 1 (Figure 4), which describes the FET of monomeric magainin molecules randomly distributed at the outer surface of the lipid vesicles, it is concluded that the existence of a significant amount of associated magainin is not likely. The high values of the ratio I_{DA}/I_D can be explained by either of the following two models of the spatial distribution of monomeric magainin analogs:

(i) Matsuzaki et al. (1995a) reported that permeabilization of lipid vesicles by magainin is associated with a translocation of peptide from the outer to the inner vesicle leaflet. This translocation could result in a uniform distribution of magainin at the inner and outer vesicle surfaces. In this case, the total vesicle surface (A_t) given by eq 6 should be used for the estimation of FET. The ratios I_{DA}/I_D calculated assuming a random distribution of magainin analogs at the inner and outer vesicle leaflets (eqs 4−6) are represented as curve 2 in Figure 4. Our experimental results agree well with this curve.

(ii) Furthermore, the observed I_{DA}/I_D ratios can be fitted by eqs 4, 5, and 7 using, however, an increased distance of closest approach, R_c. Using $R_c = 2$ nm, the calculated values for monomeric magainin randomly distributed at the outer vesicle surface are in a good agreement with the measured data (Figure 4, curve 3). An R_c of 2 nm instead of the $R_c = 1$ nm estimated from the molecular geometry would mean that at least one shell of lipids exists between two magainin molecules. The existence of peptide-associated lipid molecules was suggested by Reynaud et al. (1993). Such a lipid shell around the magainin molecules reduces the probability of direct peptide−peptide contact. It can be expected that the existence of such a lipid shell will hamper association of lipid-bound magainin.

The two proposed models explain our experimental results without requiring FET within stable associates of magainin analogs. Energy transfer within stable oligomers would depend on the percentage of Trp-Mag associated with at least one DNS-Mag and the distance between the donor and acceptor within the oligomer. A distance of about 1.4 nm was estimated for a hypothetical antiparallel dimer of helical Trp-Mag and DNS-Mag (molecular visualization software Sybyl, Tripos Inc. St. Louis, MO). The ratio $I_{DA}/I_D = D \cong 0.05$ was calculated (eq 1) assuming that all Trp-Mag molecules are involved in such dimers. In higher associates, FET should be even more efficient. For a hypothetical random dimerization of 10%, a reduction of I_{DA}/I_D by 0.07 caused by FET within dimers is predicted ($C_{DNS}/C_P = 0.9$, $C_L = 1$ mM, $C_L/C_P = 100$). Random dimerization means that the probability of heteroassociation (DNS-Mag/Trp-Mag) and homoassociation (DNS-Mag/DNS-Mag or Trp-Mag/Trp-Mag) is the same**.** Assuming a random distribution of magainin at the outer and inner leaflets (eqs 4−6, $R_C = 1$ nm) and 10% random dimerization**,** the predicted I_{DA}/I_D under various experimental conditions are given by the circled crosses in Figure 4. The significantly higher determined I_{DA}/I_D values clearly argue against substantial association.

In addition, association of peptide helices can be expected to be reduced if the helical structure of at least one of the components is disturbed. Pouny and Shai (1992) reported that association of lipid-bound pardaxin is prevented by the introduction of a D-amino acid in the helical region of the peptide. Disturbance of the amphipathic helix of magainin by double D-amino acid substitution should also significantly reduce the formation of magainin associates and consequently decrease FET caused by association. However, our results show that the FETs of Trp-Mag and DD-Trp-Mag to DNS-Mag are identical (Table 3), confirming our conclusion that the observed FET is not the result of magainin association.

Furthermore, FET within hypothetical heterodimers described above should reduce the fluorescence intensity and fluorescence lifetime by the same degree (Cheung, 1991). Different molecular species contribute to the intensity according to their number, but the mean lifetime is a value related to the contribution of all species to the total intensity. That means, differently quenched species would keep the mean lifetime nearly unchanged but would influence the mean intensity according to the number of the fluorophores. We did not find differences in the fluorescence intensities I_{DA}/I_D and lifetimes τ_{DA}/τ_D, revealing that all Trp-Mag molecules are quenched by FET to DNS-Mag in the same degree and excluding the existence of a class of highly quenched associated Trp-Mag.

Additionally, a lifetime of about 0.2 ns could be expected for Trp-Mag/DNS-Mag heteroassociates. But, the fluorescence decay of Trp-Mag in the presence of DNS-Mag does not reveal the contribution of such a short lifetime component. Lifetime measurements of a Trp standard (three exponential decay) with the used device differed by less than 1%. Furthermore, we can assume that the error of the intensity measurements is about 1% since the precision of repeated measurements of I_{DA}/I_D of one sample is better than 1%. Taken together, we suggest the amount of associated magainin to be probably smaller than 3%.

Our findings agree with results describing the randomly distributed monomeric state of cecropin P1, another helical amphipathic peptide (Gazit et al., 1995) which is also able to permeabilize lipid bilayers and shows an upward curvature in the binding isotherm. It has been suggested that cecropin P1 disrupts lipid packing in the bilayer (Gazit et al., 1995, 1996). Peptide-induced changes of lipid packing were also suggested to be an important step in bilayer permeabilization by magainin (Grant et al., 1992). It is reasonable to assume that changes within the bilayer structure at high magainin surface concentrations will influence the spectroscopic properties of bound peptides and the binding of other magainin molecules. Therefore, the changes in properties observed by Matsuzaki et al. (1994) can be explained without assuming peptide association. Since preformed stable oligomers are less likely to exist at significant concentrations, such structures cannot be used to explain the formation of multimeric pores.

The high frequency of current fluctuations observed in magainin exposed lipid bilayers (Dathe et al., 1996b) and patches of cell membranes (Haimovich, 1995) suggests that the ion-permeable membrane state is unstable and short-lived. Permeabilization in the absence of an electric potential can also be explained by assuming the presence of transient leaky patches in the bilayer (Grant et al., 1992; Matsuzaki et al., 1995b,1996).

Using time-resolved fluorescence measurements, associated structures with lifetimes significantly greater than the fluorescence lifetime in heteroassociates should be detectable.

Assuming an independent and random distribution of Trp-Mag and DNS-Mag on the bilayer surface at the surface concentration of DNS-Mag ($\Gamma_{acc} = 0.007\,07$) used for the lifetime measurements, with a probability of about 10%, Trp-Mag and DNS-Mag would be found within a distance ≤ 2.2 nm. This probability corresponds to the observed FET which we described by randomly distributed monomeric magainins.

With respect to the fact that a distance of 2.2 nm is smaller than the length of a magainin helix (3.4 nm) and taking into consideration an amount of magainin molecules in the associated state below 3% or a lifetime of the associates in the range of the fluorescence lifetime, the question arises: What are associates? Our interpretation refuses the existence of associates, but the results do not exclude transient structures consisting of several magainin molecules as result of local fluctuations of the surface concentration.

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