Interaction of Antimicrobial Dermaseptin and Its Fluorescently Labeled Analogues with Phospholipid Membranes†

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ABSTRACT: Dermaseptin, a 34 amino-acid residue antimicrobial polypeptide [Mor, A., Nguyen, V. H., Delfour, A., Migliore-Samour, D., & Nicolas, P. (1991) *Biochemistry 30*, 8824–8830] was synthesized and selectively labeled at its N-terminal amino acid with either 7-nitrobenz-2-oxa-1,3-diazole-4-yl (NBD), rhodamine, or fluorescein. The fluorescent emission spectra of the NBD-labeled dermaseptin displayed a blue-shift upon binding to small unilamellar vesicles (SUV), reflecting the relocation of the fluorescent probe to an environment of increased apolarity. Titrations of solutions containing NBD-labeled dermaseptin with SUV composed of zwitterionic or acidic phospholipids were used to generate binding isotherms, from which were derived surface partition constants of $(0.66 \pm 0.06) \times 10^4$ M^{-1} and $(2.8 \pm 0.3) \times 10^4$ M^{-1}, respectively. The shape of the binding isotherms, as well as fluorescence energy transfer measurements, suggests that some aggregation of membrane-bound peptide monomers occurs in acidic but not in zwitterionic vesicles. The preferential susceptibility of the peptide to proteolysis when bound to zwitterionic but not to acidic SUV suggests that these aggregates might then penetrate a relatively short distance into the hydrophobic region of the acidic membrane. Furthermore, the results provide good correlation between the peptide's strong binding and its ability to permeate membranes composed of acidic phospholipids, as revealed by a dissipation of diffusion potential and a release of entrapped calcein from SUV.

The naked skin of frogs plays an essential role in the defense mechanism against predators and microbes. Hence the "healing power" of frog skin, exploited even nowadays by African and American natives, is apparently due to the presence of several broad-spectrum antibiotic peptides, secreted by the dermal glands of amphibians (Nicolas et al., 1992). The first cytotropic substance discovered in the frog skin turned out to be a 24-residue hemolytic peptide isolated from the skin secretions of *Bombina varietaga* (Csordas & Michl, 1970) and named bombinin. Later, two highly homologous 23-residue antimicrobial peptides named magainin I and II (Zasloff, 1987) or, respectively, PGS and [$Gly^{10}Lys^{22}$]PGS (Terry et al., 1988), have been isolated from the skin of the African clawed frog, *Xenopus laevis*. These peptides share to a variable extent sequence homology with bombinin and were shown to be active against microorganisms including Gram-positive and Gram-negative bacteria, yeast, and protozoa.

Recently, a novel 34-residue antimicrobial peptide, termed dermaseptin,

ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ

was isolated from a skin extract of *Phyllomedusa sauvagii* and shown to be active at micromolar concentrations against pathogenic fungi (Mor et al., 1991a,b; Nicolas et al., 1992). As it was observed for other antimicrobial peptides, predictions of secondary structure and circular dichroism spectra indicated that, in hydrophobic media, dermaseptin can be configured in an amphipatic α-helix spanning over residues 1–27. As such, it was stipulated to interact with fungal membranes and perturb functions governing osmotic balance. Thus, dermaseptin may be considered part of a growing family of antimicrobial peptides which provide vertebrates, in addition to their immune system, with a cell-free, chemical defense system that allows animals to defend themselves against host invasion of occasional or obligate pathogens, as well as against uncontrolled proliferation of commensal microorganisms. The primary structures of these peptides present a large hydrophobic moment and a high propensity to adopt an α-helical conformation in hydrophobic media (Chen et al., 1988; Zasloff et al., 1988; Mor et al., 1991a). Although not proven, the available data on their mode of action suggest that they bind to the acidic components of the target cell membrane inducing permeability changes [for a recent review, see Nicolas et al. (1992)].

In this paper, we describe the mode of interaction and membrane-permeating properties of dermaseptin, tested with zwitterionic or acidic phospholipid bilayers. Labeling of the peptide at its N-terminal amino acid residue with the fluorophore 7-nitrobenz-2-oxa-1,3-diazole-4-yl (NBD)[1] allowed for examination of the location and the binding state of the peptide when bound to phospholipid bilayers, as well as for calculation of its surface partition coefficients. Furthermore, fluorescence energy transfer experiments were performed with the peptide labeled at its N-terminal amino acid residue with the fluorophores fluorescein (Flu) (energy donor) or tetramethylrhodamine (Rh) (energy ac-

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[1] Abbreviations: Fmoc, N^{α}-9-fluorenylmethyloxycarbonyl; Pfp, pentafluorophenyl; Dhbt, 3-hydroxy-2,3-dehydro-4-oxobenzotriazine; RP-HPLC, reverse-phase high-performance liquid chromatography; DIEA, diisopropylethylamine; HF, hydrogen fluoride; TFA, trifluoroacetic acid; SUV, small unilamellar vesicles; CD, circular dichroism; diS-C_2-5, 3,3′-diethylthiodicarbocyanine iodide; PC, egg phosphatidylcholine; PS, phosphatidylserine; NBD-F, 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole.

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ceptor) (Rapaport & Shai, 1992). Finally, susceptibility of membrane-bound fluorescein-labeled dermaseptin to enzymatic digestion was also assayed.

Our data show that both the partition coefficient and the membrane-permeating activity of dermaseptin obtained with acidic phospholipids bilayers are higher than those obtained with zwitterionic vesicles. Dermaseptin bound to acidic but not to zwitterionic phospholipids is resistant to proteinase digestion. Overall, these results suggest that electrostatic interactions play a central role in the mode of action of dermaseptin.

EXPERIMENTAL PROCEDURES

Materials. N^{α}-9-Fluorenylmethyloxycarbonyl-amino acids (Fmoc-amino acids) were from Milligen. HMP-linked polyamide/Kieselguhr resin (Pepsin KA) and Fmoc-amino acid pentafluorophenyl (Pfp) and 3-hydroxy-2,3-dehydro-4-oxobenzotriazine (Dhbt) esters were from Milligen/Bioresearch. Other reagents for peptide synthesis were obtained from Sigma. Calcein was purchased from Hach Chemical Co. (Loveland, CO). L-α-Phosphatidylcholine (type IVS) was obtained from Sigma and further purified by the method of Kagawa and Racker (1971). Egg phosphatidylcholine (PC) and phosphatidylserine (PS) from bovine spinal cord (sodium salt, grade I) were purchased from Lipid Products (South Nutfield, U.K.). Cholesterol (extra pure) purchased from Merck (Darmstadt, Germany) was recrystallized twice from ethanol. 3,3'-Diethylthiodicarbocyanine iodide (diS-C_2-5), 5- (and 6-) carboxyfluorescein, succinimidyl ester (Flu-Su), and 5- (and 6-) carboxytetramethylrhodamine, succinimidyl ester (Ru-Su), were obtained from Molecular Probes (Eugene, OR). 4-Chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) was obtained from Sigma. All other reagents were of analytical grade. Buffers were prepared using double-glass-distilled water.

Peptide Synthesis, Fluorescent Labeling, and Purification. Dermaseptin was synthesized by a solid-phase method, applying the Fmoc active ester chemistry on a Milligen 9050 Pepsynthesizer. After removal of the Fmoc from the N-terminal amino acid, the peptide was cleaved from the resin with a mixture of 85:5:5:5 TFA/*p*-cresol/H_2O/thioanisole (10 mg of resin-bound peptide in a 1-mL mixture). The TFA was then evaporated, and the peptide was precipitated with ether followed by washing with ether (4×). The crude peptide was extracted from the resin with 40% acetonitrile in water and was purified to chromatographic homogeneity in the range of 95% to >99% by reverse-phase HPLC on a semipreparative C_4 column using a linear gradient of 25–80% acetonitrile in water (both containing 0.1% TFA), in 40 min. The peptide was subjected to amino acid analysis and fast atom bombardment mass spectrometry (FAB) in order to confirm its composition.

Labeling of the peptide at its N-terminal amino acid with fluorescent probes was performed on resin-bound peptide as previously described (Rapaport & Shai, 1991). Briefly, 20–30 mg of resin-bound peptide (~6 μmol) was treated with piperidine in DMF in order to remove the Fmoc protecting group of the N-terminal amino acid of the linked peptide. The resin-bound peptide was then reacted with (i) 4-chloro-7-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) in DMF [containing 3% (v/v) triethylamine] (20 μmol), (ii) 5-(and 6-)-carboxyfluorescein, succinimidyl ester (Flu-Su) (10 μmol), or (iii) 5-(and 6-)-carboxytetramethylrhodamine, succinimidyl ester (Rh-Su) (10 μmol). After 24 h, the resin-bound peptides were washed thoroughly with methylene chloride, and the peptides were then cleaved from the corresponding resins with a mixture of 85:5:5:5 TFA/*p*-cresol/H_2O/thioanisole (10 mg of resin-bound peptide in a 1-mL mixture), precipitated with ether, and finally extracted from the resin with 40% (v/v) acetonitrile/water. Final purification was achieved by using reverse-phase HPLC as described above.

Preparation of Small Unilamellar Vesicles. Small unilamellar vesicles (SUV) were prepared from soybean lecithin, PC, or PC/PS (1:1 w/w) by sonication. Briefly, dry lipid and cholesterol were dissolved in $CHCl_3$/MeOH (2:1 v/v) such that the mixture contained 10% (w/w) cholesterol. The solvents were evaporated under a stream of nitrogen, and the lipids (at a concentration of 7.2 mg/mL) were resuspended in buffer by vortex mixing. The resulting lipid dispersion was sonicated (10–20 min) in a bath-type sonicator (G1125SP1 sonicator, Laboratory Supplies Company Inc., New York) until the turbidity had cleared. The lipid concentration of the solution was then determined by phosphorus analysis (Barlett et al., 1959). Vesicles were visualized as follows: A drop containing vesicles was deposited onto a carbon-coated grid and negatively stained with uranyl acetate. The grids were examined using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan). Vesicles were shown to be unilamellar with an average diameter of 20–40 nm (Papahadjopoulos & Miller, 1967).

Intrinsic Fluorescence Measurements. NBD-dermaseptin (0.1 μM) was added to 2 mL of buffer containing 100 μL (750 μM) of PC or PC/PS (1:1 w/w) SUV [containing 10% (w/w) cholesterol] to establish a lipid/peptide ratio in which most of the peptide is bound to lipid. After a 2-min incubation, the emission spectrum of the NBD group was recorded (in three separate experiments) using an SLM-8000 spectrofluorometer (SLM Instruments, Urbana, IL), with excitation at 468 nm (4-nm slit).

Binding Experiments. In order to determine the degree of NBD-dermaseptin association with lipid vesicles, PC or PC/PS (1:1 w/w) SUV were added successively to a fixed amount of NBD-dermaseptin (0.1 μM) at 24 °C. The fluorescence intensity of the mixture was measured as a function of the lipid/peptide molar ratio (in three separate experiments) on a Perkin-Elmer LS-5 spectrofluorometer, with excitation set at 468 nm, using a 10-nm slit, and with emission set at 530 nm, using a 5-nm slit. In order to determine the extent of the lipids' contribution to any given signal, the readings observed when unlabeled dermaseptin was titrated with lipid vesicles were subtracted as background from the recorded fluorescence intensity.

Resonance Energy Transfer Experiments. Flu-dermaseptin (0.22 μM) (serves as a donor) was added to a dispersion of PC or PC/PS (1:1 w/w) SUV (215 μM) in buffer (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8), followed by the addition of Rh-dermaseptin (which serves as an acceptor) in several sequential doses. Fluorescence spectra were obtained before and after the addition of the acceptor. Any changes in the fluorescence intensity of the donor due to processes other than energy transfer to the acceptor were determined by substituting unlabeled dermaseptin in place of the acceptor or by sequential addition of acceptor to vesicles alone. Fluorescence spectra were obtained at room temperature in an SLM-8000 spectrofluorometer, with the excitation monochromator set at 470 nm with a 4-nm slit width. Measurements were performed in a 1-cm path-length glass cuvette in a final reaction volume of 2 mL. Although the excitation maximum for fluorescein is 490 nm, a lower wavelength was

chosen in order to minimize the excitation of tetramethylrhodamine (Harris et al., 1991).

The efficiency of energy transfer (E) was determined by the decrease in the quantum yield of the donor as a result of the addition of acceptor. E was determined experimentally from the ratio of the fluorescence intensities of the donor in the presence (I_{da}) and in the absence (I_d) of the acceptor at the emission wavelength of the donor, after correcting for membrane light scattering and the contribution of the emission of the acceptor. The percentage value of E, is given in

$$E = (1 - I_{da}/I_d) \times 100 \quad (1)$$

The correction for light scattering was made by subtracting the signal obtained when unlabeled dermaseptin at concentrations which are equal to the sum of the donor and the acceptor were added to vesicles. Correction for the contribution of acceptor emission was made by subtracting the signal produced by the acceptor-labeled analogue alone. Correction for the displacement of Flu-dermaseptin by Rh-dermaseptin was made by subtracting the increase in the emission spectrum of Flu-dermaseptin due to the addition of unlabeled dermaseptin.

Enzymatic Digestion of Membrane-Bound Dermaseptin. Flu-dermaseptin (0.1 μM) (serves as a donor) was added to a dispersion of PC (1075 μM) or PC/PS (1:1 w/w) (215 μM) SUV, both containing 0.25% molar concentration of tetramethylrhodamine-labeled phosphatidylethanolamine (Rh-PE) (which serves as an acceptor) in buffer (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8), followed by the addition of a 20-μL solution of proteinase K (0.25 mg/mL). Fluorescence intensities as a function of time were obtained before and after the addition of the enzyme. In these experiments, the peptide/lipid molar ratio was kept at a level such that more than 90% of Flu-dermaseptin is assumed to bind to the vesicles. In a control experiment, Flu-dermaseptin (0.1 μM) was mixed with the enzyme prior to the addition of the rhodamine-labeled vesicles to the solution. Fluorescence spectra were obtained at room temperature in a Perkin-Elmer LS-5 spectrofluorometer, with excitation set at 470 nm, using a 10-nm slit, and with emission set at 530 nm, using a 5-nm slit. Measurements were performed in a 1-cm path-length glass cuvette in a final reaction volume of 2 mL.

Membrane Permeability Studies: Dissipation of Diffusion Potential from Sonicated Vesicles Induced by Dermaseptin. Membrane permeability as reflected by the dissipation of diffusion potential from vesicles (Sims et al., 1974; Loew et al., 1983) has been previously described in studies on the permeating properties of paradaxin and some of its analogues (Shai et al., 1990, 1991). In a typical experiment, 4 μL (~28 μg) of a liposome suspension [prepared from soybean lecithin, PC, or PC/PS (1:1 w/w) in K^+ buffer (50 mM K_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8)] was diluted in 1 mL of isotonic K^+-free buffer (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8) in a glass tube, to which the fluorescent, potential-sensitive dye, diS-C_2-5 (M_r = 492) was then added. A 1-μL sample of a 10^{-7} M valinomycin solution was added to the suspension in order to create a negative diffusion potential inside the vesicles, leading to fluorescence quenching of the dye. When the fluorescence had stabilized after 3–10 min, dermaseptin was added. The subsequent dissipation of the diffusion potential, reflected as an increase in fluorescence, was monitored on a Perkin Elmer LS-5 spectrofluorometer, with excitation set at 620 nm and emission at 670 nm, with gain adjusted to 100%. The percentage of fluorescence recovery, F_t, is defined as

$$F_t = (I_t - I_0/I_f - I_0) \times 100 \quad (2)$$

where I_0 = the initial fluorescence, I_f = the total fluorescence observed before the addition of valinomycin, and I_t = the fluorescence observed after adding the peptide, at time t.

Calcein Release from Vesicles Induced by Dermaseptin. Calcein (M_r = 623)-containing vesicles [formed from soybean lecithin, PC, or PC/PS (1:1 w/w), all containing 10% (w/w) cholesterol] were prepared with a self-quenching concentration of 60 mM calcein (Allen & Cleland, 1980), in 10 mM HEPES, at pH 7.4. The nonencapsulated calcein was removed from the liposome suspension by gel filtration, using a Sephadex G-50 (Pharmacia) column connected to a low-pressure LC system (Pharmacia). In a typical run, 20 μL of the liposome suspension was injected onto the column and eluted in 10 mM HEPES, SO_4^{2-}, 150 mM NaCl, pH 7.4. The eluent was monitored by UV spectroscopy (λ = 280 nm), and the vesicles peak was collected and diluted to a volume of 2 mL, in the same buffer. Dermaseptin was added to 1 mL of stirred vesicle suspensions (containing 1.3 μM liposomes). The resulting peptide-induced calcein leakage, reflected as an increase in fluorescence (Allen & Cleland, 1980), was monitored at room temperature on a Perkin-Elmer LS-5 spectrofluorometer, at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Complete dye release was obtained after disrupting the vesicles with Triton X-100 (0.1% final concentration). Under experimental conditions, in the absence of peptide, the leakage rate was less than 1% in 5 h. The percentage of fluorescence recovery is defined as described in eq 2, however, here I_f is defined as the total fluorescence observed after addition of Triton X-100.

RESULTS

In the present study, dermaseptin and its fluorescent derivatives selectively labeled at their N-terminal amino acid residue were synthesized and investigated for their interaction with membranes composed of zwitterionic or acidic phospholipids. Three fluorescent-labeled analogues were prepared by modifying the peptide with NBD (NBD-dermaseptin), with carboxyfluorescein (Flu-dermaseptin, to later serve as an energy donor), or with tetramethylrhodamine (Rh-dermaseptin, to later serve as an acceptor).

NBD Fluorescence Studies. NBD fluorescence is very sensitive to the polarity of its environment. Due to the small size of the NBD group and its small dipole moment, it has been previously utilized for polarity and binding studies without interfering with peptide–membrane interaction (Kenner & Aboderin, 1971; Frey & Tamm, 1990; Baidin & Huang, 1990; Rapaport & Shai, 1991). Accordingly, in the present study, the fluorescence emission spectrum of NBD-dermaseptin was measured either in aqueous solutions or in the presence of PC or PC/PS vesicles. The fluorescence emission spectrum of NBD-dermaseptin in the presence of PC SUV and in buffer alone is given in Figure 1. The emission spectrum in PC/PS was similar and as such is not given. The emission spectrum of NBD-aminoethanol in buffer and in the presence of vesicles is also given as a control. The labeled peptide exhibited fluorescence emission maxima at 538 ± 1 nm in buffer, a lower value than previously reported emission wavelength maxima for other NBD derivatives (Rajarathnam et al., 1989; Rapaport & Shai, 1991). However, upon addition of PC or PC/PS vesicles, NBD-dermaseptin exhibited enhanced increase in its fluorescence intensity and a blue-shift of its

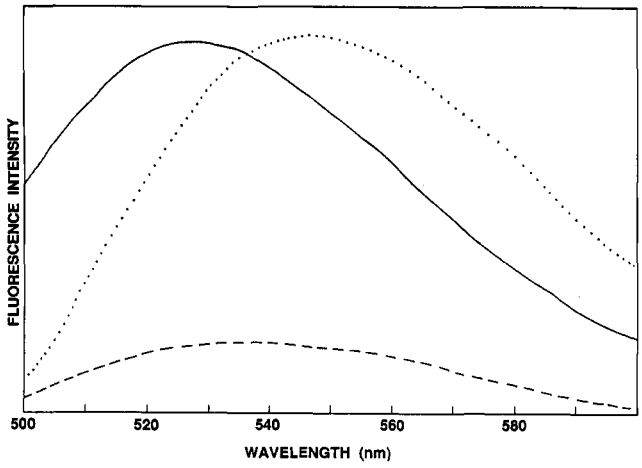

FIGURE 1: Fluorescence emission spectra of 0.1 μM NBD-labeled dermaseptin. Spectra were determined in the presence of 750 μM PC or PC/PS vesicles in buffer composed of 50 mM Na_2SO_4 and 25 mM HEPES-SO_4^{2-}, pH 6.8. The excitation wavelength was set at 468 nm. Emission was scanned from 500 to 600 nm. NBD-dermaseptin in the presence of vesicles (continuous line): NBD-dermaseptin in buffer (dashed line); NBD-aminoethanol (dotted line).

emission maxima to 528 ± 1 nm. This change reflects the relocation of the NBD group into a more hydrophobic environment. In these experiments, the lipid/peptide molar ratio was consistently maintained at an elevated level (7500:1), so that spectral contributions of the free peptide could be considered negligible.

In order to calculate the surface partition coefficient of NBD-dermaseptin, the labeled peptide, at a concentration of 0.1 μM, was titrated with PC or PC/PS SUV. The subsequent increase in the fluorescence intensity of the NBD-labeled peptide at pH 6.8 was plotted as a function of the lipid/peptide molar ratio and is reflected as traces A in Figures 2 and 3 for PC or PC/PS, respectively. SUV vesicles were used in the assay in order to minimize light scattering effects (Mao & Wallace, 1984). A control experiment was performed by titrating unlabeled dermaseptin with lipids up to the maximal concentration used in the assay. The fluorescence intensity of this solution (after subtracting the contribution of the vesicles) remained unchanged.

The binding isotherms were analyzed as a partition equilibrium (Schwarz et al., 1986, 1987; Rizzo et al., 1987; Beschiaschvili & Seelig, 1990; Rapaport & Shai, 1991), using

$$X_b = K_p C_f \quad (3)$$

where X_b is defined as the molar ratio of bound peptide per total lipid, K_p corresponds to the partition coefficient, and C_f represents the equilibrium concentration of free peptide in the solution. In order to calculate X_b, we extrapolated F_∞ (the fluorescence signal obtained when all the peptide is bound to lipid) from a double-reciprocal plot of F (total peptide fluorescence) versus C_L (total concentration of lipids) as previously suggested by Schwarz et al. (1986). Knowing the fluorescence intensities of unbound peptide, F_0, as well as bound peptide, F, the fraction of membrane-bound peptide, f_b, could be calculated using

$$f_b = (F - F_0)/(F_\infty - F_0) \quad (4)$$

Having calculated the value of f_b, it is then possible to calculate C_f, as well as the extent of peptide binding, X_b. In practice, it was assumed that the peptides were initially partitioned only over the outer leaflet of the SUV (60% of the total lipid), as had been previously suggested (Beschiaschvili & Seelig, 1990). Therefore, values of X_b were corrected as such:

$$X_b^* = X_b/0.6 \quad (5)$$

and eq 3 becomes

$$X_b^* = K_p^* C_f \quad (6)$$

The curve resulting from plotting X_b^* versus free peptide, C_f, is referred to as the conventional binding isotherm. The experimental binding isotherms resulting from plotting X_b^* versus free peptide, C_f, obtained from the interactions of NBD-dermaseptin with PC or PC/PS SUV at pH 6.8 are presented in traces B of Figures 2 and 3, respectively. The partition coefficients were estimated from the initial slopes of the curves and were found to be $(0.66 \pm 0.06) \times 10^4\ M^{-1}$ and $(2.8 \pm 0.3) \times 10^4$ (mean of three separate experiments) for PC and PC/PS, respectively. These values reflect 4-fold stronger binding to acidic than to zwitterionic phospholipid membranes.

Schwarz et al. (1986, 1987) have indicated that the shape of a binding isotherm of a peptide can provide information on the organization of the peptide within the membrane. The binding isotherm of dermaseptin in PC vesicles is a straight line, indicating a simple adhesion process, reflecting random

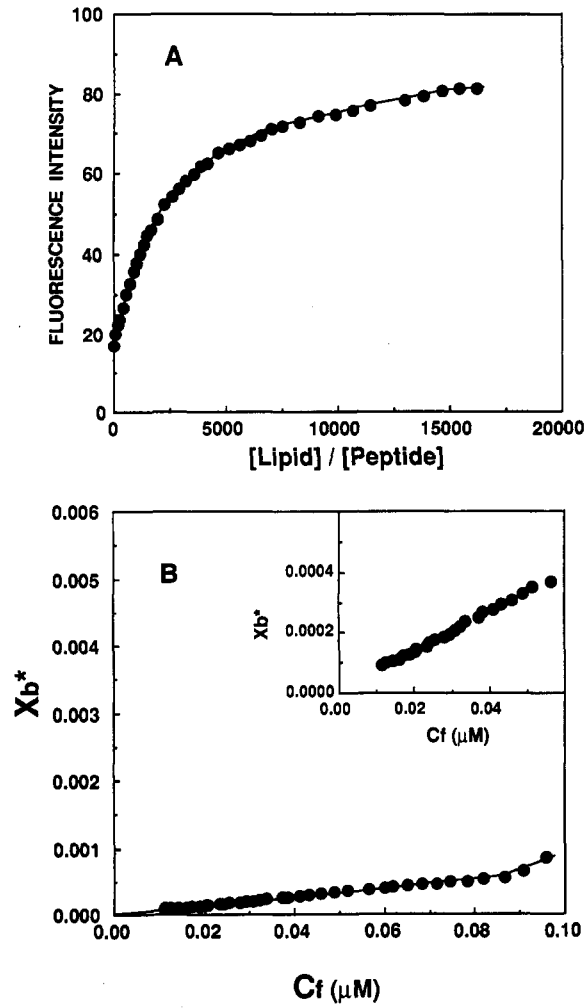

FIGURE 2: (A) Increase in the fluorescence of NBD-dermaseptin upon titration with PC vesicles. Peptide (0.1 μM total concentration) was titrated with PC vesicles with excitation set at 468 nm and emission recorded at 530 nm. The experiment was performed at 24 °C in 50 mM Na_2SO_4 and 25 mM HEPES-SO_4^{2-}, pH 6.8. (B) Binding isotherm derived from panel A by plotting X^*_b (molar ratio of bound peptide per 60% lipid) versus C_f (equilibrium concentration of free peptide in the solution). The inset shows the low concentration range of the main figure.

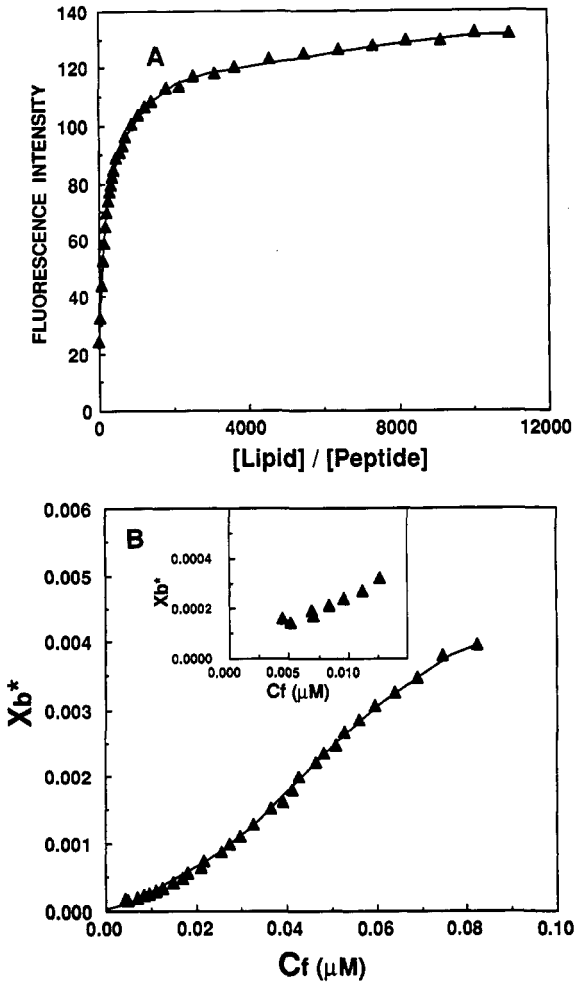

FIGURE 3: (A) Increase in the fluorescence of NBD-dermaseptin upon titration with PC/PS vesicles. Peptide (0.1 μM total concentration) was titrated with PC/PS vesicles with excitation set at 468 nm and emission recorded at 530 nm. The experiment was performed at 24 °C in 50 mM Na_2SO_4 and 25 mM HEPES-SO_4^{2-}, pH 6.8. (B) Binding isotherm derived from panel A as described for Figure 2B.

distribution of the monomers, presumably near the surface of the membrane. However, the binding isotherm in PC/PS vesicles exhibits an increase in its slope as the peptide/lipid molar ratio increases (high C_f value), implying that a slight aggregation within the lipid bilayers occurs. Similar behavior was previously described for several NBD-labeled pardaxin analogues (Rapaport & Shai, 1991).

Fluorescence Energy Transfer Studies. Intermolecular energy transfer studies between dermaseptin monomers embedded in vesicles were performed using a peptide modified with fluorescein, which served as an energy donor, or with rhodamine, which served as a fluorescence acceptor. The profiles displaying the energy transfer from Flu-dermaseptin to Rh-dermaseptin in the presence of PC phospholipid membranes are presented in Figure 4, and those in the presence of PC/PS membranes are presented in Figure 5. The continuous line represents the emission spectrum of Flu-dermaseptin in the presence of PC (Figure 4) or PC/PS (Figure 5) vesicles, at the fluorescein excitation wavelength (470 nm). When Rh-dermaseptin (final concentrations of 0.20–5.40 μM) was added sequentially to a mixture of Flu-dermaseptin (0.22 μM) with PC phospholipid vesicles (215 μM, Figure 4) or to a mixture of Flu-dermaseptin (0.22 μM) with PC/PS phospholipid vesicles (215 μM, Figure 5), marked quenching of the donor's emission, consistent with energy transfer, was observed predominantly with PC/PS vesicles (dashed and

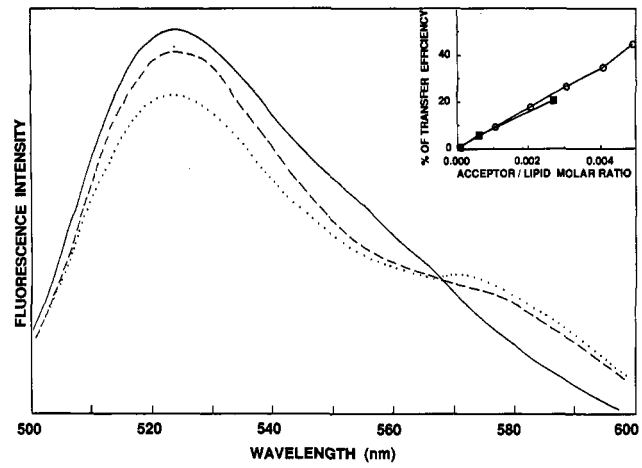

FIGURE 4: Fluorescence energy transfer dependence on Rh-dermaseptin (acceptor) concentration using PC vesicles. The spectrum of donor peptide, Flu-dermaseptin (0.22 μM), was determined in the presence or absence of various concentrations of acceptor peptide, Rh-dermaseptin. Each spectrum was recorded in the presence of 215 μM PC vesicles in 50 mM Na_2SO_4 and 25 mM HEPES-SO_4^{2-}, pH 6.8. The excitation wavelength was set at 470 nm; emission was scanned from 500 to 600 nm. The spectrum of Rh-dermaseptin in the presence of vesicles and unlabeled dermaseptin was subtracted from each spectrum. (—) 0.22 μM Flu-dermaseptin; (– – –) a mixture of 0.22 μM Flu-dermaseptin and 1.44 μM Rh-dermaseptin; (···) a mixture of 0.22 μM Flu-dermaseptin and 5.76 μM Rh-dermaseptin. (Inset) Theoretically (circles) and experimentally (filled squares) derived percentage of energy transfer versus bound acceptor/lipid molar ratio.

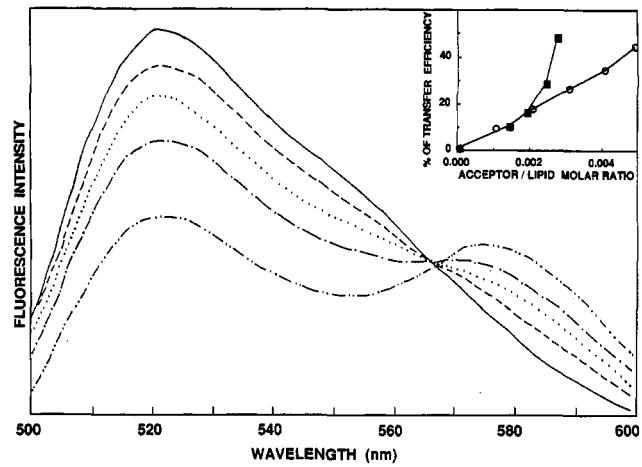

FIGURE 5: Fluorescence energy transfer dependence on Rh-dermaseptin (acceptor) concentration using PC/PS vesicles. The spectrum of donor peptide, Flu-dermaseptin (0.22 μM), was determined in the presence or absence of various concentrations of acceptor peptide, Rh-dermaseptin. Each spectrum was recorded in the presence of 215 μM PC/PS vesicles in 50 mM Na_2SO_4 and 25 mM HEPES-SO_4^{2-}, pH 6.8. The excitation wavelength was set at 470 nm; emission was scanned from 500 to 600 nm. The spectrum of Rh-dermaseptin in the presence of vesicles and unlabeled dermaseptin was subtracted from each spectrum. (—) 0.22 μM Flu-dermaseptin; (– – –) a mixture of 0.22 μM Flu-dermaseptin and 0.72 μM Rh-dermaseptin; (···) a mixture of 0.22 μM Flu-dermaseptin and 1.44 μM Rh-dermaseptin; (– · –) a mixture of 0.22 μM Flu-dermaseptin and 2.16 μM Rh-dermaseptin; (– ·· –) a mixture of 0.22 μM Flu-dermaseptin and 3.60 μM Rh-dermaseptin. (Inset) Theoretically (circles) and experimentally (filled squares) derived percentage of energy transfer versus bound acceptor/lipid molar ratio.

dotted lines in Figures 4 and 5). In control experiments, a 5–15% increase in the emission intensity of fluorescein was observed when the acceptor, Rh-dermaseptin, was replaced by an equivalent amount of unlabeled dermaseptin (data not shown). Furthermore, no decrease in the emission at 520 nm

was observed when the acceptor probe was attached to ethanolamine, which does not interact with the donor-labeled peptide. Thus, it was demonstrated that the decrease in donor emission at 520 nm was caused by a specific association of the donor- and the acceptor-labeled peptides. The insets of Figures 4 and 5 depict the curves (filled squares) of the corrected experimentally derived percentage of energy transfer versus the various molar ratios of bound acceptor (C_b)/lipid tested. The amounts of lipid-bound acceptor (Rh-dermaseptin), C_b, for the various acceptor concentrations were calculated from the binding isotherms as described below. First, the fractions of bound acceptor, f_b, were calculated for the various peptide/lipid molar ratios from Figures 4A and 5A using eq 4. Having calculated these values of f_b, it is then possible to calculate the concentration bound, c_b. The insets show, in addition, curves (open circles) corresponding to random distribution of the monomers (Fung & Stryer, 1978), assuming, R_0 of 45 Å which was previously calculated for the Flu/Rh donor/acceptor pair (Rapaport & Shai, 1992). The experiments with PC/PS vesicles show a higher value of energy transfer between lipid-embedded monomers than the theoretically derived values for random distribution, while the experiments with PC vesicles reveal random distribution.

Enzymatic Digestion of Membrane-Bound Dermaseptin. The susceptibility of membrane-bound Flu-dermaseptin to proteolytic digestion by proteinase K was investigated using PC or PC/PS vesicles labeled with rhodamine. At the lipid/peptide molar ratios tested (i.e., 10750 for PC and 2150 for PC/PS, more than 90% of Flu-dermaseptin is bound to the vesicles as revealed from the binding isotherms). Figure 6A shows the maximal fluorescence intensity of the digested labeled peptide, followed by the addition of vesicles (composed of PC or PC/PS) labeled with rhodamine. Identical profiles were obtained whether the peptide was added prior to the addition of the enzyme or after the addition of the enzyme. As seen from the figure, the addition of labeled vesicles to the digested peptide had no effect on the fluorescence intensity, demonstrating that the cleaved peptide lost its affinity to the membrane. However, a decrease in the fluorescence of Flu-dermaseptin was observed when the peptide was mixed with rhodamine-labeled vesicles prior to the addition of the enzyme. The addition of the enzyme to the mixture caused an increase in the fluorescent intensity when PC vesicles were used (Figure 6B). However, when the acidic PC/PS vesicles were used, only a slight increase in the fluorescent intensity was observed (Figure 6C). As seen from the figures, the enzyme could digest Flu-dermaseptin efficiently only when bound to PC vesicles but not when bound to PC/PS vesicles.

Membrane Permeability Studies: Valinomycin-Mediated Diffusion Potential Assay. Dermaseptin and its fluorescently labeled analogues were tested for their abilities to dissipate the diffusion potential in sonicated SUV prepared from PC, PC/PS (1:1 w/w), or soybean lecithin. Peptides, at increasing concentrations, were mixed with the SUV (at constant concentration), pretreated with the fluorescent dye (diS-C_2-5) and valinomycin. Peptide/lipid molar ratios ranging from 0.002:1 to 0.05:1 were used. The maximal activity for each peptide/lipid molar ratio tested was determined by monitoring the fluorescence recovery until a plateau was observed (usually after 15 min). Figure 7 shows a plot of the maximal activity versus peptide/lipid molar ratio for dermaseptin and Rh-dermaseptin. Each point represents the mean of three separate experiments with standard deviation calculated to be ~5%. The other labeled analogues showed similar activities and as such are not given. The results show in Figure 7 a high

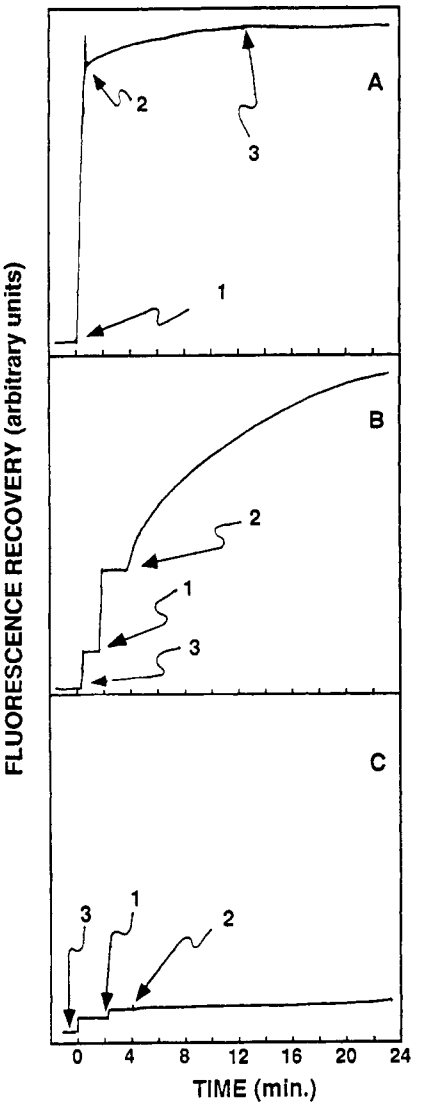

FIGURE 6: Digestion of Flu-dermaseptin in the presence of vesicles by proteinase K. Flu-dermaseptin (0.1 μM, label 1), Rh-labeled vesicles (label 3), and proteinase K (20-μL solution of 0.25 mg/mL, label 2) were mixed together in the following order: (A) first peptide (1), then enzyme (2), and finally vesicles (3); (B) first PC vesicles (3), then peptide (1), and finally enzyme (2); (C) first PC/PS vesicles (3), then peptide (1), and finally enzyme (2).

perturbing activity of dermaseptin with PC/PS and soybean vesicles, in marked contrast to the low level observed with PC vesicles. As an example, the lipid/peptide molar ratios at which 20% activity is observed was found to be 0.0045, 0.008, and 0.04 for PC/PS, soybean, and PC vesicles, respectively. Moreover, there exists a range of peptide/lipid molar ratio (up to 0.012) in which the peptide is almost inactive with PC yet reaches 90% of its activity in soybean or PS/PC vesicles.

Calcein Leakage from Vesicles. Dermaseptin and its fluorescently labeled analogues were tested for their potencies to evoke calcein release from SUV composed of PS/PC (1:1 w/w), soybean, or PC at pH 7.4. Increasing amounts of peptides were added to a vesicles suspension at fixed concentration. The ability of dermaseptin and its analogues to permeate the membrane was elucidated by monitoring the fluorescence recovery until a plateau was observed (usually after 10–20 min). This level was taken as the maximal activity attainable at each concentration of the tested analogues (see Figure 8 for dermaseptin and Rh-dermaseptin). The other labeled analogues showed similar activities and as such are not given. Figure 8 shows that dermaseptin has high activity

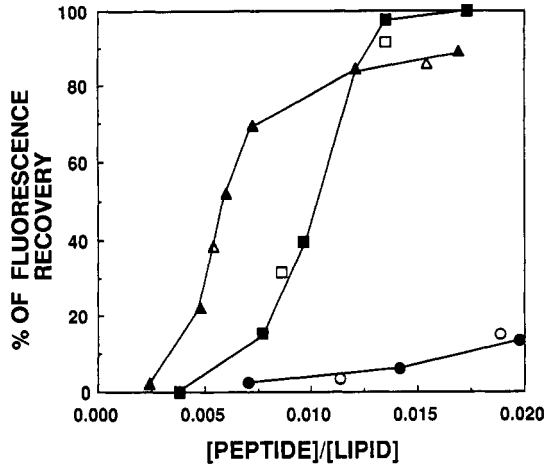

FIGURE 7: Dissipation of diffusion potentials induced by dermaseptin in PC, PC/PS, or soybean vesicles. Peptides were added to 1 mL of buffer containing a constant concentration of vesicles (phospholipid concentration = 38 μM), preequilibriated with the fluorescent dye diS-C_2-5 and valinomycin. Fluorescence recovery was measured after 10–20 min and is plotted as a function of the peptide/lipid molar ratio. (Filled triangles) Dermaseptin in PC/PS; (filled squares) dermaseptin in soybean; (filled circles) dermaseptin in PC; (open triangles) Rh-dermaseptin in PC/PS; (open squares) Rh-dermaseptin in soybean; (open circles) Rh-dermaseptin in PC.

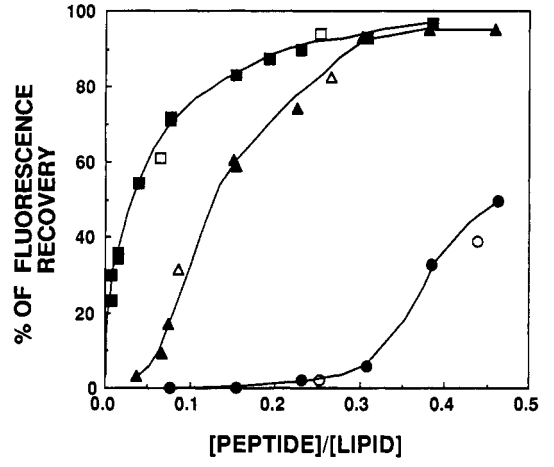

FIGURE 8: Calcein release induced by dermaseptin. Increasing amounts of peptide were added to 2.5 μM of PC, soybean, or PC/PS vesicles containing entrapped calcein at a self-quenching concentration in 1 mL of buffer (10 mM Hepes, 150 mM NaCl, pH 7.4). Fluorescence recovery was measured after 10 min and is plotted as a function of the peptide/lipid molar ratio. Designations are as in Figure 6.

in inducing calcein release from acidic PC/PS as compared to PC vesicles, similar to the preference which was observed in the diffusion potential experiments. As an example, the lipid/peptide molar ratios at which 20% activity is observed were found to be 0.005, 0.075, and 0.350 for soybean, PC/PS, and PC vesicles, respectively. These numbers reflect 70- or 5-fold lower activity with the zwitterionic versus the acidic soybean or PC/PS vesicles, respectively. Moreover, as seen in the diffusion potential experiments, there is a range of peptide/lipid molar ratios (up to 0.2) in which the peptide has no activity with PC yet reaches 90% of its activity in soybean vesicles.

DISCUSSION

Herein, a fluorometric approach was utilized to investigate the mode of action of dermaseptin with phospholipid membranes. For this purpose, dermaseptin was synthesized and selectively labeled at its N-terminal amino acid with the fluorescent probes NBD, Flu, or Rh. Four major characteristics were then determined: (i) the partition coefficients of NBD-dermaseptin with phospholipid vesicles composed of zwitterionic PC or acidic PC/PS vesicles; (ii) the organization state of dermaseptin when bound to PC or PC/PS vesicles; (iii) the susceptibility of membrane-bound dermaseptin to proteolysis; and (iv) the ability of dermaseptin to permeate phospholipid vesicles composed of PC, PC/PS, or soybean lecithin.

The data presented in this article show a direct correlation between the ability of the peptide to bind to phospholipid membranes and its efficacy to permeate them. When partition coefficients were estimated from the binding isotherms of NBD-labeled dermaseptin analogue, the partition coefficient obtained with acidic vesicles (PC/PS) was 4-fold higher than that obtained with zwitterionic vesicles (PC). Thus, for similar concentrations of dermaseptin, the bound peptide/lipid molar ratio is higher with PC/PS than with PC vesicles. This might explain the high effectiveness of dermaseptin to permeate vesicles composed of acidic rather than zwitterionic phospholipids, as indicated by diffusion potential experiments (Figure 7) and by the calcein release assay (Figure 8). Since the induction of calcein release require extremely high peptide/lipid molar ratios in both cases, the peptide might behave as a detergent at these conditions. However, the dose-dependent behavior at these high peptide/lipid molar ratios, i.e., the incomplete release of calcein, suggests a nondetergent mechanism. That a slight self-association of peptide monomers occurs in acidic, but not in zwitterionic, SUV was indicated by the shape of the binding isotherm (straight line with PC and slightly bent upward with PC/PS) and from FET experiments. The self-association of dermaseptin in acidic membranes could be explained by the fact that negative and positive charges counteract each other in an acidic PC/PS–dermaseptin system but not in the zwitterionic PC–dermaseptin system. In other words, the negatively charged headgroups of the acidic vesicles would compensate for the electrostatic repulsions generated between positive charges of dermaseptin, when several peptide monomers are brought into close proximity. However, the presence of positive charges is not the only prerequisite for the peptide's specific binding to acidic phospholipid membranes, since other highly positively charged amphiphilic peptides did not bind preferentially to acidic vesicles. One such highly positively charged peptide is the S-4 segment of the sodium channel of *Electrophourus electricus* electric organ, exhibiting similar partition coefficients in acidic and in zwitterionic vesicles (Rapaport et al., 1992). Other examples are analogues of the shark repellent neurotoxin, pardaxin, in which similar partition coefficients are obtained independent of the molecule's charge. Furthermore, the induction of the dissipation of diffusion potential from acidic SUVs obtained with positively (+5 net charge) or negatively charged (–5 net charge) pardaxin analogues was similar (Shai et al., 1990, 1991; Rapaport & Shai, 1991).

Although there is no sequence homology between dermaseptin and magainin, an antimicrobial peptide, isolated from the skin of *X. laevis* (Zasloff, 1987), they both contain six positive and two negative charges along their backbone, and both peptides adopt amphiphilic α-helical structure in hydrophobic surroundings (Matsuzaki et al., 1991; Mor et al., 1991a). Magainin, like dermaseptin, can permeate vesicles composed of acidic, but not of zwitterionic, phospholipids. A proposed mechanism for magainin antimicrobial activity involves the disruption of energy metabolism in the target

organism by increasing the permeability of energy-transducing membranes (Westerhoff et al., 1989a,b). According to this mechanism, magainin would first disrupt the outer membrane (Matsuzaki, et al., 1991) and then transverse it to reach the inner membrane (Rana et al., 1991). A channel-like mechanism was also proposed to account for magainin's activity (Cruciani et al., 1988). However, biophysical studies revealed that magainin could not penetrate deeply into the hydrophobic region of the membrane (Matsuzaki et al., 1991). Our results with dermaseptin support a mechanism in which the peptide is adsorbed onto the surface of the membrane, with its amphiphilic N-terminal segment buried within the acyl-chain region of the membrane. This conclusion is based on the observation that the environment encountered by the N-terminal is more hydrophobic (emission maximum of 528 nm) than that detected with an NBD probe located on the surface of the membrane (emission maximum 533 nm) yet less hydrophobic than that detected within the acyl chain (emission maximum of 522 nm) (Chattopadhyay & London, 1987; Rapaport & Shai, 1991). Moreover, the susceptibility of dermaseptin to rapid proteolysis when bound to PC, and to a very slow proteolysis when bound to PC/PS, confirms that, at least with PC SUV, a portion of dermaseptin is located on the surface of the membrane and is exposed to the solution. In acidic PC/PS SUV, the positively charged α-helical amphiphilic dermaseptin (Mor et al., 1991a) could lie parallel to the surface of the membrane, tightly bound to the acidic phospholipid headgroups, and self-associate with other peptide monomers, in a "carpet"-like manner. This aggregate is probably oriented with its hydrophobic face embedded relatively shallowly within the hydrophobic region of the membrane and its positive charges directed toward the hydrophilic region. In such an orientation, dermaseptin would be protected from proteolysis and could perturb the structural organization of the membrane, leading to content leakage from the vesicles. Although a channel formation mechanism is theoretically possible, it is less likely. First, such channels would probably have to consist of a bundle of amphiphilic α-helices, with their hydrophilic faces forming the lumen of the channel (Lear & DeGrado, 1988). Although dermaseptin aggregated slightly within acidic phospholipid membranes, its aggregation state was much less than that observed for channel forming peptides such as alamethicin or the shark repellent neurotoxin, pardaxin. The slope of the binding isotherm of dermaseptin increased 2-fold as the peptide/lipid molar ratio increased, while the slopes of the binding isotherms of alamethicin (Rizzo et al., 1987) and pardaxin (Rapaport & Shai, 1991) increased $\sim$100-fold (reflecting a higher partitioning of the peptide within the lipid bilayers, with the later two). Second, a bundle of dermaseptin monomers transversing the lipid bilayers seem to be unfavorable due to the electrostatic repulsion between the positive charges in the lumen of dermaseptin channel. This electrostatic repulsion probably accounts for the inability of dermaseptin to form aggregates when bound to PC membranes, where no counteracting negative charges are present. However, in order to verify the possibility of a channel-formation mechanism, single-channel experiments are currently being performed.

REFERENCES

Allen, T. M., & Cleland, L. G. (1980) *Biochim. Biophys. Acta 597*, 418–426.

Baidin, G., & Huang, J. R. (1990) *FEBS Lett. 259*, 254–256.

Bartlett, G. R. (1959) *J. Biol. Chem. 234*, 466–468.

Beschiaschvili, G., & Seelig, J. (1990) *Biochemistry 29*, 52–58.

Chattopadhyay, A., & London, E. (1987) *Biochemistry 26*, 39–45.

Chen, H. C., Brown, J. H., Morell, J. L., & Huang, C. M. (1988) *FEBS Lett. 236*, 461–466.

Cruciani, R. A., Stanley, E. F., Zasloff, M., Lewis, D. L., & Barker, J. L. (1988) *Biophys. J. 53*, 9a.

Csordas, A., & Michl, H. (1970) *Monatsh. Chem. 101*, 182–189.

Frey, S., & Tamm, L. K. (1990) *Biochem. J. 272*, 713–719.

Fung, B. K.-K., & Stryer, L. (1978) *Biochemistry 17*, 5241–5248.

Harris, R. W., Sims, P. J., & Tweten, R. K. (1991) *J. Biol. Chem. 266*, 6936.

Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.

Kenner, R., & Aboderin, A. (1971) *Biochemistry 10*, 4433–4440.

Lear, J. D., Wasserman, Z. R., & DeGrado, W. F. (1988) *Science 240*, 1177–1181.

Loew, L. M., Benson, L., Lazarovici, P., & Rosenberg, I. (1985) *Biochemistry 24*, 2101–2104.

Mao, D., & Wallace, B. A. (1984) *Biochemistry 23*, 2667–2673.

Matsuzaki, K., Harada, M., Handa, T., Funahoshi, S., Nobutaka, F., Yajima, H., & Miyajima, K. (1989) *Biochim. Biophys. Acta 981*, 130–134.

Matsuzaki, K., Harada, M., Funakoshi, S., Fujii, N., & Miyajima, K. (1991) *Biochim. Biophys. Acta 1063*, 162–170.

Mor, A., Nguyen, V. H., Delfour, A., Migliore-Samour, D., & Nicolas, P. (1991a) *Biochemistry 30*, 8824–8830.

Mor, A., Nguyen, V. H., & Nicolas, P. (1991b) *J. Mycol. Med. 1*, 216–220.

Nicolas, P., Mor, A., & Delfour, A. (1992) *Medicine/Sciences 8*, 423–431.

Papahadjopoulos, D., & Miller, N. (1967) *Biochim. Biophys. Acta 135*, 624–638.

Rajarathnam, K., Hochman, J., Schindler, M., & Ferguson-Miller, S. (1989) *Biochemistry 28*, 3168–3176.

Rana, F. R., Macias, E. A., Sultany, C. M., Modzrakowski, M. C., & Blazyk, J. (1991) *Biochemistry 30*, 5858–5866.

Rapaport, D., & Shai, Y. (1991) *J. Biol. Chem. 266*, 23769–23775.

Rappaport, D., & Shai, Y. (1992) *J. Biol. Chem. 267*, 6502–6509.

Rappaport, D., Danin, M., Gazit, E., & Shai, Y. (1992) *Biochemistry 31*, 8868–8875.

Rizzo, V., Stankowsky, S., & Schwarz, G. (1987) *Biochemistry 26*, 2751–2759.

Schwarz, G., Stankowsky, S., & Rizzo, V. (1986) *Biochim. Biophys. Acta 861*, 141–151.

Schwarz, G., Gerke, H., Rizzo, V., & Stankowsky, S. (1987) *Biophys. J. 52*, 685–692.

Shai, Y., Bach, D., & Yanovsky, A. (1990) *J. Biol. Chem. 265*, 20202–20209.

Shai, Y., Hadari, Y. R., & Finkels, A. (1991) *J. Biol. Chem. 266*, 22346–22354.

Sims, P. J., Waggoner, A. S., Wang, C. H., & Hoffmann, J. R. (1974) *Biochemistry 13*, 3315–3330.

Terry, S. A., Poulter, L., Williams, D. H., Nutkins, J. C., Giovannini, M. G., Moore, C. H., & Gibson, B. W. (1988) *J. Biol. Chem. 263*, 5745.

Westerhoff, H. A., Hendler, R. W., Zasloff, M., & Juretic, D. (1989a) *Biochim. Biophys. Acta 975*, 361–369.

Westerhoff, H. A., Juretic, D., Hendler, R. W., & Zasloff, M. (1989b) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6597–6601.

Zasloff, M. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5449–5453.

Zasloff, M., Martin, B., & Chen, H. C. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 910–913.