Spectrum of Antimicrobial Activity and Assembly of Dermaseptin-b and Its Precursor Form in Phospholipid Membranes†

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ABSTRACT: Dermaseptins are 27–34 amino acid antimicrobial peptides that irreversibly inhibit growth of pathogenic filamentous fungi, in addition to their ability to inhibit the growth of bacteria, yeasts, and protozoa. Synthetic peptides, with sequences corresponding to dermaseptin-b (DS-b) and its N-terminal extended precursor form dermaseptin-B (DS-B), were synthesized and investigated with respect to their spectrum of antimicrobial activity and their mode of interaction with model membranes composed of PS or PC/PS phospholipids. We found that DS-B is much more potent than DS-b against all microorganisms tested. Furthermore, despite significant structural identity between DS-B and DS-S (Pouny et al., 1992), only the former is highly effective at inhibiting the growth of filamentous fungi. The peptides were labeled selectively at their N-terminal amino acid with either 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) or rhodamine fluorescent probes, which facilitated the determination of their partition coefficients with phospholipid membranes and their organization in their membrane-bound state. The partition coefficients of DS-B are 10-fold higher than those of DS-b and DS-S, with both acidic and zwitterionic phospholipid vesicles. This may explain the ability of DS-B to permeate both types of vesicles efficiently. Furthermore, while both DS-b and DS-B interact with phospholipid membranes in a noncooperative manner, they are self-associated in their membrane-bound state. This noncooperative binding probably prevents aggregation of the peptides on the surface of outer bacterial membranes, and assists them in efficiently diffusing into the inner target membranes. The exceptional property of DS-B to bind strongly to phospholipid membranes and to form small bundles correlates with its high potential to kill yeast and filamentous fungi. As a molecular model, dermaseptins may be of potential interest in drug design, particularly in antifungal warfare.

The immune response of vertebrates to invasion by pathogenic microorganisms includes cell-free defense mechanisms, in which small-sized and positively charged amphiphilic peptides are directly involved. A considerable knowledge of this molecular immunity is derived from the study of antimicrobial peptides from insects or amphibians (Boman & Hultmark, 1987; Bevins & Zasloff, 1990; Nicolas et al., 1992). Among these peptides, a five-membered novel family of broad-spectrum antimicrobial peptides, termed dermaseptins, was recently identified in skin extracts of the South American arboreal frog *Phyllomedusa sauvagii* (Mor et al., 1991; Mor & Nicolas, 1994a). Dermaseptins differ from the hitherto known antimicrobial peptides in that they irreversibly inhibit growth of pathogenic fungi, in addition to their ability to inhibit the growth of Gram-negative and Gram-positive bacteria, as well as of yeasts and protozoa. Dermaseptins do not lyse erythrocytes or other mammalian cells, which is a characteristic of other animal- and insect-originated antibacterial peptides, such as magainins (Zasloff, 1987) and cecropins (Hultmark et al., 1980; Steiner et al., 1981). Prediction of secondary structure and circular dichroism spectral analysis indicated that in hydrophobic media dermaseptins, like magainins, cecropins, and other antibacterial peptides, are configured as amphipathic α-helixes (Mor et al., 1991; Mor & Nicolas, 1994a). Although the precise mechanism by which dermaseptins and other antibacterial peptides exert their toxic effect is yet not fully understood, it becomes clear that lipid–peptide interaction has an important role in this effect, and that specific receptors are not involved. This conclusion has been supported by the finding that all D-amino acid analogues of cecropin and magainin possess antibacterial activity similar to that of the parent molecule (Wade et al., 1990; Bessalle et al., 1990). Previous studies aimed at elucidating initial steps involved in the interaction of dermaseptin S (isolated from *Phyllomedusa sauvagii*) with model phospholipid membranes revealed that the peptide is not self-associated in its membrane-bound state (Pouny et al., 1992). Therefore, it has been suggested that the peptide permeates phospholipid vesicles via membrane disintegration rather than via ion channel or pore formation as described by the "barrel stave" model (Ehrenstein & Lecar 1977; Rapaport & Shai, 1991). Further studies have indicated that the molecular elements responsible for the antimicrobial potency of the various dermaseptins can be traced to the N-terminal α-helical amphipathic segment of the molecule (Mor & Nicolas, 1994b).

Recently, a new 27 amino acid residue dermaseptin-like peptide, termed dermaseptin-b (DS-b),[1] was isolated from the skin of *Phyllomedusa bicolor* (Mor et al., 1994). Among the noticeable differences between DS-b and the dermaseptins from *Phyllomedusa sauvagii* are its naturally occurring carboxyamidated carboxy terminus, an extra negative charge at its NH_2-terminus, and that both termini are shorter than in DS-S (Table 1). Although DS-b exhibits broad-spectrum

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Table 1: Amino Acid Sequences of the Peptides and Their Fluorescently Labeled Analogues

Peptide No.	Designation	Sequence
1	DS-b (X=H)	X-NH-DVLKKIGTVALHAGKAALGAVADTISQ-NH_2
2	NBD-DS-b (X=NBD)	
3	Flu-DS-b (X=Flu)	
4	Rho-DS-b (X=Rho)	
5	DS-B (X=H)	X-NH-AMWKDVLKKIGTVALHAGKAALGAVADTISQ-NH_2
6	NBD-DS-B (X=NBD)	
7	Rho-DS-B (X=Rho)	
8	[a]DS-S (X=H)	X-NH-ALWKTMLKKLGTMALHAGKAALGAADTISQGTQ-COOH
9	NBD-DS-S (X=NBD)	
10	[a]Rho-DS-S (X=Rho)	

[a] Taken from Pouny et al. (1992). Identical amino acids are underlined.

antimicrobial action, compared to DS-S its potency is inferior with respect to most organisms tested, particularly against yeast and fungi. Interestingly, however, the molecular cloning of the biosynthetic precursor DS-b (Amiche et al., 1994) revealed that its sequence is preceded, in the NH_2 end of the precursor molecule, by the sequence AMWK which is juxtaposed to a cleavage site commonly found in the processing of amphibian skin polypeptide precursors. This sequence is highly homologous to that found in the N-terminus of DS-S (i.e., ALWK). This suggests that during the maturation of this precursor, a 31-residue peptide, whose sequence will be that of DS-b, elongated with the tetrapeptide AMWK at its NH_2 end, can be released.

To investigate the implication of the N-terminal extension (AMWK) in the antimicrobial activity and the mode of action of DS-b with phospholipid membranes, DS-b, its longer version referred to as dermaseptin-B (DS-B), and their fluorescent derivatives were synthesized and investigated for their interaction with phospholipid membranes, their antimicrobial potencies against pathogenic microorganisms, and their lytic effect toward erythrocytes. The peptides were labeled with 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), or with rhodamine, which facilitated examination of the initial steps involved in the interaction of DS-b and DS-B with phospholipid membranes at a very high lipid:peptide molar ratio (~6000:1). These included determination of the location of the N-terminus of the peptides when bound to phospholipid bilayers, calculation of their surface partition coefficients, and determination of their organizational state when bound to phospholipid membranes.

We found that DS-B is much more potent than DS-b against all microorganisms tested. Moreover, despite a significant identity between DS-B and DS-S (Pouny et al., 1992), only the former is highly effective at inhibiting the growth of some pathogens especially *Cryptococcus* and filamentous fungi. Our data reveal that both DS-b and DS-B interact with phospholipid membranes in a noncooperative manner, as was found for DS-S. However, unlike DS-S, both DS-b and DS-B are self-associated in their membrane-bound state. Furthermore, the partition coefficient of DS-B is 10-fold higher than those obtained for DS-b and DS-S, with both acidic and zwitterionic phospholipid vesicles, which may explain the ability of DS-B to permeate both types of vesicles efficiently. The results are discussed in line of proposed mechanisms for antibacterial activity of other polypeptides as well.

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 used for peptide synthesis and fluorescent labeling included trifluoroacetic acid (TFA, Sigma) and *N*,*N*-diisopropylethylamine (DIEA, Aldrich, distilled over ninhydrin). 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) was supplied by Merck (Darmstadt, Germany) and recrystallized twice from ethanol. 3,3′-Diethylthiodicarbocyanine iodide (diS-C_2-5), 5(6)-carboxyfluorescein succinimidyl ester, and 5(6)-carboxytetramethylrhodamine succinimidyl ester, were obtained from Molecular Probes (Eugene, OR). 4-Fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) was obtained from Sigma. All other reagents were of analytical grade. Buffers were prepared in double glass-distilled water.

Peptide Synthesis and Fluorescent Labeling. DS-b and DS-B were prepared by a stepwise solid phase synthesis using *N*-9-fluorenylmethyloxycarbonyl (Fmoc) polyamide active ester chemistry on a Milligen 9050 PepSynthesizer. Side chain protection used were *tert*-butyl for Asp and Thr and *tert*-butyloxycarbonyl for Lys and His. After removal of Fmoc from the N-terminal amino acid, the peptides were cleaved from the resins with a mixture of 85:5:5:5 TFA/*p*-cresol/H_2O/thioanisole (10 mg of resin-bound peptide in 1 mL of mixture). The TFA was then evaporated, and the peptides were precipitated with ether followed by washing with ether (4×). Crude peptides were extracted from the resins with 40% acetonitrile in water, and were purified to a chromatographic homogeneity of >96% 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 peptides were subjected to amino acid analysis, solid phase sequencing, and fast atomic bombardment mass spectrometry to confirm their composition.

Labeling of the peptides at their N-terminal amino acids with fluorescent probes was performed on resin-bound peptides 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-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-F) in DMF, (ii) 5(6)-carboxytetramethylrhodamine succinimidyl ester (Rho-Su) (10 μmol) in DMF containing 5% diisopropylethylamine, and (iii) 5(6)-carboxyfluorescein succinimidyl ester (Flu-Su) (10 μmol) in DMF containing 5% diisopropylethylamine. After 24 h, the resin-bound peptides were washed thoroughly, with DMF followed by methylene chloride, and 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 1 mL of mixture), precipitated with ether, and finally extracted from the resin with 40% v/v acetonitrile/water. Final purification was achieved using RP-HPLC as described above.

Antimicrobial and Hemolytic Assays. Antimicrobial assays were performed in sterilized 96-well plates (Nunc F96

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

microtiter plates) in a final volume of 100 μL as follows. Fifty microliters of suspension containing fungi or bacteria at 1×10^6/mL in culture medium (Sabouraud's glucose broth or LB medium, respectively) was added to 50 μL of water containing the peptide in serial 2-fold dilutions in water. Inhibition of growth was determined by measuring the absorbance at 492 nm with a Titertek Multiskan MCC after an incubation time of 48 h at 30 °C (37 °C for bacteria). Hemolytic activity was assayed with heparinized fresh human red blood cells rinsed 3 times with PBS by centrifugation for 15 min at 900*g*. Red blood cells were then incubated under agitation at 37 °C in distilled water for 100% hemolysis, in PBS for control, or in PBS containing various concentrations of the peptide in a final volume of 100 μL. Release of hemoglobin was monitored by measuring the absorbance of the supernatant at 541 nm after centrifugation for 15 min at 900*g*, after 1 h of incubation.

Preparation of Liposomes. Small unilamellar vesicles (SUV) were prepared by sonication of PC or PC/PS (1:1 w/w). Briefly, dry lipid and cholesterol (10:1 w/w) were dissolved in a $CHCl_3$/MeOH mixture (2:1 v/v). The solvents were then evaporated under a stream of nitrogen and the lipids (at a concentration of 7.2 mg/mL) were subjected to a vacuum for 1 h and then resuspended in the appropriate buffer, by vortexing. The resultant lipid dispersions were then sonicated for 5–15 min in a bath type sonicator (G1125SP1 sonicator; Laboratory Supplies Co., Inc., New York, NY) until clear. The lipid concentrations of the resulting preparations were determined by phosphorus analysis (Bartlett, 1959). Vesicles were visualized using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo, Japan) as follows. A drop of vesicle suspension was deposited on a carbon-coated grid and negatively stained with uranyl acetate. Examination of the grids demonstrated that the vesicles were unilamellar with an average diameter of 20–50 nm (Papahadjopoulos & Miller, 1967).

NBD Fluorescence Measurements. NBD-labeled peptide (0.04 nmol) was added to 0.4 mL of buffer (50 mM Na_2SO_4/25 mM HEPES–sulfate, pH 6.8) containing 160 nmol of PC or PS/PC SUV, to establish a lipid:peptide ratio (4000:1) at which most of the peptide is bound to lipids. After a 2 min incubation, the emission spectrum of the NBD group was recorded (in three separate experiments) using a Perkin-Elmer LS-50B spectrofluorometer, with the excitation set at 470 nm (10 nm slit).

Determination of Partition Constants of NBD-Labeled Peptides with Phospholipid Vesicles. Binding experiments were conducted as previously described (Rapaport & Shai, 1991). In a typical experiment, PC or PC/PS SUV were added successively to 0.1 μM fluorescently-labeled peptide at 25 °C. Fluorescence intensity was measured as a function of the lipid:peptide molar ratio on a Perkin-Elmer LS-50B spectrofluorometer, with the excitation set at 470 nm, using a 10 nm slit, and the emission set at 530 nm, using a 5 nm slit, in three separate experiments. Plotting the fluorescence intensity versus the lipid:peptide molar ratio yields titration curves. To correct for background, the readings obtained when unlabeled peptide was titrated with lipid vesicles were subtracted from each recording of fluorescence intensity.

The binding isotherms obtained from the titration curves were analyzed as a partition equilibrium as described previously (Schwarz et al.,1987; Rizzo et al., 1987; Beschiaschvili & Seelig, 1990; Rapaport & Shai, 1991), using the formula:

$$X^*_b = K^*_p C_f$$

where X^*_b is defined as the molar ratio of bound peptide (C_b) per 60% of total lipid [assumed to be initially partitioned only over the outer leaflet of the SUV, as had been previously suggested (Beschiaschvili & Seelig, 1990)], K^*_p corresponds to the experimental partition coefficient, and C_f represents the equilibrium concentration of free peptide in the solution.

The curve resulting from plotting X^*_b versus free peptide, C_f, is referred to as the conventional binding isotherm.

Resonance Energy Transfer (RET) Measurements of Membrane-Embedded Donor-/Acceptor-Labeled Dermaseptin. In a typical experiment, donor (NBD/Flu-labeled) peptide at a final concentration of 0.02, 0.04, or 0.1 μM was added to a dispersion of PC or PS/PC SUV (33.5, 67, or 100 μM, respectively) in buffer (50 mM Na_2SO_4/25 mM HEPES–sulfate, pH 6.8), followed by the addition of acceptor (Rho-labeled) peptide in several sequential doses. Fluorescence spectra were obtained before and after the addition of the acceptor peptide. Experiments were performed at room temperature using a Perkin-Elmer LS-50B spectrofluorometer, with the excitation monochromator set at 460 nm, to minimize the excitation of tetramethylrhodamine, and with a 5-nm slit width. Measurements were performed in a 0.5-cm path-length glass cuvette and a final reaction volume of 0.4 mL.

The percentage of energy transfer (E) was determined by measuring 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 donor's emission wavelength, after correcting for membrane light scattering and the contribution of acceptor emission. The percentage of transfer efficiency (E) is defined as

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

The correction for light scattering was made by subtracting the signal obtained when unlabeled analogues were added to vesicles containing the donor molecule. Correction for the contribution of acceptor emission was made by subtracting the signal produced by the acceptor-labeled analogue alone.

Membrane Permeation Induced by the Peptides. Membrane permeation was assessed utilizing the diffusion potential assay (Loew et al., 1983; Sims et al., 1974) as previously described (Shai et al., 1990, 1991), except that in these experiments a total volume of 0.1 mL instead of 1 mL was used. In a typical experiment, in a glass tube, 4 μL (final concentration of 37 μM phospholipids) of a liposome suspension, prepared in a K^+-containing buffer (50 mM K_2SO_4/25 mM HEPES–sulfate, pH 6.8), was diluted with 1 mL of an isotonic K^+-free buffer (50 mM Na_2SO_4/25 mM HEPES–sulfate, pH 6.8), to which the fluorescent, potential-sensitive dye diS-C_2-5 was then added. Valinomycin (1 μL of 10^{-7} M) was added to the suspension in order to slowly create a negative diffusion potential inside the vesicles, which led to a quenching of the dye's fluorescence. Once the fluorescence had stabilized, 3–10 min later, peptides were added to 0.1 mL of this solution. The subsequent dissipation of the diffusion potential, as reflected by an increase in fluorescence, was monitored on a Perkin Elmer LS-50B spectrofluorometer, with excitation set at 620 nm and emission at 670 nm, and the gain was adjusted to 100%. The percentage of fluorescence recovery, F_t, was defined as

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

where I_0 = the initial fluorescence, I_f = the total fluorescence

Table 2: Spectrum of the Antimicrobial Activity of Dermaseptin Sauvagii (DS-S), Precursor Dermaseptin Bicolor (DS-B1), and Dermaseptin Bicolor (DS-b1)

organism	MIC[a] (μM) DS-S	DS-B	DS-b
Microsporum canis (IP1194)	14.5	0.63	18.9
Tricophyton rubrum (IP1400-82)	29.0	1.60	37.8
Arthroderma simii (IP1063-74)	29.0	3.20	37.8
Cryptococcus neoformans (IP960-67)	4.30	0.63	56.7
Cryptococcus neoformans (IP962-67)	4.30	1.20	56.7
Candida albicans (IP886-65)	7.30	9.60	56.7
Aeromonas caviae (IP67-16T)	14.5	7.90	56.7
Escherichia coli (IP76-24)	1.45	4.70	28.3
Nocardia brasiliensis (IP16-80)	58.0	7.90	56.7
Staphylococcus aureus (IP76-25)	4.30	4.70	>75.0
hemolysis of human erythrocytes[b] (μg/mL)	>58.0	>40.0	>367

[a] MIC is defined as the peptide concentration at which 100% inhibition of growth was observed after 48 h incubation in culture media. [b] Hemolytic activity after 1 h of incubation.

observed before the addition of valinomycin, and I_t = the fluorescence observed after adding the peptide at time t.

RESULTS

DS-b, its precursor form DS-B, and their fluorescent derivatives were synthesized and characterized spectroscopically and functionally for their interaction with phospholipid membranes, their antimicrobial potencies toward pathogenic microorganisms, and their lytic effect on human erythrocytes. The fluorescent analogues, selectively labeled at their N-terminal amino acid residues, were prepared by modifying the peptides with either one of the following fluorescent probes: NBD (to serve as an energy donor and to facilitate binding experiments), fluorescein (to serve as an alternative energy donor); or tetramethylrhodamine (to serve as an energy acceptor). The peptides and their designations are listed in Table 1.

Dermaseptins-b and -B Are Cytolytic for Pathogenic Microorganisms. The effects of DS-b, DS-B, and their fluorescent derivatives over various pathogenic microorganisms were assessed using minimal inhibitory concentration (MIC) experiments. MIC is defined as the minimal peptide concentration for which 100% inhibition of growth is observed after 48 h incubation. Both peptides inhibited the proliferation of most microorganisms tested by 100%, including Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi, at concentrations ranging from 0.63 to 58 μM depending on the strain tested. The cytotoxicity of the dermaseptins to mammalian cells was assessed on human red blood cells. The results are summarized in Table 2, which also shows results obtained with DS-S for comparison (Mor & Nicolas, 1994b). The data reveal the following: (i) The extended form DS-B is more potent than the shorter version DS-b toward all of the organisms tested; (ii) DS-B inhibits the growth of fungi at concentrations that are 7–25-fold lower than those required for either DS-b or DS-S; (iii) both DS-B and DS-S are highly active against bacteria, but with different specificities; and (iv) the fluorescently labeled analogues retained 70–100% of the activities of the corresponding parent molecules when tested against some microorganisms (*Cryptococcus neoformans* and *Staphylococcus aureus*, data not shown).

Localization of the Environment of the NBD Moiety. The fluorescence of NBD reflects its location due to its sensitivity to the dielectric constant of its environment (Kenner & Aboderin, 1971). As such, the NBD probe was utilized for binding experiments (Frey & Tamm, 1990; Rapaport & Shai, 1991). Herein, the fluorescence emission spectra of NBD-DS-b, NBD-DS-B, and NBD-aminoethanol (a control) were monitored in aqueous solutions, at pH 6.8, and in the presence of vesicles composed of either PC or PC/PS. In these studies, SUV were used to minimize light scattering effects (Mao & Wallace, 1984), and the lipid:peptide molar ratio was elevated (~4000:1) so that the spectral contributions of free peptide would be negligible. In buffer, both peptides exhibited fluorescence emission maxima at 545 ± 1 nm (Table 3), which indicate that the NBD moiety is in a hydrophilic environment (Rajarathnam et al., 1989). However, when PC or PC/PS SUV vesicles were added to the aqueous solutions containing NBD-DS-b or NBD-DS-B, both a blue shift in the emission maxima and an increase in the fluorescence intensity of the NBD group were observed for the two peptides. Yet, both the blue shift and the enhancement in the fluorescence intensity were larger with NBD-DS-B than with NBD-DS-b (Table 3). The change in the spectrum of the NBD group reflects its relocation to a more hydrophobic environment (Chattopadhyay & London, 1987). This blue shift suggests an interface localization of the NBD group in the case of NBD-DS-b (λ_{max} = 533 or 540 nm with PC or PS/PC, respectively), or a slight penetration of the N-terminal end of the peptide into the phospholipid environment in the case of NBD-DS-B (λ_{max} = 527 nm with both PC and PS/PC) (Chattopadhyay & London, 1987). No shift was observed with the control, NBD-aminoethanol. The fact that the N-terminus of NBD-DS-b is more exposed to the solution when bound to PS/PC (λ_{max} = 540 nm) than when bound to PC vesicles may result from electrostatic repulsion between the negatively charged aspartic acid located at the N-terminus of NBD-DS-b and the acidic head groups of PS.

Characterization of Binding Isotherms and Determination of Partition Constants. The fluorescence properties of the NBD moiety facilitated the generation of binding isotherms for NBD-labeled peptides, from which partition coefficients could be calculated as previously described (Rapaport & Shai, 1991). First, a fixed concentration (0.1 μM) of NBD-labeled peptide was titrated with the desired vesicles (e.g., PC or PS/PC). Plotting of the resulting increases in the fluorescence intensities of NBD-labeled peptides as a function of lipid:peptide molar ratios yielded conventional binding curves (Figure 1A,C for NBD-DS-b with PC/PS and PC, respectively, and Figure 2A,C for NBD-DS-B with PC/PS and PC,

Table 3: Fluorescence Increases, Emission Maxima, and Calculated Partition Coefficients of Dermaseptins in the Presence of Phospholipid Vesicles

peptide designation	Fl_{lipid}/Fl_{buffer} PC	PS/PC	λ_{max} (nm) buffer	PC	PS/PC	K^*_p (M^{-1}) PC	PS/PC
DS-b	2.3	3.0	545 ± 1	533 ± 1	540 ± 1	8.7 (±0.9) × 10^3	8.7 (±0.6) × 10^4
DS-B	4.1	4.8	545 ± 1	527 ± 1	527 ± 1	8.3 (±1.5) × 10^4	8.0 (±1.0) × 10^5
DS-S[a]			545 ± 1	528 ± 1	528 ± 1	6.6 (±0.6) × 10^3	2.8 (±0.5) × 10^4
NBD-aminoethanol	1.0	1.0	545 ± 1	545 ± 1	545 ± 1	not bind	not bind

[a] Taken from Pouny et al. (1992).

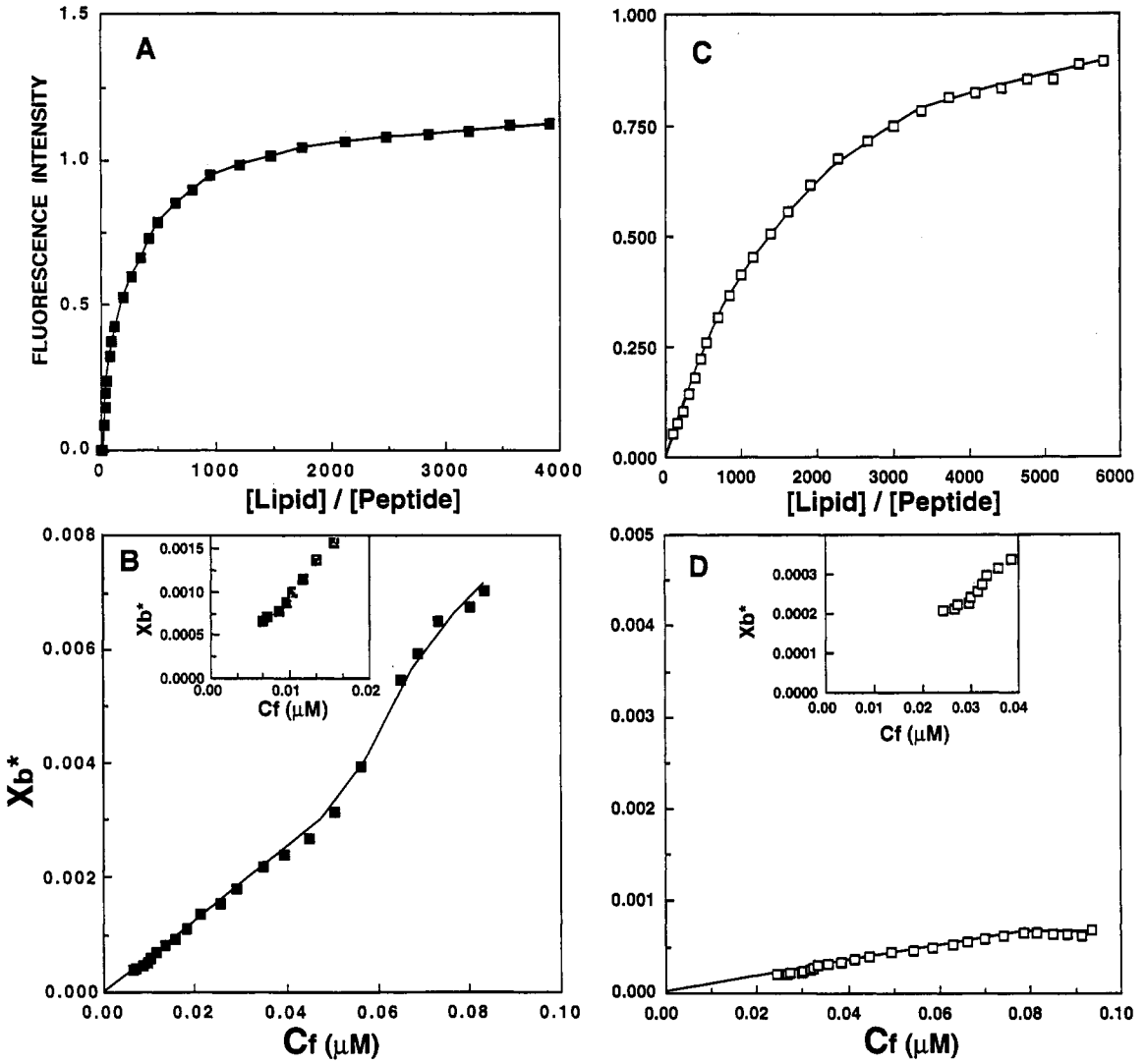

FIGURE 1: Increases in the fluorescence of NBD-DS-b upon titration with PC/PS (A) or PC (C) vesicles, and the resulting binding isotherm (B and D, respectively). NBD-DS-b (0.1 μM) was titrated with SUV with excitation set at 467 nm and emission recorded at 530 nm. The experiment was performed at room temperature in buffer composed of 50 mM Na_2SO_4/25 mM HEPES–sulfate, pH 6.8. The binding isotherms were derived from (A) or (C) 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.

respectively). Since the concentrations of the NBD-labeled peptides in the mixtures were low and the peptide:lipid molar ratios were also very low, it was assumed that the peptides did not disrupt the bilayer structure. When unlabeled peptides were titrated with lipids, up to the maximal concentration used with NBD-labeled peptides, the fluorescence intensities of the solutions, after subtracting the contribution of the vesicles, remained unchanged.

Binding isotherms were constructed by plotting X^*_b (the molar ratio of bound peptide per 60% of the total lipid) versus C_f (the equilibrium concentration of free peptide in the solution) (see Figure 1B,D for NBD-DS-b with PC/PS and PC, respectively, and Figure 2B,D for NBD-DS-B with PC/PS and PC, respectively). The surface partition coefficients were estimated by extrapolating the initial slopes of the curves to C_f values of zero and are given in Table 3 (3–4 measurements each). The data presented in Table 3 reveal that the K^*_p values with the acidic PS/PC vesicles are 10-fold higher than those obtained with the zwitterionic PC vesicles, for both NBD-DS-b and NBD-DS-B. The stronger binding of the peptides to the acidic vesicles may result from electrostatic interaction between the acidic head groups of PS and the positive charges of the peptides. Interestingly, however, with both types of vesicles, the K^*_p values of NBD-DS-B are 10-fold higher than those obtained for NBD-DS-b, or for the previously studied NBD-DS-S (Pouny et al., 1992). The K^*_p values obtained for both dermaseptins are within the range of those obtained for other membrane-permeating bioactive peptides, such as melittin and its derivatives (Stankowski and Schwarz, 1990), the *Staphylococcus* δ-toxin (Thiaudière et al., 1991), and pardaxin and its analogues (Rapaport & Shai, 1991).

It has been suggested that the shape of a binding isotherm of a peptide can provide information on the organization of the peptide within the membrane (Schwarz et al., 1987). The binding isotherms of NBD-DS-b and NBD-DS-B with both PC and PC/PS vesicles are straight lines, indicating a noncooperativity in the binding process. These binding isotherms are similar to those obtained with the previously reported DS-S (Pouny et al., 1992), or cecropin (Gazit et al., 1994).[2] However, they are different from those obtained with the following pore-forming polypeptides: alamethicin (Rizzo et al., 1987), pardaxin and its analogues (Rapaport & Shai, 1991), the α-5 segment of *Bacillus thuringiensis* CryIIIA δ-endotoxin (Gazit & Shai, 1993a), and helix-2 of *Bacillus thuringiensis* var. *israelensis* cytolytic toxin (Gazit & Shai,

[2] Preliminary results were present at the Ciba Foundation Symposium, 186, on Antibacterial Peptides, London, 1994.

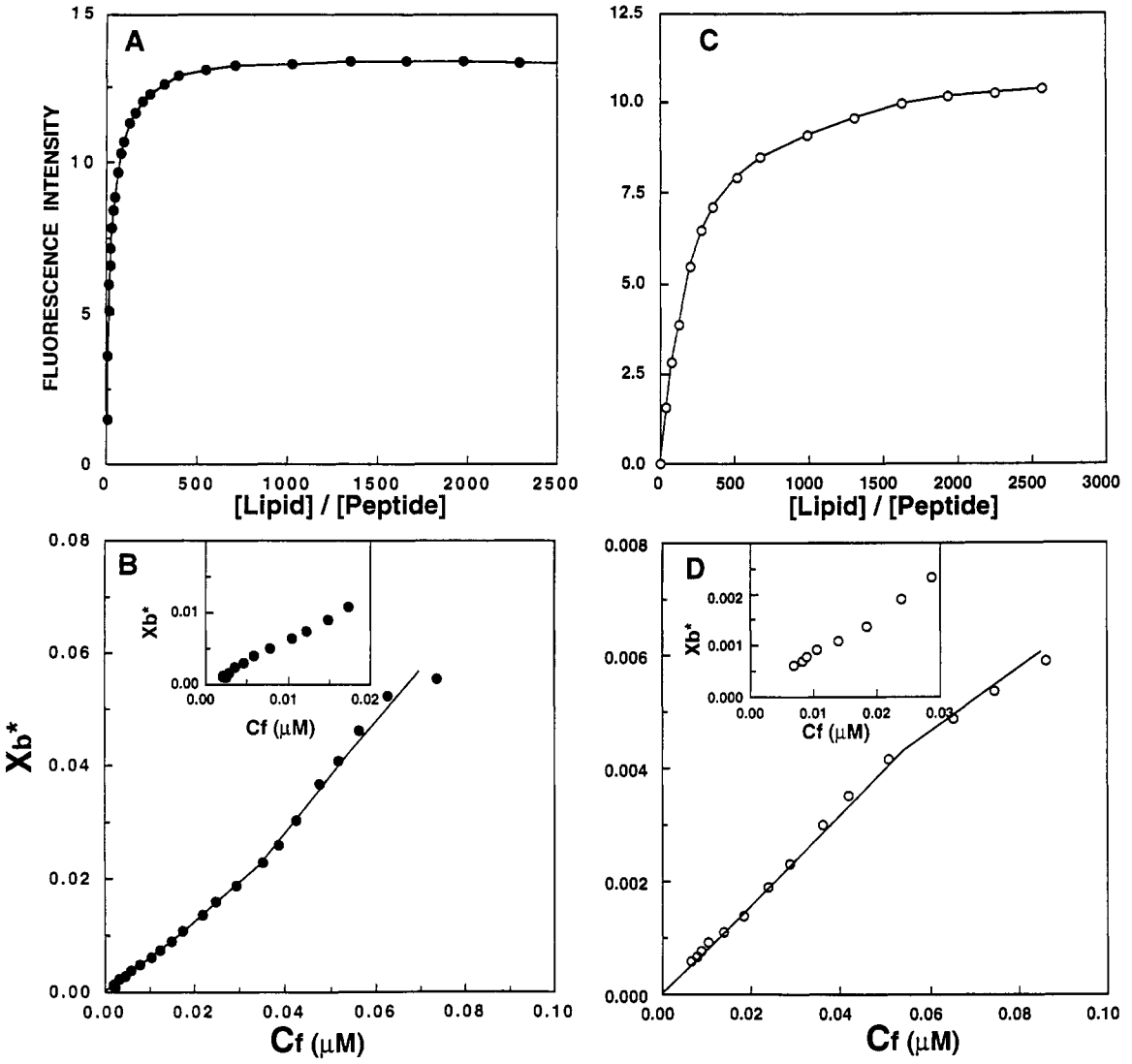

FIGURE 2: Increases in the fluorescence of NBD-DS-B upon titration with PC/PS (A) or PC (C) vesicles, and the resulting binding isotherm (B and D, respectively). NBD-DS-B (0.1 μM) was titrated with SUV with excitation set at 467 nm and emission recorded at 530 nm. The experiment was performed at room temperature in buffer composed of 50 mM Na_2SO_4/25 mM HEPES–sulfate, pH 6.8. The binding isotherms were derived from (A) or (C) as described in the legend of Figure 1.

1993b). The isotherms of the latter pore-forming peptides display an initial "lag", i.e., initially the curves are flat, but rise sharply once a threshold concentration is achieved, indicating a cooperativity in the binding process (Schwarz et al., 1987).

Resonance Energy Transfer (RET) Experiments. The shape of a binding isotherm may indicate whether or not a particular peptide binds the membranes in a cooperative manner to form large aggregates in its membrane-bound state. However, such experiments cannot indicate self- or hetero-association of polypeptides which could form small-sized bundles. To evaluate whether such bundles are formed, RET measurements were performed as described previously (Pouny et al., 1992). In these experiments, DS-b and DS-B were used either as donors (NBD- or Flu-labeled forms) or as acceptors (Rho-labeled forms). Similar results were obtained whether Flu- or NBD-labeled dermaseptin was used as a donor. Examples of typical profiles of the energy transfer from Flu-DS-b to Rho-DS-b, in the presence of PC phospholipid vesicles, are depicted in Figure 3A, and those showing the energy transfer from NBD-DS-B to Rho-DS-B, in the presence of PC phospholipid vesicles, are depicted in Figure 3B. In each case, the addition of Rho-labeled peptide (final concentrations of 0.02–0.2 μM) to NBD-labeled peptide (0.02 μM or 0.04 μM) preincubated with phospholipid vesicles (33.5–100 μM), quenched the donor's emission and increased the acceptor's emission, which is consistent with energy transfer. Similar experiments were performed using mixtures of NBD-labeled peptides and Rho-labeled pardaxin as a control. To determine the actual percentage of energy transfer, the amounts of lipid-bound acceptors (Rho-peptides, termed "bound-acceptor") at the various acceptor-peptide concentrations were calculated from the binding isotherms of the corresponding NBD-labeled peptides as previously described (Pouny et al., 1992). The curves of the experimentally derived percentage of energy transfer versus the bound-acceptor:lipid molar ratio are depicted in Figure 4. A curve corresponding to a random distribution of monomers (Fung & Stryer, 1978), assuming an R_0 (distance at which 50% RET occurs) of 51 Å, which was previously calculated for the NBD/Rho-donor/acceptor pair (Gazit & Shai, 1993b), is also depicted. A high percentage of energy transfer was obtained with NBD-(or Flu-)DS-b/Rho-DS-b only in the presence of the acidic PS/PC vesicles, and with NBD-DS-B/Rho-DS-B in the presence of both the acidic PS/PC and the zwitterionic PC vesicles. These values are markedly higher than those expected for random distribution of monomers (Figure 4). However, efficiencies of energy transfer between either NBD-DS-b or NBD-DS-B, serving

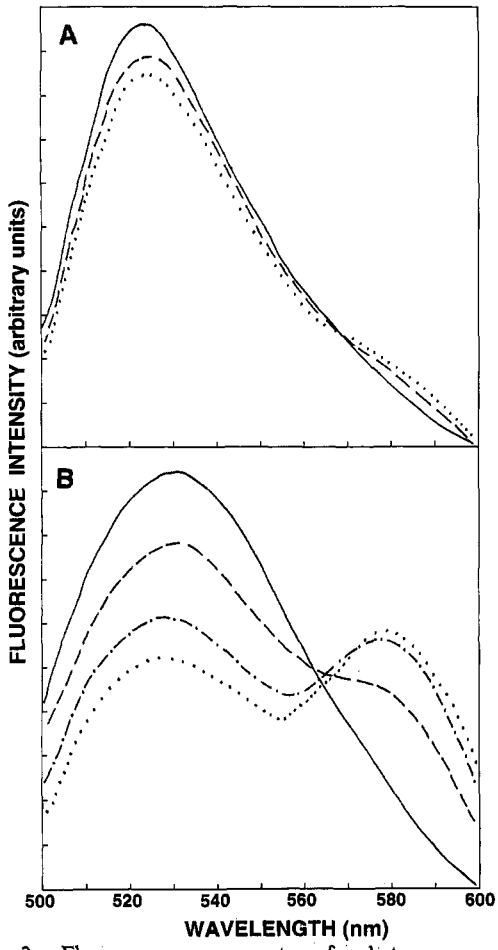

FIGURE 3: Fluorescence energy transfer between membrane-embedded donor/acceptor-peptides using PC vesicles. The spectra of Flu-DS-b (0.02 μM, panel A) or NBD-DS-B (0.04 μM, panel B), the donor peptides, were determined in the presence or absence of various concentrations of the acceptor-peptides, Rho-DS-b (panel A) and Rho-DS-B (panel B). Each spectrum was recorded in the presence of PC vesicles (33.5 μM with Flu-DS-b and 67 μM with NBD-DS-B) in 50 mM Na_2SO_4/25 mM HEPES–sulfate, pH 6.8. The excitation wavelength was set at 470 nm; emission was scanned from 500 to 600 nm. The spectra of Rho-DS-b and Rho-DS-B in the presence of vesicles and unlabeled DS-b or DS-B were subtracted from the corresponding spectra. Panel A: (—) 0.02 μM Flu-DS-b; (– –) a mixture of 0.02 μM Flu-DS-b and 0.1 μM Rho-DS-b; (···) a mixture of 0.02 μM Flu-DS-b and 0.2 μM Rho-DS-b. Panel B: (—) 0.04 μM NBD-DS-B; (– –) a mixture of 0.04 μM NBD-DS-B and 0.027 μM Rho-DS-B; (– · –) a mixture of 0.04 μM NBD-DS-B and 0.067 μM Rho-DS-B; (···) a mixture of 0.04 μM NBD-DS-B and 0.094 μM Rho-DS-B.

as a donor, and the pore-forming peptide Rho-pardaxin are similar to those observed for random distribution (data not shown). Thus, DS-B appears to associate rather than distribute randomly in both acidic and zwitterionic phospholipid membranes, while DS-b appears to associate only in acidic ones. Previous studies, utilizing Flu-DS-S as a donor at 0.1 μM concentration, showed that DS-S is not self-associated in its membrane-bound state (Pouny et al., 1992). Experiments were repeated herein with NBD-DS-S serving as a donor at 0.04 μM concentration, with the same results.

Further experiments were performed to examine whether DS-b, DS-B, and DS-S can form heteroaggregates in their membrane-bound state. In separate experiments each one of the three NBD-labeled peptides was incubated with the vesicles followed by the addition of either one of the two other Rho-labeled peptides, and the percentage of RET was measured. Figure 5 summarizes the results. The data show that

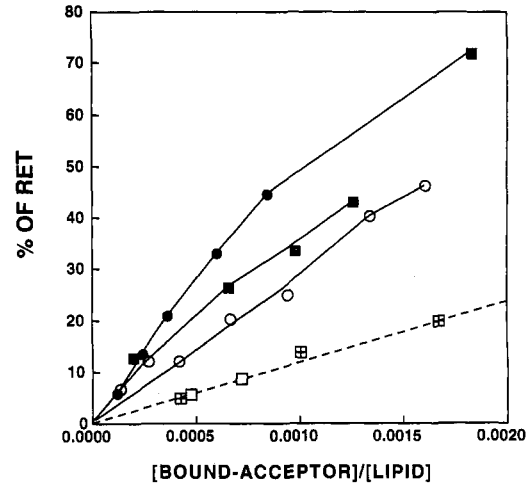

FIGURE 4: Theoretically and experimentally derived percentage of energy transfer versus bound-acceptor:lipid molar ratio, due to self-assembly. The amounts of lipid-bound acceptor (Rho-peptides), C_b, at various acceptor concentrations were calculated from the binding isotherms (Pouny et al., 1992). Symbols: filled circles, NBD-DS-B/Rho-DS-B in PS/PC vesicles; filled squares, NBD-DS-b/Rho-DS-b in PS/PC vesicles; open circles, NBD-DS-B/Rho-DS-B in PC vesicles; open squares, NBD-DS-b/Rho-DS-b in PC vesicles; crossed squares, NBD-DS-S/Rho-DS-S in PS/PC vesicles; dashed line, random distribution of the monomers (Fung & Stryer, 1978), assuming an R_0 of 51 Å.

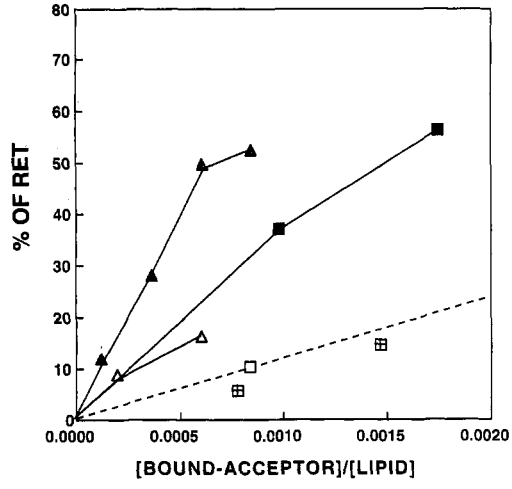

FIGURE 5: Theoretically and experimentally derived percentage of energy transfer versus bound-acceptor:lipid molar ratio due to heteroaggregate formation. The amounts of lipid-bound acceptor (Rho-peptides), C_b, at various acceptor concentrations were calculated from the binding isotherms (Pouny et al., 1992). Symbols: filled triangles, NBD-DS-B/Rho-DS-b in PS/PC vesicles; filled squares, NBD-DS-B/Rho-DS-S in PC vesicles; open triangles, NBD-DS-B/Rho-DS-b in PC vesicles; open squares, Flu-DS-b/Rho-DS-S in PS/PC vesicles; crossed squares, Flu-DS-b/Rho-DS-S in PC vesicles; dashed line, random distribution of the monomers (Fung & Stryer, 1978), assuming an R_0 of 51 Å.

significant RET occurs between the two pairs, DS-b/DS-B and DS-B/DS-S, but not with the DS-b/DS-S pair.

Membrane Permeability Induced by the Peptides. The efficacy of DS-b, DS-B, and their fluorescent derivatives to perturb lipid packing and to cause leakage of vesicular contents was examined by utilizing the dissipation of diffusion potential assay. Increasing concentrations of the peptides or their fluorescent analogues were mixed with either PC or PS/PC SUV that had been pretreated with the fluorescent potential-sensitive dye (diS-C_2-5) and valinomycin, and the recovery of fluorescence was monitored as a function of time. Maximal activity of the peptides was plotted versus peptide:lipid molar

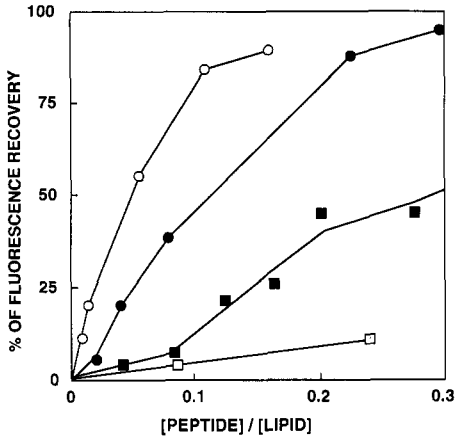

FIGURE 6: Dissipation of diffusion potentials in PC or PC/PS vesicles. Peptides were added to 0.1 mL of buffer containing a constant concentration of vesicles (36 μM) preequilibrated with the fluorescent dye diS-C_2-5 and valinomycin. Fluorescence recovery was measured after 10 min and is plotted as a function of the peptide:lipid molar ratio. Open circles, DS-B with PC vesicles; filled circles, DS-B with PC/PS vesicles; filled squares, DS-b with PC/PS vesicles; open squares, DS-b with PC vesicles.

ratios (Figure 6). Each point represents the mean of three separate experiments with standard deviations of ~5%. The fluorescently labeled analogues retained the full activity of the unlabeled peptides (data not shown) as was shown also with other membrane-permeating polypeptides (Rapaport & Shai, 1991; Pouny et al., 1992; Gazit & Shai, 1993a,b). A high perturbing activity of DS-B was observed with both PC and PS/PC vesicles. However, DS-b was significanly active only with the acidic PS/PC vesicles. These results correlate with the observed higher antimicrobial potency and with the larger partition coefficients of DS-B as compared to DS-b in both types of vesicles. Thus, at the specific peptide:lipid molar ratio tested, more DS-B was bound to the vesicles as compared to DS-b.

DISCUSSION

Dermaseptins, a family of antimicrobial peptides, are members of a group of short α-helical peptides with a unique property of being water soluble while also being able to interact strongly with phospholipid membranes (designated as WMSP for water-membrane soluble polypeptides). This group includes antimicrobial polypeptides such as alamethicin (Boheim, 1974), magainins (Zasloff, 1987), and cecropins (Hultmark et al., 1980; Steiner et al., 1981; Boman, 1991), as well as the toxins melittin (Hanke et al., 1983; Tosteson et al., 1987) and pardaxin (Shai et al., 1990). In spite of the similar structural features (e.g., high α-helical structure in hydrophobic environment) and comparable membrane-permeating properties of WMSP, they differ markedly in their functions. For example, melittin and pardaxin are highly hemolytic, whereas dermaseptin, cecropin, and magainin are not hemolytic. Although the precise mechanism by which WMSP exert their activities is yet unknown, accumulating data suggest that peptide–lipid interactions are an essential step in their function. In the case of antibacterial polypeptides, it is proposed that they permeate the inner wall of the bacteria, and therefore destroy the energy metabolism of the target organism (Okada & Natori, 1985; Zasloff, 1987; Westerhoff et al., 1989). Whether all antibacterial polypeptides exert their toxic activity via a similar mechanism is yet unclear.

In this study, DS-b and its precursor form DS-B were synthesized, fluorescently labeled, tested *in vitro* against a broad array of microorganisms, and investigated for their mode of action using simple models of phospholipid vesicles that varied with respect to their lipid composition. DS-b and DS-B were compared using the following criteria: (i) their antimicrobial spectrum of activity; (ii) their ability to bind vesicles composed of zwitterionic PC or acidic PS/PC phospholipids; (iii) their organization state when bound to PC or PS/PC vesicles, and (iv) their ability to permeate phospholipid vesicles composed of PC or PS/PC. The modes of action of DS-b and DS-B on model membranes were also compared with that of DS-S described previously (Pouny et al., 1992) and discussed with regard to the proposed antibacterial mechanisms of other antibacterial peptides.

The data presented herein reveal that the extension by four amino acids at the N-terminus of DS-b, found in its precursor form, markedly increases the antimicrobial activity of the resultant DS-B toward all the organisms tested. DS-S was the first vertebrate peptide that was shown to be active against pathogenic fungi, and DS-S is highly homologous to DS-B (Table 2). The most striking finding is that DS-B is 10–25-fold more active than DS-S against filamentous fungi. Furthermore, DS-B is 2–7-fold more active than DS-S against *Cryptococcus*, which is a major pathogen in AIDS patients (Table 2). The higher antimicrobial activity of DS-B may be in part due to its high membrane-permeating activity with both PC and PC/PS vesicles (Figure 6), while DS-b (Figure 6) and DS-S (Pouny et al., 1992) are able to permeate efficiently only the acidic PC/PS vesicles. This property is probably the result of its higher partition coefficients, as compared to DS-b and DS-S, with both PC and PC/PS vesicles (10-fold higher, Table 3). The higher partition coefficient of DS-B as compared to DS-S with the acidic phospholipids is quite surprising, since DS-B has an additional acidic amino acid in the N part of the molecule. Although DS-b has a partition coefficient with PC/PS vesicles that is similar to that of DS-B with PC vesicles, the latter is more effective in membrane permeation. This may be due in part to the low ability of DS-b to penetrate the hydrophobic interior of the membrane as reflected by the interface location of its N-terminus (Table 3). Furthermore, although DS-B binds PC/PS vesicles more efficiently than PC vesicles, it permeates PC vesicles better than PC/PS vesicles (Figure 6). This may be due in part to the repulsive forces between the acidic charge at the N-terminus of DS-B and the acidic head groups of the phospholipids, which make it difficult for the N-terminus of DS-B to penetrate into the hydrophobic interior of the membrane. It also should be noted that DS-S (Pouny et al., 1992) is more potent than DS-B in permeation of PS/PC vesicles, although its partition coefficient is lower than that of DS-B. This may also be due in part to the presence of an additional acidic charge at the N-terminus of DS-B, as compared to DS-S, which makes it difficult to penetrate through the acidic head groups of the phospholipids. However, a difference in the batches of phospholipids used in the two experiments could also contribute to this effect.

Despite differences in the partition coefficients of the three dermaseptins, the data suggest that both DS-b and DS-B bind to phospholipid membranes in a noncooperative manner, as was previously found for DS-S (Pouny et al., 1992) and cecropin B2 (Gazit et al., 1994).[2] This suggestion is based on the shape of the binding isotherms (i.e., straight lines) of NBD-labeled dermaseptins obtained with both the acidic (PS/PC) and zwitterionic (PC) phospholipid vesicles (Figures 1B, 1D, 2B, and 2D). These binding isotherms are different from those observed for pore-forming peptides, such as pardaxin

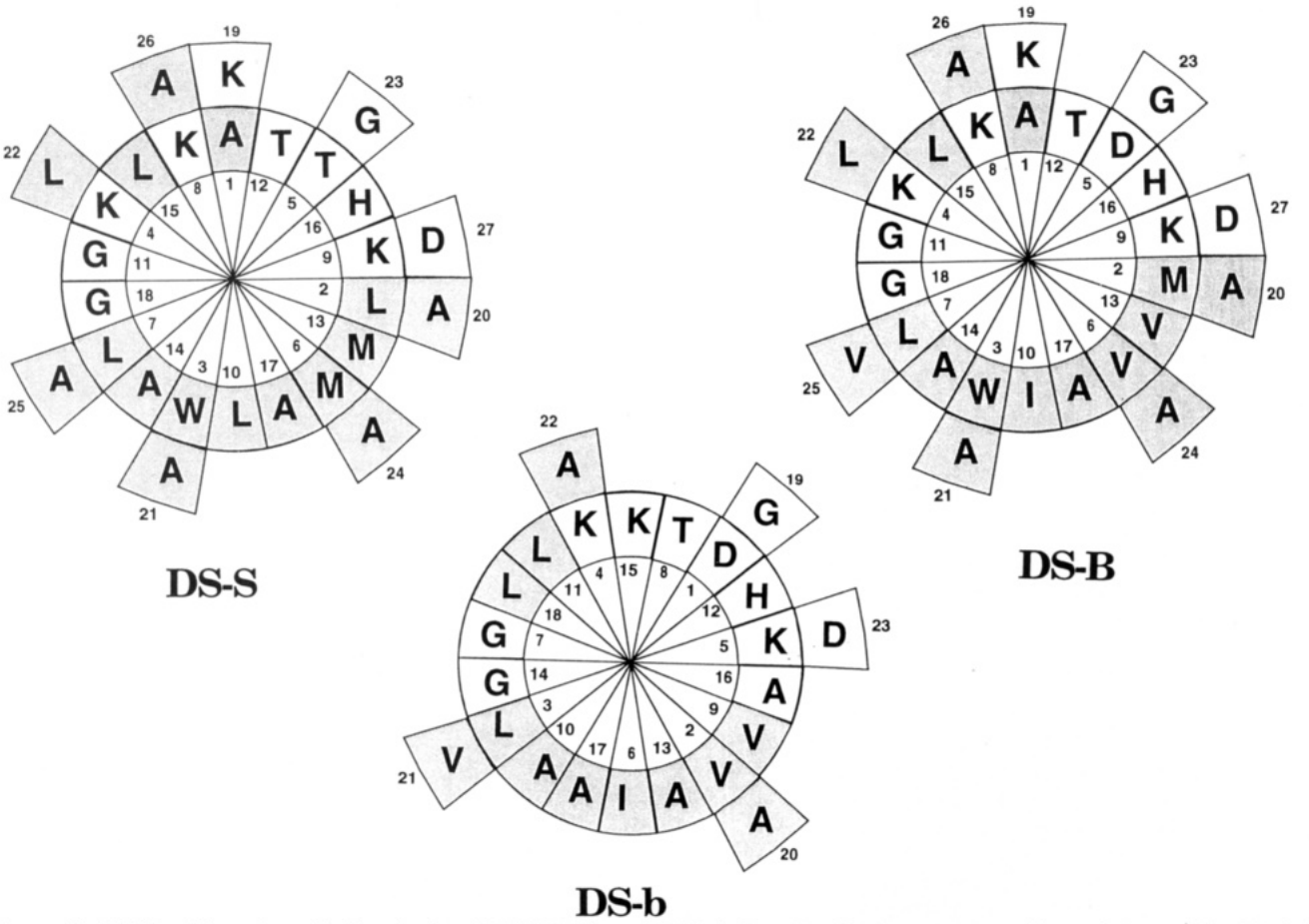

FIGURE 7: Schiffer–Edmundson wheel projection of DS-S, DS-b, and DS-B helices. Number 1 represents residue 1 of the peptides. Shaded areas indicate hydrophobic amino acids.

(Rapaport & Shai, 1991) and helix-II of Bti δ-endotoxin (Gazit & Shai, 1993b). The binding slopes of these pore-forming peptides increased sharply upon reaching a threshold concentration, suggesting a "barrel stave" mechanism (Ehrenstein & Lecar, 1977) for their pore-forming properties.

We propose that antibacterial polycationic polypeptides may noncooperatively bind with phospholipid membranes, thus acquiring their efficient toxic activity (Shai, 1994). A logical explanation is as follows. The site most likely to be the target of membrane-permeating antibacterial peptides is the inner membrane of bacteria, which typically contains the electron-transport chain and the enzymatic apparatus necessary for oxidative phosphorylation (Westerhoff et al., 1989). To reach the target membrane, the peptides have to traverse the bacterial wall [composed of lipopolysaccarides (LPS) in the case of Gram-negative bacteria] and the peptidoglycan layer, both of which are negatively charged. The net positive charge of the antibacterial peptides should facilitate their binding to bacteria. A noncooperative, as opposed to cooperative, binding of the peptides on the outer surface of bacteria wall will make it easier for the peptide molecules to diffuse into the inner membrane and to permeate it, rather than to remain in the outer surface as large aggregates.

Interestingly, however, although the sequences of DS-b, DS-B, and DS-S are almost identical (Table 1), only the two former can self-assemble in their membrane-bound state at very low peptide:lipid molar ratios, as revealed in the RET experiments (Figure 4). DS-B self-assembles in both PC and PC/PS vesicles while DS-b self-assembles only in PC/PS. Dermaseptins have been shown previously to adopt predominantly α-helical structures in hydrophobic environments (Mor et al., 1991, 1994; Mor & Nicolas, 1994b). Inspection of the amphiphilic structure of the three dermaseptins as envisioned in their Schiffer and Edmondson (1967) wheel projection (Figure 7) may explain this property. Both DS-b and DS-B contain an additional aspartic acid at position 1 or 5, respectively, which is on the hydrophilic surface of the peptide. This aspartic acid may partially reduce the repulsive forces between the other positive charges located within the same surface. The noncooperativity in binding of DS-b and DS-B to membranes, and their ability to self-aggregate, suggests that they are present as small bundles in their membrane-bound state. Such bundles can either lay on the surface or form small channels. Although it has not been proven, it is tempting to suggest that both the self-assembly and the efficient membrane-permeating activity of DS-B are responsible for its very high potency against filametous fungi and yeasts as compared to the other two dermaseptins.

The RET experiments (Figure 5) suggest that the extra negative charge at the N part of DS-b or DS-B is reponsible for their ability to assemble or to coassemble as well as the ability of DS-S to coassemble with the precursor DS-B.

In summary, the noncooperativity in the binding of dermaseptins to phospholipid membranes appears to be used by other amphiphilic α-helical antibacterial peptides, but not by membrane-permeating amphiphilic α-helical peptides that do not manifest efficient antibacterial activity. This noncooperative binding probably prevents the aggregation of antibacterial peptides on the surface of outer bacterial membranes, and assists them in efficiently diffusing into the inner target membranes. The exceptional property of DS-B to form small bundles correlates with its high potency to kill

yeast and filamentous fungi. These results also confirm that dermaseptins are a family of broad-spectrum antimicrobial drugs and that they are unique vertebrate peptides that exhibit potent yet selective action against microorganisms that are responsible for severe pathologies (e.g., candidosis, cryptococcosis, and nocardiosis) against which the therapeutic armamentarium is not very effective. As a molecular model, dermaseptins may thus be of potential interest in particular for drug design in antifungal warfare.

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