Structural and Functional Characterization of the α5 Segment of *Bacillus thuringiensis* δ-Endotoxin†

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ABSTRACT: One of the most conserved sequences in various δ-endotoxins is the 30 amino acid long block I. Block I of cryIIIA δ-endotoxin contains a 23 amino acid amphiphilic α-helix termed α5. The potential involvement of this α5 helix in the toxic mechanism of δ-endotoxin was examined. For this purpose, a peptide corresponding to the α5 segment and its proline incorporated analogue (P-α5) were synthesized and characterized. The α-helical content of the peptides, assessed in methanol by circular dichroism (CD), was 58% and 24% for α5 and P-α5, respectively. To monitor the interaction of α5 peptides with phospholipid membranes, they were selectively labeled at their N-terminal amino acids with the fluorescent probes 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) or carboxyfluorescein. Fluorometric studies allowed the calculation of membrane surface partition constants, which were about 10^4 M^{-1} for both α5 and P-α5, and revealed that their N-terminals are located within the lipid bilayers. The shape of the binding isotherms indicated that α5 aggregated in both zwitterionic and acidic vesicles. Functional characterization of the α5 peptides was determined by assessing their ability to dissipate a diffusion potential from sonicated small unilamellar vesicles (SUV) composed of zwitterionic or acidic phospholipids and to lyse human erythrocytes. α5 was much more active than P-α5 in both assays. Moreover, membrane-bound α5 was more protected from enzymatic proteolysis than P-α5. The results reveal a good correlation between the structure and function of α5 and experimentally support the hypothesis that α5 is a structural component of the pores formed by δ-endotoxins via aggregation of amphiphilic α-helices.

The Gram-positive soil bacteria *Bacillus thuringiensis* produce the δ-endotoxin insecticidal proteins during sporulation. Different strains of *B. thuringiensis* produce δ-endotoxins that differ in their toxic spectra (for review, see Höfte and Whiteley (1989), Aronson et al. (1986), and Whiteley and Schnepf (1986)). Commercial preparations of *B. thuringiensis* have been widely use as pesticides for more than 2 decades. The use of δ-endotoxins rather than conventional chemical pesticides is preferable, both because of their high specificity and efficiency and because of their environmental safety. The insecticidal properties of δ-endotoxins are thought to be due to the formation of pores in the cell membranes of the midgut epithelium of insects. This results in osmotic shock to the cells and ultimately causes the death of the insects (Knowles & Ellar, 1987; Sacchi et al., 1986; Wolfersberger, 1989). Different δ-endotoxins form similar cation-selective channels in planar lipid bilayers (Slatin et al., 1990), reflecting the permeation properties of the toxins. The high specificity of the various δ-endotoxins is assumed to be primarily due to the high affinity of the toxins for specific receptors in the midgut epithelium of susceptible insect species (Van Rie et al., 1990).

Although the action of the various δ-endotoxins is highly insect species specific, they share homologous sequences. One of the δ-endotoxins' most conserved motifs is the 30 amino acid long block I. X-ray crystallography of the cryIIIA δ-endotoxin showed that block I contains a 23 amino acid long α-helix, termed α5 (Li et al., 1991). The α5 region is thought to play a major role in the toxic activity of δ-endotoxins

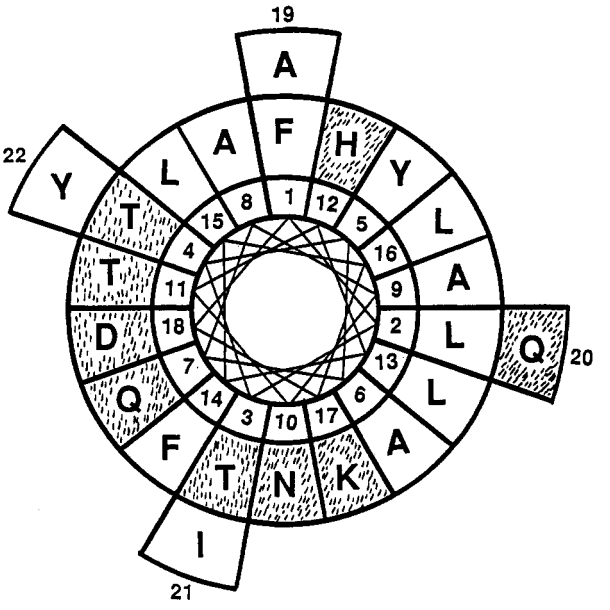

FIGURE 1: Schiffer–Edmundson wheel projection of the α5 helix. Projection of amino acid residues 193–214 of cryIIIA δ-endotoxin. Number 1 represents residue 193 of cryIIIA δ-endotoxin. Shaded areas indicate hydrophilic amino acids.

both because of its predicted amphiphilic α-helical structure (as envisioned by a plot of its Schiffer–Edmundson wheel projection shown in Figure 1) and because various mutations in this region, including introduction of proline assumed to break its α-helical structure, reduced the toxic effect of the intact molecule (Ahmad & Ellar, 1990; Wu & Aronson, 1992). A model in which amphiphilic α-helices are potential pore-forming units is based on the abundance of such structures in protein ion channels (e.g., Na^+, K^+, and Ca^{2+} channels) and membrane-permeating naturally occurring polypeptides (melit-

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tin, cecropins, and pardaxin). According to this model, trans-membrane amphiphilic α-helices form bundles in which outwardly directed hydrophobic surfaces interact either with other hydrophobic trans-membrane segments or with the lipid constituents of the membrane, while inwardly facing hydrophilic surfaces produce a pore (Inouye, 1974; Guy & Seetharamulu, 1986; Greenblatt et al., 1985; Lear et al., 1988; Oiki et al., 1988a,b; Tosteson et al., 1989; Ghosh & Stroud, 1991; Langosch et al., 1991). The trigger for the insertion of the α-helices of δ-endotoxins into the epithelium cell's membrane is assumed to be a conformational change of the toxin, which occurs when the δ-endotoxin binds to a membrane receptor via a domain other than that involved in pore formation (Ahmad & Ellar, 1990).

Herein the synthesis and functional characterization of two 23 amino acid residue polypeptides and their fluorescently labeled analogues are described. The sequence of one peptide is identical to amino acid residues 193–215 of δ-endotoxin, i.e., α5 segment, while the other is its proline^{201}-incorporated analogue. The secondary structure, membrane interaction, membrane permeability, and hemolytic activity toward human erythrocytes of these α5 peptides were examined. Our results experimentally support the hypothesis that α5 is involved in the toxic activity of δ-endotoxin, probably as a component of a homomolecular or heteromolecular bundle of trans-membrane amphiphilic α-helices that line the pore formed by the toxin.

EXPERIMENTAL PROCEDURES

Materials. BOC-Gly-PAM[1] resin and dimethylformamide (peptide synthesis grade) were purchased from Applied Biosystems (Foster City, CA), and BOC amino acids were obtained from Peninsula Laboratories (Belmont, CA). Other reagents for peptide synthesis included trifluoroacetic acid (TFA) (Sigma), *N,N*-diisopropylethylamine (DIEA) (Aldrich, distilled over ninhydrin), dicyclohexylcarbodiimide (DCC) (Fluka), and 1-hydroxybenzotriazole (HOBT) (Pierce). Dermaseptin was available in our lab (Mor et al., 1991; Pouny et al., 1992), and magainin was purchased from Sigma. 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], NBD-F (4-fluoro-7-nitrobenz-2-oxa-1,3-diazole), Rh-PE [*N*-(lissamine rhodamine B-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt], and carboxyfluorescein succinimido ester were obtained from Molecular Probes (Eugene, OR). All other reagents were of analytical grade. Buffers were prepared in double-glass-distilled water.

Peptide Synthesis and Purification. The peptides were synthesized by the solid-phase method on BOC-Gly resin (0.15 mequiv) (Merrifield et al., 1982). The resin-bound peptides were reacted with 10 equiv of thiophenol in order to remove the dinitrophenol (DNP) protecting group from histidine, cleaved from the resins by HF, and finally extracted with a mixture of acetonitrile/water (1:1, v/v) after HF evaporation, followed by solvent lyophilization. Both crude peptides contained one major peak (as revealed by RP-HPLC), which was shown to be >70% pure peptide by weight. The synthesized peptides were purified by RP-HPLC on a C_4 reversed-phase Vydac column (300 Å pore size). The column was eluted in 40 min, at a flow rate of 0.6 mL/min, using a linear gradient of 25–80% acetonitrile in water in the presence of 0.1% TFA (v/v). The purified peptides were shown to be homogeneous (~99%) by analytical HPLC. The peptides were subjected to amino acid analysis in order to confirm their composition.

Fluorescent Labeling of Peptide. Labeling of the N-terminus of the peptides with fluorescein was achieved as previously described (Rapaport and Shai, 1992). Briefly, 30–70 mg of resin-bound peptides (10–25 μmol) were treated with TFA (50% v/v in methylene chloride) in order to remove the BOC protecting group from the N-terminal amino group of the linked peptides. The resin-bound peptides were then reacted with 5(and6)-carboxyfluorescein succinimido ester (Flu-Su) (3–4 equiv) in dimethylformamide (containing 2.5% (v/v) diisopropylethylamine). After 24 h, the mixtures were washed thoroughly with methylene chloride, and the peptides were then reacted with 10 equiv of thiophenol. The peptides were then cleaved from the resins by HF and extracted with acetonitrile/water (1:1, v/v) followed by solvent evaporation.

Labeling of the N-terminus of the peptides with NBD was performed as previously described (Rapaport & Shai, 1991). Briefly, HF-cleaved peptides (~1–2 mg, HF salt) were reacted with NBD-F (2 μmol), in dry DMF, for 2–5 h. Product formation was followed by HPLC. Under these conditions the NBD moiety was attached preferentially to the peptides' N-terminus amino groups. The mixtures were then treated with 10 equiv of thiophenol, followed by precipitation with ether. All peptides were purified using RP-HPLC as described in the previous section.

Preparation of Liposomes. Small unilamellar vesicles (SUV) were prepared by sonication of PC or a PS/PC (1:1, w/w) mixture. 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 put under vacuum for 1 h and then resuspended in the appropriate buffer, via vortex mixing. The resultant lipid dispersion was then sonicated for 10–30 min in a bath type sonicator (G1125SP1 sonicator, Laboratory Supplies Company Inc., Hicksville, NY) until clear. The lipid concentration of the supernatant was determined by phosphorus analysis (Bartlett et al., 1959). Vesicles were visualized by using a JEOL JEM 100B electron microscope (Japan Electron Optics Laboratory Co., Tokyo) as follows: a drop of vesicles was deposited on a carbon-coated grid and negatively stained with uranyl acetate. The grids were examined and the vesicles were shown to be unilamellar with an average diameter of 20–50 nm (Papahadjopoulos & Miller, 1967).

CD Spectroscopy. CD spectra of the peptides were measured with a JASCO J-500A spectropolarimeter. The spectra were scanned in a capped quartz optical cell with 0.5 mm path length, at 23 °C. Spectra were obtained at wavelengths of 250 to 190–200 nm. Seven scans were taken at a scan rate of 20 nm/min. The peptides were scanned at concentrations of 2.0×10^{-5} M, in methanol. Fractional

[1] Abbreviations: BOC, butyloxycarbonyl; CD, circular dichroism; diS-C_2-5, 3,3′-diethylthiodicarbocyanine iodide; DIEA, diisopropylethylamine; DNP, dinitrophenol; Flu, fluorescein; HF, hydrogen fluoride; NBD-F, 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole; PAM, (phenylacetamido)methyl; PC, egg phosphatidylcholine; PS, phosphatidylserine; Rh-PE, *N*-(lissamine rhodamine B-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt; RP-HPLC, reversed-phase high-performance liquid chromatography; SUV, small unilamellar vesicles; TFA, trifluoroacetic acid.

helicities (Wu et al., 1981) were calculated as follows:

$$f_h = \frac{([\theta]_{222} - [\theta]^0_{222})}{[\theta]^{100}_{222}} \quad (1)$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm, and values for $[\theta]^0_{222}$and $[\theta]^{100}_{222}$, correspond to 0% and 100% helix content at 222 nm, estimated at 2000 and 30 000 deg·cm²/dmol, respectively (Chen et al., 1974; Wu et al., 1981).

Fluorometric Detection of Membrane Pores. Pore-mediated diffusion potential assays (Sims et al., 1974; Loew et al., 1983) were performed as previously described (Shai et al., 1990, 1991). In a typical experiment, 4 μL (28.8 μg) of a liposome suspension, prepared in K^+ buffer (50 mM K_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8), were 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 slowly create a negative diffusion potential inside the vesicles, leading to a quenching of the dye's fluorescence. Once the fluorescence had stabilized 3–10 mins later, α5 peptides were 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 monitored at 670 nm, with gain adjusted to 100%. The percentage of fluorescence recovery, F_t, is defined as

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

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

Hemolytic Assay. The hemolytic activity of the peptides was ascertained as previously described (Weaver et al., 1989; Shai et al., 1990). A 0.2-mL sample of human blood was mixed with 15 mL of PBS (pH 7.3), separated by centrifugation, and washed three times in PBS. The separated red blood cells were diluted 1:1800 with PBS (pH 7.3), and 400 μL of this suspension (~5500 cells/mm³) was used for light scattering experiments. Light scattering of the suspension was measured both prior to and after the addition of the peptides, and the decrease in light scattering was recorded using a Sienco Tm dual sample aggregation meter DP-247E at 37 °C. At the end of the experiment, Triton X-100 (0.1% final concentration) was added to obtain the maximal decrease of light scattering.

Intrinsic Fluorescence Measurements. Changes in the intrinsic fluorescence of NBD-labeled peptides were measured upon their binding to vesicles. NBD-labeled peptide (0.1 μM) was added to 2 mL of buffer containing 120 μL (430 μM) of SUV composed of either PC or PC/PS to establish a lipid/peptide ratio in which most of the peptide is bound to lipid. After a 2-min incubation, emission spectra were recorded with an SLM-8000 spectrofluorimeter (SLM Instruments, Urbana, IL), with excitation set at 468 nm (4-nm slit).

Binding Experiments. Binding experiments were conducted as previously described (Rapaport & Shai, 1991). Briefly, SUV (prepared as described above) were added successively to 0.1 μM peptide at 24 °C. Fluorescence intensity was measured as a function of the lipid/peptide molar ratio on a Perkin-Elmer LS-5 spectrofluorometer with excitation set at 468 nm using a 10-nm slit and emission monitored at 530 nm using a 5-nm slit, in three separate experiments. In order to account for the background signal contributed by the lipids to any given signal, the readings observed when unlabeled peptide was titrated with lipid vesicles were subtracted from a given recording of fluorescence intensity.

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 the following formula:

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

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. To calculate X_b, F_∞ (the fluorescence signal obtained when all of the peptide is bound to lipid) was extrapolated from a double reciprocal plot of F (total peptide fluorescence) versus C_L (total concentration of lipids) (Schwarz et al., 1986). As the fluorescence intensities of unbound peptide, F_0, as well as bound peptide, F, known, the fraction of membrane-bound peptide, f_b, could be calculated using the formula:

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

After calculation of 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 and 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.

Enzymatic Digestion of Membrane-Bound Peptides. Flu-α5 or Flu-P-α5 (0.1 μM) (serving as donors) was added to a 430 μM dispersion of PC or PC/PS (1:1, w/w) SUV labeled with 0.25% Rh-PE (serving as an acceptor) in buffer (50 mM Na_2SO_4, 25 mM HEPES-SO_4^{2-}, pH 6.8), followed by the addition of 20 μL of a 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 a control experiment, Flu-α5 or Flu-P-α5 (0.1 μM) was mixed with the enzyme prior to the addition of the rhodamine-labeled vesicles to the solution. Fluorescence intensities were recorded at room temperature in a Perkin-Elmer LS-5 spectrofluorometer, with excitation set at 470 nm using a 10-nm slit and with emission monitored 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.

RESULTS

To evaluate a potential role of the α5 helix in δ-endotoxin's toxicity, a peptide whose sequence is identical to that of the α5 helix segment of cryIIIA δ-endotoxin (residues 193–215), its proline-incorporated analogue, and their fluorescently labeled forms were synthesized by a solid phase method and characterized by a variety of biophysical methods. Table I lists the peptides and their designations.

CD Spectroscopy. The extent of α5 peptides' α-helical secondary structure was estimated from their CD spectra in hydrophobic environment (methanol) (Figure 2). α5 exhibited

Table I: Amino Acid Sequences of α5 Peptides and Their Fluorescently Labeled Analogues[a]

peptide no.	peptide designation	sequence
1	α5	H_2N-FLTTYAQAANTHLFLLKDAQIYG-COOH
2	NBD-α5	**NBD**-FLTTYAQAANTHLFLLKDAQIYG-COOH
3	Flu-α5	**Flu**-FLTTYAQAANTHLFLLKDAQIYG-COOH
4	P-α5	H_2N-FLTTYAQAPNTHLFLLKDAQIYG-COOH
5	NBD-P-α5	**NBD**-FLTTYAQAPNTHLFLLKDAQIYG-COOH
6	Flu-P-α5	**Flu**-FLTTYAQAPNTHLFLLKDAQIYG-COOH

[a] Modifications are indicated by boldface type.

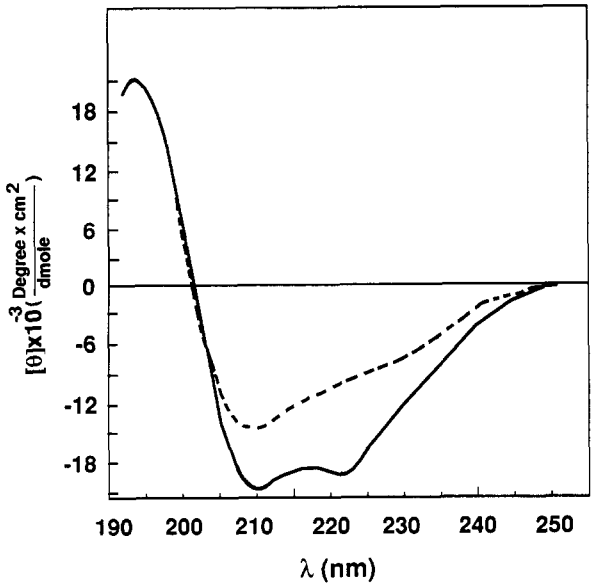

FIGURE 2: CD spectra of α5 (—) and P-α5 (- - -) in methanol. Spectra were taken as described under the Experimental Procedures section at a peptide concentration of 2.0×10^{-5} M.

a mean residual ellipticity $[\theta]_{222}$ of 19 330 ± 690 deg·cm^2/dmol, corresponding to the relatively high fractional helicity value of 57.8 ± 2.3% (Wu et al., 1981). Similarly, high α-helical contents are present in naturally occurring membrane-permeating polypeptides, such as the bee venom melittin (Vogel, 1981), bombinin-like peptides (Gibson et al., 1991), pardaxin and its analogues (Shai et al., 1990, 1991), and the antimicrobial peptides alamethicin (Rizzo et al., 1987), magainin (Chen et al., 1988), cecropins (Andreu et al., 1985), and dermaseptin (Mor et al., 1991). P-α5 exhibited a mean residual ellipticity $[\theta]_{222}$ of 9120 ± 910 deg·cm^2/dmol, corresponding to a helicity of 23.7 ± 3.1%, a value which is less than 50% of that obtained for α5, thus reflecting a distortion of α5 peptide helical structure due to the incorporation of proline.

Valinomycin-Mediated Diffusion Potential Assay. To examine the efficacy of α5 peptides to perturb the lipid packing and to cause leakage of vesicular contents, the peptides' ability to dissipate a diffusion potential of SUV was examined. Increasing concentrations of α5 peptides or their fluorescently labeled analogues were mixed with SUV composed of either phosphatidylcholine (PC) or a 1:1 mixture of phosphatidylcholine and phosphatidylserine (PC/PS) that had been pretreated with the fluorescent, potential-sensitive dye diethylthiodicarbocyanine iodide (diS-C_2-5) and valinomycin. Recovery of fluorescence was monitored with time, with peptide/lipid molar ratios of 0.01:1 to 0.2:1. The maximal potential of the peptides to permeate the membranes was elucidated by monitoring the fluorescence recovery until a plateau was observed, usually 10–30 min (Figure 3). The

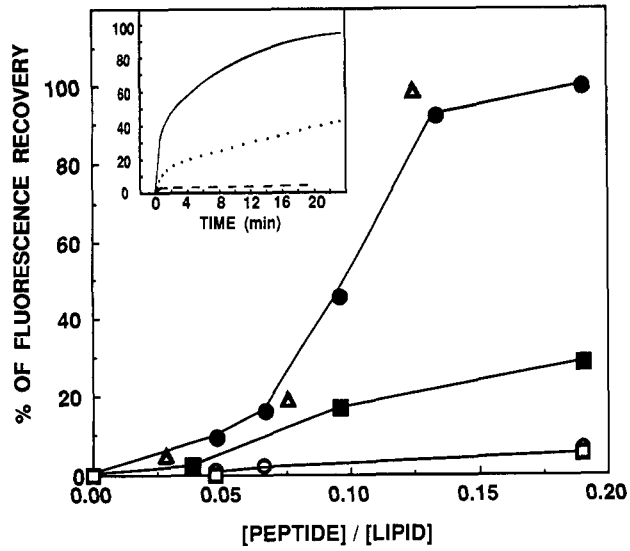

FIGURE 3: Maximal dissipation of the diffusion potential in vesicles induced by α5 and P-α5. The peptides were added to isotonic K^+-free buffer containing SUV, preequilibrated with the fluorescent dye diS-C_2-5 and valinomycin. Fluorescence recovery, measured 30 min after mixing the peptides with the vesicles, is depicted. Designations are as follows: ●, α5 with PC/PS SUV; ■, α5 with PC SUV; ▲, NBD-α5 with PC/PS SUV; ○, P-α5 with PC/PS SUV; □, P-α5 with PC SUV. Inset: Typical profiles of the kinetics of the dissipation of diffusion potential in PC/PS. Peptide designation and peptide/lipid molar ratios are as follows: ···, α5 0.095; —, α5 0.133; - - -, P-α5 0.19.

inset shows typical profiles of fluorescence recovery as a function of time for α5 and P-α5 using PC/PS vesicles, at various lipid/peptide molar ratios. Similar profiles were obtained using the fluorescently labeled α5 analogues. The results demonstrate that α5 has a higher permeating activity with PC/PS vesicles than with PC vesicles, while P-α5 has very low permeating activity with both zwitterionic and acidic vesicles.

Hemolytic Activity. The efficacy of α5 peptides and other membrane-permeating control peptides to lyse human erythrocytes was assessed by monitoring the light scattering of human erythrocytes, as previously described (Weaver et al., 1989; Shai et al., 1990). Peptide-induced lysis of erythrocytes by the α5 peptides and by the naturally occurring membrane-permeating peptides, magainin II and dermaseptin, was examined (Figure 4). The results reveal that α5 is able to effectively lyse human erythrocytes, whereas P-α5 is only moderately active.

Location of the NBD Moiety in the Hydrophobic Environments. Since the fluorescence of NBD is sensitive to its environment, the probe is suitable for polarity and binding studies (Kenner & Aboderin, 1971; Frey & Tamm, 1990; Rapaport & Shai, 1991; Pouny & Shai, 1992). Herein, the fluorescence emission spectra of NBD-α5, NBD-P-α5, and the control NBD-ethanolamine were monitored in aqueous solutions or in the presence of vesicles composed of either PC or PC/PS at pH 6.8. In these fluorometric studies, SUV were used to minimize differential light scattering effects (Mao & Wallace, 1984), and the lipid/peptide molar ratio was elevated (4300:1) so that the spectral contributions of free peptide would be negligible. The NBD-α5 and NBD-P-α5 peptides exhibited fluorescence emission maxima at 547 ± 1 nm in buffer, reflecting a hydrophilic environment for the NBD moiety (Rajarathnam et al., 1989; Rapaport and Shai, 1991). However, upon addition of vesicles (either PC or PC/PS) to the solutions containing NBD-α5 or NBD-P-α5, a blue shift in the emission maxima (toward 527–528 nm) (data

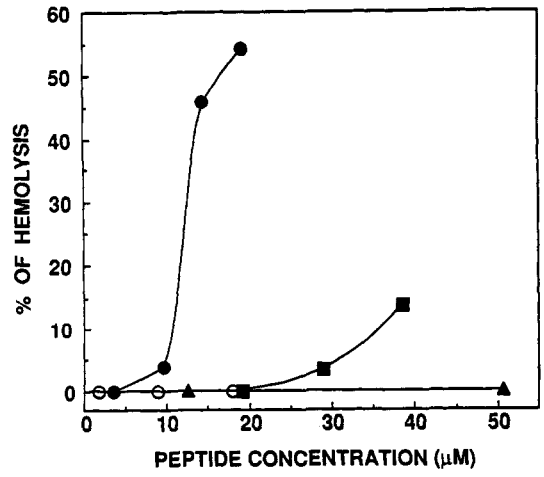

FIGURE 4: Hemolytic activity of α5, P-α5 and other membrane-permeating peptides. α5 (●), P-α5 (■), magainin II (▲), or dermaseptin (○) was added to 400 μL of an erythrocyte suspension, and light transmission was recorded for 15 min. The percent of increase in light transmission (decrease in light scattering) corresponds with the percent of hemolysis.

not shown) and an increase in the fluorescence intensities of the NBD group were observed. The changes in the spectra reflect relocation of the NBD group into the hydrophobic environment of the lipid bilayer (Chattopadhyay & London, 1987). No shift was detected when the control NBD-ethanolamine was used.

Characterization of Binding Isotherms and Determination of Partition Constants. The fluorescent properties of the NBD moiety enabled the generation of binding isotherms, from which partition coefficients could be calculated as previously described (Rapaport & Shai, 1991). First, a fixed concentration (0.1 μM) of NBD-α5 or NBD-P-α5 was titrated with PC or PS/PC (Figures 5–8) SUV. Plots of the resulting increases in the fluorescence intensities of NBD-labeled peptides as a function of lipid/peptide molar ratios yield conventional binding curves (Figures 5A, 6A, 7A, and 8A). Since the concentrations of the NBD-labeled peptides in the mixtures were low, they were assumed not to disrupt the bilayer structure. When unlabeled α5 or P-α5 was titrated with lipids up to the maximal concentration used with NBD-labeled peptides, the fluorescence intensities of these solutions (after subtracting the contribution of the vesicles) remained unchanged.

The curves obtained by plotting X_b* (molar ratio of bound peptide per 60% of the total lipid) versus C_f (the equilibrium concentration of free peptide in the solution) are referred to as the conventional binding isotherms. The experimental binding isotherms of the interactions of NBD-α5 and NBD-P-α5 with PC (Figures 5B and 7B) and PC/PS (Figures 6B and 8B) SUV were determined. The surface partition coefficients were estimated by extrapolating the initial slopes of the curves to zero C_f values. The estimated surface partition coefficients, K_p*, of NBD-α5 were $(1.0 \pm 0.2) \times 10^4$ and $(2.4 \pm 0.4) \times 10^4$ M^{-1} with PC and PC/PS vesicles, respectively (four measurements); the estimated surface partition coefficients, K_p*, of NBD-P-α5 were $(1.2 \pm 0.2) \times 10^4$ and $(2.8 \pm 0.5) \times 10^4$ M^{-1} with PC and PC/PS vesicles, respectively (four measurements). These K_p* values are within the range of those obtained for membrane-permeating bioactive peptides such as melittin and its derivatives (Stankowski & Schwarz, 1990), the *Staphylococcus* δ-toxin (Thiaudière et al., 1991), the antibiotic dermaseptin (Pouny et al., 1992), and pardaxin analogues (Rapaport & Shai, 1991).

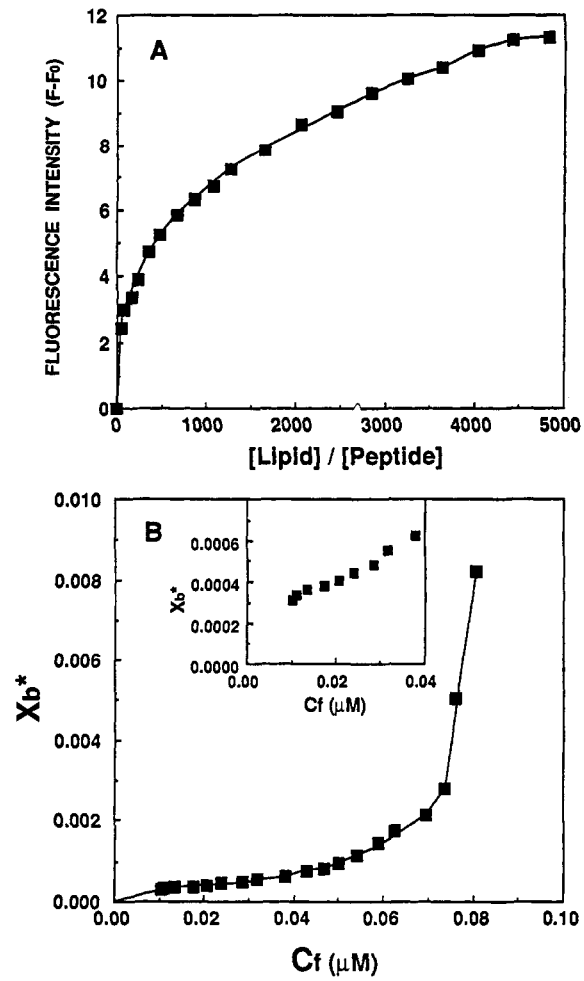

FIGURE 5: Increase in the fluorescence of NBD-α5 upon titration with PC vesicles (A) and the resulting binding isotherm (B). NBD-α5 (0.1 μM) was titrated with PC vesicles, with the excitation wavelength set at 468 nm and emission monitored at 530 nm. The binding isotherm was derived from A by plotting X_b* (molar ratio of bound peptide per 60% of lipid) versus C_f (equilibrium concentration of free peptide in the solution). Inset: Values at low C_f concentrations.

The shapes of the binding isotherms of NBD-α5 and NBD-P-α5 are rather different. With NBD-α5, the isotherms obtained with either PC or PS/PC vesicles displayed an initial "lag", i.e., initially the curves were flat, but they rose once a threshold concentration was achieved. This behavior is consistent with a process whereby peptides first incorporate into the membrane and then once inside the membrane aggregate to form a pore (Schwarz et al., 1986, 1987). Those shapes correlated with the organizational state of the following membrane-bound peptide monomers: alamethicin (Rizzo et al., 1987); the shark repellent neurotoxin, pardaxin, and its analogues (Rapaport & Shai, 1991, 1992); and the S-4 segment of the sodium channel (Rapaport et al., 1992). The binding isotherms of NBD-P-α5 are more flat, reflecting the presumably less aggregated state within the membrane.

Enzymatic Digestion of Membrane-Bound Flu-α5. The susceptibility of membrane-bound Flu-α5 or Flu-P-α5 to proteolytic digestion by proteinase-K was investigated using PC or PC/PS vesicles labeled with rhodamine (Rh). At the lipid/peptide molar ratios tested (i.e., 4300 for PC and PC/PS), more than 95% of Flu-α5 or Flu-P-α5 is bound to the vesicles, as revealed from their binding isotherms. Maximal fluorescence intensities were obtained when the labeled peptides and proteinase-K were co-incubated for 5–10 min prior to the addition of rhodamine-labeled vesicles (Figure

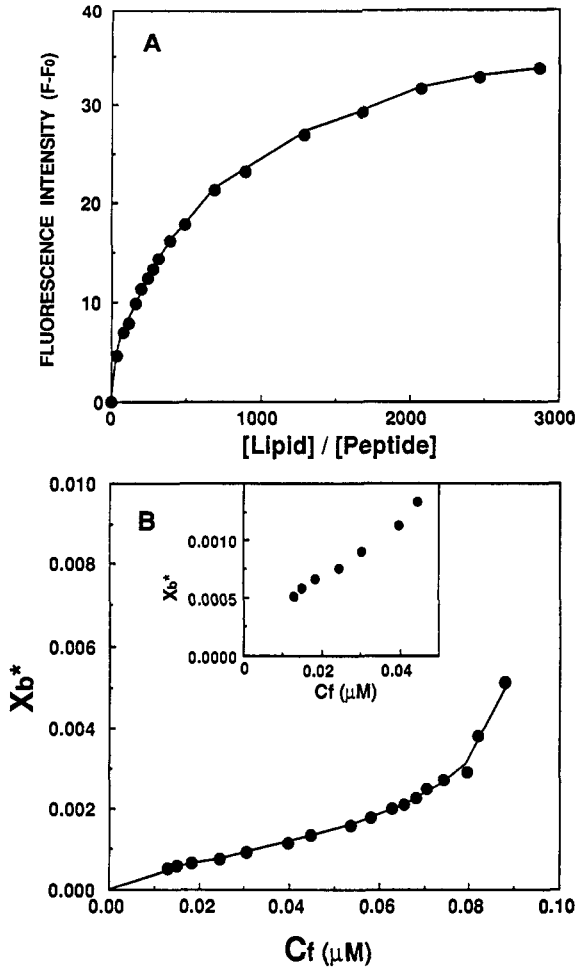

FIGURE 6: Increase in the fluorescence of NBD-α5 upon titration with PS/PC vesicles (A) and resulting binding isotherm (B). NBD-α5 was titrated with PS/PC vesicles, with the excitation wavelength set at 468 nm and emission monitored at 530 nm. The binding isotherm was derived from A, as described in the legend of Figure 5.

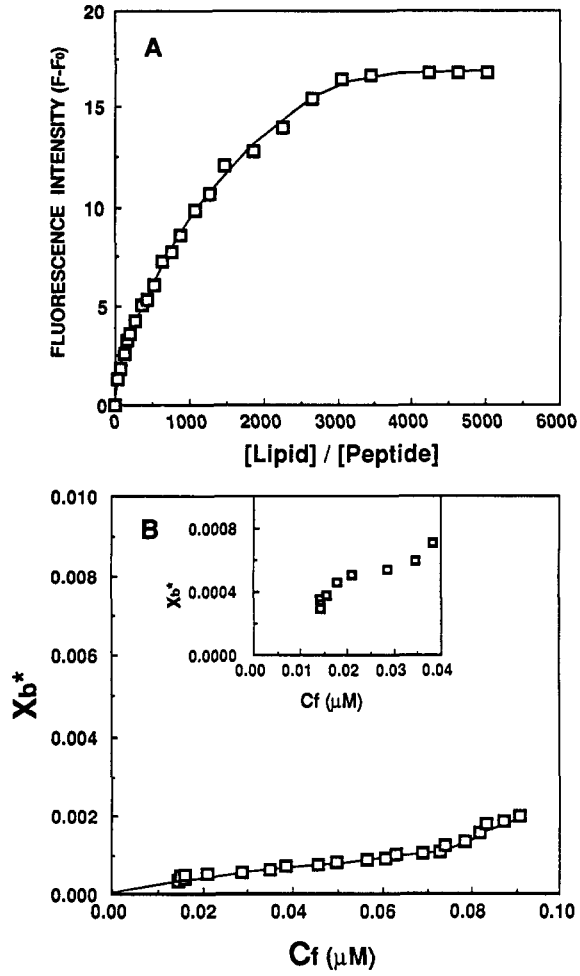

FIGURE 7: Increase in the fluorescence of NBD-P-α5 upon titration with PC vesicles (A) and the resulting binding isotherm (B). NBD-P-α5 was titrated with PC vesicles, with the excitation wavelength set at 468 nm and emission monitored at 530 nm. The binding isotherm was derived from A, as described in the legend of Figure 5.

9A). Thus, once the fluorescein-labeled peptides are digested, the addition of Rh-labeled vesicles does not affect their fluorescence intensities. In contrast, when Flu-α5 was mixed with Rh-labeled vesicles [PC (Figure 9B) or PC/PS (Figure 9C)] prior to the addition of the enzyme, virtually no increase in fluorescence occurred. Thus, demonstrating that when bound to phospholipid membranes, the α5 peptide cannot be digested by proteinase-K, which suggests that it is either embedded within the phospholipid bilayers or, as result of conformational changes in its membrane-bound state, is less available for proteolysis. However, when P-Flu-α5 was assayed some degree of digestion occurred (Figure 9E,F).

DISCUSSION

The sequence encoding the α5 helix is highly conserved in the δ-endotoxin family of insecticidal toxins. This amphiphilic α-helix (Figure 1) is postulated to play a major role in the toxic activity of δ-endotoxins (Ahmad & Ellar, 1990; Wu & Aronson, 1992). In order to evaluate the involvement of this segment in the toxic mechanism of δ-endotoxins, the structure–function relationship of the α5 segment was characterized. For this purpose, 23 amino acid peptides, corresponding to the α5 domain of the cryIIIA coleoptera-specific δ-endotoxin and its proline-incorporated analogue, were synthesized and fluorescently labeled.

δ-Endotoxins were previously shown to interact nonspecifically with phospholipid vesicles (Haider & Ellar, 1989; Yunovitz & Yawetz, 1988). Herein, the α5 peptide interacted with phospholipid vesicles with high affinity and permeating potency comparable to those of naturally occurring membrane-seeking peptides, such as melittin and its derivatives (Stankowski & Schwarz, 1990), the *Staphylococcus* δ-toxin (Thiaudière et al., 1991), the antibiotic alamethicin, and the shark repellent neurotoxin, pardaxin, and its analogues (Rapaport & Shai, 1991). Both the binding and the permeating properties of α5 were higher with acidic vesicles (PS/PC SUV, Figures 3, 5, and 7) than with zwitterionic ones (PC SUV, Figures 3, 6, and 8). These findings were similar to those observed with highly positively charged amphiphilic peptides, such as the antimicrobial peptides magainin (Zasloff, 1987) and dermaseptin (Pouny et al., 1992) and the S-4 segment of the sodium channel (Rapaport et al., 1992). However, NBD-α5 has a net neutral charge, so that electrostatic interactions, thought to be involved in the phospholipid membrane binding of charged peptides, are probably not the predominant components involved in its binding.

The maximum emission wavelength observed for NBD-α5 in the presence of vesicles (527–528 nm) is significantly lower than that observed for vesicle-incorporated NBD-phosphatidylethanolamine (NBD-PE) (533 nm) (Chattopadhyay & London, 1987). Since the NBD group in NBD-PE is believed

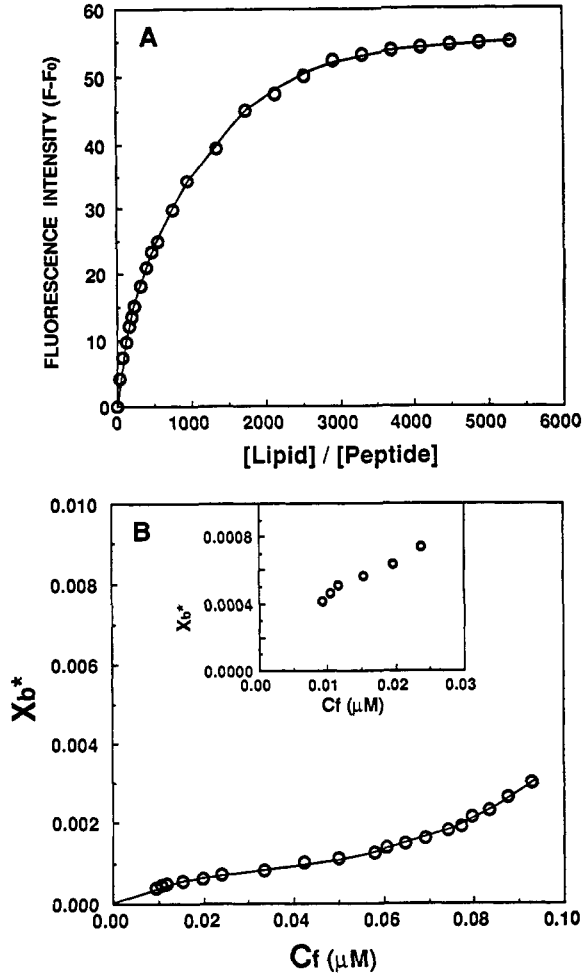

FIGURE 8: Increase in the fluorescence of NBD-P-α5 upon titration with PS/PC vesicles (A) and the resulting binding isotherm (B). NBD-P-α5 was titrated with PS/PC, with the excitation wavelength set at 468 nm and emission monitored at 530 nm. The binding isotherm derived from A, as described in the legend of Figure 5.

to lie at the surface of the vesicles, it can be concluded that the N-terminus of NBD-α5 is located under the surface of the vesicles. One interpretation is that the N-terminus of the peptide is located in close proximity either to the inner or to the outer surface of the vesicles. However, further investigation is required to clarify this point. That membrane-bound α5 is not digested by proteinase-K (Figure 9) might also indicate that vesicle-associated α5 is localized within the lipid bilayers. Such results could also be explained by a conformational change of the peptide from a presumably unordered structure in aqueous solution to an α-helical structure in a hydrophobic environment (Figure 2). However, the upward curvature of NBD-α5's binding isotherms (Figures 5 and 6), which are similar to those obtained for channel-forming peptides such as pardaxin and its derivatives (Rapaport & Shai, 1991) and alamethicin (Rizzo et al., 1987), argues in favor of a process whereby peptides first incorporate into the membrane and then aggregate.

Proline is an imino acid incapable of participating in hydrogen bonding and thus can introduce structural distortion into trans-membranal helices. Indeed, P-α5 exhibits a lower value of α-helical structure than α5 (Figure 2). Nevertheless, this reduced α-helicity does not affect its ability to bind to phospholipid membranes, since its partition coefficient is similar to that of α5. However, the decrease in P-α5's α-helical structure causes a reduction in its permeating and hemolytic abilities (Figures 3 and 4). These reductions are in agreement

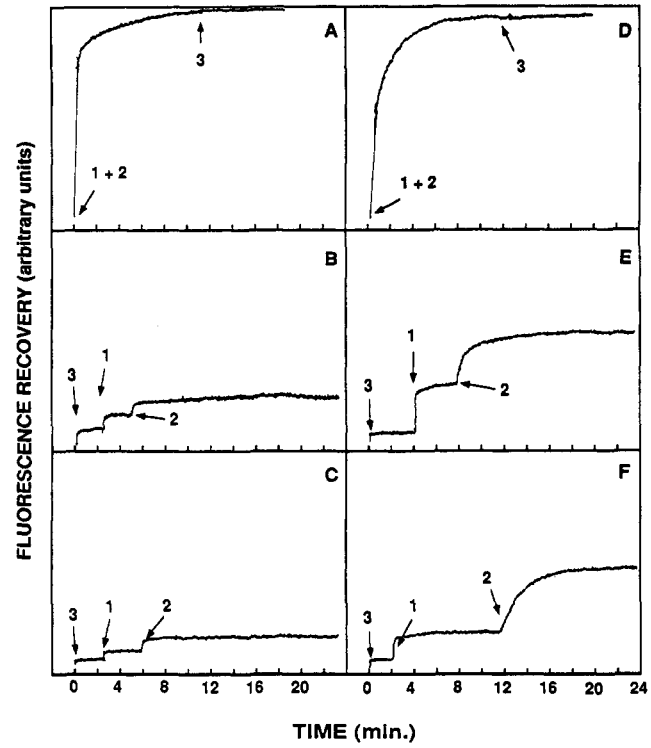

FIGURE 9: Digestion by proteinase-K of Flu-α5 or Flu-P-α5 in the presence of vesicles: Flu-α5 (0.1 μM, 1 in panels A–C) or Flu-P-α5 (0.1 μM, 1 in panels D–F), PC vesicles containing 0.25% Rh-PE (430 μM, 3 in panels B and E) or PS/PC vesicles containing 0.25% Rh-PE (430 μM, 3 in panels C and F), and proteinase-K (5 μg, 2) were mixed together (final volume, 2 mL) at room temperature at the indicated times in the following order: (A) enzyme (2) plus α5 (1), PC or PS/PC vesicles (3); (B) PC vesicles (3), α5 (1), enzyme (2); (C) PC/PS vesicles (3), α5 (1), enzyme (2); (D) enzyme (2) plus P-α5 (1), PC or PS/PC vesicles (3); (E) PC vesicles (3), P-α5 (1), enzyme (2); (F) PC/PS vesicles (3), P-α5 (1), enzyme (2). The excitation wavelength was set at 470 nm and the emission was monitored at 530 nm.

with the lower degree of P-α5's self-aggregation within phospholipid vesicles as compared to that of α5, as revealed by the differences in the shapes of their binding isotherms (Figures 5–8). Therefore, α-helical structure appears to be important for the effective aggregation of monomers that results in the formation of a pore. Similar effects were obtained using D-amino acid incorporated analogues of pardaxin (Pouny & Shai, 1992). Our results are consistent with the in vivo and in vitro losses of toxicity of site-directed mutants of cryIA$_c$ (Wu & Aronson, 1992) and sotto (Ahmad & Ellar, 1990) δ-endotoxins, in which proline-substituted alanines occur at the same or nearby positions within the α5 segment.

The results herein are consistent with current models explaining δ-endotoxin's toxicity (review by Gill et al. (1992)). According to these models, δ-endotoxins are composed of two major domains, one involved in receptor binding and one responsible for their toxic effects. Receptor binding causes conformational changes, thus exposing δ-endotoxin's pore-forming domain, which contains the α5 helix. The subsequent interaction of the toxic domain with target cell membranes facilitates pore formation in the midgut epithelium of susceptible species. That site-directed mutagenesis of the α5 region of δ-endotoxin alters its toxicity, but not its membrane-receptor binding (Ahmad & Ellar, 1990; Wu & Aronson, 1992), further suggests that α5 is not involved in the binding of δ-endotoxins to the midgut membranes of susceptible species, but rather in pore formation.

In summary, our data experimentally support the hypothesis that the α5 helix is a constituent of the bundles of trans-

membrane α-helices of δ-endotoxin pores. The δ-endotoxin pore might be formed by either (i) the toxic domains of several δ-endotoxin molecules aggregating such that a bundle of $\alpha 5$ helices forms a intermolecular pore or (ii) aggregation of several α-helices within the same toxic domain of a δ-endotoxin molecule to form an intramolecular pore.

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