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Specific antimicrobial and hemolytic activities of 18-residue peptides derived from the amino terminal region of the toxin pardaxin

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Peptides are part of the host defense system against bacteria and fungi in species right across the evolutionary scale. However, endogenous antibacterial peptides are often composed of 25 residues or more and, therefore, are not ideal for therapeutic use. Hence it is of considerable interest to design and engineer short peptides having antimicrobial activity. Peptides composed of 18 amino acids, derived from the N-terminal region of the 33-residue toxin pardaxin (PX), GFFALIPKIISSPLFKTLLSAVGSALSS-SGEQE, were synthesized and examined for biological activities. Peptide corresponding to the 1–18 stretch of PX exhibited antimicrobial activity only against *Escherichia coli* and not against Gram-positive microorganisms. The peptide also did not possess hemolytic activity. Replacement of P7 by A resulted in a peptide possessing both antibacterial and hemolytic activity. Substitution of both K residues by Q in the 'A' analog resulted in a peptide having only hemolytic activity. Conformational analysis of these peptides and investigation of their model membrane permeabilizing activities indicated that selective activity can be explained by their biophysical properties. Hence, by a rational design approach based on biophysical principles, it should be possible to generate short peptides having specific biological activity.

Keywords: antimicrobial activity/hemolysis/pardaxin/synthetic peptides

Introduction

There has been considerable interest in recent years in various aspects of the structure and biological activities of endogenous antibacterial and hemolytic peptides which form part of the host defense mechanism of species right across the evolutionary scale (Boman and Hultmark, 1987; Bevins and Zasloff, 1990; Boman, 1991; Lehrer *et al.*, 1991; Hultmark, 1993). Detailed investigations have revealed how these endogenous peptides exert their activity and also structural requirements necessary for activity (Christensen *et al.*, 1988; Duclohier *et al.*, 1989; Kagan *et al.*, 1990; Hill *et al.*, 1991; Sansom, 1991). Based on this knowledge, there have been attempts to synthesize 'designer peptides' with improved biological activity (DeGrado and Lear, 1985; Lee *et al.*, 1986; Boman *et al.*, 1989; Blondelle and Houghten, 1992). An important aspect in the design of such model peptides has been to explore whether short peptides (~15–18 residues) have biological activity (Houghten *et al.*, 1991; Andreu *et al.*, 1992; Blondelle *et al.*, 1994) as the endogenous peptides are often >25 residues in length (Boman and Hultmark, 1987; Bevins and Zasloff, 1990; Boman, 1991; Lehrer *et al.*, 1991; Hultmark, 1993).

Our approach to generating designer peptides has involved the analysis of the sequences of endogenous antibacterial or hemolytic peptides in order to delineate regions that may contribute to activity, synthesize peptides corresponding to these regions as well as their variants and examine their biological activity (Saberwal and Nagaraj, 1989, 1993; Sitaram and Nagaraj, 1990; Sitaram *et al.*, 1992, 1993, 1995; Dhople and Nagaraj, 1995; Thennarasu and Nagaraj, 1995). Based on the activities observed, they were further 'engineered' in order to generate analogs with improved and/or specific activity and also delineate the features required for optimal activity (Dhople and Nagaraj, 1995; Sitaram *et al.*, 1995). By this approach we have obtained 13-residue peptides corresponding to the most hydrophobic region of a 47-residue antibacterial protein, seminalplasmin, having antibacterial activity (Sitaram and Nagaraj, 1990; Sitaram *et al.*, 1992, 1993, 1995). Likewise, we have also obtained 16-residue peptides related to the α-helical region of the hemolytic toxin δ-hemolysin having hemolytic and/or antibacterial activity (Dhople and Nagaraj, 1995). In this study, we chose the 33-residue toxin pardaxin as the target molecule. Pardaxin is secreted by the sole fish of the genus *Pardachirus* (Lazarovici *et al.*, 1986). The toxin is presumed to play a role in the defense mechanism of the fish against predators. Lipid bilayers doped with pardaxin at concentrations below 10^{-7} M show single-channel conductance behavior (Morah *et al.*, 1984). At concentrations of ~10^{-4} M, pardaxin causes cytolysis (Lazarovici *et al.*, 1986). The peptide also exhibits several other pharmacological effects, including exocytosis of bovine adrenal medullary chromatin granules (Lazarovici and Lelkes, 1993) and release of acetylcholine from electric organ synaptosomes (Arribas *et al.*, 1993). Pardaxin binds to and permeates phospholipid vesicles and its biological activities appear to stem from these properties (Shai, 1994). We show in this paper that the peptide corresponding to the N-terminal 1–18 segment, GFFALIPKIISSPLFKTL-amide, does not show cytolytic activity like pardaxin but has antibacterial activity against *Escherichia coli*. When the proline at position 7 is substituted by alanine, the resultant peptide shows both cytolytic and antibacterial activity. Replacement of K by Q in this peptide results in the peptide possessing only hemolytic activity. The primary structures of pardaxin and peptides described in this study are shown in Table I.

Materials and methods

Materials

Fmoc amino acids, NovaSyn KR resin and dimethylaminopyridine (DMAP) were obtained from Nova Biochem (UK). Trifluoroacetic acid (TFA) was purchased from Aldrich (USA). Synthesis-grade piperidine was procured from Merck (India). Diisopropylethylamine (DIPEA) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Applied Biosystems (USA). Synthesis-grade *N*-methylpyrrolidone (NMP) was purchased from LOBA Biochem (India). Lipids were obtained from Avanti Polar

Table I. Primary structure of pardaxin and related peptides

Peptide	Sequence[a]
Pardaxin (PX)	GFFALIPKIISSPLFKTLLSAVGSALSSGEQE
18P	GFFALIPKIISSPLFKTL-amide
18A	GFFALIAKIISSPLFKTL-amide
18Q	GFFALIAQIISSPLFQTL-amide

[a]Amino acid residues that are underlined reflect changes from the pardaxin sequence.

Lipids (Alabaster, AL, USA). Carboxyfluorescein (sodium salt) was procured from Molecular Probes (USA). All other reagents were of analytical grade. Unless stated otherwise, all the experiments were carried out in 150 mM saline buffered to pH 7.4 by 10 mM HEPES.

Peptide synthesis

The peptides were synthesized on polyamide resins using Fmoc chemistry (Atherton and Sheppard, 1989) on a Biolynx 4175 Pharmacia LKB Peptide Synthesizer. Fmoc-Leu was converted into the corresponding symmetrical anhydride and coupled to NovaSyn KR resin in NMP using DMAP as catalyst. All the other amino acids were coupled as HBTU active esters in the presence of an equivalent amount of HOBT using DIPEA as the catalyst. At the end of the synthesis, the resin was washed thoroughly with DMF and diethyl ether and dried *in vacuo*. The peptides were cleaved from the resin using a mixture containing TFA, thioanisole, ethanedithiol and phenol (8.75:0.5:0.5:0.25, v/v) at room temperature for 12 h. All the peptides contained one major peak when checked on a Pharmacia Fast Performance Liquid Chromatography (FPLC) system using a PepRPC reversed-phase column, which was >85% pure by weight. The peptides were purified using a linear gradient system consisting of 0.1% (v/v) TFA in water and 0.1% (v/v) TFA in acetonitrile. The compositions of the peptides were confirmed by amino acid analysis on an LKB Alpha Plus 4151 Amino Acid Analyzer after hydrolysis *in vacuo* by 6 M HCl.

Antibacterial activity

Different bacterial strains from mid-logarithmic phase cultures were inoculated into LB medium (Miller, 1972) to a concentration of ~10^5 CFU/ml. After 6–7 h of incubation at 37°C, the absorbance of the cultures at 600 nm (A_{600}) were determined. The A_{600} value observed for the control culture for which no peptide was added was taken as 100 for the purpose of calculation of percentage inhibition. Aliquots which showed maximum inhibition of growth were appropriately diluted (~15 000 cells/ml), and 20 µl of each of these diluted culture media were plated onto nutrient agar plates and incubated at 37°C for 15 h. Peptide concentrations of aliquots which showed no colony-forming units were taken as the minimal inhibitory concentrations (MIC). The experiments were performed in duplicate and triplicate independently.

Outer membrane (OM) and inner membrane (IM) permeabilization

Escherichia coli transformed with plasmid pBR322 was used for the permeabilization experiments. The strain was constitutive for β-galactosidase, a cytoplasmic enzyme and β-lactamase, a periplasmic enzyme. For the experiments, bacteria were prepared by inoculating 10 ml of LB medium containing 0.5 mg of ampicillin with organisms picked from a single colony. After overnight (16 h) incubation at 37°C, the culture was washed three times with 10 mM sodium phosphate buffer (PBS), pH 7.4 and resuspended in PBS to a concentration of ~10^8 CFU/ml (OD = 0.35). A stock solution of 7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethylaminophenylazo)pyridinium-methyl]-3-cephem-4-carboxylic acid (PADAC) (from Calbiochem, USA) was prepared by dissolving the compound in phosphate buffer to a concentration of 0.5 mg/ml. An *o*-nitrophenyl-β-D-galactoside (ONPG) stock solution was prepared at a concentration of 3 mg/ml in PBS. Assay mixtures contained ~10^6 CFU of *E.coli* cells per ml of phosphate buffer containing PADAC or ONPG and peptides. The chromogenic substrate PADAC is impermeable to the OM and is accessible to β-lactamase only when the OM is permeabilized. This is reflected by a decrease in OD at 570 nm (Lehrer *et al.*, 1988, 1989). The increase in the OD at 420 nm (in the presence of peptides) over and above the control, in the absence of peptide was taken as a measure of IM permeabilization (Lehrer *et al.*, 1988, 1989).

Lysis of erythrocytes

Human erythrocytes were used for the experiment. The buffy coat was removed by centrifugation of freshly collected blood and washing three times with isotonic saline. Erythrocytes (~2×10^7 cells) were incubated at 37°C in 10 mM HEPES buffered saline with different concentrations of test peptides for 30 min, centrifuged and the absorbance at 540 nm of the supernatant was measured. The absorbance obtained by treatment of erythrocytes with 0.1% Triton X-100 was taken as 100%.

Circular dichroism (CD)

CD measurements were carried out on a Jobin Yvon dichrograph V spectropolarimeter which was calibrated using 10-(+)-camphorsulfonic acid (Chen and Yang, 1977). Spectra were obtained on samples containing 5 and 10 µM peptide at various water:TFE proportions. No concentration dependence was observed in the spectra in the indicated range. The spectra were normalized for concentration and path length to obtain the mean residue ellipticity after baseline correction. All the measurements were done at 25°C.

Vesicle preparation and model-membrane permeabilization experiments

Small unilamellar vesicles (SUV) made of pure lipid, dioleoylphosphatidylcholine (DOPC), were essentially prepared by sonication. The lipid solution in chloroform was taken to dryness and then dispersed in buffer or buffer containing carboxyfluorescein (CF) (50 mM CF, 100 mM NaCl, 10 mM HEPES, pH 7.4) by vortex mixing. The resulting liposome suspension was sonicated using a sonfier (Branson Sonic Power, Danbury, CT) until clear solutions were obtained. The sonicated solution was passed through a Sephadex G-75 column (40×1.5 cm i.d.) to separate CF-entrapped lipid vesicles from free CF. The elution buffer contained 150 mM NaCl. The concentration of lipid was estimated by the method of Stewart (1980). The peptides were added from a stock solution using a Hamilton syringe to lipid vesicles and the release of CF as a function of time was recorded continuously.

Results and discussion

Choice of peptides

The peptides chosen for the study are listed in Table I. Preliminary investigations had indicated that the C-terminal

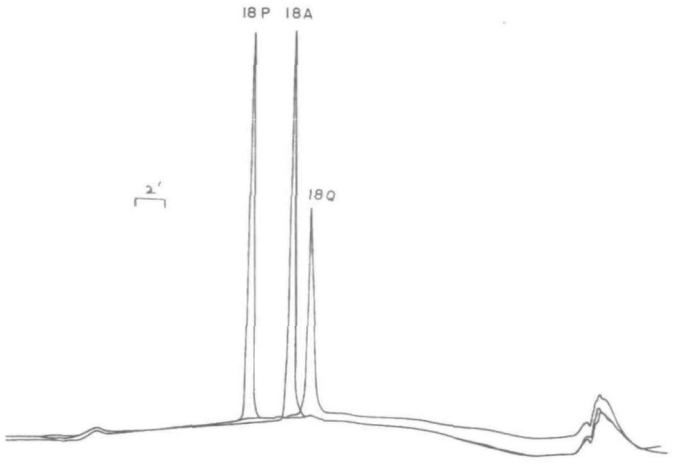

Fig. 1. FPLC of purified peptides derived from pardaxin. The peptides were analysed on a Pharmacia C_{18} PepRPC HR 5/5 column Solvent 1: 0.1% TFA in water Solvent 2: 0.1% TFA in acetonitrile. Elution conditions: linear gradient from 0% acetonitrile in water containing 0.1% TFA at 5 min to 100% acetonitrile containing 0.1% TFA at 45 min. Detection at 214 nm.

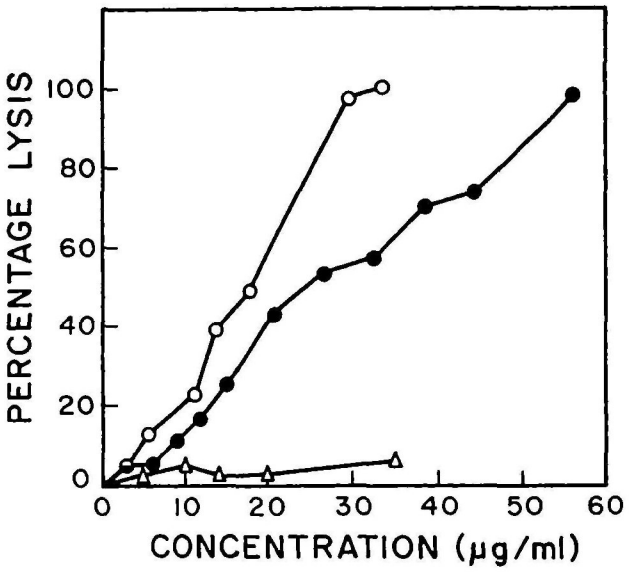

Fig. 2. Hemolysis of human erythrocytes as a function of peptide concentration: (○), 18A; (o), 18Q; (△), 18P. Data points correspond to means of two independent observations. The variation was <10%

segment of pardaxin, KTLLSAVGSALSSSGEQE, did not have membrane permeabilizing activity. Since positive charges are presumed to play an important role in determining the membrane activity of peptides related to the N-terminal segment of pardaxin (Saberwal and Nagaraj, 1993), the N-terminal 18-residue segment which has two K residues was synthesized and checked for activity. In pardaxin, P13 causes structural distortion in the helix backbone as suggested by CD and NMR studies (Shai *et al.*, 1990; Zagorski *et al.*, 1991). This distortion is not present when P13 was replaced by A (Shai *et al.*, 1990). Since P7 in 18P can also bring about structural distortion and thereby modulate its activities, an analog wherein P7 was replaced by A was synthesized. It has been observed that Gln has an important role in the channel formation of peptides devoid of charged residues (Fox and Richards, 1982; Mathew and Balaram, 1983; Karle *et al.*, 1991). Hence, an analog of 18A was synthesized wherein the two lysine residues were replaced by Q. The grand average hydropathy (GRAVY) values calculated by the program SOAP developed by Kyte and Doolittle (1982) for 18P, 18A and 18Q are 11.88, 13.77 and 14.22, respectively. These values indicate that the order of hydrophobicity amongst the peptides is 18P < 18A < 18Q. Since retention time on a reversed phase matrix is determined to a large extent by hydrophobic interaction, the retention times of the peptides were checked on a C_{18} reversed-phase FPLC column. The profiles of the purified peptides shown in Figure 1 indicate that the order of hydrophobicity is the same as indicated by the calculation based on Kyte and Doolittle's method.

Hemolytic activity

The hemolytic activity of peptides against human erythrocytes was examined. Peptide 18P did not exhibit hemolytic activity even at a concentration of 150 μg/ml, whereas 18A and 18Q were hemolytic. The activities of 18A, 18Q and 18P are shown in Figure 2; 100% hemolysis is observed for 18A at a concentration of 30 μg/ml. 18Q also shows considerable hemolysis but is relatively less active, as 100% lysis is observed at 55 μg/ml. In these concentration ranges, 18P is totally inactive. Thus, a single switch of P to A has profound effect on activity. Since 18Q can cause hemolysis, the requirement of positively charged residues for hemolytic activity is clearly not essential.Peptide 18A was, however, less active than PX, which showed 100% hemolysis at 13 μg/ml.

Table II. Antibacterial activity of peptides related to pardaxin

Peptide[a]	Range of concentration in which growth inhibition was observed (μg/ml)	MIC against *E.coli* (μg/ml)	100% hemolysis (μg/ml)
PX	3.4–34	24	14
18P	4.0–40	34	NA
18A	10–50	40	33
18Q	NA	NA	56

[a]MIC represents mean values of three independent experiments performed in duplicate. Peptide 18Q did not inhibit microbial growth at a concentration of ~50 μg/ml. NA = not active.

Antibacterial activity

The antibacterial activity of the peptides listed in Table I was examined against *E.coli*, a Gram-negative bacterium, and the activities of the peptides are presented in Table II. It was found that 18P and 18A exhibit activity with comparable MIC, which is higher than that of PX. 18P, which is not hemolytic, can inhibit the growth of *E.coli*; however, 18Q, which is hemolytic, has no antibacterial activity. The peptides exhibited activity specifically only against *E.coli*. Although PX has potent antibacterial and hemolytic activities, its N-terminal 18-residue segment has only antibacterial activity. The lack of activity observed for 18Q suggests the importance of charged residues as determinants of antibacterial activity, unlike in the case of hemolysis. The peptides did not show antibacterial activity against *Staphylococcus aureus*, a Gram-positive micro-organism.

Mechanism of antibacterial activity

Since the peptides in Table I are too large to pass through porin channels in the OM of *E.coli*, their mode of entry is likely to be by perturbation of the OM as in the case of defensins (Lehrer *et al.*, 1989). Hence, the OM permeability in *E.coli* transformed with pBR322 was examined in the presence of 18A, 18P and 18Q. The bacterial cells had

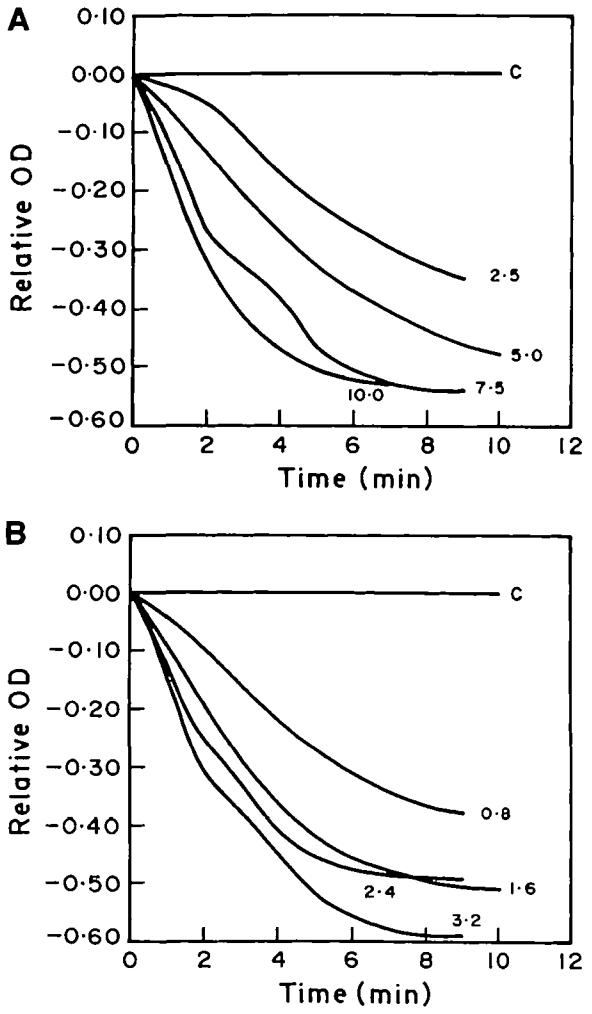

Fig. 3. Permeabilization of *E.coli* outer membrane assessed by influx of PADAC, a chromogenic substrate for the periplasmic enzyme β-lactamase. Profiles of membrane permeabilization shown by peptides: (A) 18P and (B) 18A. The numbers next to the lines correspond to peptide concentration (μg/ml) and c indicates control where no peptide is present. Details of the experimental protocol are given in Materials and methods.

β-lactamase in the periplasmic space, which was cryptic for PADAC unless the OM was permeabilized. This resulted in hydrolysis of PADAC, an event that can be followed by UV spectroscopy. Figure 3 shows the hydrolysis of PADAC in the presence of 18P and 18A. Both peptides cause fairly rapid hydrolysis of PADAC, indicating the ability of the peptides to permeabilize the OM. It was found that 18A is more effective than 18P in this process; 18Q did not have the ability to cause the influx of PADAC, indicating a requirement for positive charges for the process. However, the activities of 18A and 18P indicate that even two lysine residues are sufficient for this purpose. Figure 4 shows the effect of the peptides 18A and 18P on ONPG (a substrate for β-galactosidase) influx across the bacterial plasma membrane. Both the peptides permeabilize the IM at comparable efficiencies.

Studies on N-terminal peptides related to PX reveal that the N-terminal 18-residue peptide has selective antibacterial activity. A single amino acid replacement of P to A results in a peptide possessing both antibacterial and hemolytic activities. The importance of cationic residues for antibacterial activity but not for hemolytic activity is borne out by the properties of 18Q. In order to rationalize the activities of the peptides in

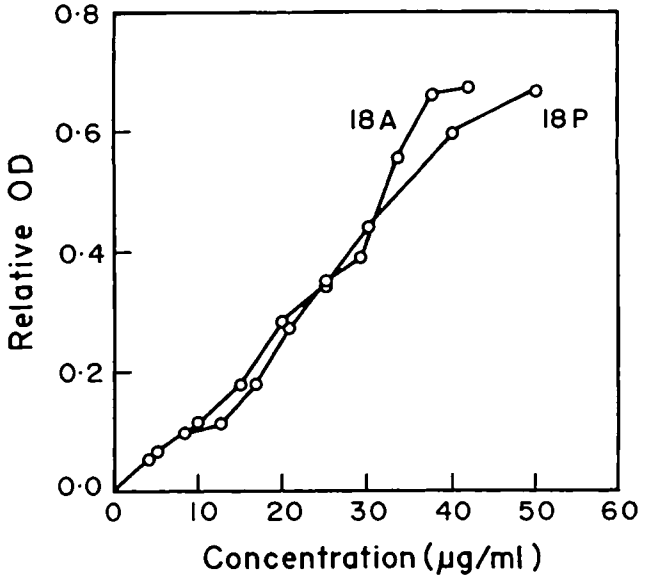

Fig. 4. Permeabilization of *E.coli* inner membrane by peptides assessed by influx of ONPG, a substrate for cytoplasplasmic β-galactosidase.

terms of structure and membrane permeabilizing abilities, the conformations of the peptides and activities on model membranes were examined.

Conformation of peptides

The conformations of 18P, 18A and 18Q were examined in aqueous medium at pH 7.4 and varying proportions of TFE. The results are presented in Figure 5. In an aqueous environment, 18P and 18A do not have a significant population in ordered conformation. With increasing TFE, spectra with double minima characteristic of a helical conformation are observed. The contribution of various secondary structures was estimated by the method of convex constraint analysis (Perczel *et al.*, 1991, 1992). Only an ~21% helical content is observed for 18P even in neat TFE. For 18A, the analysis indicates ~40% helical content in neat TFE with a random contribution of ~60%. The lower helical content observed for 18P probably arises owing to the presence of P, especially P7, as a replacement of P7 by A results in an increased helical content. Alternatively, it is possible that the helical structure in 18P is distorted, as distortions from regular helix or β structures are known to result in spectra different from those for idealized helix or β structures (Manning *et al.*, 1988). Peptide 18Q adopts a β structure in an aqueous environment. With increasing amounts of TFE, the single minimum persists until ~65% TFE. At 100% TFE, a spectrum with double minima is observed. Convex constraint analysis of the spectra with a single minimum indicates β and random structures with no helical content. Even in neat TFE, β structure (31%) predominates over helical structure (19%). Random structure contributes 50%. Now, although TFE promotes helical conformation, it does so only if the peptide has an intrinsic propensity to form a helix (Dyson *et al.*, 1988, 1992). CD results indicate that only 18A has a propensity for helix conformation. In 18P, the presence of Pro prevents the formation of a long helical stretch and in neat TFE, the fraction of molecules in ordered conformation probably exists as a distorted helix. The CD spectra of 18Q in micelles and lipid vesicles are shown in Figure 6. The spectra indicate that the peptide adopts a β-structure in lipid vesicles.

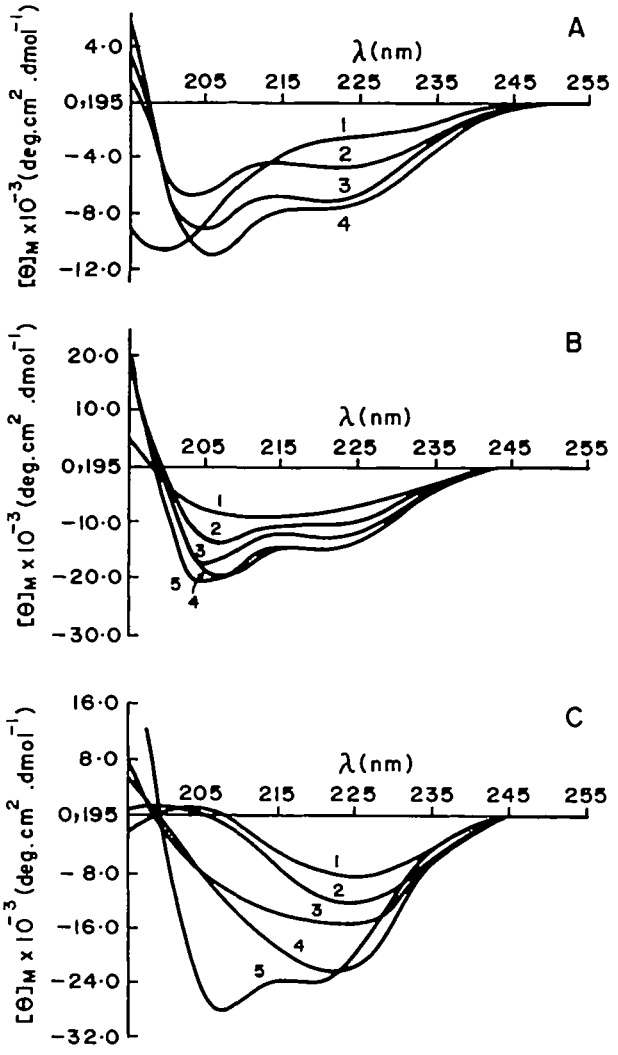

Fig. 5. Circular dichroism spectra of peptides in buffer at pH 7.4 and various amounts of trifluoroethanol (TFE). (A) 18P (10 μM); 1, 2 and 3 correspond to 10, 20 and 30% (v/v) TFE, respectively, and 4 to neat TFE. (B) 18A (10 μM); 1, 2, 3, 4 and 5 correspond to 10, 20, 30, 50 and 100% TFE, respectively. (C) 18Q (5 μM); 1, 2, 3, 4 and 5 correspond to 10, 20, 30, 65 and 100% TFE, respectively.

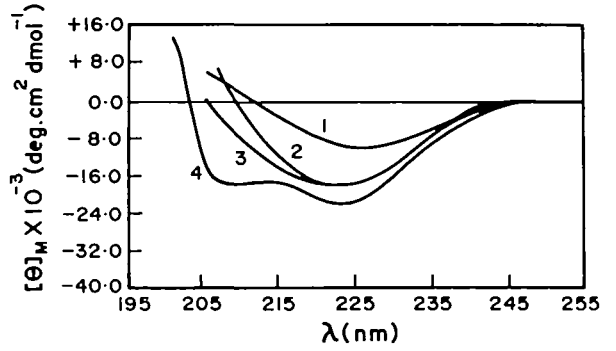

Fig. 6. Circular dichroism spectra of 18Q (5μM) in the presence of micelles and small unilamellar lipid vesicles: (1) DOPC; (2) PI; (3) PS; (4) SDS micelles (30 mM). Lipid concentration: 100 μM.

Model membrane permeabilization

The ability of 18P, 18A and 18Q to perturb model membranes was assessed by monitoring the release of entrapped solutes such as CF. This method has been used extensively to obtain details about the channel-forming properties of peptides in

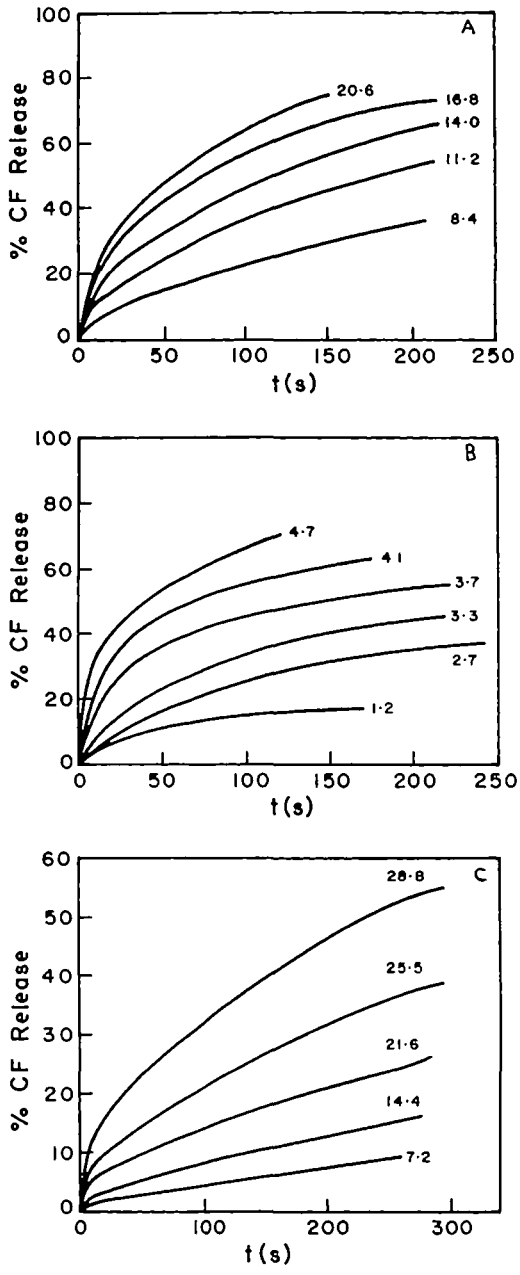

Fig. 7. Ability of peptides to cause the release of entrapped CF from DOPC vesicles: (A) 18P; (B) 18A; (C) 18Q. Numbers adjacent to the lines correspond to the concentration (μg/ml) of peptides added. Lipid = 100 μM.

lipid vesicles (Menestrina, 1988; Schwarz and Robert, 1990; Shai *et al.*, 1990; Vaz *et al.*, 1993). The CF release profiles from DOPC vesicles in the presence of peptides are shown in Figure 7. The profiles indicate that 18A is the most effective in permeabilizing the vesicles, followed by 18P and 18Q. The membrane activity of 18Q and the CD data together indicate that a helical conformation is not an absolute prerequisite for membrane association. Although our CD data do not permit us to arrive at a molecular structure for 18Q, it is likely that it forms an amphipathic β-sheet, with peptide molecules connected by intermolecular hydrogen bonding. The greater membrane-permeabilizing activity of 18A over 18P correlates with their relative hydrophobicities. However, 18Q, although more hydrophobic than 18A or 18P, is less effective in

permeabilizing model membranes. This observation suggests that correlation of membrane activity and hydrophobicity may be valid only in cases where peptide structures are similar as with some model peptides (Blondelle and Houghten, 1992).

In our search for peptides possessing specific biological activity, we have found that 18P, the N-terminal segment of PX, has only antibacterial activity with no hemolytic activity even at high concentrations. The properties of other analogs generated from this peptide as well as conformational and membrane permeabilization studies suggest requirements for specific activity. The analog 18A, which has both antibacterial and hemolytic activities, is hydrophobic and cationic, has a strong propensity to occur in helical conformation and also permeabilizes model membranes most effectively. The antibacterial peptide 18P is cationic and less hydrophobic, has a low propensity to occur in a helical conformation and is less effective in permeabilizing lipid membranes than 18A. The analog 18Q, which has only hemolytic activity, is the most hydrophobic and devoid of net charge. Thus, an 'inefficient' membrane permeabilizing peptide has selective biological activity. In red blood cells and other eukaryotic cells, the volume-regulating ability of Na^+ pumps in the plasma membrane has to be overcome for lysis to occur by a colloid osmotic mechanism (Pasternak *et al.*, 1985). If the peptide cannot perturb the membrane to an extent where the volume-regulating ability is not overcome, no lysis occurs, which is the case with an 'inefficient' channel former. The bacterial plasma membrane has all the respiratory enzymes and a membrane potential is necessary for proper functioning (Lughtenberg and Van Alphen, 1983). Even a slight perturbation would result in loss of membrane functions. Hence, an 'inefficient' channel former can have antimicrobial activity, provided, of course, it can gain access to the IM. The activities of the peptides used in this study indicate that while positive charges are essential, even two lysines are sufficient for OM perturbation.

In summary, our studies indicate that selective antibacterial or hemolytic activities of peptides may be explained by their biophysical properties. It should be possible rationally to design and engineer short peptides having selective activity based on biophysical principles such as conformation, membrane permeabilizing ability, hydrophobicity and net charge.

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