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Identification and characterization of a primary antibacterial domain in CAP18, a lipopolysaccharide binding protein from rabbit leukocytes

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Abstract

Secondary structure prediction studies on CAP18, a lipopolysaccharide binding protein from rabbit granulocytes, identified a highly cationic, 21-residue sequence with the tendency to adopt an amphipathic α-helical conformation, as observed in many antimicrobial peptides. The corresponding peptide was chemically synthesized and shown to exert a potent bactericidal activity against both Gram-negative and Gram-positive bacteria, and a rapid permeabilization of the inner membrane of *Escherichia coli*. Five analogues were synthesized to elucidate structure/activity relationships. It was found that helix disruption virtually eliminates antibacterial activity, while the degree of amphipathicity and the presence of an aromatic residue greatly affect the kinetics of bacterial inner membrane permeabilization.

Key words: Antibacterial peptide; CAP18; Amphipathic helix; Membrane permeabilization; Leukocyte

1. Introduction

A number of antimicrobial polypeptides involved in host defense have been isolated from various animal species [1–5]. In general, they are highly cationic and act by perturbing the membranes of target microorganisms. This perturbation very likely depends on the adoption of amphipathic conformations, such as those observed for cecropins [6], magainins [7], and defensins [8]. In mammals, the cytoplasmic granules of polymorphonuclear leukocytes are a major store of defense polypeptides, which show diverse primary structures and a varied spectrum of antibacterial activity [2–5].

Recent cloning of three structurally unrelated antibacterial peptides from bovine neutrophils have shown that their proforms share a highly conserved pro region [9–11]. This region is remarkably homologous to cathelin, a cathepsin L inhibitor previously isolated from pig leukocytes [12,13], and has to be proteolytically removed to convert the inactive proform to the mature antibacterial peptide [14]. The presence of a cathelin-like region was also identified in two other polypeptides from rabbit neutrophils, namely CAP18 [9], from 18 kDa cationic antimicrobial protein [15], and p15, from 15 kDa protein [16]. CAP18 was reported to bind lipopolysaccharide (LPS) [17], but to our knowledge its antimicrobial activity has not yet been described despite its name.

By analogy with the bovine antibacterial peptides located C-terminal to a cathelin-like domain [9–11], we inspected the C-terminal region of CAP18 for features typical of antibacterial peptides. A highly cationic sequence, corresponding to residues 106–125, was identified and predicted to assume an amphipathic α-helical conformation. These convergent indications induced us to synthesize the corresponding peptide and to test its antibacterial activity.

In this paper we show that this synthetic peptide, termed C18, exerts a potent bactericidal activity against both Gram negative and Gram positive microorganisms, and a remarkable permabilizing effect on the inner membrane of *E. coli*. The bioactivity of five synthetic analogues of C18, designed to probe how structural characteristics, charge and individual residues affect its biological activity, is also reported.

2. Materials and methods

2.1. Materials

Fmoc-amino acids and reagents for peptide synthesis were obtained from Millipore (Bedford, USA) and Novabiochem (Laufelfingen, Switzerland). HPLC-grade acetonitrile was from Lab-Scan (Dublin, Ireland). Trifluoroacetic acid (TFA), *N*-methylmorpholine and trifluoroethanol (TFE) were from Janssen Chimica (Beerse, Belgium). Mueller- Hinton broth and Bacto-Agar were purchased from Difco Laboratories (Detroit, USA). Melittin and *o*-nitrophenyl-β-D-galactopyranoside (ONPG) were from Sigma (St. Louis, USA). All other chemicals were of analytical grade.

2.2. Structure prediction methods

Prediction of the secondary structure of CAP18, based on the algorithms of Garnier et al. [18] and Chou and Fasman [19], was obtained using the 'PeptideStructure' and 'Pep' programs in the GCG and Intel-

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ligenetics suites, respectively. The same programs were used to predict the effect of amino acid substitutions on the structure of the C18 peptide.

2.3. Peptide synthesis

Solid-phase peptide synthesis was carried out with a Milligen 9050 synthesizer using Fmoc-Ile-PEG-PS resin (0.13 mmol/g). A fivefold excess of an equimolar mixture of Fmoc-amino acid, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetraflouroborate and *N*-hydroxybenzotriazole, dissolved in dimethylformamide containing *N*-methylmorpholine (tenfold excess), was used for each coupling step. Double couplings were carried out for all peptides at residues 5, 12, 16 and 19. Side-chain protecting groups were as follows: trityl (Asn, Gln), *t*-butyl (Glu), *t*-butyloxycarbonyl (Lys) and 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Arg). Deprotection/cleavage was carried out with a mixture of TFA, phenol, ethanedithiol, methanol and water (92:2:2:2:2) for 3 h at room temperature. The free peptides were then filtered, repeatedly extracted with ethyl ether, and purified by preparative RP-HPLC (1.9 × 30 cm Delta Pak column from Waters, Millipore) eluting the column with a 0–60% gradient of acetonitrile in 0.05% TFA. The homogeneity of each purified peptide was assayed by analytical RP-HPLC (0.4 × 25 cm Pep-S column, Pharmacia LKB Biotechnology, Uppsala, Sweden) and acid urea-PAGE [20].

2.4. Analytical assays

Peptide concentrations were determined by absorbance at 214 nm as reported by Buck et al. [20]. Amino acid analyses and mass determinations were performed using the Pico-Tag system (Millipore) [21], and an LDI 1700 laser desorption mass spectrometer (Linear Scientific Inc., Reno, USA), respectively.

CD spectra were recorded at 20°C on a Jasco J-600 spectropolarimeter with a cell path length of 2 mm. Peptide samples (0.1 mg/ml) were dissolved in 5 mM sodium phosphate buffer, pH 7.0, containing 0 to 50% (vol/vol) TFE and three scans were averaged over the wavelength range 190–240 nm. The α-helical content of the peptides was estimated with the CONTIN program [22] and by the method of Wu et al. [23], using mean molar residue ellipticities of −2000 and −32000 deg · cm^2 · dmol^{-1} at 222 nm for 0% and 100% helix content, respectively.

2.5. Biological activity assays

The minimal inhibitory concentration (MIC) of the synthetic peptides was determined by a microdilution susceptibility test as previously described [24], using the following strains of bacteria: *Escherichia coli* ML-35 and ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus megaterium* (local isolate), and *Staphylococcus aureus* ATCC 25923. To evaluate the bactericidal activity, aliquots of the medium taken from peptide-containing wells with no visible bacterial growth at the end of the MIC assay, were plated on nutrient agar and incubated for 16–18 h to allow colony counts. The minimal bactericidal concentration (MBC) was defined as the lowest concentration of peptide causing at least a 99.9% reduction of the number of microorganisms present at the beginning of the MIC determination [25].

The effect of the synthetic peptides on the permeability of the bacterial inner membrane was evaluated as previously described [26], by following the unmasking of cytoplasmic β-galactosidase activity, using ONPG as substrate. For these experiments the lactose permease deficient, β-galactosidase constitutive *E. coli* ML-35 strain was used. Total β-galactosidase activity, corresponding to 100% permeabilization, was determined with bacteria lysed by ultrasonication.

The hemolytic activity of the peptides was determined with human erythrocytes [27]. The blank was evaluated in the absence of additives and 100% hemolysis in the presence of 0.2% Triton X-100. Melittin was used as a positive control, at concentrations between 1 and 10 μM.

3. Results

Inspection of the sequence of CAP18, as deduced from cDNA [15], showed a highly cationic region between residues 106 and 125 (Table 1), with 11 cationic residues out of 20. Secondary structure prediction studies indicated a high potential for this region to adopt an α-helical conformation, which clearly shows an amphipathic nature in a helical wheel projection (Fig. 1). The polar face by far dominates the projection, subtending an angle of 260° perpendicular to the helical axis, which is unusually high compared to known α-helical antibacterial peptides such as magainins and cecropins, where it subtends angles of 100–180° [6,7].

By analogy with known antibacterial peptides, the cationicity of this sequence, its location after a cathelin-like domain, and its predicted α-helical, amphipathic nature strongly suggested that the corresponding peptide might possess antibacterial activity. This peptide, termed C18, was synthesized by the solid-phase method, purified by preparative RP-HPLC, and the major peak shown to contain a virtually homogeneous peptide by analytical RP-HPLC and acid urea-PAGE (not shown). The correct structure of the peptide was confirmed by mass determination (Table 1) and by its amino acid composition (not shown).

The peptide displayed a spectrum typical of an unordered conformation in 5 mM phosphate buffer, pH 7.0 (not shown), while at greater than 20% TFE, the increase in θ_{222} and the shape of the spectrum indicated that it had acquired a significant level of α-helical conformation (Fig. 2, inset). The helical content, calculated with both the Contin program [22] and the method of Wu et al. [23], is about 55% at 30% TFE.

The antibacterial activity of C18 was tested by the MIC and MBC assays which give the minimal bacteriostatic and bactericidal concentrations, respectively. Three Gram-negative (*E. coli*, *S. typhimurium* and *P. aeruginosa*) and two Gram-positive (*B. megaterium* and *S. aureus*) species were selected as test organisms. The results (Table 2) show that C18 is active against all of the five species at concentrations ranging from 0.5 to 4.0 μM, with a considerable activity against *P. aeruginosa* (MIC of 0.5 μM), a species often resistant to antimicro-

Table 1
Amino acid sequences and masses of the synthetic peptide C18 and its analogues

Peptide	Amino acid sequence	Mass	
		Measured[a]	Calculated
	1 10 20		
C18	GLRKRLRKFRNKIKEKLKKI	2553.72	2553.25
C18P	--------P---P-------	2488.47	2487.14
C18K	-----K------K-------	2583.81	2583.27
C18AA	----A----------A----	2412.30	2411.04
C18A	--------A----------	2476.82	2477.15
C18Q	----------Q-----	2553.07	2552.27

[a]The mass of each peptide was determined with a laser desorption mass spectrometer on samples (1–2 pmol) crystallized under low vacuum in the presence of 50 mM sinapinic acid directly on the probe tip of the spectrometer.

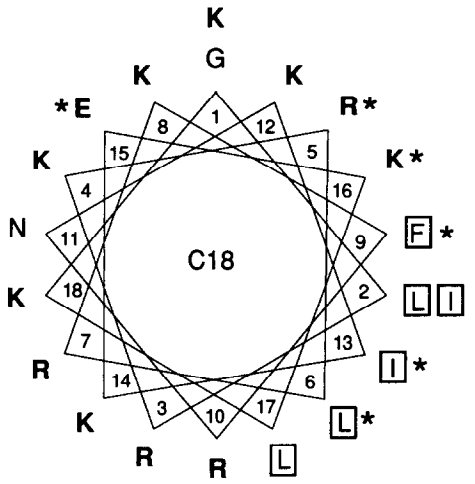

Fig. 1. Helical wheel representation of the C18 peptide. Charged residues are in bold and strongly hydrophobic residues are boxed. The asterisks indicate those residues that have been replaced in the C18 analogues.

bial peptides [24,27,28]. For all strains tested, the MBC of C18 is equal to or at most twofold higher than the MIC (data not shown), indicating that this peptide is bactericidal and not only bacteriostatic. The amphipathic α-helical structure of C18 suggested that it would act by permeabilizing biological membranes. Potential damage to the inner membrane of the *E. coli* ML-35 strain and to the membrane of human erythrocytes was thus evaluated. The peptide rapidly increased the inner membrane permeability of *E. coli* at a concentration as low as 1 μM (Fig. 3). At this concentration, accessibility of the cytosolic β-galactosidase to its substrate was already evident after 90 s and the reaction reached a steady state within 10 min. The rate of reaction was about 50% when compared to that of bacteria completely lysed by sonication. The kinetics of permeabilization is thus considerably faster than that reported for the bactenecins Bac5 and Bac7, which require a tenfold higher concentration to obtain comparable results [26], or that for human defensins, which is even lower [28]. β-galactosidase activity was found in the supernatant after bacterial sedimentation, indicating that the peptide had induced a sufficient alteration of the membrane to allow leakage of the enzyme. Conversely, C18 did not exert any lytic effect on human erythrocytes, even at a concentrations of 50 μM, while melittin, a hemolytic peptide known to assume an amphipathic α-helical conformation [30], lysed over 90% of the erythrocytes at a concentration of 3 μM in parallel experiments.

In order to determine how the structure of C18 affects its biological activity, five analogues (Table 1) were synthesized, purified and analyzed in a manner similar to the parent peptide. Appropriate amino acid substitutions were introduced to vary charge and amphiphilicity or to test the importance of individual residues towards the activity.

The analogue C18P (Phe-9 and Ile-13 changed to proline, see Table 1) was designed to greatly reduce the propensity for α-helix formation without affecting charge. C18K (Leu-6 and Ile-13 replaced by lysine residues) was designed to increase cationicity while reducing amphiphilicity. The opposite effect was obtained in C18AA by replacing Arg-5 and Lys-16 with alanine. C18Q, (Glu-15 replaced by glutamine) and C18A (Phe-9 replaced by alanine), were designed to respectively test the role of the single anionic and aromatic residues present in C18.

The correct identity and homogeneity of the five analogues were confirmed as for C18 (Table 1). As predicted, CD spectra of C18P were characteristic of an unordered conformation even at 30–50% TFE (Fig. 2), indicating a greatly reduced ability to assume an α-helical conformation. This peptide was inactive against each strain tested up to 64 μM (Table 2). C18K also showed a reduced propensity for α-helical conformation as compared to C18 (only about 20% helical content at 30% TFE, Fig. 2) and showed no antibacterial activity up to 64 μM against most strains (Table 2). Not surprisingly, neither C18P nor C18K permeabilized the inner membrane of *E. coli* ML-35 at concentrations up to 50 μM (Fig. 3), nor did they display hemolytic activity at this concentration.

The hydrophobic surface was increased to subtend an angle of 140° in C18AA when compared to that of 100° in C18. An analogue with three leucines and a hydrophobic surface subtending an angle of 160° was discarded due to low solubility. CD spectra indicated an increased tendency of C18AA to adopt an α-helical conformation as compared to C18 (over 70% α-helix at 30% TFE, Fig. 2). As reported in Table 2, C18AA showed a two- to fourfold increase in antibacterial activity with respect to

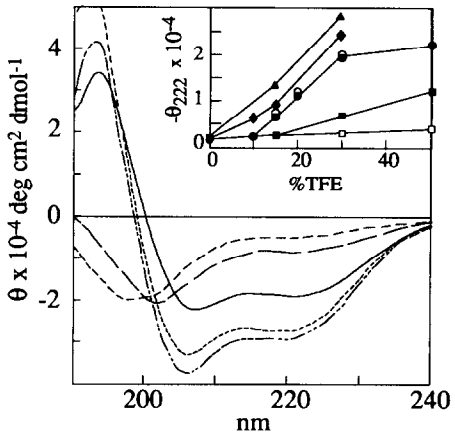

Fig. 2. Circular dichroism spectra of C18 and of its analogues. The spectra were measured at a peptide concentration of 0.1 mg/ml in 5 mM phosphate buffer, pH 7.0, in the presence of 30% TFE: C18 or C18Q (——), C18P (– – –), C18K (— – —), C18AA (– – – –) and C18A (— – —). The inset shows the variation of the mean molar residue ellipticity at 222 nm ($-\theta_{222}$) with increasing TFE concentrations: C18 (●), C18Q (○), C18P (□), C18K (■), C18AA (◆) and C18A (▲).

the parent peptide, with the exception of *P. aeruginosa*, against which it was sligthly less effective. C18AA permeabilized *E. coli* ML-35 with a faster kinetics than C18 (reaction rate about 80% that of sonicated bacteria at steady state, Fig. 3) while still not exerting any hemolytic activity up to a concentration of 50 μM.

Finally, the role played in determining the activity of C18 by the single anionic and aromatic residues was investigated with C18Q and C18A, respectively (Table 1). C18Q displayed a structural behaviour and biological activities remarkably similar to C18 (Figs. 2 and 3, and Table 2). Substitution of Phe-9 with alanine in C18A appeared to increase the α-helical propensity of this analogue with respect to the parent peptide (over 80% helical content at 30% TFE, Fig. 2). The antibacterial activity is sligthly lower than that of C18, as the MIC and MBC values doubled for most strains (Table 2). A twentyfold greater concentration was however required to give a permeabilization behaviour comparable to that of the parent peptide (Fig. 3, trace d vs. trace f). At 1 μM (Fig. 3, trace c), a similar steady state was observed only after fourty minutes.Thus the presence of the aromatic residue appears to affect the kinetics rather than the degree of permeabilization. Neither C18Q nor C18A displayed hemolytic activity up to a concentration of 50 μM.

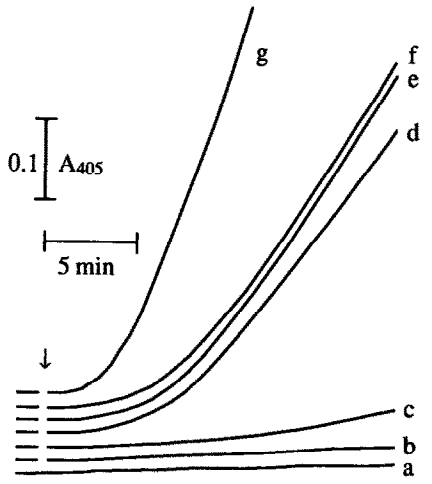

Fig. 3. Kinetics of permeabilization of *E. coli* ML-35 inner membrane by C18 and its analogues. Permeabilization was determined by following the unmasking of cytoplasmic β-galactosidase activity spectrophotometrically at 405 nm. Bacteria (about 10^7 colony forming units/ml) were suspended in 10 mM sodium phosphate buffer, pH 7.5, containing 100 mM NaCl and 1.5 mM substrate. Trace a, untreated bacteria; trace b, C18P or C18K at 50 μM; trace c, C18A at 1 μM; trace d, C18A at 20 μM; trace e, C18Q at 1 μM; trace f, C18 at 1 μM; trace g, C18AA at 1 μM. The arrow indicates the time of addition of peptides. Results are representative of three very similar, independent experiments.

4. Discussion

The present study, based on a secondary structure prediction/peptide synthesis approach, has allowed the identification of a primary antimicrobial domain in CAP18 from rabbit leukocytes. Results show that the corresponding peptide, C18, displays a conformational change from an unordered to a predominantly α-helical conformation when the polarity of the environment is decreased, a behaviour typical of an amphipathic helix, and exerts a potent antibacterial activity against Gram-negative and Gram-positive microorganisms.

Table 2
Antibacterial activity of C18 and of its analogues

Organism and strain	MIC (μM)					
	C18	C18P	C18K	C18AA	C18A	C18Q
Escherichia coli ML 35	2	> 64	> 64	0.5	4	2
Escherichia coli ATCC 25922	4	> 64	> 64	2	4	4
Salmonella typhimurium ATCC 14028	2	> 64	> 64	0.5	16	2
Pseudomonas aeruginosa ATCC 27853	0.5	> 64	> 64	2	1	0.5
Bacillus megaterium (local isolate)	4	> 64	64	2	8	4
Staphylococcus aureus ATCC 25923	4	> 64	> 64	2	8	4

MIC was defined as the lowest concentration of peptide preventing visible growth after 18 h incubation at 37°C in Mueller-Hinton broth [24]. Results, determined with approximately 1.5×10^5 colony forming units/ml, are the mean of at least six independent determinations with a divergence of not more than one MIC value with respect to those reported here.

Amphipathic helices have been observed for a wide variety of biologically active peptides whose function is related to the binding of lipids and cell membranes [31], and is a feature common to many antimicrobial peptides, such as insect cecropins [6], amphibian magainins [7,32], PGLa [32] and dermaseptin [33], and other membrane interacting peptides, such as melittin [30]. The C18 peptide here described, with the analogous PMAP-36(1–20), derived from a precursor cloned from pig myeloid cells (Storici, P. et al., unpublished), and cecropin P1 from pig intestine [34,35] show that the amphipathic α-helix is a conformational motif represented also among the mammalian antibacterial peptides. It is interesting to note that C18 and PMAP-36(1–20), as opposed to insect and amphibian peptides, are characterized by an unusually high content of cationic residues (55–60%) and a large hydrophilic face of the helix, which points to the considerable flexibility in the make up of α-helices with antibacterial activity.

Membrane permeabilization experiments show that C18 rapidly permeabilizes the inner membrane of the *E. coli* ML-35 strain. As damage to the membrane is sufficient to allow the leakage of a relatively large enzyme, it is likely that this damage is the primary event in killing bacteria. The importance of α-helical conformation in the antibacterial and membrane permeabilizing activities

in C18 is shown by the ineffectiveness of analogues in which this conformation is disrupted or greatly reduced (C18P and C18K). The analogue C18AA displays a greater antibacterial activity and a faster kinetics of permeabilization, so that a moderate increase in amphipathicity at the expense of charge can potentiate the activity of the parent peptide. Conversely, replacement of the only aromatic residue in C18A results in a much slower kinetics of permeabilization, while the degree of permeabilization at steady state and the antibacterial activity are not greatly affected. In this respect, aromatic residues are nearly always present in α-helical antibacterial peptides, and have been shown to play an important role for activity. In fact, replacement of Trp-2 of cecropin A with Glu, but not with Phe, results in a sharp drop in antibacterial activity [36], as does the replacement of Phe-5 and Phe-12 with Thr or Ser in magainin 2 [37]. While C18A, in which Phe-9 is replaced with the hydrophobic residue Ala, does not show the dramatic decrease in antibacterial activity of the above cases, it suggests that the presence of an aromatic residue is important in mediating the interaction with bacterial membranes.

We have observed that α-helical antibacterial peptides, such as magainins and cecropins, very often contain a glutamic acid residue. This induced us to test whether this residue had any significant role in the biological activity of C18, by synthesizing the C18Q analogue. Our results indicate that it is replaceable by glutamine without any measurable effect, at least as far as helix formation, antibacterial activity and membrane permeabilization kinetics or specificity are concerned.

Our study shows how simple considerations based on sequence homology (presence of a cathelin-like pro region) and structure prediction analysis, followed by peptide synthesis, have allowed the successfull identification of an antibacterial sequence in the LPS binding polypeptide CAP18. Moreover, it has provided information on structure/activity relationships which may be useful in the design of novel antibiotic peptides.

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