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Functional domain and poly-L-proline II conformation for candidacidal activity of bactenecin 5

Periathamby Antony Raj*, Mira Edgerton

Department of Oral Biology, Dental Research Institute, Periodontal Disease Clinical Research Center and Prosthodontics, State University of New York at Buffalo, 109 Foster Hall, Buffalo, NY 14214, USA

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Abstract The functional domain for candidacidal activity of bactenecin 5 has been determined by synthesizing bactenecin 5 and its fragments [1–22 (BN22), 7–22 (BN16) and 20–43 (BC24)]. The N-terminal sequence BN16 retained the candidacidal potency of the parent molecule and this region appears to be the candidacidal domain. The circular dichroism spectra of these peptides indicate the presence of largely poly-L-proline II conformations in aqueous solutions and in lipid vesicles. The coupling constant ($J_{NH\text{-}C\alpha_H}$) values, and a set of medium- and short-range nuclear Overhauser effects observed for the N-terminal peptide (BN16) in the two-dimensional nuclear magnetic resonance suggest that poly-L-proline II helix could be the biologically active conformation.

Key words: Antimicrobial peptide; Candidacidal activity; Peptide conformation; Circular dichroism; 2D NMR

1. Introduction

Bactenecins belong to the family of proline- and arginine-rich antibiotics found in the granules of bovine neutrophils [1,2]. Two polypeptides designated as bactenecin 5 (Bac 5) and bactenecin 7 (Bac 7) have been purified and examined for antibacterial activity [1,3]. They have been reported to be synthesized in immature myeloid cells of the bone marrow and stored as inactive probactenecins in the large granules [4]. Neutrophil stimulation with microbes has been shown to trigger the removal of the proportion and activation of these peptides [5]. The primary structure determination using plasma desorption-mass spectrometry has shown Bac 5 and Bac 7 sequences to contain 42 and 59 residues, respectively [3]. However, it has recently been established from cloning and sequence analyses that Bac 5 is a 43-residue polypeptide [6]. The mechanism of cidal action of bactenecins has been reported to involve their interaction with the outer membrane of susceptible microorganisms followed by a rapid translocation to the inner membrane and impairment of energy-dependent membrane activities [2,3,7]. Despite the importance of bactenecins in the nonimmune host defense systems, there are relatively few reports on the secondary structure and structure–function analyses of these pharmacologically active polypeptides. Though the mechanism of cidal action of bactenecins has been suggested to involve membrane interaction [7], the conformations they acquire in lipid environment have not yet been reported. Bactenecins have been shown to be active against a broad range of bacteria and enveloped viruses [3]. However, their effects on fungal organisms have not been reported. In this paper, we report the functional domain and the conformational requirements for candidacidal activity of Bac 5. We also describe the 2D-NMR of the active region 7–22 of Bac 5.

2. Materials and methods

2.1. General materials and methods

Amino acid derivatives, DPPC, phenylacetamidomethyl and 4-methylbenzhydrylamine resins were purchased from Sigma (St. Louis, MO) and Bachem (Torrance, CA). All peptides were synthesized using a Beckman System 990 synthesizer. HPLC was carried out on a Gilson Rabbit-HP system interfaced to an Apple IIe microcomputer using a Rainin Dynamax-60A reversed-phase C18 column (10 × 250 mm) coupled to a guard column (10 × 50 mm) employing acetonitrile-water (each containing 0.1% trifluoroacetic acid) linear gradient elution (flow rate 1.5–2.0 ml min^{-1}) mode with detection at 230 nm. Amino acid analysis was performed on a Beckman System 6300 amino acid analyser. The amino acid sequences of peptides were verified by sequence analysis using an Applied Biosystems Model 471A Protein Sequencer interfaced with a Hewlett Packard HP3394A integrator.

2.2. Peptide synthesis and purification

Bactenecin 5 and its fragments shown in Fig. 1 were synthesized by standard solid-phase procedures using 4-methylbenzhydrylamine and phenylacetamidomethyl resins (for Bac 5 and its fragments, respectively) and *N-tert*-butyloxycarbonyl (t-Boc) amino acids as described in our previous publications [8,9]. The side-chain protecting groups were the following: *N*-tosyl (Arg) and *O*-2-bromobenzyloxycarbonyl (Tyr). Peptides were purified by reversed-phase HPLC and fully characterized by sequence and amino acid analyses and examined in cationic [10] and SDS [11] PAGE systems which showed a single band. The HPLC retention time, amino acid composition and migration in the cationic and SDS PAGE systems of synthetic Bac 5 were found to be in good agreement with those of the native molecule isolated from bovine neutrophils using the previously reported procedures [1,12].

2.3. Measurement of candidacidal activity

Candida albicans strain obtained from a clinical isolate from a denture-induced stomatitis (DIS) patient was used. The identity of the clinical isolate was verified by the Yeast System (Flow Laboratories, McLean, VA). Organisms were streaked onto Sabouraud Dextrose Agar plates (Difco Laboratories, Detroit, MI) and maintained at 4°C. One colony of *C. albicans* from this plate was inoculated into 10 ml of yeast synthetic growth media containing sucrose, salts and biotin and incubated for 48 h at 25°C in a shaker rotating at 200 rpm. After this period, the population of yeast cells was in the late logarithmic phase growth. The cell morphology was determined and found to be uniformly blastospores by phase-contrast microscopy. The antifungal activity of Bac 5 and its fragments were assessed in vitro against *C. albicans*. The assay was performed as described previously for salivary histatin 5 and its fragments [8]. We refer to the candidacidal action of peptides as a loss of viability of cells, since the inability of the yeast

*Corresponding author. Fax: (1) (716) 829 3942.

Abbreviations: CD, circular dichroism; DPPC, dipalmitoylphosphatidylcholine; HPLC, high-performance liquid chromatography; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; PAGE, polyacrylamide gel electrophoresis; TOCSY, total correlated spectroscopy.

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to replicate following removal of peptides is considered non-viable. Thus, the assay used in this study measured only fungicidal activity. The candidacidal activity of Bac 5 and its fragments has been found to be influenced by peptide concentration and incubation time. The time-dependent loss of viability of cells induced by Bac 5 and its fragments at different concentrations have shown that maximal loss in cell viability could be reached within 50 min after incubation for all peptides.

2.4. CD

CD spectra were recorded at 30°C with a JASCO J-600 spectropolarimeter interfaced to an IBM PS/2 microcomputer. Measurements were carried out in cells with 0.1 cm path length using a peptide concentration of 0.03–0.05 mM. The peptide in aqueous DPPC dispersion was prepared as described previously [13]. The lipid peptide molar ratio was 20:1. CD band intensities are expressed as molar ellipticities, $[\Theta]_M$ in $deg \cdot cm^2 \cdot dmol^{-1}$.

2.5. NMR measurements

The purified BN16 (7 mg) was dissolved in 630 μl of double distilled water and 70 μl of ^2H_2O (Cambridge Isotope Laboratories, Woburn, MA). All 1D- and 2D-NMR experiments used for conformational analyses were performed at 30°C using a Varian 500 MHz VXR-500 spectrometer equipped with a SUN Sparcstation 2 as described previously [14]. The TOCSY [15,16] experiments were recorded using a MLEV-16 pulse sequence for the spin lock with a field strength of ~5.6 kHz and a trim pulse of 2 ms using an isotropic mixing period of 65 ms. The 2D-NOE experiments [17–18] were performed with a mixing time of 150 ms after ascertaining that spin diffusion is not significant at this mixing time. The coupling constant ($J_{NH\text{-}C^aH}$) values were determined from the 1D spectrum with a high digital resolution of 0.1 Hz. Hydrogen–deuterium (^1H–^2H) exchange of the amide groups and variable temperature experiments have been performed as described previously [14]. Computer modeling of the molecular conformation of BN16 was carried out using SYBYL molecular modeling software version 5.5 (Tripos Associates, St. Louis, MO) on an Evans & Sutherland Workstation 3–32 as described in our previous publication [14].

3. Results and discussion

3.1. Candidacidal activity

The candidacidal activity of Bac 5 and its fragments for the DIS strain are summarized in Fig. 2. The EI_{50} (molar concentration of peptide which would induce half-maximal loss in cell viability) value of synthetic Bac 5 is in good agreement with that of the native molecule. The candidacidal potency of the N-terminal fragments, BN22 and BN16, are comparable to that of Bac 5 whereas the C-terminal sequence (BC24) appears to be significantly less active (Fig. 2). The fragment BC24 has diminished positive charge at the N-terminus as compared with Bac 5, BN16 and BN22. Hence, the lowered activity observed for BC24 indicates that the positive charge at the N-terminus is crucial for high candidacidal activity. The EI_{50} values of BN16 and BN22 suggest that the first six residues (R-F-R-P-P-I) at the N-terminal of BN22 (Fig. 1) may not be essential for candidacidal activity. Bac 5 has an amide function at the C-terminal whereas the fragments have a carboxyl function. The fact that the N-terminal fragments retain the activity of the whole molecule suggests that the amide function at the C-terminal of Bac 5 may not be essential for fungicidal activity. Collectively, the data suggest that the region 7–22 of Bac 5 could be the functional domain for fungicidal activity. A hydrophobic sequence at the C-terminus and a positively charged N-terminus as in the sequence of 7–22 (BN16) of Bac 5 appear critical to elicit appreciable candidacidal activity.

3.2. CD studies

The CD spectra of Bac 5 in sodium phosphate buffer shown in Fig. 3 has a strong broad negative π-π^* band at ~202 nm ($[\Theta]_M = -67.82 \times 10^4 deg \cdot cm^2 \cdot dmol^{-1}$) with the n-$\pi^*$ band approaching towards the positive minimum in aqueous solution. The CD spectrum of synthetic Bac 5 in aqueous buffer is in good agreement with that of the native sequence suggesting the optical purity of the synthesized peptide. A strong negative band at ~206 nm with a weak positive band at ~229 nm is characteristic of poly-L-proline II helical structure [19]. The absence of the weak positive band expected for poly-L-proline II helix and the shift in the strong negative π-π^* band from ~206 to 202 nm is attributable to the presence of only one half of the tertiary amide groups [20] or to a slight deviation from the ideal poly-L-proline II ($\phi = -78°$, $\psi = 149°$ and $\omega = 180°$) helical conformation. The presence of minor populations of *cis* conformations (poly-L-proline I, $\omega = 0°$) may also cause a change in the positive n-π^* transition and a shift in the π-π^* transition towards shorter wavelength. The CD spectrum of synthetic Bac 5 in aqueous buffer is in good agreement with that of the native sequence. The CD spectra of the fragments of Bac 5 also resemble the CD spectra of Bac 5 with lowered ellipticity values (figure not shown) which could be attributed to their shorter

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                    5         10        15        20        25        30        35        40
Bac7:  R-R-I-R-P-R-P-P-R-L-P-R-P-R-P-R-P-L-P-F-P-R-P-G-P-R-P-R-P-I-P-R-P-L-P-F-P-R-P-G-P-R-P-I-

          45        50        55    59
       P-R-P-L-P-F-P-R-P-G-P-R-P-I-P-R-P

                    5         10        15        20        25        30        35        40    43
Bac 5: R-F-R-P-P-I-R-R-P-P-I-R-P-P-F-Y-P-P-F-R-P-P-I-R-P-P-I-F-P-P-I-R-P-P-F-R-P-P-L-G-P-F-P

BN22:  R-F-R-P-P-I-R-R-P-P-I-R-P-P-F-Y-P-P-F-R-P-P

BN16 :           R-R-P-P-I-R-P-P-F-Y-P-P-F-R-P-P

BC24 :                                                R-P-P-I-R-P-P-I-F-P-P-I-R-P-P-F-R-P-P-L-G-P-F-P
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Fig. 1. Amino acid sequences of Bac 7, Bac 5 and its fragments. Synthetic Bac 5 has the amide function at the C-terminal whereas the fragments have the carboxyl function. The one letter symbol used for amino acids in the sequences are described as in IUPAC-IUB Commission on Biochemical Nomenclature [31].

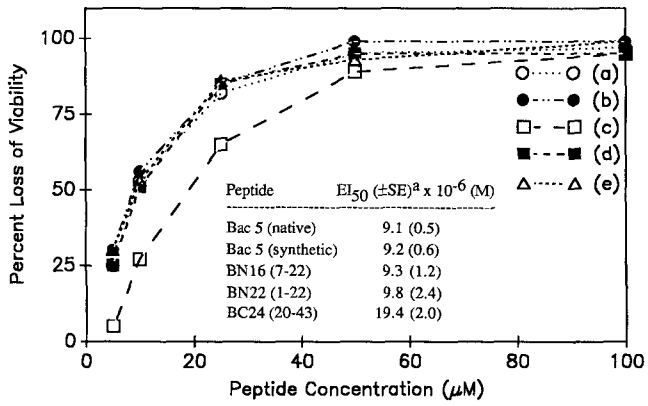

Fig. 2. Dependence of % loss of viability of *C. albicans* (DIS) on peptide concentration. (a) synthetic Bac 5, (b) native Bac 5, (c) BC24, (d) BN22 and (e) BN16. The EI_{50} = molar concentration of peptide required to induce half-maximal loss in cell viability as determined from the concentration–effect curve.

chain length [8,9]. In DPPC vesicles (Fig. 3), the intensity of the π-π^* transition increases with a shift in the band position towards longer wavelength as compared with that in phosphate buffer suggesting that peptide molecules may associate in lipid bilayer to form a higher order polyproline helical structure. The CD data of bactenecin peptides reflect the existence of a major population of poly-L-proline II conformations in aqueous solution and in lipid vesicles.

3.3. NMR studies

The high biological activity of BN16 provides a strong stimulus to determine its conformation in aqueous solution using 2D-NMR. This peptide is unique with two non-proline residues flanked by two adjacent prolines in the sequence (Fig. 1). Moreover, both the in vivo and in vitro activity of Bac 5 could reflect the candidacidal potency of the active fragments, as the parent molecule is highly susceptible to proteolytic degradation. The observed ^{1}H NMR resonances are due to a major population (95%) of exclusively *trans* prolines with additional resonances (5%) that correspond to *cis* prolines. Conformational analysis has been carried out only for the major *all trans* peptide molecules. The 2D-TOCSY and NOESY spectra allowed unambiguous assignment of resonances to individual amino acids as shown for the backbone amide resonance connectivities to the respective side-chain protons (Fig. 4). The αN (i, i+1) and $\alpha\delta$ (i, i+1) NOE connectivities have been used to identify the non-proline and proline residues, respectively, in the sequence. The $J_{NH\text{-}C\alpha_H}$ values for the non-proline residues (6.9–7.8 Hz) provided in Fig. 5 suggest ϕ values of ~ −81–89° or ~ −151–159° [21] for non-proline residues. However, the stereochemical constraints imposed on the non-proline residues flanked by Pro residues indicate only the lower ϕ values of −81–89° as the compatible conformational angles [22–24] which are close to the poly-L-proline II conformation (ϕ = −78°, ψ = 149° and ω = 180°).

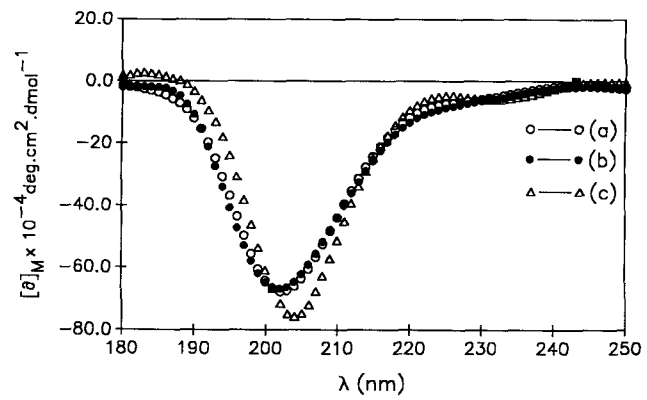

Fig. 3. The CD spectra of synthetic (a) and native (b) Bac 5 recorded at 30°C in sodium phosphate buffer at pH 7.4 and the CD spectrum of synthetic Bac 5 (c) in DPPC vesicles (peptide : lipid molar ratio 1 : 20).

The medium-range αN (i, i) NOEs observed for most of the non-proline residues (Fig. 5) suggest a spatial distance of 3.0–3.2Å between the respective protons and appear to be consistent with large ψ (~140–150°) values for these residues. The strong αN (i, i+1), βN (i, i), $\alpha\delta$ (i, i+1) NOE connectivities generate a distance of 2.2–2.4Å between the respective protons. The weak $\alpha\alpha$ (i, i+1) NOEs (Fig. 5) are consistent with a distance of 4.2–4.6Å. The 2D-NOE spectrum does not show any observable NN (i, i+1) NOEs for non-proline residues indicating spatial distances >4.8Å between the protons [25]. These interproton distances deduced from the NOE data are fully consistent with a poly-L-proline II conformation. In addition, the absence of intramolecularly hydrogen bonded NH groups as shown by the high temperature coefficients (≥0.0042 ppm K^{-1}) of NH resonances and the fast ^1H–^2H exchange on amide groups (Fig. 5) are also in agreement with this semi-extended conformation. Collectively, the NMR and NOE data provide evidence that BN16 predominantly exists as poly-L-proline II conformers in aqueous solutions. The stereochemical preference of proline, the repetitive occurrence of adjacent Pro residues in the sequence and the high Chou–Fasman propensity of Arg residues for poly-L-proline structure lend further support to the interpretations.

3.4. Conformation and biological activity

A family of 16 closely related structures generated for BN16 using molecular modeling techniques and the NMR constraints

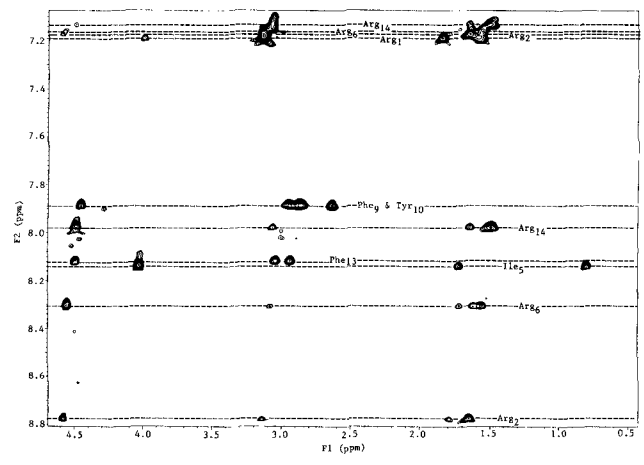

Fig. 4. Part of the TOCSY spectrum of BN16 showing the backbone amide and Arg side-chain amino resonance connectivities to $C^{\alpha}H$, $C^{\beta}H$, $C^{\gamma}H$ and $C^{\delta}H$ resonances. The spectrum was recorded in $H_2O/^2H_2O$ at pH 3.8 and 30 °C.

are consistent with polyproline II helices. The average structure provided in Fig. 6 has ϕ values (~−75°) for all Pro residues very close to the allowed upper limit. The average backbone conformational angles of the structure are $\phi = -85.2°$, $\psi = 148.5°$ and $\omega = 179.7°$. It is a single-stranded, extended helix with the backbone C = O and N-H groups projecting outward. The N-terminal of the BN16 helix is polar and charged due to the presence of Arg_1, Arg_2 and Arg_6, whereas the C-terminal consists of mostly hydrophobic residues such as Pro, Phe, Ile and Tyr. The interaction of the bactenecin peptides with the microbial membranes appears to involve the electrostatic forces between the positively charged residues and the negatively charged head groups of the membrane bilayer. Our results indicate that the positively charged Arg residues at the N-terminus seem to be important for candidacidal activity. Even the replacement of one Arg residue at the N-terminal diminishes the candidacidal activity dramatically as observed for the C-terminal fragment BC24. The Influence of positively charged residues at the N-terminus on the orientation of the hydrophobic domains in membrane proteins has recently been reported [26]. Most antimicrobial cationic polypeptides have been reported to form amphiphilic α-helical or β-sheet structures [27–30]. The spontaneous insertion of these amphiphilic peptides into cell membranes have been suggested as the probable mechanism of their candidacidal action [27,29]. The high candidacidal potency observed for Bac 5 and the N-terminal BN16 fragment emphasizes the importance of poly-L-proline II helical structure for microbial interaction and antifungal activity.

In conclusion, our results demonstrate for the first time that bactenecin peptides are powerful candidacidal agents which prefer to adopt poly-L-proline II conformation in aqueous solution and in lipid vesicles. The N-terminal 7–22 sequence of Bac 5 appears to be the functional domain for candidacidal activity. Alternate occurrence of two adjacent Pro residues flanking two non-proline residues (basic residues, such as Arg, at the N-terminus and mostly hydrophobic residues at the C-terminus) and a chain length of 16 residues seem to be the structural requirements for eliciting appreciable candidacidal activity. The positive charge at the N-terminus appears to be essential for high candidacidal potency. The tendency of bactenecin peptides to associate in lipid bilayer suggests the possibility of their formulation in liposome drug carriers and their potential application in antimicrobial therapy.

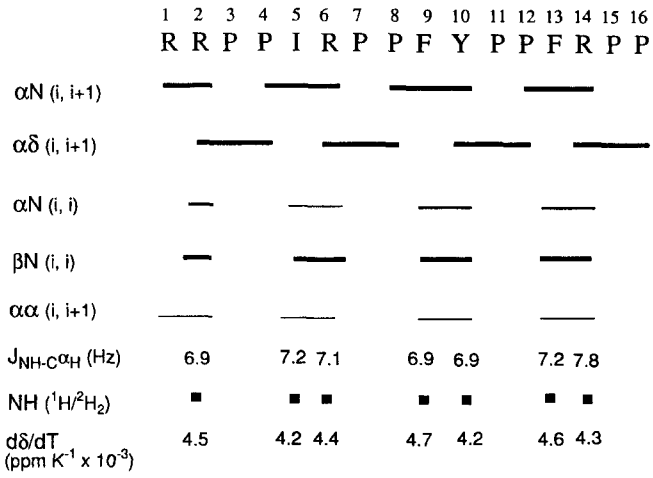

Fig. 5. Summary of sequential and medium-range NOE data compiled from the NOESY spectra of BN16 recorded at 30°C in (a) $H_2O/^2H_2O$. The thickness of the bars indicates that the NOE is strong, medium and weak, respectively. ■ indicates fast-exchanging amide NHs.

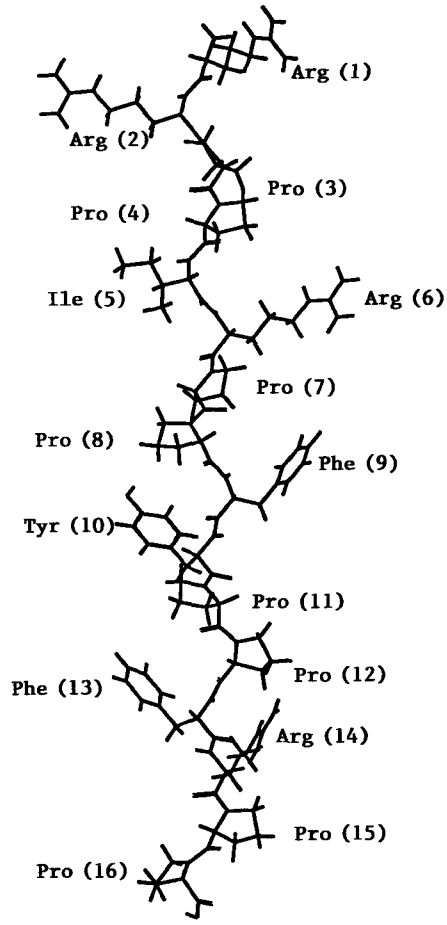

Fig. 6. A perspective view of the poly-L-proline II structure of BN16 with the N-terminus at the top and the C-terminus at the bottom, deduced from molecular modeling using the NMR and NOE data recorded at 30°C in $H_2O/^2H_2O$.

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