BTM-P1 polycationic peptide biological activity and 3D-dimensional structure

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Abstract

The novel BTM-P1 peptide interferes with energetic processes in mitochondria; its antimicrobial activity against Gram-positive and Gram-negative bacteria is described here. BTM-P1 three-dimensional structure was determined by ^{1}H NMR to explain its biological mechanisms and membrane activity. Structural data indicated that BTM-P1 can form an α-helix; circular dichroism analysis confirmed the peptide's propensity to behave as a typical transmembrane helix in a lipidic environment. According to the structural characteristics of the polycationic BTM-P1 peptide so revealed, its biological activity can be explained by a mechanism involving the formation of ion-permeable channels in biomembranes.
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Cationic antimicrobial peptides are generally peptides having less than 50 amino acid residues with an overall positive charge imparted by the presence of multiple lysine and arginine residues and a substantial portion (50% or more) of hydrophobic residues [1]. These peptides are produced by all kinds of organism (from plants and insects to humans) [2] as a major part of their innate effective non-specific defences against infection [3]. They are preferentially amphipatic allowing the peptide to interact with plasma membrane phospholipids [4]. Several of these molecules have been isolated, amino acid sequenced and described in the literature. Along with their antimicrobial activity, these peptides have been shown to inhibit certain viruses [5], have anticancer activity [6], promote wound healing [7] and cause depolarisation of isolated mitochondria's inner membrane [8]. These properties make cationic peptides very promising candidates for developing new therapeutic agents.

It is generally assumed that most antimicrobial peptides disrupt or permeate the target cell membrane, resulting in irreversible damage of membrane functionality [9]. Most cationic peptides seem to adopt an amphipathic arrangement with opposing hydrophobic and positively charged faces when coming into contact with the bacterial membrane [10]. Accumulating data suggests that the permeating pathway depends on both the peptide and the membrane. More specifically, membrane-bound peptides recognise each other, oligomerise and form transmembranal pores. Permeation/disruption mechanism can vary amongst different peptides.

The Cry toxins' pore-forming domain consists of seven alpha helices presenting lytic peptide features [11]. One of

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the peptides (BTM-P1), synthesised based on Cry11Bb1 *Bacillus thuringiensis* serovar. *Medellin* toxin α2 helix, has been described [12] and its activity on isolated mitochondria has been studied recently [13]. BTM-P1's ability to kill bacteria was evaluated, the 3D-peptide structure was solved, its conformation in solution and in hydrophobic environment determined and a mechanism for its biological activity proposed.

Materials and methods

Chemical synthesis of peptides. All the peptides were synthesised by the solid-phase method [14] according to good manufacturing practices (GMP). A *p*-methyl benzhydrylamine-resin (MBHA) (0.7 M_{eq}/g), *t-Boc* aminoacids (Bachem, USA) and low-high cleavages were used in the process [15]. Once synthesised, the peptides were extracted with 10% (v/v), acetic acid and water. The peptides were purified by reverse-phase HPLC, lyophilised, and dissolved in bidistilled water at 1 mg/mL concentration.

Mitochondria isolation. White male rats were decapitated and their livers homogenised in 45 mL ice-cold medium composed of 210 mM mannitol, 70 mM sucrose, 5 mM Hepes–Tris, and 1 mM EGTA, pH 7.4, using a glass Dounce homogeniser and Teflon pestle. The homogenate was centrifuged at 600g for 10 min at 4 °C (Jouan centrifuge, type MR1812). The supernatant was again centrifuged at 10,000g for 10 min at 4 °C. The mitochondrial pellet was suspended in 30 mL ice-cold 210 mM mannitol, 70 mM sucrose, 5 mM Hepes–Tris, and 20 μM EGTA, pH 7.4 (MSHE) and further centrifuged at 10,000g for 10 min at 4 °C. The final mitochondrial pellet was suspended in MSHE buffer at 60–80 mg/mL protein concentration. All analytical grade reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Mitochondrial swelling assays. Mitochondrial swelling was monitored as changes in apparent absorbance at 540 nm (OD_{540}) on a Turner spectrophotometer (model SP-850, USA) modified with a mini-magnetic stirrer device and a thermostated chamber. The curves were recorded on a graphic register (Linseis model L250E, USA). 0.5 mg/mL mitochondria, 2.5 μM rotenone and 1 μM oligomycin (RO) were simultaneously added to 1 mL of different isotonic media: 125 mM NH_4NO_3, 5 mM Hepes–Tris, pH 7.4; 125 mM Tris-NO_3, 5 mM Hepes–Tris, pH 7.4; 125 mM $NaNO_3$, 5 mM Hepes–Tris, pH 7.4; and 5 mM KNO_3, pH 7.4.

Antimicrobial activity assays. The peptide's antimicrobial activity was tested against different bacteria (see Table 2). Bacterial growth proceeded until logarithmic phase (4–6 h) at 1.0 OD_{600}. Spent culture medium was removed by spinning at 10,000 rpm for 10 min at 4 °C (International Equipment Company IEC, Centra MP4R; rotor 851, USA); the bacterial pellet was washed twice in 25 mM Na_2PO_4, 25 mM $NaHPO_4$, 150 mM NaCl, and 5 mM KCl, pH 7.4 (PBS). Bacterial concentration was adjusted to a 0.2 OD_{600} in the same buffer (5000–10,000 cells/mL). The peptide's *in vitro* antibacterial activity was measured as follows: 500 μL bacterial suspension were treated with 7.1 μM of BTM-P1 peptide for 2 h at 37 °C in 1.5 mL Eppendorf tubes. Following incubation, cells were washed in the same volume of buffer and then plated onto nutrient agar Petri dishes; colony forming units (CFU) were determined after 24 h incubation at 37 °C. Percentage inhibition was calculated by comparing the number of CFU obtained when incubated with and without the peptide.

Circular dichroism (CD). Spectra were performed as described. [16] Measurements were taken at room temperature on nitrogen-flushed cells using a Jasco J-810 spectropolarimeter (Jasco, Japan). Spectra were recorded at 190–250 nm wavelength interval using a 1-mm path-length rectangular cell. Each spectrum was obtained by averaging three scans taken at a 20 nm/min scan-rate with 1 nm spectral bandwidth; Jasco software was used for correcting the baseline. The CD profiles were done by dissolving lyophilised purified peptides in 0–50% aqueous 2,2,2-trifluoroethanol (TFE) in a final 500 μL volume. A typical 0.2 μM peptide concentration in TFE-water mixture stabilised but did not induce secondary structure in peptides, as described elsewhere [17]. The results were expressed as molar ellipticity.

Liposome preparation. Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cholesterol (CH) were dissolved in chloroform at 1:1:1 PC:PE:CH and 1:1 PC:CH molar ratios. Solvent was then removed by evaporation and dried at high vacuum for 8 h. The lipid mixture was rehydrated to final 100 mM concentration in 25 mM Na_2PO_4, 25 mM $NaHPO_4$, 150 mM NaCl, and 5 mM KCl, pH 7.4 (PBS). Liposomes were homogenised by sonication (Sonicator Branson, USA) for 1 h. Fifty millimolar phospholipids (liposomes) and 0.2 μM peptide in PBS were mixed for 1 h at 5 °C and analysed by CD at room temperature, as described above.

NMR analysis. NMR samples were prepared by dissolving 7–10 mg of peptide in 500 μL TFE-d3 (Cambridge Isotope, 99.94%)/H_2O mixture (30/70 v/v). NMR spectra were recorded on a Bruker DRX-600 spectrometer (Bruker, Billerica, USA) at 295 K. Basic NMR structure determination protocol for all peptides was as follows: proton spectra were assigned by DQF-COSY [18], TOCSY [19] and NOESY [20]; TOCSY and DQF-COSY spectra were then used for identifying individual spin systems (amino-acids) and NOESY (350 ms mixing time) for amino-acid stretches within a given primary structure (sequential assignment). Three-dimensional structure was assigned by Wüthrich's method [21]. 2D NMR data were processed with XWIN-NMR software. TOCSY spectra, recorded at different temperatures (285–315 K), were used to obtain amide temperature coefficients for predicting hydrogen bonds ($-\Delta H^N$/ppb/K).

Structure calculations. Peptide structure was determined by Accelrys Inc., software. NOESY peak signals were classified as strong, medium and weak according to their relative intensity corresponding to 1.8–2.5, 2.5–3.5 and 3.5–5.0 Å interproton distances, respectively. Hydrogen bond constraints were introduced for low amide temperature coefficients; only <4 and $-\Delta\delta H^N$/ppb/K were used in structure calculations. Distance ranges involving these likely NH···O hydrogen bonds were set at 1.8–2.5 Å, between residue acceptor oxygen (i-4) and residue donor amide hydrogen (i). All peptide bonds were forced to trans and Cα chirality to L during calculations. Distance Geometry (DGII) software was used for providing a family of 50 structures. These structures were refined by using simulated annealing protocol (Discover software). Structures having reasonable geometry and few violations were then selected.

Results

Effect of Cry11Bb1-derived peptides on mitochondrial swelling

Only the BTM-P1 peptide displayed significant ionophore activity in the inner mitochondrial membrane amongst the peptides tested here (Table 1, Fig. 1A and B). BTM-P1's ionophoric activity was determined in mitochondria suspended in isotonic 125 mM KNO_3, 5 mM Hepes–Tris pH 7.4 (Fig. 1A) and 125 mM NH_4NO_3, 5 mM Hepes–Tris pH 7.4 (Fig. 1B) media at 1.8, 0.36 and 0.18 μM concentration of BTM-P1 (Fig. 1A and B, traces a–c, respectively). The rate of swelling (slopes) induced by BTM-P1 in 125 mM KNO_3, 5 mM Hepes–Tris, pH 7.4 was comparable to that of valinomycin (−1.6 slope with 0.18 μM BTM-P1 and −1.4 slope with 120 nM Valinomycin) (Fig. 1A, traces c and e, respectively). A significant increase in mitochondrial swelling rate was observed when mitochondria was energised (−9.3 slope with 0.18 μM BTM-P1 and 2.5 mM succinate) (Fig. 1A, trace d). BTM-P1 swelling rate in 125 mM NH_4NO_3, 5 mM Hepes–Tris, pH 7.4, was comparable to that of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) (−2.1 slope with 0.36 μM BTM-P1 and −3.5 slope with 2 μM FCCP) (Fig. 1B traces b and f, respectively). Swelling