Capped acyclic permutants of the circular protein kalata B1

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Abstract The cyclotides are a family of head-to-tail cyclized peptides that display exceptionally high stability and a range of biological activities. Acyclic permutants that contain a break in the circular backbone have been reported to be devoid of the haemolytic activity of the prototypic cyclotide kalata B1, but the potential role of the charges at the introduced termini in this loss of membraneolytic activity has not been fully determined. In this study, acyclic permutants of kalata B1 with capped N- and C-termini were synthesized and found to adopt a native fold. These variants were observed to cause no measurable lysis of erythrocytes, strengthening the connection between backbone cyclization and haemolytic activity.

Keywords: Cyclotide; Cyclic peptide; Haemolysis; Acetylation; Amidation

1. Introduction

The cyclotides are a fascinating family of plant-derived peptides characterized by their knotted arrangement of three disulfide bonds and head-to-tail cyclization [1]. They possess a wide array of biological activities but their natural function in plants appears to be as defence molecules [2]. More than 50 members of the family are currently known and their rate of discovery has increased in recent years [3–5]. The first cyclotide to be structurally characterized, kalata B1 (KB1) [6,7], is the most thoroughly studied peptide in this family. Fig. 1 shows a schematic representation of the macrocyclic backbone and cystine knot [8,9], and indicates the nomenclature for defining the six backbone loops between the cysteine residues.

Some members of this family possess moderate haemolytic activity, with EC_{50} values in the 30–1500 μM range [10–13]. The mechanism for haemolysis is unknown, but it has been suggested that it is related to cell membrane disruption based on the hydrophobic nature of these peptides [12]. The extremely high stability of the cyclotides suggests that they might make useful scaffolds for bearing bioactive peptide epitopes in order to improve stability and bioavailability for pharmaceutical applications [14]. Therefore, understanding the basis for undesirable activities such as haemolytic activity is essential to their successful utilization.

The haemolytic activity appears to be linked to the cyclic backbone based on a study of synthetic derivatives where a break was introduced at different sites in the cyclic backbone. Several acyclic permutants of the prototypic cyclotide kalata B1 were demonstrated to have lost all haemolytic activity despite having native global folds [10]. However, the precise relationship between cyclization and haemolytic activity is not clear from this single study. The introduction of free termini into acyclic permutants adds charges to the peptides at physiological pH, rendering significant differences in the electrostatic profile of their surfaces relative to the native cyclic peptides. This is especially relevant if their hydrophobic nature is essential to their activity. Although earlier acyclic permutants with C-terminal amides have been synthesized and found to lack haemolytic activity [10], the effects of introducing N-terminal positive charges have not been investigated.

To address these issues, a series of acyclic permutants of kalata B1 were synthesized with capped amino and carboxyl termini to mask the introduced charges. In particular, combinations of N-terminal acetylation and C-terminal amidation were synthesized as shown in Fig. 1. The folding and activity of these peptides were assessed to gain a more detailed understanding of the relationship between backbone cyclization and haemolytic activity.

2. Materials and methods

2.1. Synthesis

Capped acyclic permutants of kalata B1 were assembled using manual solid phase peptide synthesis with Boc chemistry. Compounds with a free C-terminus (N-KB1-C, Ac-KB1-C) were synthesized on a 0.35 mmol scale using Pam-Asn resin. The resin was split in half for the final N-terminal acetylation of Ac-KB1-C with acetic anhydride/pyridine. Compounds with an amidated C-terminus (N-KB1-Am, Ac-KB1-Am and Ac-[des-Gly]-KB1-Am) were synthesized on a 0.5 mmol scale. The resin was split into thirds after the coupling of the penultimate leucine. One third was acetylated to give Ac-[des-Gly]-KB1-Am, one third was coupled to glycine to give N-KB1-Am and one third was coupled to glycine then acetylated to give Ac-KB1-Am.

Cleavage of the peptides from the resin was conducted using hydrogen fluoride in the presence of *p*-cresol and *p*-thiocresol (18:1:1 v/v). The reaction proceeded at 0 °C for 90 min. The peptides were then dissolved in 50% acetonitrile/0.05% TFA and lyophilized. The crude reduced peptides were purified using preparative RP-HPLC on a Vydac C-18 column. Gradients of 1% per minute of 90% acetonitrile/10% H_2O/0.05% TFA against 0.05% TFA/H_2O at a flow rate of 8 ml/min were employed and the eluant profile was monitored at 230 nm. Similar conditions were used for subsequent purifications. Mass analysis was performed on a Sciex triple quadrupole mass spectrometer (Thornhill, Ontario) using electrospray ionization.

Oxidation of the peptides was achieved by dissolving the purified reduced peptides in 2-propanol and 0.1 M ammonium bicarbonate (pH 8.0) (50:50 v/v) with 10 mM glutathione and stirring at room temperature overnight. The solutions were acidified with TFA and the isopropanol removed under vacuum before RP-HPLC. Final peptide

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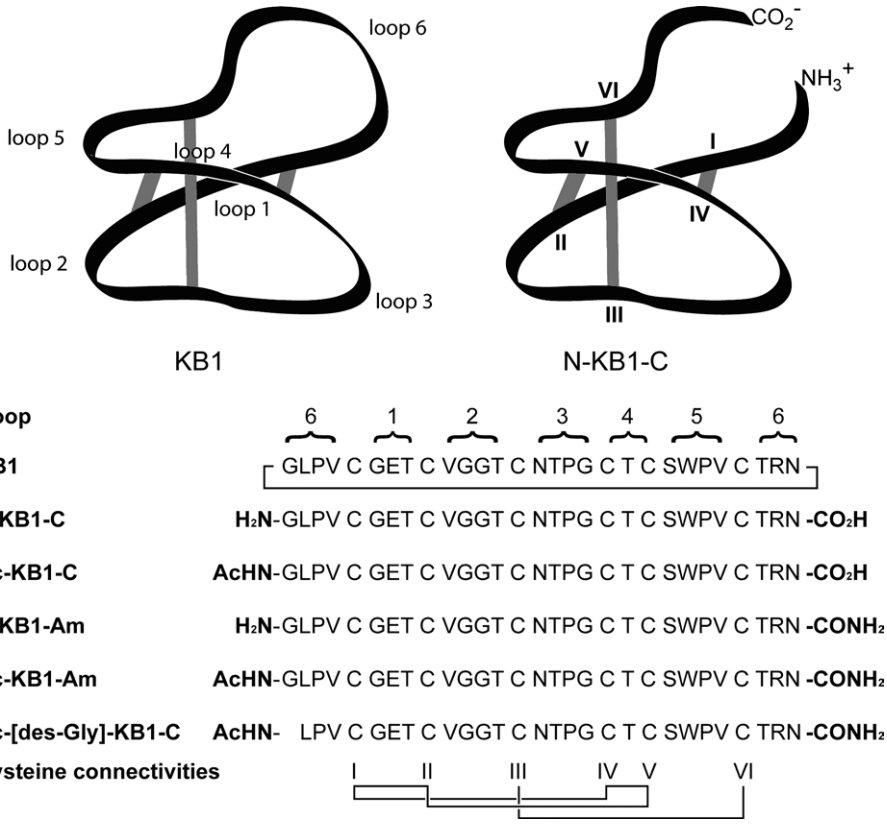

Fig. 1. Schematic representation of the cyclotide framework and sequences of kalata B1 and capped acyclic permutants. The backbone is shown in black ribbon and the disulfide bonds in grey shading, Cysteine residues are indicated with Roman numerals. The sequences of various acyclic permutants are shown in one-letter amino acid codes. Capping functionalities are indicated in bold on the sequences. In the compound names, N refers to a free amino terminus, C to a free carboxyl, Ac to an N terminal acetyl and Am to a C terminal amidation. The numbering of backbone loops between the cysteine residues is indicated in the schematic diagram and above the tabulated sequences.

yields (reduced/oxidized) in mg were: N-KB1-C (48/14), Ac-KB1-C (oxidized as crude to mitigate low solubility/3), N-KB1-Am (15/3), Ac-KB1-Am (10/0-the peptide was lost during HPLC due to low solubility) and Am-[des-Gly]-KB1-Ac (11/2).

2.2. NMR spectroscopy

Samples for 1D ^{1}H NMR measurements contained ~1 mg peptide in 120 μl of 10% D_2O/H_2O at pH 7. Spectra were recorded at 290 K on a Bruker DMX-750 MHz spectrometer equipped with a shielded gradient unit using a 2.5 mM probe. A sample of Am-[des-Gly]-KB1-Ac for 2D ^{1}H NMR was dissolved in 500 μl of 10% D_2O/H_2O at pH 4, and NOESY and TOCSY spectra were acquired on a Bruker-AMX 600 MHz spectrometer at 290 K. The spectra were assigned using the standard sequential assignment strategy to derive ^{1}H chemical shifts for all residues [15]. D_2O was obtained from Cambridge Isotope Laboratories, Woburn, MA. Spectra were processed on a Silicon Graphics Indigo workstation using XWINNMR (Bruker) software.

2.3. Haemolytic activity assays

Sheep erythrocytes were washed six times with a 10-fold excess of PBS by serial centrifugation at 4000 rpm, then a 1% (v/v) solution of red blood cells in PBS was prepared. Test peptides were added to 20 μl of 1% red blood cell solution to make a total sample volume of 100 μl and final peptide concentrations of 50, 100 and 200 μM. The samples were incubated for one hour at room temperature, centrifuged and the absorbance at 415 nm of 20 μl of supernatant in 600 μl water was measured. Controls of 1% SDS for complete haemolysis and PBS for zero haemolysis were run concurrently.

3. Results

Synthesis of the capped acyclic permutants presented some minor difficulties. The yields of the compounds with a free C-terminus were reduced by the formation of a C-terminal succinimide of Asn during HF cleavage. This was ameliorated by ensuring that the reaction was kept below 0 °C and not allowed to proceed for more than 90 min. Two of the variants with N-terminal acetylation (Ac-KB1-C and Ac-KB1-Am) had low solubility in the reduced and oxidized forms that was manifest as an incompatibility with ultrafiltration membranes and HPLC guard columns, causing them to rapidly clog. This occurred even at high dilutions when there was no visible precipitate and led to the loss of Ac-KB1-Am during HPLC. Consequently, the oxidization of Ac-KB1-N was done in an unpurified state and final purification was done without ultrafiltration. A third acetylated analogue, Ac-[des-Gly]-KB1-Am, that was lacking the N-terminal glycine residue was also prepared and displayed normal solubility. Despite the difficulties, the final yields of purified, oxidized peptides were sufficient to allow NMR analysis and haemolytic assays for the remaining peptides. Oxidation trial profiles for the acyclic permutants were similar to those previously observed for native cyclotides, showing a characteristic increase in retention time upon forming the native oxidized fold [10].

NMR spectroscopy of the peptides indicated that they were correctly folded. Fig. 2 shows partial 1D ^{1}H NMR spectra of the native peptide and capped acyclic variants at pH 7, with each showing similar amide fingerprint regions and a characteristic upfield proline β-proton shift (−0.25 ppm [7]). The sparsity of peaks in the amide region of the variants is due to rapid amide exchange at this pH resulting in a loss of some

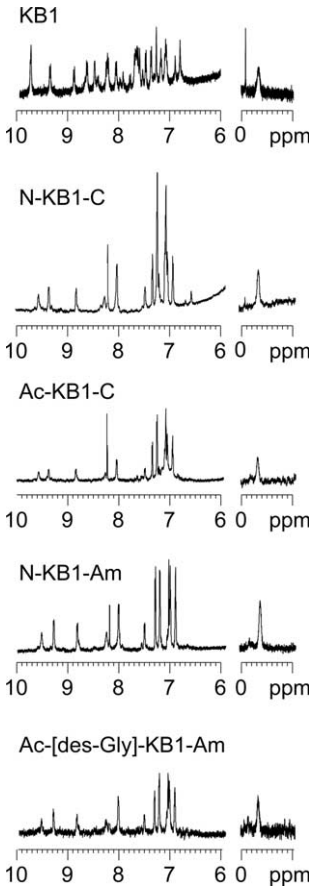

Fig. 2. ^{1}H NMR spectra of selected regions of native and acyclic analogues of kalata B1.

signals, with only those amide protons involved in long-lived intramolecular hydrogen bonds visible. Further analysis of Ac-[des-Gly]-KB1-Am at lower pH showed that the αH chemical shifts were consistent with the peptide adopting the same global fold as native cyclic KB1. The differences in αH chemical shifts of the acyclic permutant Ac-[des-Gly]-KB1-Am relative to the native cyclic KB1 are summarized in Fig. 3. The chemical shifts in loop 6 differ from those in the native peptide due to the introduced break in the backbone, but the shifts are closely comparable between the two peptides for the rest of the molecules.

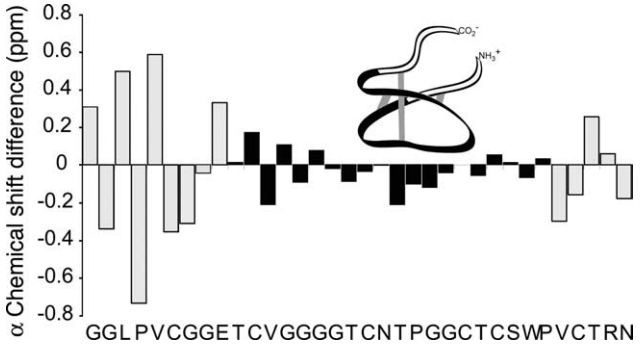

Fig. 3. αH Chemical shift differences between Ac-[des-Gly]-KB1-Am and kalata B1 at pH 4. White bars correlate with white regions on the inset schematic and indicate structurally perturbed residues.

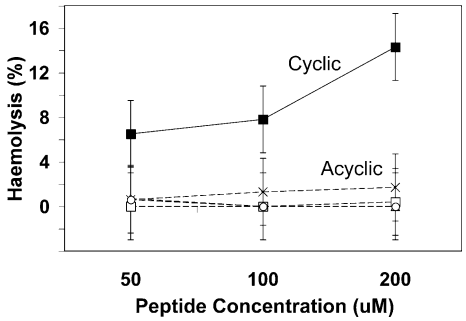

Fig. 4. Haemolytic activity for cyclic kalata B1 (solid line) and acyclic derivatives (dashed lines); ×, N-KB1-C; △, Ac-KB1-C; □, N-KB1-Am and ○, Ac-[des-Gly]-KB1-Am.

Haemolytic assays showed a concentration dependent erythrocyte lysis for the wild-type cyclic kalata B1, as summarized in Fig. 4. Sheep blood was found to be more resilient than human blood [11], with a maximum of 14% haemolysis observed at 200 μM peptide concentration. In contrast with the wild type peptide, all acyclic permutants had no observable haemolytic activity up to 200 μM.

4. Discussion

In this study, we have explored the connection between cyclization, terminal charges and loss of haemolytic activity in the prototypic cyclotide kalata B1. Capped acyclic analogues of kalata B1 were synthesized and shown to adopt essentially the same fold as the native cyclic peptide but they displayed no haemolytic activity, in contrast to the native peptide.

Acyclic permutants of kalata B1 in which the C- and N-terminal charges were eliminated underwent oxidation in a manner similar to previously prepared linear and cyclic analogues. Unusual solution properties of two N-terminally acetylated variants were observed. Their reduced and oxidized forms were incompatible with microfiltration, even when in an apparently soluble state. The capped acyclic analogue Ac-[des-Gly]-KB1-Am was considered to be a suitable replacement for the Ac-KB1-Am that was lost due to its unusual solution properties. The observation that Ac-[des-Gly]-KB1-Am had more favourable solution properties than the other acetylated permutants is noteworthy. In this compound the N terminal Gly was omitted, allowing the N terminal acetyl group to fill the space normally occupied by this amino acid. This compound represents an acyclic mimic of kalata B1 with the smallest possible steric and electrostatic modification, being in effect a version of the wild-type peptide where the only modification is the hydrogenation of the N-Cα bond of the glycine in loop 6, as indicated in Fig. 5. The difficulties experienced with the two full-length acetylated analogues suggest that the acetyl group introduces a significant steric clash.

NMR analysis of the peptides clearly indicated that they adopt a global fold comparable to the native cyclic peptide. An analysis of the chemical shifts of the acyclic mimic Ac-[des-Gly]-KB1-Am, as shown in Fig. 3, indicated only minor αH chemical shift variations relative to the native peptide in the majority of the sequence. As expected, those residues closest to the introduced termini were more highly disrupted. Inspection of the

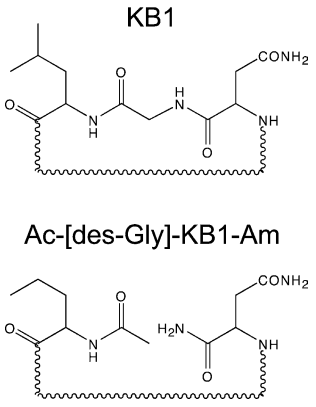

Fig. 5. Chemical structures of break region of loop 6 of kalata B1 and acyclic analogue Ac-[des-Gly]-KB1-Am, illustrating the isosteric nature of the analogue compared to the native peptide.

structure of the wild-type peptide [7] shows that the disrupted region of the backbone is involved in a hydrogen bond between residues 24 and 27. Cleavage of the backbone at this position will clearly disrupt this hydrogen bond and destabilize the loop. Similar results were seen in a previously studied acyclic permutant of loop 6 with a free N-terminus [10]. This reinforces the suggestion that a cyclic backbone is not essential for the formation of the native global fold and further establishes that terminal charges are also not essential for folding. Although native-like structures are generally maintained, the spectra show some subtle differences in the acyclic permutants compared to the cyclic peptide. In particular, the paucity of amide peaks in spectra measured on the acyclic permutants at pH 7 indicated that as expected, many amide protons undergo significant exchange at this pH. The retention of a larger number of amide protons at this pH in the wild type peptide [16] suggests that the intramolecular hydrogen bond network observed in the native is not fully maintained in the analogues. This is consistent with earlier observations that acyclic permutants of kalata B1 displayed enhanced amide exchange rates, relative to the native cyclic peptide, at pH 4 for the majority of amide protons in the β-sheet region C15-V29 [10].

The activity of wild-type kalata B1 on sheep erythrocytes was observed to be significantly weaker than previous observations on human blood, with an $EC_{50} > 1$ mM compared to as potent as 30 μM for human erythrocytes. This is consistent with prior observations of the sensitivity of haemolytic assays to variations in the nature of the blood used [10]. In contrast with the native peptide, the acyclic permutants were found to have no observable haemolytic activity at the concentrations used here, as shown in Fig. 4. Masking of the C-terminal carboxylic acid or the N-terminal amine did not reintroduce haemolytic activity. In addition, the preparation of a compound with effectively only hydrogenation of the N-C bond gave a comparable result. This shows that the introduction of terminal charges is not responsible for the loss of haemolytic activity in acyclic permutants of kalata B1 and establishes a clear link between backbone cyclization and haemolysis. The possibility exists though that a capped acyclic permutant of another loop may retain some haemolytic activity. However, this seems inconsistent with the emerging pattern of delocalized activity dependent on structural stability.

Although this study strengthens the connection between backbone cyclization and haemolytic activity, the prospective use of the cyclotide framework as a scaffold for carrying bioactive peptide epitopes still remains promising. This work has demonstrated that haemolytic activity is perturbed by minor modifications to the molecule, suggesting that changing amino acid side chains without breaking the cyclic backbone or compromising the stability of the framework should be capable of reducing haemolytic activity to acceptable levels.

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