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Role of individual disulfide bridges in the conformation and activity of spinoxin (α-KTx6.13), a potassium channel toxin from *Heterometrus spinifer* scorpion venom

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ABSTRACT

Spinoxin (SPX; α-KTx6.13), isolated from venom of the scorpion *Heterometrus spinifer*, is a K^+ channel-specific peptide toxin (KTx), which adopts a cysteine-stabilized α/β scaffold that is cross-linked by four disulfide bridges (Cys1–Cys5, Cys2–Cys6, Cys3–Cys7, and Cys4–Cys8). To investigate the role of the individual disulfide bonds in the structure-activity relationship of SPX, we synthesized four SPX analogs in which each pair of cysteine residues was replaced by alanine residues. The analysis of circular dichroism spectra and inhibitory activity against Kv1.3 channels showed that the SPX analogs lacking any of three specific disulfide bonds (Cys1–Cys5, Cys2–Cys6, and Cys3–Cys7) were unable to form the native secondary structure and completely lost inhibitory activities. Thus, we conclude that Cys1–Cys5, Cys2–Cys6, and Cys3–Cys7 are required for the inhibition of the Kv1.3 channel by SPX. In contrast, the analog lacking Cys4–Cys8 retained both native secondary structure and inhibitory activity. Interestingly, one of the isomers of the analog lacking Cys1–Cys5 also showed inhibitory activities, although its inhibition was ~18-fold weaker than native SPX. This isomer had an atypical disulfide bond pairing (Cys3–Cys4 and Cys7–Cys8) that corresponds to that of maurotoxin (MTX), another α-KTx6 family member. These results indicate that the Cys1–Cys5 and Cys2–Cys6 bonds are important for restricting the toxin from forming an atypical (MTX-type) disulfide bond pairing among the remaining four cysteine residues (Cys3, Cys4, Cys7, and Cys8) in native SPX.

1. Introduction

A number of peptides that target a variety of subtypes of voltage-gated potassium ion channels (Kv) have previously been isolated from venomous animals such as scorpions, bees, marine cone snails, snakes, and sea anemones. These toxins consist of 22–60 amino acid residues and are cross-linked by two to four disulfide bonds (Jouirou et al., 2004). Given the physiological and clinical significance of Kv channels in cellular electrical excitability, cell proliferation, apoptosis, and volume regulation, and their potential (Kv1.3 in particular) as molecular targets for the therapeutic modulation or diagnosis of autoimmune disorders and some

Abbreviations: Acm, acetamidomethyl; CSαβ scaffold, cysteine-stabilized α/β scaffold; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; EDTA, ethylenediamine-*N,N,N′,N′*-tetraacetic acid; GSH, reduced glutathione; GSSG, oxidized glutathione; HBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxy-1H-benzotriazole; HsTX1, *Heterometrus spinifer* scorpion toxin 1; KTx, K^+ channel-specific scorpion toxins; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; MTX, maurotoxin; NMP, 1-methyl-2-pyrrolidone; Pi1, potassium channel-blocking toxin 1; RP-HPLC, reversed phase high performance liquid chromatography; SPX, spinoxin; TFA, trifluoroacetic acid; Trt, triphenylmethyl.

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cancers (Beeton et al., 2006; Bielanska et al., 2009; Teisseyre et al., 2015), the study of the structure-function relationships of toxins that interact with Kv channels has generated considerable interest.

We have previously isolated and characterized spinoxin (SPX) from venom of the Malaysian black scorpion *Heterometrus spinifer* (Scorpionidae) (Sugahara et al., 2004). SPX is a 34-residue peptide neurotoxin that inhibits Kv1.2 and Kv1.3 channels, but has no activity on Kv1.1 channels. It is classified as α-KTx6.13 according to the unified nomenclature of K^+ channel scorpion toxins (Peigneur et al., 2016). Using an alanine-scanning approach, we determined that three amino acid residues (Lys^{23}, Asn^{26}, and Lys^{30}) in SPX are the most important for its inhibitory activity against Kv1.3 channels (Peigneur et al., 2016). Furthermore, Arg^{7}, Met^{14}, Lys^{27}, and Tyr^{32}—residues for which replacements largely reduced inhibitory activity—are the second-most important residues required for inhibition (Peigneur et al., 2016).

Using homology modeling, the secondary structure of SPX is predicted to form the cysteine-stabilized α/β (CSαβ) scaffold typical of scorpion toxins that act on Kv channels; this scaffold contains an α-helix (Ser^{6} to Gln^{16}) and a two-stranded antiparallel β-sheet (Cys^{24} to Asn^{26} and Cys^{29} to Cys^{31}; Fig. 1) (Peigneur et al., 2016). In SPX, the N-terminal region (Ile^{1} to Pro^{20}) that includes the α-helix, and the C-terminal region (Asn^{21} to Cys^{34}) that includes the β-hairpin region, are connected by four disulfide bonds. We previously determined that the disulfide bonding pattern of SPX is Cys1–Cys5, Cys2–Cys6, Cys3–Cys7, and Cys4–Cys8 using enzymatic digestion (Peigneur et al., 2016). This disulfide pairing is commonly found in most toxins belonging to the α-KTx6 subfamily (Fig. 1). The role of disulfides in peptide toxins has been extensively studied (Zhu et al., 2002; Khoo et al., 2009; Sato et al., 2014), and the possible activities of different disulfide isomers has been recently reported (Dutton et al., 2002; Wu et al., 2014; Carstens et al., 2016). However, the specific roles of the four disulfide bonds in scorpion toxin peptides such as SPX remain relatively unknown (Ojeda et al., 2014). Analyses of disulfide bonds in scorpion toxins targeting Kv channels have been reported previously, but these studies either described toxins that have only three native disulfide bonds (charybdotoxin), or focused only on the fourth disulfide bond (Cys4–Cys8) in other four-disulfide-bridged toxins (Pi1 and HsTX1) (Drakopoulou et al., 1998; Carrega et al., 2005). A comprehensive analysis using α-KTx analogs to study the role of each of the four disulfide bonds in the structure and function of a four-disulfide-bridged toxin has not been reported to date.

In this study, in order to investigate the role of each disulfide bond in SPX, we synthesized SPX analogs in which two cysteine residues of each disulfide bond in SPX were systematically replaced by two alanine residues. We determined that three specific disulfide bonds (Cys1–Cys5, Cys2–Cys6, and Cys3–Cys7) in SPX are required for inhibitory activity against Kv1.3 channels. Furthermore, we found that two disulfide isomers of the SPX analog lacking either the first (Cys1–Cys5) or the second disulfide bond (Cys2–Cys6) exhibited an uncommon disulfide bond pairing, Cys3–Cys4 and Cys7–Cys8, which resembled maurotoxin (MTX; α-KTx6.2), an unusual member of the α-KTx6 scorpion toxin family. Interestingly, the analog lacking Cys1–Cys5 showed effective Kv1.3 channel inhibition. Our results provide valuable insight into the roles of the four individual disulfide bonds in the conformational stability, Kv1 channel-blocking activity, and folding of four-disulfide-bridged scorpion toxins in the α-KTx6 family.

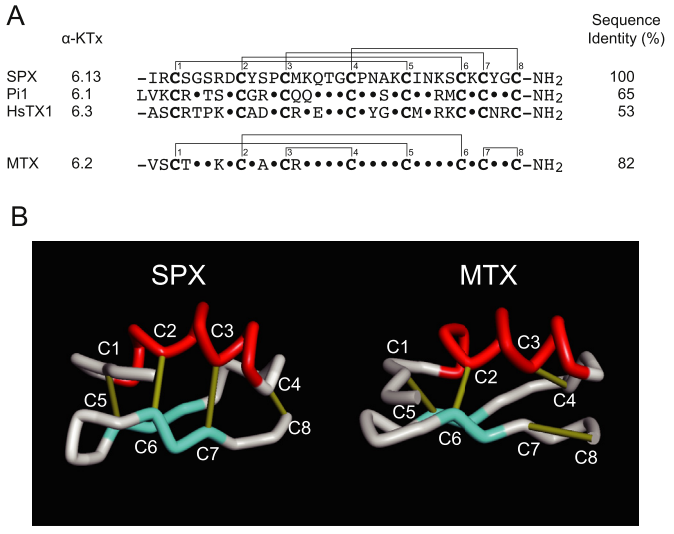

Fig. 1. Sequence and structure of SPX. A. Multiple sequence alignment of α-KTxs. Cysteine residues are in bold type, and dots indicate conserved residues. Pi1 is from *Pandinus imperator* (Olamendi-Portugal et al., 1996), HsTX1 from *Heterometrus spinifer* (Lebrun et al., 1997), and MTX from *Scorpio maurus palmatus* (Kharrat et al., 1996). B. Structures of SPX and MTX. A model of SPX was constructed by homology modeling using MODELLER in the Discovery Studio software package (Sali and Blundell, 1993) using the solution structure of HsTX1 determined by NMR (PDB 1QUZ, Savarin et al., 1999) as a template. A model of MTX was created using PDB 1TXM. Disulfide bonds are shown in yellow, α-helix in red, and β-sheet in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Material and methods

2.1. Materials

All Fmoc-amino acids, Fmoc-NH-SAL resin, dichloromethane (DCM), *N,N*-diisopropylethylamine (DIEA), pyperidine, 1-hydroxy-1H-benzotriazole (HOBt), 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and 1-methyl-2-pyrrolidone (NMP) were purchased from Watanabe Chemical Industries, LTD. (Japan). Other reagents were obtained from Kokusan Chemical Works, Ltd. (Japan) and Wako Pure Chemical Industries, Ltd. (Japan).

2.2. Peptide synthesis and purification

Solid phase peptide synthesis was performed on an Applied Biosystems 431A peptide synthesizer (Applied Biosystems Inc., USA). Linear precursors of the analogs were synthesized using the solid phase method with Fmoc chemistry starting from Fmoc-NH-SAL Resin (Watanabe Chemical Industries, LTD). We used the regioselective two-step disulfide bonds formation method (Nishiuchi and Sakakibara, 1982) with Trt and Acm protecting groups for cysteine residues to synthesize the analogs **A1**, **A2**, and **A3**, which lack Cys1–Cys5, Cys2–Cys6, and Cys3–Cys7, respectively (Fig. 2). Fmoc-Cys(Acm)-OH was used as follows: **A1** (**A1-C4,8-Acm**); Cys4 and Cys8, **A2** (**A2-C1,5-Acm**) and **A3** (**A3-C1,5-Acm**); Cys1 and Cys5 (Fig. 2). In the synthesis of **A4** which lacks Cys4–Cys8, only Fmoc-Cys(Trt)-OH was used. The synthesis scheme of **A1** is shown as a representative example in Fig. 3.

2.2.1. Cleavage and air oxidation

The protected peptide resin of **A1-C4,8-Acm** (737 mg, 0.10 mmol) was treated with TFA (8 mL) in the presence of thioanisole (0.5 mL), H_2O (0.5 mL), phenol (0.75 mL), and 1,2-ethanedithiol (0.25 mL) at 0 °C for 5 min followed by an incubation at room temperature for 1.5 h to remove the resin and protecting group (except Acm) from the synthetic peptides. After precipitation by the addition of an excess of diethyl ether, the crude linear peptide was collected by filtration and extracted with 0.1%