Structure Report

Structure of the scorpion toxin BmBKTtx1 solved from single wavelength anomalous scattering of sulfur

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Abstract

This report describes the crystal structure of the K⁺ channel-blocking toxin, BmBKTtx1, isolated recently from the venom of the scorpion Buthus martensi Karsch. This is only the second structure of the short-chain K⁺ channel-blocking toxin from scorpion solved by means of X-ray crystallography. Additionally, reductive dimethylation of folded BmBKTx1 employed to induce its crystallization and solution of the structure based on the anomalous signal from the sulfur atoms make this example quite unique. The monomer of BmBKTx1 is formed by 31 amino acid residues, including 6 cysteines connected in 3 disulfide bridges. Crystals of this toxin belong to the space group P2₁ with two molecules present in the asymmetric unit. The unit cell parameters are a = 21.40 Å, b = 39.70 Å, c = 29.37 Å, and β = 94.13°. Based on the high-quality dataset (anomalous signal) collected to the resolution 1.72 Å using the conventional X-radiation generator (λCuKα = 1.5478 Å), the positions of sulfur atoms contributed by 12 cysteine residues have been identified, and subsequent improvement of the experimental phases have allowed structure solution. The final model was refined to the crystallographic R-factor of 0.166. The methyl groups on several lysine residues could be easily modeled into the electron density.

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1. Introduction

The scorpion Buthus martensi Karsch (BmK)¹ is widely distributed in some parts of Asia, such as Mongolia, Korea and China, where the whole body and especially its tail have been used in traditional medicine for thousands of years (Song, 1983). Although over 1500 different species of scorpions have been identified, and it is estimated that, on average, their venoms consist of between 50 and 100 polypeptide toxins (Lourencio, 1994), detailed studies have been conducted for only about 30 species and have resulted in characterization of 210 bioactive proteins or peptides. Among them, a substantial fraction consists of neurotoxic proteins or peptides (Martin-Eauclaire and Couraud, 1995). Most of those toxins interact specifically with various ion channels in excitable membranes (Cattarall, 1980; Garcia et al., 1997; Gordon et al., 1998). Composition of the BmK venom was extensively studied during the last several years, and about 80 different proteins or peptides were isolated and characterized on the basis of their amino acid sequences, physiological functions, and their corresponding receptors (reviewed in Goudet et al., 2002).

Solution of the crystal structure of BmBKTx1 toxin required the use of several non-standard approaches. Among the obstacles preventing formation of crystals suitable for X-ray diffraction experiments was its high solubility in various precipitant solutions (in excess of 100 mg/ml). This property is often observed for proteins composed of many charged, surface-exposed amino acid residues. One of the methods used for reducing solubility of lysine-rich proteins is reductive methylation of the primary amino groups (Rayment, 1997). Despite a relatively harsh chemical environment associated with

¹ Abbreviations used: BmK, Buthus martensi Karsch.

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redductive methylation, successful results were previously reported for several proteins, such as insecticyanin (Holden et al., 1987), myosin (Rayment et al., 1993), and lysozyme (Rayment, 1997; Rypniewski et al., 1993). Whereas appearance of the diffracting protein crystals is necessary for the structural characterization by means of X-ray crystallography, the process of the structure solution can frequently be tedious and may require preparation of derivatized variants. In such cases, one of the most popular approaches is to prepare the seleno-methionine derivative of the target protein, and to collect X-ray data at the wavelength (about 0.98 Å) corresponding to the absorption edge of Se. Such experiments, however, often can only be conducted at the synchrotrons at the expense of efficiency and cost-effectiveness of structural studies. For well-diffracting crystals of proteins containing immobilized sulfur atoms, as is the case with cysteine residues forming disulfide bridges, an anomalous component of the diffraction intensities contributed by the sulfurs may be detected and utilized in the process of structure solution (Dauter et al., 2002). Due to weak signal, structure determinations based solely on the anomalous signal from S-atoms are rare and usually are based on the experiments conducted using the synchrotron radiation sources (Dauter et al., 2002).

In this report we describe the X-ray structure of the recently identified novel toxin from scorpion B. martensi Karsh, designated as BmBKTx1. BmBKTx1 is active on BKCa, calcium-activated potassium channel, and shares no sequence homology with other toxins except for the cystine framework (C.X., personal communication).

2. Materials and methods

2.1. Synthesis, refolding, and purification

BmBKTx1 was synthesized on Boc-Tyr(2-Br-Z)-OCH2-PAM resin using a custom-modified form of the recently identified K+ channels-blocking, machine-assisted chemistry tailored from the published in situ DIEA neutralization/HBTU activation protocol for Boc solid phase peptide synthesis (Schnolzer et al., 1992). The following side-chain protections were used: Cys(4-MeBzl), Glu(OcHx), His(Bom), Lys(2-Cl-Z), Asn(Xan), Arg(Tos), Ser(Bzl), Thr(Bzl), and Tyr(2-Br-Z). After chain assembly, the peptide was cleaved and deprotected by HF in the presence of 5% p cresol/thiocresol (1:1) at 0°C, followed by precipitation with cold ether. The crude product was purified by reverse-phase HPLC to homogeneity, and its molecular weight was ascertained by electrospray ionization mass spectrometry. Oxidative folding of purified BmBKTx1 was performed by dissolving the peptide at 3 mg/ml in 6 M GuHCl containing 18 mM reduced glutathione and 1.8 mM oxidized glutathione, followed by a rapid 6-fold dilution with 0.25 M NaHCO3. The folding/disulfide formation proceeded quantitatively at room temperature overnight, and the final product was purified by HPLC and lyophilized. The oxidative folding of BmBKTx1 was confirmed by both analytical reverse-phase chromatography and spectrometry. Decrease in the retention time during chromatography as well as loss of 6 molecular mass units indicated folding of a compact structure and formation of three disulfide bonds, as expected for BmBKTx1.

2.2. Reductive methylation of BmBKTx1

The reaction was performed according to the protocol described by Rayment (1997). Since the native protein consists of six primary amino groups (five lysine residues and the N-terminus), its molecular mass (3504.2 Da) was expected to increase by 168.3 Da upon methylation, while the net charge would change from +5 to −1. After 24 h of reaction, subsequent mass spectrometry analysis (TOF-ES, model 1100 Series LC/MSD, Agilent) showed that BmBKTx1 was nearly quantitatively dimethylated. The pure (diMe)BmBKTx1 was isolated from the reaction mixture by reverse-phase chromatography, lyophilized, and stored at −80°C.

2.3. Crystallization, X-ray data collection, and processing

Originally, crystallization experiments with BmBKTx1 were conducted at room temperature using the sparse-matrix crystallization screens (Hampton Research, Emerald BioStructures). It was found that even for the solutions of BmBKTx1 at concentrations of up to 100 mg/ml, droplets stayed clear (free of precipitant or oily residue) over many weeks. Therefore, in the subsequent experiments all crystallization trials were performed using the derivative of BmBKTx1, with all primary amino groups dimethylated. Although initially the behavior of droplets prepared with (diMe)BmBKTx1 seemed to mirror that observed earlier for the native protein, after about 8 weeks a cluster of plate-shaped crystals appeared in the presence of 4 M sodium formate. Crystals grew to the size of 0.15 mm × 0.15 mm × 0.08 mm. For the X-ray experiments, one of the crystals was briefly transferred to the solution containing 3.6 M sodium formate and 10% (v/v) of glycerol and subsequently frozen at the temperature of 100 K.

The X-ray data set was collected using a rotating anode (λ = 1.5478 Å mounted on the Rigaku-Ru200 X-ray generator operated at 100 kV and 50 mA. The intensities were recorded using a Mar345 (Mar Research) image plate detector. Although the preliminary exposures to the X-rays showed the diffraction pattern extending to ~1.45 Å, we reduced the exposure time (100 s per 1° image) to avoid oversaturation for the
lower-resolution reflections. The complete and very redundant X-ray data set, extending to the resolution of 1.72 Å, resulted from merging of 600 consecutive images. The experimental images were indexed and integrated and scaled with the program HKL2000 (Otwinowski and Minor, 1997). The anomalous differences, however, were extracted after scaling of the non-reduced data with the program XPREP (Bruker Nonius, 2001). Anomalous differences obtained from this experiment were subsequently used for the solution of the positions of sulfur atoms, while the scaled intensities (from HKL2000) were used during the model building and initial structure refinement. The representative statistics are shown in Table 1.

### 3. Results

#### 3.1. Structure solution, model building, and refinement

Based on the high quality of the experimental data (see Table 1) as well as the presence of anomalous signal (assessed with programs HKL2000 and XPREP), we attempted to find the positions of twelve sulfur atoms (six cysteine residues in each of two independent monomers of BmBKTx1) using only the anomalous differences. Calculations were performed with the program SHELXD (Usón and Sheldrick, 1999) using all ($AF_{\text{anom}}$) values within the resolution range 30–1.8 Å. Readily, within 40–50 cycles of each run, the program converged to the solution characterized by the values of Patterson figure of merit (FOM) and the correlation coefficient exceeding 0.38 and 0.35, respectively. We found that the top 12 putative sulfur atoms were arranged into two structurally very similar clusters of three duplets, each resembling a single disulfide bond, further confirming the correctness of the structure solution. Interestingly, we made an analogous observation previously for BmK38 (Szyk et al., 2003). In the next step, positions of the 12 putative sulfur atoms as well as the experimental intensities and anomalous differences were fed into the program SHARP (de La Fortelle and Bricogne, 1997) for subsequent phase refinement according to the maximum-likelihood principle. The refined phases were characterized by the overall values of FOM, Cullis $R$-factor and the phasing power of 0.54, 0.57, and 2.34, respectively, for all data within the resolution range 30–1.8 Å. The refined phases were then modified with the program Solomon (Abrahams and Leslie, 1996), leading to further improvement, as reflected by the mean FOM of 0.75. Finally, using the refined phases but without the information about non-crystallographic symmetry, even though the latter was apparent from the positions of sulfur peaks, the program wARP/ARP (Lamzin and Wilson, 1997) was used to build the initial model for BmBKTx1. This program easily traced two polyalanine chains with a connectivity index of 0.93, corresponding to the two molecules of BmBKTx1 present in the asymmetric unit, reporting the final values of $R$ and free-$R$ of 0.237 and 0.340, respectively. This preliminary model was subjected to rigid-body refinement and energy minimization with CNS (Brünger et al., 1998). Visual inspection of the $2F_o-F_c$ and $F_o-F_c$ electron density maps with program O (Jones et al., 1991) allowed for easy introduction of the correct amino acid sequence. In the next several cycles, the continuously improving model was subjected to the structural refinement (CNS) and manual corrections (O) with simultaneous extension of the resolution of the X-ray data. At the very final stages, the dimethylated lysine residues, the solvent molecules, and all observed multiple conformations of protein fragments were modeled into the high-quality electron density maps (Fig. 1). The $B$-factor values of all atoms were refined isotropically. The final statistics from the structural refinement are shown in Table 1.
4. Discussion

4.1. The structure of BmBKTx1

The asymmetric unit of BmBKTx1 crystals consists of two molecules, A and B, for which all the residues are well defined by the electron density (see Fig. 1). The root-mean-square (r.m.s.) deviation for the equivalent 30 Cα atoms (excluding the first residue) of the two monomers is 0.36 Å. The side chains of three residues, SerA6, SerB6, and MetB15, accommodate two conformations, while some dynamic disorder is observed for the side chains of a few other residues. The protein molecules are surrounded by 108 waters. The whole structure is characterized by a rather low average B-factor (17.3 Å²). Modest interactions between different molecules indicate that BmBKTx1 likely does not form specific oligomers. The monomer of BmBKTx1 (Fig. 2) has an overall topology common to other K⁺ channel-blocking toxins. The 4 residue-long coiled N-terminus is followed by three turns of an α-helix (Ser5–Ala14). The second half of the molecule forms a hairpin, contributed by two 5 residue-long β-strands (Ser19–Ile23 and Lys26–Tyr30). These two β-strands are interspersed by a type IV β-turn (Cys22–Ser25) (Chan et al., 1993). The structure of this small molecule is stabilized by three disulfide...
bridges (Cys3–Cys22, Cys8–Cys27, and Cys12–Cys29), which have conformations of the left-, right-, and left-hand spiral (Chan et al., 1993), respectively. These disulfide bonds, conserved in all short-chain K⁺ channel-blocking toxins, link the N-terminal fragment of BmBKTx1 with each of the two β-strands.

During the preparation of this paper, the coordinates of the NMR structure of BmBKTx1 in solution have been deposited in the Protein Data Bank (1Q2K) although an associated publication was not referenced. The solution structure also describes the monomer with topology very similar to the structure reported here, yet several minor differences can be described. The r.m.s. deviations between X-ray structure and the individual NMR models varies between 0.75 and 1.2 Å. The largest discrepancies between both the X-ray and NMR models are found for the protein termini and for the type IV β-turn (Cys22–Ser25). Also, the second disulfide bridge (Cys8–Cys27) in some of the individual NMR models has a left-handed conformation. Overall, however, a high consistency of the solutions obtained with two methods indicates that the structure of BmBKTx1 is not affected by either the crystallization or methylation of the primary amino groups. Such a rigid topology of BmBKTx1, mostly determined by the presence of three disulfide bridges, is likely to be preserved in the complex of this toxin with a channel molecule.

4.2. Structure solution

Only a handful of protein structures have been solved to date on the basis of an anomalous signal of sulfur, detected with data collected using a conventional source of X-radiation (Dauter et al., 2002; Szyk et al., 2003). This work demonstrates another example proving that the quantitative chemical modification of lysines is straightforward and can be crucial for successful crystallization of proteins. Since lysines frequently determine the surface properties (shape, charge) in proteins, they are often a major factor during aggregation processes, i.e., crystallization. For small, basic proteins [i.e., toxins (Goudet et al., 2002), defensins (Hoover et al., 2001), or chemokines (Rossi and Zlotnik, 2000)], high solubility and failure to form crystals are quite frequent. As shown in Fig. 3, five lysine residues in addition to one arginine and the N-terminus, form a relatively uniform distribution of positive charges on the molecular surface, most likely preventing stable oligomerization of this protein. In this and similar cases, the reductive methylation of the primary amino groups should be considered as one of the simplest alternatives of alteration the molecular surface.

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References


