Crystallization of a potassium ion channel and X-ray and neutron data collection

Patricia S. Langan,^{a,‡} Venu Gopal Vandavasi,^{a,§} Brendan Sullivan,^a Joel Harp,^b Kevin Weiss^a and Leighton Coates^{a,*}

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‡ Present address: Lentigen Technologies, 910 Clopper Road, Gaithersburg, MD 20878, USA.

§ Present address: Department of Chemistry, Princeton University, Princeton, NJ 08540, USA.

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^aNeutron Scattering Division, Neutron Sciences Directorate, Oak Ridge National Laboratory, 1 Bethel Valley Road, Oak Ridge, TN 37831, USA, and ^bDepartment of Biochemistry and Center for Structural Biology, Vanderbilt University School of Medicine, 1161 21st Avenue South, Nashville, TN 37232, USA. *Correspondence e-mail: coatesl@ornl.gov

The mechanism by which potassium ions are transported through ion channels is currently being investigated by several groups using many different techniques. Clarification of the location of water molecules during transport is central to understanding how these integral membrane proteins function. Neutrons have a unique sensitivity to both hydrogen and potassium, rendering neutron crystallography capable of distinguishing waters from K⁺ ions. Here, the collection of a complete neutron data set from a potassium ion channel to a resolution of 3.55 Å using the Macromolecular Neutron Diffractometer (MaNDi) is reported. A room-temperature X-ray data set was also collected from the same crystal to a resolution of 2.50 Å. Upon further refinement, these results will help to further clarify the ion/water population within the selectivity filter of potassium ion channels.

1. Introduction

Potassium ion channels are essential elements in cellular function, regulating both electrical excitability and resting potential in non-excitable cells (Miller, 2001). The signature amino-acid sequence in potassium ion selectivity is the TVGYG sequence, which makes up the selectivity filter of the potassium ion channel (Doyle *et al.*, 1998). This sequence imparts a high selectivity for potassium ions over sodium ions and is formed from four continuous and equivalent binding sites. The filter orients several carbonyl O atoms into the ion pore, each with a partial negative charge. This structure would be unstable were it not for the presence of positively charged potassium ions within the selectivity filter.

The TVGDG filter sequence of the NaK channel from *Bacillus cereus* forms a nonselective specificity filter which allows the transport of potassium and sodium ions through the channel (Sauer *et al.*, 2013). The NaK channel shares a high amino-acid sequence homology and a similar structure (Shi *et al.*, 2006) with the bacterial KcsA potassium ion channel (Doyle *et al.*, 1998; Zhou *et al.*, 2001), but their selectivity filters adopt different conformations (Shi *et al.*, 2006). Both NaK and KcsA are tetrameric ion channels composed of four identical polypeptide chains which form a fourfold symmetrical structure, at the center of which an ion pore lined with the conserved residues makes up the selectivity filter. The mutation of two residues, Asp66Tyr and Asn68Asp, in the NaK amino-acid sequence causes main-chain conformational changes in the selectivity filter. This mutant, NaK2K, possesses

the selectivity-filter sequence TVGYG; it is almost identical (r.m.s.d. of 0.16 Å) to that found in the potassium ion channel KcsA (Sauer *et al.*, 2013) and is highly selective for potassium ions (Sauer *et al.*, 2013). The removal of the first 19 amino acids from the wild-type sequence which form the interfacial helix places the ion channel in an open conformation (Alam & Jiang, 2009).

It has been proposed that water and potassium ions co-translocate through the selectivity filter together (Zhou *et al.*, 2001; Zhou & MacKinnon, 2003) in an alternating K⁺, water, K⁺, water arrangement through the four binding sites in the selectivity filter (Fig. 1). However, others have proposed that potassium ions translocate through the selectivity filter using direct Coulomb knock-on and that no water molecules are present in the selectivity filter (Kopec *et al.*, 2018; Köpfer *et al.*, 2014). To date, the composition of the selectivity filter has been studied using anomalous X-ray scattering (Langan *et al.*, 2018; Zhou & MacKinnon, 2003) and QM/MM computational simulation (Köpfer *et al.*, 2014; Kopec *et al.*, 2018). In contrast to X-ray diffraction, neutrons have very different scattering properties between elements and even between isotopes of the same element. For instance, heavy water (D₂O) contains three atoms, all with a coherent scattering cross-section of around +5 barns (1 barn = 10⁻²⁴ cm²), giving a characteristic boomerang-shaped nuclear density (Afonine *et al.*, 2010). On the other hand, a lone potassium ion scatters neutrons significantly less well, with a coherent scattering cross-section of +1.7 barns. This difference in coherent scattering cross-section makes it feasible to distinguish waters from K⁺ ions within the selectivity filter even at modest resolution. Here, we report the collection of complete neutron and X-ray crystallo-

graphic data sets for the NaK2K mutant obtained from the same crystal.

2. Materials and methods

2.1. Protein purification

A plasmid containing the NaK mutant NaK2K from *B. cereus* m1550 in the pD441 vector was purchased from ATUM (Newark, California, USA) and transformed into *Escherichia coli* BL21 competent cells. This plasmid contained the two point mutations Asp66Tyr and Asn68Asp, and had the first 19 amino acids of the wild-type sequence which form the interfacial helix removed, placing the ion channel in an open conformation (Alam & Jiang, 2009). Cultures were inoculated with colonies from transformation plates into lysogeny broth medium at 37°C. After the culture reached an optical density of 0.6 at λ = 600 nm, the temperature was reduced to 25°C and isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM. After 18 h of induction, the cells were centrifuged and the pellets were collected and weighed. For every gram of cells, 5 ml lysis buffer (50 mM Tris pH 7.8, 100 mM KCl), protease-inhibitor tablets (Millipore Sigma), 1 mg ml⁻¹ lysozyme and Benzonase nuclease were added. Resuspension of cells was achieved by slow stirring at room temperature for 30 min. The cells were further lysed by sonication and cell debris was removed by centrifugation at 10 000g. NaK2K was then solubilized by incubation of the supernatant at room temperature for 2 h with 40 mM Sol-grade *n*-decyl-β-D-maltopyranoside (DM). The lysate was further clarified by centrifugation at 21 000g for 35 min. The

Figure 1
The protein structure of the NaK2K mutant; the four ion/water-binding sites within the selectivity filter are shown as orange spheres. (a) A top view of the protein in which each of the four subunits of the ion channel is colored separately. (b) A side view of the protein in which one of the four subunits has been removed for clarity. The PDB entry used to generate the figure was 6dz1 (Langan *et al.*, 2018).

protein was then purified using TALON (Clontech) metal-affinity resin with buffers containing 4 mM DM. Fractions containing NaK2K were pooled and the 6×His purification tag was removed by the addition of 1 U thrombin per milligram of NaK2K and incubation at room temperature for 16 h. After concentration using a 30 kDa cutoff ultrafiltration device, the protein was further purified on a Superdex 200 Increase 10/300 GL column using 20 mM Tris–HCl pH 7.8, 100 mM KCl, 4 mM Anagrade DM.

2.2. Protein crystallization

A solution of NaK2K was concentrated to 14 mg ml^{−1} using a 50 kDa cutoff concentrator. The crystal used for data collection was grown in a sitting drop prepared by mixing equal volumes of protein solution in buffer (20 mM Tris–HCl pH 7.8, 200 mM KCl, 4 mM Anagrade DM) with well solution (72.5% MPD, 100 mM KCl, 200 mM MES pH 6). A large crystal of NaK2K of around 0.3 mm³ in volume (Fig. 2) was removed from a sitting drop and mounted in a VitroCom fused-quartz capillary along with a slug of deuterated mother liquor.

3. Results and discussion

3.1. Neutron and X-ray room-temperature data collection

Neutron diffraction data were collected using the time-of-flight (TOF; Langan *et al.*, 2008) technique on the Macromolecular Neutron Diffractometer (MaNDi) instrument (Coates *et al.*, 2015, 2018) at the Spallation Neutron Source (SNS) to give wavelength-resolved Laue diffraction data. The crystal was held stationary at room temperature and diffraction data were collected for 21 h using neutrons of between 2 and 4 Å wavelength. Following this, the crystal was rotated by $\Delta\varphi = 10^\circ$ and the subsequent diffraction pattern was collected. A total of eight data frames were collected to form the complete neutron data set. Diffraction data were reduced

Figure 2

A large protein crystal of NaK2K which was roughly $0.8 \times 0.7 \times 0.5$ mm in size, giving a total crystal volume of around 0.3 mm³, was used for the neutron data collection on MaNDi. After the neutron data collection had been completed, a room-temperature X-ray data set was collected from the same crystal.

Table 1

NaK2K room-temperature neutron and X-ray data-collection statistics.

	X-ray	Neutron
Unit-cell parameters (Å, °)	$a = 67.86, b = 67.86, c = 92.03, \alpha = \beta = \gamma = 90$	
Space group	<i>I</i> 4	
Estimated twin fraction	0.3	
Wavelength(s) (Å)	1.00	2–4
No. of unique reflections	7400 (725)	2510 (235)
Resolution range (Å)	16.72–2.50 (2.59–2.50)	13.68–3.55 (3.68–3.55)
Multiplicity	4.93	3.56 (3.31)
$\langle I/\sigma(I) \rangle$	8.9 (2.1)	11.8 (3.60)
R_{merge}	0.114 (0.615)	0.176 (0.300)
$CC_{1/2}$	0.995 (0.845)	0.955 (0.497)
$R_{\text{p.i.m.}}$	0.057 (0.313)	0.105 (0.173)
Data completeness (%)	99.42 (100)	95.33 (93.63)

using the *Mantid* package (Arnold *et al.*, 2014) with integration performed by three-dimensional TOF profile fitting (Sullivan *et al.*, 2018). Wavelength-normalization of the Laue data was performed using *LAUENORM* from the *LAUEGEN* suite (Campbell *et al.*, 1998). The neutron data collection and processing statistics are given in Table 1. Using the same crystal, X-ray diffraction images were recorded using a Bruker D8 VENTURE with an Excillum MetalJet diffractometer and a PHOTON II detector at room temperature. The crystal was rotated by a $\Delta\omega$ of 0.25° for 520 frames, each with an exposure time of 2.5 s. The diffraction data frames were reduced and scaled using *PROTEUM3* (Bruker AXS, Madison, Wisconsin, USA). The X-ray data-collection and processing statistics are given in Table 1.

The *phenix.xtriage* program from the *PHENIX* suite (Adams *et al.*, 2010) was used to check for signs of merohedral twinning, which is a distinct possibility in space group *I*4 and is often seen in tetrameric membrane proteins. An initial twin fraction of 0.3 was estimated from the X-ray data set. This will be refined during joint neutron/X-ray refinement with *phenix.refine* (Afonine *et al.*, 2010) using data sets taken from the same crystal.

This work marks the first successful neutron crystallographic data collection from an integral membrane protein. Owing to the high-symmetry space group and large area-detector coverage present on the MaNDi instrument, we were able to collect a neutron data set with high data completeness. The resolution of the diffraction data that we obtained using neutrons (3.55 Å) is limited both by the size of the crystal used and its diffraction quality, as shown by the resolution of the X-ray data (2.50 Å). It is envisioned that improvements in the deuterium labeling of membrane proteins and crystal growth will allow us to collect future neutron data sets to a higher resolution.

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