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Expression, purification, crystallization and preliminary crystallographic study of the carboxyl-terminal domain of the human voltage-gated proton channel Hv1

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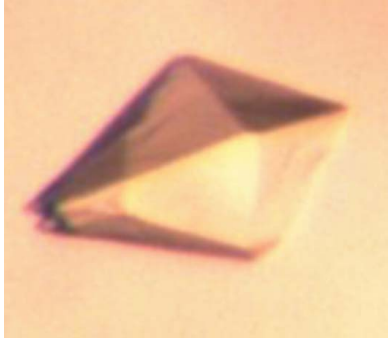

The voltage-gated proton channel Hv1 is essential to proton permeation and contains a voltage-sensor domain without a pore domain. It contains three predicted domains: an N-terminal acid and proline-rich domain, a transmembrane voltage-sensor domain and a C-terminal domain that is responsible for the dimeric architecture of Hv1. Here, the C-terminal domain of the human voltage-gated proton channel Hv1 (C-Hv1) was overexpressed in *Escherichia coli*, purified and crystallized using the hanging-drop vapour-diffusion method. The crystals have a tetragonal form and diffraction data were collected to 2.5 Å resolution in-house. The crystal belongs to space group $P4_12_12$, with unit-cell parameters $a = b = 37.76$, $c = 137.52$ Å. Structural determination of C-Hv1 is in progress.

1. Introduction

Voltage-gated proton channels have been observed in a variety of organisms since first being documented in snail neurons more than 25 years ago (Thomas & Meech, 1982), but it is only relatively recently that they have been identified at a molecular level (Ramsey *et al.*, 2006; Sasaki *et al.*, 2006). Voltage-gated proton channels (Hv channels) have been also reported in the plasma membranes of many blood cells that undergo phagocytosis, including macrophages, neutrophils and eosinophils (Okamura, 2007). The Hv channels in mammalian phagocytes were originally proposed to be responsible for the proton-transport pathway that regulates intracellular pH during oxygen consumption associated with phagocytosis, which is known as 'respiratory burst' (Henderson *et al.*, 1987). Hv channels are extremely selective for H^+, with no detectable permeability to other cations (DeCoursey, 1991; DeCoursey & Cherny, 1994; Cherny *et al.*, 1995) and the voltage–activation relationship depends strongly on both the intracellular pH (pH_i) and the extracellular pH (pH_o). Increasing pH_o or lowering pH_i promotes proton-channel opening by shifting the activation threshold to a more negative potential (Eder & DeCoursey, 2001; DeCoursey, 2003). Furthermore, Hv currents are known to be inhibited by submillimolar concentrations of Zn^{2+}, Cd^{2+} and other divalent cations (Byerly & Suen, 1989; DeCoursey, 2003; Cherny & DeCoursey, 1999).

The human and mouse proton channels (Hv1 and mVSOP, respectively) were identified using bioinformatics searches based on known cation channels for Hv1 and the voltage-sensor domain of *Ciona intestinalis* VSP for mVSOP (Ramsey *et al.*, 2006; Sasaki *et al.*, 2006). Hv1 and mVSOP contain three predicted domains: an N-terminal acid and proline-rich domain, a transmembrane voltage-sensor domain and a C-terminal domain that is responsible for the dimeric architecture of Hv1, according to hydropathy analysis (Koch *et al.*, 2008; Lee *et al.*, 2008; Tombola *et al.*, 2008). The proteins show homology to the voltage-sensor domain (VSD), but lack both the pore domain and the phosphatase domain. The structure of the protein remains unknown. Here, we report the crystallization and preliminary X-ray diffraction analysis of the carboxyl-terminal domain of the human voltage-gated proton channel Hv1 (C-Hv1).

2. Materials and methods

2.1. Cloning and expression

The target gene of human C-Hv1 encoding residues 221–273 was PCR-amplified using IMAGE clone 6424182. The PCR product was cloned into pGEX6P-1 with *Bam*HI and *Eco*RI sites to create a fused protein with glutathione *S*-transferase (GST) and a PreScission protease cleavage site for removal of the GST moiety. The construct was transformed into *Escherichia coli* strain BL21(DE3). The transformant was cultured in LB medium containing 100 µg ml^{-1} ampicillin at 303 K to an absorbance of 1.0 at 600 nm and then induced with 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) at 298 K for 20 h. Cells were harvested by centrifugation at 5000*g* for 10 min, washed with wash buffer (50 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl) and then pelleted again for protein purification.

2.2. Purification

The harvested cells were resuspended in lysis buffer containing 50 m*M* Tris–HCl pH 8.0, 0.5 *M* NaCl, 1.0 m*M* EDTA, 5 m*M* *β*-mercaptoethanol, 200 mg l^{-1} lysozyme, 1.0 m*M* phenylmethylsulfonyl fluoride (PMSF), 3 µg ml^{-1} leupeptin, 3 µg ml^{-1} pepstatin A and disrupted by sonication. Cell debris was discarded after centrifugation at 30 000*g* for 60 min. The supernatant was loaded onto a Glutathione-Sepharose 4B affinity column equilibrated with cleavage buffer containing 50 m*M* Tris–HCl pH 8.0, 0.5 *M* NaCl, 1 m*M* EDTA and 5 m*M* *β*-mercaptoethanol. The resin was washed with cleavage buffer. The resin with bound fused protein was digested using preScission protease in cleavage buffer at 277 K for 16 h. The digested C-Hv1 protein was eluted and concentrated using Amicon Ultra-4 (10 kDa cutoff, Millipore). A HiTrap Desalting column (5 ml, GE Healthcare) was used to change the buffer to 20 m*M* MES pH 6.0. The protein solution was then loaded onto a High S Cartridge column (5 ml, Bio-Rad) pre-equilibrated with buffer *A* (20 m*M* MES pH 6.0). Target protein was eluted with an NaCl linear gradient (0.0–1.0 *M*). Most of the target protein was eluted at 1 *M* NaCl. The fractions containing C-Hv1 were collected by monitoring the absorbance at 214 nm and checked by 12.5% SDS–PAGE (Fig. 1). The concentrated sample was then applied onto a Superdex 75 10/300 Prep-Grade gel-filtration column (GE Healthcare) which was equilibrated with 20 m*M* MES pH 6.0, 150 m*M* NaCl. Purified C-Hv1 was concentrated to 10 mg ml^{-1} and stored in liquid nitrogen. The concentrations of protein solution were determined by the BCA method using BSA as a standard (Smith *et al.*, 1985).

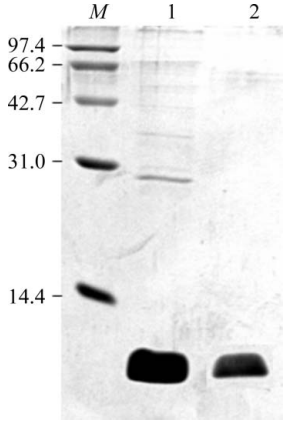

Figure 1
SDS–PAGE of purified C-Hv1 stained using Coomassie Brilliant Blue. Lane *M* shows the positions of the migrations of standard proteins and their molecular weights (in kDa); lane 1 contains C-Hv1 isolated from the GST moiety by preScission protease and lane 2 contains C-Hv1 finally purified using an ion-exchange column.

Table 1
Summary of data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418
Space group	$P4_12_12$
Unit-cell parameters (Å)	$a = b = 37.74$, $c = 137.33$
Resolution range (Å)	50–2.50 (2.59–2.50)
No. of unique reflections	3873 (355)
Completeness (%)	99.5 (97.0)
$R_{\rm merge}$†	0.074 (0.448)
$I/\sigma(I)$	31.3 (2.7)
Redundancy	11.2 (4.9)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th observation and $\langle I(hkl)\rangle$ is the mean intensity of the reflection.

2.3. Crystallization

C-Hv1 was crystallized using the hanging-drop vapour-diffusion method in 24-well Linbro tissue-culture plates at 289 K. The protein was initially screened with Index Screen, Crystal Screen and Crystal Screen 2 from Hampton Research. Crystals appeared in two conditions, Index No. 9 and No. 11, in which sodium chloride was used as the precipitant. Further conditions were explored by varying the buffer type, the pH and the NaCl concentration. Larger crystals were obtained by optimization using the hanging-drop method by mixing 1 µl protein solution with 1 µl reservoir solution and equilibrating against 1 ml reservoir solution. Crystals grew in one month at 289 K and the final crystallization solution contained 0.1 *M* sodium citrate pH 5.0, 3.4 *M* NaCl.

2.4. Data collection and processing

Prior to data collection, all crystals were transferred to a cryoprotectant solution (mother liquor supplemented with 20% glycerol) and flash-cooled in a nitrogen-gas stream. Preliminary diffraction data were collected at 100 K on a 300 mm R-AXIS IV++ image-plate detector using Cu $K\alpha$ radiation (1.5418 Å) generated from an FR-E SuperBright rotating-anode generator operated at 45 kV and 45 mA (Rigaku, Tokyo, Japan). The crystal-to-detector distance, the exposure time and the oscillation range were 180 mm, 3 min and 0.5°, respectively. 360 diffraction images were collected and integrated using *MOSFLM* (Collaborative Computational Project, Number 4, 1994). The data were then scaled and merged to give the final data set characterized in Table 1 using *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Purification of a GST-fused recombinant protein is not a complex procedure and we found that C-Hv1 was more soluble and stable in buffer containing 20 m*M* MES pH 6.0. During further purification with a High S Cartridge column, most of the target protein was eluted at 1 *M* NaCl in 20 m*M* MES pH 6.0. The final C-Hv1 protein purified using our protocol is very pure, as judged by SDS–PAGE with a 12.5%(*w*/*v*) acrylamide gel under reduced conditions (Fig. 1) (Laemmli, 1970).

The initial screening for the crystallization conditions of C-Hv1 was performed using three commercial crystallization kits (Hampton Research). Small crystals appeared and the crystallization conditions were optimized. We obtained colourless crystals from a crystallization solution containing 0.1 *M* sodium citrate pH 5.0, 3.4 *M* NaCl after 6 d

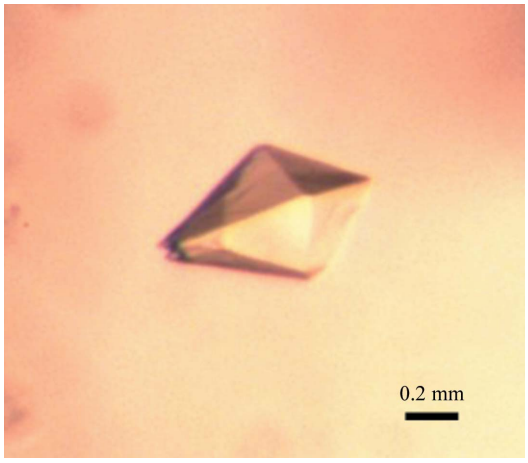

Figure 2
Photograph of a single C-Hv1 crystal obtained in 3.4 *M* NaCl, 0.1 *M* citrate buffer pH 5.0. The maximum dimension of the crystal is 0.8 mm.

at 289 K (Fig. 2). Similar crystals were also obtained in conditions consisting of 0.1 *M* sodium citrate pH 5.0, 3.2 *M* NaCl or 3.6 *M* NaCl a few days later.

X-ray diffraction data were collected from native C-Hv1 crystals and the data-processing statistics are summarized in Table 1. The crystal has a tetragonal lattice with space group $P4_12_12$ and unit-cell parameters $a = b = 37.74$, $c = 137.33$ Å. Based on the Matthews parameter (Matthews, 1968; Collaborative Computational Project, Number 4, 1994), we estimated that there are two molecules in one asymmetric unit (Matthews coefficient 1.83 Å^3 Da^{-1}; 32.7% solvent). We are currently solving the structure of the carboxyl-terminal domain of the human voltage-gated proton channel using the method of isomorphous replacement. The structural details will be available shortly.

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