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Crystallization and preliminary X-ray diffraction studies of the tetramerization domain derived from the human potassium channel Kv1.3

Andreas Winklmeier,[a] Michael Weyand,[b] Christina Schreier,[a] Hans Robert Kalbitzer[a] and Werner Kremer[a]*

[a]Department of Biophysics and Physical Biochemistry, University of Regensburg, Universitätsstrasse 21, D-93040 Regensburg, Germany, and [b]Max-Planck-Institut für Molekulare Physiologie, Otto-Hahn-Strasse 11, 44227 Dortmund, Germany

Correspondence e-mail: werner.kremer@biologie.uni-regensburg.de

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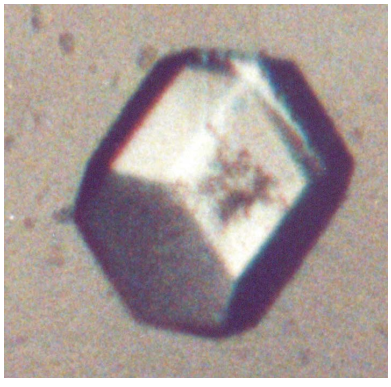

The tetramerization domain (T1 domain) derived from the voltage-dependent potassium channel Kv1.3 of *Homo sapiens* was recombinantly expressed in *Escherichia coli* and purified. The crystals were first grown in an NMR tube in 150 m*M* potassium phosphate pH 6.5 in the absence of additional precipitants. The crystals showed *I*4 symmetry characteristic of the naturally occurring tetrameric assembly of the single subunits. A complete native data set was collected to 1.2 Å resolution at 100 K using synchrotron radiation.

1. Introduction

Eukaryotic voltage-gated potassium channels (Kv family) participate in a variety of cellular functions ranging from the regulation of cardiac electrical patterns to signal transduction pathways in non-neural cells. A common feature of the Kv channel family is the highly controlled passage of potassium ions across the lipid bilayer of the cell, which is regulated by the membrane potential as well as by intermolecular interactions. The overall architecture of voltage-gated potassium channels consists of an N-terminal cytosolic domain containing the tetramerization domain (T1 domain), which is essential for the assembly as well as the regulation of the channel, followed by the transmembrane domain (TM domain) and a C-terminal cytosolic domain containing a PDZ motif at the end.

The human voltage-gated potassium channel Kv1.3 belongs to the Shaker subfamily. It is not ubiquitously distributed in the body, but is specifically expressed in lymphocytes, the central nervous system, kidney, liver, skeletal muscle, testes, sperm and osteoclasts (Xu *et al.*, 2004; Chen *et al.*, 1999; Grunnet *et al.*, 2003; Escobar *et al.*, 2004). Kv1.3 is mainly expressed in epithelial cells and more than 50% of all Kv1 subunits found in tissues can be assigned to subgroup 3 (Kv1.3; Grunnet *et al.*, 2003).

There are indications that the N-terminal inactivation is missing in Kv1.3 and inactivation occurs only *via* the slower C-type mechanism. It has recently been shown that Kv1.3 also regulates peripheral insulin sensitivity and thus might serve as a pharmacological target for the treatment of diabetes (Xu *et al.*, 2004). In addition, Kv1.3 plays a role in the activation of human T lymphocytes. These findings may help in the future to suppress the immune response in organ transplantations by inhibition of Kv1.3 (Shab *et al.*, 2003).

Currently, there are no structural data available for any domains of mammalian Kv1.3 potassium channels. However, a number of structural studies on a KcsA-Kv1.3 chimeric transmembrane domain were carried out by Baldus and coworkers using solid-state NMR spectroscopy (Lange *et al.*, 2006; Ader *et al.*, 2008 and citations therein).

Several three-dimensional structures of the T1 domain from channels other than Kv1.3 are known: the X-ray structures of the T1 domains from *Aplysia* Kv1.1 (Kreusch *et al.*, 1998; Cushman *et al.*, 2000), from rat Kv1.1 in complex with the β-subunit (Gulbis *et al.*, 2000), from mammalian brain and heart Kv1.2 (Minor *et al.*, 2000) and from Kv4.3 in complex with the Kv channel interacting protein

KChIP1 (Pioletti *et al.*, 2006). MacKinnon and coworkers were able to elucidate the structure of the transmembrane domain of the bacterial potassium channel KcsA first (MacKinnon *et al.*, 1998; Doyle *et al.*, 1998; Zhou *et al.*, 2001). A similar structure has been confirmed for three other bacterial potassium channels, MthK (Jiang *et al.*, 2002), KvAP (Jiang *et al.*, 2003) and KirBac1.1 (Kuo *et al.*, 2003). Recently they also elucidated the structure of the complete mammalian Kv1.2–β subunit complex (Long *et al.*, 2005a). This allowed them to characterize the structural basis of the electromechanical coupling of the voltage sensor in Kv1.2 (Long *et al.*, 2005b) and even to report the atomic details of the lipid membrane-like environment of the voltage-sensor paddle (Long *et al.*, 2007).

The T1 domain forms a symmetric tetramer located coaxial to the pore and close to the cytoplasmic channel exit (Kreusch *et al.*, 1998; Cushman *et al.*, 2000; Bixby *et al.*, 1999; Gulbis *et al.*, 2000; Minor *et al.*, 2000; Long *et al.*, 2005*a*). The structure of the T1 domain is already formed within the ribosomal tunnel (Kosolapov *et al.*, 2004). In three of the four families of voltage-gated potassium channels (all except Shaker) the polar interaction is coordinated by a Zn atom (Bixby *et al.*, 1999). Deletion of the T1 domain leads to malfunction of the channel (Zerangue *et al.*, 2000).

The T1 tetramer forms a central pore with a diameter of about 4 Å and a length of 20 Å which is too narrow for the passage of hydrated potassium ions (Kreusch *et al.*, 1998). Hence, it is still an open question how the potassium ion reaches the membrane pore. Current hypotheses are either that the T1 domain might switch into an open conformation in which the potassium ion can pass through or that the potassium ion enters through the side *via* an opening between the T1 domain and transmembrane domain to the pore (Kreusch *et al.*, 1998; Choe *et al.*, 1999; Gulbis *et al.*, 2000; Long *et al.*, 2005*a*). Experiments with single amino-acid mutations in the area of the T1–T1 interaction surfaces showed effects on the opening of the channel, although the structure of the T1 domain was virtually unchanged. The T46V mutant led to the loss of one single hydrogen bond and slowed down the activation rate. This mutation stabilizes the closed state of the channel (Minor *et al.*, 2000). Minimal changes in the interaction surface of the individual subunits showed a clear effect on the channel properties (Yi *et al.*, 2001). Covalent modifications or the binding of the kV-β subunit to the T1 domain can also cause such conformational changes (Minor *et al.*, 2000; Cushman *et al.*, 2000). The T1 domain is likely to modulate the channel behaviour and is involved in the gating mechanism (Minor *et al.*, 2000; Cushman *et al.*, 2000).

Since the structure of the tetramerization domain of Kv1.3 channels is still unknown, it seems worthwhile to try to solve this structure by either X-ray crystallography or NMR spectroscopy. Here, we report the successful crystallization of the T1 domain of human Kv1.3 corresponding to the 118 amino acids MERVVINISGLRFETQL-KTLCQFPETLLGDPKRRMRYFDPLRNEYFFDRNRPSFDAIL-YYYQSGGRIRRPVNVPIDIFSEEIRFYQLGEEAMEKFREDE-GFLREEKLAAALEHHHHHH with a molecular mass of 14 320.24 Da. The crystals diffracted to a resolution of 1.2 Å.

2. Materials and methods

2.1. Subcloning and expression of the T1 domain derived from Kv1.3

The cDNA (gene KCNA3, GeneID 3738) of Kv1.3 from *Homo sapiens* was obtained from the German Resource Center for Genome Research (RZPD). Using the primer sequences 5′-GGAATTCCA/TATGGAGCGCGTGGTCATCAAC-3′ and 5′-CCCCCA/AGCTT-CTCCTCCCGCAGGAAGC-3′, the cDNA corresponding to amino acids 51–155 of the T1 domain of Kv1.3 [T1(51–155)] was amplified by PCR. Ligation was performed into the plasmid pET20b purchased from Novagen (T7-RNA polymerase heterologous expression control). All cloning steps and amplification of the vector were carried out in *Escherichia coli* DH5α. The heterologous protein expression was tested and carried out in the commercially available expression strain Rosetta(DE3)pLysS (Novagen) carrying the pLysSRARE plasmid supplying tRNA sequences that are often used in mammalian sequences but rarely in *E. coli* sequences. Protein expression was induced by adding 1 m*M* IPTG. This allowed us to obtain up to 300 mg purified recombinant protein from 1 l cell culture. The expression media were either LB (Bertani, 1951), TB (Tartof & Hobbs, 1987) or NMM medium (Budisa *et al.*, 1995).

2.2. Purification and refolding of the T1 domain derived from Kv1.3

The expression of the protein was monitored by SDS–PAGE. The identity of the protein was confirmed by Western blotting and subsequent N-terminal sequencing and mass spectrometry. Since our construct contained a C-terminal His_6 tag, we were able to perform a single purification step from the crude lysate under denaturing

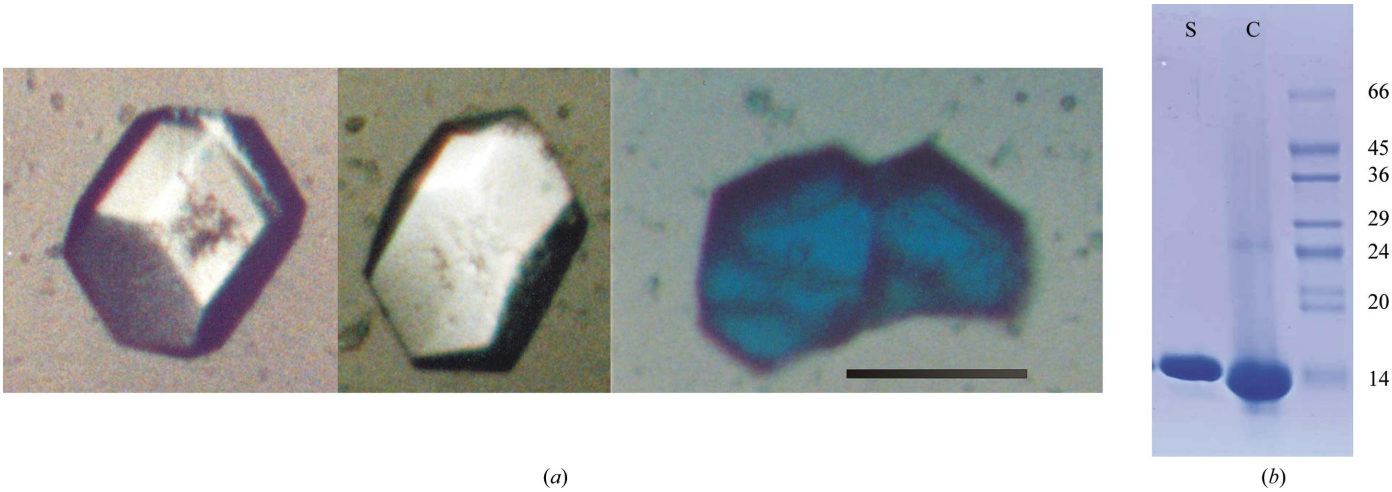

Figure 1
Crystals of T1(51–155). Left and middle, native crystals; right, crystals stained with Izit™ crystal dye. The bar corresponds to 0.2 mm. (*b*) Gel electrophoresis of the supernatant (S) and a crystal redissolved in the electrophoresis buffer (C). Markers (labelled on the right) are in kDa.

conditions according to the 'The QIAexpressionist' handbook. The cells were harvested and resuspended in modified lysis buffer *A* (100 m*M* NaH_2PO_4, 10 m*M* Tris–HCl, 6 *M* GdmHCl, 10 m*M* β-mercaptoethanol and 5 m*M* imidazole pH 8.0) with a ratio of 10 ml buffer per 1 g of wet bacterial pellet. This solution was stirred for 3 h at room temperature. A subsequent centrifugation step (30 000*g*, 30 min at room temperature) of the lysate was used to pellet the insoluble particles. The supernatant was collected and used for further purification. An Ni–NTA column (Qiagen) was equilibrated with three column volumes of modified buffer *A*. The lysate was loaded onto the resin at a flow rate of 2 ml min^{-1}. The column was washed with ten column volumes of modified buffer *C* (100 m*M* NaH_2PO_4, 10 m*M* Tris–HCl, 8 *M* urea and 10 m*M* β-mercaptoethanol pH 6.3) and three column volumes of modified buffer *D* (100 m*M* NaH_2PO_4, 10 m*M* Tris–HCl, 8 *M* urea and 10 m*M* β-mercaptoethanol pH 5.9). Elution of the protein was finally achieved with three column volumes of modified buffer *E* (100 m*M* NaH_2PO_4, 10 m*M* Tris–HCl, 8 *M* urea and 10 m*M* β-mercaptoethanol pH 4.5), collecting fractions of 5–10 ml. Potassium phosphate refolding buffer (150 m*M*) was generated by adjusting the pH to 6.5 through mixing of 1 *M* stock solution containing either KH_2PO_4 or K_2HPO_4. Refolding of the protein was performed by diluting the protein in 150 m*M* potassium phosphate refolding buffer additionally containing 6 *M* GdmHCl to a final protein concentration of 0.5 mg ml^{-1}, 3 h dialysis at 277 K against 150 m*M* potassium phosphate refolding buffer containing 3 *M* GdmHCl and subsequent dilution in continuous drop dilution dialysis with 150 m*M* potassium phosphate refolding buffer down to 0.3 *M* GdmHCl. Finally, dialysis was performed for 12 h at 277 K against refolding buffer (150 m*M* potassium phosphate pH 6.5). Protein aggregates were removed by centrifugation at 35 000g (30 min, 277 K). The supernatant was concentrated at 5000*g* at 277 K with a molecular-mass cutoff of 8000–10 000 Da. A second purification step consisted of gel-filtration chromatography with a Superdex 200 column (GE Healthcare). The correct molecular mass of the protein was confirmed by mass spectrometry and the identity of the first residues was confirmed by N-terminal sequencing.

2.3. Crystallization

Initially, we wanted to use the protein in 150 m*M* potassium phosphate buffer pH 6.5 in 5 mm NMR tubes for characterization using NMR spectroscopy. During testing, we observed the formation of crystals in the NMR tubes. An initial test of the crystals showed an excellent diffraction pattern, so we decided to continue with crystallization optimization in NMR tubes, resulting in very good crystals.

2.4. Data collection

X-ray diffraction data were collected at 100 K on beamline X10SA (PXII) of the Swiss Light Source (SLS, Villigen, Switzerland) equipped with a MAR 225 CCD detector. The *XDS* package (Kabsch, 1993) was used to process, integrate and scale the collected data.

3. Results and discussion

The tetramerization domain T1(51–155) of Kv1.3 crystallized in an NMR tube in a buffer solution consisting of 150 m*M* potassium phosphate buffer pH 6.5 at 277 K. The crystallization was performed under nearly physiological buffer conditions and did not require the presence of any additional precipitants. Thus, the obtained crystals do not contain small molecules other than potassium ions and phosphate anions. Crystallization could be reproduced in a sitting drop under identical conditions (Hampton Research).

Table 1
Characterization of the crystals of T1(55–151).

Values in parentheses are for the highest resolution shell.

Crystallization conditions	150 m*M* potassium phosphate buffer pH 6.5, 277 K
Space group	*I*4
Unit-cell parameters (Å, °)	$a = b = 59.8$, $c = 62.3$, $\alpha = \beta = \gamma = 90$
X-ray source	SLS, X10SA (PX-II)
Wavelength (Å)	0.95007
Resolution (Å)	20–1.2 (1.3–1.2)
Unique reflections	33942 (7143)
Observations	124301 (21102)
Completeness (%)	99.2 (98.5)
$R_{\rm mrgd\text{-}F}$† (%)	3.9 (13.1)
$\langle I\rangle/\sigma(I)$	17.01 (8.59)

† $R_{\rm mrgd\text{-}F}$ is the quality of amplitudes *F* in the scaled data set according to Diederichs & Karplus (1997). $R_{\rm mrgd\text{-}F} = \sum(A_{I_{h,P}} - A_{I_{h,Q}})/0.5\sum A_{I_{h,P}} + A_{I_{h,Q}}$ with pseudo-amplitudes $A_I = I^{1/2}$ if $I \geq 0$ and $-I^{1/2}$ if $I < 0$.

Fig. 1 shows a selection of the crystals that were typically obtained. Their size, which is of the order of 0.3 mm, is sufficient for structural analysis. Treatment of the crystals with Izit gives the blue colour typical of protein crystals (Fig. 1*a*). Analysis of the mother liquor and the crystal itself by SDS–PAGE showed that both samples contained the same protein (Fig. 1*b*). The molecular mass of approximately 14 kDa corresponds closely to the molecular mass of 14 320 Da calculated from the sequence (with the inclusion of the His tag).

X-ray diffraction experiments were performed at the SLS synchrotron source. Analysis of the obtained data showed that the crystals belonged to space group *I*4, with unit-cell parameters $a = 59.8$, $c = 62.3$ Å (Table 1). Assuming the presence of one molecule in the asymmetric unit, the solvent content of the crystals is 58.2%, corresponding to a Matthews coefficient of 1.94 $Å^3$ Da^{-1}. The crystals diffracted very well to 1.2 Å resolution, promising a very well resolved X-ray structure of the tetramerization domain.

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