Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray crystallographic characterization of a cyclic nucleotide-binding homology domain from the mouse EAG potassium channel

Maria João Marques-Carvalho[a,b] and João Henrique Morais-Cabral[a]*

[a]IBMC – Instituto de Biologia Molecular e Celular, Rua do Campo Alegre 823, 4150-180 Porto, Portugal, and [b]ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Largo Professor Abel Salazar 2, 4099-003 Porto, Portugal

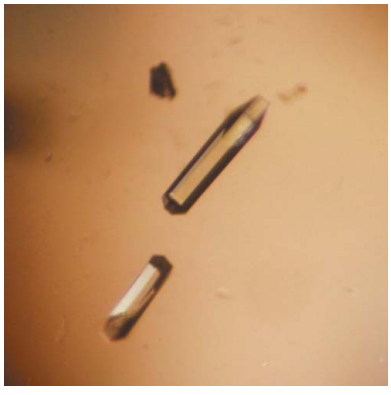
Correspondence e-mail: jcabral@ibmc.up.pt

Received 2 January 2012
Accepted 1 February 2012

The members of the family of voltage-gated KCNH potassium channels play important roles in cardiac and neuronal repolarization, tumour proliferation and hormone secretion. These channels have a C-terminal cytoplasmic domain which is homologous to cyclic nucleotide-binding domains (CNB-homology domains), but it has been demonstrated that channel function is not affected by cyclic nucleotides and that the domain does not bind nucleotides *in vitro*. Here, the crystallization and preliminary crystallographic analysis of a CNB-homology domain from a member of the KCNH family, the mouse EAG channel, is reported. X-ray diffraction data were collected to 2.2 Å resolution and the crystal belonged to the hexagonal space group $P3_121$.

1. Introduction

The eukaryotic voltage-gated KCNH potassium channel family includes the *ether-à-go-go* (EAG), EAG-related gene (ERG) and EAG-like (ELK) channels (Warmke & Ganetzky, 1994). These channels are involved in cardiac and neuronal repolarization (Sanguinetti & Tristani-Firouzi, 2006), hormone secretion and cell proliferation (Pardo & Sühmer, 2008). KCNH channels contain four subunits, each comprising six transmembrane (TM) helices and large N-terminal and C-terminal cytoplasmic regions (Warmke & Ganetzky, 1994). At the C-terminus, immediately after the last TM helix, there is a domain homologous to cyclic nucleotide-binding (CNB) domains. CNB domains are regulatory domains that participate in many signalling pathways in prokaryotes and eukaryotes. The ligand cAMP or cGMP binds to these domains and induces a conformational change that is propagated to an effector domain such as a kinase or an ion channel (Rehmann *et al.*, 2007). In cyclic nucleotide-regulated channels there are extra helices that connect the channel pore to the CNB domain and are responsible for transmitting the cAMP-induced conformational change to the pore; this region is known as the C-linker (Craven & Zagotta, 2006). Importantly, it has been well demonstrated that KCNH channel function is not affected by cyclic nucleotides and that the CNB-homology domain from KCNH channels does not bind nucleotides (Brelidze *et al.*, 2009). Here, we describe the protocols for purification and crystallization of the CNB-homology domain from one of the members of the KCNH family, the mouse EAG channel. This structure will help to understand the structural features that make these domains not bind cyclic nucleotides.



2. Materials and methods

2.1. Cloning of mouse EAG CNB-homology domain

Specific primers (forward, 5′-CCCGGATCCGCTGGTGCCGCGCGGCAGCACAGAGAAGGTCCTGC-3′; reverse, 5′-CCGCTCGAGCTACTAGAACACAATCCTCTTCCTCAG-3′) were designed to amplify the open reading frame encoding residues 552–707 of the mouse EAG potassium channel isoform 1 (mEAG1; NP_034730.1) by PCR with Pfu polymerase (Fermentas) according to the manufacturer's specifications. The PCR product was cloned into pET-15b (Novagen) between *Nde*I and *Xho*I restriction sites. The expression construct encodes an N-terminal His_6 tag and thrombin cleavage site

(corresponding to the amino-acid sequence MGSSHHHHHHSS-GLVPRGSHM) followed by residues 552–707 of the mEAG1 channel, as confirmed by DNA sequencing.

2.2. Expression and purification of recombinant mEAG1 CNB-homology domain

Escherichia coli BL21 (DE3) competent cells transformed with the expression vector were grown in Luria broth medium supplemented with ampicillin (100 mg l^{-1}) at 310 K with agitation until the optical density at 600 nm reached 0.6–0.8. At this point, the cultures were placed on ice for 30 min (to induce cold-shock chaperones), after which ethanol was added dropwise to a final concentration of 2%(*v*/*v*) (to induce heat-shock chaperones); IPTG was then added to a final concentration of 0.5 m*M* for overnight induction at 291 K (12–16 h). The cultures were harvested by centrifugation at 4785*g* for 20 min at 277 K and the resulting pellet was either stored at 253 K or immediately resuspended in buffer *A* (1 l pellet in 25 ml 20 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl) supplemented with protease inhibitors: 1 m*M* PMSF, 1 µg ml^{-1} leupeptin, 1 µg ml^{-1} pepstatin. Cell lysis was performed in a cooled cell disruptor (Emulsiflex-C5, Avestin) and the lysate was then centrifuged at 32 800*g* for 45 min at 277 K to remove cell debris. The supernatant was loaded onto Talon Metal Affinity Resin (Clontech; 2 ml slurry per 25 ml supernatant) pre-equilibrated with buffer *A* supplemented with protease inhibitors (as above) and washed with buffer *A* until the optical density at 280 nm (OD_{280}) stabilized. While monitoring the OD_{280} of the eluate, the beads were sequentially washed with buffer *A* containing 5 m*M* imidazole and buffer *A* containing 20 m*M* imidazole; His-tagged protein was eluted with buffer *A* containing 150 m*M* imidazole (∼7 ml elution volume). Thrombin (at 1.2 U per milligram of protein) was added to the eluted protein to cleave the His_6 tag and the protein was dialysed overnight at 277 K against buffer *A* containing 5 m*M* DTT (the volume ratio of protein solution to buffer was 1:∼150) using a dialysis membrane with a molecular-weight cutoff of 10 000 Da. The dialysed nontagged protein (residues 552–707 preceded by the amino-acid extension GSHM) was concentrated using a 10 000 Da cutoff spin concentrator. Further purification was achieved by size-exclusion chromatography at 277 K on a Superdex 200 column equilibrated with buffer *A* containing 5 m*M* DTT. Fractions containing the CNB-homology domain were concentrated to 12 mg ml^{-1} in the buffer used for size-exclusion chromatography using a 10 000 Da molecular-weight cutoff device.

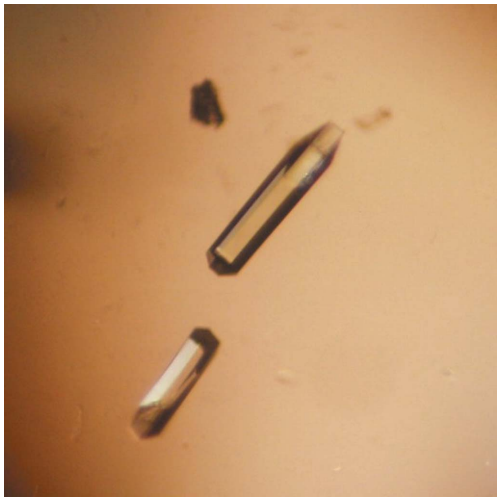


Figure 1
Crystals of the mEAG1 CNB-homology domain belonging to space group $P3_121$. The approximate dimensions of the crystals were 400 µm in length and 80 µm in width.

Table 1
Crystal and data-collection statistics for the mEAG1 CNB-homology domain.

Values in parentheses are for the outer resolution shell.

Crystal data	
Crystal system	Hexagonal
Space group	$P3_121$
Unit-cell parameters (Å, °)	$a = b = 60.3$, $c = 85.4$, $\alpha = \beta = 90$, $\gamma = 120$
Matthews coefficient ($Å^3$ Da^{-1})	2.49
Solvent content (%)	50
Data-collection details	
X-ray source	ESRF beamline ID14-4
Detector	ADSC Quantum Q315r
Wavelength (Å)	0.9765
Temperature (K)	100
Resolution range (Å)	52.3–2.2 (2.31–2.20)
No. of unique reflections	9566 (1356)
No. of measured reflections	59240 (7118)
Multiplicity	6.2 (5.2)
Completeness (%)	99.4 (97.5)
$\langle I/\sigma(I)\rangle$	4.8 (2.2)
R_{merge} (%)†	8.9 (25.1)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity of multiple observations of symmetry-related reflections.

2.3. Crystallization, data collection and processing

Initial crystallization conditions were screened at 277 and 293 K using the sitting-drop method with commercial sparse-matrix crystallization screens from Emerald BioSystems, Hampton Research and Qiagen. Axygen 96-well sitting-drop plates were manually filled with 100 µl of the commercial solution in the reservoir and a mixture of 1 µl precipitant solution plus 1 µl protein solution (at 12 mg ml^{-1}) in the drop. Crystals were obtained within 3 d in a condition consisting of 0.2 *M* trisodium citrate dihydrate pH 7.8, 20%(*w*/*v*) PEG 3350 at 277 K and did not require further optimization. Crystals were cooled and stored in liquid nitrogen after rapid transfer into a cryoprotectant solution consisting of 25%(*w*/*v*) PEG 3350, 0.2 *M* trisodium citrate, 10%(*v*/*v*) glycerol.

X-ray diffraction data were collected on beamline ID14-4 at the European Synchrotron Radiation Facility (ESRF; Grenoble, France) using an ADSC Quantum Q315r detector. Two data sets comprising 270 images of 1° oscillation each were collected at 100 K from a single crystal of mEAG1 552–707 with a crystal-to-detector distance of 290 mm. To record the high-angle diffraction spots (at 2.2 Å; high resolution) the first data set was collected with a beam transmission of 100%. For the low-angle diffraction spots (low resolution), which were overloaded on the previous data set, a second data set was collected with an attenuated beam (transmission of 10%). 90 images from each data set were integrated in space group $P3_121$ with *MOSFLM* (Leslie, 2006) and were scaled with *SCALA* (Evans, 2006) from the *CCP*4 program suite (Winn *et al.*, 2011). Homology models obtained from the *Phyre* protein structure-prediction server (Kelley & Sternberg, 2009) were used as the search model for molecular replacement with *Phaser* (McCoy *et al.*, 2007) from the *CCP*4 program suite and a solution was obtained.

3. Results and discussion

The mEAG1 CNB-homology domain was successfully purified, with a yield of 7 mg pure protein per litre of culture (molecular weight of

17 995 Da), and crystallized, resulting in crystals (Fig. 1) that diffracted to 2.2 Å resolution. Data-collection statistics are shown in Table 1. Molecular replacement was attempted with *Phaser* using the mHCN2 CNBD structure (PDB code 1q3e; Zagotta *et al.*, 2003) as a search model (23% identity to mEAG1 552–707), but failed. A molecular-replacement solution (log-likelihood gain = 28, *Z* score = 5.1) was only obtained when an ensemble of six superimposed models created by the *Phyre* server was provided to *Phaser*. The six search homology models were created from CNB-domain structures with PDB codes 1q3e, 1vp6 (21% identity; Clayton *et al.*, 2004), 2ptm (17% identity; Flynn *et al.*, 2007), 1cx4 (13% identity; Diller *et al.*, 2001), 1ne6 (18% identity; Wu *et al.*, 2004) and 2pqq (18% identity; Midwest Center for Structural Genomics, unpublished work). Electron-density maps calculated after partial refinement of the molecular-replacement solution in *PHENIX* (Adams *et al.*, 2010) showed features that were not present in the search models, confirming the validity of the solution. The structure is currently in the final stages of refinement.

We acknowledge the ESRF for providing access to X-ray beamlines and thank the ESRF staff for support. This work was supported by grants from EMBO (installation grant) awarded to JHMC.

References

Adams, P. D. *et al.* (2010). *Acta Cryst.* D**66**, 213–221.
Brelidze, T. I., Carlson, A. E. & Zagotta, W. N. (2009). *J. Biol. Chem.* **284**, 27989–27997.
Clayton, G. M., Silverman, W. R., Heginbotham, L. & Morais-Cabral, J. H. (2004). *Cell*, **119**, 615–627.
Craven, K. B. & Zagotta, W. N. (2006). *Annu. Rev. Physiol.* **68**, 375–401.
Diller, T. C., Madhusudan, Xuong, N.-H. & Taylor, S. S. (2001). *Structure*, **9**, 73–82.
Evans, P. (2006). *Acta Cryst.* D**62**, 72–82.
Flynn, G. E., Black, K. D., Islas, L. D., Sankaran, B. & Zagotta, W. N. (2007). *Structure*, **15**, 671–682.
Kelley, L. A. & Sternberg, M. J. (2009). *Nature Protoc.* **4**, 363–371.
Leslie, A. G. W. (2006). *Acta Cryst.* D**62**, 48–57.
McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
Pardo, L. A. & Sühmer, W. (2008). *Expert Opin. Ther. Targets*, **12**, 837–843.
Rehmann, H., Wittinghofer, A. & Bos, J. L. (2007). *Nature Rev. Mol. Cell Biol.* **8**, 63–73.
Sanguinetti, M. C. & Tristani-Firouzi, M. (2006). *Nature (London)*, **440**, 463–469.
Warmke, J. W. & Ganetzky, B. (1994). *Proc. Natl Acad. Sci. USA*, **91**, 3438–3442.
Winn, M. D. *et al.* (2011). *Acta Cryst.* D**67**, 235–242.
Wu, J., Jones, J. M., Nguyen-Huu, X., Ten Eyck, L. F. & Taylor, S. S. (2004). *Biochemistry*, **43**, 6620–6629.
Zagotta, W. N., Olivier, N. B., Black, K. D., Young, E. C., Olson, R. & Gouaux, E. (2003). *Nature (London)*, **425**, 200–205.