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Crystallization and preliminary X-ray crystallographic analysis of Ca^{2+}-free primary Ca^{2+}-sensor of Na^{+}/Ca^{2+} exchanger

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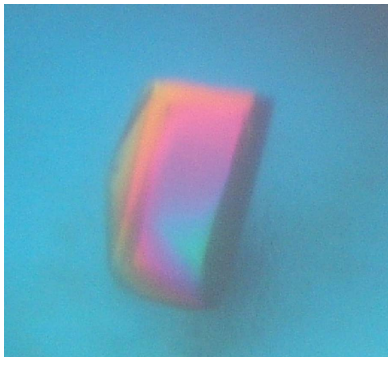

The plasma-membrane Na^{+}/Ca^{2+} exchanger (NCX) regulates intracellular Ca^{2+} levels in cardiac myocytes. Two Ca^{2+}-binding domains (CBD1 and CBD2) exist in the large cytosolic loop of NCX. The binding of Ca^{2+} to CBD1 results in conformational changes that stimulate exchange to exclude Ca^{2+} ions, whereas CBD2 maintains the structure, suggesting that CBD1 is the primary Ca^{2+}-sensor. In order to clarify the structural scaffold for the Ca^{2+}-induced conformational transition of CBD1 at the atomic level, X-ray structural analysis of its Ca^{2+}-free form was attempted; the structure of the Ca^{2+}-bound form is already available. Recombinant CBD1 (NCX1 372–508) with a molecular weight of 16 kDa was crystallized by the sitting-drop vapour-diffusion method at 293 K. The crystals belonged to the hexagonal space group $P6_222$ or $P6_422$, with unit-cell parameters $a = b = 56.99$, $c = 153.86$ Å, $\beta = 120^{\circ}$, and contained one molecule per asymmetric unit ($V_M = 2.25$ Å^3 Da^{-1}) with a solvent content of about 55% ($V_S = 45.57\%$). Diffraction data were collected within the resolution range 27.72–3.00 Å using an R-AXIS detector and gave a data set with an overall R_{merge} of 10.8% and a completeness of 92.8%.

1. Introduction

The Na^{+}/Ca^{2+} exchanger (NCX) is a plasma-membrane protein that extrudes one intracellular Ca^{2+} ion in exchange for three extracellular Na^{+} ions and regulates intracellular Ca^{2+} levels in many mammalian cell types (Blaustein & Lederer, 1999; Philipson & Nicoll, 2000; Shigekawa & Iwamoto, 2001). The activity of NCX is modulated by the binding of Ca^{2+} to a high-affinity regulatory site on an intracellular loop portion of the protein; the regulatory Ca^{2+} is not transported but potentially activates the exchange activity. Two Ca^{2+}-binding domains (CBD1 and CBD2) in the intracellular loop have been identified as being responsible for regulation of activity (Philipson *et al.*, 2002; Hilge *et al.*, 2006). NMR studies (Hilge *et al.*, 2006) have shown that the Ca^{2+}-free (apo) structure of CBD1 differs significantly from that of the Ca^{2+}-bound state, while CBD2 maintains its structural integrity in the Ca^{2+}-free state. A fluorescence resonance energy-transfer study indicated that binding elicits a conformational change in CBD1 (Ottolia *et al.*, 2004). These observations indicate that CBD1 is the primary Ca^{2+}-sensor.

The crystal structure of Ca^{2+}-bound CBD1 (Nicoll *et al.*, 2006) revealed a novel Ca^{2+}-binding site consisting of four Ca^{2+} ions arranged in a tight planar cluster, which may represent a general platform for Ca^{2+}-sensing. The structure of Ca^{2+}-free CBD1 is not available at present. Therefore, the X-ray structural analysis of the Ca^{2+}-free CBD1 of NCX1, one of three mammalian isoforms (Nicoll *et al.*, 1990), was attempted in order to clarify the structural scaffold for the conformational transition of CBD1 induced by Ca^{2+} binding and the structural difference from CBD2 at the atomic level.

2. Materials and methods

2.1. Expression and purification

The construct NCX1CBD1 corresponds to Val371–Glu508 of NCX1 plus an eight-residue N-terminal His tag that facilitated

purification using Ni-chelate chromatography. The expression plasmid pHisNCX1CBD1 coding for NCX1CBD1 was constructed as follows. Two oligonucleotide primers were synthesized with *Nhe*I and *Bam*HI restriction sites to facilitate cloning in frame into the His-tag fusion-protein expression vector pET-19a. PCR was performed with the canine NCX1 plasmid as a template for 30 cycles (367 K for 15 s, 330 K for 30 s and 341 K for 30 s). The amplified DNA was digested by *Nhe*I and *Bam*HI and the resulting fragment (411 bp) was inserted into the corresponding sites of pET19a. *Escherichia coli* BL21 (DE3) cells harbouring pHisNCX1CBD1 were induced with 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) at the mid-exponential growth phase (OD_{600} = 0.5) and incubated for a further 3 h at 310 K. Cells were harvested by centrifugation, washed and resuspended in 50 m*M* Tris–HCl pH 7.5 containing 150 m*M* NaCl. The cells were disrupted by sonication and the lysate was centrifuged at 15 000 rev min^{-1} for 60 min. The supernatant fraction was loaded onto a Chelating Sepharose Fast Flow column equilibrated with 50 m*M* Tris–HCl pH 7.5 containing 150 m*M* NaCl and 10 m*M* imidazole. After washing the column with 50 m*M* Tris–HCl pH 7.5 containing 150 m*M* NaCl and 60 m*M* imidazole, His-tag fusion protein (HisNCX1CBD1) was eluted with 50 m*M* Tris–HCl pH 7.5 containing 150 m*M* NaCl and 300 m*M* imidazole. The eluted fraction containing HisNCX1CBD1 was dialysed overnight into 20 m*M* HEPES–NaOH pH 7.5 and was applied onto a MonoQ 5/50 GL column (GE Healthcare). HisNCX1CBD1 was eluted with a linear gradient of NaCl from 0 to 0.5 *M* in 20 m*M* HEPES–NaOH pH 7.5 and dialysed overnight into 5 m*M* Tris–HCl pH 7.5. The sample was concentrated to approximately 13 mg ml^{-1} using Centricon centrifugal concentrators (Millipore) and EDTA was added to a final concentration of 10 m*M*. The purity of the final preparation was estimated to be greater than 95% by SDS–PAGE. The size distribution of the protein molecule in solution was shown to be monodisperse by dynamic light-scattering measurements.

2.2. Crystallization

Initial crystallization screening took place using the sitting-drop vapour-diffusion method at 293 K and crystallization kits from Hampton Research. Crystallization drops consisting of 1 µl protein solution (13 mg ml^{-1} in 5 m*M* Tris–HCl, 10 m*M* EDTA pH 7.5) and 1 µl reservoir solution were equilibrated against 100 µl reservoir solution. Small hexagonal rod-shaped crystals were obtained using a reservoir condition of 30%(*w*/*v*) PEG 4000, 0.2 *M* ammonium sulfate and 0.1 *M* citrate buffer pH 5.0. To improve the quality of the crystals, we applied the micro-stirring technique. We shook the sitting-drop vapour-diffusion crystallization plates with a rotating shaker using a speed of 50 rev min^{-1}. Details of this technique have been described elsewhere (Adachi, Matsumura *et al.*, 2004; Adachi, Takano *et al.*, 2004; Adachi *et al.*, 2005). After screening the crystallization conditions and using the micro-stirring technique, diffraction-quality crystals were obtained using 22%(*w*/*v*) PEG 4000, 0.2 *M* ammonium sulfate and 0.1 *M* citrate buffer pH 5.0. Crystals grew within a few weeks to dimensions of approximately 0.2 × 0.1 × 0.1 mm (Fig. 1).

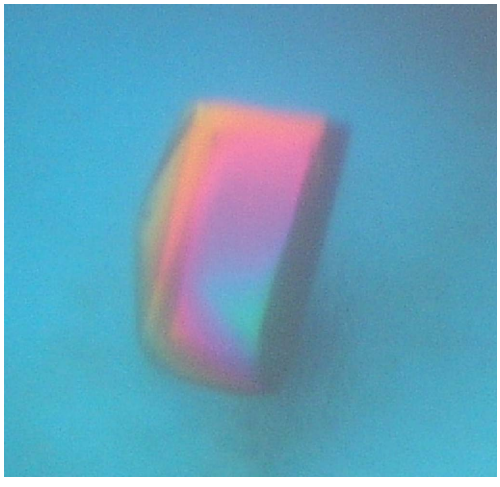

Figure 1
Native crystal of NCX1CBD1. The crystal has approximate dimensions of 0.2 × 0.1 × 0.1 mm.

2.3. Data collection and processing

Native data were collected at 100 K using Cu $K\alpha$ radiation from an FR-E rotating-anode X-ray generator (Rigaku Corp.) equipped with CMF optics (Osmic Inc.) and an R-AXIS VII detector (the crystal-to-detector distance was 135 mm). 90° of data were collected as 180 diffraction images using a 0.5° oscillation angle and an exposure time of 60 s (Fig. 2). The data were processed using the program *CrystalClear* (Rigaku MSC; Pflugrath, 1999).

3. Results and discussion

Recombinant CBD1 was prepared in *E. coli* using biotechnology. Our initial crystallization trials with Ca^{2+}-free CBD1, which were performed in a manner similar to that used to obtain the Ca^{2+}-bound form, failed. However, the results of dynamic light-scattering experiments suggested a high likelihood of crystallization as the size distribution of the CBD1 molecules in the sample solution was narrow and monomodal. Thus, rescreening with various screening kits was continued and the micro-stirring method (Adachi *et al.*, 2005) resulted in well shaped hexagonal plate-shaped crystals of Ca^{2+}-free CBD1 at 293 K (Fig. 2). The crystals belonged to the hexagonal space group $P6_222$ or $P6_422$, with unit-cell parameters $a = b = 56.99$, $c = 153.86$ Å, $\beta = 120°$. The V_M value, the crystal volume per unit of protein molecular weight, was calculated to be 2.25 Å^3 Da^{-1}, indi-

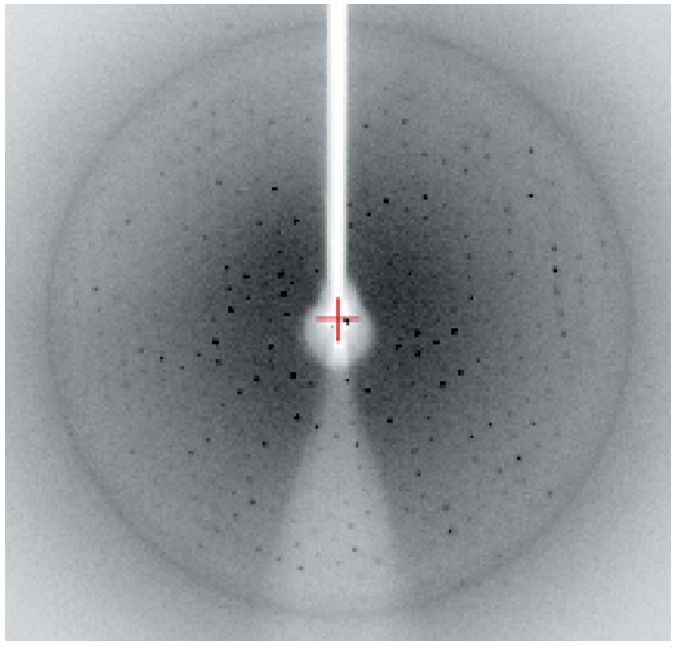

Figure 2
0.5° oscillation image of a native NCX1CBD1 crystal.

Table 1
Statistics for the native NCX1CBD1 diffraction data.

Values in parentheses are for the highest resolution shell.

Space group	$P6_222$ or $P6_422$
Unit-cell parameters	
$a = b$ (Å)	56.99
c (Å)	153.86
β (°)	120
Resolution range (Å)	26.72–3.00 (3.11–3.00)
No. of unique reflections	25923 (3102)
Average redundancy	8.36 (8.45)
Completeness (%)	92.8 (90.1)
$R_{\rm merge}$†	0.108 (0.351)
Average $I/\sigma(I)$	14.9 (5.7)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the observed intensity and mean intensity of related reflections, respectively.

cating the presence of one molecule in an asymmetric unit, and a solvent content of about 55% was indicated from the V_S value of 45.57% (Matthews, 1968); these V_M and V_S values are within the range usually observed for protein crystals.

A total of 25 923 reflections in the resolution range 26.72–3.00 Å were collected with 92.8% completeness and an $R_{\rm merge}$ of 10.8%. We are attempting phase determination by molecular-replacement methods using the coordinates of Ca^{2+}-bound CBD1 [PDB codes 2dpk (crystal structure; Nicoll *et al.*, 2006) and 2fws (NMR structure; Hilge *et al.*, 2006)] and Ca^{2+}-free/Ca^{2+}-bound CBD2 (crystal structure; Besserer *et al.*, 2007) as a search model. Structure determination of CBD1 using heavy-atom derivatives is also in progress. The X-ray structure analysis is promising for the elucidation of the structural scaffold for the Ca^{2+}-dependent structural transformation, which is an essential component of the Ca^{2+}-signalling pathway.

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