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# Crystallization and preliminary crystallographic analysis of human $Ca^{2+}$-loaded calbindin-D28k

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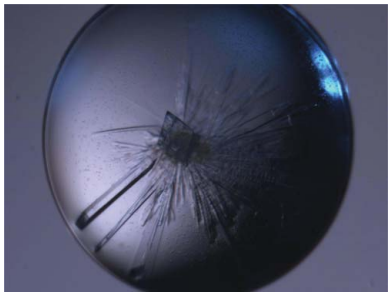
Calbindin-D28k is a calcium-binding protein that belongs to the troponin C superfamily. It is expressed in many tissues, including brain, intestine, kidney and pancreas, and performs roles as both a calcium buffer and a calcium sensor and carries out diverse physiological functions of importance. In order to resolve the crystal structure of human calbindin-D28k and to gain a better understanding of its biological functions, recombinant human calbindin-D28k was crystallized at 291 K using PEG 3350 as precipitant and a 2.4 Å resolution X-ray data set was collected from a single flash-cooled crystal (100 K). The crystal belonged to space group $C2$, with unit-cell parameters $a$ = 108.1, $b$ = 28.2, $c$ = 70.6 Å, $\beta$ = 107.8°. The presence of one molecule per asymmetric unit is presumed, corresponding to a Matthews coefficient of 1.75 $Å^3$ $Da^{-1}$.

## 1. Introduction

Human calbindin-D28k (261 amino-acid residues) is a ubiquitous calcium-binding protein that performs diverse roles in numerous tissues. It is now widely accepted that calbindin-D28k carries out physiological functions as both a calcium buffer and a calcium sensor.

As a calcium-binding protein, calbindin acts in $Ca^{2+}$-transporting epithelia as a dynamic $Ca^{2+}$ buffer (Lambers *et al.*, 2006). It can also control the rate of insulin release *via* the regulation of $Ca^{2+}$ in pancreatic islet cells (Sooy *et al.*, 1999). Simultaneously, calbindin-D28k provides protection against toxic high $Ca^{2+}$ levels by buffering the cytosolic $Ca^{2+}$ concentration; it can thus inhibit apoptosis induced by various pro-apoptotic stimuli, such as presenilin-1, which is linked to familial Alzheimer's disease. By inhibiting free-radical formation, calbindin-D28k can protect islet $\beta$ cells from autoimmune destruction in type 1 diabetes. It has been reported that the expression of calbindin-D28k in a pancreatic islet $\beta$-cell line protects against cytokine-induced apoptosis and necrosis (Christakos & Liu, 2004; Rabinovitch *et al.*, 2001). Calbindin-D28k seems to act as a neuroprotective agent; decreased calbindin expression leads to a failure of calcium buffering or intraneuronal calcium homeostasis, which contributes to calcium-mediated cytotoxic events during aging and to the pathogenesis of neurodegenerative diseases such as Parkinson's, Huntington's and Alzheimer's diseases (Iacopino & Christakos, 1990).

In addition to acting as a calcium-buffering protein, recent reports have revealed that calbindin undergoes a large conformational change upon $Ca^{2+}$ loading and interacts with other proteins, thus endowing calbindin with a calcium-sensing function. It has been shown that calbindin-D28k interacts with Ran-binding protein M (RanbpM; Lutz *et al.*, 2003), a protein that has been shown to play a role in microtubule function. High-resolution NMR methods showed that a RanbpM-derived peptide interacted with several regions of calbindin-D28k. Calbindin-D28k also interacts with *myo*-inositol monophosphatase (IMPase), a key enzyme in the regulation of the activity of the phosphatidylinositol signalling pathway. Both apo and $Ca^{2+}$-bound calbindin were found to activate IMPase by up to



250-fold, depending on the pH and the substrate concentration (Berggard *et al.*, 2002; Schmidt *et al.*, 2005). This activity is of interest given that IMPase is one of the assumed targets of $Li^+$ in the treatment of bipolar disorder. Additionally, calbindin-D28k protects against apoptosis in bone cells by interacting with and inhibiting caspase-3 activity, a property that is independent of its calcium-binding capability, providing a new anti-apoptotic mechanism other than calcium buffering (Bellido *et al.*, 2000).

Although calbindin carries out very important physiological functions, knowledge of its overall structure remained limited until the recent NMR solution structure of $Ca^{2+}$-loaded calbindin from *Rattus norvegicus* (PDB code 2g9b; Kojetin *et al.*, 2006; Linse *et al.*, 1997). Previous research has mainly focused on the general conformational effects of $H^+$ concentration (Berggard *et al.*, 2000), $Ca^{2+}$ binding (Berggard *et al.*, 2000; Venters *et al.*, 2003; Venyaminov *et al.*, 2004), deamidation (Vanbelle *et al.*, 2005), disulfide-bond formation (Cedervall *et al.*, 2005), *S*-nitrosation (Tao *et al.*, 2002; Tao & English, 2003) and the binding of peptides derived from the protein targets RanBPM and IMPase3 with the aid of NMR and CD (Kordys *et al.*, 2007).

Here, we have cloned the human CALB1 gene encoding the calbindin-D28k protein using the polymerase chain reaction (PCR) amplification method. In order to understand the structural details of calbindin-D28k and the structural basis of the mechanism of enhancement of activity of IMPase induced by calbindin-D28k, in this study we report the crystallization and preliminary crystallographic studies of human $Ca^{2+}$-loaded calbindin-D28k protein.

## 2. Materials and methods

### 2.1. Cloning, expression and purification

The primers 5′-CT **GGA TCC** ATG GCA GAA TCC CAC CTG-3′ and 5′-CCG **CTC GAG** CTA GTT ATC CCC AGC ACA-3′ were used to amplify the CaLB1 gene from *Homo sapiens* liver cDNA. The primers included *Bam*HI and *Xho*I restriction sites (shown in bold). The human CALB1 gene was amplified by PCR cloned into the pGEX-6p-1 vector (GE Healthcare). The sequence of the insert was verified by sequencing. The recombinant plasmid was transformed into *Escherichia coli* strain BL21 (DE3). Transformed cells were then cultured at 310 K in LB medium containing 50 µg ml$^{-1}$ ampicillin. When the culture density reached an $A_{600}$ of 0.6–0.7, induction was performed with 1 mmol l$^{-1}$ IPTG and cell growth was continued for 12–14 h at 289 K. Cells were harvested by centrifugation, resuspended in 1×PBS buffer (140 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* $Na_2HPO_4$, 1.8 m*M* $KH_2PO_4$ pH 7.3) and then homogenized by sonication. The lysate was centrifugated at 20 000*g* for 30 min to remove the cell debris. The supernatant was collected and the fusion protein was purified by GST-glutathione affinity chromatography. The GST tag of the fusion protein was cleaved with GST-rhinovirus 3C protease for more than 12 h, leaving an extra sequence GPLGS at the N-terminus of the native protein. The following day, the target calbindin-D28k protein was eluted with 1×PBS buffer. The protein was further purified on a Resource Q (GE Healthcare) ion-exchange chromatography column run in 20 m*M* Tris pH 8.0, 5 m*M* $CaCl_2$ and developed with a 0–400 m*M* NaCl gradient. The purity of the calbindin-D28k was estimated to be greater than 95% by SDS–PAGE. The SDS–PAGE, which suggests human calbindin-D28k to have a molecular weight of 28 kDa, and mass-spectrometry results, which show the molecular weight to be 30 kDa, are provided as supplementary material[1].

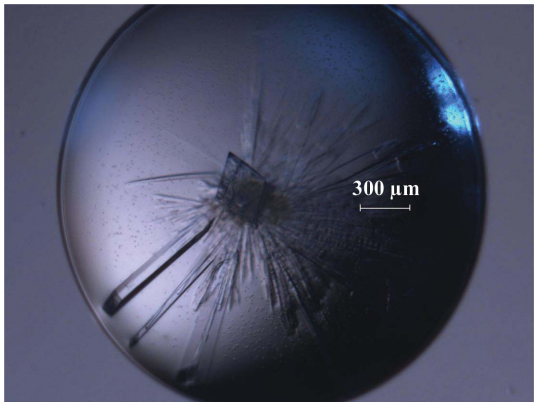



**Figure 1**
A single crystal of human calbindin-D28k.

**Table 1**
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

| | |
|---|---|
| Space group | *C*2 |
| Unit-cell parameters (Å, °) | $a$ = 108.1, $b$ = 28.2, $c$ = 70.6, $\beta$ = 107.8° |
| Resolution range (Å) | 50.0–2.4 (2.5–2.4) |
| Total reflections | 41257 (2682) |
| Unique reflections | 8058 (671) |
| Redundancy | 5.2 (4.0) |
| Average $I/\sigma(I)$ | 9.6 (4.1) |
| $R_{\rm merge}$† (%) | 6.5 (23.2) |
| Data completeness (%) | 97.2 (83.3) |

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

### 2.2. Crystallization

The purified calbindin-D28k protein was concentrated to ∼60 mg ml$^{-1}$ in 5 m*M* Tris pH 8.0, 1 m*M* $CaCl_2$. Crystallization was performed by the hanging-drop vapour-diffusion method at 291 K in 16-well plates. Each drop contained 1.5 µl protein solution and 1.5 µl reservoir solution with 500 µl reservoir solution in the well. Initial screening was carried out using Hampton Research Crystal Screen kits and positive hits were then optimized. The optimized reservoir solution consisted of 24% PEG 3350, 0.5 *M* ammonium acetate, 0.1 *M* bis-Tris pH 6.5. Crystals of good quality were obtained within 5 d (Fig. 1).

### 2.3. Data collection and processing

Diffraction data were collected on a MAR345 (MAR Research, Hamburg) image-plate detector at 100 K using a Rigaku MM007 rotating-anode home X-ray generator operated at 40 kV and 20 mA (λ = 1.5418 Å). The crystal was mounted on a nylon loop and flash-cooled in a cold nitrogen-gas stream at 100 K using an Oxford Cryosystems Cryostream without any cryoprotectant. A total of 360 frames of data were collected (Fig. 2). All intensity data were indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997).

[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: EN5282).

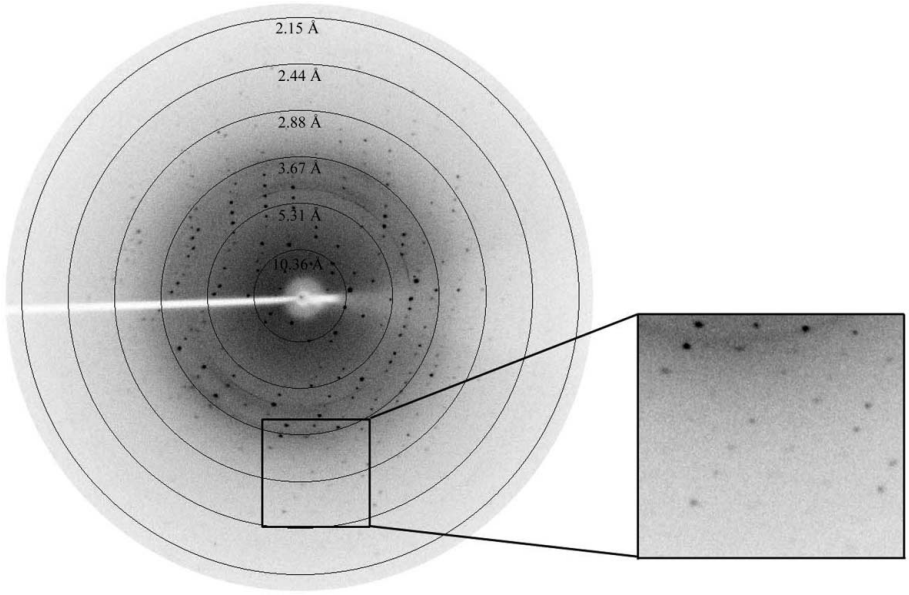



**Figure 2**
A typical diffraction pattern of a human calbindin-D28k crystal. The exposure time was 180 s, the crystal-to-detector distance was 100 mm and the oscillation range per frame was 1°. The diffraction image was collected on a MAR345 image-plate detector. An enlarged image is shown on the right.

## 3. Results and discussion

Initial crystals were obtained from condition No. 79 of the Index kit (Hampton Research) containing 25% PEG 3350, 0.2 *M* ammonium acetate, 0.1 *M* bis-Tris pH 6.5. The obtained crystals were twinned, which made them unsuitable for X-ray diffraction. Further crystallization optimization was performed by carefully adjusting the concentration of PEG 3350 and ammonium acetate together with hair seeding to eliminate twinning. Several single crystals were obtained from the optimized reservoir solution (24% PEG 3350, 0.5 *M* ammonium acetate, 0.1 *M* bis-Tris pH 6.5) and diffracted to 2.4 Å at the home X-ray generator. The crystal belonged to space group *C*2, with unit-cell parameters $a = 108.1$, $b = 28.2$, $c = 70.6$ Å, $\beta = 107.8^\circ$. We assume the presence of one molecule per asymmetric unit, which gives a Matthews coefficient of 1.75 Å$^3$ Da$^{-1}$ and 40% solvent content. Complete data-collection statistics are given in Table 1.

Initial molecular replacement was performed with the solution structure of $Ca^{2+}$-loaded calbindin D28k (Kojetin *et al.*, 2006; PDB code 2g9b; 98% identity) and the crystal structure of an EF-hand protein from *Danio rerio* Dr.36843 (G. E. Wesenberg, G. N. Phillips Jr, B. W. Han, E. Bitto, C. A. Bingman & E. Bae, unpublished work; PDB code 2be4; 40% identity) as the initial search models. This procedure was performed using *CNS* v.1.2 (Brünger *et al.*, 1998) and *Phaser* (McCoy *et al.*, 2007), but no obvious correct solution was found according to the rotation and translation functions. Although calbindin-D28k and the search models for MR have relatively high sequence identities, the relative positions of the domains could change under different conditions and cause the failure of MR. Heavy-atom and selenomethionine derivatives have been produced in order to obtain the correct phases. Structure determination is under way.

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