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Crystallization and preliminary X-ray diffraction studies of the calcium-binding protein CalD from *Streptomyces coelicolor*

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Calcium ions play an important regulatory role in eukaryotes. However, the regulatory roles of Ca^{2+} in prokaryotes are poorly understood. CalD, an 18 kDa calcium-binding protein from the model actinomycete *Streptomyces coelicolor* A3(2), was purified and crystallized for structure determination by X-ray crystallography. Crystals of CalD that were suitable for X-ray diffraction were obtained using the hanging-drop vapour-diffusion method and diffraction data were collected in-house to 1.56 Å resolution. The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 32.9, b = 51.0, c = 87.0 Å, $\alpha = \beta = \gamma = 90.0^\circ$. There is one protein molecule per asymmetric unit.

1. Introduction

The important and diverse regulatory roles of Ca^{2+} in eukaryotic cells are mediated by a large family of EF-hand calcium-binding proteins (CaBPs; Clapham, 1995; Strynadka & James, 1989). These helix–loop–helix EF-hand-containing proteins (Babu *et al.*, 1988) can be divided into two groups according to the function induced by Ca^{2+}: calcium sensors convey the Ca^{2+} signal through an ion-induced conformational change for the recognition of target molecules (Ikura *et al.*, 1992), while calcium buffers interact with the metal ions for transportation roles in eukaryotes that are less sensitive to Ca^{2+} loading (Ikura, 1996).

In contrast to eukaryotes, calcium-mediated regulatory roles are less evident in prokaryotes, despite accumulated evidence that Ca^{2+} is involved in a myriad of physiologically important bacterial activities, including cell division (Yu & Margolin, 1997), chemotaxis (Tisa & Adler, 1995; Watkins *et al.*, 1995) and cell differentiation (Norris *et al.*, 1991; Michiels *et al.*, 2002; Yonekawa *et al.*, 2001). Calerythrin, a well studied CaBP from *Saccharopolyspora erythraea*, was the first prokaryotic calcium-binding protein identified to contain four canonical calcium-binding EF-hand motifs (Leadlay *et al.*, 1984; Swan *et al.*, 1987) and was proposed to function as a Ca^{2+} buffer rather than as a Ca^{2+} sensor (Cox & Bairoch, 1988).

It has recently been reported that the CaBP from *Streptomyces coelicolor*, a model actinomycete responsible for producing most natural antibiotics used in medicine, is involved in the regulation of spore germination and aerial hypha formation (Wang *et al.*, 2008). Cell-differentiation processes have been established to be calcium-dependent and calcium-mediated in actinomycetes (Natsume *et al.*, 1989; Natsume & Marumo, 1992). The preliminary X-ray diffraction analysis of an 18 kDa calcium-binding protein from *S. coelicolor* A3(2) named CalD (SCO4411) offers a fundamental structural basis for understanding the possible regulatory roles of Ca^{2+} in prokaryotes. The low sequence similarity (less than 28%) between CalD and other prokaryotic EF-hand proteins (Bylsma *et al.*, 1992; Michiels *et al.*, 2002; Swan *et al.*, 1987; Tossavainen *et al.*, 2003; Yonekawa *et al.*, 2001) suggests that it has a potentially distinct and significant function in physiological processes. Here, we report the expression, purification and crystallization of CalD. Preliminary crystallographic analysis shows that CalD is a stable monomer and is suitable for X-ray crystallographic studies.

2. Materials and methods

2.1. Protein expression and purification

We obtained the original pET-44b-CalD plasmid used to express the CalD gene (SCO4411) from Shenglan Wang (Institute of Microbiology, Chinese Academy of Sciences) and subcloned the full-length CalD gene (residues 1–170) into the bacterial expression vector pGEX-6p-1 (GE Healthcare) with *Bam*HI and *Eco*RI restriction sites. The new recombinant plasmid pGEX-6p-1-CalD, which introduced a N-terminal GST tag for protein purification by glutathione *S*-transferase affinity chromatography, was transformed into *Escherichia coli* strain BL21 (DE3).

Cells were grown in Luria–Bertani (LB) medium with 100 µg ml^{-1} ampicillin at 310 K and were induced with 0.2 m*M* IPTG at an OD_{600} of ∼0.6 for functional expression. After growth for a further 4 h, the cells were harvested by centrifugation. After resuspension in 40 ml 1×PBS (phosphate-buffered saline; 10 m*M* sodium phosphate pH 7.4, 150 m*M* NaCl), cells were lysed by sonication on ice (medium power output). The debris was removed by centrifugation (30 min at 27 000*g*, 277 K) and the supernatant was loaded onto a glutathione column filled with Glutathione Sepharose 4B (GE Healthcare) equilibrated with 1×PBS. The GST-tagged protein was eluted with 300 m*M* NaCl in 1×PBS buffer and cleaved with 200 µg GST-rhinovirus 3C protease at 277 K overnight, which left five residues (GPLGS) at the N-terminus before the CalD protein. Fractions containing CalD lacking the GST tag were pooled and desalted by ultrafiltration for further purification using Resource Q anion-exchange chromatography columns (Amersham-Pharmacia Biotech) pre-equilibrated with 20 m*M* HEPES pH 7.5. The protein was washed with five column volumes of 20 m*M* HEPES pH 7.5 and eluted with a linear 0–1.0 *M* gradient of NaCl in 20 m*M* HEPES pH 7.5. The flowthrough containing CalD fractions with $A_{280}/A_{254} > 1.25$ was collected and concentrated. The target proteins were then further applied onto a Superdex 75 gel-filtration column (Pharmacia) pre-equilibrated and eluted with 20 m*M* HEPES pH 7.5, 150 m*M* NaCl. Fractions containing CalD (tested by SDS–PAGE) were collected and concentrated to 20 mg ml^{-1} by ultrafiltration (10 kDa molecular-weight cutoff). The purified CalD with high purity and homogeneity was analyzed by 15% SDS–PAGE (Fig. 1) and stored in 20 m*M* HEPES pH 7.5.

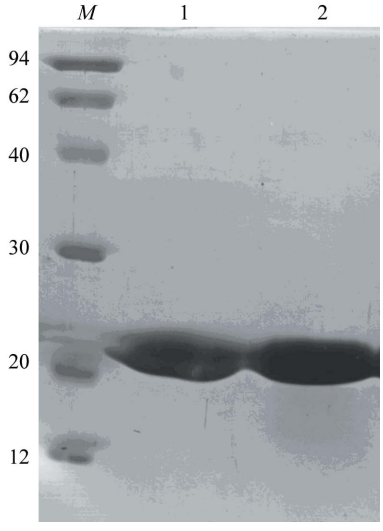

Figure 1
15% SDS–PAGE analysis of CalD purified using a Superdex 75 column. Lanes 1 and 2 contain protein at different concentrations. Lane *M* contains molecular-weight markers (kDa).

Table 1
Data-collection statistics.

Values in parentheses are for the outer resolution shell.

Wavelength (Å)	1.5418
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 32.9, b = 51.0, c = 87.0, α = 90.0, β = 90.0, γ = 90.0
Resolution range (Å)	50–1.56 (1.61–1.56)
Total reflections	91632
Unique reflections	21409 (1683)
Mean redundancy	4.3 (3.5)
Completeness (%)	98.9 (94.6)
$R_{\rm merge}$† (%)	5.4 (33.7)
Average $I/\sigma(I)$	11.9 (2.8)

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

2.2. Crystallization

Crystallization trials were carried out in custom-made 16-well plates at 291 K using the hanging-drop vapour-diffusion method. Initial crystallization screening was carried out with Crystal Screens I and II (Hampton Research). A 1 µl droplet consisting of 20 mg ml^{-1} protein in 20 m*M* HEPES pH 7.5 was mixed with an equal volume of reservoir solution and equilibrated against 200 µl reservoir solution in a sealed chamber. Initial needle-like crystals of CalD appeared in Crystal Screen I condition No. 45 (18% PEG 8000, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* zinc acetate) after 1 d and this condition was used for further optimization by variation of the precipitant concentration, buffer pH and protein concentration. Following optimization, thin tablet-like CalD crystals which diffracted well were obtained using the following conditions: 14%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* zinc acetate (Fig. 2).

2.3. Data collection and processing

A single CalD crystal was harvested using a nylon loop (Hampton Research), flash-cooled to 100 K in a nitrogen-gas stream and used for data collection. A cryo-solvent was not used in the flash-cooling of the CalD crystals, since the precipitant in the crystallization solution was able to serve as a cryo-solvent. Complete diffraction data were collected at 100 K with 1° oscillation per image on an R-AXIS IV^{++} image-plate detector using an in-house Rigaku MicroMax-007 rotating-anode X-ray generator (Fig. 3). The data were indexed and scaled using the programs *DENZO* and *SCALEPACK* from the *HKL*-2000 package (Table 1).

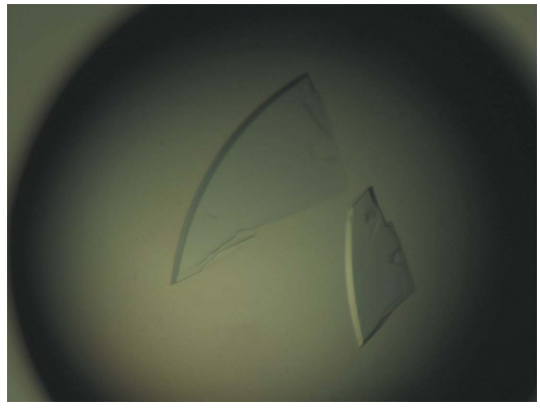

Figure 2
Crystal of CalD from *S. coelicolor* grown by the hanging-drop vapour-diffusion method in 14%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* zinc acetate.

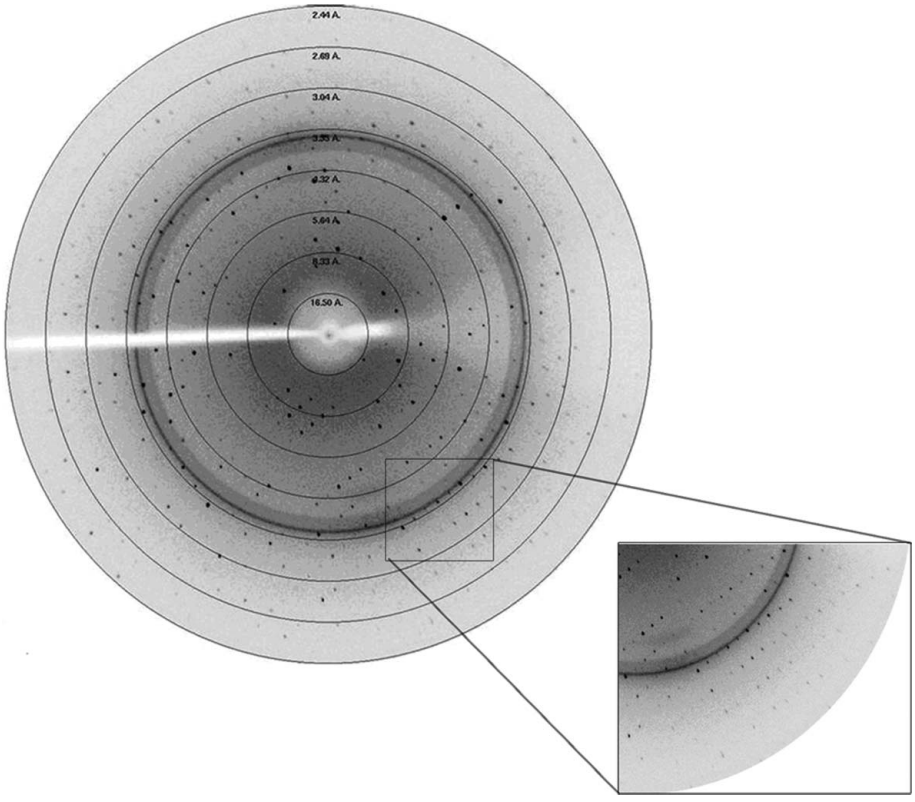

Figure 3
A typical X-ray diffraction pattern from a CalD crystal collected on an R-AXIS IV^{++} image-plate detector.

3. Results and discussion

The initial needle-like crystals exhibited high mosaicity and were not good enough for data collection. Following optimization of the initial conditions, plate-like CalD crystals which diffracted well and were suitable for data collection were obtained by reducing the concentration of the precipitant and lowering the protein concentration to 10 mg ml^{-1}. Diffraction data were collected in-house from the plate-like crystals to 1.56 Å resolution (Fig. 3, Table 1). The crystals belonged to space group $P2_12_12_1$, with unit-cell parameters a = 32.9, b = 51.0, c = 87.0 Å, $\alpha = \beta = \gamma = 90.0^\circ$ (Table 1). Initial analysis of the crystal solvent content using the Matthews coefficient (Matthews, 1968) suggested that the asymmetric unit contains one molecule with 39.4% solvent content (Matthews coefficient 2.03 Å^3 Da^{-1}). Attempts at MAD phasing based on selenomethionine substitution are in progress.

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