

## Crystallization and preliminary X-ray analysis of two new crystal forms of calmodulin

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### Abstract

Two new crystal forms of calmodulin from *Gallus gallus* are reported. Crystals in space group *P1* (cell dimensions  $a = 59.7$ ,  $b = 53.1$ ,  $c = 24.6$  Å,  $\alpha = 93.2$ ,  $\beta = 96.7$ ,  $\gamma = 89.2^\circ$  and  $Z = 2$ ), grow as long thin needles. Water content on density considerations is  $\sim 50\%$ . They diffract to  $\sim 2.0$  Å, but give wide multiply peaked spot profiles. Crystals in space group *P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>* (cell dimensions  $a = 32.2$ ,  $b = 56.0$ ,  $c = 67.3$  Å and  $Z = 4$ ), grow as clusters of thin tablets and contain  $\sim 30\%$  water by volume. These small crystals ( $\sim 0.4 \times 0.15 \times 0.1$  mm) diffracted well to  $\sim 1.4$  Å and some appreciable intensities were observed at resolutions better than 1.2 Å.

### 1. Introduction

Calmodulin (CAM) is a highly conserved eukaryotic protein that plays an important role in Ca-dependent signal transduction pathways (Schutt, 1985; Finn & Forsén, 1995). Its structure was first solved at 3.0 Å resolution (Babu *et al.*, 1985) using multiple isomorphous derivatives of rat testis CAM. Structures of various other CAM's have since been reported: bovine brain at 2.2 Å (Babu, Bugg & Cook, 1988), recombinant *Drosophila melanogaster* (Taylor, Sack, Maune, Beckingham & Quiocho, 1991) and recombinant *Paramecium tetraurelia* (Rao *et al.*, 1993). All these studies reported triclinic crystals by precipitation with 2-methyl 2,4-pentanediol (MPD), with resolution limits between 3.0 and 1.8 Å. Monoclinic crystals grown by precipitation with polyethylene glycol (PEG) have also been described (Kretsinger, Rudnik, Sneden & Schatz, 1980), but were not conducive to structure determination owing to a diffraction limit of  $\sim 5$  Å.

In *P1* crystals, CAM adopts a dumbbell shape with two lobes connected by a central 21-residue helix. NMR studies in solution (Barbato, Ikura, Kay, Pastor & Bax, 1992), indicate a flexible region in this helix allowing it fold around target peptides, as observed by Meador, Means & Quiocho (1992). A mutant CAM (des-Glu84) described by Raghunathan *et al.* (1993) provides evidence in support of this flexible tether. Since all previously reported native CAM's are essentially isostructural in *P1*, investigation of other crystal forms is important.

### 2. Experimental methods

#### 2.1. Extraction and purification

CAM was purified at 277 K from chicken gizzard according to the method of Marshak, Lukas & Waterson (1985), modified as

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follows. Chicken gizzards (Pel-Freez, Inc., Rogers, Arkansas) were minced in  $\sim 500$  g batches into 3–5 mm pieces after removal of all connective tissue. These batches were homogenized in a buffer (pH 8.0) containing 50 mM Tris-HCl, 2 mM EDTA, 1 mM 2-mercaptoethanol using 2 ml of buffer per gram of tissue. After centrifugation, the pellet was re-homogenized in buffer (1 ml per gram of original tissue weight) and centrifugation was repeated. The pooled supernatants were brought to 60% saturation by addition of  $(\text{NH}_4)_2\text{SO}_4(\text{s})$ , stirred and centrifuged. This supernatant was acidified to pH 4.05 with 50% sulfuric acid, stirred, centrifuged and the supernatant discarded. The pellet was resuspended in deionized water and Tris-base was added to pH 8.0. The solution was dialyzed against deionized water twice for 2–3 h each, followed by overnight dialysis against buffer *B* (10 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 1 mM EGTA, 1 mM 2-mercaptoethanol). It was then centrifuged, and the supernatant was loaded on a column (5 × 25 cm, DEAE Sephadex A-50 equilibrated in buffer *B*). This column was washed with buffer *B*, until the effluent absorbance at 280 nm returned to baseline. The protein was eluted with a linear gradient (21) of buffer *B* to a final salt concentration of 0.7 M. Fractions with an abundance of protein were pooled and dialyzed overnight against buffer *F* (10 mM Tris-HCl, pH 8.0, 2 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 1 mM 2-mercaptoethanol). It was then loaded on a column (2.5 × 6 cm, phenyl-Sepharose 4B, Pharmacia), equilibrated in buffer *F* and washed first with buffer *F* and then with buffer *F* containing 0.2 M NaCl. It was eluted by buffer *E* (10 mM Tris-HCl, pH 8.0, 2 mM MgCl<sub>2</sub>, 1 mM EGTA, 1 mM 2-mercaptoethanol). Further dialysis against 10 mM NH<sub>4</sub>HCO<sub>3</sub>(aq) and distilled water (overnight) was followed by lyophilization. Typical protein yield was 25–30 mg for 500 g of gizzard.

The CAM was characterized [mass spectrometry, high-pressure liquid chromatography, peptide maps following trypsin digestion, partial sequence analysis, activator activity using myosin light chain kinase (a gift from Thomas J. Lukas, Vanderbilt University), and polyacrylamide gel electrophoresis] and appeared homogeneous by all criteria.

#### 2.2. Crystallization of *P1* crystals

*P1* crystals of CAM were difficult to grow by reported methods. The procedure of Cook & Sack (1983) gave thin needles diffracting to  $\sim 2.5$  Å, but were unsuitable for data collection. The procedure was modified similar to that described by Barford, Gilliland & Morgan (1986). Crystals grew at room temperature within 2 d in 10 µl hanging drops [6 µl CAM (12.5 mg ml<sup>-1</sup> in 4 mM CaCl<sub>2</sub>), 4 µl reservoir (10 mM NaOAc, pH 4.0, 25% MPD, 15% iso-propanol)]. Small well formed seed crystals were washed in a different reservoir (10 mM NaOAc, pH 4.0, 30% MPD, 15% iso-propanol), and

Table 1. Data-collection statistics for *P1* crystals

Resolution (Å)	∞–1.80	∞–3.08	3.08–2.44	2.44–2.13	2.13–1.94	1.94–1.80
$R_{\text{merge}}^*$ (%)	3.64	3.01	6.17	8.41	12.39	17.90
Completeness (%)	71.5	87.7	75.8	68.7	63.8	59.9
$\langle I \rangle / \langle \sigma(I) \rangle$	10.9	26.4	9.90	5.61	3.03	1.78

$$*R_{\text{merge}} = \sum |I_i - \bar{I}| / \sum I_i$$

Table 2. Data-collection statistics for *P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>* crystals in the 'low'- and high-resolution shells

'Low'-resolution shell						
Resolution (Å)	∞–1.68	∞–2.87	2.87–2.28	2.28–1.99	1.99–1.81	1.81–1.68
$R_{\text{merge}}^*$ (%)	2.76	2.27	4.19	5.68	9.70	12.87
Completeness (%)	71.1	94.6	82.7	74.4	68.3	34.6
$\langle I \rangle / \langle \sigma(I) \rangle$	14.3	30.9	12.9	8.42	4.86	3.59
High-resolution shell						
Resolution (Å)	∞–1.17	∞–2.00	2.00–1.58	1.58–1.38	1.38–1.26	1.26–1.17
$R_{\text{merge}}^*$ (%)	2.91	1.74	9.51	14.2	20.9	25.8
Completeness (%)	47.3	26.9	46.6	64.2	58.9	39.3
$\langle I \rangle / \langle \sigma(I) \rangle$	6.77	38.9	4.65	2.50	1.47	1.18

$$*R_{\text{merge}} = \sum |I_i - \bar{I}| / \sum I_i$$

placed in drops of 12  $\mu\text{l}$  CAM and 8  $\mu\text{l}$  of reservoir. Some crystals grew large within a few days, but then stopped growing. These lancet-like crystals ( $\sim 2 \times 0.2 \times 0.1$  mm), were fragile and delaminated into flat shreds parallel to the long growth direction.

### 2.3. Crystallization of *P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>* crystals

A mini-screen (Jancarik & Kim, 1991) containing a subset of the Hampton Research Crystal Screen I reagents was set up to determine alternate crystallization conditions. Only condition 31 [30% PEG 4000, no buffer, 0.2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>(aq)] delivered crystals, but these were tiny and embedded in a cloudy precipitate. A 4  $\times$  6 screen of hanging drops was set up at room temperature as follows: 4  $\mu\text{l}$  of CAM (15 mg ml<sup>-1</sup> in 4 mM CaCl<sub>2</sub>) mixed with 2.7  $\mu\text{l}$  of reservoir containing 10 mM of NaOAc (pH 3.7–4.4) and 5–20% PEG 4000, pH in the wells was not adjusted. Within 2 d prismatic crystal clusters appeared at PEG concentrations of 15 and 20% in the pH range 3.85–4.14 (*i.e.* around the isoelectric point).

## 3. Results and discussion

### 3.1. Preliminary X-ray analysis of *P1* crystals

The MPD concentration in the mother liquor was sufficient to form a protective glass upon cooling. Crystals of varying quality were mounted in fine glass loops embedded in copper mounting pins, and quickly transferred to the (initially deflected) cold stream of a modified Siemens LT-2 low-temperature apparatus. The crystals had wide ( $\sim 2^\circ$  base-to-base) multiply peaked spot profiles. Eventually, a 1.0  $\times$  0.2  $\times$  0.1 mm fragment broken from a large aggregate gave single-peaked diffraction maxima that were  $\sim 1.5^\circ$  base-to-base and  $\sim 0.6^\circ$  full-width-at-half-maximum (FWHM) in  $\omega$ . The tendency to splinter is a likely cause of the wide multiply peaked diffraction spot profiles.

The crystals were triclinic ( $a = 59.7$ ,  $b = 53.1$ ,  $c = 24.6$  Å,  $\alpha = 93.2$ ,  $\beta = 96.7$ ,  $\gamma = 89.2^\circ$ ,  $Z = 2$ ), and appear to be related to other *P1* CAM crystals with  $Z = 1$ , but with the  $a$

axis doubled. On density considerations, solvent content in these 'cell doubled' *P1* crystals is estimated at about 50% by volume. A data set to 1.8 Å was collected at 130 K on a Xuong-Hamlin area detector, statistics are given in Table 1. Phasing experiments are underway.

### 3.2. Preliminary X-ray analysis of *P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>* crystals

These crystals could not be mounted in their mother liquor. After surgery to break the clusters, cooling was successful after a brief wash in unbuffered aqueous 50% PEG 4000. Visual inspection after some minutes suggested that little could be gained by longer soaks. The crystals were mounted in glass loops as described above. All gave sharp single-peaked diffraction spots with base-to-base and FWHM profiles of  $\sim 0.8$  and  $0.3^\circ$ , respectively. They diffracted well to around 1.4 Å, and in all cases, some appreciable intensities were observed above 1.2 Å. A crystal approximately 0.30  $\times$  0.15  $\times$  0.10 mm was indexed as orthorhombic, space group *P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub>* with  $a = 32.2$ ,  $b = 56.0$ ,  $c = 67.3$  Å,  $Z = 4$ . On density considerations, solvent content is about 30%, much lower than the triclinic form. This may be linked to the dramatically higher resolution, and hence to a more ordered structure. Data extending to 1.2 Å were collected at 130 K in separate low- and high-resolution shells, statistics are given in Table 2. Better counting statistics at high resolution should be possible when crystallization conditions have been further optimized. In the combined set [ $R_{\text{merge}} = 4.12\%$ ,  $\langle I \rangle / \langle \sigma(I) \rangle = 11.5$ ], completeness to 1.7 Å is 83.6%, while at the highest resolution it is  $\sim 40\%$ . Diffractometer modifications made since these experiments will allow recording of a full high-resolution data set. Phasing experiments are underway.

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