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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, $\alpha = 93.2$, $\beta = 96.7$, $\gamma = 89.2°$ and $Z = 2$), grow as long thin needles. Water content on density considerations is ~ 50%. They diffract to ~ 2.0 Å, but give wide multiply peaked spot profiles. Crystals in space group $P2_12_12_1$ (cell dimensions $a = 32.2$, $b = 56.0$, $c = 67.3$ Å and $Z = 4$), grow as clusters of thin tablets and contain ~ 30% water by volume. These small crystals (~ 0.4 × 0.15 × 0.1 mm) diffracted well to ~ 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 ~ 5 Å.

In $P1$ crystals, CAM adopts a dumbell shape with two lobes connectd 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 puried at 277 K from chicken gizzard according to the method of Marshak, Lukas & Waterson (1985), modified as follows. Chicken gizzards (Pel-Freez, Inc., Rogers, Arkansas) were minced in ~ 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 m*M* Tris–HCl, 2 m*M* EDTA, 1 m*M* 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 $(NH_4)_2SO_4$(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 m*M* Tris–HCl, pH 8.0, 0.2 *M* NaCl, 1 m*M* EGTA, 1 m*M* 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 (2 l) 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 m*M* Tris–HCl, pH 8.0, 2 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1 m*M* 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 m*M* Tris–HCl, pH 8.0, 2 m*M* $MgCl_2$, 1 m*M* EGTA, 1 m*M* 2-mercaptoethanol). Further dialysis against 10 m*M* NH_4HCO_3(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 ~ 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^{-1} in 4 m*M* $CaCl_2$), 4 µl reservoir (10 m*M* NaOAc, pH 4.0, 25% MPD, 15% iso-propanol)]. Small well formed seed crystals were washed in a different reservoir (10 m*M* NaOAc, pH 4.0, 30% MPD, 15% iso-propanol), and

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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_{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

* $R_{merge} = \sum |\langle I \rangle - I| / \sum \langle I \rangle$.

Table 2. *Data-collection statistics for $P2_12_12_1$ 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_{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_{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

* $R_{merge} = \sum |\langle I \rangle - I| / \sum \langle I \rangle$.

placed in drops of 12 µl CAM and 8 µl of reservoir. Some crystals grew large within a few days, but then stopped growing. These lancet-like crystals (~ 2 × 0.2 × 0.1 mm), were fragile and delaminated into flat shreds parallel to the long growth direction.

2.3. *Crystallization of $P2_12_12_1$ 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* $(NH_4)_2SO_4$(aq)] delivered crystals, but these were tiny and embedded in a cloudy precipitate. A 4 × 6 screen of hanging drops was set up at room temperature as follows: 4 µl of CAM (15 mg ml^{-1} in 4 m*M* $CaCl_2$) mixed with 2.7 µl of reservoir containing 10 m*M* 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 (~2° base-to-base) multiply peaked spot profiles. Eventually, a 1.0 × 0.2 × 0.1 mm fragment broken from a large aggregate gave single-peaked diffraction maxima that were ~ 1.5° base-to-base and ~ 0.6° full-width-at-half-maximum (FWHM) in ω. 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, $\alpha = 93.2$, $\beta = 96.7$, $\gamma = 89.2°$, $Z = 2$), and appear to be related to other *P*1 CAM crystals with $Z = 1$, but with the *a* axis doubled. On density considerations, solvent content in these 'cell doubled' *P*1 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 $P2_12_12_1$ 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 ~ 0.8 and 0.3°, 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 × 0.15 × 0.10 mm was indexed as othorhombic, space group $P2_12_12_1$ 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 [$R_{merge} = 4.12\%$, $\langle I \rangle / \langle \sigma(I) \rangle = 11.5$], completeness to 1.7 Å is 83.6%, while at the highest resolution it is ~ 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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