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, α = 93.2, β = 96.7, γ = 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 multiplet peaked spot profiles. Crystals in space group P212121 (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 (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 precipita-

tion 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, Snedek & Schatze, 1980), but were not conducive to structure determination owing to a diffraction limit of ~ 5 Å.

In P1 crystals, CAM adopts a dumbbell shape with two lobes connected by a central 21-residue helix. NMR studies in solution (Barford, 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 Marshall, 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 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 (NH4)2SO4(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 x 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 mM Tris–HCl, pH 8.0, 2 mM MgCl2, 1 mM CaCl2, 1 mM 2-mercaptoethanol). It was then loaded on a column (2.5 x 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 MgCl2, 1 mM CaCl2, 1 mM 2-mercaptoethanol). Further dialysis against 10 mM NH4HCO3(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 i 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 mM CaCl2), 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

<table>
<thead>
<tr>
<th>Resolution (Å)</th>
<th>∞-1.80</th>
<th>∞-3.08</th>
<th>3.08-2.44</th>
<th>2.44-1.94</th>
<th>2.13-1.94</th>
<th>1.94-1.80</th>
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<tbody>
<tr>
<td>Rmerge* (%)</td>
<td>3.64</td>
<td>3.01</td>
<td>6.17</td>
<td>8.41</td>
<td>12.39</td>
<td>17.90</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>71.5</td>
<td>87.7</td>
<td>75.8</td>
<td>68.7</td>
<td>63.8</td>
<td>59.9</td>
</tr>
<tr>
<td>(I)/(o(I))</td>
<td>10.9</td>
<td>26.4</td>
<td>9.90</td>
<td>5.61</td>
<td>3.03</td>
<td>1.78</td>
</tr>
</tbody>
</table>

\[ \text{R}_{\text{merge}} = \frac{\sum |I(I) - I(E)|}{\sum I(I)} \]

Table 2. Data-collection statistics for P212121 crystals in the ‘low’- and high-resolution shells

<table>
<thead>
<tr>
<th>Resolution (Å)</th>
<th>∞-1.68</th>
<th>∞-2.87</th>
<th>2.87-2.28</th>
<th>2.28-1.99</th>
<th>1.99-1.81</th>
<th>1.81-1.68</th>
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<tbody>
<tr>
<td>Rmerge* (%)</td>
<td>2.76</td>
<td>2.27</td>
<td>4.19</td>
<td>5.68</td>
<td>9.70</td>
<td>12.87</td>
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<tr>
<td>Completeness (%)</td>
<td>71.1</td>
<td>94.6</td>
<td>82.7</td>
<td>74.4</td>
<td>68.3</td>
<td>34.6</td>
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<tr>
<td>(I)/(o(I))</td>
<td>14.3</td>
<td>30.9</td>
<td>12.9</td>
<td>8.42</td>
<td>4.86</td>
<td>3.59</td>
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</table>

<table>
<thead>
<tr>
<th>Resolution (Å)</th>
<th>∞-1.17</th>
<th>∞-2.00</th>
<th>2.00-1.58</th>
<th>1.58-1.38</th>
<th>1.38-1.26</th>
<th>1.26-1.17</th>
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</thead>
<tbody>
<tr>
<td>Rmerge* (%)</td>
<td>2.91</td>
<td>1.74</td>
<td>9.51</td>
<td>14.2</td>
<td>20.9</td>
<td>25.8</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>47.3</td>
<td>26.9</td>
<td>46.6</td>
<td>64.2</td>
<td>58.9</td>
<td>39.3</td>
</tr>
<tr>
<td>(I)/(o(I))</td>
<td>6.77</td>
<td>38.9</td>
<td>4.65</td>
<td>2.50</td>
<td>1.47</td>
<td>1.18</td>
</tr>
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</table>

\[ \text{R}_{\text{merge}} = \frac{\sum |I(I) - I(E)|}{\sum I(I)} \]

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 x 0.2 x 0.1 mm), were fragile and delaminated into flat shreds parallel to the long growth direction.

2.3. Crystallization of P212121 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 (NH₄)₂SO₄(aq)] delivered crystals, but these were tiny and embedded in a cloudy precipitate. A 4 x 6 screen of hanging drops was set up at room temperature as follows: 4 µl of CAM (15 mg ml⁻¹ in 4 mM CaCl₂) mixed with 2.7 µl 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 (~2° base-to-base) multiply peaked spot profiles. Eventually, a 1.0 x 0.2 x 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, α = 93.2°, β = 96.7°, γ = 89.2°, Z = 2), and appear to be related to other P1 CAM crystals with Z = 1, but with the α 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 P212121 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 x 0.15 x 0.10 mm was indexed as orthorhombic, space group P212121 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 [Rmerge = 4.12%, (I)/(o(I)) = 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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References


