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Preliminary X-ray analysis of a C2-like domain from protein kinase C-δ

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Abstract

C2 domains are intracellular modules of approximately 130 residues that are found in many proteins involved in membrane trafficking and signal transduction. They are known to serve a variety of roles including binding ligands such as calcium, phospholipids and inositol polyphosphates as well as interacting with larger macromolecules. Although originally identified in the Ca^{2+}-dependent protein kinase C isoforms (PKC), initially no C2 domain was evident within the Ca^{2+}-independent isoenzymes. A recent study identified a divergent C2 domain in several novel, Ca^{2+}-independent PKCs (δ, ε, η and θ), located at their N-termini in a region previously referred to as a variable domain zero (Vo) [Ponting & Parker (1996). *Protein Sci*. **5**, 2375–2390]. The functional importance of this domain in the context of the novel PKCs is at present not well understood though it has been implicated in substrate recognition. The expression, crystallization and preliminary crystallographic analysis of recombinant Vo domain (residues 1–123) from PKC-δ is reported here. Crystals were obtained from incomplete factorial screens after removal of the histidine tag used to aid purification. These crystals diffracted to Bragg spacings of approximately 3 Å using a rotating-anode source and to 1.9 Å using synchrotron radiation. The crystals have cell parameters of $a = 60.7$, $b = 120.9$ and $c = 40.7$ Å and systematic absences consistent with the orthorhombic space group $P2_12_12_1$. To facilitate structure determination we have prepared, characterized and crystallized selenomethionine-substituted material.

1. Introduction

Despite the large size and apparent complexity of many eukaryotic proteins, a reductionist approach to understanding their structure and function has been highly successful primarily as a result of their modular character. Genetic, biochemical and structural studies on isolated modules derived from larger macromolecules have defined their biological function and sequence motifs or molecules recognised by these domains; recent examples include the src homology-2 (SH2) (Waksman *et al.*, 1993) and pleckstrin-homology (PH) domains (Ferguson *et al.*, 1995).

Derivation of sequence templates for these modules has been used to establish their widespread distribution in many intracellular eukaryotic signalling proteins (Pawson, 1995). An example of a ubiquitous multi-functional module is the C2 domain named after conserved region 2 of the lipid and calcium-activated protein kinase C family (PKC).

Within the conventional, Ca^{2+}-dependent PKCs, the C2 domain is responsible for Ca^{2+}-dependent phospholipid binding necessary for proper activation of this class of PKCs (Luo & Weinstein, 1993). Subsequent sequence analyses have revealed a more widespread distribution of C2 domains in a variety of eukaryotic proteins including synaptotagmins, phospholipases and more recently, the product of *unc*13 gene, various phosphatidylinositol 3-kinases, and the inositol 1,3,4,5 tetrakisphosphate binding protein from the GAP1 family (Nalefski & Falke, 1996).

Three-dimensional structures for two C2 domains have been determined indicating a β-sandwich tertiary fold comprised of two four-stranded antiparallel β-sheets (Sutton *et al.*, 1995; Essen *et al.*, 1996). Interestingly, these structures superposed very closely even though each represents a distinct circular permutation of this fold. Comparison of these structures has allowed a classification of C2 sequences into either S-type (synaptotagmin type) or P-type (phospholipase C-δ type) variants. Functionally important calcium-binding ligands for both variants are located at topologically equivalent positions within two extended loops. Recent NMR and crystallographic data have suggested the presence of multiple calcium-binding sites within these two loops (Shao *et al.*, 1996; Essen *et al.*, 1997).

Of the 65 C2-like sequences identified to date (Nalefski & Falke, 1996), a subclass of C2 sequences lack the amino acids essential for a functional calcium-binding site. One example is the C2-like domains found in the novel PKC isoforms PKCs (δ, ε, η and θ) (Ponting & Parker, 1996) predicted to adopt a P-type topology (Nalefski & Falke, 1996). A functional role for this C2-like domain has been established for PKC-δ, where it binds to a PKC neuronal substrate, GAP-43, in a calcium-dependent manner (16) despite the absence of conserved ligands essential for calcium coordination present in other C2 domains.

Here we report the crystallization of a recombinant C2-like domain from PKC-δ (residues 1–123), and describe the X-ray diffraction properties of crystals derived from wild-type and selenomethionine-labelled material.

2. Materials and methods

2.1. *Cloning, expression and purification*

A polymerase chain reaction product coding for residues 1–123 of rat PKC-δ was subcloned into the *Nde*I and *Xho*I sites of the translation vector pET14b (Novagen) and expressed as a thrombin-cleavable histidine-tagged fusion protein using the *E. coli* host strain B834(DE3)pLysS. Cells were grown in NZCYM media containing M9 salts and 1 mg ml^{-1} CAS amino acids at 310 K to an optical density of 0.8 at 600 nm prior to induction. The cultures were induced with 50 μM isopropyl-β-

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D-thiogalactopyranoside (IPTG) and expression of recombinant material proceeded for 6 h at room temperature, 300 K.

Selenomethionine-substituted protein was prepared by growing the methionine auxotroph B834(DE3) cells on minimal media in the presence of selenomethionine. Optimal yields of the selenomethionine-substituted protein were obtained using starter cultures grown overnight in 5–10 ml of minimal media containing 3 m*M* methionine. The minimal media consisted of M9 medium supplemented with 1 mg l^{-1} thiamine, 250 mg l^{-1} $MgSO_4.7H_2O$, 14 mg l^{-1} $CaCl_2.2H_2O$, 5 mg l^{-1} $FeCl_3$, 10 g l^{-1} glucose, 200 µg ml^{-1} ampicillin, 18 amino acids (lacking methionine and cysteine) at 50 µg ml^{-1} each (Hendrickson *et al.*, 1990). The overnight culture was centrifuged and the cell pellet resuspended in a minimal volume of LB. The cell suspension was used to inoculate 1 l of minimal media prepared as described above supplemented with 50 µg ml^{-1} selenomethionine. Under these conditions, the cells had a doubling time of 90 min. Induction with 50 µ*M* IPTG was initiated when the cell density reached an OD of 0.8 at 600 nm. Expression of the selenomethionine-substituted protein continued overnight at 300 K. Both native and selenomethionine-substituted PKCδ123 were purified as described below with the only exception that 10 m*M* DTT was present in all the solutions during the purification of the selenomethionine substituted protein to prevent oxidation.

Pelleted cells from 1 l culture were lysed by mild sonication in 50 m*M* Tris–HCl, 300 m*M* NaCl, 10% glycerol, 5 m*M* β-ME, 0.25 m*M* PMSF pH 7.9. Any insoluble material and cell debris was removed by centrifugation at 20 370*g* for 30 min at 277 K and the soluble histidine-tagged fusion protein was batch absorbed onto Ni-NTA-agarose (Qiagen). The resin was washed twice with the sonication buffer described above, followed by two washes with 50 m*M* Tris–HCl, 300 m*M* NaCl, 1 m*M* imidazole, 20% glycerol, 5 m*M* β-ME pH 7.9. The protein was eluted from the resin with 50 m*M* Tris–HCl, 100 m*M* NaCl, 20% glycerol, 300 m*M* imidazole, 5 m*M* β-ME, pH 7.9. Removal of the affinity tag was performed using 6 units of thrombin at 287 K overnight. The untagged protein was subsequently purified from other minor contaminants on a Superdex S-100 gel-filtration column equilibrated in 20 m*M* Tris–HCl, 150 m*M* NaCl, 2 m*M* DTT, pH 8.0. The molecular weight of the recombinant material as well as the degree of substitution by selenomethionine was confirmed by mass spectroscopy. N-terminal sequencing of material in solution and from crystals confirmed the first 20 residues of the construct (data not shown).

2.2. *Crystallization and characterization*

Conditions for crystallization were initially screened through the use of the sparse-matrix approach and vapour diffusion (Jancarik & Kim, 1991). Crystals were obtained using polyethylene glycol (PEG 4000, Sigma) as precipitant. Diffraction-quality crystals were obtained in sitting drops at room temperature by mixing an equal volume of the protein solution, 2 µl of approximately 5 mg ml^{-1} PKCδ123 and reservoir solution.

A native data set was measured at station 9.6 of the SRS synchrotron, Daresbury, using a 30 cm MAR image plate and a wavelength of 0.87 Å. A crystal-to-detector distance of 250 mm was used and data were collected with 1.5° oscillations at room temperature. Selenomethionine data were recorded at station 9.5 at a wavelength of 0.979 Å, close to the selenium *K* edge. Bijvoet differences were recorded using mirror plane symmetry to minimize systematic error from radiation decay. Data were reduced using the *DENZO*/*SCALEPACK* crystallographic data-reduction package.

3. Results

As part of a study to examine the function and structure of regulatory regions of PKC we observed high expression levels and protease resistance of PKCδ123 in bacteria in contrast to a series of larger constructs (Dekker & Parker, 1997). In order to produce sufficient quantities of this domain for crystallization trials, we have overexpressed and purified PKCδ123 using a pET vector that added a histidine-affinity tag to the N-terminus of the protein. The protein was produced in high yields, greater than 20% of the soluble protein in cell extracts and readily purified by a two-step purification procedure described in §2. The molecular weight of the protein was estimated by electrospray mass spectrometry and was found to be 14 228 ± 3 Da in close agreement with a calculated value of 14 215 Da, modified to include three additional vector-derived amino acids Gly-Ser-His that precede the N-terminal methionine.

Since the sequence of the PKCδ123 domain is relatively abundant in methionines (six methionines in 123 residues), we prepared selenomethionine-labelled material. Successful incorporation of selenium was obtained using the B834(DE3) strain instead of the B834(DE3)pLysS cells since we were unable to grow transformed B834(DE3)pLysS cells in the presence of minimal media. We also found that reproducibly high yields of selenomethionine-substituted material were obtained only when the starter cultures were supplemented with methionine in the presence of minimal media. We also observed a much shorter lag phase when the cells were transferred to selenomethionine containing minimal media relative to the lag phase observed from cells grown overnight in rich cultures. Yields for the seleno-protein were comparable to those for wild-type material and complete selenium substitution was confirmed by mass spectrometry. The selenomethionine substituted PKCδ123 had a molecular weight of 14 493 ± 5 Da consistent with selenium being incorporated into all six methionines. Occupancies below 99% were observed only when the cells were grown in the minimal media

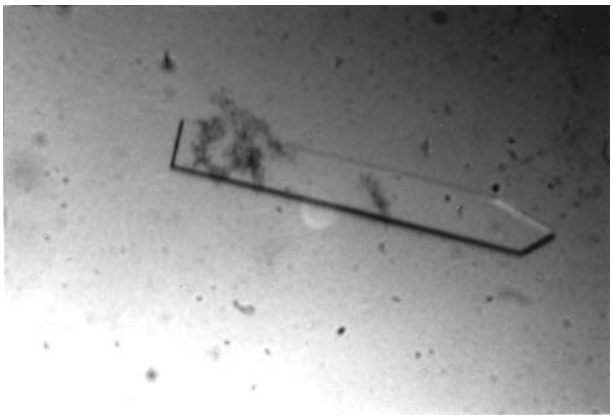

Fig. 1. Crystals of PKCδ123 obtained by microseeding.

Table 1. *Data-collection statistics for native and selenomethionine-substituted PKCδ123*

Statistical values for the highest resolution shell are shown in parentheses corresponding to 2.4–2.3 Å for the native and 2.9–2.8 Å for the selenium data.

Diffraction data	Native	Selenomethionine
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell (Å)	60.7 × 120.9 × 40.7	60.6 × 120.9 × 40.7
Resolution (Å)	2.3	2.8
Total observations	36783	35815
Unique reflections	13223	7565
Intensity $I/\sigma(I)$	7.9 (3.6)	7.3 (5.1)
Completeness (%)	96.0 (91.4)	97.1 (93.0)
R_{sym} (%)	7.1 (19.0)	7.9 (14.3)
R_{ano} (%)	—	5.6 (9.7)

supplemented with LB at concentrations higher than 10%(*v/v*).

Crystals of wild-type PKCδ123 were initially obtained using a reservoir solution of 30% PEG 4000, 0.1 *M* Na citrate, 0.2 *M* NH_4 acetate, pH 5.6 at room temperature. These crystals grew as twinned stacks of thin plates and were not suitable for data collection. Single X-ray quality crystals were obtained only after reducing the precipitant concentration to 20% PEG 4000 and using microseeding techniques (Fig. 1). The addition of 10 m*M* $CaCl_2$ did not visibly perturb the crystals. Sitting drops were microseeded by adding 0.5 µl of diluted seed solution. The seed solution was made by crushing clusters of twinned crystals in 30% PEG 4000, 0.1*M* Na citrate, 0.2 *M* NH_4 acetate, pH 5.6. The microseed solution could be stored indefinitely at 277 K. After seeding, crystals appeared overnight but reached a size appropriate for data collection only after one to two weeks.

The largest single crystals grew as rectangular plates (1.0 × 0.4 × 0.1 mm) and although they diffracted X-rays to 3 Å using a rotating-anode X-ray source, we were able to collect a complete native data set at 2.3 Å at the Daresbury synchrotron station 9.6 on a single unfrozen crystal (Fig. 2*a*). Diffraction data could be observed extending out to 1.9 Å Bragg spacings (Fig. 2*b*). A summary of the quality of the native data is shown in Table 1. The refined unit-cell dimensions are $a = 60.7$, $b = 120.9$ and $c = 40.7$ Å and $\alpha = \beta = \gamma = 90°$. Crystals displayed systematic absences of $h = 2n + 1$, $k = 2n + 1$, $l = 2n + 1$ for axial reflections indicating they belong to space group $P2_12_12_1$. Using a partial specific volume of 0.74 $cm^3\ g^{-1}$ for PKCδ123 and assuming a dimer per asymmetric unit, the solvent content of these crystals is estimated at 49%. No non-crystallographic symmetry was evident from self-rotation function or native Patterson syntheses.

Selenomethionine-substituted crystals were obtained using the same method but after reducing the precipitant concentration to 10% PEG 4000 as a consequence of the lower solubility generally observed for selenomethionyl proteins. The selenomethionine substituted crystals are suitable for a multiwavelength anomalous diffraction (MAD) experiment at the selenium *K* edge from a single crystal. Therefore, suitable cryo conditions were established as 25% PEG 4000, 0.1 *M* Na citrate, 0.2 *M* NH_4 acetate, pH 5.6. However, on cryocooling a large irreproducible shift in the unit-cell parameters was observed preventing the scaling of data recorded from different crystals. Although this will not hinder a cryo-MAD experiment, in parallel we are screening for isomorphous heavy-atom derivatives by recording data at room temperature. This may be important should the large number of anticipated selenium Harker vectors (12 expected sites per asymmetric unit) derived from MAD or from single isomorphous differences prove problematic in identifying the selenium positions.

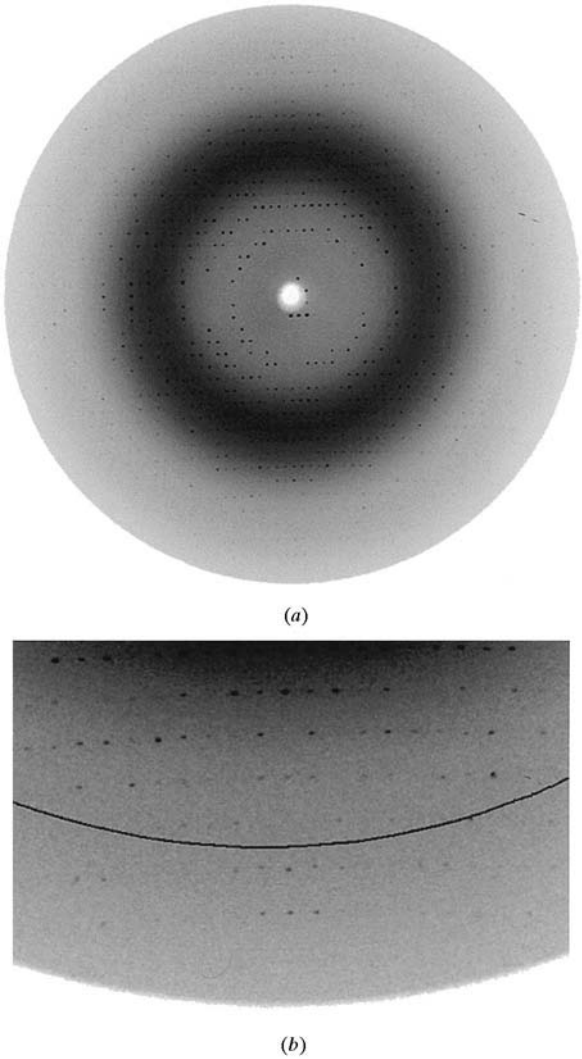

Fig. 2. Oscillation image for a native PKCδ123 crystal. Inset shows data extend to reciprocal lattice spacings equivalent to 1.9 Å (black line indicates 2.2 Å).

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