Microcrystals of the β_1 isoenzyme of protein kinase C: an electron microscopic study

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Microcrystals of protein kinase C β_1 have been grown from solutions of poly(ethylene glycol). Image analysis of electron micrographs of the protein crystals, which diffracted to 5.0 nm, revealed p3 symmetry with a unit cell of about 10.3 nm $\times$ 10.3 nm. The electron stain-excluding densities showed a three-domain

ring structure in projection, giving kidney bean-shaped molecules of about 7.0 nm $\times$ 4.5 nm diameter, packed as trimers. The implications of these observations for the function of the enzyme are discussed.

INTRODUCTION

The protein kinase C (PKC) family comprises at least ten isoenzymes which phosphorylate their target proteins on serine and threonine residues (Nishizuka, 1989; Stabel and Parker, 1991). The members of this family are single polypeptide chains, with molecular masses of around 80 kDa, containing two functional domains. The larger domain (of approx. 50 kDa) contains the catalytic site, which binds MgATP and the substrate, and shows sequence similarity with other protein kinases including the catalytic subunit of cyclic AMP (cAMP)-dependent protein kinase. The crystal structure of this latter kinase has been solved to atomic resolution (Knighton et al., 1991). The approx. 30 kDa cysteine-rich domain of PKC contains the regulatory region to which diacylglycerols specifically bind (reviewed in Nishizuka, 1989; Stabel and Parker, 1991).

In this study we report the first visualization by electron microscopy of crystals of PKC, obtained by the vapour-diffusion 'hanging-drop' technique and negatively stained. The molecular shape and structure-function aspects of these findings are discussed.

MATERIALS AND METHODS

PKC β_1 (this corresponds to PKC- β_{11}) was purified from a baculovirus expression system essentially as described previously (Stabel et al., 1991), and final protein concentrations were determined by the Bradford protein assay (Bradford, 1976). The purified protein appeared as a single band on SDS/PAGE when stained with Coomassie Blue.

Crystallization was carried out by the 'hanging-drop' method (McPherson, 1976). Samples of PKC were concentrated to 12 mg/ml in 2 mM Hepes buffer (pH 7.0) containing 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.05% n-octyl glucoside and 1 mM MgCl₂. A 3 μ l sample of protein was mixed with an equal volume of reservoir solution on a coverslip and suspended over the reservoir, which contained 1.0 ml of 0.048 M sodium phosphate buffer, pH 7.4, 20% poly(ethylene glycol) 4000 and 1 mM MgCl₂. The trays were sealed and allowed to equilibrate by vapour diffusion at 4 °C. Crystals formed within 7–9 days but

were too small for X-ray analysis. Crystals grew as triangular plates with sides up to 20 μ m in length and with cross-sections up to 5 μ m. Thin crystals were used for transmission electron microscopy (see Figure 1a). Negatively stained specimens for electron microscopy were prepared as follows. Microcrystals of PKC produced by the hanging-drop method that were suitable for electron microscopy were collected on air-glow-discharged carbon coated 400 mesh nickel grids and floated on a drop of 1% (w/v) uranyl acetate for 20 s. Excess stain was blotted and the grid was dried in air prior to examination in a Philips 301 transmission electron microscope at 100 kV. Micrographs of negatively stained crystals were taken at 39000 $\times$ and areas (approx. 10 mm $\times$ 10 mm) suitable for analysis were selected by optical diffraction and grabbed and digitized using a charge-coupled device (CCD) camera attached to a Compaq 386 (U.K.) microcomputer running Semper software from Synoptics (Cambridge, U.K.). The areas were Fourier-transformed and phases $a(h,k,l)$ (in the case of a two-dimensional crystal, $l = 0$) were extracted for reflections significantly above background.

Stain-excluding density maps were calculated using the fast Fourier transform program algorithm (Ten Eyck, 1973) as implemented in the CCP4 program FFT (CCP4, 1979). Model building was carried out using the interactive graphics program FRODO (Jones, 1978).

RESULTS AND DISCUSSION

When observed directly in negative stain, the PKC crystals appeared triangular with truncated vertices (Figure 1a). A clear lattice can be seen that, on analysis by optical diffraction (Figure 1b), indicated a resolution of about 5.0 nm and gave lattice constants, in projection, of $a, b = 10.30 \pm 0.45$ nm; $\gamma = 120^\circ$. Analysis of the phases of nine clear peaks (Friedel pairs) in the Fourier transform gave a phase residual of 8.21° assuming p3 symmetry, and of 19.08° assuming p6 symmetry. The lower phase residual and inspection of the noise-filtered image (Figure 1c) indicated trigonal rather than hexagonal packing. We therefore carried out further averaging of the noise-filtered image assuming p3 symmetry (Figure 1d). Rings of protein (white) surround a central hole and the protein ring is made up of three

Abbreviations used: PKC, protein kinase C; cAMP, cyclic AMP.

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Figure 1 Analysis of PKC β microcrystals

(a) A negatively stained crystal of PKC β grown from a hanging drop. The lattice of $10.3 \text{ nm} \times 10.3 \text{ nm}$ is visible. Scale bar = $1.0 \mu\text{m}$. (b) An optical transform of the microcrystal shown in (a). Reflections significantly above background show resolution to about 5 nm in a hexagonal lattice. Scale bar = 0.2 nm^{-1} . (c) Unsymmetrized, noise-filtered image of a crystalline region of (a). Rings of protein (white) are made up of three domains. (d) Noise-filtered image after applying p3 symmetry (in this case the protein has been contoured). The p3 symmetrized image does not show perfect 3-fold symmetry because the program we used must first 'distort' the filtered image before re-interpolation. The unit cell is outlined.

Figure 2 Superposition of the catalytic core of cAMP-dependent protein kinase (all atoms) on the PKC β stain-excluding density

molecules, arranged as discrete domains. The distribution of stain-excluding density indicated that under these conditions the protein monomer has dimensions of about $7.0 \text{ nm} \times 4.5 \text{ nm}$.

Carefully washed crystals (48 mM sodium phosphate, $\text{pH } 7.4$, containing 1 mM MgCl_2) were analysed by SDS/PAGE and stained with Coomassie Blue. The gel revealed a single protein band whose mobility corresponded to a molecular mass of approx. 80 kDa , confirming that the whole protein had been crystallized (results not shown).

The crystals analysed showed p3 symmetry, with three distinct domains of stain-excluding density. The primary sequence for PKC β_1 predicts a catalytic domain (approx. 50 kDa) and a regulatory domain (approx. 30 kDa). The size of the cAMP-dependent protein kinase is known from the atomic structure to be approximately $6.5 \text{ nm} \times 4.5 \text{ nm} \times 4.5 \text{ nm}$ (Knighton et al., 1991). Superposition of the three-dimensional structure of the cAMP-dependent kinase catalytic domain (which has $> 35\%$ identity with the catalytic domain of PKC) and the two-dimensional stain-excluding density from the above crystals (Figure 2) shows correspondence, in projection, with the mono-

Figure 3 Schematic representation of the proposed domain structure of PKC β , in relation to the stain-excluding density maps and alignments shown in Figures 1 and 2

This model includes the predicted pseudosubstrate site interaction with the catalytic domain.

mer. As the whole of the PKC molecule was crystallized, the cAMP-dependent kinase catalytic domain superposition indicates that the PKC molecule in the crystal most probably lies with its catalytic domain parallel to the regulatory domain. This implies that the regulatory domain of PKC lies above the catalytic domain, and could be represented by a sphere of approx. 2.5 nm diameter (Figure 3). The above superposition of the cAMP-dependent kinase catalytic domain on the PKC stain-excluding density was in our judgement the most probable; however, the possibility, given the low resolution of the stain-excluding density map, of other orientations of the cAMP-dependent kinase catalytic domain cannot be discounted. However, the premise that the regulatory domain lies above or below the catalytic domain would not be negated, as there is not room

for the regulatory domain in the projected trimeric molecular arrangement. Unfortunately the multilayered nature of the crystals precludes the possibility of obtaining a three-dimensional data set which would help to clarify further the location of the regulatory domain.

The catalytic C-terminal domain contains binding sites for the protein substrate and ATP. All PKC isoenzymes contain, within their N-terminal regulatory domain, sequences which resemble their substrate phosphorylation sites (House and Kemp, 1987). These pseudosubstrate sites appear to interact with the enzyme's active site and thus prevent substrate access (Pears et al., 1990). Additionally, the regulatory domain contains binding sites for Ca^{2+} , phospholipid and the physiological activator diacylglycerol (Nishizuka, 1989; Stabel and Parker, 1991). These cofactors, when bound, presumably induce a conformational change which exposes the catalytic site and renders the enzyme active.

The proposed overall shape of the PKC molecule, in which there could be close proximity of the regulatory N-terminal pseudosubstrate and the catalytic C-terminal domain substrate binding site, lends support to the above model for PKC function.

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