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Crystallization and preliminary X-ray analysis of two inhibitor complexes of the catalytic domain of death-associated protein kinase

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The catalytic domain of death-associated protein kinase (DAPK) has been overexpressed, purified and crystallized using the sitting-drop vapour-diffusion method with PEG 8000 and magnesium acetate as precipitants. Complexes with the inhibitor staurosporine and its analogue BDB402 were also crystallized in the presence of PEG 400 and PEG 8000, respectively. Diffraction data were collected to 2.4 Å for the native catalytic domain, to 2.9 Å for the staurosporine complex and to 2.7 Å for the BDB402 complex. All three crystals belong to space group $P2_12_12_1$, with unit-cell parameters a = 77.992, b = 109.909, c = 50.063 Å for the catalytic domain, a = 78.911, b = 113.162, c = 50.658 Å for the staurosporine complex and a = 77.337, b = 108.869, c = 50.186 Å for the BDB402 complex. In both complexes the inhibitor molecule was clearly assigned in the difference Fourier map calculated on the basis of the phases obtained from the structure of the catalytic domain.

1. Introduction

Death-associated protein kinase (DAPK) is a multidomain calmodulin-regulated serine/threonine protein kinase (Deiss *et al.*, 1995) that has been implicated in apoptosis and tumour suppression (Raveh *et al.*, 2001). Although the catalytic domain of DAPK has been considered to be responsible for its cellular functions, endogenous protein substrates for DAPK have not yet been identified and no inhibitors are available. As recent work has suggested that DAPK is involved in ischaemia-induced neuronal cell death (Yamamoto *et al.*, 1999), the search for substrates and specific inhibitors of DAPK has important clinical implications. Staurosporine is a natural product originally isolated from the bacterium *Streptomyces staurosporeus* (Omura *et al.*, 1977) and first characterized as an inhibitor of protein kinase C (Tamaoki *et al.*, 1986). It has subsequently been shown to be a potent but non-specific protein kinase inhibitor (Omura *et al.*, 1995). Staurosporine also shows high affinity for DAPK, suggesting that understanding of the DAPK–staurosporine structure may promote the discovery of new drugs targeting DAPK. In this paper, we describe the crystallization and data collection of the DAPK catalytic domain (DK1) and two inhibitor complexes.

2. Materials and methods

2.1. Protein expression and purification

A DK1 construct (amino acids Met1–Thr280 of DAPK) cloned into the pET31 vector was transformed into BL21 (DE3). The protein was isolated from *Escherichia coli* and purified by anion-exchange and hydrophobic interaction chromatography.

The purity of DK1 was checked by SDS–PAGE. Staurosporine (Sigma No. S4400) was purchased from Sigma Co. Ltd BDB402, a staurosporine analogue, was a generous gift from Mitsubishi Pharma Corporation.

2.2. Crystallization

The purified protein solution was concentrated to 6.87 mg ml^{-1} with a Centricon microconcentrator (10 kDa molecular-weight cutoff, Millipore). Crystallization of DK1 was carried out using the sitting-drop vapour-diffusion method. Protein droplets prepared by mixing 2 µl DK1 solution and 2 µl reservoir buffer solution (27 m*M* sodium cacodylate pH 6.5, 5.3% PEG 8000, 53 m*M* magnesium acetate, 6.7% glycerol) were set up in CrystalClear Strips (Hampton Research) with 100 µl reservoir solution at 283 K.

Crystals of the DK1–inhibitor complex were prepared as follows. Staurosporine and BDB402 were dissolved in dimethyl sulfoxide (to a final concentration of 15.3 m*M*). The staurosporine solution was stored in the dark at 277 K until use. The DK1 solution was mixed with aliquots of the respective inhibitor solutions so that the inhibitor was present in a two- to threefold molar excess over DK1 (final concentration 0.2 m*M*). The solutions were incubated at 277 K for 1 h and then centrifuged at 15 000 rev min^{-1} for 5 min to remove any precipitate. Crystals of the complexes were

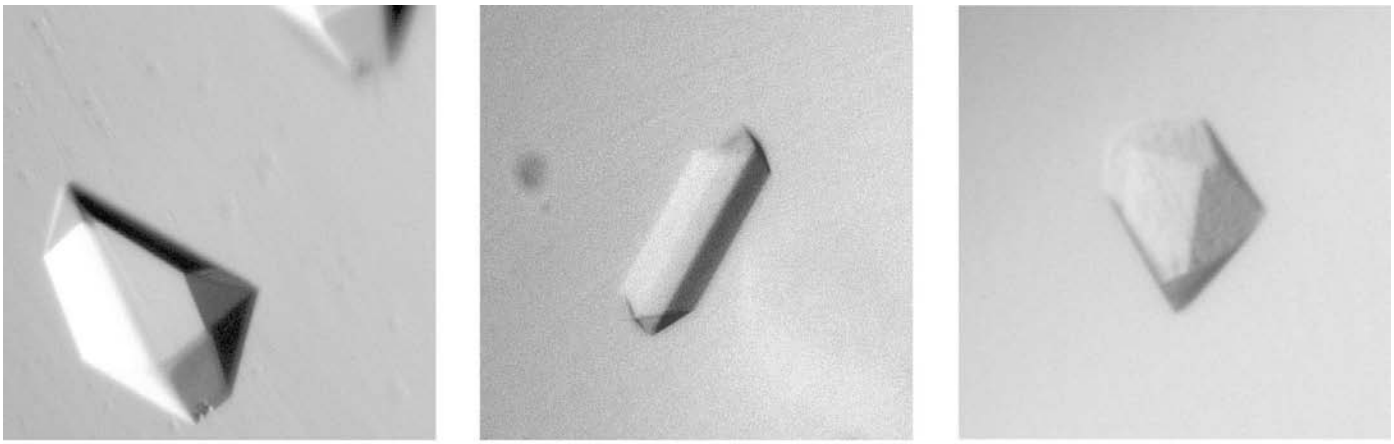

Figure 1
Crystals of (*a*) DK1 native, (*b*) DK1–staurosporine and (*c*) DK1–BDB402 complexes.

Table 1
Crystallographic data statistics.

Values in parentheses are for the highest resolution shell.

Crystal	DK1	DK1–staurosporine	DK1–BDB402
Wavelength (Å)	0.9000	0.7000	0.7100
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters (Å)			
a	77.992	78.911	77.337
b	109.909	113.162	108.869
c	50.063	50.658	50.186
Resolution (Å)	31.8–2.4 (2.53–2.40)	42.6–2.9 (3.06–2.90)	63.0–2.7 (2.85–2.70)
No. observations	92558	71763	83631
No. unique reflections	17309 (2433)	10601 (1496)	12223 (1747)
Completeness (%)	99.3 (99.3)	99.9 (99.9)	99.9 (100.0)
$R_{\rm merge}$† (%)	11.8 (34.6)	10.3 (39.1)	8.2 (40.2)
$I/\sigma(I)$	4.5 (1.7)	6.4 (2.0)	8.1 (1.9)

† $R_{\rm merge} = 100\sum|I_i - \langle I\rangle|/\sum I_i$, where I_i is the intensity of the ith observation.

grown from these solutions using 0.1 *M* Tris–HCl pH 8.5, 15% PEG 400, 5% PEG 8000, 15% glycerol (DK1–staurosporine complex) and 0.1 *M* Tris–HCl pH 8.0, 20% PEG 400, 5% PEG 8000, 15% glycerol (DK1–BDB402 complex) as reservoir solution.

2.3. Data collection and analysis

Diffraction data were collected from three crystals at 100 K using synchrotron radiation with an ADSC Quantum (4R) CCD detector on beamline BL40B2 at SPring-8 (Hyogo, Japan). Data collection for the DK1–staurosporine complex was carried out in the dark. The wavelengths employed for these diffraction experiments were 0.7000–0.9000 Å and the crystal-to-detector distances were 250–280 mm. Diffraction images were indexed with the program *MOSFLM* (Leslie, 1994) and processed using programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The detailed conditions of the diffraction experiments for the three crystals are summarized in Table 1.

3. Results

The purity of the protein samples used in the crystallization experiments was confirmed by SDS–PAGE. All crystals were grown over 7–14 d. The typical dimensions of the crystals were 0.20 × 0.10 × 0.04 mm (DK1), 0.20 × 0.05 × 0.05 mm (DK1–staurosporine) and 0.14 × 0.09 × 0.03 mm (DK1–BDB402) (Fig. 1). Diffraction data were collected to 2.4 Å for DK1, to 2.9 Å for DK1–staurosporine and 2.7 Å for DK1–BDB402. Statistics for each crystal are shown in Table 1. All three crystals belong to space group $P2_12_12_1$. The unit-cell parameters of the complex crystals (a = 78.911, b = 113.162, c = 50.658 Å for DK1–staurosporine and a = 77.337, b = 108.869, c = 50.186 Å for DK1-BDB402) are also similar to those of the DK1 crystal (a = 77.992, b = 109.909, c = 50.063 Å). The unit-cell parameters of the DK1 crystal in this study are considerably different from those reported previously (a = 62.55, b = 88.56, c = 46.93 Å; Tereshko *et al.*, 2001). Assuming one protein molecule per asymmetric unit of the crystal, the calculated solvent content per asymmetric unit was 63% ($V_{\rm M}$ = 3.3 Å^3 Da^{-1}) for DK1, 65% ($V_{\rm M}$ = 3.5 Å^3 Da^{-1}) for DK1–staurosporine and 63% ($V_{\rm M}$ = 3.3 Å^3 Da^{-1}) for DK1–BDB402 (Matthews, 1968).

The molecular-replacement method (*AMoRe*; Navaza, 1994) was applied for phase determination with the reflection data set of DK1 using the structure of twitchin kinase (PDB code 1kob; Kobe *et al.*, 1996) as a search model. One set of parameters for the rotational and translational searches gave a correct solution and the structure model was fitted with the electron-density map calculated on the basis of the initial phases. The model was improved by manual fitting in *XtalView* (McRee, 1999) and refined to a resolution of 2.4 Å with an R value of 24.0% (free R value = 28.0%) using the *CNS* package (Brünger *et al.*, 1998). The refined DK1 structure was then used for structure determination with two inhibitor complexes. By calculating the difference Fourier ($F_{\rm o} - F_{\rm c}$) maps with the phases of DK1, a residual electron-density peak was clearly identified for both DK1–inhibitor complexes. Model fitting of the inhibitor molecules in these maps and refinement are now in progress.

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