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Crystallization and preliminary crystallographic analysis of 3′-aminoglycoside kinase type IIIa complexed with a eukaryotic protein kinase inhibitor, CKI-7

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3′-Aminoglycoside kinase type IIIa [APH(3′)-IIIa] catalyzes the transfer of γ-phosphate from ATP to the 3′-hydroxyl of many aminoglycoside antibiotics, abolishing their bactericidal effects. Despite very low sequence identity, APH(3′)-IIIa and eukaryotic protein kinases share structural and functional similarities, including a sensitivity to isoquinolinsulfonamide-type inhibitors. APH(3′)-IIIa has been cocrystallized with CKI-7, a casein kinase 1 inhibitor. These crystals were grown using PEG 3000 as precipitant and required consecutive cycles of microseeding. Data were collected to 2.5 Å. The crystals belong to space group $P2_12_12_1$, with unit-cell parameters $a = 49.84$, $b = 91.90$, $c = 131.2$ Å.

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1. Introduction

The evolution of bacterial resistance to multiple antibiotics has greatly compromised our ability to combat infectious diseases using existing drugs. Hence, there is marked interest in delineating the mechanisms of antibiotic resistance and in identifying ways in which antibiotic resistance can be overcome. Aminoglycosides are a group of antibiotics that are widely used for treating nosocomial infections. Despite their toxicity to the kidneys and the inner ear, they are commonly prescribed owing to their low cost and high efficacy (Forge & Schacht, 2000). Aminoglycosides exert their bactericidal effects by targeting the A-site of the 16S ribosomal RNA (Moazed & Noller, 1987). Aminoglycoside binding induces conformational changes in the decoding region of the ribosome that promotes the coupling of mRNA to near-cognate or non-cognate tRNA, resulting in erroneous protein translation (Pape *et al.*, 2000). However, the effectiveness of aminoglycosides has been seriously undermined owing to the emergence of resistant bacterial strains. The majority of these resistant strains harbour plasmid-mediated factors encoding enzymes that catalyze the covalent modification of aminoglycosides (Davies, 1991). Covalent modification results in reduced affinity of these antibiotics for their target and they therefore lose their bactericidal properties (Llano-Sotelo *et al.*, 2002). For example, 3′-aminoglycoside phosphotransferase type IIIa [APH(3′)-IIIa], found in many Gram-positive bacteria such as enterococci and staphylococci, catalyzes the ATP-dependent phosphorylation of various clinically used aminoglycosides. APH(3′)-IIIa is perhaps the most studied aminoglycoside-resistance factor, since one of its distinguishing features is that it has an unusually broad substrate profile (McKay *et al.*, 1994).

Crystal structures of APH(3′)-IIIa in the apo form, in complex with phosphonucleotides and several ternary complexes have been determined (Hon *et al.*, 1997; Burk *et al.*, 2001; Fong & Berghuis, 2002). These structural studies reveal that despite very low sequence identity, the overall structure of APH(3′)-IIIa is remarkably similar to that of serine/threonine and tyrosine eukaryotic protein kinases (ePKs), specifically around the nucleotide-binding pocket. Subsequently, it was shown that APH(3′)-IIIa is capable of phosphorylating the serine residues of some peptide substrates of ePKs (Daigle *et al.*, 1999); more importantly, it can also be inhibited by protein kinase inhibitors of the isoquinolinesulfonamide family which are competitive against ATP binding (Daigle *et al.*, 1997). For example, the casein kinase 1 inhibitor CKI-7 has an inhibition constant of $\sim$65 μM for APH(3′)-IIIa. These inhibitors can serve as starting models for the design and development of inhibitors for APH(3′)-IIIa, which could be exploited as adjuvants in antimicrobial therapies (Burk & Berghuis, 2002; Fong *et al.*, 2004). Here, we report the crystallization of the APH(3′)-IIIa–CKI-7 complex.

2. Experimental procedures

2.1. Purification and crystallization

APH(3′)-IIIa was expressed and purified using previously established procedures (McKay *et al.*, 1994). The pure protein was then dialysed in 25 mM sodium cacodylate pH 7.0 and its concentration adjusted to 10 mg ml^{-1}. CKI-7 was purchased from Seikagaku America (MJS BioLynx Inc., Ontario, Canada) and

dissolved in dimethyl sulfoxide to make a 10 m*M* stock. A fivefold molar excess of CKI-7 was added to the protein solution. Initial screening was carried out at 277 and 295 K using commercial sparse-matrix screens (Crystal Screens I and II, Hampton Research; Wizard Screens I and II and Cryo Screens I and II, Emerald Biostructures; Jancarik & Kim, 1991). A 4 µl drop consisting of equal volumes of the protein–inhibitor solution and the reservoir solution was equilibrated against a 0.7 ml reservoir using the hanging-drop vapour-diffusion method. The procedure for refinement of the preliminary condition is described in §3.

2.2. Data collection and processing

Data from a single crystal were collected under cryogenic conditions (110 K) at beamline X8C of the National Synchrotron Light Source, Brookhaven National Laboratory equipped with an ASDC Quantum CCD detector. The crystal was soaked for approximately 2 min in mother liquor supplemented with 12.5%(*v*/*v*) 2-methyl-2,4-pentanediol (MPD) and 12.5%(*v*/*v*) PEG 600 before being flash-frozen in a cold stream for data collection. The crystal-to-detector distance was set at 200 mm and the data were collected with an oscillation angle of 1.0° and a wavelength of 1.072 Å. Intensities were integrated using *HKL*2000 and scaled using the *HKL* program suite (Otwinowski & Minor, 1997).

3. Results and discussion

Past experience has shown that APH(3′)-IIIa can crystallize in a variety of crystal forms depending on the presence and/or absence of substrates or substrate analogues. The APH(3′)-IIIa apo form crystallizes in space group $P4_32_12$ with unit-cell parameters $a = b = 55$, $c = 185$ Å (Burk *et al.*, 2001), the nucleotide-bound state crystallizes in space group $P2_12_12_1$ with unit-cell parameters $a = 50$, $b = 91$, $c = 132$ Å (Hon *et al.*, 1997; Burk *et al.*, 2001) and the ternary complex crystals belong to either space group $P4_322$ with unit-cell parameters $a = b = 47$, $c = 301$ Å (Fong & Berghuis, 2002) or space group $P4_22_12$ with unit-cell parameters $a = b = 80$, $c = 110$ Å (Fong & Berghuis, unpublished data). The initial strategy for crystallizing APH(3′)-IIIa in complex with CKI-7 was to pursue crystallization conditions and procedures akin to those used for obtaining nucleotide-bound or ternary complex crystals, substituting the casein kinase 1 inhibitor for the nucleotide. These crystallization trials proved to be completely fruitless and therefore a sparse-matrix screening approach was taken in order to obtain suitable crystallization conditions.

One condition from the preliminary sparse-matrix crystallization experiments [20%(*w*/*v*) polyethylene glycol (PEG) 3000, 0.1 *M* Tris pH 7.0 and 0.2 *M* calcium acetate at 277 K] produced thin plate-shaped crystals with uneven edges and surfaces (Fig. 1*a*). Extensive fine-screening by varying the protein concentration and the amounts of precipitant, salt and pH did not significantly improve the crystal quality (Fig. 1*b*). Subsequently, the microseeding method was attempted. A crystal was placed in a stabilizing solution (25–35% PEG 3000, 0.1 *M* Tris pH 7.5–8.0 and 0.2 *M* calcium acetate) and crushed using a Seed Bead (Hampton Research Corporation). This microseed stock was then diluted tenfold to 1000-fold and 1 µl of the microseed slurry was added to drops containing reduced concentrations of precipitant and protein. Crystals appeared after approximately one week. Although these plate-shaped crystals were small and thin (Fig. 1*c*), some had edges and surfaces that were much sharper and smoother than those grown in the absence of seeds. This procedure was repeated, with an improved crystal being used as seed in the following cycle. Each subsequent round of microseeding produced single rod-shaped crystals that were progressively larger and thicker (Figs. 1*d*–1*f*). The reservoir solutions for the fourth and final round of microseeding contained 10–12%(*w*/*v*) PEG 3000,

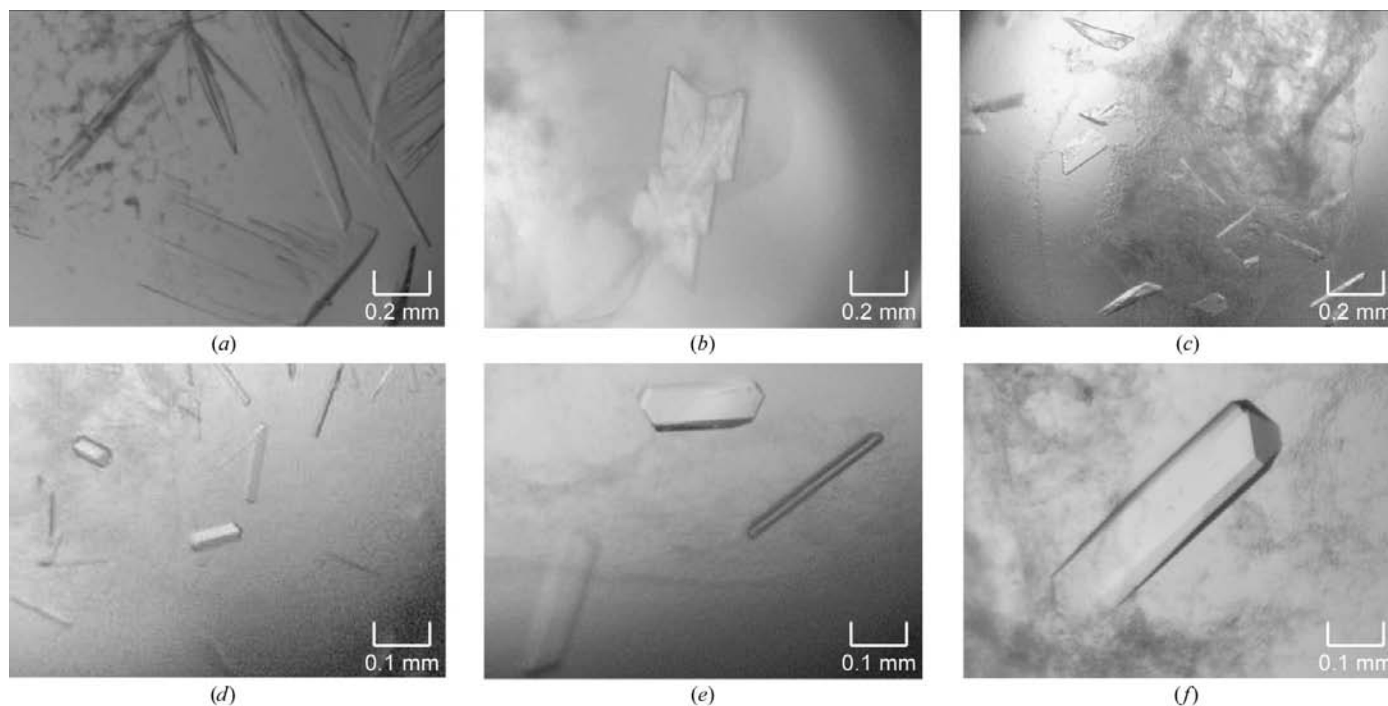

Figure 1
Typical crystals of the APH(3′)-IIIa–CKI-7 complex at various stages of optimization. (*a*) Crystals of APH(3′)-IIIa with CKI-7 obtained from sparse-matrix screening. (*b*) Crystals observed after refining the protein and precipitant concentrations, as well as the pH. (*c*) Crystals grown by employing the microseeding technique. (*d*)–(*f*) Typical crystals obtained from subsequent successive cycles of microseeding. Photographs were taken under polarized light.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 49.84, b = 91.90, c = 131.2, $\alpha = \beta = \gamma$ = 90
Resolution (Å)	
Overall	50–2.50
Lowest resolution shell	50–5.38
Highest resolution shell	2.59–2.50
Reflections observed	101620
Unique reflections	21525
Completeness (%)	99.6 (99.8)
$I/\sigma(I)$	32.24 (9.233)
R_{sym}	0.045 (0.124)

0.1 *M* Tris pH 7.0–8.5 and 0.2 *M* calcium acetate. Crystals grew to approximately 0.55 × 0.15 × 0.05 mm in about four weeks in drops containing 3.5 µl reservoir solution, 3.5 µl APH(3′)-IIIa–CKI-7 solution at 6 mg ml^{-1} and 1 µl of the microseed slurry diluted 1000-fold in a stabilizing solution containing 25%(*w*/*v*) PEG 3000, 0.1 *M* Tris pH 8.0 and 0.2 *M* calcium acetate.

Relevant data-collection statistics are summarized in Table 1. Assuming the presence of two molecules per asymmetric unit, the Matthews coefficient (V_M; Matthews, 1968) has a value of 2.4 $Å^3$ Da^{-1} and the solvent content is about 49%. These crystals are isomorphous with the nucleotide-bound crystals of APH(3′)-IIIa as shown by preliminary rigid-body refinement in the *Crystallography and NMR System* (*CNS*) program (Brünger *et al.*, 1998) using ADP-bound APH(3′)-IIIa (PDB code 1j7l; Burk *et al.*, 2001) as the model, which produced an R factor and R_{free} value of 34.6 and 35.3%, respectively. However, the crystallization conditions of the APH(3′)-IIIa–CKI-7 complex are sufficiently different from those previously reported that it is understandable that our initial attempts to exploit previous crystallization conditions proved unsuccessful.

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