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Crystallization and preliminary crystallographic analysis of an aminoglycoside kinase from *Legionella pneumophila*

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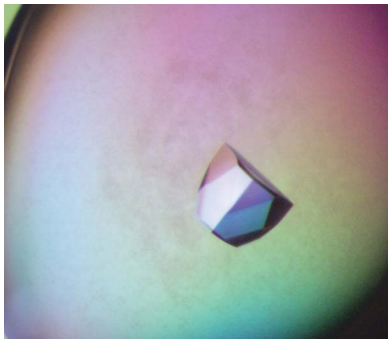


9-Aminoglycoside phosphotransferase type Ia [APH(9)-Ia] is a resistance factor in *Legionella pneuemophila*, the causative agent of legionnaires' disease. It is responsible for providing intrinsic resistance to the antibiotic spectinomycin. APH(9)-Ia phosphorylates one of the hydroxyl moieties of spectinomycin in an ATP-dependent manner, abolishing the antibiotic properties of this drug. Here, the crystallization and preliminary X-ray studies of this enzyme in two crystal forms is reported. One of the these crystal forms provides diffraction data to a resolution of 1.7 Å.

1. Introduction

Concomitant with the increased use of antibiotics for medical and agricultural purposes, there has been a rise in resistance to these drugs (Cizman, 2003). The mechanisms by which bacteria overcome the effects of bactericidal and bacteriostatic compounds are diverse and include up-regulation of active efflux pumps and methylation of antibiotic targets (Walsh, 2000; Wright, 2003). For aminoglycosides such as kanamycin and gentamicin, the mechanism that is predominantly observed clinically is chemical alteration of the drug catalyzed by aminoglycoside-modifying enzymes. The modified antibiotic has a dramatically reduced affinity for its target, the 30S ribosome, resulting in a resistance phenotype. Three different classes of enzymes that confer resistance to aminoglycoside antibiotics have thus far been identified in pathogenic bacteria: aminoglycoside N-acetyltransferases, O-adenyltransferases and O-phosphotransferases (kinases) (Azucena & Mobashery, 2001; Wright *et al.*, 1998). Most of these enzymes are encoded on plasmids, consistent with the rapid dissemination of resistance factors among bacteria. However, some aminoglycoside-modifying enzymes are chromosomally encoded, thus providing intrinsic resistance to certain antibiotics (Suter *et al.*, 1997; Wybenga-Groot *et al.*, 1999).

Structural studies of all three classes of aminoglycoside-modifying enzymes have been reported (Smith & Baker, 2002; Wright *et al.*, 1998). However, for aminoglycoside kinases, this information is limited to the crystal structures of two closely related enzymes: APH(3′)-IIIa (Burk *et al.*, 2001; Fong & Berghuis, 2002; Hon *et al.*, 1997) and APH(3′)-IIa (Nurizzo *et al.*, 2003), which share 33% identity in amino-acid sequence and are structurally highly homologous. These studies have shown that aminoglycoside kinases are structurally related to eukaryotic protein kinases. In fact, inspired by this homology, it has been demonstrated that some protein kinase inhibitors of the isoquinolinesulfonamide family, which are competitive with ATP, are also capable of inhibiting aminoglycoside kinases (Daigle *et al.*, 1997).

Here, we report the crystallization and preliminary diffraction studies of 9-aminoglycoside phosphotransferase type Ia [APH(9)-Ia], also known as spectinomycin kinase, from *Legionella pneumophila*, the bacteria responsible for legionnaires' disease. This enzyme provides resistance to only one antibiotic, spectinomycin, and is encoded on the chromosome (Suter *et al.*, 1997; Thompson *et al.*, 1998). These properties make APH(9)-Ia unlike most other aminoglycoside kinases, including APH(3′)-IIIa and APH(3′)-IIa, which provide resistance to multiple antibiotics and are plasmid-encoded (Fong & Berghuis, 2002; Nurizzo *et al.*, 2003). The atypical properties

of APH(9)-Ia are also mirrored in the sequence, which does not reveal significant similarity to either APH(3′)-IIIa or APH(3′)-IIa as assessed by the *BLAST* algorithm (Altschul *et al.*, 1990). Furthermore, multiple sequence alignment of aminoglycoside kinases suggests that there is less than 4% identity between APH(9)-Ia and APH(3′)-IIIa or APH(3′)-IIa (Wright *et al.*, 1998).

2. Materials and methods

2.1. Construction of the APH(9)-Ia expression vector

The APH(9)-Ia coding sequence was amplified by PCR such that *Nde*I and *Xho*I restriction sites were introduced at the N- and C-termini, respectively. The PCR product was ligated into the pET21b expression vector (Novagen) at the corresponding restriction sites such that a six-histidine tag linked by the dipeptide LE was appended to the C-terminus of the enzyme. DNA sequencing was performed on the coding region of the resulting construct to ensure that the correct nucleic acid sequence had been conserved through the PCR and subsequent genetic manipulations. The resulting expression vector, pET21b-APH(9)H, was transformed into *Escherichia coli* strain BB101 {genotype *ara* Δ(*lac proAB*) Δ*slyD* (*kan37*^{r}) *nalA argEam rif thi* F′[*lacI*^{q} *proAB*$^{+}$] (λDE3)}. Starter cultures were prepared by inoculating 5 ml Luria–Bertani (LB) medium containing 100 µg ml^{-1} ampicillin and incubating overnight at 310 K. Each overnight growth was then pelleted and resuspended in 1 ml fresh medium and then diluted in 1 l LB containing 100 µg ml^{-1} ampicillin. This culture was grown at 310 K to an OD_{600} of 0.2, at which point the temperature was lowered to 289 K at a rate of approximately 0.25 K min^{-1}. When the culture achieved an OD_{600} of 0.6, protein expression was induced by the addition of IPTG to a final concentration of 1 m*M*. Orbital shaking was maintained at 240 rev min^{-1} throughout bacterial growth. The cells were harvested 12–16 h post-induction by centrifugation (5000 rev min^{-1}, 277 K, 15 min).

2.2. Purification

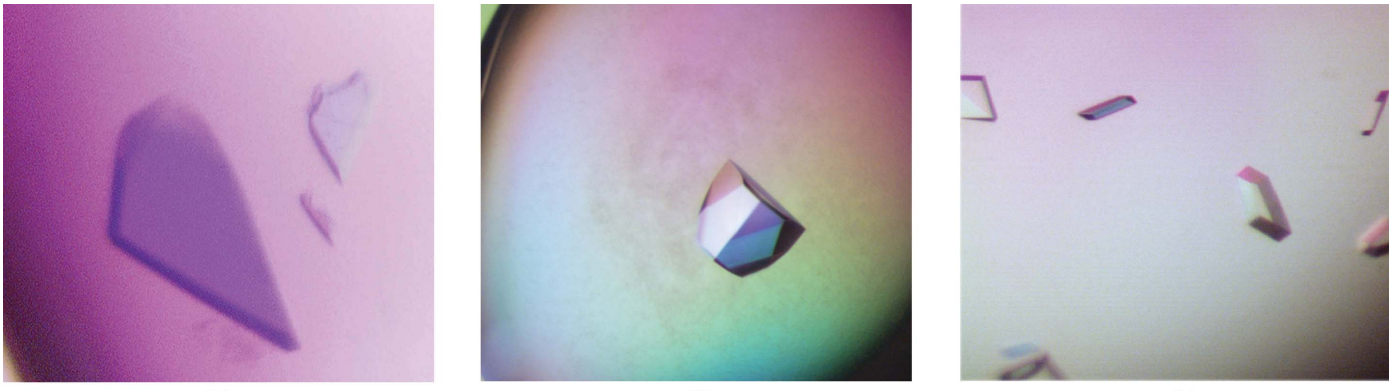
The cell pellet was resuspended in 50 ml buffer *A* (300 m*M* NaCl, 50 m*M* Tris–HCl pH 8.0) supplemented with 1 mg ml^{-1} lysozyme and incubated for 30 min at 277 K with mild agitation. The cells were then lysed by 20 cycles of sonication, each consisting of a 10 s pulse followed by 30 s of cooling on ice. The soluble fraction of the lysate was decanted after further centrifugation (15000*g*, 277 K, 30 min) and loaded directly onto 3 ml nickel–NTA resin (Qiagen) previously equilibrated with buffer *A*. The column was washed with 30 ml buffer *A* containing 5 m*M* imidazole and then with 20 ml buffer *A* containing 40 m*M* imidazole. The protein was eluted from the column with buffer *A* containing 300 m*M* imidazole in a single 15 ml fraction. The buffer was changed to 25 m*M* HEPES pH 7.5 and the protein was concentrated to approximately 20 mg ml^{-1} using an Ultrafree-15 Biomax-10K NMWL membrane concentrator (Millipore). Protein purity was assessed using SDS–PAGE and a single band corresponding to the APH(9)-Ia protein (molecular weight 39.6 kDa) was observed.

2.3. Crystallization

Crystallization of APH(9)-Ia was pursued under three different conditions, with the objective of ultimately gaining insight into the three-dimensional structures of the apoenzyme, the enzyme in complex with substrates and the enzyme in complex with an isoquinolinesulfonamide protein kinase inhibitor. Crystallization experiments were performed using the hanging-drop method, where drops consisting of equal volumes (2 µl) of protein and precipitant solutions were suspended from a siliconized glass cover slip over a reservoir containing 0.6–0.7 ml precipitant solution. The protein solution consisted of 10–15 mg ml^{-1} protein in 25 m*M* HEPES pH 7.5 and when appropriate a five-molar excess of ADP (Sigma) and spectinomycin (Sigma) or a five-molar excess of the kinase inhibitor CKI-7 (MJS Biolynx Inc.). In order to identify approximate conditions suitable for crystal growth, various precipitant solutions were tested at two temperatures (277 and 293 K) using commercial screening kits from Hampton Research, Emerald BioStructures and Nextal Biotechnologies. Subsequently identified conditions were optimized *via* a combination of various approaches, including variation of pH, alteration of the concentration of constituents, screening of additives (Hampton Research) and the employment of seeding.

2.4. Diffraction data collection

Diffraction data for APH(9)-Ia crystals grown under apoenzyme conditions and in the presence of substrates were collected at beamline X8C of the National Synchrotron Light Souce, Brookhaven National Laboratories, which is equipped with an ASDC Quantum CCD detector. Crystals were flash-frozen in a cold stream of nitrogen gas and data collection was performed at 100 K using an oscillation angle of 1.0° and a wavelength of 1.100 or 1.008 Å. Intensities were integrated and scaled using *HKL*2000 (Otwinowski & Minor, 1997). Diffraction data for APH(9)-Ia crystals grown in the presence of the inhibitor CKI-7 were collected on a Rigaku rotating copper-anode



(*a*) (*b*) (*c*)

Figure 1
Micrographs of APH(9)-Ia crystals grown in apoenzyme conditions (*a*), in the presence of ADP and spectinomycin (*b*) and in the presence of the protein kinase inhibitor CKI-7 (*c*). The largest crystal dimensions for each were ∼0.8, 0.6 and 0.5 mm, respectively.

Table 1
Crystallization conditions and diffraction data and processing statistics.

Values in parentheses are for the highest resolution shell.

	Apoenzyme	Enzyme + ADP + spectinomycin	Enzyme + CKI-7
Crystallization conditions	0.1 *M* MES pH 6.5, 30% PEG 200, 3% PEG 3000, 3% aminocaproic acid, 3% DMSO	0.1 *M* bicine pH 8.0, 8% PEG 8000, 0.2 *M* magnesium acetate, 10 m*M* manganese chloride	0.1 *M* HEPES pH 7.0, 10% MPD
Space group	$P2_1$	$P3_121/P3_221$	$P2_1$
Unit-cell parameters (Å, °)	a = 47.4, b = 70.7, c = 99.3, β = 95.9	$a = b$ = 75.0, c = 140.1	a = 47.7, b = 70.4, c = 100.2, β = 96.4
V_M (Å^3 Da^{-1})	2.09	2.87	2.11
Molecules per AU	2	1	2
Solvent content (%)	41.3	57.1	41.7
Radiation source	BNL-NSLS X8C	BNL-NSLS X8C	Rotating anode
Wavelength (Å)	1.100	1.008	1.5418
Resolution range (Å)	50–1.7 (1.76–1.7)	50–2.4 (2.49–2.4)	50-2.7 (2.8–2.7)
Unique reflections	71575 (7004)	17846 (1785)	17847 (2113)
Redundancy	4.3 (3.5)	9.3 (6.5)	3.5 (2.7)
Completeness (%)	99.3 (97.9)	95.3 (87.5)	96.4 (78.4)
$I/\sigma(I)$	16.3 (3.9)	15.0 (4.2)	8.1 (3.0)
R_{merge}† (%)	6.1 (43.4)	9.0 (37.1)	6.3 (25.0)

† $R_{merge} = \sum|I_i - \langle I_i\rangle|/\sum\langle I_i\rangle$, where I_i is the intensity of reflection i and $\langle I_i\rangle$ is the average intensity for that reflection.

generator equipped with Osmic confocal optics and an R-AXIS IV^{++} image-plate detector system. Analogous to data collection for other APH(9)-Ia crystals, crystals were flash-frozen in a cold stream and data collection was conducted at 100 K using an oscillation angle of 1.0°. Data processing of measured intensities was performed using *MOSFLM* (Leslie, 2003).

3. Results and discussion

Recombinant APH(9)-Ia from *L. pneumophila* was overexpressed in *E. coli* and purified to homogeneity with a yield of approximately 15–20 mg from 1 l bacterial culture. This is comparable to what others have achieved for this enzyme (Thompson *et al.*, 1998). Crystallization experiments identified three distinct conditions for crystal growth dependent on the presence or absence of substrates and/or inhibitor (Fig. 1). Analysis of the diffraction data reveals that APH(9)-Ia crystallized in two different crystal forms (Table 1). Crystals grown under apoenzyme conditions and in the presence of CKI-7 both belong to the monoclinic space group $P2_1$, with very similar unit-cell parameters. This is surprising given the significant differences in crystallization conditions. However, this observation may imply that APH(9)-Ia does not undergo a significant conformational change upon inhibitor binding, as has been suggested for APH(3′)-IIIa (Fong & Berghuis, 2002, 2004), but unlike what has frequently been observed for protein kinases (Cherry & Williams, 2004). Alternatively, it may be that CKI-7 is not bound to the enzyme in this crystal form.

We have attempted to determine the structure of APH(9)-Ia by molecular-replacement methods using APH(3′)-IIIa as a search model. Specifically, the following programs were tried: *CNS* (Brünger *et al.*, 1998) and *MOLREP* (Vagin & Teplyakov, 2000). However, none of our attempts provided a clear solution. This is not very surprising given the less than 4% identity between the search model and APH(9)-Ia, which is well below the limit of what has proven feasible (Schwarzenbacher *et al.*, 2004). The structure-determination strategy will therefore focus on using selenomethionine-substituted enzyme and multiwavelength anomalous dispersion experiments (Hendrickson, 1991).

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