New crystal structures of adenylate kinase from *Streptococcus pneumoniae* D39 in two conformations

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Adenylate kinases (AdKs; EC 2.7.3.4) play a critical role in intercellular homeostasis by the interconversion of ATP and AMP to two ADP molecules. Crystal structures of adenylate kinase from *Streptococcus pneumoniae* D39 (SpAdK) have recently been determined using ligand-free and inhibitor-bound crystals belonging to space groups $P2_1$ and $P1$, respectively. Here, new crystal structures of SpAdK in ligand-free and inhibitor-bound states determined at 1.96 and 1.65 Å resolution, respectively, are reported. The new ligand-free crystal belonged to space group $C2$, with unit-cell parameters $a = 73.5$, $b = 54.3$, $c = 62.7$ Å, $\beta = 118.8^\circ$. The new ligand-free structure revealed an open conformation that differed from the previously determined conformation, with an r.m.s.d on C^α atoms of 1.4 Å. The new crystal of the complex with the two-substrate-mimicking inhibitor P^1, P^5 -bis(adenosine-5'-)pentaphosphate (Ap5A) belonged to space group $P1$, with unit-cell parameters $a = 53.9$, $b = 62.3$, $c = 63.0$ Å, $\alpha = 101.9$, $\beta = 112.6$, $\gamma = 89.9^\circ$. Despite belonging to the same space group as the previously reported crystal, the new Ap5A-bound crystal contains four molecules in the asymmetric unit, compared with two in the previous crystal, and shows slightly different lattice contacts. These results demonstrate that SpAdK can crystallize promiscuously in different forms and that the open structure is flexible in conformation.

1. Introduction

Streptococcus pneumoniae, a Gram-positive pathogen, causes more than 1.6 million deaths worldwide per year (Liu *et al.*, 2012). Regulation of the intracellular ATP level plays a critical role in the survival of all organisms (Haase *et al.*, 1989; van Horssen *et al.*, 2009), including pneumococci. Adenylate kinases (AdKs) catalyze the formation of two ADP molecules from ATP and AMP in the presence of Mg^{2+} or other divalent metal ions (Dzeja & Terzic, 2009). The reaction catalyzed by AdKs is essential for metabolic monitoring and signalling pathways in living cells (Dzeja & Terzic, 2009).

AdKs are known to assume open, ligand-free and closed, inhibitor-bound conformations (Henzler-Wildman *et al.*, 2007; Müller & Schulz, 1992; Müller *et al.*, 1996). Conformational changes between the open and closed forms of AdKs have served as a model system to understand the mechanism of the reversible conformational transition (Jana *et al.*, 2011; Whitford *et al.*, 2007). Only three open, ligand-free AdK structures, from *Escherichia coli*, *Desulfovibrio gigas* and *Aquifex aeolicus*, have been reported (Müller *et al.*, 1996; Gavel *et al.*, 2004; Henzler-Wildman *et al.*, 2007), while a few dozen closed, inhibitor-bound structures are known.

Adenylate kinase from *S. pneumoniae* D39 (SpAdK) shares a common architecture with other known AdKs: an NMP-binding domain at the N-terminus and a core domain in the centre followed by a lid domain at the C-terminus. Recently, we reported the crystal structure of SpAdK in two conformations: a ligand-free structure in the open conformation from a crystal belonging to space group $P2_1$ (PDB entry 4ntz) and a structure with the two-substrate-mimicking inhibitor P^1, P^5 -bis(adenosine-5'-)pentaphosphate (Ap5A) bound in the closed conformation from a crystal belonging to space group $P1$ (PDB entry 4nu0) (Thach *et al.*, 2014). Here, we report new crystal structures of SpAdK in two conformations, ligand-free and

Table 1

Macromolecule information.

Source organism	<i>S. pneumoniae</i> D39
DNA source	Genomic DNA
Forward primer	GST tag, <i>Tobacco etch virus</i> protease cleavage site, <i>Bam</i> HI
Reverse primer	<i>Eco</i> RI
Cloning vector	Parallel GST2
Expression vector	Parallel GST2
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct	GANGSMNLLIMGLPGAGKGTQAAKIVEQFHVAHISTGDMFRA-AMANTQEMVLAKSYIDKGLVPDEVNTGIVKERLSQDDI-KETGFLLDGYPRTIEQAHALDKTLAELGIELEGIINIEVN-PDSLLERLSGRIIHRVTGETFHKVFNPVDYKEEDYYQRE-DDKPETVKRRLDVNIAQGEPIIAHYRAKGLVHD

Ap5A-bound SpAdK, along with biochemical characterizations of SpAdK and the SpAdK–Ap5A complex.

2. Materials and methods

2.1. Macromolecule production

SpAdK was cloned, expressed and purified as described elsewhere (Thach *et al.*, 2014). Briefly, the gene encoding AdK from *S. pneumoniae* D39 (accession No. NC_008533.1) was amplified by PCR from genomic DNA and inserted into a parallel GST2 vector (Sheffield *et al.*, 1999) using *Bam*HI and *Eco*RI restriction enzymes to yield pGST2–SpAdK (Table 1). GST–SpAdK was expressed in *E. coli* strain BL21(DE3) and purified by affinity chromatography followed by size-exclusion chromatography. The GST moiety was cleaved using *Tobacco etch virus* protease (Wu *et al.*, 2009).

Protein polydispersity was checked by dynamic light scattering on a DynaPro 100 system (Protein Solutions). Experiments were conducted at 295 K. Optimal buffer conditions for monodisperse SpAdK were screened by combining the following parameters: four pH values (6.5, 7.5, 8.0 and 8.5), six NaCl concentrations (0.1, 0.5, 1.0, 1.5, 2.0 and 2.5 M) and two divalent cations (Mg^{2+} and Mn^{2+}) at 1 mM. The concentration of the SpAdK protein was 10 mg ml^{−1} in a buffer with one of four pH values (50 mM NaCl, 50 mM Tris–HCl pH 6.5, 7.5, 8.0 or 8.5).

To determine the binding affinity of SpAdK to Ap5A, isothermal titration calorimetry (ITC) experiments were performed on a VP-ITC instrument (MicroCal). All buffer, protein and ligand solutions were degassed before use. 0.2 mM SpAdK in buffer A (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$) was placed in the sample cell and titrated against 2 mM Ap5A in buffer B (50 mM Tris–HCl pH 7.5, 50 mM NaCl) in a 292 µl injection syringe at 295 K. The total number of injections was 25, each of 10 µl in volume. The sample cell was stirred at 300 rev min^{−1}. In the reference cell, Ap5A was injected into buffer A.

2.2. Crystallization

Crystallization screening was carried out with protein at 18 mg ml^{−1} by the microbatch-under-oil method using screening kits from Hampton Research and Molecular Dimensions at 295 K. Initial conditions were further optimized by the hanging-drop vapour-diffusion method. Streak-seeding was used to enhance the size of the initial crystals to a size suitable for diffraction studies. Crystal morphology was employed as primary criterion to screen new crystal forms, along with determination of the unit-cell parameters. A diffraction-quality crystal of ligand-free SpAdK was obtained by mixing 2.5 µl protein solution with 2.5 µl reservoir solution consisting of 1.0 M sodium citrate, 0.1 M HEPES pH 7.5. A crystal of SpAdK–

Table 2

Crystallization.

Crystal	SpAdK	SpAdK–Ap5A
Method	Hanging-drop vapour diffusion	Hanging-drop vapour diffusion
Plate type	24-well plate	24-well plate
Temperature (K)	295	295
Protein concentration (mg ml ^{−1})	18	18
Buffer composition of protein solution	50 mM Tris–HCl pH 7.5, 50 mM NaCl	50 mM Tris–HCl pH 7.5, 50 mM NaCl
Composition of reservoir solution	1.0 M sodium citrate, 0.1 M HEPES pH 7.5	0.2 M sodium acetate pH 7.2, 30% PEG 8K
Volume and ratio of drop	2.5 µl protein solution and 2.5 µl reservoir solution	2.5 µl protein solution and 2.5 µl reservoir solution
Volume of reservoir (µl)	500	500

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Crystal	SpAdK	SpAdK–Ap5A
Diffraction source	Beamline 7A, PAL	BL26B1, SPring-8
Wavelength (Å)	1.0	1.0
Temperature (K)	100	100
Detector	ADSC Quantum Q270r	Rigaku Jupiter 210
Crystal-to-detector distance (mm)	200	200
Rotation range per image (°)	1	1
Total rotation range (°)	360	360
Exposure time per image (s)	1	1
Space group	C2	P1
<i>a</i> , <i>b</i> , <i>c</i> (Å)	73.5, 54.3, 62.7	53.9, 62.3, 63.0
α , β , γ (°)	90, 118.8, 90	101.9, 112.6, 89.9
Mosaicity (°)	0.72	1.06
Resolution range (Å)	29.06–1.96 (1.99–1.96)	46.99–1.65 (1.68–1.65)
Total No. of reflections	70292	319630
No. of unique reflections	15454 (754)	85180 (4245)
Completeness (%)	98.9 (96.1)	95.9 (94.5)
Multiplicity	4.5 (3.7)	3.8 (3.7)
$\langle I/\sigma(I) \rangle$	12.3 (6.3)	39.0 (4.4)
<i>R</i> _{int} (%)	9.5 (21.2)	8.8 (38.6)
Overall <i>B</i> factor from Wilson plot (Å ²)	21.4	9.9

Ap5A was obtained by mixing 2.5 µl protein solution with 2.5 µl reservoir solution consisting of 0.2 M sodium acetate pH 7.2, 30% PEG 8K at 295 K. Detailed crystallization information is summarized in Table 2.

2.3. Data collection and processing

The crystals were transferred into a cryoprotectant solution containing 12% and 10% glycerol for SpAdK and SpAdK–Ap5A, respectively. Diffraction data were collected from a single cooled crystal in a 100 K gaseous nitrogen stream on BL-5A at Photon Factory, beamline BL26B1 at SPring-8 and beamlines 5C and 7A at Pohang Accelerator Laboratory. Diffraction data from the ligand-free and AP5A-bound crystals were collected over a range of 360° with a rotation angle per image of 1.0°. The native and complex crystals diffracted to maximum resolutions of 1.96 and 1.65 Å, respectively, using a crystal-to-detector distance of 200 mm. Data processing and reduction were carried out using *HKL-2000* (Otwinowski & Minor, 1997) and *POINTLESS* (Evans, 2006). The data-collection and processing statistics are summarized in Table 3.

2.4. Structure solution and refinement

The ligand-free SpAdK structure was solved by molecular replacement using *PHENIX* (Adams *et al.*, 2010) with the open SpAdK structure in space group *P*₂₁ (PDB entry 4ntz; Thach *et al.*, 2014) as

Table 4

Structure-refinement statistics.

Values in parentheses are for the outer shell.

Crystal	SpAdK	SpAdK–Ap5A
Resolution range (Å)	29.06–1.96	46.99–1.65
Completeness (%)	98.9 (94.0)	95.7 (90.0)
σ Cutoff	0	0
No. of reflections, working set	15309 (491)	84877 (2499)
No. of reflections, test set	1676 (138)	4002 (124)
Final R_{cryst}	0.184	0.259
Final R_{free}	0.217	0.287
No. of non-H atoms		
Protein	1627	6576
Ion	—	4
Ligand	31	324
Water	169	582
Total	1827	7486
R.m.s. deviations		
Bonds (Å)	0.020	0.007
Angles (°)	0.699	1.916
Average B factors (Å ²)		
Protein	41.74	30.45
Ion	—	22.00
Ligand	79.17	27.75
Water	44.20	34.72
Ramachandran plot		
Favoured regions (%)	96.57	98.10
Additionally allowed (%)	2.94	0.95
Outliers (%)	0.49	0.95

a search model. Iterative model building and refinement were performed using *Coot* (Emsley & Cowtan, 2004) and *PHENIX*. The structure of the SpAdK–Ap5A complex was solved by molecular replacement using the same complex structure (PDB entry 4nu0; Thach *et al.*, 2014) as a search model. Structure-refinement statistics are summarized in Table 4.

3. Results and discussion

3.1. Protein characterization and crystallization

The purified SpAdK showed a single band of approximately 24 kDa on SDS–PAGE, consistent with the calculated molecular mass of SpAdK (23 721 Da; Supplementary Figs. S1a and S1b¹). Mono-dispersity of the protein is often believed to be critical for successful crystallization (Proteau *et al.*, 2010). The optimal buffer condition was found to include 50 mM Tris–HCl pH 7.5, 150 mM NaCl and 1 mM MgCl₂. SpAdK in the optimal buffer showed 9.2% polydispersity and a hydrodynamic radius of 2.07 nm as judged by DLS (Supplementary Fig. S1c). Ap5A is known to inhibit AdKs in general (Müller & Schulz, 1992). The interaction of SpAdK with Ap5A was characterized by isothermal titration calorimetry (ITC). The ITC data were best fitted with a two-site model ($\Delta H_1 = -2175 \pm 975$ kcal mol^{−1} and $\Delta H_2 = 570 \pm 368$ kcal mol^{−1}). This supports juxtaposition of the lid domain with the core domain to embrace a substrate followed by its mediation of binding between the substrate and the NMP domain (Jana *et al.*, 2011). The binding affinities of SpAdK for Ap5A were 57 ± 2 nM for K_{d1} and 177 ± 53 nM for K_{d2} (Supplementary Fig. S1d).

New ligand-free SpAdK and SpAdK–Ap5A crystals appeared in one week. The crystal morphology of the new crystals was different from those reported previously (Thach *et al.*, 2014) in that the new crystals of both types were plate-shaped compared with the previous needle-shaped ligand-free crystals and trigonal-shaped SpAdK–Ap5A crystal.

¹ Supporting information has been deposited in the IUCr electronic archive (Reference: FT5051).

3.2. Crystal structures in two conformations

The new ligand-free SpAdK crystal diffracted to 1.96 Å resolution and the SpAdK–Ap5A crystal diffracted to 1.65 Å resolution. The new ligand-free crystal belonged to space group *C2*, which is different from the previously reported crystal in space group *P2*₁. Five residues of the loop in the lid domain (Lys140–Pro144) are missing in the final model because of a lack of electron density. The new ligand-free structure (PDB entry 4w5h) adopts an open conformation similar to that of the previous open structure (PDB entry 4ntz). Superposition of these structures showed that the largest shifts of C α atoms are localized in the lid domain, with an overall r.m.s.d. of 1.4 Å on C α atoms (Fig. 1). This result supports the importance of the relative

Figure 1

New ligand-free SpAdK crystal structure in the open conformation. (a) Local conformational changes of the new ligand-free SpAdK structure (PDB entry 4w5h) in comparison to the previously reported structure (PDB entry 4ntz). The r.m.s.d. on C α atoms in the two structures is shown as a sausage representation, with red colour indicating the largest r.m.s.d. and blue the smallest. The lid domain (arrow) undergoes the largest local conformational change. Tris and HEPES molecules are shown as stick models. (b) Top, SpAdK domain architecture. The boundaries of the domains are shown. Bottom, r.m.s.d. on C α atoms by residue between the new (PDB entry 4w5h) and previously reported (PDB entry 4ntz) SpAdK structures.

Figure 2

Lattice contacts in (a) the new SpAdK–Ap5A crystal structure (PDB entry 4w5j) and (b) the previously reported structure (PDB entry 4nu0). The N-termini of the SpAdK molecules in the asymmetric unit are labelled. Four SpAdK molecules are depicted as surface representations and are coloured blue, cyan, orange and green. Unit cells are shown in red lines.

orientation of the lid and NMP domains in binding the substrates of AdK (Henzler-Wildman *et al.*, 2007). The new ligand-free structure also suggests that the open conformation of SpAdK is dynamic and pH-dependent. The previous crystal structure was obtained in basic buffer (pH 9.5), while the ligand-free structure reported here was determined from a crystal grown in neutral buffer (pH 7.5). The different pH values may have captured SpAdK in slightly different conformations.

Although the SpAdK–Ap5A crystal reported here belonged to the same space group (*P1*) as the previous crystal, the new SpAdK–Ap5A crystal contains four molecules in the asymmetric unit instead of two molecules. The new SpAdK–Ap5A structure (PDB entry 4w5j) assumed a closed conformation, as did the previously reported complex structure (PDB entry 4nu0), with an r.m.s.d of 0.4 Å on C α atoms. The conditions for the new SpAdK–Ap5A crystal are different from those for the previous crystal in that the new crystallization conditions lack the 50 mM NaF (Thach *et al.*, 2014). Not only is the number of molecules in the asymmetric unit different, but the lattice contacts are slightly changed. The dimensions of the unit cell in the new crystal are different, with *a*, *b* and *c* being 14, 14 and 11 Å longer, respectively. This difference in unit-cell dimensions appears to be caused by a slight distortion of symmetry-related molecules in three directions (Fig. 2). The four molecules in the asymmetric unit form two planes with two molecules per one plane. The two planes are rotated from each other by 12° (Supplementary Fig. S2). In summary, we show here that SpAdK can crystallize in different forms and that the open structure can assume multiple conformations.

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