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Structure of AadA from *Salmonella enterica*: a monomeric aminoglycoside (3")\(9\) adenylyltransferase

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Aminoglycoside resistance is commonly conferred by enzymatic modification of drugs by aminoglycoside-modifying enzymes such as aminoglycoside nucleotidyltransferases (ANTS). Here, the first crystal structure of an ANT(3")\(9\) adenylyltransferase, AadA from *Salmonella enterica*, is presented. AadA catalyses the magnesium-dependent transfer of adenosine monophosphate from ATP to the two chemically dissimilar drugs streptomycin and spectinomycin. The structure was solved using selenium SAD phasing and refined to 2.5 Å resolution. AadA consists of a nucleotidyltransferase domain and an \(\alpha\)/C\(11\) helical bundle domain. AadA crystallizes as a monomer and is a monomer in solution as confirmed by small-angle X-ray scattering, in contrast to structurally similar homodimeric adenylylating enzymes such as kanamycin nucleotidyltransferase. Isothermal titration calorimetry experiments show that ATP binding has to occur before binding of the aminoglycoside substrate, and structure analysis suggests that ATP binding repositions the two domains for aminoglycoside binding in the interdomain cleft. Candidate residues for ligand binding and catalysis were subjected to site-directed mutagenesis. *In vivo* resistance and *in vitro* binding assays support the role of Glu87 as the catalytic base in adenylation, while Arg192 and Lys205 are shown to be critical for ATP binding.

1. Introduction

Ever since the discovery of the first aminoglycoside antibiotic, streptomycin (Schatz *et al.*, 1944), which was isolated from *Streptomyces griseus*, these broad-spectrum antibiotics have been widely used in the treatment of bacterial infections. Aminoglycosides have been shown to increase misreading and to inhibit translocation in bacterial translation as well as to damage the cell membrane (Davis, 1987). Crystal structures of aminoglycosides bound to the 30S ribosomal subunit (Brodersen *et al.*, 2000; Carter *et al.*, 2000; Demirci *et al.*, 2013) shed new light on their mechanisms of increasing errors in decoding. Streptomycin has been shown to stabilize the ribosomal ambiguity (\(\text{ram}\)) or error-prone state of the 30S subunit as well as to perturb the structure of the decoding centre, while other aminoglycosides such as paromomycin instead influence the conformations of the 16S rRNA bases directly involved in decoding (Carter *et al.*, 2000; Demirci *et al.*, 2013). Spectinomycin is an aminoglycoside-like aminocyclitol that inhibits translocation through preventing conformational changes of the head domain of the 30S subunit (Borovinskaya *et al.*, 2007).

Resistance to aminoglycosides can be acquired by four general mechanisms: reduction of the drug concentration in the cell by efflux pumps, decreased uptake of aminoglycosides into the cell via decreased cell-membrane permeability,
Aminoglycoside nucleotidyltransferase AadA

2. Materials and methods

2.1. Cloning of aadA into pEXP5-CT

Bacterial strains and plasmids are listed in Supplementary Table S1. The aadA gene was PCR-amplified from a colony suspension of S. enterica serovar Typhimurium strain LT2 using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) according to the manufacturer’s instructions with the primers aadA_start_Fwd and aadA_CT_Rev (Supplementary Table S2) and was cloned into the pEXP5-CT/TOPO vector (Invitrogen), which cut within the aadA gene. 5 ng of the linearized plasmid was used as template in a PCR reaction using screened for the correct insert by PCR and sequencing using the primers T7_Forward and T7_Term_Reverse (Supplementary Table S2). The resulting plasmid pEXP5-CT-aadA encodes the complete AadA sequence followed by a C-terminal linker and hexahistidine tag (KGHHHHHH).

2.2. Construction of aadA point mutations

The eight point mutations in aadA were generated in two steps. A cat-sacB-T0 cassette (GenBank KM018298) containing the cat gene (conferring chloramphenicol resistance) and the Bacillus subtilis sacB gene (conferring sensitivity to sucrose) was inserted at the five target codons (codons 87, 112, 182, 192 and 205) using λ-Red recombinering (Datsenko & Wanner, 2000; Datta et al., 2006), selecting chloramphenicol-resistant colonies. In the second step, a 70-mer oligonucleotide containing the designed mutation in the middle was used in a λ-Red transformation to replace the cat-sacB-T0 cassette, selecting sucrose-resistant cells that had lost the sacB gene. Sucrose-resistant, chloramphenicol-sensitive transformants were verified by PCR and sequencing of the aadA gene.

2.3. Gap-repair cloning of mutant aadA alleles

To transfer the mutant aadA alleles from the chromosome to the pEXP5-CT-aadA plasmid, a gap-repair cloning strategy was used. The pEXP5-CT-aadA plasmid containing the wild-type aadA gene was linearized using Stul and PvuII (Thermo Scientific), which cut within the aadA gene. 5 ng of the linearized plasmid was used as template in a PCR reaction using

![Figure 1](image)

Aminoglycoside substrates of the ANT(3')(9) AadA. (a) Streptomycin with the adenylation-site 3'-hydroxyl in bold. (b) Spectinomycin with the adenylation-site 9-hydroxy in bold.
2.4. MIC determinations (E-tests)

Determination of the minimum inhibitory concentrations (MICs) of streptomycin, spectinomycin, amikacin, tobramycin, gentamicin and kanamycin were performed using E-tests (bioMérieux). As AadA is not expressed during growth on rich medium (Koskiniemi et al., 2011), the tests were performed using minimal medium. Cultures grown overnight at 37°C in liquid M9 + 0.2% glycerol medium were diluted 500-fold and swabbed onto M9 + 0.2% glycerol agar plates using sterile cotton swabs. E-test strips were applied to the plates, which were incubated at 37°C for approximately 24 h. The MICs were read as the lowest concentration of antibiotic at which no bacterial growth was visible.

2.5. Expression and purification of Aad protein

pEXP5-CT-aadA was transformed into Escherichia coli BL21 Star cells. To express native wild-type or mutant AadA protein, a 1 l culture in LB with 100 μg ml⁻¹ ampicillin was inoculated with 10 ml overnight culture and incubated at 37°C until an OD₆₀₀ of 0.6 was reached, and then chilled to 16°C before induction with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 24 h. Expression of selenomethionine-substituted AadA was performed according to a standard protocol (Van Duyne et al., 1993). Cells were harvested by centrifugation and stored at -20°C.

All AadA variants were purified using the same protocol. Cells were resuspended in buffer A (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 50 mM imidazole, 5 mM β-mercaptoethanol) containing DNase, RNase, lysozyme and complete protease inhibitor (Roche) and were lysed using a cell disruptor (Constant Systems). After centrifugation in an SS34 rotor at 16 000 rev min⁻¹ for 30 min, the supernatant was loaded onto a pre-equilibrated Ni Sepharose gravity column and incubated under slow rotation at 4°C for 1 h. The column was washed extensively with buffer A and with buffer B containing 500 mM NaCl, and AadA was eluted with buffer B (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 500 mM imidazole, 5 mM β-mercaptoethanol). Protein-containing fractions were loaded onto a HiLoad 16/60 Superdex 75 gel-filtration column equilibrated with buffer C (50 mM Tris–HCl pH 7.5, 200 mM NaCl, 5 mM β-mercaptoethanol). Peak fractions were concentrated to 10 mg ml⁻¹ and used directly for crystallization or stored at -80°C after shock-freezing in liquid nitrogen.

2.6. Crystallization

Crystallization was performed using the sitting-drop method at 8°C. Crystals appeared in 24 h in the Morpheus screen (Molecular Dimensions) with a drop size of 2 μl and a reservoir solution consisting of 0.12 M salts, 0.1 M Morris buffer system 1 pH 6.5 and 30% ethylene glycol/PEG 8000. Most crystals grew as thin plates with dimensions of around 50 x 100 μm, while a few appeared as thin rods. Plate-shaped crystals were fished out directly from the drop and vitrified in liquid nitrogen for data collection.

2.7. Data collection and structure determination

All data were collected on beamline ID14-4 at ESRF, Grenoble, France. Initial phases were obtained by single-wavelength anomalous diffraction (SAD) phasing using crystals of selenomethionine-substituted protein and data collected at the peak wavelength of 0.9793 Å, as determined by a fluorescence scan. Data were integrated and scaled using XDS (Kabsch, 2010) and AIMLESS (Evans & Murshudov, 2013) (Table 1) and suggested one molecule per asymmetric unit, with 51% solvent content and a Matthews coefficient of 2.53 Å³ Da⁻¹ (Matthews, 1968).

Three Se sites were identified using AutoSol implemented in PHENIX (Adams et al., 2010). The figure of merit was 0.37 (0.72 after density modification). An initial model with an R_work and R_free of 0.38 and 0.40, respectively, and 200 out of
262 residues (44 with unassigned sequence) was built and refined using AutoBuild in PHENIX. Further manual rebuilding was aided by B-factor map sharpening in Coot (Emsley et al., 2010). A model consisting of 252 amino acids was built and refined using phenix.refine (Afonine et al., 2012) to an $R_{\text{work}}$ and $R_{\text{free}}$ of 0.23 and 0.26, respectively (Table 1). TLS refinement was implemented in the last round of refinement. Atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession code 4cs6.

2.8. Structure analysis

Detailed structure comparisons were performed using SSM in Coot (Emsley et al., 2010) and the LSQ commands in O (Jones et al., 1991; Kleywegt & Jones, 1997), which were also used as the basis for structure-based sequence alignment. Surface-conservation analysis was performed using the ConSurf server (Celniker et al., 2013; Ashkenazy et al., 2010). Structure figures were made using PyMOL (v.1.2r3pre, Schrödinger). Multiple sequence alignment was performed using ClustalW (Larkin et al., 2007). The EsPript server (Gouet et al., 2003) was used to prepare sequence-alignment figures.

2.9. Isothermal titration calorimetry (ITC) binding experiments

Binding studies were performed at 25°C using a MicroCal iTC200 instrument (GE Healthcare). Wild-type or mutant AadA at 20–30 µM concentration was dialyzed overnight against 50 mM Tris–HCl pH 7.5, 200 mM NaCl, 5 mM MgCl$_2$, 1 mM tris(2-carboxyethyl)phosphine and titrated with 500–1000 µM ATP, streptomycin (Sigma) or spectinomycin (Sigma) freshly dissolved in the same batch of dialysis buffer prior to each experiment. For titration of streptomycin/spectinomycin in the presence of ATP, a first titration of AadA with ATP to saturation was followed by a second titration with streptomycin/spectinomycin in buffer containing an equivalent concentration of ATP as in the cell. In this way, heats of dilution of ATP were avoided. At least 36 consecutive injections of 2 µl were applied at 2 min intervals. The data were analyzed using the MicroCal Analysis plugin in Origin. All ITC data were analyzed assuming one set of binding sites. Each experiment was performed at least twice. For spectinomycin, the concentration used to fit the data was adjusted to the estimated active concentration in binding to AadA, assuming 1:1 binding.

2.10. SAXS measurements and analysis

SAXS data were collected on beamline P12 at the PETRA synchrotron, EMBL, Hamburg, Germany (Blanchet et al., 2015). For data collection, 1 mg ml$^{-1}$ AadA was dialyzed against buffer C and was further concentrated to 10 mg ml$^{-1}$. The concentration was determined using a Rudolph Research Analytical J357 refractometer.

Data were measured at concentrations of 1, 2, 5 and 10 mg ml$^{-1}$ and normalized to the intensity of the transmitted beam, and the scattering of the buffer was subtracted. Data processing was performed using the ATSAS software package (Petoukhov et al., 2012). Theoretical scattering curves were calculated from PDB coordinates and fitted to the experimental scattering curve at 5 mg ml$^{-1}$ concentration using CRYSOL (Svergun et al., 1995). The radius of gyration was computed using GNOM (Svergun, 1992). The molecular weight was estimated from the Porod volume using bovine serum albumin as a standard.

3. Results and discussion

3.1. Structure determination of AadA

AadA crystals grew in space group $P2_12_12$ and diffracted to 2.5 Å resolution (Table 1). Trials to perform molecular
replacement using search models with low sequence identity failed, and the structure was solved using SAD phasing with selenomethionine-substituted AadA crystals. In order to assist in model building, a DALI search (Holm & Rosenström, 2010) was performed with the incomplete autobuilt N-terminal domain. The top hit, with an r.m.s.d. of 2.6 Å over 96 Cα atoms, was a hypothetical protein from Haemophilus influenzae (PDB entry 1no5; Lehmann et al., 2005) classified as a nucleotide-binding domain of a two-protein nucleotidyltransferase. Although this structure was not successful as a molecular-replacement search model, the connectivity was similar and it could be used to guide the manual building of the remaining parts of the N-terminal domain of AadA. The final model contains residues 3–262 and only lacks the first two N-terminal residues and the His tag. The loop regions 97–103 and 235–240 display weak density, indicating flexibility.

3.2. Overall structure of AadA

AadA consists of two domains that together form a bi-lobed 55 × 40 × 35 Å structure (Fig. 2a). The N-terminal domain (residues 3–157) forms a nucleotidyltransferase fold according to the SCOP database (Murzin et al., 1995) and has a central five-stranded mixed β-sheet surrounded by six α-helices. The β-strands β2 and β3 are parallel and the others are anti-parallel. The long α-helices α1, α2, α4 and α5 surround the central β-sheet, whereas α3 is a single-turn helix following β3 and α6 is another short helix that follows β5 before continuing to the C-terminal domain. The C-terminal domain (residues 158–262) consists of five α-helices forming an up-and-down α-helical bundle.

3.3. Comparison of AadA to similar structures

A search for similar structures in the PDB was performed using the DALI server (Holm & Rosenström, 2010). In a DALI search with the entire AadA molecule, the top hit, with a Z-score of 9.5, was the ANT(4) kanamycin nucleotidyltransferase (KNTase) from S. aureus (PDB entry 1kny; Pedersen et al., 1995; r.m.s.d. of 4.8 Å for 187 Cα atoms), which showed 14% amino-acid sequence identity to AadA. Several hypothetical predicted nucleotidyltransferases proteins also show similarities to AadA, with Z-scores from 8.9 to 6.7. Among these, the most similar is the hypothetical protein HI0073 (PDB entry 1no5; Lehmann et al., 2005), which was used as a guide for building the N-terminal domain of AadA. The lincosamide nucleotidyltransferase LinB from Enterococcus faecium (PDB entry 3jz0; Morar et al., 2009) shows a lower similarity to AadA, with a Z-score of 6.7 and an r.m.s.d. of 5.6 Å for 173 Cα atoms, with only 9% sequence identity. Other available ANT structures displayed low structural similarity to AadA. Of the DALI hits, S. aureus KNTase (Chen-Goodspeed et al., 1999; Pedersen et al., 1995) and E. faecium LinB (Morar et al., 2009) were biochemically characterized as nucleotidyltransferases acting on drug substrates, and structures were available in complex with the ATP analogue AMPCPP and the drug substrate. Further comparisons were performed with these two structures, both of which consist of N-terminal nucleotidyltransferase domains and C-terminal helical bundle domains. However, both of these proteins crystallize as homodimers, and the orientation between the two domains is distinct from that in AadA. Careful domain-by-domain superpositioning between AadA and KNTase shows that in the N-terminal domain helix α1 and the central β-sheet formed by β1–β5 of AadA superpose well on their equivalent secondary-structure elements in KNTase (r.m.s.d. of 1.59 Å for 81 Cα atoms; Fig. 2b), and in the C-terminal domain helices α8–α11 of AadA superpose on their equivalents (r.m.s.d. of 2.23 Å for 57 Cα atoms; Supplementary Fig. S1). In the superpositioning of AadA with LinB, in the N-terminal domain helix α1 and strands β1–β3 from AadA superpose on LinB (r.m.s.d. of 2.05 Å for 73 Cα atoms).
and in the C-terminal domain helices α7, α8 and α11 superpose on LinB (r.m.s.d. of 2.02 Å for 49 Cα atoms). Helices α2 and α4 in the N-terminal domain of AadA do not have any equivalents in the KNTase or LinB structures. Also, the connectivity of β2 and β3, as well as β4 and β5, in AadA is distinct from the other two structures.

3.4. Oligomeric state of AadA

3.4.1. Structure comparison with KNTase and LinB. Both KNTase and LinB function as homodimers with very similar dimerization contacts and two active sites located at the dimer interfaces. *S. aureus* KNTase was believed to be monomeric from an early study using native gels (Sadaie et al., 1980). Yet, the protein purified using the same procedure crystallized as a homodimer. Each monomer has a very extended conformation, and the binding pockets for kanamycin and ATP are formed by both subunits (Supplementary Fig. 2a). *S. enterica* AadA was purified as a monomer in size-exclusion chromatography and crystallized as a monomer. It has a more closed domain arrangement compared with KNTase (Fig. 2b) and more extensive interdomain interactions within the monomer. In KNTase, the conserved ligand-binding surface displays a negative charge, whereas the conserved dimerization surface
is uncharged (Supplementary Fig. S2b). Could the AadA structure potentially open up to also form a homodimer with two active sites? We find this unlikely for two reasons. Firstly, the surface corresponding to the dimer interface in KNTase is negatively charged and nonconserved, making it unlikely to be involved in the formation of a homodimer. Secondly, the highly conserved helix \( \eta2 \) preceding \( \alpha4 \) in AadA that has no equivalent in KNTase (see below) would clash with the packing of helix \( \alpha6 \) in the C-terminal domain of KNTase against the \( \beta \)-sheet of the N-terminal domain. Thus, we propose that AadA represents an ANTase that functions as a monomer.

3.4.2. SAXS studies of AadA in solution. To confirm the oligomeric state of AadA in solution, we performed a SAXS experiment on apo AadA. There were no signs of concentration-dependent protein aggregation at 1–5 mg ml\(^{-1} \) concentration. The resulting Guinier plot was linear, consistent with a monodisperse protein preparation (Fig. 3a). The molecular mass calculated from the SAXS data was 29 kDa. Comparison of the experimental SAXS data with the scattering curve predicted from the crystal structure (Fig. 3b) gave an excellent fit with a \( \chi \) value of 1.9, while the predicted scattering curves from the KNTase monomer and dimer (PDB entry 1kny) gave poor fits, with \( \chi \) values of 11.3 and 35.5, respectively. The radius of gyration (\( R_g \)) determined from the Guinier plot was 23 Å, which agrees well with the \( R_g \) of 20 Å calculated from the AadA crystal structure. Thus, the SAXS data confirm that AadA is indeed a monomer in solution and that the crystal structure agrees well with the solution structure.

3.5. Multiple sequence alignment

A BLAST search of the UniRef90 database identified full-length homologues of \( S. \) enterica AadA mainly in enterobacteria, proteobacteria and firmicutes. A representative set of these, displaying 36–81% sequence identity to the search sequence, was used for multiple sequence alignment (Fig. 4a). In the N-terminal domain, residues involved in the hydrophobic packing between the \( \beta \)-sheet and the four long helices are well conserved, and the same is true for the interface between helices \( \alpha 8, \alpha 9 \) and \( \alpha 10 \) in the C-terminal domain. Among the conserved residues in AadA, Ser36 and Asp47 in the \( \alpha 2 \)–\( \alpha 3 \) loop, Asp49 in \( \alpha 3 \), Glu87 and Thr89 in \( \alpha 3 \), Trp112 in \( \alpha 4 \), Asp182 and Arg192 in \( \alpha 9 \) and Lys205 in the \( \alpha 9 \)–\( \alpha 10 \) loop.
are exposed at the surface and point towards the interdomain space.

3.6. Ligand-binding and catalytic sites of AadA

We attempted without success to co-crystallize and soak AadA with its substrates streptomycin or spectinomycin together with the nonhydrolysable ATP analogue AMPCPP and magnesium. AadA has a pI of 5 and the electrostatic surface potential shows that the cleft between the two domains displays a strong negative charge (Fig. 4b). Mapping of surface conservation using ConSurf (Ashkenazy et al., 2010; Celniker et al., 2013) shows that this is also where the AadA sequence displays the highest conservation (Fig. 4c; see below). The negative charge may mimic the nucleic acid environment that the aminoglycosides bind to in the ribosome (Romanowska et al., 2013) and is required for binding of the positively charged drug molecules.

3.6.1. Comparative analysis of ligand-binding sites

The structure of KNTase has been solved in the presence of kanamycin A (Pedersen et al., 1995) and the structure of the more distantly related LinB has been solved in complex with clindamycin (Morar et al., 2009), in both cases in the presence of AMPCPP and magnesium, thus allowing comparison of these structures with the apo structure of AadA. In both of these structures the ligands bind between the N-terminal domain of one monomer and the C-terminal domain of the second monomer in the dimer.

In KNTase, residues from both subunits make up the nucleotide-binding site (Fig. 5a). From one subunit, Ser39 and Ser49 coordinate to the β/C13-phosphate of AMPCPP, Arg42 forms hydrogen bonds to the β/C12-phosphate of AMPCPP and the ribose, Asp50 and Glu52 coordinate to the Mg²⁺ ion and Thr187 forms a hydrogen bond with the β-phosphate. From the other subunit, Glu145 and Lys149 form hydrogen bonds to the α-phosphate of AMPCPP. KNTase can use also other nucleotides as substrates, and there is no specific interaction between the KNTase and the base (Pedersen et al., 1995). The nucleotide-binding pocket of LinB is very similar to that of KNTase (Fig. 5b) and is formed by Ser29, Ser39, Asp40, Glu42 and Glu89 from one subunit and Arg165 and Arg170 from the other subunit (Morar et al., 2009). The phosphate-coordinating serine residues and the magnesium-chelating acidic residues in KNTase and LinB (Morar et al., 2009; Pedersen et al., 1995; Figs. 5a and 5b) are conserved in AadA, where the corresponding residues that are likely to adopt the same roles are Ser36, Ser46, Asp47, Asp49 and Glu87 (Fig. 4c). Apart from these residues, there is almost no sequence conservation between AadA and LinB. Therefore, structure-based sequence alignment (Supplementary Fig. S3) was only performed between AadA and KNTase.

In KNTase, Glu145 from the second subunit was proposed to be the catalytic base (Pedersen et al., 1995), but there is no structurally equivalent residue in AadA (Supplementary Fig. S3). In LinB Glu89 was proposed to be the catalytic base, and it was confirmed by mutagenesis that this residue is essential for catalysis (Morar et al., 2009). The equivalent residue in AadA, Glu87, is strictly conserved (Fig. 4a), making it a good candidate for the catalytic base. In KNTase, this corresponds to Glu76, which is located in close proximity to the substrate kanamycin but does not have a clear role. However, in the overlay based on the N-terminal domain, the carboxylic O atoms of Glu145 of KNTase are within 2.5–5 Å of those of Glu87 in AadA, suggesting that these residues may play the same role in these enzymes, catalyzing the same reaction at different positions of different substrates.

Figure 6

ITC curves for the titration of (a) ATP, (b) streptomycin and (c) spectinomycin with wild-type AadA. The top panels show the raw data and the bottom panels show the binding isotherms.
A clear chemical explanation for why only about half of the potent spectinomycin molecules bind to AadA, but it could possibly be related to the carbonyl–diol equilibrium of the drug in aqueous solution (Bryskier, 2005).

These results indicate that binding of ATP and magnesium between the two domains of AadA will orient the two domains for binding of either of the aminoglycoside substrates to the intersubunit pocket and that the O atom between the α- and β-phosphates forms a critical interaction with AadA. Thus, AadA binds ATP before the aminoglycoside substrate, in contrast to KNTase, where kanamycin first binds to a lower affinity nonspecific binding site and then relocates to the final binding cleft when a nucleotide is present (Matesanz et al., 2012). This also agrees with the pre-formed ATP-binding site in the dimeric apo KNTase structure and the closed ATP-binding site of the present apo AadA structure. It is most likely that ATP binding will induce an open conformation of the structure in which residues from the two domains are correctly positioned for substrate recognition.

### 3.7. Mutational studies of AadA

To test our hypotheses about the roles of the conserved amino acids Glu87, Trp112, Asp182 and Lys205 in ligand binding and catalysis, we generated the following AadA mutants by mutating the chromosomal *aadA* gene: E87A, E87Q, W112A, W112F, D182A, D182N, R192A and K205A.

**Table 2**

| Strain         | *aadA* genotype/ amino-acid substitution | Streptomycin | Spectinomycin | Kanamycin | Tobramycin | Amikacin | Gentamicin |
|---------------|-----------------------------------------|-------------|---------------|-----------|------------|----------|------------|
| DA6192        | Wild type                               | 128         | 192           | 6         | 3          | 4        | 0.75       |
| DA8900        | *aadA::cat*                              | 4           | 24            | 6         | nd         | nd       | nd         |
| DA29580       | E87A                                    | 4           | 24            | 6         | nd         | nd       | nd         |
| DA29582       | E87Q                                    | 4           | 24            | 6         | nd         | nd       | nd         |
| DA29584       | W112A                                   | 16          | 24            | 6         | nd         | nd       | nd         |
| DA29586       | W112F                                   | 48          | 24            | 6         | nd         | nd       | nd         |
| DA29588       | D182A                                   | 32          | 48            | 6         | nd         | nd       | nd         |
| DA29590       | D182N                                   | 24          | 48            | 6         | nd         | nd       | nd         |
| DA29592       | R192A                                   | 6           | 24            | 6         | nd         | nd       | nd         |
| DA29594       | K205A                                   | 4           | 24            | 6         | nd         | nd       | nd         |

† MICs were determined with E-tests (bioMérieux). All culture media were M9 plus 0.2% glycerol. ‡ The *cat* cassette replaced the entire *aadA* gene.

comparison (Fig. 5) suggests that Lys205 may be available for interaction with the phosphates of ATP. The remaining strictly conserved exposed residues in the AadA homologues (Thr89, Trp112, Asp182 and Arg192) are not conserved in KNTase or LinB. Therefore, these residues are more likely to be responsible for substrate interactions and specificity.

In the AadA structure, several of the residues predicted to participate in ATP binding are involved in interactions with the C-terminal domain: Ser36 forms a hydrogen bond to Asp206, Ser46 hydrogen-bonds to the amino group of Lys43, and Asp47 and Asp49 form salt bridges with Lys205. Thus, in the conformation observed in the apo AadA structure the nucleotide-binding site is blocked by interdomain interactions.

In vivo ligand-binding studies using ITC. To test the binding affinity of ATP and aminoglycoside ligands for AadA, ITC experiments were performed (Table 3). We observed that ATP binds to wild-type AadA with a *K*ₐ of 13 μM (Fig. 6a), while the nonhydrolysable ATP analogue AMP allophosphate did not show any detectable binding. This indicates that in contrast to the observations for KNTase and LinB, an interaction with the O atom linking the α- and β-phosphates of ATP may be essential for ATP binding in AadA. Streptomycin and spectinomycin did not display detectable binding to AadA in the absence of ATP (data not shown). In the presence of ATP, both streptomycin and spectinomycin bind to AadA with an estimated *K*ₐ of 0.5 μM (Figs. 6b and 6c). ATP and streptomycin showed binding to AadA with an approximate 1:1 stoichiometry. While the spectinomycin was sold as having a potency of 603 μg mg⁻¹ (as estimated from a bacterial growth assay), the fit of the spectinomycin ITC data showed that its activity in binding to AadA was only 31% of the assumed active concentration. The data only indicate one type of binding site and there is no indication that AadA or any related adenylytransferase would have a different stoichiometry to one ATP and one adenylation substrate per enzyme. Therefore, the spectinomycin concentration was adjusted to 31% of the concentration based on the dry weight of the powder to fit the data, yielding an N value of 1. We do not have...
from specific effects such as loss of catalytic function or binding affinity.

3.7.2. In vitro ligand-binding studies of mutants using ITC. The effect of mutations on ligand binding was tested in ITC experiments (Table 3). E87Q and E87A displayed a threefold to fourfold lower affinity for ATP compared with the wild type, consistent with a role of the deprotonated Glu87 in magnesium-mediated coordination of the α-phosphate of ATP. For the R192A and K205A mutants no ATP binding could be detected, suggesting critical roles of these residues in the coordination of ATP. Comparison with KNTase and LinB suggests that Lys205 could form an interaction with the phosphates (Fig. 5).

The Glu87 mutants had a 20–40-fold lowered affinity for streptomycin and displayed no measurable binding of spectinomycin, agreeing with a direct or magnesium-mediated role of Glu87 in substrate coordination. Surprisingly, the W112F mutant did not affect the affinity for ATP or substrates, suggesting that the in vivo observation may be owing to effects on folding or on the correct orientation of the substrate for modification. The D182N mutation only diminished the binding of spectinomycin, suggesting a direct interaction. While the R192A mutant had a dramatically lowered affinity for both substrates, the K205A mutant could still bind both substrates, suggesting that this mutation either made the enzyme capable of binding the substrate in the absence of ATP or capable of binding substrate and ATP at the same time.

3.7.3. Recognition of different adenylation substrates of AadA. Streptomycin and spectinomycin are chemically very different, yet both are substrates of AadA and bind to the wild-type enzyme with similar affinities. What is common to the two modification sites? Both involve hydroxyl groups at positions next to a methylamine group on six-membered rings (Fig. 1), suggesting that this part of the two substrates could form similar interactions with the enzyme. At present we do not know whether the enzyme specifically binds these two molecules or whether the enzyme binds a broader range of aminoglycosides but only positions the appropriate hydroxyl groups of these two molecules for adenylation, in analogy with what has been described for the aminoglycoside-2”-phosphotransferase family (Young et al., 2009).

Expression of the aadA gene in S. enterica is only turned on under certain environmental conditions and is positively regulated by the stringent response regulator (p)ppGpp (Koskiniemi et al., 2011). The presence of the aadA gene in the genome of S. enterica, a species that to our knowledge has not been under selection for aminoglycoside resistance (Crump et al., 2015), suggests that there may also be an alternative adenylation substrate in the cell that remains to be identified.

4. Conclusions
We have presented the first crystal structure of an ANT(3’)(9) adenylyltransferase: AadA from S. enterica. The crystal structure together with SAXS data shows that in contrast to the structurally similar kanamycin nucleotidyltransferase, AadA functions as a monomer in magnesium-dependent adenyl transfer.

We have shown using ITC that ATP binds to AadA before the aminoglycoside substrate and positions the two domains for aminoglycoside binding in the interdomain cleft. Candidate residues for ligand binding and catalysis were subjected to site-directed mutagenesis and assayed for effects on resistance in vivo and ligand binding in vitro. The assays support a role for Glu87 as the catalytic base in adenylation, while Arg192 and Lys205 are critical for ATP binding and Asp182 is more important for the binding of spectinomycin than streptomycin. The details of substrate binding and catalysis remain to be clarified in future studies.

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