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Aminoglycoside binding and catalysis specificity of aminoglycoside 2″-phosphotransferase IVa: A thermodynamic, structural and kinetic study

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ABSTRACT

Background: Aminoglycoside *O*-phosphotransferases make up a large class of bacterial enzymes that is widely distributed among pathogens and confer a high resistance to several clinically used aminoglycoside antibiotics. Aminoglycoside 2″-phosphotransferase IVa, APH(2″)-IVa, is an important member of this class, but there is little information on the thermodynamics of aminoglycoside binding and on the nature of its rate-limiting step.
Methods: We used isothermal titration calorimetry, electrostatic potential calculations, molecular dynamics simulations and X-ray crystallography to study the interactions between the enzyme and different aminoglycosides. We determined the rate-limiting step of the reaction by the means of transient kinetic measurements.
Results: For the first time, K_d values were determined directly for APH(2″)-IVa and different aminoglycosides. The affinity of the enzyme seems to anti-correlate with the molecular weight of the ligand, suggesting a limited degree of freedom in the binding site. The main interactions are electrostatic bonds between the positively charged amino groups of aminoglycosides and Glu or Asp residues of APH. In spite of the significantly different ratio K_d/K_m, there is no large difference in the transient kinetics obtained with the different aminoglycosides. We show that a product release step is rate-limiting for the overall reaction.
Conclusions: APH(2″)-IVa has a higher affinity for aminoglycosides carrying an amino group in 2′ and 6′, but tighter bindings do not correlate with higher catalytic efficiencies. As with APH(3′)-IIIa, an intermediate containing product is preponderant during the steady state.
General significance: This intermediate may constitute a good target for future drug design.

1. Introduction

Bacterial infections are the cause of diseases that are often fatal and highly contagious. The generalized introduction of antibiotics after the Second World War was one of the most important therapeutic progresses of the 20th century. However, the misuse and over use of antibiotics in the past decades have led to the emergence of several resistant bacteria strains [1]. Bacteria have developed several strategies to combat antibiotics. Of these, the mechanisms whereby bacteria express enzymes that chemically inactivate antibiotics such as aminoglycosides are of particular interest. There are more than 120 bacterial enzymes that inactivate aminoglycosides by *N*-acetylation, *O*-adenylation or *O*-phosphorylation [2]. Aminoglycoside phosphotransferases, APHs, catalyze the transfer of a phosphate to a hydroxyl group of the antibiotic. The location of the carbon atom carrying the modified group is specific to each subclass of APH. The most common subclasses are APH(3′) that modify the hydroxyl on the carbon 3 of ring A of the aminoglycoside, and APH(2″) on carbon 2 of the ring C. The name of the subclass of APH is followed by a roman numeral that distinguishes enzymes

Abbreviations: APH(2″), aminoglycoside 2″-phosphotransferases; APH(3′), aminoglycoside 3′,5″-phosphotransferases; *LE*, ligand efficiency; N_{CNO}, number of non-hydrogen atoms of the ligand; PMF, potential of mean force.

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with different phospho-acceptor and -donor specificity [3]. Finally, isozymes are indicated by a lower case letter.

APH(2″)-IVa confers a high level of resistance in several *Enterococcus* strains towards various aminoglycosides that are prescribed in clinics, and it can use either ATP or GTP as phospho-donor. The first report of the gene encoding this enzyme was by Tsai *et al.* [4]. By the use of an enzyme coupled assay, Toth *et al.* showed that APH(2″)-IVa can phosphorylate all 4,6-disubstituted aminoglycosides, but not the 4,5-disubstituted ones [3]. In a subsequent paper, Toth *et al.* reported the steady state parameters, k_{cat} and K_m, of this enzyme with several aminoglycosides and they solved the crystal structure of the apoprotein [5]. They attempted to explain its aminoglycoside specificity by a molecular modeling study that involved the homologous APH(2″)-IIa. One year later, Shi *et al.* determined the crystal structures of APH(2″)-IVa complexed with tobramycin or kanamycin A [6].

Here, we obtained information on the thermodynamics of binding of different aminoglycosides to APH(2″)-IVa by isothermal titration calorimetry. We used crystal structures, complemented with surface potential calculations and molecular dynamics simulation of aminoglycoside release, to describe important interactions between the antibiotics and the enzyme.

To date, there is little information on the reaction pathway of APH(2″)-IVa, apart from the studies of Toth *et al.* [5] who showed that the reaction of APH(2″)-IVa proceeds by a Bi–Bi mechanism in which aminoglycoside and ATP or GTP bind randomly. To fully understand the mechanism of action of an enzyme, one must obtain information on the nature and the rate of interconversion of the intermediates that make up its reaction pathway. This can hardly be obtained from steady state and equilibrium studies alone — it requires transient kinetics [7]. Here, we used a direct quench–flow method, allowing both steady state and transient kinetic measurements, to obtain the time course of total ADP production (APH-bound plus free). Combining this method with free ADP time course measurements using a fluorescent biosensor in a stopped–flow apparatus, has allowed us to determine the nature of the rate-limiting step of the reaction.

2. Materials and methods

2.1. Chemicals

All aminoglycosides, ATP and other chemicals were obtained from Sigma-Aldrich at the highest purity grade. For control experiments, the sulfate ions contained in the commercialized aminoglycoside powders were removed by $Ba(OH)_2$ treatment as described elsewhere [8]. However, the presence of sulfate did not affect the thermodynamic and kinetic parameters. Therefore, sulfate was not removed for subsequent experiments. Equimolar concentrations of $MgCl_2$ were added to ATP stock solutions. In the text, unless otherwise stated, ATP refers to MgATP. Aminoglycoside and ATP stock solutions were prepared in 50 mM Tris–HCl, 40 mM KCl and 1 mM free $MgCl_2$ and were stored at −20 °C after adjusting pH to 7.5.

2.2. Protein purification

Recombinant APH(2″)-IVa from *Enterococcus casseliflavus* was produced in *Escherichia coli* BL21 (DE3) transformed with pET15b plasmid encoding for APH(2″)-IVa with a 6His-tag in N-terminal. *aph(2″)-IVa* gene was the generous gift of Professor Vakulenko, Notre Dame, USA.

Two liters of Luria Broth media, LB, supplemented with 0.1 mg/mL of Ampicillin, was inoculated at a final $OD_{600\ nm}$ of 0.1 with a 100 mL overnight starting culture. Cells were grown at 37 °C with an agitation of 180 rpm. Protein expression was induced at the late mid log phase ($OD_{600\ nm} = 0.9$) with 1 mM final concentration of IPTG. After an overnight incubation at 20 °C, cells were harvested by centrifugation and the pellet was solubilized in 40 mL of lysis buffer (50 mM NaH_2PO_4 at pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM DTT and 1 tablet of complete™ EDTA-free protease inhibitor). Bacteria were lysed on ice by sonication. Cell debris were removed by centrifugation and ultracentrifugation steps before loading the supernatant on a HisTrap FF 5 mL column in an Aktä Purifier system (GE Healthcare). The enzyme was eluted from the column with lysis buffer complemented with a linear gradient up to 500 mM of imidazole. Pure fractions were pooled and the protein was concentrated to 30–50 mg/mL on a 30 kDa Centricon® device (Millipore). The elution buffer was exchanged by a storage buffer containing 50 mM Hepes at pH 7.5, 50% glycerol and 1 mM DTT. The protein was stored at −20 °C. Typical production yield was 80 mg/L of culture.

To improve protein purity for the ITC experiments and crystallogenesis, a supplementary gel filtration step was carried out on a HiLoad 16/60 Superdex 75 column (GE Healthcare) in either 50 mM Tris–HCl pH 7.5, 40 mM KCl, 1 mM $MgCl_2$ for ITC or in 50 mM Hepes pH 7.5, 10 mM $MgCl_2$ for crystallization assays.

The catalytic activity of APH(2″)-IVa was independent of the presence of the 6His-tag, as controlled by comparing the steady state rates obtained with kanamycin A before and after cleavage of the tagged protein with thrombin (data not shown). The 6His-tag has also no effect on the crystallogenesis of the protein. Therefore, in all experiments the 6His-tag was kept at the N-terminal part of the enzyme.

2.3. Isothermal titration calorimetry experiments

The enzyme was extensively dialyzed against buffer containing 50 mM Tris–HCl at pH 7.5, 40 mM KCl, 1 mM $MgCl_2$ and ligand solutions were prepared at 5 mM in the final dialysate with readjusting pH to 7.5. The enzyme was used at a final concentration of 150 μM. ITC experiments were carried out at 25 °C in a VP-ITC calorimeter (MicroCal, GE Healthcare). Titrations consisted of 40 injections of 5 μL of ligand every 300 s. The cell stirring speed was 300 rpm. To obtain reliable dissociation constants, the *c*-value, a unitless parameter obtained by the multiplication of the association constant and the total concentration of ligand binding sites, was kept between 1.8 and 100 for all titrations. Control runs were performed by titrating ligands to buffer, and the resulting background signal was subtracted from the corresponding experimental data. Experiments were carried out at 1 mM free Mg^{2+}.

Binding and thermodynamic parameters K_a (association constant), ΔH (enthalpy change) and stoichiometry were obtained by nonlinear least-squares fitting of experimental data using a single-site binding model of the Origin software package (version 5.0) provided with the instrument. The free energy of binding (ΔG) and entropy change at 25 °C (ΔS) were obtained using the following equations:

$$\Delta G = -RT \ln(K_a) \quad (1)$$

$$\Delta G = \Delta H - T\Delta S \quad (2)$$

The experiments were performed in triplicate (except with G418 and kanamycin B, in duplicate) and the error values given in the tables are standard deviations.

2.4. Electrostatic potential and free energy calculations

Electrostatic potential surfaces were computed with the APBS program [9]. The charges and radius of all atoms were calculated using PDB2PQR software [10]. The linearized traditional Poisson–Boltzmann equation was solved with APBS using a cubic spline charge discretization and with dielectric constants of 2.0 for the solute and 78 for the solvent at 300 K. Electrostatic potential is represented by a positive and a negative isosurface at $\pm 10\ K_bT/e$ (K_b, Boltzmann's constant; T, temperature and e, charge of an electron).

All molecular dynamics simulations were performed using the NAMD 2.10 software [11] in the isobaric–isothermal ensemble. The pressure (1 atm) and temperature (300 K) were kept constant using