Published in final edited form as:

Mol Cell Biochem. 2014 February ; 387(0): 207–216. doi:10.1007/s11010-013-1886-1.

Purification and Characterization of Aminoglycoside Phosphotransferase APH(6)-Id, a Streptomycin Inactivating Enzyme

Meseret Ashenafi¹, Tatiana Ammosova², Sergei Nekhai², and W. Malcolm Byrnes^{1,*}

¹Department of Biochemistry and Molecular Biology, College of Medicine, Howard University, Washington, DC

²Center for Sickle Cell Disease and Department of Medicine, College of Medicine, Howard University, Washington, DC

Abstract

As part of an overall project to characterize the streptomycin phosphotransferase enzyme APH(6)-Id, which confers bacterial resistance to streptomycin, we cloned, expressed, purified and characterized the enzyme. When expressed in *E. coli*, the recombinant enzyme increased by up to 70-fold the minimum inhibitory concentration (MIC) needed to inhibit cell growth. Size exclusion chromatography gave a molecular mass of 31.4 ± 1.3 kDa for the enzyme, showing that it functions as a monomer. Activity was assayed using three methods: (1) an HPLC-based method that measures the consumption of streptomycin over time; (2) a spectrophotometric method that utilizes a coupled assay; and (3) a radioenzymatic method that detects production of ³²P-labeled streptomycin phosphate. Altogether, the three methods demonstrated that streptomycin was consumed in the APH(6)-Id-catalyzed reaction, ATP was hydrolyzed, and streptomycin phosphate was produced in a substrate-dependent manner, demonstrating that APH(6)-Id is a streptomycin phosphotransferase. Steady state kinetic analysis gave the following results: $K_m(\text{streptomycin})$ of 0.38 ± 0.13 mM, $K_m(\text{ATP})$ of 1.03 ± 0.1 mM, $V_{\max}$ of 3.2 ± 1.1 $\mu\text{mol}/\text{min}/\text{mg}$ and k_{cat} of 1.7 ± 0.6 s^{-1} . Our study demonstrates that APH(6)-Id is a *bona fide* streptomycin phosphotransferase, functions as a monomer, and confers resistance to streptomycin.

Keywords

streptomycin; antibiotic resistance; aminoglycoside phosphotransferase; APH(6)-Id

Introduction

The aminoglycoside antibiotic streptomycin (Sm), discovered by Selman Waksman in the 1940s [1], has been used clinically in combination with other drugs for the treatment of pulmonary tuberculosis caused by *Mycobacterium tuberculosis* infection. Although Sm is still used in some developing countries, its use has diminished in recent years due to its toxicity and the fact that many bacteria have become resistant to it. Sm acts by binding to the bacterial 16S ribosomal RNA (rRNA), thereby interfering with both the binding of transfer RNA (tRNA) to the ribosomal A-site and the proofreading step of protein translation [2]. The disruption of protein biosynthesis, in turn, causes permeabilization of the cell membrane, which is responsible for much of streptomycin's bactericidal effects [3].

*Corresponding Author: W. Malcolm Byrnes, Department of Biochemistry and Molecular Biology, Howard University College of Medicine, 520 W Street, NW, Washington, DC 20059; wbyrnes@howard.edu; Phone: 202-806-9749.

Resistance can arise through mutations in protein or RNA components of the ribosome, but more commonly resistance to Sm (and other aminoglycoside antibiotics) occurs due to modification of the antibiotic through phosphorylation, acetylation or adenylation mediated by a specific kinase (aminoglycoside *O*-phosphotransferase, APH), *N*-acetyltransferase (AAC) or *O*-nucleotidyltransferase (ANT), respectively, encoded by genes present in the resistant bacteria [4].

Sm is produced by the soil-dwelling gram-positive bacterium *Streptomyces griseus*, which also contains the gene for an enzyme, APH(6)-Ia, that protects the organism against the toxic effects of its own antibiotic [5, 6]. The recombinant APH(6)-Ia protein has been expressed in *Escherichia coli* [7] and the native enzyme has been isolated from *S. griseus*, purified, and characterized [8]. Streptomycin's three-ring structure consists of N-methyl-L-glucosamine, streptose and streptidine rings; it is the 6-hydroxyl group of the streptidine ring that is phosphorylated by APH(6)-Ia. *S. griseus* also contains a second Sm-phosphorylating enzyme, APH(3'')-Ia, which catalyzes the addition of a phosphate group to the hydroxyl at position 3'' of its N-methyl-L-glucosamine ring [9]. However, whereas the gene for APH(6)-Ia (*aph(6)-Ia*) is located within the Sm biosynthetic gene cluster, *aph(3'')-Ia* is not [5].

There are three APH(6) enzymes in addition to APH(6)-Ia. Like APH(6)-Ia, each inactivates Sm by catalyzing addition of a phosphate group at position 6 of the streptidine ring. These enzymes are APH(6)-Ib, -Ic and -Id [10]. APH(6)-Ib is found within *Streptomyces glaucescens*, a species closely related to *S. griseus*, which produces 5'-hydroxystreptomycin (HSm); APH(6)-Ib can phosphorylate and inactivate HSm (and possibly Sm as well). The gene for APH(6)-Ic, on the other hand, is found within the transposable element Tn5 [11], while that for APH(6)-Id (*strB*) has been found linked to *strA* (encoding an APH(3'')-Ib that also inactivates Sm) as an *strA-strB* pair within the small broad-host-range non-conjugative plasmid RSF1010 obtained from bacterial isolates in humans and other animals [12]. In plant pathogenic bacteria such as the *Pseudomonas syringae* strain from which the *strB* (*aph(6)-Id*) gene used in this study was isolated, however, the *strA-strB* pair is found within transposon Tn5393 on large conjugative plasmids [13-15]. There is now a significant body of evidence showing the presence of the *strA-strB* pair (containing *aph(6)-Id*) in a range of unrelated bacteria in the environment and the clinic, underscoring the wide distribution of these streptomycin resistance genes [16-19].

The reaction catalyzed by APH(6)-Id (and the other APH(6) enzymes), which involves the transfer of a γ -phosphate from ATP to the hydroxyl group at position 6 of the streptidine ring of streptomycin, is shown in Fig. 1. Adenosine diphosphate (ADP), a hydrogen ion and streptomycin 6-phosphate are products of the reaction. As with other kinases, magnesium ion is an essential cofactor.

Previously we reported the nucleotide sequences of the *aph(6)-Id* (and *aph(6)-Ia*) genes, and demonstrated that recombinant APH(6)-Id (and APH(6)-Ia) could be over-expressed in *Escherichia coli* [20]. In the work presented here, we have purified the recombinant APH(6)-Id protein and characterized it in terms of its oligomeric state and steady-state kinetic properties. We show that APH(6)-Id is a *bona fide* streptomycin phosphotransferase, functions as a monomer, and confers resistance to streptomycin in *E. coli* cells in which it is expressed.

Materials and Methods

Chemicals

Streptomycin, ampicillin, chloramphenicol, adenosine-5'-triphosphate (ATP), pyruvate kinase/lactate dehydrogenase, nicotinamide adenine dinucleotide (NADH), isopropyl- β -

D-1-thiogalactopyranoside (IPTG), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), and the P81 phosphocellulose paper used for the bio-dot assay were all obtained from Sigma Chemical Co. (St. Louis, MO). Bacto-Tryptone, Bacto-Agar, Bacto-Yeast Extract, Mueller Hinton Agar, and Mueller Hinton Broth were from Becton, Dickinson and Co. (Sparks, MD). γ -³²P-labeled ATP was from Perkin-Elmer Corp. (Waltham, MA). The plasmid containing APH(6)-Id gene (pSM1; ref. 13) was a generous gift from Dr. George W. Sundin (Michigan State University).

Purification of recombinant APH(6)-Id

The *aph(6)-Id* gene was PCR-amplified from pSM1 and subcloned into the expression vector pET15b (Novagen), and the His-tagged recombinant APH(6)-Id protein was expressed in *Escherichia coli* Rosetta(DE3)pLysS cells as previously described [20]. Cells were harvested by centrifugation. Cell pellets were stored overnight at -20°C , resuspended in Lysis Buffer (100 mM Tris-HCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 15 mM imidazole) and sonicated. The lysate was centrifuged at 11,000 rpm for 20 min at 4°C . The supernatant was removed and mixed with Ni-NTA resin that had been equilibrated with Binding Buffer (300 mM NaCl, 5 mM β -mercaptoethanol, 20 mM imidazole and 5% glycerol in 50 mM Tris-HCl, pH 7.5) and gently mixed on a shaker at 4°C for 60 minutes. The cleared lysate-Ni-NTA mixture was loaded onto a disposable chromatographic column (Novagen) and allowed to flow by gravity. Less tightly-bound proteins were washed stepwise off the column with Column Buffer (300 mM NaCl, 5 mM β -mercaptoethanol and 5% glycerol in 50 mM Tris-HCl, pH 7.5) containing 30 and 100 mM imidazole, respectively. The more tightly-bound APH(6)-Id protein was eluted with Column Buffer containing 250 mM imidazole. A PD-10 desalting column (GE Healthcare) was used to remove imidazole from the protein preparation. Desalted protein preparations were dialyzed against a buffer containing 1 mM DTT, 1 mM PMSF and 50% glycerol in 100 mM Tris-HCl, pH 7.5, and stored at minus 20°C until ready for use.

Determination of Minimum Inhibitory Concentrations

The antimicrobial potency of streptomycin against *E. coli* cells expressing APH(6)-Id was determined as Minimum Inhibitory Concentrations (MICs) using two methods: the broth macro-dilution method and the disk diffusion method. Protocols used were those developed by the National Committee for Clinical Laboratory Standards [21, 22]. To prepare inocula, *E. coli* Rosetta(DE3)pLysS cells containing recombinant plasmid pET15b-*aph(6)-Id* and, as controls, Rosetta(DE3)pLysS (no added plasmid) and standard *E. coli* strain MM294 (ATCC# 33625), were streaked out and grown on LB agar containing appropriate antibiotics (none for MM294). Single colonies were picked and transferred to overnight tubes containing 2 ml of Mueller-Hinton Broth (MHB; Difco BBL Microbiology), grown to a cell density of $\text{OD}_{625} = 0.3$, diluted with MHB to adjust to a 0.5 McFarland standard (a measure of turbidity; it corresponds to a cell density of approximately 1.5×10^8 CFU/ml) and then further diluted to give a final density of 10^5 - 10^6 CFU/ml. For the broth dilution method, 100 μl of cell culture were added to each of a series of tubes containing two-fold dilutions of streptomycin in 2 ml of MHB. Isopropyl- β -D-1-thiogalactoside (IPTG, 100 μM) was added to a subset of the tubes to induce expression of APH(6)-Id and test for its effect on streptomycin susceptibility. After incubating the cultures at room temperature ($\sim 25^{\circ}\text{C}$) with shaking for 18 hours, MIC-values were determined as the lowest concentration of streptomycin that resulted in complete inhibition of growth (as determined spectrophotometrically).

For the disk diffusion method, Mueller-Hinton Agar (MHA) plates were inoculated by uniformly coating them with one or the other of the bacterial cultures at 10^5 - 10^6 CFU/ml. Paper disks containing streptomycin at various concentrations were placed onto the MHA

plates, which were then incubated at 35°C for 18 hours. The diameters of the inhibitory zones, i.e., areas around the disks in which there was no cell growth, were measured.

Enzyme Activity Measurements

Three different methods were used to measure APH(6)-Id enzyme activity. These were (i) a spectrophotometric method that involved the coupling of ADP released from the ATP-dependent phosphorylation reaction to the oxidation of NADH which, in turn, could be followed by measuring the decrease in absorbance at 340 nm; (ii) a reversed-phase high performance liquid chromatography (HPLC)-based method that was developed to detect streptomycin consumed in the reaction; and (iii) a bio-dot assay in which ^{32}P -labeled streptomycin phosphate produced in the reaction was trapped in phosphocellulose paper through which the reaction mixtures were filtered.

Spectrophotometric Assay—The standard 100 μl assay was carried out at 25°C in 50 mM HEPES buffer, pH 7.5, containing ATP (2 mM), streptomycin (20 mM), MgCl_2 (12.5 mM), KCl (40 mM), phosphoenolpyruvate (PEP; 2 mM), coupling enzymes pyruvate kinase/lactate dehydrogenase (~2 units of each), NADH (0.2 mg/ml), and APH(6)-Id enzyme (2 μg). A dual-beam UV-visible spectrophotometer (Shimadzu) was used to measure the oxidation of NADH (decrease in absorbance at 340 nm), which was coupled to the production of ADP in the APH(6)-Id-catalyzed reaction via pyruvate kinase and lactate dehydrogenase. The assay was found to be linear for ~1.5 minutes.

RP-HPLC-Based Assay—Chromatographic analysis was performed using a Waters Breeze HPLC system with binary pump, an AtlantisTM dC18 column (20 $\times$ 4.6 mm, 5 μm pore size) and a SoftA Model 100 evaporative light-scattering detector (ELSD). A separations method developed by Waters Corp. for analyzing aminoglycoside antibiotics (AtlantisTM Columns Applications Notebook Code 720000472EN) that involved two mobile phases was employed. Phase A contained 0.1% heptafluorobutyric acid in water, and phase B contained 0.1% heptafluorobutyric acid in acetonitrile. After column equilibration with mobile phase A, a 20 μl sample of reaction mixture was injected and the column was run with a flow rate of 1 ml/min using the following gradient: 5-60 % of B in 9 min. The analyte (streptomycin) was detected by ELSD, and a decrease in peak area was indicative of consumption of streptomycin in the enzyme-catalyzed reaction. The composition of the enzyme reaction mixture was the same as that above for the spectrophotometric activity assay, except that PEP, NADH and the coupling enzymes were omitted. The reactions were carried out at 37°C for 10 minutes and were stopped by placing the reaction tubes on ice. Decrease in peak area over time was converted to enzyme activity using a standard curve constructed by plotting peak area versus streptomycin concentration for standard solutions of known concentration.

Radioenzymatic γ - ^{32}P Bio-Dot Assay—The protocol used was a modified version of a dot-blot assay developed by Michael et al. [23]. The standard 60 μl assay sample contained ATP (100 μM), streptomycin (5 mM), MgCl_2 (12.5 mM), KCl (40 mM), APH(6)-Id enzyme (100 ng) and γ - ^{32}P -labeled ATP (2.5 μCi) in 50 mM HEPES buffer, pH 7.5. The reaction, which was determined to be linear for three minutes, was carried out and stopped at the 3-minute point by adding 150 μl of hot deionized water (80°C) to the reaction mixture and then incubating it at 80°C for 5 min. After cooling the reaction mixture on ice, it was centrifuged briefly and filtered through P81 phosphocellulose paper (Whatman) using a bio-dot apparatus (Bio-Rad) attached to a vacuum trap. Non-bound γ - ^{32}P -labeled nucleotide was removed from the filter by washing it three times with 150 ml of deionized water; the filter was then air dried at room temperature. The amount of γ - ^{32}P -labeled streptomycin bound to the P81 phosphocellulose paper was measured using a filmless phosphor imager screen

(Perkin-Elmer) that was scanned in Cyclone Model B431220 Phosphor Imager/Scanner (Packard) and quantified using OptiQuant software. Note that ATP, which is negatively charged, does not adhere to the filter. Measurements were converted to enzyme activity by comparing (under identical conditions) the “counts” obtained to those obtained for a reaction allowed to go to completion using a known concentration of streptomycin, so that all of the streptomycin was converted to labeled product.

Determination of Steady-State Kinetic Constants

The radioenzymatic bio-dot assay was found to be the most reliable to use for steady-state kinetic analysis of the enzyme. Six or seven different substrate concentrations were used in initial velocity experiments to determine Michaelis constants (K_m) and maximum velocity (V_{max}) values with respect to each of the two substrates, streptomycin and Mg-ATP. The experiments were done by increasing the concentration of one substrate while keeping the other constant at a saturating or near-saturating level. In order to obtain ATP initial velocity curves, it was necessary to keep the ratio of ^{32}P -labeled ATP to unlabeled ATP constant, so that the concentration of radioactive label (which made a miniscule molar contribution) increased in step with the concentration of cold ATP substrate. Experimental insights were gleaned from a paper by Luedtke and Schepartz [24]. The important aspect of the ratio was that it had to be such that the radioactivity from a spot obtained using low ATP concentration was measurable, but the radioactivity from a spot obtained using high ATP was not so great that it exceeded linearity (and therefore was not quantifiable). Based on these experimental limitations, a workable molar ratio of hot-to-cold ATP was found to be 1:6,000. (Nevertheless, as discussed below (see *Discussion* section), these experimental constraints in some cases served to limit the concentration of ATP that could be used to a below-saturating level.) The initial velocity data were fitted to the Michaelis-Menten equation ($V_i = V_{max} [S] / (K_m + [S])$) using SigmaPlot software (Systat, Inc).

Gel Filtration Chromatography

A Sephacryl S-200 column (C 16/40, GE Healthcare Life Sciences) was equilibrated overnight at 4°C in 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM DTT. A BioLogic LP low pressure chromatography system with UV detection (BioRad) was used. APH(6)-Id protein sample was concentrated to 4 mg/ml, loaded onto the column, and eluted at a flow rate of 0.5 ml/min. Fractions (1 ml) were collected and analyzed for protein (absorbance at 280 nm) and streptomycin phosphotransferase activity. The following standard proteins were used to calibrate the column: ribonuclease A (13.7 kDa), carbonic anhydrase (29 kDa), conalbumin (75 kDa) and aldolase (158 kDa).

Results

Purification of the Recombinant APH(6)-Id Enzyme

We previously reported the expression of a His-tagged APH(6)-Id protein from pET-15b in *E. coli* Rosetta(DE3)pLysS cells [20]. In the present study, using the same expression protocol, we obtained approximately 4 mg of soluble protein per liter of cell culture. The enzyme was purified from this preparation using Ni-NTA affinity chromatography as described in *Materials and Methods*. SDS-PAGE analysis of the recombinant His-tagged APH(6)-Id protein (Fig. 2) indicated purification to near-homogeneity and a subunit molecular mass of ~30 kDa, which matches that predicted from the amino acid sequence (32 kDa). Gel filtration analysis yielded a molecular mass of 31.4 ± 1.3 kDa; this shows that APH(6)-Id, like the similar enzyme APH(6)-Ia from *S. griseus* [8], is monomeric.

APH(6)-Id Expression Protects *E. coli* Cells against Streptomycin

In order to determine whether or not our recombinant protein was active *in vivo*, i.e., able to protect cells in which it was expressed against the growth-inhibitory effects of streptomycin, we carried out antibiotic susceptibility experiments. We determined the Minimum Inhibitory Concentrations (MICs) of streptomycin required to effectively inhibit cell growth using two methods: disk diffusion and broth dilution. The results, presented in Table 1, show that expression of the recombinant protein in the *E. coli* Rosetta(DE3)pLysS cells protects the cells against streptomycin toxicity. In the disk diffusion experiments, the concentration of streptomycin that gave a zone of inhibition 16 mm in diameter increased from 3.1 µg/ml in control cells to 50 µg/ml in cells induced to express APH(6)-Id. This represents a 16-fold decrease in streptomycin toxicity in the presence versus the absence of the expressed protein. Similar but more dramatic results were observed in the broth dilution experiments. Here, induction of APH(6)-Id expression increased the MIC from 3 µg/ml to 200 µg/ml; this represents a 67-fold decrease in the toxic effects of streptomycin. Note that the MIC value obtained for the *E. coli* control strain MM294 (a standard strain) as well as for Rosetta(DE3)pLysS cells lacking the plasmid was 3 µg/ml (Table 1), a value that closely matches that of 2 – 4 µg/ml reported in the literature for *E. coli* [25].

The difference in the increase in MIC value obtained using the two antibiotic susceptibility methods (67-fold increase versus 16-fold increase) may reflect differences in the ease of diffusion through the two different types of physical media used: liquid media versus agar. Because of these differences, the results of the two methods cannot be compared directly. Nonetheless, both show that the APH(6)-Id enzyme expressed in the *E. coli* cells is active *in vivo* and effectively protects the cells against streptomycin.

APH(6)-Id Enzymatic Activity

With evidence in hand from the antibiotic susceptibility experiments that recombinant APH(6)-Id was active in cells in which it is expressed (i.e., *in vivo*), we set out to measure the activity of the purified enzyme *in vitro*. Three different assay methods were employed to do this: a spectrophotometric assay in which ADP produced in the reaction was coupled to the oxidation of NADH, an HPLC-based assay, and a bio-dot assay that used radioactive γ -³²P-labeled ATP. The methods yielded similar values for the specific activity of the enzyme (see $V_{\max}$ values in Table 2). An average value of 3.2 ± 1.1 µmol/min/mg was calculated. Using this value together with results from gel filtration analysis showing that APH(6)-Id is monomeric with a molecular mass of 31.4 kDa, the catalytic rate constant of 1.7 ± 0.6 s⁻¹ was calculated.

All reaction mixtures used in the assays contained HEPES buffer rather than Tris-HCl. This is because earlier experiments in our laboratory had shown that the substrate, streptomycin, was not stable in Tris-HCl but was stable in HEPES, remaining so for several hours at 30°C [26]. The enzyme, on the other hand, was equally stable in both buffers. Since removal of the N-terminal 6xHis tag did not alter the enzyme's activity (data not shown), the tag was left on for all subsequent experiments. Nonetheless, the possibility remains that the presence of the tag may have subtly affected the kinetic properties of the enzyme.

Although the spectrophotometric coupled assay did allow us to measure enzyme activity and demonstrate that activity was linear with respect to enzyme concentration (not shown), we found that it was not useful for conducting initial velocity experiments. In the course of attempting to perform initial velocity experiments using the coupled assay, we discovered that the activity of the enzyme did not appear to change with streptomycin concentration, and that concentrations as low as 0.5 µM and as high as 20 mM gave similar activities. In order to measure activity at even lower concentrations of streptomycin (0.1 µM), we

employed a sensitive fluorescence-based coupled assay developed by Perlin et al. [27] in which a decrease in the fluorescence of NADH ($\lambda_{\text{ex}} = 340 \text{ nm}$; $\lambda_{\text{em}} = 450 \text{ nm}$) is measured over time. However, this assay gave results similar to those of the spectrophotometric assay: activity did not consistently increase with streptomycin concentration. Similar results were also obtained when ATP was the variable substrate: activity did not appear to change with ATP concentration from low micromolar to high millimolar amounts. Finally, since we had obtained results similar to these when using crude cell extracts containing the enzyme APH(6)-Ia [26], we speculated that this phenomenon was not unique to APH(6)-Id but was a feature common among APH(6) enzymes. Notably, no enzyme activity was observed in the absence of streptomycin.

To confirm that the reason for the failure of the coupled assay was not because the PEP or coupling enzyme components were rate-limiting, we performed experiments to optimize the amounts of these components in the assay. Moreover, we ran the enzyme hexokinase (from baker's yeast purchased from Sigma) as a positive control, optimizing assay conditions and then applying these same conditions directly to the APH(6)-Id enzyme assay. The $K_m(\text{glucose})$ -value we obtained was similar to a value reported in the literature ($\sim 0.3 \text{ mM}$ (this study) versus 0.17 mM (ref. [28])), suggesting that the assay itself was not flawed. Despite this, APH(6)-Id activity, as measured in the assay, still did not increase with streptomycin concentration.

One possible explanation for the failure of the coupled assay could have been that the K_m with respect to substrate was very low, and that, as a result, the amounts of substrate that were used were all too high (saturating), so that activity would not increase as substrate concentration increased. However, this explanation appears unlikely in our case for two reasons. First, streptomycin concentrations as low as $0.1 \mu\text{M}$ gave apparent activities similar to those obtained using concentrations as high as 20 mM . It would be unlikely that the $K_m^{\text{streptomycin}}$ for APH(6)-Id would be lower than $1 \mu\text{M}$ since even the most sensitive aminoglycoside phosphotransferases studied to date, i.e., APH(3')-Ia [27, 29], have K_m -values of at least $1 \mu\text{M}$. And secondly, the bio-dot assay, performed later, eventually confirmed that the K_m -values for APH(6)-Id were, in fact, within the sub-millimolar range, not the sub-micromolar range (see results section). Thus, the reason for the failure of the coupled assay remains elusive.

Since the coupled assay is dependent only on the generation of ADP in the reaction, we wondered if the recombinant APH(6)-Id enzyme we had purified might be acting merely as a streptomycin-activated ATPase, one that does not consume streptomycin and is fully activated at very low (micromolar) concentrations of streptomycin. It was important for us to test this possibility because at least one other aminoglycoside phosphotransferase, e.g., APH(3')-Ia, had been shown to possess an intrinsic ATPase activity, albeit one that was seen only in the absence of aminoglycoside [29], not in its presence, as would be the case here. Therefore, we measured APH(6)-Id activity by analyzing reaction mixtures using reversed-phase HPLC with evaporative light scattering detection of streptomycin. Aliquots ($20 \mu\text{l}$) were injected and run at time = 0 and again after 10 minutes of reaction. The results, presented in Fig. 3, show that streptomycin (retention time 2.2 min) is consumed in the reaction. Notably, when a solution of streptomycin alone (no enzyme) is incubated under the same conditions for the same length of time (10 minutes), no consumption of streptomycin is evident, i.e., peak area did not decrease, indicating that the decrease seen in Fig. 3 was mediated by the presence of the enzyme and was not due to any intrinsic instability of streptomycin. From this experiment and three additional ones, using the reduction in streptomycin peak area over time, we were able to calculate a specific activity for the enzyme (Table 2). This value ($2.0 \pm 0.9 \mu\text{mol/min/mg}$) is similar to those obtained by the other two assays methods employed (Table 2). However, the HPLC method (in our hands at

least) was not suitable for obtaining data for initial velocity plots. This led us to the use of a radioenzymatic method for assaying activity.

Radioenzymatic Assay Allows Detection of the Phosphorylated Product and Determination of Steady-State Kinetic Properties

Whereas the HPLC-based assay proved that streptomycin was being consumed in the APH(6)-Id-catalyzed reaction, it did not address the question of whether or not a phosphorylated product was being formed. To answer this question and to obtain initial velocity data, we turned to a radioenzymatic (bio-dot) assay that measures incorporation of a ^{32}P label into a streptomycin phosphate product. The assay is based on the principle that streptomycin phosphate, which is positively-charged, will adhere to the negatively-charged phosphocellulose paper through which it is being filtered whereas ATP, which is negatively-charged, will not and can be washed away. Thus, it selectively measures accumulation of product in the reaction. Using this assay, initial velocity experiments were successfully performed. Fig. 4a gives representative bio-dot data from experiments in which streptomycin is the variable substrate. The initial velocity curve obtained from the data is displayed in Fig. 4b. Similarly, Figs. 4c and d show analogous results with Mg-ATP as the variable substrate. Steady-state kinetic constants (K_m and V_{max} values) were obtained from the plots. These are presented in Table 2.

Discussion

Here we report the expression, purification and characterization, in terms of oligomeric state and steady state kinetic properties, of the streptomycin-inactivating enzyme APH(6)-Id (see Table 2). The kinetic properties can be compared to those of other known aminoglycoside phosphotransferases (see Table 3; note that different types of activity assays were used in studies from different labs). Sugiyama et al. [8] reported values of 3.5 mM for $K_m^{\text{streptomycin}}$, 0.4 mM for $K_m^{\text{Mg-ATP}}$, and 28.6 $\mu\text{mol/min/mg}$ for V_{max} for native APH(6)-Ia from *S. griseus* (the k_{cat} would be 17.6 s^{-1} , which is approximately ten-fold higher than the value we obtained for APH(6)-Id). The $k_{cat}/K_m^{\text{streptomycin}}$ is $5.0 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$, compared to $4.5 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ for APH(6)-Id (see Table 3). Thus, although the K_m and V_{max} values differ somewhat between APH(6)-Id and APH(6)-Ia, the catalytic efficiencies (represented by $k_{cat}/K_m^{\text{streptomycin}}$ -values) of the two enzymes are similar. A comparison of these k_{cat}/K_m -values to those for the APH(3') phosphotransferases APH(3')-Ia and -IIIa, which inactivate kanamycin A and structurally-similar aminoglycosides (Table 3), reveals that the APH(6) enzymes are considerably less sensitive to aminoglycoside concentration and less efficient in their ability to inactivate the antibiotic through phosphorylation. The ecological and clinical relevance of this is not known, but APH(6)-Id (encoded by *strB*) is often found in environmental and clinical isolates paired with the streptomycin phosphotransferase APH(3'')-Ib (encoded by *strA*) [13]. Moreover, *aph(6)-Id* was found to be frequently paired with *aph(3'')* in streptomycin-resistant bacteria isolated from a variety environmental sites (rhizosphere soil, manure, activated sludge, seawater) in Northern Europe [30]. In some of these isolates, an *ant(3'')* gene was also present, giving the three-gene combination *aph(6)-Id*, *aph(3'')* and *ant(3'')*, but in no isolate was *aph(6)-Id* found by itself, although in one, it was found paired with *aph(6)-Ic* [30]. Finally, as mentioned above, *aph(6)-Ia* is found together with the gene for a corresponding APH(3'')-type phosphotransferase (*aph(3'')-Ia*) within *S. griseus*, the streptomycin-producing strain.

Altogether, these observations suggest that a 3''-phosphotransferase or 3''-adenylyltransferase activity, in addition to the 6-phosphotransferase activity of APH(6)-Id or APH(6)-Ia, may be required for effective inactivation of streptomycin. Note that although the regiospecificities of APH(3'')-Ia and a related enzyme, APH(3'')-Ic (isolated from

Mycobacterium fortuitum), have been confirmed [9, 31], neither enzyme has been characterized biochemically. Until such characterization is accomplished, it will remain difficult to speculate on what the putative complementary roles of APH(6) and APH(3'') (or ANT(3'')) might be in the streptomycin inactivation process.

A caveat regarding the $K_m^{\text{streptomycin}}$ obtained in this study is that, in part due to the practical limitations imposed by the bio-dot radioenzymatic assay, high levels of ATP were not used in some of the initial velocity experiments. Thus, saturating levels of Mg-ATP were not used in assays for the streptomycin initial velocity experiments. For this reason, the value of $K_m^{\text{streptomycin}}$ obtained should be regarded as approximate. Nonetheless, if APH(6)-Id obeys a kinetic mechanism similar to those of the aminoglycoside phosphotransferases APH(3')-IIIa and APH(3')-Ia, in which the value of $K_m^{\text{aminoglycoside}}$ is independent of Mg-ATP concentration (because the enzyme obeys a Theorell-Chance mechanism in the former case and a rapid-equilibrium random mechanism in the latter [29, 32]), then the value we have obtained for $K_m^{\text{streptomycin}}$ will be close to, if not the same as, the true value. Lending support to this conclusion is evidence suggesting that all APHs appear to follow a similar mechanism of aminoglycoside inactivation [33].

This work on APH(6)-Id expands the list of aminoglycoside phosphotransferases that have been characterized to date. Enzymatic studies of this sort are valuable for at least three reasons. First, they biochemically confirm the functionality of the expression products of genes found in clinical and other bacterial isolates. Without such biochemical confirmation, there inherently is a degree of uncertainty regarding whether the presence of the genetic signature for a resistance enzyme indicates that an active enzyme is actually present. This uncertainty hampers the ability to confidently attach functional significance to streptomycin resistance genes found in environmental and clinical isolates. Second, there is strong evidence that genes for enzymes involved in streptomycin resistance and streptomycin biosynthesis are widely distributed in soil, and are transferred within populations of soil streptomycetes [30, 34-37]. Although it has been hypothesized that aminoglycoside resistance genes in the environment, particularly those associated with antibiotic production, are the ultimate source of the genes found in clinical isolates [38, 39], the mechanisms by which the genes have moved from environmental to clinical isolates remain unclear. The biochemical and kinetic characterization of aminoglycoside resistance enzymes such as APH(6)-Id (and APH(6)-Ia), which involves determination of the properties of particular gene products, may help to shed light on these mechanisms. Finally, in an era of increased resistance to antibiotics, it is generally important to understand the mechanisms by which resistance moves from environment to clinic in order to devise strategies to combat the problem and to better plan for the future industrial development of new antibiotics.

Acknowledgments

We gratefully acknowledge support from the U. S. National Institutes of Health (NIH) RCMI program (grant number 8G12MD007597) and MBRS-SCORE program (grants number 3S06GM0816-33S1, SC3GM083752 and SC1GM08232). We especially thank Ms. Myrna Stupart of the Department of Microbiology at Howard University for help with the antibiotic susceptibility protocols and for providing *E. coli* control strain MM294.

References

1. Jones D, Metzger HJ, Schatz A, Waksman SA. Control of gram-negative bacteria in experimental animals by streptomycin. *Science*. 1944; 100:103–105. [PubMed: 17788929]
2. Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature*. 2000; 407:340–348. [PubMed: 11014183]

3. Davis BD. Mechanism of bactericidal action of aminoglycosides. *Microbiol Rev.* 1987; 51:341–350. [PubMed: 3312985]
4. Wright GD. Aminoglycoside-modifying enzymes. *Curr Opin Microbiol.* 1999; 2:499–503. [PubMed: 10508725]
5. Piepersberg, W. Streptomycin and related aminoglycosides. In: Vining, LC.; Stuttard, C., editors. *Genetics and biochemistry of antibiotic production*. Butterworth Heinemann, Halifax; Nova Scotia, Canada: 1996. p. 531-570.
6. Distler J, Mansouri K, Mayer G, Stockmann M, Piepersberg W. Streptomycin biosynthesis and its regulation in *Streptomyces*. *Gene.* 1992; 115:105–111. [PubMed: 1377151]
7. Lim C-K, Smith MCM, Petty J, Baumberg S, Wootton JC. *Streptomyces griseus* streptomycin phosphotransferase: expression of its gene in *Escherichia coli* and sequence homology with other antibiotic phosphotransferases and with eukaryotic protein kinases. *J Gen Microbiol.* 1989; 135:3289–3302. [PubMed: 2561487]
8. Sugiyama M, Sakamoto M, Mochizuki H, Nimi O, Nomi R. Purification and characterization of streptomycin 6-kinase, an enzyme implicated in self-protection of a streptomycin-producing microorganism. *J Gen Microbiol.* 1983; 129:1683–1687. [PubMed: 6313852]
9. Heinzel P, Werbitzky O, Distler J, Piepersbert W. A second streptomycin resistance gene from *Streptomyces griseus* codes for streptomycin-3"-phosphotransferase. Relationships between antibiotic and protein kinases. *Arch Microbiol.* 1988; 150:184–192. [PubMed: 2844130]
10. Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. *Microbiol Rev.* 1993; 57:138–163. [PubMed: 8385262]
11. Mazodier P, Cossart P, Giraud E, Gasser F. Completion of the nucleotide sequence of the central region of Tn5 confirms the presence of three resistance genes. *Nucleic Acids Res.* 1985; 13:195–205. [PubMed: 3889831]
12. Scholz P, Haring V, Wittmann-Liebold B, Ashman D, Bagdasarian M, Scherzinger E. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. *Gene.* 1989; 75:271–288. [PubMed: 2653965]
13. Sundin GW, Bender CL. Ecological and genetic analysis of copper and streptomycin resistance in *Pseudomonas syringae* pv. *Syringae*. *Appl Environ Microbiol.* 1993; 59:1018–1024.
14. Chiou C-S, Jones AL. Nucleotide sequence analysis of a transposon (Tn5393) carrying streptomycin resistance genes in *Erwinia amylovora* and other gram-negative bacteria. *J Bacteriol.* 1993; 175:732–740. [PubMed: 8380801]
15. Chiou C-S, Jones AL. Expression and identification of the *strA-strB* gene pair from streptomycin-resistant *Erwinia amylovora*. *Gene.* 1995; 152:47–51. [PubMed: 7828927]
16. Sundin GW, Bender CL. Dissemination of the *strA-strB* streptomycin-resistance genes among commensal and pathogenic bacteria from humans, animals, and plants. *Mol Ecol.* 1996; 5:133–143. [PubMed: 9147689]
17. Sundin GW. Examination of base pair variants of the *strA-strB* streptomycin resistance genes from bacterial pathogens of humans, animals and plants. *J Antimicrob Chemother.* 2000; 46:848–849.
18. Pezella C, Ricci A, DiGiannatale E, Luzzi I, Carattoli A. Tetracycline and streptomycin resistance genes, transposons, and plasmids in *Salmonella enterica* isolates from animals in Italy. *Antimicrob Agents Chemother.* 2004; 48:903–908. [PubMed: 14982782]
19. Shaw PC, Liang AC, Kam KC, Ling JM. Presence of *strA-strB* gene within a streptomycin-resistance operon in a clinical isolate of *Shigella flexneri*. *Pathology.* 1996; 28:356–358. [PubMed: 9007957]
20. Collins AC, Ashenafi M, Saunders AA, Byrnes WM. Cloning and expression of streptomycin-inactivating enzymes APH(6)-Ia and APH(6)-Id. *Cell Mol Biol.* 2007; 53:74–79. [PubMed: 17531152]
21. National Committee for Clinical Laboratory Standards, NCCLS. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved Standard M7-A2. NCCLS; Villanova, PA: 1990.
22. National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests. Approved Standard M2-A4. NCCLS; Villanova, PA: 1990.

23. Michael SS, Duch M, Paludan K, Jorgensen P, Finn SP. Measurement of hygromycin B phosphotransferase activity in crude mammalian cell extracts by a simple dot-blot assay. *Gene*. 1992; 112:257–260. [PubMed: 1313387]
24. Luedtke NW, Schepartz A. Lanthanide-mediated phosphoester hydrolysis and phosphate elimination from phosphopeptides. *Chem Commun*. 2005; 43:5426–5428.
25. Martindale, W. The Extra Pharmacopoeia. 28th. Pharmaceutical Press; London: 1982.
26. Collins, AC. Dissertation. Howard University; 2007. Cloning, sequencing and expression of the gene for streptomycin-inactivating enzyme APH(6)-Ia from *Streptomyces griseus*.
27. Perlin MH, McCarty SC, Greer JP. Coupled spectrofluorometric assay for aminoglycoside phosphotransferases. *Anal Biochem*. 1988; 171:145–149. [PubMed: 2841886]
28. Fromm HJ, Zewe V. Kinetic studies of yeast hexokinase. *J Biol Chem*. 1962; 237:3027–3032. [PubMed: 13945795]
29. Siregar JJ, Miroshnikov K, Mobashery S. Purification, characterization, and investigation of the mechanism of aminoglycoside 3'-phosphotransferase type Ia. *Biochemistry*. 1995; 34:12681–12688. [PubMed: 7548020]
30. van Overbeek LS, Wellington EMH, Egan S, Smalla K, Heuer H, Collard J-M, Guillaume G, Karagouni AD, Nikolakopoulou TL, van Elsas JD. Prevalence of streptomycin-resistance genes in bacterial populations in European habitats. *FEMS Microbiol Ecol*. 2002; 42:277–288. [PubMed: 19709288]
31. Ramón-García S, Ota I, Martín C, Gómez-Lus R, Aínsa JA. Novel streptomycin resistance gene from *Mycobacterium fortuitum*. *Antimicrob Agents Chemother*. 2006; 50:3920–3922. [PubMed: 16954315]
32. McKay GA, Wright GD. Kinetic mechanism of aminoglycoside phosphotransferase type IIIa: evidence for a Theorell-Chance mechanism. *J Biol Chem*. 1995; 270:24686–24692. [PubMed: 7559583]
33. Thompson PR, Hughes DW, Cianciotto NP, Wright GD. Spectinomycin kinase from *Legionella pneumophila*: characterization of substrate specificity and identification of catalytically important residues. *J Biol Chem*. 1998; 273:14788–14795. [PubMed: 9614079]
34. Tolba S, Egan S, Kallifidas D, Wellington EHM. Distribution of streptomycin resistance and biosynthesis genes in streptomycetes recovered from different soil sites. *FEMS Microbiol Ecol*. 2002; 42:269–276. [PubMed: 19709287]
35. Wiener P, Egan S, Wellington EMH. Evidence for transfer of antibiotic resistance genes in soil populations of streptomycetes. *Mol Ecol*. 1998; 7:120501216.
36. Egan S, Wiener P, Kallifidas D, Wellington EMH. Transfer of streptomycin biosynthesis gene clusters within Streptomycetes isolated from soil. *Appl Environ Microbiol*. 1998; 64:5061–5063. [PubMed: 9835611]
37. Anderson AS, Clark DJ, Gibbons PH, Sigmund JM. The detection of diverse aminoglycoside phosphotransferases within natural populations of actinomycetes. *J Ind Microbiol Biotech*. 2002; 29:60–69.
38. Benveniste R, Davies J. Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. *Proc Natl Acad Sci USA*. 1973; 70:2276–2280. [PubMed: 4209515]
39. Davies J, Wright GD. Bacterial resistance to aminoglycoside antibiotics. *Trends Microbiol*. 1997; 5:234–240. [PubMed: 9211644]
40. Todar, KG. Todar's online textbook of bacteriology. 2012. <http://textbookofbacteriology.net/index.html>. Accessed 3 May 2012
41. McKay GA, Thompson PR, Wright GD. Broad spectrum aminoglycoside phosphotransferase type III from *Enterococcus*: overexpression, purification, and substrate specificity. *Biochemistry*. 1994; 33:6936–6944. [PubMed: 8204627]

Fig. 1.

The streptomycin 6-phosphotransferase-catalyzed reaction. Modified with permission from a figure showing the structure of streptomycin in ref. [40] using MarvinSketch version 6.1.3 (ChemAxon, 2013, <http://www.chemaxon.com>)

Fig. 2. SDS-PAGE analysis of recombinant His-tagged APH(6)-Id. *M*, molecular mass markers; sizes indicated at left (in kDa).

Fig. 3. Reversed-phase HPLC analysis of reaction mixture showing consumption of streptomycin in the APH(6)-Id-catalyzed reaction. *Peak A*: reaction at time = 0; *Peak B*: reaction at time = 10 min. The assay was carried out as indicated in the *Materials and Methods* section, except that [streptomycin] was 0.5 mM and [Mg-ATP] was 2 mM. Data are representative of four independent experiments

a

b

C

d

Fig. 4.

Dependence of enzyme initial velocity on concentration of substrates. **Streptomycin:** *a*, bio-dot experimental data showing an increase in spot intensity with streptomycin concentration (0.15, 0.31, 0.63, 1.20, 2.50 and 5.00 mM). *b*, plot of initial velocity versus streptomycin concentration. **Mg-ATP:** *c*, bio-dot data showing an increase in spot intensity with Mg-ATP concentration (0.05, 0.10, 0.20, 0.40, 0.80, 1.60 and 3.20 mM). *d*, plot of initial velocity versus Mg-ATP concentration. The results in panel A were used for B, and panel C were used for D. Data are representative of three (A, B) or two (C, D) independent experiments

Table 1

Minimum Inhibitory Concentration (MIC) Values

Method	MIC Values ($\mu\text{g/ml}$)					
	Cells Used					
	MM294 or Rosetta (DE3)pLysS		Rosetta(DE3)pLysS-pET15b- <i>aph(6)</i> - <i>ld</i> (no induction)		Rosetta(DE3)pLysS-pET15b- <i>aph(6)</i> - <i>ld</i> (induced with IPTG)	
Broth dilution	3.1		25		200	
Disk diffusion ^a	[Streptomycin] ($\mu\text{g/ml}$)	Zone Diameter (mm)	[Streptomycin] ($\mu\text{g/ml}$)	Zone Diameter (mm)	[Streptomycin] ($\mu\text{g/ml}$)	Zone Diameter (mm)
	10	20	25	25	400	23
	6.25	18	12.5	23	200	20
	3.1 ←	16	6.25	20	100	17
	1.5	12	3.1 ←	18	50	15
	0.08	0	1.5	12	25	0

^aArrows indicate the concentration of streptomycin that gave a zone of inhibition of ~16

mm in the disk diffusion method

Table 2

Physicochemical and Steady-State Kinetic Properties of APH(6)-Id

Property	Value
$V_{\max}$ ($\mu\text{mol}/\text{min}/\text{mg}$)	3.1 $\pm$ 1.1 (coupled assay) 2.0 $\pm$ 0.9 (RP-HPLC-based assay) 4.4 $\pm$ 1.4 (bio-dot radioenzymatic assay) 3.2 $\pm$ 1.1 (average)
Molecular mass (kDa)	31.4 $\pm$ 1.3
k_{cat} (s^{-1}) [*]	1.7 $\pm$ 0.6
$K_{\text{m}}^{\text{streptomycin}}$ (mM) [*]	0.38 $\pm$ 0.04
$K_{\text{m}}^{\text{Mg-ATP}}$ (mM) [*]	1.03 $\pm$ 0.06
$k_{\text{cat}}/K_{\text{m}}^{\text{streptomycin}}$ ($\text{M}^{-1}\text{s}^{-1}$) [*]	4.5×10^3

^{*} determined using the bio-dot radioenzymatic assay method

Table 3

Comparison to Other Aminoglycoside Phosphotransferases

Enzyme	k_{cat} (s^{-1}) ^a	K_{m} aminoglycoside (μM)	K_{m} Mg-ATP (μM)	$k_{\text{cat}}/K_{\text{m}}$ aminoglycoside ($\text{M}^{-1}\text{s}^{-1}$)	Type of Assay Used
APH(6)-Id	1.7 ± 0.6	380 ± 40	$1,030 \pm 60$	4.5×10^3	radioenzymatic assay (this study)
APH(6)-Ia	17.6	3,500	400	5.0×10^3	bioassay of Sm remaining after reaction (ref. [8])
APH(3')-Ia	102 ± 14	1.2 ± 0.2	19.7 ± 2.5	8.5×10^7	spectrofluorimetric assay (ref. [29])
APH(3')-IIIa	1.79 ± 0.09	12.6 ± 2.6	30	1.4×10^5	coupled assay (ref. [41])

^aThe calculation of k_{cat} for APH(6)-Ia assumes that the protein's molecular mass is ~37 kDa, as reported by Sugiyama et al. [8]