Broad Spectrum Aminoglycoside Phosphotransferase Type III from *Enterococcus*: Overexpression, Purification, and Substrate Specificity†

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ABSTRACT: The aminoglycoside phosphotransferases (APHs) are responsible for the bacterial inactivation of many clinically useful aminoglycoside antibiotics. We report the characterization of an enterococcal enzyme, APH(3′)-IIIa, which inactivates a broad spectrum of aminoglycosides by ATP-dependent *O*-phosphorylation. Overproduction of APH(3′)-IIIa has permitted the isolation of 30–40 mg of pure protein/(L of cell culture). Purified APH(3′)-IIIa is a mixture of monomer and dimer which is slowly converted to dimer only over time. Dimer could be dissociated into monomer by incubation with 2-mercaptoethanol, suggesting that dimerization is mediated by formation of disulfide bond(s). Both monomer and dimer show K_m values in the low micromolar range for good substrates such as kanamycin and neomycin, and k_{cat} values of 1–4 s^{-1}. All aminoglycosides show substrate inhibition except amikacin and kanamycin B. Determination of minimum inhibitory concentrations indicates a positive correlation between antibiotic activity and k_{cat}/K_m, but not with K_m or k_{cat}. NMR analysis of phosphorylated kanamycin A has directly demonstrated regiospecific phosphoryl transfer to the 3′-hydroxyl of the 6-aminohexose ring of the antibiotic. Analysis of structure–activity relationships with a variety of aminoglycosides has revealed that the deoxystreptamine aminocyclitol ring plays a critical role in substrate binding. This information will form the basis for future design of inhibitors of APH(3′)-IIIa.

Bacterial resistance to antibiotics has been recently declared a public health emergency (Cohen, 1992; Berkelman & Hughes, 1993; Kunin, 1993). Bacteria evade toxic compounds by a number of different mechanisms including changes in cell permeability, alteration of targets, and chemical modification of foreign molecules (Brighty et al., 1993). The latter method predominates in bacterial resistance to the aminoglycoside/aminocyclitol group of antibiotics (Davies, 1991). These compounds act in part by binding to the bacterial 30s ribosomal subunit thereby initiating a complex series of events which lead to cell death (Davis, 1987). The aminoglycoside antibiotics include such compounds as streptomycin (the first compound successfully used against *Mycobacterium tuberculosis*, the causative agent for tuberculosis), kanamycin, amikacin, and gentamicin, all of which find clinical use throughout the world (Edson & Terrell, 1991). Bacteria have responded to the evolutionary pressure brought about by the introduction of these drugs by expressing a panoply of enzymes which covalently modify aminoglycosides, thereby rendering them inoffensive (Umezawa & Kondo, 1982). These detoxifying enzymes fall into three classes, the *O*-phosphotransferases (APH),[1] the *O*-adenyltransferases, and the *N*-acetyltransferases (Umezawa & Kondo, 1982). The expression of at least seven distinct classes of APHs (Shaw et al., 1993) which are capable of modifying aminoglycosides at position 3′ (denoted as APH(3′)s) (Figure 1) by many pathogenic bacteria has virtually eliminated the clinical utility of previously useful compounds possessing a 3′-hydroxyl such as kanamycin.

Enterococci are second only to *Escherichia coli* in the frequency of nosocomial infections necessitating antibiotic therapy (Spera & Farber, 1992). While the enterococci are intrinsically resistant to low levels of aminoglycosides and β-lactams, treatment with a combination of both drugs results in synergistic killing of the cells (Moellering, 1991). The widespread dissemination of aminoglycoside resistance in enterococci has gravely affected the ability to use aminoglycoside/β-lactam synergism as a treatment for enterococcal infections (Shlaes et al., 1993). The only remaining option is the glycopeptide antibiotic vancomycin, which has also recently fallen to resistance in enterococci (Wright & Walsh, 1992). Treatment of individuals infected with enterococci resistant to aminoglycosides and vancomycin has therefore become very difficult. Two recent studies have indicated that 23–37% of *Enterococcus faecalis* isolates are resistant to at least one aminoglycoside and that 22–47% of these harbored *aph(3′)* genes (van Asselt et al., 1992; Weems et al., 1989).

The 3′-phosphotransferase activity in enterococci confers resistance to a broad spectrum of aminoglycoside antibiotics including kanamycin, amikacin, neomycin, paromomycin, ribostamycin, lividomycin, and butirosin (Figure 2) (Leclercq et al., 1992). The fact that resistance to lividomycin, a compound which lacks a 3′-hydroxyl (Figure 2), is observed has been interpreted as evidence for 5″-phosphotransferase activity. The gene encoding the broad spectrum enterococcal APH(3′) (designated APH(3′)-IIIa) has been cloned and sequenced from plasmid pJH1, a 72.6-kb R plasmid isolated from a clinical isolate (Jacob & Hobbs, 1974; Trieu-Cuot & Courvalin, 1983). The 792-bp *aph(3′)-IIIa* gene encodes an open reading frame corresponding to a protein of 264 amino acids in length with a predicted molecular mass of 30 978 Da which shows significant homology to other APH(3′) proteins (Shaw et al., 1993; Trieu-Cuot & Courvalin, 1983). Sequence analysis of the *aph(3′)* gene from *Staphylococcus aureus*, the third most frequent nosocomial pathogen, has revealed that the amino acid sequence differs from the enterococcal gene

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[1] Abbreviations: APH, aminoglycoside phosphotransferase; MIC, minimal inhibitory concentration; MALDI-Tof, matrix-assisted laser desorption time of flight.

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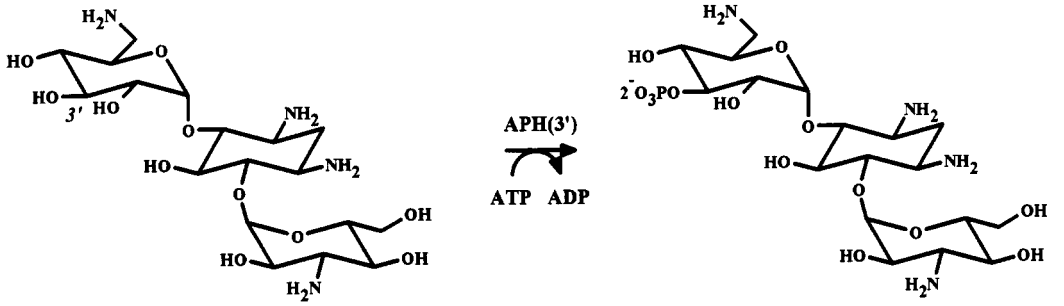
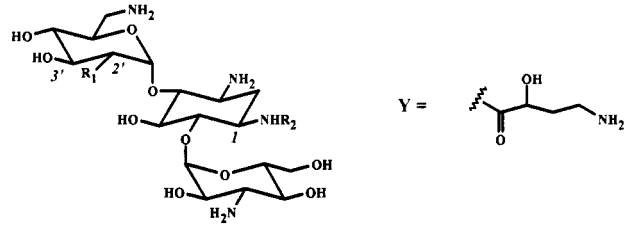


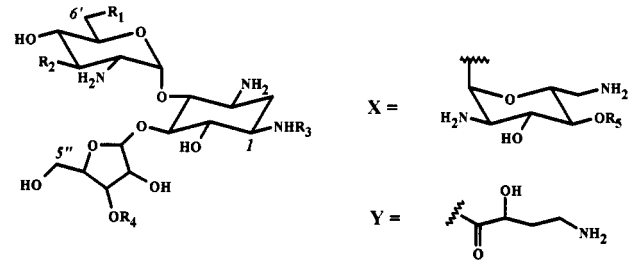
FIGURE 1: Reaction catalyzed by APH(3')-IIIa.

A.



	R1	R2
Kanamycin A	OH	H
Kanamycin B	NH_2	H
Amikacin	NH_2	Y

B.



	R1	R2	R3	R4	R5
Neomycin B	NH_2	OH	H	X	H
Paromomycin	OH	OH	H	X	H
Lividomycin A	OH	H	H	X	mannose
Ribostamycin	NH_2	OH	H	H	
Butirosin	NH_2	OH	Y	H	

FIGURE 2: Structure of aminoglycoside antibiotics: (A) 4,6-disubstituted deoxystreptamine; (B) 4,6-disubstituted deoxystreptamine.

by deletion of one amino acid out of 264 (Val35), while all remaining amino acids are identical (Gray & Fitch, 1983).

The alarming status of antibiotic resistance in enterococci and staphylococci deserves an immediate response. In order to effectively address aminoglycoside resistance, for example by the synthesis of inhibitors of aminoglycoside modification enzymes, a thorough understanding of the mechanism of resistance is required. We report herein the overproduction of APH(3')-IIIa and characterization of its physical and kinetic properties.

MATERIALS AND METHODS

Chemicals

Kanamycin A, kanamycin B, neomycin B, paromomycin, lividomycin A, butirosin, ribostamicin, amikacin, ATP, and pyruvate kinase/lactate dehydrogenase were obtained from Sigma (St. Louis, MO). MacroPrep Q was from Bio-Rad (Mississauga, ON, Canada). Methyl 6-amino-6-deoxyglucopyranoside was prepared by sodium borohydride reduction of methyl 6-azido-α-glucopyranoside. The latter was prepared from methyl 6-tosyl-α-glucopyranoside (Cramer, 1963) as previously described (Cramer, 1962).

Plasmid pAT21-1 was the generous gift of Dr. P. Courvalin, Institut Pasteur, Paris.

Preparation of Overproducing Constructs

A plasmid designed to overproduce APH(3')-IIIa was constructed using the polymerase chain reaction (PCR) to amplify the gene. PCR primers were designed to create a unique *Nde*I (5' GC TCT AGA CAT ATG GCT AAA ATG AGA 3') site 5' to the initiation codon and a unique *Hin*dIII (5' CG AAG CTT GGA CTA AAA CAA TTC ATC CAG 3') site 3' of the termination codon. These primers were used to amplify the aminoglycoside kinase gene (*aph(3')-IIIa*) from plasmid pAT21-1. The isolated fragment was digested with *Nde*I and *Hin*dIII and ligated into pET-22b(+) (Novagen, Madison, WI) cut with the same restriction endonucleases. The gene was then sequenced in order to ensure that no mutations occurred during the PCR procedure. This construct places the *aph(3')-IIIa* gene under control of a bacteriophage T7 promoter and the lac operator. The new plasmid, pPCRG6, was transformed into *E. coli* BL21 (DE3).

Protein Purification

All purification steps were carried out at 4 °C. All Tris buffers are Tris·HCl.

(i) APH(3')-IIIa from E. coli JM105/pAT21-1. A 2-L solution of Luria broth (LB) containing 100 μg/mL ampicillin was inoculated with 20 mL of an overnight culture of *E. coli* JM105/pAT21-1 which caries the *aph(3')-IIIa* gene from *E. faecalis* inserted in the unique *Cla*I of pBR322 (Trieu-Cuot & Courvalin, 1983). Cells were grown at 37 °C at 250 rpm to late log phase (6 h), harvested by centrifugation at 3000*g* for 10 min, and washed with ice cold 0.85% NaCl. Cells were resuspended in 20 mL of lysis buffer (50 mM Tris pH 7.5, 5 mM EDTA, 200 mM NaCl, 1 mM PMSF, 0.1 mM DTT) and lysed by two passages through a French pressure cell at 20 000 psi followed by removal of cell debris by centrifugation at 10000*g* for 20 min. The supernatant was applied to a column containing MacroPrep Q (2 × 10 cm) equilibrated with buffer A (50 mM Tris pH 8.0, 1 mM EDTA). APH(3')-IIIa was eluted by a 0–50% linear gradient in buffer B (50 mM Tris

pH 8.0, 1 mM EDTA, 1000 mM NaCl). Fractions containing APH(3′)-IIIa were identified by enzyme assay, pooled, and applied to a Mono Q column (HR5/5) (Pharmacia) equilibrated with buffer A. Enzyme was recovered by a linear gradient to 50% in buffer B. Kanamycin kinase activity eluted in two distinct peaks at ≈34% B and ≈39% B. Each of these was concentrated over Centricon 10 (Amicon) filters and applied separately to a Superdex 200 column (HR 10/30) (Pharmacia) equilibrated with 50 mM Tris pH 8.0, 1 mM EDTA, 200 mM NaCl. Fractions containing APH(3′)-IIIa were identified by enzyme activity, pooled, and stored at 0 °C.

(ii) APH(3′)-IIIa from E. coli BL21 (DE3)/pPCRG6. Cells were grown from overnight inocula at 37 °C (250 rpm) in Luria broth containing 100 μg/mL ampicillin to an absorbance of 0.5 at 600 nm. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM, and the cells were grown for an additinal 2 h. Cells were harvested at 3000*g* for 10 min, washed with 0.85% NaCl, and lysed as described above. The lysate was loaded onto a MacroPrep Q column (2 × 10 cm) equilibrated with buffer A, and APH(3′)-IIIa was eluted by a 0–50% linear gradient in buffer B over 70 mL (fast gradient). Fractions containing kanamycin kinase activity were pooled, reapplied to the MacroPrep Q column, and eluted with a gradient to 50% in buffer B over 150 mL (slow gradient). The enzyme is stable at 0 and −20 °C (in the presence of 5% glycerol) for several months.

(iii) Determination of the Native Molecular Weight of Purified APH(3′)-IIIa. Purified samples of APH(3′)-IIIa were analyzed by gel filtration using a Superdex 200 (HR10/30) at a flow rate of 0.4 mL/min in 50 mM Tris pH 8.0, 1 mM EDTA, 200 mM NaCl. Samples of β-amylase, alcohol dehydrogenase, bovine albumin, carbonic anhydrase, cytochrome *c*, and blue dextran were used as standards to obtain a calibration curve from which an estimate of the molecular weight of APH(3′)-IIIa could be obtained.

Enzyme Assay

Phosphorylation of aminoglycoside antibiotics was monitored by coupling the release of ADP to a pyruvate kinase/lactate dehydrogenase (PK/LDH) reaction. The oxidation of NADH was followed by continuously monitoring the absorbance at 340 nm using a Cary 3E UV–vis spectrophotometer. In a typical experiment 975 μL of assay buffer (50 mM Tris pH 7.5, 40 mM KCl, 10 mM $MgCl_2$, 0.5 mg/mL NADH, 2.5 mM phosphoenolpyruvate, 1 mM ATP) was mixed with 10 μL of aminoglycoside solution and 5 μL of PK/LDH enzyme solution. The mixture was preincubated for 15 min at 37 °C and the assay initiated by rapid addition of 10 μL of purified APH(3′)-IIIa (3.5 μg/μL stock solution).

Assays on partially purified enzyme, e.g., during enzyme purification, were carried out with 0.1 mM kanamycin A. The values indicated have been normalized for background ATPase activity, which was monitored by assay in the absence of kanamycin A.

All antibiotic stock solutions (for both kinetics and minimal inhibitory concentration experiments) were titrated using this assay under the assumption that addition of a phosphate group to the antibiotic was a 1:1 stoichiometric event (*vide infra*).

Initial rates were obtained directly from progress curves and analyzed by nonlinear least squares fitting of eq 1,

$$v = V_{max}S/(K_m + S) \quad (1)$$

or eq 2 for substrate inhibited reactions,

$$v = V_{max}S/(K_m + S + S^2/K_i) \quad (2)$$

Determination of Minimal Inhibitory Concentrations (MICs)

MICs for various aminoglycosides were determined according to the National Committee for Clinical Laboratory Standards (1990). MICs were obtained using *E. coli* BL21/pSACG1 which incorporates a silent *Sac*I restriction site in the *aph(3′)-IIIa* gene (P. R. Thompson and G. D. Wright, unpublished results). Briefly, cells were grown in Mueller–Hinton broth (MHB) (Difco) to an absorbance of 0.3–0.35 at 600 nm followed by subsequent dilution with MHB to give a final concentration of 5 × 10^6 colony forming units/mL. A volume of 100 μL of this culture was added to 2 mL of MHB with increasing concentrations of aminoglycosides and grown for 24 h at 37 °C. MIC end points were determined to be the concentration of aminoglycoside where no visible growth was obtained.

Large-Scale Preparation and Purification of Phosphorylated Kanamycin A

Large-scale production of phosphorylated kanamycin A was performed by modification of the method of Coombe and George (1981). Incubations consisted of 100 mg of kanamycin A in 50 mM Tris pH 7.5, 40 mM KCl, 10 mM $MgCl_2$ and used 1 mg of purified APH(3′)-IIIa (an additional 1 mg of enzyme was added after 20 h to drive the reaction to completion). The reactions were carried out at 37 °C with 250 rpm shaking and in sufficient volume such that the final concentration of kanamycin A was an order of magnitude below its K_i. A 2-fold molar excess of ATP was added to ensure complete phosphorylation of the aminoglycoside.

The progress of the reaction was monitored by measuring the amount of active antibiotic by a *Bacillus subtilis* susceptibility disk assay. Reaction mixture (15 μL) was applied to filter paper disks (6.4 mm) at various time points, and these disks were overlaid onto an agar plate freshly inoculated with 200 μL of an overnight culture of *B. subtilis*. Plates were incubated at 37 °C overnight, and the amount of remaining antibiotic was estimated by measuring the radius of the clear zone surrounding the disk. Once the *B. subtilis* showed no further sensitivity to the samples, the reaction was deemed complete (the MIC of kanamycin A for *B. subtilis* is 1 μg/mL).

The reaction mixture was then applied to an AG50W-X8 (Bio-Rad) (NH_4^+ form) column (10 × 3 cm). The column was washed with 200 mL of H_2O, and bound phosphorylated aminoglycoside was eluted by the addition of 200 mL of 0.1% NH_4OH. Fractions (4 mL) were collected and analyzed by TLC on silica plates (Merck) using either methanol/ammonium hydroxide (5:2) or *n*-butyl alcohol/ethanol/chloroform/ammonium hydroxide (4:5:2:8) as mobile phases. Aminoglycosides were visualized by ninhydrin spray. Fractions containing phosphorylated aminoglycoside were pooled and lyophilized, and the residue was dissolved in 2 mL of H_2O.

The product was then applied to a BioRex 70 (Bio-Rad) (NH_4^+ form) column (11 × 3 cm) preequilibrated with 200 mL of H_2O. The column was washed with a further 200 mL of H_2O, and the product was eluted using a linear gradient of 0–1 M NH_4OH. The presence of phosphorylated drug was determined by TLC analysis of the fractions and the pooled lyophilized material dissolved in 500 μL of H_2O.

The sample was applied to a Sephadex G-25 column (82 × 2 cm) equilibrated in H_2O, and the phosphorylated drug was desalted at a flow rate of 1.5 mL/min. Fractions were monitored by TLC as described above, and those containing phosphorylated aminoglycoside were pooled and lyophilized.

NMR and Mass Spectral Analysis of Phosphokanamycin A

All NMR spectra were obtained at ambient temperature at the McMaster magnetic resonance facility. ^{31}P and ^{1}H NMR spectra were obtained on a Bruker AL 300 instrument operating at 300 MHz with a field strength of 7.05 T. ^{1}H spectra, both 1D and 2D, were obtained on compounds dissolved in $^{2}H_2O$, and samples were presaturated to suppress the HOD resonance. ^{31}P NMR spectra were obtained using an external reference of 85% H_3PO_4 in $^{2}H_2O$. *J*-Modulated ^{13}C spin-echo experiments were performed on a Bruker AM 500 instrument operating at 11.745 T with compounds dissolved in $^{2}H_2O$.

Pneumatically assisted electrospray mass spectra of purified aminoglycosides were obtained in the presence of trifluoroacetic acid on a Fisons platform quadrupole mass spectrometer.

Miscellaneous Methods

(i) Protein Determination. Protein concentration was estimated by Bradford assay using bovine serum albumin as standard (Bradford, 1976).

(ii) Mass Spectrometry of Purified APH(3′)-IIIa. Matrix-assisted laser desorption time of flight (MALDI-Tof) mass spectra were obtained on a VG/Fisons TofSpec (Manchester, England) linear time of flight mass spectrometer equipped with a nitrogen laser (337 nm, 5-ns pulse). The accelerating voltage in the ion source was 25 kV. Data were acquired with a transient recorder with sampling at 250 MHz. Mass resolution was 300 (full width at half maximum). The spectra were produced by accumulating 64 laser shots, and the matrix used was α-cyano-4-hydroxycinnamic acid. Time to mass conversion was done by internal calibration using bovine serum albumin.

RESULTS

APH(3′)-IIIa Is Isolated as a Mixture of Monomer and Dimer

Purification of APH(3′)-IIIa from *E. coli* JM105/pAT21-1 was followed using a coupled assay to monitor kanamycin-dependent ADP release. We initially attempted to use trapping of [^{32}P]phosphokanamycin with phosphocellulose and scintillation counting, an assay which is frequently used to monitor aminoglycoside modification (Haas & Dowding, 1975); however, this technique proved unreliable for kinetic experiments in our hands and could not be used to compare results obtained with other aminoglycosides.

Purification of APH(3′)-IIIa resulted in two different protein peaks with kanamycin kinase activity off the Mono Q column (Table 1). These fractions were applied separately to a sizing column, Superdex 200, and kanamycin kinase activity eluted at different times, 41 min for Mono Q peak 1 and 37 min for Mono Q peak 2 (Figure 3). Rechromatography over Superdex 200 following standardization with proteins of known molecular weight revealed an apparent molecular mass of 30 kDa for peak 1 and 60 kDa for peak 2, yet they appeared as single bands of 30 kDa by SDS–PAGE (Figure 4). This indicated that APH(3′)-IIIa was purified as a mixture of monomer and dimer.

Table 1: Purification of APH(3′)-IIIa from *E. coli* JM105/pAT21-1

fraction	protein (mg)	act. (units)	sp act. (units/mg)	purifn (-fold)	recov (%)
cell extract	299	–[a]	–	–	–
MacroPrep Q	13.5	15.6	1.2	–	100
Mono Q					
fraction 1	2.6	5.9	2.3	1.9	54
fraction 2	1.3	2.5	1.9	1.6	
Superdex 200					
fraction 1	1.0	2.5	2.5	2.1	
fraction 2	0.37	1.6	4.3	3.6	26

[a] Enzyme activity could not be accurately determined do to high background ATPase activity in whole cell extracts.

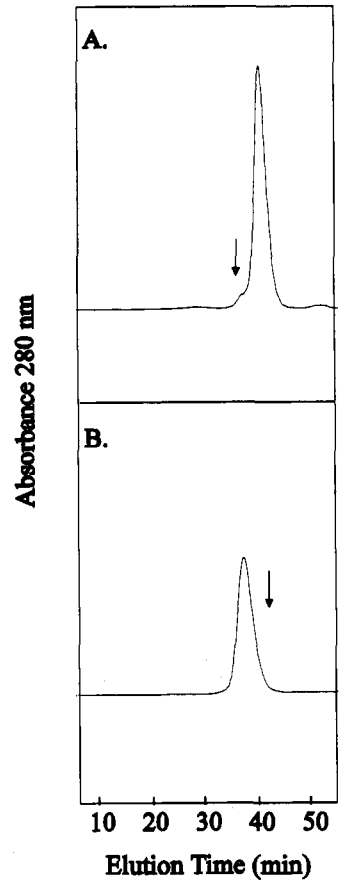


FIGURE 3: Chromatography of Mono Q peak 1 (A) and peak 2 (B) over Superdex 200. Arrows indicate the positions of dimer (A) and monomer (B) for comparison. The column was run with a mobile phase consisting of 50 mM Tris pH 8.0, 200 mM NaCl, 1 mM EDTA at a flow rate of 0.4 mL/min.

Both forms of the enzyme had identical kinetic constants within experimental error, which indicates that they are catalytically identical and demonstrates that there are at least two active sites per dimer (for kanamycin, monomer K_m = 18 ± 5.3 μM, k_{cat} = 1.63 ± 0.15 s^{-1}; dimer K_m = 16.3 ± 3.8 μM, k_{cat} = 1.60 ± 0.11 s^{-1}). APH(3′)-IIIa dimer could not be dissociated by dilution or high salt, and purified monomer slowly converted to dimer at 0 °C, although dimer was stable under the same conditions. Dimer could be completely converted to monomer by the addition of 2-mercaptoethanol followed by incubation at 0 °C for several days.

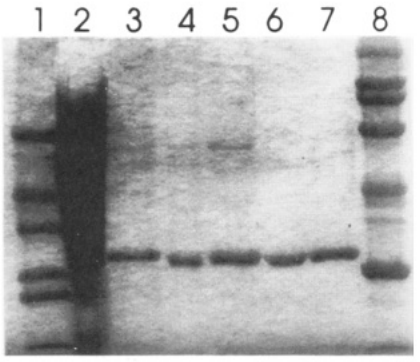


FIGURE 4: Purification of APH(3′)-IIIa from *E. coli* JM105/pAT21-1. The SDS–polyacrylamide gel (11%) was stained with Coomassie blue. Lanes: 1, low molecular weight markers (66, 45, 36, 29, 24, 20, and 14.2 kDa); 2, crude extract; 3, MacroPrep Q eluent; 4, Mono Q peak 1; 5, Mono Q peak 2; 6, Superdex 200 peak 1; 7, Superdex 200 peak 2; 8, high molecular weight markers (205, 116, 97.4, 66, 45, and 29 kDa). Conditions are detailed in Materials and Methods.

Overproduction of APH(3′)-IIIa

While purification of APH(3′)-IIIa from construct pAT21-1 afforded sufficient enzyme for initial studies, we required substantial quantities of enzyme to determine kinetic properties of many aminoglycosides and regiospecificity. We therefore designed and prepared a construct, pPCRG6, which gave excellent overproduction of APH(3′)-IIIa (Figure 5A). A two-step purification yielded 30–40 mg of enzyme/(L of cell culture) (Table 2, Figure 5A). The MALDI-Tof mass spectrum indicated significant peaks at m/z = 31 030 and 61 834 indicative of monomer and dimer, respectively (predicted 30 978 and 61 956) (Figure 5B). This overproduced enzyme has catalytic properties identical with those of APH(3′)-IIIa purified from the pAT21-1 construct.

Kinetic Properties of APH(3′)-IIIa

Purified overexpressed APH(3′)-IIIa was used to determine the reactivity of a series of 4,5- and 4,6-disubstituted aminoglycosides by ADP release coupled assay (Table 3). Most aminoglycoside substrates demonstrated substrate inhibition, a property which has been noted for aminoglycoside acetyltransferases and aminoglycoside adenyltransferases (Gates & Northrop, 1988; Radika & Northrop, 1984a). Substrate inhibition was obvious but weak, being some 27 (paromomycin) to 490 (kanamycin A) times greater than K_m. Substrate inhibition was more pronounced in the 4,5-disubstituted aminoglycosides than the 4,6-disubstituted drugs, and two of these, kanamycin B and amikacin, did not show signs of inhibition. As the 3′-hydroxyl was predicted to be phosphorylated, we prepared methyl 6-amino-α-glucoside as a potential substrate. Neither this amino sugar nor methyl α-glucoside nor ribose was a substrate or inhibitor at 10 mM concentration. Tobramycin (3-deoxykanamycin B) was not a substrate but was a competitive inhibitor for kanamycin A with a K_i of 2 μM.

Relationship between MIC and Kinetic Parameters

MICs were determined for a representative group of aminoglycosides using our overproducing construct in *E. coli* BL21(DE3) (Table 4). While we should expect no expression of APH(3′)-IIIa in this construct due to the requirement of a T7 RNA polymerase for transcription, there is some basal expression as indicated by the resistance shown to various aminoglycosides in Table 4. No resistance was observed in the absence of the plasmid. We examined the relationship between MIC and K_m, k_{cat}, and k_{cat}/K_m (Figure 6), omitting the MIC data from paromomycin and lividomycin as only lower limits for MIC could be determined. The only significant relationship is between MIC and k_{cat}/K_m, which shows a positive correlation with r = 0.703. Correlations with k_{cat} and K_m were 0.269 and –0.654, respectively.

Regiospecificity of APH(3′)-IIIa

APH(3′)-IIIa resistance to kanamycin but not tobramycin implies that this enzyme has aminoglycoside 3′-phosphotransferase activity. Titration of aminoglycosides with purified APH(3′)-IIIa in the presence of the PK/LDH coupled assay system gave apparent 1:1 stoichiometries for ADP hydrolysis with the amount of aminoglycoside added (as determined by the mass of aminoglycoside prior to stock solution preparation).[2] Electrospray mass spectral analysis of purified phosphorylated kanamycin A revealed a prominent peak at 565.3 Da/e in both the positive and negative ion modes consistent with an M + 4 (due to protonation of the four amino groups on kanamycin A) molecular ion peak confirming the expected 1:1 stoichiometry. In order to explore the regioselectivity of the enzyme, we performed large-scale inactivations of kanamycin A, purified the phosphorylated product, and examined the NMR spectra of various nuclei (Figure 7). The ^{31}P spectra of the phosphorylated aminoglycosides show a single peak at 4.89 ppm (Figure 7A) indicative of a single tetrahedral phosphate monoester (Gorenstein, 1984). Comparison of the 1D ^{1}H spectrum of kanamycin and kanamycin phosphate reveals one significant difference, which is the presence of a multiplet at 4.1 ppm in the phosphorylated compound (Figure 7B). This pattern is consistent with splitting of axial 3′H with axial 2′H and 4′H and a three-bond coupling with phosphorus (P–O–C–3′H) of 9 Hz, and has been previously reported (Naganawa et al., 1971). DQF-COSY of kanamycin and kanamycin phosphate allowed unambiguous assignment of 3′H of kanamycin at 3.59 ppm (d, J = 10 Hz), which disappears upon phosphorylation. The resonance of 1′H of phosphokanamycin at 5.33 ppm (Naganawa et al., 1971) permitted identification of 2′H at 3.52 ppm, which splits 3′H at 4.1 ppm (Figure 7C). The J-modulated ^{13}C spin-echo spectrum of phosphokanamycin revealed a negative doublet at 75.5 ppm (J = 5 Hz) as predicted for a P–O–CHR$_2$ coupling (not shown). The observed ^{13}C chemical shifts are consistent with the previously determined ^{13}C spectrum of 3′-phosphokanamycin A obtained by incubation with an unspecified APH (Coombe & George, 1981).

DISCUSSION

Antibiotic resistance has severely impacted on current antimicrobial therapy. Treatment of enterococcal infections has especially become difficult as these organisms are sensitive only to combination therapy with β-lactams and aminoglycosides, or with vancomycin. Resistance to all these drugs has been reported in clinical enterococcal isolates, so treatment of individuals infected by bacterial strains which harbor resistance to all three classes of antibiotics, or treatment of individuals who are allergic to β-lactams and are infected with vancomycin-resistant organisms, is problematic in the extreme. One solution to this impasse is the coadministration of inhibitors of antibiotic resistance enzymes with antibiotics to permit regaining of the original antimicrobial index. Such

[2] The exceptions to this observation are neomycin and ribostamycin, both of which gave stoichiometries of >1; the precise mechanism of this anomaly is currently being investigated.

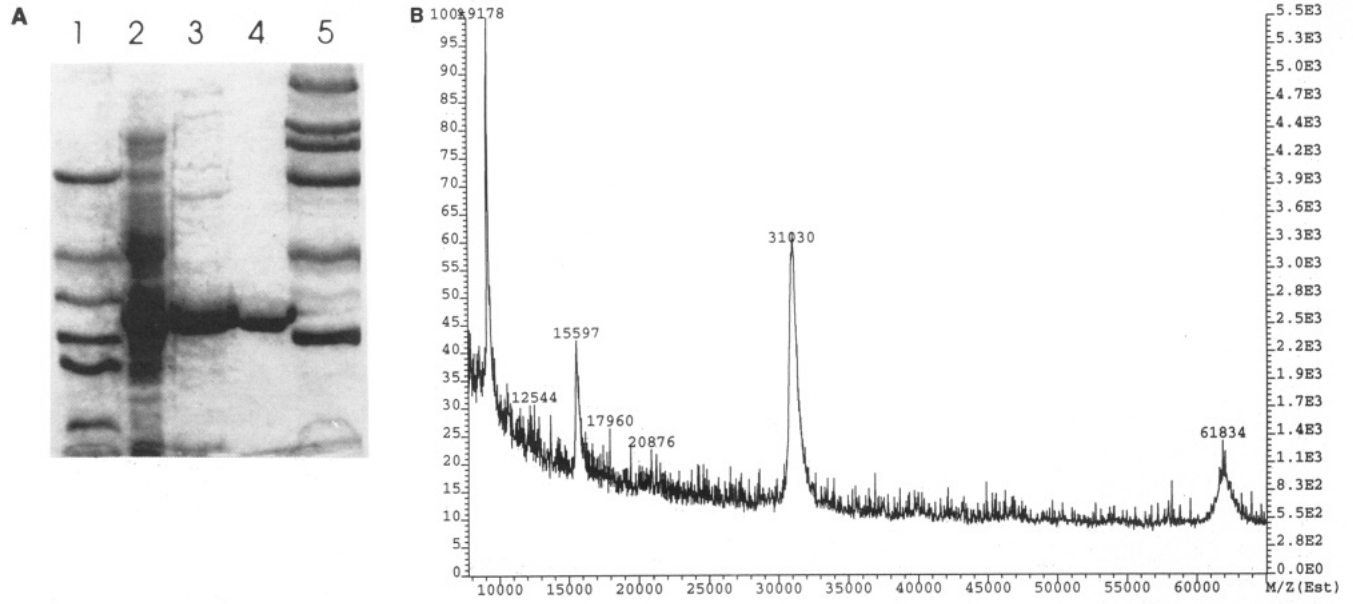


FIGURE 5: Purification of APH(3′)-IIIa from *E. coli* BL21(DE3)/pPCRG6. (A) The SDS–polyacrylamide gel (11%) was stained with Coomassie blue. Lanes: 1, low molecular weight markers (66, 45, 36, 29, 24, 20, and 14.2 kDa); 2, crude extract; 3, MacroPrep Q fast gradient; 4, MacroPrep Q slow gradient; 5, high molecular weight markers (205, 116, 97.4, 66, 45, and 29 kDa). (B) MALDI-Tof spectrum of purified overproduced APH(3′)-IIIa.

Table 2: Purification of APH(3′)-IIIa from *E. coli* BL21(DE3)/pPCRG6

fraction	protein (mg)	act. (units)	sp act. (units/mg)	purifn (-fold)	recov (%)
cell extract	600	280	0.47		100
MacroPrep Q					
fast gradient	155	248	1.6	3.4	88
slow gradient	76	167	2.2	4.7	60

Table 3: Kinetic Parameters of Purified Overexpressed APH(3′)-IIIa[a]

substrate	K_m (μM)	k_{cat} (s^{-1})	K_i (mM)	k_{cat}/K_m (M^{-1} s^{-1})
kanamycin A	12.6 ± 2.6	1.79 ± 0.09	6.38 ± 1.67	1.43×10^5
kanamycin B	19.4 ± 2.2	3.51 ± 0.19	nd[b]	1.81×10^5
amikacin	245 ± 27	2.46 ± 0.11	nd[b]	1.00×10^4
neomycin B	7.72 ± 0.9	2.08 ± 0.07	2.65 ± 0.59	2.69×10^5
paromomycin	19.5 ± 3.5	3.62 ± 0.25	0.53 ± 0.11	1.86×10^5
lividomycin A	31.6 ± 5.1	3.97 ± 0.25	1.53 ± 0.42	1.26×10^5
ribostamycin	9.30 ± 1.8	1.89 ± 0.10	1.73 ± 0.66	2.03×10^5
butirosin	34.3 ± 3.1	2.02 ± 0.07	2.17 ± 0.41	5.87×10^4
ATP	27.7 ± 3.7	1.76 ± 0.08	nd[b]	6.37×10^4

[a] Kinetic parameters were determined using purified overexpressed APH(3′)-IIIa at 37 °C as described in Materials and Methods using 1 mM ATP. The data for ATP were collected using 100 μM kanamycin A. [b] Substrate inhibition was not detected at the highest concentration of aminoglycoside tested (2–2.5 mM).

Table 4: MICs of Aminoglycosides with *E. coli* BL21(DE3)/pSACG1

aminoglycoside	MIC (μg/mL)
kanamycin A	350
kanamycin B	350
amikacin	7.5
neomycin B	220
paromomycin	>1320
lividomycin A	>1240
butirosin	20

an approach has met with success in treatment of penicillin-resistant organisms with a combination of a β-lactam and a β-lactamase inhibitor such as clavulanic acid (Sutherland, 1991). In order for such a strategy to be used, a thorough understanding of resistance enzyme mechanism is desired. As a first step in such a program we have overproduced and characterized the broad spectrum aminoglycoside kinase from *E. faecalis*.

Aminoglycoside phosphotransferases have been purified previously largely by the use of immobilized aminoglycoside columns (e.g., Sarwar & Akhtar, 1990; Lee et al., 1991), and an APH(3′)-III has been purified from *S. aureus* and *Staphylococcus epidermidis* using sisomycin Sepharose 4B (Ubukata et al., 1984). We have found that enterococcal kanamycin kinase, APH(3′)-IIIa, is readily purified from *E. coli* extracts by anion exchange chromatography. APH(3′)-IIIa purifies as a mixture of monomer and dimer which are catalytically indistinguishable. While monomer will dimerize over time, dimer is quite stable but will be converted to monomer by the addition of 2-mercaptoethanol. This suggests that the interaction is due to the formation of one or more intraprotein disulfide bonds. The site of dimerization must occur at a catalytically inert protein region as kanamycin phosphorylation is unaffected by the dimeric state of the enzyme. It is therefore unlikely that the substrate binding site or catalytically important residues occur at the interface of the dimeric complex as has been noted for chloramphenicol acetyltransferase (Leslie et al., 1988). Overproduced enzyme also behaves in an identical fashion, indicating that dimerization is a fundamental property of the APH(3′)-IIIa polypeptide chain.

The overproduction construct, pPCRG6, has enabled access to large quantities of purified enzyme. This has permitted structure–function analysis of APH(3′)-IIIa as summarized in Table 5. An initial examination of this data indicates significant differences in K_m for aminoglycosides differing only by one substituent, suggesting a difference in binding to the enzyme, assuming that the reaction proceeds under rapid equilibrium conditions. However, Williams and Northrop have presented evidence to suggest that, for an aminoglycoside acetyltransferase, rapid equilibrium cannot be assumed for all substrates (Williams & Northrop, 1978a). These authors have argued that, under nonrapid equilibrium conditions, different changes in V and V/K reflect a change in substrate

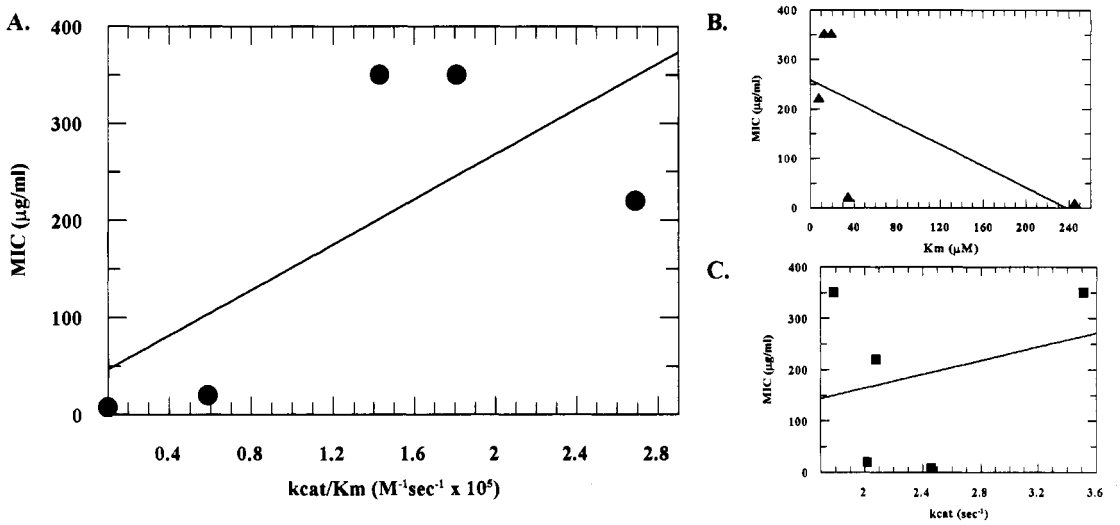


FIGURE 6: Relationship between MIC and kinetic parameters of APH(3′)-IIIa. Data were obtained from Tables 3 and 4.

binding while similar changes in V and V/K are indicative of changes in catalysis (Williams & Northrop, 1978b). We have not yet determined whether APH(3′)-IIIa functions under rapid equilibrium conditions; however, we note from inspection of Table 5, and assuming either rapid or nonrapid equilibrium conditions, that K_m remains a good indicator of binding.

A general observation is that APH(3′)-IIIa has similar affinities for 4,6-disubstituted aminoglycosides such as kanamycin and 4,5-disubstituted drugs such as neomycin, indicating that the active site shows little stringency for either 4,6- or 4,5-substitution of the aminocyclitol ring. At the same time, methyl 6-amino-α-glucoside and ribose, which incorporate the sites of phosphorylation for kanamycin A and lividomycin A, respectively, are not substrates or inhibitors of the enzyme, suggesting that the 2-deoxystreptamine ring plays an important role in substrate binding. Underlining this fact is a 14-fold decrease in specificity (k_{cat}/K_m) for amikacin, which is kanamycin A substituted with a (*S*)-4-amino-2-hydroxybutyryl group (AHB) at the aminocyclitol N-1 (Figure 2), over the parent compound (Table 5). Similarly, a 3.4-fold decrease in k_{cat}/K_m is observed between the 4,5-disubstituted deoxystreptamine antibiotics, butirosin and ribostamicin, which also differ by the presence of an AHB group at the aminocyclitol N-1. In both cases, the effect is primarily found on K_m, suggesting a binding phenomenon. This evidence unambiguously identifies the aminocyclitol ring as an important component in substrate orientation in APH(3′)-IIIa.

Changes in the 6-aminohexose ring of both 4,6-disubstituted and 4,5-disubstituted aminoglycosides result in small changes in affinity (compare kanamycin A and B, neomycin B, and paromomycin in Table 5). In both cases, substitution of an amino group for a hydroxyl results in a modest decrease in affinity for APH(3′)-IIIa.

Many aminoglycosides show weak but measurable substrate inhibition, while ATP does not. Such inhibition has been noted previously with aminoglycoside acetyltransferases and adenyltransferase where it is more prominent (Gates & Northrop, 1988; Radika & Northrop, 1984a). The structural basis for this inhibition is unclear. One possibility is that improper binding of the drug may be analogous to such binding in lysozyme where there are distinct binding sites for the saccharide units on either site of the scissile glycosidic bond (Sharon, 1967). If aminoglycoside-modifying enzymes exploit such an arrangement where productive binding results in phosphate transfer, substrate inhibition could occur when, for example in the case of kanamycin A, the two deoxystreptamine ring or the 3-aminoglucose ring binds in the 6-aminoglucose binding site, in a fashion related to nonproductive binding in lysozyme. Clearly, however, the very large values for K_i with APH(3′)-IIIa suggest that substrate inhibition has no biological significance.

An important consideration is the biological consequence of the variety of kinetic constants measured. It has been noted elsewhere that one can consider inhibition of cell growth by an aminoglycoside antibiotic as being dependent on two rates, that of entry of the drug into the cell and that of detoxification by the presence of an aminoglycoside-modifying enzyme (Davies & Kagan, 1977; Radika & Northrop, 1984b). It is therefore possible that some correlation may exist between the measured kinetic constants of purified APH(3′)-IIIa and inhibition of cell growth by cells harboring the phosphotransferase gene. Others have reported a positive correlation between MIC and K_m only for aminoglycoside 2″-adenyltransferase, 3-acetyltransferase, and 2′-acetyltransferase (Vastola et al., 1980), with K_m and V/K for aminoglycoside 2″-adenyltransferase (DeHertogh & Lerner, 1985) and V/K only for 6′-acetyltransferase (Radika & Northrop, 1984b) and 2″-nucleotidyltransferase (Bongaerts & Molendijk, 1984). We note that there does exist a positive correlation between MIC and k_{cat}/K_m, the apparent second-order rate constant for the enzyme reaction with APH(3′)-IIIa. We see no significant correlation with K_m as expected for an equilibrium phenomenon, nor do we see a correlation with k_{cat}, indicating that the rate at saturating substrate conditions does not contribute significantly to resistance. The positive, though albeit modest, correlation we see with k_{cat}/K_m, which is the rate of phosphoryl transfer at sub-K_m substrate concentrations, suggests a state where there is a contribution to resistance by the rate of entry of the aminoglycoside into the cell. When this latter rate is very low, the correlation breaks down, which we predict dominates the apparently anomalous MIC values seen with lividomycin A and paramomycin. We note, however, that the correlation which we observe (0.703) is weaker than that reported for other aminoglycoside-modifying enzymes, e.g., 0.818 for 6′-acetyltransferase (Radika & Northrop, 1984b). This suggests that for APH-III the relationship may be more complex than a simple direct cause and effect correlation.

Patterns of drug resistance had indicated that APH(3′)-IIIa would phosphorylate kanamycin at the 3′-hydroxyl. We have shown that the ^{31}P, ^{1}H, and ^{13}C NMR and mass spectra of purified kanamycin A phosphorylated by APH(3′)-IIIa

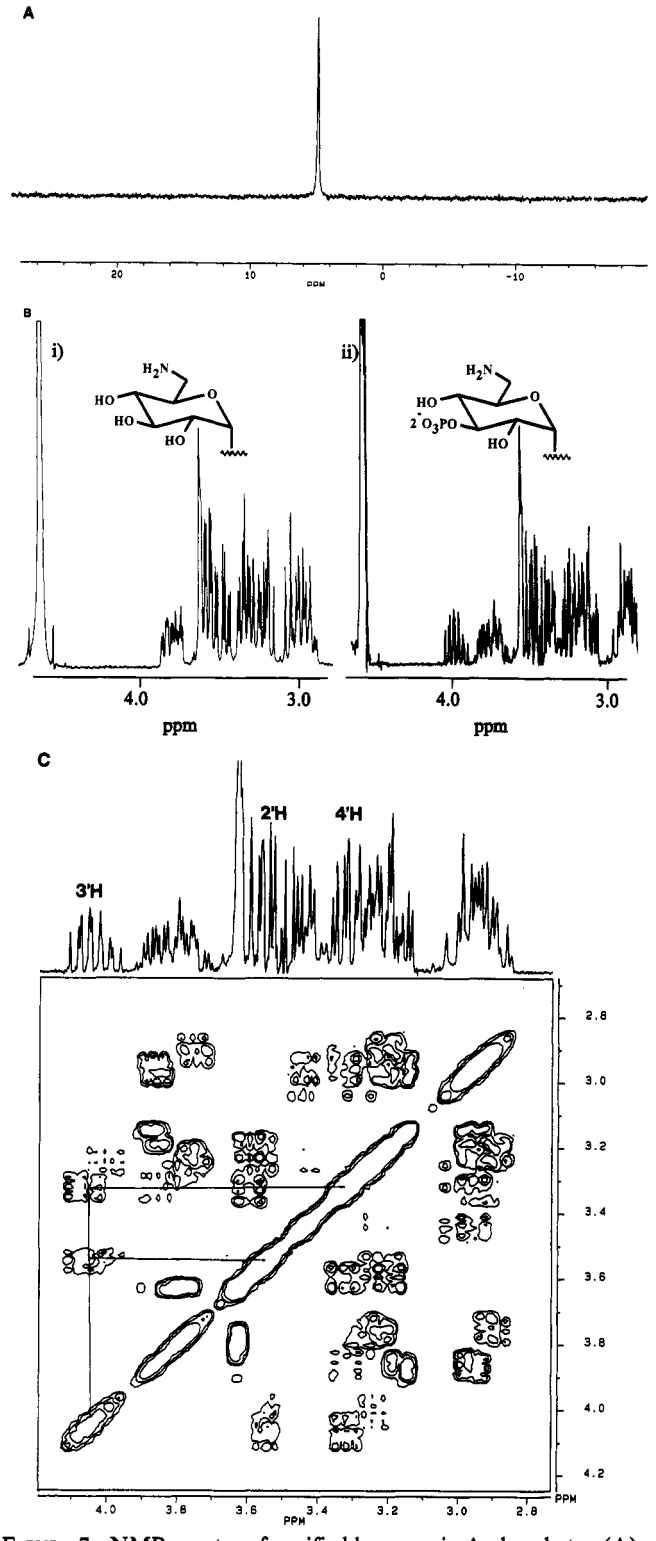


FIGURE 7: NMR spectra of purified kanamycin A phosphate: (A) ^{31}P spectrum (referenced to external H_3PO_4 (85%) in D_2O); (B) comparison of the 1D ^1H spectra for kanamycin A (i) and kanamycin A phosphate (ii); (C) portion of the ^1H DQF-COSY spectrum of kanamycin A phosphate.

are consistent with this hypothesis. Furthermore, tobramycin (3-deoxykanamycin B) was not a substrate and was a potent competitive inhibitor with a K_i of 2 μM. APH(3′)-IIIa therefore is regiospecific with respect to phosphorylation of kanamycin A despite the fact that there exist seven chemically equivalent hydroxyl groups in the drug (Figure 1). Therefore, while the substrate binding site has some flexibility, as evidenced by the range of aminoglycosides phosphorylated,

Table 5: Structure–Activity Analysis of APH(3′)-IIIa[a]

aminoglycoside	position[b]	group	ΔK_m	Δk_{cat}	$\Delta k_{cat}/K_m$
kanamycin A	2′	OH	1	1	1
kanamycin B	2′	NH_2	1.5	1.96	0.8
kanamycin A	1	NH_2	1	1	1
amikacin	1	NH-AHB[c]	19	1.37	0.07
neomycin B	6′	NH_2	1	1	1
paromomycin	6′	OH	2.5	1.74	0.69
ribostamycin	1	NH_2	1	1	1
butirosin	1	NH-AHB	3.6	0.94	0.29

[a] Differences are indicated as ratios. [b] See Figure 2 for chemical structures of aminoglycosides. [c] AHB = (*S*)-4-amino-2-hydroxybutyryl (group Y in Figure 2).

there is also rigidity in that phosphorylation is regiospecific for kanamycin A. Further work on elucidation of the substrate binding site is in progress and will be required for more extensive analysis.

The net result of this single regiospecific phosphorylation is the ability to completely abolish drug potency. This has manifested itself in the virtual elimination of 3′-hydroxylated aminoglycosides (with the exception of amikacin) from clinical use. Consequently, antibiotics such as tobramycin and gentamicin C, both of which are 3′-deoxyaminoglycosides, have emerged as clinically useful agents; however, these are inactivated by a number of other aminoglycoside-modifying enzymes (Shaw et al., 1993). Furthermore, a recent report has noted a potential problem caused by the overproduction of APH(3′)-I in *E. coli* (Menard et al., 1993). Tobramycin is not a substrate for this enzyme; however, an increase in MIC was observed when APH(3′)-I was overproduced presumably by interaction of the drug and enzyme in a noncovalent fashion with free drug thereby sequestering it in the cell. We note that tobramycin binds tightly to APH(3′)-IIIa and that overexpression of this gene in a clinical isolate could result in resistance to tobramycin in the absence of ATP-dependent phosphorylation.

In order to meet the challenge of the rapid dissemination of antibiotic resistance, new strategies including the search for novel antibiotics, the design of inhibitors of resistance enzymes, and the judicious use of current therapeutics must be pursued. This study marks a first step in the understanding of the molecular basis of aminoglycoside phosphorylation by APH(3′)-IIIa and as such should serve as a basis for further analysis of this class of resistance enzymes.

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