Aminoglycoside 2''-phosphotransferases mediate high-level resistance to aminoglycoside antibiotics in Gram-positive microorganisms, thus posing a serious threat to the treatment of serious enterococcal infections. The manuscript reports on cloning, purification and detail mechanistic characterization of aminoglycoside 2''-phosphotransferase known as type Ic enzyme. In an unexpected finding, the enzyme exhibits strong preference for guanosine triphosphate over adenosine triphosphate as the phosphate donor, a unique observation among all characterized aminoglycoside phosphotransferases. The enzyme phosphorylates only certain 4,6-disubstituted aminoglycosides exclusively at the 2''-hydroxyl with $k_{cat}$ values of 0.5-1.0 s$^{-1}$ and $K_m$ values in the nanomolar range for all substrates but kanamycin A. Based on this unique substrate profile, the enzyme is renamed aminoglycoside 2''-phosphotranserase type IIIa. Product and dead-end inhibition patterns indicated a random sequential Bi Bi mechanism. Both the solvent viscosity effect and determination of the rate constant for dissociation of guanosine triphosphate indicated that at pH 7.5 the release of guanosine triphosphate is rate-limiting. A computational model for the enzyme is presented, which sheds light on the structural aspects of interest to this family of enzymes.

The main mechanism of resistance of bacteria to aminoglycoside antibiotics is the enzymatic modification of the amino or hydroxyl groups of these drugs (1). Aminoglycoside phosphotransferases (APHs)$^2$ catalyze the regiospecific transfer of the γ-phosphoryl group of ATP to one of the aminoglycoside hydroxyls. This mechanism of resistance is particularly relevant for clinical enterococcal and staphylococcal isolates (2). Despite the widespread occurrence of aminoglycoside phosphotransferases in pathogenic bacteria, only few of these enzymes have been characterized mechanistically (3-6). The most prevalent aminoglycoside phosphotransferases are the APH(3')s, which transfer a phosphate group to the 3'-hydroxyl of kanamycin A (7). From this class, APH(3’)-IIIa has been the best studied (4,8-10). Product and dead-end inhibition, solvent isotope and viscosity effect experiments have shown that this enzyme follows a Theorell-Chance mechanism for turnover chemistry, with ordered substrate binding (ATP prior to aminoglycoside) and sequential product release (the release of ADP occurs last in the rate-limiting step) (4). It has also been suggested that this enzyme involves a loose, or dissociative, transition state for the phosphate transfer (9), in agreement with the findings for the non-enzymatic phosphate transfer in monoesters (11). The presence of what came to be known as the aph(2'') antibiotic resistance genes ($aph(2'')$-Ia,-Ib,-Ic and -Id) in enterococci was shown to eliminate the synergistic killing achieved by the combination of gentamicin with a cell-wall active antimicrobial agent such as ampicillin (12). The results that are described herein reveal for the first time that the mechanistic information precludes the grouping of these enzymes collectively as APH(2'') type I (a, b, c, and d subgroups) enzymes. The enzyme previously designated as APH(2'')-Ic, the subject of our study, is classified as APH(2'')-IIIa in this report. We report the
cloning, purification and mechanistic investigations of this enzyme from Enterococcus spp.

**EXPERIMENTAL PROCEDURES**

**Cloning, protein expression and purification** - The aph(2")-IIIa gene, previously cloned into the pBluescript II KS(+) vector (12) was used as a template for cloning into the expression vector. The plasmid pBluescript::aph(2")-Ic was digested with *Nde*I and *Hind*III and the aph(2")-IIIa gene was cloned into the unique *Nde*I-*Hind*III sites of pET22a vector (Novagen) to produce pET22a::aph(2")-IIIa. Chemically competent *Escherichia coli* BL21(DE3) cells were transformed with this plasmid and selection was performed on LB agar supplemented with 100 µg/mL of ampicillin. A solution of 1.4 L of LB broth containing 100 µg/mL ampicillin and 25 mM L-proline was inoculated with 15 mL of an overnight culture of *E. coli* BL21(DE3) harboring the pET22a::aph(2")-IIIa plasmid. The medium was incubated at 37 °C in a shaking bath until the culture reached an optical density of 1.25 at 600 nm. The culture was cooled to 10 °C and IPTG was added to a final concentration of 0.8 mM. The cells were grown at 10 °C (200 rpm) for 16 h and were harvested by centrifugation at 10000 x g. The cell pellet was resuspended in 40 mL of buffer A (25 mM HEPES, pH 7.5, 0.2 mM DTT, 1 mM EDTA) and the bacteria were disrupted by sonication. The lysate was centrifuged at 21000 x g for 30 min and streptomycin sulfate (1.5%) was added to the supernatant to precipitate nucleic acids. The resultant solution was centrifuged at 3500 x g for 20 min, followed by dialysis of the supernatant against two changes of buffer A. The dialysate was centrifuged at 3500 x g for 10 min and the supernatant was loaded onto a kanamycin affinity (Affi-Gel 15, BioRad) column (2.5 x 10 cm) prepared as previously described (13). APH(2")-IIIa was eluted by a linear gradient of buffer B (25 mM HEPES, pH 7.5, 1 M NaCl, 0.2 mM DTT, 1 mM EDTA) in buffer A from 25 to 40% over two column volumes. Fractions were analyzed by assay of dibekacin phosphorylation and by SDS-PAGE for enzyme purity. Peak fractions were pooled, concentrated to 30 mL, dialyzed overnight against buffer A and applied to a gentamicin affinity column (2.5 x 10 cm) prepared as previously described (13). APH(2")-IIIa was eluted with a 55–75% gradient of buffer B in buffer A over two column volumes. Fractions of >95% purity of APH(2")-IIIa were pooled, concentrated to 0.35 mg/mL, dialyzed against the storage buffer (25 mM HEPES, pH 7.5, 1 mM DTT) and stored at -80 °C, without significant loss of activity over four months. The protein concentration was determined using the calculated extinction coefficient at 280 nm (23 000 M⁻¹cm⁻¹), using the program from www.expasy.org.

**Kinetic studies** - Enzyme activity was monitored (unless otherwise stated) by coupling the release of NDP (GDP, ADP, UDP, IDP, CDP or TDP) from the APH(2")-IIIa - catalyzed phosphorylation of the aminoglycosides to pyruvate kinase and lactate dehydrogenase, as previously described (5). In a typical experiment, 480 µL of assay mixture (100 mM buffer, 100 mM NaCl, 10 mM MgCl₂, 20 mM KCl, 0.2 mM NADH, 2 mM PEP, 12 units of pyruvate kinase, 27 units of lactate dehydrogenase) was mixed with NTP and APH(2")-IIIa (23-230 nM); the reaction was started by adding the aminoglycoside. When CTP and TTP were used as the phosphate donors, higher levels of pyruvate kinase were used, i.e. 60 and 120 units, respectively, due to the poor turnover of CDP and TDP by this enzyme. All experiments were carried out at 25 °C. The buffers used for the pH profiles were: MES (pH = 5.8-6.7), HEPES (pH = 6.8-7.8) and TAPS (pH = 7.9-8.6). The pH values were taken as pH meter reading + 0.4.

For the inhibition experiments with GDP and also when using GTP-γ-S as a substrate, the regeneration of GTP from the product (GDP) was interrupted by decoupling the pyruvate kinase/lactate dehydrogenase system (PEP was excluded from the assay buffer). After different incubation times, the reaction was quenched by the addition of 1 mM neomycin; PEP was then added and the change in absorbance at 340 nm (Δεₒ = 6700 M⁻¹cm⁻¹) (8) gave the amount of product formed at different times (the background absorbance change due to GDP present in the system was always subtracted). For measuring the thio effect, defined as k₈/k₅, where k₅ and k₈ are the rate constants for GTP and GTP-γ-S, respectively, kanamycin A was used at a saturating concentration.
value of 50 μM and GTP (or GTP-γ-S) at four different concentrations (5, 10, 20 and 40 μM).

Isolation of turnover product of kanamycin A - Phosphorylated kanamycin A was synthesized in vitro by incubating 40 mg of kanamycin A in 10 mL reaction buffer (100 mM HEPES, 20 mM MgCl₂, 20 mM KCl, pH 7.5, 40 mM PEP, 7 mM GTP, 3.4 μM APH(2″)-IIIa, 540 units of pyruvate kinase) for 6 h at 25 °C, after which additional pyruvate kinase (400 units) was added, and the reaction was allowed to proceed for a further 14 h. The reaction mixture at that point contained less than 1% kanamycin A, as determined from a kinetic assay. The solution was filtered through a 10 kDa cutoff membrane to remove the proteins and the filtrate was applied to a silica gel column; the column was washed with H₂O/MeOH (1 : 1) mixture (200 mL), followed by a H₂O/MeOH/NH₄OH (1 : 1 : 0.5) mixture (80 mL). The latter contained the phosphorylated kanamycin A, as shown by TLC (EtOH/MeOH/H₂O/MeOH/H₂O/MeOH (1 : 1 : 0.9 : 0.9), Rf = 0.57). The 80 mL fraction was dried by evaporation in vacuo and the residue was resuspended in water (5 mL). The insoluble matter was removed by centrifugation and the supernatant was loaded onto an Amberlite CG 50 ion exchange resin (Sigma) column (2.5 × 10 cm), previously activated with 1% NH₄OH and equilibrated with water. The column was washed with water (200 mL) and the sample was eluted stepwise with different concentrations of NH₄OH in water (0.2, 0.4, 0.6, 0.8 and 1% (v/v)) -30 mL (3 × 10 mL fractions) for each concentration). Phosphokanamycin A eluted at 0.6-0.8% NH₄OH over 30 mL (TLC). The fractions containing phosphokanamycin A were dried by evaporation in vacuo, the residue was resuspended in water (1 mL) and the solution was centrifuged to remove the insoluble impurities. The supernatant was loaded onto a G10 Sephadex (Sigma) column (1 × 120 cm) pre-equilibrated with water. It was eluted with water and fractions (1 mL) were collected; phosphokanamycin A eluted at 35-50 mL. The fractions were monitored by TLC as described above, and those containing phosphokanamycin A were concentrated to dryness in vacuo. ¹H-NMR of purified 2″′-phosphokanamycin A has been reported previously in conjunction with the reaction of APH(2″′)-lb (14) and the spectrum of our sample was identical to the literature report.

Data analysis - For determining k_cat and k_cat/K_m for the aminoglycoside and NTP substrates the concentration of the second substrate was kept constant and at saturating levels and the kinetic data were fit to the simple Michaelis-Menten equation (1):

\[
v = \frac{V_m[A]}{K_a + [A]}
\]

where V_m is the maximum velocity, [A] and K_a are the concentration and the Michaelis constant of the variable substrate.

The true dissociation constants of the substrates from the free enzyme (Kₐ for substrate A and Kₐ for substrate B) were determined by fitting the initial velocities to equation (2), corresponding to a random mechanism (where KₐKₖ = KₐKₖ).

\[
v = \frac{V_m[A][B]}{K_aK_b + K_a[B] + K_b[A] + [A][B]}
\]

The inhibition constants, Kₐ (corresponding to the dissociation of the EI complex) and Kₐ (corresponding to the dissociation of the EIB complex) of the substrate analogues (aminoglycosides and GMP-PNP) were determined from fitting the initial velocities obtained at several concentrations of inhibitor to equation (3) for a non-competitive inhibition of a substrate A analogue against substrate B at fixed concentrations of substrate A, as described by Morrison (15).

\[
v = \frac{V_m[A][B]}{K_aK_i\left(1 + \frac{[I]}{K_a}\right) + K_a[A] + K_b[B] + \left(1 + \frac{[I]}{K_b}\right) + [A][B]}
\]

For the competitive inhibition of a substrate A analogue against substrate A, the apparent competitive inhibition constant, K_i, was determined, and was found to be in good agreement with the calculated value \(K_i = (K_{ib}/[B] + 1)/(K_{ia}/K_{ib}[B] + 1/K_{ia})\) using the Kₐ and Kₐ values determined from the non-competitive plots. We have used this approach rather than just determining the competitive and non-competitive inhibition constants, because for a random mechanism the latter are not true dissociation constants (15). The same procedure was used for calculating Kₐ and Kₐ for the product inhibition of GDP (for a product inhibition pattern
with one dead-end complex in a random mechanism).

For 2’”-phosphokanamycin A, the $K_a$ was calculated from equation (4) (for a product inhibition pattern with no dead-end complexes in a random mechanism).

$$v = \frac{V_0[A][B]}{K_a K_b \left( 1 + \frac{[I]}{K_{is}} \right) + K_b [A] + K_a [B] + [A][B]}$$

(4)

Viscosity effects - The effect of solvent viscosity was determined in the presence of sucrose and PEG 8000. Sucrose was used at concentrations of 14, 22 and 30% (w/v) that correspond to relative viscosities ($\eta/\eta^0$) of 1.5, 2.0 and 3.2 (16). PEG 8000 was used at a concentration of 6.7% (w/v) ($\eta/\eta^0 = 3.6$, (15)). The assays were performed at fixed concentrations of kanamycin A (50 µM) or dibekacin (100 µM) and variable concentrations of GTP, GTP-γ-S (5–400 µM), ITP (20–400 µM) and ATP (0.5–2.0 mM). The viscosity effect is given by the slope of a plot of $k^0/k$ against relative viscosity, i.e., $(k^0/k)^\eta$, where $k^0$ and $k$ are the rate constants in the absence and presence of visciosigen, respectively.

Molecular modeling - A three-stage molecular modeling was performed to generate the computational model of APH(2”)-IIIa. The analysis is based on the structure of the homologous APH(2”)-IIa (previously described as APH(2”)-Ib), whose x-ray structure was recently solved in collaboration with Dr. C. Smith (unpublished data). Primary alignment using the web-based BLAST program (available at http://www.ncbi.nlm.nih.gov/BLAST/) shows that the sequence identity is 26% and gaps is 7% at an expectation value (E-value) of $10^{-15}$. The critical residues visualized in Sybyl (SYBYL, version 7.0, Tripos Inc., St. Louis, MO) are conserved in these two enzymes, as will be described. The positions of several conserved residues were used to thread in the Swiss-model (17) the sequence for APH(2”)-IIIa to that of APH(2”)-IIa and enabled to wrap amino acids one by one to the backbone residues of APH(2”)-IIa. The gaps in both target and template sequences required further processing of the computational model in the second stage of modeling. We carried out a template library search based on the hidden Markov method (18) and aligned the initial structure of APH(2”)-IIIa from the first-stage to the template of APH(3´)-IIa (Protein Data Bank code in RCSB: 1n4d). The resulting E-value improved to $2.95 \times 10^{-18}$. The third stage of modeling was the optimization of the solvated structure at room temperature (300 K). This was performed by the Amber software package of molecular dynamics programs (19). With atoms restrained in harmonic potential, the model was energy minimized with 250,000 steps in Cartesian space, using the steepest descent method. Subsequently, the six individual molecular dynamics simulations were carried out by gradually relaxing the restraints on the entire APH(2”)-IIIa model, expressed in force constant from 500 kcal mol$^{-1}$ A$^2$, to 100, 50, 10, 2, and 0 kcal mol$^{-1}$ A$^2$. The fully relaxed system was then subjected to a molecular dynamics run of 12 ps during heating from 0 to 100 K and 4 ps equilibrated at 100 K. The procedure was repeated for temperatures ranging between 100-200 K and 200-300 K. At 300 K, a 2000-step equilibrating process was completed and the averaged structure was selected for imaging.

RESULTS AND DISCUSSION

Substrate profile. We investigated ATP, GTP, ITP, UTP, CTP and TTP as phosphate donors for the reaction with kanamycin A, kanamycin B, dibekacin and tobramycin. CTP and TTP are poor substrates for APH(2”)-IIIa for phosphorylation of dibekacin and tobramycin with $k_{cat}/K_m$ values smaller than 10 M$^{-1}$s$^{-1}$ (Table 1). Among the other four nucleotide triphosphates, GTP had the lowest $K_m$ value (4.0 µM), followed by ITP ($K_m = 39$ µM). UTP and ATP have $K_m$ values of 1.3 and 1.6 mM, respectively, at pH 7.5. Whereas the $k_{cat}$ values are similar for GTP, ITP, ATP and ATP with kanamycin B, dibekacin and tobramycin, there was larger difference in catalytic efficiency of the enzyme for kanamycin A when different NTPs were used as phosphate donor (Table 1). The highest $k_{cat}$ for kanamycin A phosphorylation was seen with ITP as a phosphate donor and it was 2.5-fold higher than with GTP and 15- and 20-fold higher than with UTP and ATP, respectively. However, the $K_m$ for kanamycin A increases with increases of the $K_m$ for NTP (i.e., from GTP to ITP to ATP to UTP), which makes GTP the most efficient second substrate for kanamycin A phosphorylation in terms of $k_{cat}/K_m$ values. This
trend—increase in $K_m$ of kanamycin A with increase in the $K_m$ for the NTP—might suggest a positive cooperativity effect in substrate binding.

The preference for GTP over ATP is unusual for aminoglycoside phosphotransferases (4-6,8,9). However, the ATP and GTP concentrations in bacteria have been estimated to be approximately 3 mM and 1 mM, respectively (20). Therefore, given the more ready saturation of APH(2")-IIIa by GTP ($K_m = 4.0 \mu M$) over ATP ($K_m = 1.6 \mu M$), it is likely that this enzyme uses GTP as the phosphate donor in vivo. Consequently, the steady-state kinetic parameters for catalysis by APH(2")-IIIa and the mechanistic model were determined with GTP as the second substrate.

The aminoglycoside substrate profile for APH(2")-IIIa with GTP is shown in Table 2. These studies reveal that only the 4,6-disubstituted aminoglycosides: kanamycin A, kanamycin B, dibekacin, tobramycin, gentamicin C and netilmicin showed significant turnover with $k_{cat}$ values in the range of 0.7-0.9 s⁻¹ at pH 7.5. The $K_m$ value could only be determined accurately for kanamycin A, as for the rest of these aminoglycosides the rate is independent of their concentrations from 2.5 to 100 µM, indicative of saturation at lower concentrations. Sensitivity of the assay did not permit us to use lower concentrations of the aminoglycosides in these experiments.

For the other types of aminoglycosides (the 4,5-disubstituted and the atypical ones), their dissociation constants from the free and GTP-complexed enzyme ($K_i$ and $K_{in}$, respectively), were determined by treating them as inhibitors of enzyme-catalyzed phosphorylation of kanamycin A. For two of these inhibitors, apramycin and neamine, the slope and intercept replots of the Lineweaver-Burk analysis were not linear at higher concentrations of inhibitor. We established that this nonlinearity is due to a residual GTPase activity (GTP hydrolase) of the enzyme ($k_{cat}$ of 0.012 ± 0.002 s⁻¹ and a $k_{cat}/K_m$ of $(5.7 \pm 0.8) \times 10^3$ M⁻¹s⁻¹ at pH 7.5). This activity is actually enhanced in the presence of apramycin and neamine ($k_{cat} = 0.038 \pm 0.004$ s⁻¹, $k_{cat}/K_m = (3.4 \pm 0.6) \times 10^3$ M⁻¹s⁻¹ with apramycin and $k_{cat} = 0.041 \pm 0.005$ s⁻¹, $k_{cat}/K_m = (5.7 \pm 0.7) \times 10^3$ M⁻¹s⁻¹ with neamine). The $K_m$ parameter for GTP in the GTPase activity is decreased from 21 ± 3 µM (in the absence of the aminoglycosides) to 11 ± 1 and 7.2 ± 1 µM in the presence of apramycin and neamine, respectively. The numbers in the presence of the two aminoglycosides for the $K_m$ of GTP approach the $K_m$ value determined in the aminoglycoside phosphorylation reaction (4.0 ± 0.5 µM). Therefore, the dissociation constants for these antibiotics were determined by treating them as activators of the enzyme-catalyzed GTP hydrolysis (21). In fact, apramycin and neamine were the only non-phosphorylatable aminoglycosides studied that showed activation of the GTPase activity at concentrations below 100 µM, possibly due to a conformational change on the part of the enzyme combined with the accessibility of a water molecule to the catalytic site.

It is worth noting that the kinetic parameters for kanamycin B with the four NTPs studied are significantly different than those for kanamycin A and closer to those for tobramycin and dibekacin (Table 1). The only structural difference between kanamycin A and B is the presence of an amino group on the 2’ position in kanamycin B, which replaces the hydroxyl group in kanamycin A (Figure 1). The 2’ amino group is present in all other aminoglycoside substrates of APH(2")-IIIa (Table 2). Hence, it appears that this amino group has a significant contribution in lowering the $K_m$ values of dibekacin, tobramycin, gentamicin C and netilmicin with GTP as a phosphate donor (Table 2). The presence of the 2’ amino group also increases the turnover numbers and lowers aminoglycoside $K_m$ values for APH(2")-IIIa with ATP and UTP as second substrates (Table 1).

Our data demonstrate that APH(2")-IIIa has a narrow aminoglycoside substrate profile compared to other enzymes of its class. The APH(2")-Ia domain of AAC(6)-APH(2")-Ia has a broad substrate profile, including the 4,6- and 4,5-disubstituted aminoglycosides, however it exhibits somewhat modest $k_{cat}/K_m$ values ($10^3-10^5$ M⁻¹s⁻¹) (14). APH(2")-IIa (formerly known as APH(2")-Ib) has high specificity for the 4,6-disubstituted aminoglycosides (it does not phosphorylate the 4,5-disubstituted antibiotics), but with much higher $k_{cat}/K_m$ values ($10^7-10^8$ M⁻¹s⁻¹) (19).

APH(2")-IIIa only catalyzes phosphorylation of certain 4,6-disubstituted aminoglycosides (Figure 1). It does not phosphorylate amikacin or isepamicin, which have a 4-amino-2-
hydroxybutyryl/propionyl substituent on the nitrogen at position 1 of the aminocyclitol ring. They act as inhibitors, although with high dissociation constants (Table 2), indicating that the presence of the bulky substituent at N1 is detrimental to aminoglycoside binding to APH(2")-IId.

The $K_m$ parameters for all aminoglycoside substrates but kanamycin A were below 1 µM; another unique feature distinguishing this enzyme from other studied aminoglycoside phosphotransferases. As indicated earlier, the enzyme also binds 4,5-disubstituted substrates and some atypical aminoglycosides (Table 2), but it is unable to turn them over. The narrow aminoglycoside substrate profile, together with poor saturation by ATP ($K_m = 1.6 \pm 0.2$ mM)—the usual phosphate donor in enzymatic aminoglycoside phosphorylation reactions—and the strong preference for GTP ($K_m = 4.0 \pm 0.5$ µM) suggest that APH(2")-IId has evolved differently than other known APHs.

The MIC values (12) parallel better with the $k_{cat}/K_m$ than with the $k_{cat}$ values, as the $k_{cat}$ values are roughly constant for the five substrates studied. Interestingly, the $K_m$ values are mostly in the nanomolar range (< 0.3 µM) for the best aminoglycoside substrates. This too is unusual for the family of APHs and might imply that either the concentration of the antibiotic that penetrates enterococci is low and/or that the manifestation of resistance occurs by enzyme merely binding the aminoglycosides without turnover. This mode of resistance finds precedent in certain aminoglycoside phosphotransferases and acetyltransferases (22,23). The latter possibility is supported by the fact that neomycin shows elevated MIC values for the strains harboring the aph(2")-IId gene (12), although it is not turned over by the enzyme.

*Initial velocity pattern, dead-end and product inhibition.* The initial velocity pattern of the APH(2")-IId-catalyzed phosphorylation of kanamycin A was determined at several fixed concentrations of GTP. The double reciprocal plots of reaction velocity versus concentrations of kanamycin A exhibits intersecting lines, indicative of a sequential kinetic mechanism (see Figure 1 of Supplemental Data). We hasten to add that the sequential mechanism appears to be common to all classes of aminoglycoside-modifying enzymes (5,9,24-27).

To further distinguish between a random and an ordered sequential mechanism, dead-end and product inhibition studies were carried out with substrate analogues of aminoglycosides and GTP and with 2"-phosphokanamycin and GDP, respectively. The 4,5-disubstituted aminoglycoside neomycin, which is not phosphorylated by the enzyme, is a competitive inhibitor of kanamycin A and a non-competitive inhibitor of GTP with $K_{i_s} = 8.1 \pm 1.0$ µM and $K_{ii} = 4.2 \pm 0.5$ µM (Table 3; Figures 2 and 3 of Supplemental Data). The non-hydrolizable GTP analogue, GMP-PNP ($\beta,\gamma$-imidoguanosinetriphosphate) is a competitive inhibitor versus GTP and a non-competitive inhibitor versus kanamycin A with $K_{i_s} = 77 \pm 10$ µM and $K_{ii} = 46 \pm 6$ µM (Table 3; Figures 4 and 5 of Supplemental Data). A dead-end inhibition pattern, where the non-reactive analogue of each substrate is a competitive inhibitor of its reactive counterpart and a non-competitive inhibitor of the other substrate (Table 3) is indicative of a random Bi Bi mechanism (28). The fit of the initial velocities at various concentrations of aminoglycoside and GTP to equation (2), using the $k_{cat}$ and $K_m$ parameters determined independently, yielded the true dissociation constants of the substrates from the complex with the free enzyme. It was found that $K_{i_s} = 8.7 \pm 1.0$ µM for kanamycin A and $K_{ib} = 8.2 \pm 0.8$ µM for GTP, which are two fold higher than the corresponding $K_m$ values. The fact that binding of the first substrate lowers the $K_m$ for the second substrate, indicates positive cooperativity in substrate binding to the enzyme.

To further characterize the kinetic mechanism, we performed inhibition studies with the products of the APH(2")-IId-catalyzed reaction. The product of kanamycin A phosphorylation by APH (2")-IId and GTP was isolated (as described in the Experimental Procedures). Analyses of this enzymic product and comparison with the product of kanamycin A modification by APHI(2")-IId (19), revealed them to be identical, with phosphorylation at the 2" position. Inhibition profiles of 2"-phosphokanamycin versus kanamycin A and GTP were both competitive at sub-saturating concentrations of the fixed substrate ([kanamycin A] = 4 µM and [GTP] = 5 µM), with
$K_{is} = 550 \pm 90 \mu M$, while no inhibition was observed at saturating concentrations of the fixed substrate (Table 3; Figures 6 and 7 of Supplemental Data). The second product of the reaction, GDP, was found to be a competitive inhibitor of GTP and a non-competitive inhibitor of kanamycin A with $K_i = 1.0 \pm 0.3 \mu M$ and $K_i = 2.5 \pm 0.8 \mu M$, respectively (Table 3; Figures 8 and 9 of Supplemental Data). The product inhibition pattern for a random Bi Bi mechanism shows competitive inhibition of both products against their corresponding substrates. If no dead-end complexes are formed, the product of the first substrate will also show competitive inhibition against the other substrate. The inhibition will be removed at saturating levels of the fixed substrate. However, if dead-end complexes form, the product inhibition against the complementary substrate becomes non-competitive. Our product inhibition data are consistent with a random Bi Bi mechanism, where one dead-end complex forms, i.e., enzyme-GDP-kanamycin A. A random sequential mechanism has been proposed also for two other APH(2″) enzymes studied so far, AAC(6)-APH(2″)-Ia (3) and APH(2″)-IIa (14), and for APH(3′)-Ia (6).

**Viscosity effects.** To determine whether a diffusion process is controlling the rate of the APH(2″)-IIIa-catalyzed phosphorylation, the reaction was studied in solvents with different viscosities. For this purpose, two types of viscosogenic agents have been used: microviscosigen, glycerol and sucrose, which affect both the measured viscosity and the rates of the diffusive processes, and a polymeric macroviscosigen, PEG 8000, which increases viscosity without affecting the diffusion of small molecules (29). An important issue with the experiments involving the modification of solution viscosity is that, apart from affecting the diffusional steps, viscosogens can have non-specific effects on the enzymatic reactions (29). Interpretation of viscosity effects as diffusional must be based on controlling these complex interactions by using “poor substrates”, or “poor enzymes”, for which the chemical step is rate-limiting and the change in the diffusion rate is not expected to change the overall rate of the reaction (29). GTP-γ-S, a GTP analogue where a non-bridging oxygen from the γ-phosphate is replaced by sulfur, was tested as a substrate for APH(2″)-IIIa with kanamycin A. The experimentally determined $k_{cat}$ and $k_{cat}/K_{m}$ values for GTP-γ-S were 54- and 74-fold smaller than the corresponding values for GTP at pH 7.5. Thus, GTP-γ-S is a poorer substrate for APH(2″)-IIIa than GTP and can be used as a control in the viscosity experiments.

The experiments with both macroviscosigen and microviscosigen were performed at pH 6.5 and 7.5, in an attempt to see if diffusion can become the rate-limiting step with the change of the pH of solution. At both pH values the macroviscosity effect on $k_{cat}$ and $k_{cat}/K_{m}$ were not significant ($\leq 0.1$); thus it can be concluded that macroviscosity of the solution does not affect the reaction rates.

Initially we had used glycerol as a microviscosigen, but its use required correction factors complicating analysis, so we opted to use sucrose instead. The microviscosity control experiments with GTP-γ-S in sucrose gave very small effects (within experimental error) for both $k_{cat}$ and $k_{cat}/K_{m}$ at pH 7.5 and $k_{cat}$ at pH 6.5, with a slightly negative effect on $k_{cat}/K_{m}$ of $-0.2 \pm 0.1$ at pH 6.5. Hence, sucrose proved to be a suitable microviscosigen for the study of diffusion-controlled steps in APH(2″)-IIIa-catalyzed reaction.

The viscosity effect on $k_{cat}$ (at saturating levels of kanamycin A and GTP) at pH 7.5 has a value of $1.0 \pm 0.1$. This indicates that product release is solely rate-limiting with GTP as the substrate at this pH. The enzyme has a much higher affinity for GDP ($K_i = 1.0 \pm 0.3 \mu M$) than for 2″-phosphokanamycin A ($K_i = 550 \pm 90 \mu M$), indicating that the release of GDP is the rate-limiting step of the reaction. Nucleotide diphosphate release as the rate-limiting step has been previously reported for APH(3′)-IIIa, which has been shown to follow a special case of an ordered (Theorell-Chance) mechanism (9). However, at pH 6.5, the viscosity effect on $k_{cat}$ decreased to a value of $0.4 \pm 0.1$, indicating that at this pH product release is only partially rate-limiting and the chemical step also influences $k_{cat}$.

The observed microviscosity effects on $k_{cat}/K_{m}$ for GTP (at saturating levels of kanamycin A) are $0.1 \pm 0.1$ at pH 7.5 and $-0.1 \pm 0.1$ at pH 6.5. Even if the viscosity effect at pH 6.5 is corrected for the value found with the control substrate GTP-γ-S (i.e., $-0.2 \pm 0.1$), the overall effect will still be within the experimental error of $0.1 \pm 0.1$. The
lack of a viscosity effect on the second-order rate constant is consistent with a rapid-equilibrium mechanism, where the release of the substrate (GTP) is more rapid than its turnover (30).

The microviscosity data indicate that for GTP and kanamycin A as substrates, changing the pH from 7.5 to 6.5 results in a change in the rate-limiting step of the enzymatic reaction from solely product (GDP) dissociation at pH 7.5 to both product dissociation and a chemical step at pH 6.5.

We were also interested in correlating the affinity of the enzyme for the NTP with the nature of the rate-limiting step, as it has been shown that for some protein kinases factors other than physical release of nucleotide diphosphate could limit turnover (31). For this purpose we have investigated the viscosity effects on $k_{\text{cat}}$ for GTP, ITP and ATP using dibekacin as the second substrate at pH 7.5. We have used dibekacin in these experiments because it readily saturates the enzyme (at submicromolar concentrations) with all three NTPs. The viscosity effects, $(k^0/k)^n$, increase with increasing affinity of the enzyme for the NTP in the order GTP $(0.96 \pm 0.05) >$ ITP $(0.48 \pm 0.05) >$ ATP $(0.13 \pm 0.05)$ (Figure 2). From these viscosity effects the rates of product release, $k_3$ (Scheme 1), can be estimated using equations (5) and (6) and the $k_{\text{cat}}$ values at pH 7.5 for GTP, ITP and ATP with dibekacin (Table 1).

\[
(k^0/k)^n = \frac{k_2}{k_2 + k_3} \quad (5)
\]

\[
k_{\text{cat}} = \frac{k_2 k_3}{k_2 + k_3} \quad (6)
\]

The rate constant for product release, $k_3$, was found to be $0.7 \text{ s}^{-1}$ for GTP, $3.2 \text{ s}^{-1}$ for ITP and $>8 \text{ s}^{-1}$ for ATP. These results indicate that GDP release for APH(2")-IIIa is the rate-limiting step for turnover at pH 7.5 due to the higher affinity of the enzyme for GDP. It would appear that the presence of both a carbonyl (H-bond acceptor) rather than an amino group on the C6 position and an amino group in the C2 position of the purine are important for NTP binding (Figure 3).

The pH-rate profile and kinetic solvent isotope effect. In light of the findings with the viscosity effects with different NTPs, we were interested to investigate the pH profiles of the $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ in order to determine possible titrating groups on the enzyme that are responsible for binding and turnover. All the experiments were performed at saturating concentrations of dibekacin.

The pH profiles of $k_{\text{cat}}/K_m$ for GTP, ITP and ATP are all sigmoidal in the pH range of 6.0-8.5, and can be explained by the ionization of a non-critical group of $pK_a$ around 7.5 on the enzyme (Figure 4A). The fact that the ionization is not critical (i.e. titrations one way or the other do not lead to impairment of catalysis) is indicated by the fact that log $k_{\text{cat}}/K_m$ decreases with decreasing pH with a slope smaller than one and then levels off at around pH 6. The data were fit to equation (7), where $(k_{\text{cat}}/K_m)^n$ and $(k_{\text{cat}}/K_m)^0$ are the second-order rate constants for the protonated and deprotonated enzyme forms, respectively. The parameters obtained are given in Table 4.

\[
\left(\frac{k_{\text{cat}}}{K_m}\right)^{\text{app}} = \frac{(k_{\text{cat}}/K_m)^0 + [H^+] (k_{\text{cat}}/K_m)^n}{1 + [H^+] k_a}
\]  \quad (7)

The pH-$k_{\text{cat}}$ profile for ATP is sigmoidal, with one group of $pK_a$ around 7.5 required in the deprotonated form for activity (Figure 4B). For GTP and ITP the pH profiles of $k_{\text{cat}}$ are bell-shaped (Figure 4B) and the experimental data for the two NTPs were fit to a double ionization model (equation (8)) where $pK_a^1$ and $pK_a^2$ correspond to the groups required for catalysis in deprotonated and protonated form, respectively. The $pK_a$ values determined from the three profiles are not identical (Table 4). It would appear that perturbation of the active site based on the variation of the structure of the nucleic bases in the NTP influences the requirement for ionization of the titratable residues. Whereas for GTP $k_{\text{cat}}$ decreases with the ionization of a group of $pK_a = 7.6 \pm 0.1$, $pK_a$, for ATP there is an increase in $k_{\text{cat}}$ with the ionization of a group of similar $pK_a$, and for ITP $k_{\text{cat}}$ is independent of pH within the pH range of 6.5 and 8. A group of $pK_a = 6.0 \pm 0.2$, is required in the deprotonated form for activity with both ITP and GTP and another group of $pK_a = 8.4 \pm 0.1$ is required in protonated form with ITP.

\[
k_{\text{cat}}^{\text{app}} = \frac{k_{\text{cat}}}{1 + [H^+] + K_a^{pK_a} + [H^+]^{pK_a}}
\]  \quad (8)

The pH profiles of $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ fit a model with three important ionizations, as shown in...
Scheme 2. Whereas the model of Scheme 2 can accommodate three titratable residues in the enzyme-antibiotic-NTP complex, in actuality analysis of Figure 4 allows us to physically evaluate only two ionizations for each NTP. The fit is such that the effect of the third titratable residue is marginal and obscured. A group of $pK_a = 6.0 \pm 0.2$ is required in deprotonated form for catalytic activity and possibly corresponds to a general base that activates the 2'' OH group for nucleophilic attack ($K_a^3$ in Scheme 2) such as Asp-190 (vide infra). Another group of $pK_a = 8.4 \pm 0.1$ is required in protonated form for activity and may correspond to a general acid involved in the NDP formation ($K_a^a$ in Scheme 2). Interestingly, a group of $pK_a = 7.5 \pm 0.2$ ($K_a^a$ in Scheme 2) gives an increase in the second-order rate constants for all three nucleotides studied, i.e. $(k_{cat}/K_m)^H < (k_{cat}/K_m)^b$ (Table 4), but has different effects on their $k_{cat}$ values. For ATP $k_{cat}^H \ll k_{cat}^0$, while for ITP $k_{cat}^a$ is very similar for ESH and ESH$_2$ and for GTP only ESH$_2$ appears to be catalytically active ($k_{cat}^H \gg k_{cat}^0$). The fact that ESH has lower $K_a$ for GTP than does ESH$_2$ but ESH has very slow (or no) turnover, implies that ESH-GTP is a non-productive enzyme complex. Considering that the rate-limiting step of APH(2'')-IIIa-catalyzed phosphorylation with GTP is GDP release at pH 7.5, it is not surprising that a higher affinity of the enzyme for the nucleotide results in lower turnover rates. Our results indicate that the group of $pK_a = 7.5 \pm 0.2$ is involved in nucleotide binding and is probably responsible for the unusual nucleotide specificity of APH(2'')-IIIa.

The pH/pD vs. $k_{cat}$ profile was also determined with GTP-$\gamma$-S as a substrate, where the chemical step is most likely rate limiting, in order to ascertain if there is significant proton transfer in the transition state. The pH profile of $k_{cat}$ (Figure 4C) is also bell-shaped with a maximum value of 0.023 s$^{-1}$ at pH 7.0. The data were fit to a two-ionization model (equation (8)) and the values evaluated for the parameters were: $k_{cat}^{H_2O} = 0.036 \pm 0.005$ s$^{-1}$, $k_{cat}^{D_2O} = 0.023 \pm 0.004$ s$^{-1}$, $pK_a^{H_2O} = 6.5 \pm 0.2$, $pK_a^{D_2O} = 8.0 \pm 0.2$. The $pK_a$ shift from H$_2$O to D$_2$O for both titrating groups is in the range expected for a weak acid (32), i.e. 0.6 \pm 0.2. The calculated kinetic solvent isotope effect (KSIE), $k_{cat}^{H_2O}/k_{cat}^{D_2O}$, for the APH(2'')-IIIa-catalyzed phosphorylation of kanamycin A using GTP-$\gamma$-S as a substrate is 1.6, which suggests a contribution from a proton transfer step to the transition state of APH(2'')-IIIa-catalyzed reaction with GTP-$\gamma$-S and kanamycin A.

**Insights from the homology model** – The computational model for APH(2'')-IIIa was based on the X-ray structure of the related enzyme APH(2'')-IIa, which was generously shared with us by Dr. Clyde Smith prior to publication. The molecular model for the entire protein is depicted as Fig. 5A. The close up of the active site is given as Fig. 5B. Of all the amino acid residues participating in hydrogen bond formation with kanamycin in the APH(3')-IIIa and APH(3'')-IIa enzymes only one, Asp190, is also absolutely conserved in the APH(2'') phosphotransferases (Figure 6 and Figure 10 of Supplemental Data). Conservation of Asp190 is absolute, not only in aminoglycoside phosphotransferases but also in the eukarotic protein kinases (33). Aspartate-190 is the likely active site general base that promotes the 2''-hydroxyl of the antibiotic in the course of the transfer of phosphate. The homologous conserved aspartate in APH(3')-IIIa is implicated for the same role in promotion of the 3'-hydroxyl group (34). As shown in Fig. 5C, which depicts the active site of APH(3')-IIa in complex with kanamycin A, the side chain of Asp190 is in contact with the 3'-hydroxyl of the antibiotic. This arrangement allows Asp-190 to promote the hydroxyl for the transfer of the phosphate from nucleotide triphosphate. A similar role for Asp-190 of APH(2'')-IIIa is likely. As revealed in Fig. 5B and 5C, the positions of the residues in the two enzymes are same.

Four (Lys44, Glu60, Asn195, and Asp208) out of five amino acid residues that are conserved among eukaryotic and bacterial protein kinases and in both the APH(3') and APH(2'') phosphotransferases are located within the nucleotide-binding regions of the proteins. One could speculate that upon evolution of aminoglycoside phosphotransferases towards enzymes with different regiospecificity of phosphoryl transfer (to the 3' or 2'') they retained these critical residues for interaction with nucleotide triphosphate. However, the enzymes
evolved in the regions involved in positioning of various aminoglycosides to ensure modification of the proper hydroxyl group. Lys44 is located on the β strand 3 and strategically positioned over the ATP binding site, where it forms hydrogen bonds with both the α and β phosphate groups of ATP, while Glu60 forms a salt bridge with invariant Lys44. In addition to the interaction with the enzyme, phosphate groups of ATP form hydrogen bonds with water molecules and are coordinated to two magnesium ions in the active site of APH(3′)-IIIa. These magnesium ions, commonly referred to as Mg1 and Mg2 are, in turn, coordinated to two residues, Asn195 (coordinates Mg2 ion) and Asp208 (coordinates both Mg1 and Mg2 ions). Both Asn195 and Asp208 are fully conserved in APH(3′)s and bacterial and eukaryotic Ser/Thr and Tyr kinases, an observation that underlines their importance for enzyme function. Mutation of Asp208 to Ala, Glu, or Asn in APH(3′)-IIIa resulted in complete loss of enzyme activity, indicating that this residue is critical for catalytic mechanism of APH(3′)-IIIa(33). On the other hand, the Asn195Ala mutant demonstrated a five-fold increase in $K_m$ for ATP and no change in $k_{cat}/K_m$ for most of aminoglycoside substrates. The moderate decrease in affinity for ATP indicates that Asn195, though not critical for APH(3′)-IIIa aminoglycoside kinase activity, plays a role in ATP binding by optimally coordinating the Mg$^{2+}$ ion.

The model also educates on the preference for GTP over ATP in APH(2′′)s. The region spanning amino acids 86-94 is defining the pyrimidine binding site. We surmise that this stretch of amino acids is tailored for interaction with GTP in preference to ATP, but the definitive answer on this has to wait till the actual crystal structure for this enzyme is in hand. The work on crystallization of this enzyme is in progress.

Concluding Remarks - The enzyme designated as APH(2′′)-IIIa here belongs to a class of proteins that confers bacterial resistance to aminoglycosides by the phosphorylation of the 2′′ position of these antibiotics. Two other enzymes of this class, the APH domain of bifunctional AAC(6′)-APH(2′′)-Ia and APH(2′′)-IIa (formerly APH(2′′)-Ib), have also been characterized and despite the low sequence similarity among the three proteins, they all phosphorylate kanamycin A at the 2′′ position (14,35). However, they vary significantly with respect to their substrate profiles and catalytic efficiencies. The detailed kinetics reported herein for APH(2′′)-IIIa and previously for AAC(6′)-APH(2′′)-Ia and APH(2′′)-IIa have been critically useful in defining the properties of these enzymes with some precision. As outlined in this report, APH(2′′)-IIIa distinguished itself among these enzymes by exhibiting a narrow substrate profile, with unusually low $K_m$ for its aminoglycoside substrates (nanomolar for all substrates, expect kanamycin A) and an unexpected 200-fold preference for GTP over ATP as the phosphate donor.

Prior to our work, the phenotypic profiles of aminoglycoside phosphotransferases, and to a lesser extent the small sequence similarities, have been used for classification of these enzymes. It is clear that the breadth of activity and exploration of the nature of both substrates have to be investigated to draw meaningful conclusions. Yet, the detailed kinetics also reveal the points of divergence among these enzymes. Strong preference for GTP in APH(2′′)-IIIa and the observation of low $K_m$ are two points that argue for this divergent tangent of evolution. Why preference for GTP has been selected is not obvious at the presence. We venture to speculate that the low $K_m$ for the antibiotics might be a necessity for the organism in which aph(2′′)-IIIa gene evolved and might have to do with low penetration of the antibiotic into the cytoplasm. Hence, the enzyme had to reach saturation at lower concentrations of the antibiotic for it to be effective, presenting a selection advantage to the resistant organism.

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FOOTNOTES

1 This work was supported by a grant from the National Institutes of Health.

2 The abbreviations used are: AAC, aminoglycoside acetyltransferase; APH, aminoglycoside phosphotransferase; LB, Luria-Bertani medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; GMP-PNP, guanosine 5'-O-([β,γ-imido]triphosphate; GTP-γ-S, guanosine 5'-O-(3-thiotriphosphate); KSIE, kinetic solvent isotope effect; MES, 2-(N-morpholino)ethanesulfonic acid; MIC, minimal inhibitory concentration; PEP, phosphoenolpyruvate; TAPS, 3-[tris(hydroxymethyl)methyl]-3-amino]propanesulfonic acid;

FIGURE LEGENDS

Fig. 1. Kanamycin A and its phosphorylation product by APH(2′′)-IIIa.

Fig. 2. Viscosity effects on $k_{\text{cat}}$ for the APH(2′′)-IIIa-catalyzed phosphorylation of dibekacin with GTP (×), ITP (■) and ATP (▲), at [Dibekacin] = 100 µM, in 100 mM HEPES, pH 7.50, 100 mM NaCl, 10 mM MgCl$_2$, 20 mM KCl, at 25 ºC.

Fig. 3. The chemical structures of the nucleoside triphosphates used in this study.

Fig. 4. (A) Plot of log $k_{\text{cat}}/K_m$ versus pH for APH(2′′)-IIIa phosphorylation of dibekacin with GTP (×), ITP (■) and ATP (▲) determined at fixed, saturating concentration of dibekacin (100 µM), in 100 mM buffer, pH 7.5, 100 mM NaCl, 10 mM MgCl$_2$, 20 mM KCl, at 25 ºC. The solid line is the fit based on equation (7) and the parameters in Table 4. (B) Plot of log $k_{\text{cat}}$ versus pH for APH (2′′)-IIIa phosphorylation of dibekacin with GTP (×), ITP (■) and ATP (▲) at fixed, saturating concentration of dibekacin (100 µM). The solid line is the fit based on equation (8) and the parameters in Table 4. (C) Plot of log $k_{\text{cat}}$ versus pH (■)/pD(▲) for APH(2′′)-IIIa phosphorylation of kanamycin A with GTP-γ-S, at fixed, saturating concentration of kanamycin A (100 µM). The solid line is the fit based on a double-ionization equation (8) and the parameters given in the text.

Fig. 5 (A) The stereo representation of the computational model of APH(2′′)-IIIa, with the black arrow pointing to the active site of the enzyme. The protein is depicted in green. (B) The close up of the active site of APH(2′′)-IIIa, highlighting the positions of Asp-190 and Asp-208, two active site residues within the aminoglycoside- and nucleotide-binding sites, respectively. The sequence numbering is based on APH(3′)-IIIa. (C) The x-ray structure for the active site of APH (3′)-IIa in its complex with kanamycin A (PDB code: 1nd4) is depicted for comparison to panel B. The 3'-hydroxyl is in contact with the side chain of Asp-190, which promotes the hydroxyl for the transfer of phosphate to it. Kanamycin A, and the two active site residues are represented in capped-stick. Carbons are colored in grey, nitrogens in blue, and oxygens in red. The PyMOL (0.99rev8) program (DeLano Scientific LLC, Palo Alto, CA, USA) was used in generation of these structures.
Table 1. NTP substrate profile of APH(2")-IIIa, determined in 100 mM HEPES, pH 7.50, 100 mM NaCl, 10 mM MgCl₂, 20 mM KCl, at 25 °C. The \( K_m \) and \( k_{cat}/K_m \) values were determined for aminoglycosides at fixed concentrations of NTP.

| NTP      |  \( k_{cat} \) |  \( K_m \) | \( k_{cat}/K_m \) |  \( k_{cat} \) |  \( K_m \) | \( k_{cat}/K_m \) |  \( k_{cat} \) |  \( K_m \) | \( k_{cat}/K_m \) |
|----------|----------------|------------|-----------------|----------------|------------|-----------------|----------------|------------|----------------|
| GTP (100 µM) | 0.8 ± 0.1 | 4.5 ± 0.5 | (1.8 ± 0.3)×10⁵ | 0.8 ± 0.1 | <0.5 | (1.6 ± 0.2)×10⁶ | 0.7 ± 0.1 | <0.3 | 0.7 ± 0.1 | <0.3 |
| ITP (1.0 mM) | 2.1 ± 0.2 | 39 ± 4 | (5.6 ± 0.5)×10⁴ | 1.7 ± 0.2 | <1 | (1.7 ± 0.2)×10⁶ | 1.5 ± 0.1 | <0.5 | 1.5 ± 0.1 | <0.5 |
| ATP (3.0 mM) | 0.09 ± 0.02 | 90 ± 10 | (1.0 ± 0.1)×10³ | 0.6 ± 0.1 | 2.4 ± 0.4 | (2.5 ± 0.5)×10⁵ | 0.9 ± 0.1 | <1 | 0.9 ± 0.1 | <1 |
| UTP (3.0 mM) | 0.14 ± 0.02 | 100 ± 10 | (1.4 ± 0.2)×10³ | 0.7 ± 0.1 | 5.0 ± 0.6 | (1.3 ± 0.2)×10⁵ | 0.9 ± 0.1 | <1 | 0.9 ± 0.1 | <1 |
Table 2. Steady-state kinetic parameters and dissociation constants for APH(2")-IIIa, determined in 100 mM HEPES, pH 7.50, 100 mM NaCl, 10 mM MgCl₂, 20 mM KCl, at 25 °C. For the phosphorylation reaction, GTP concentration was held constant at saturating levels (100 µM), while for the inhibition studies GTP concentration was varied around the $K_m$ value (4.0 µM).

| Aminoglycoside  | MIC$^a$ | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $K_i^e$ | $K_{ii}^e$ |
|-----------------|---------|-----------|-------|---------------|---------|-----------|
|                 | µg/ml   | s⁻¹       | µM    | M⁻¹s⁻¹        | µM      | µM        |
| 4,6-disubstituted aminoglycosides |         |           |       |               |         |           |
| Kanamycin A     | 4.0/0.8 | 0.8 ± 0.1 | 4.5 ± 0.5 | (1.8 ± 0.3)×10⁵ |         |         |
| Dibekacin       | 128/0.3 | 0.7 ± 0.1 | < 0.3  | > 2.3×10⁶     |         |         |
| Tobramycin      | 48/0.3  | 0.7 ± 0.1 | < 0.3  | > 2.2×10⁶     |         |         |
| Gentamicin C    | 32/0.3  | 0.5 ± 0.1 | < 0.3  | > 1.8×10⁶     |         |         |
| Netilmicin      | 2.0/0.2 | 0.7 ± 0.1 | < 1    | > 6.7×10⁵     |         |         |
| Amikacin        | 1.0/0.8 | No reaction | 800 ± 100 | 900 ± 100     |         |         |
| Isepamicin      | 0.5/0.5 | No reaction | > 5000 | 2100 ± 300    |         |         |
| 4,5-disubstituted aminoglycosides |         |           |       |               |         |           |
| Neomycin        | 1.0/0.4 | No reaction | 8.1 ± 1.0 | 4.2 ± 0.5     |         |         |
| Butirosin       | No reaction | 900 ± 100 | 800 ± 100     |         |         |
| Lividomycin A   | No reaction | 18 ± 2 | 17 ± 2     |         |         |
| Paromomycin     | No reaction | 78 ± 10 | 47 ± 5     |         |         |
| Atypical aminoglycosides |         |           |       |               |         |           |
| Spectinomycin   | No reaction | No inhibition up to 1mM |         |         |
| Streptomycin    | No reaction | 14 ± 2 | > 1000     |         |         |
| Apramycin       | No reaction | 6.0 ± 0.6$^b$ | 3.0 ± 0.4$^b$ |         |         |
| Hygromycin C    | No reaction | No inhibition up to 1mM |         |         |
| Neamine         | No reaction | 50 ± 5$^b$ | 15 ± 2$^b$ |         |         |

$^a$E coli JM83 with pBluescript::aph(2")-IIIa plasmid / E coli JM83 with pBluescript plasmid as described by Lee et al. (12). $^b$Dissociation constants determined for GTPase activation. $^cK_i$ and $K_{ii}$ are the dissociation constants of the antibiotic from free enzyme and enzyme-GTP complex, respectively.
Table 3. Dead-end and product inhibition of APH(2’’)-IIIa, determined in 100 mM HEPES, pH 7.50, 100 mM NaCl, 10 mM MgCl₂, 20 mM KCl, at 25 °C. The concentration of the fixed substrate was around the $K_m$ value (4.0 µM for kanamycin A and 5.0 µM for GTP).

| Inhibitor            | Variable substrate | Inhibition pattern | $K_{is}$ (µM) | $K_{ii}$ (µM) | $K_{ic}$ (µM) |
|----------------------|--------------------|--------------------|---------------|---------------|---------------|
| Neomycin             | Kanamycin A        | Competitive        | 6.2 ± 0.8     |               |               |
|                      | GTP                | Non-competitive    | 8.1 ± 1.0     | 4.2 ± 0.5     |               |
| GMP-PNP              | Kanamycin A        | Non-competitive    | 77 ± 10       | 46 ± 6        | 65 ± 8        |
|                      | GTP                | Competitive        |               |               |               |
| 2’’-phosphokanamycin A | Kanamycin A      | Competitive        | 550 ± 90      |               |               |
|                      | GTP                | Competitive        | 500 ± 80      |               |               |
| GDP                  | Kanamycin A        | Non-competitive    | 1.0 ± 0.3     | 2.5 ± 0.8     | 1.4 ± 0.3     |
|                      | GTP                | Competitive        |               |               |               |
Table 4. Rate and ionization constants for APH(2′′)-IIIa-catalyzed phosphorylation of dibekacin with different NTPs determined by fitting the data in Figures 4A and 4B to equations 7 and 8, respectively. The parameters in parenthesis correspond to the mechanism depicted in Scheme 2.

|                | GTP                        | ITP                        | ATP                        |
|----------------|---------------------------|----------------------------|----------------------------|
| \( \frac{k_{\text{cat}}}{K_m} \) (M\(^{-1}\)s\(^{-1}\)) | \( (2.0 \pm 0.4) \times 10^4 \) | \( (2.3 \pm 0.2) \times 10^3 \) | \( 10 \pm 3 \) |
| \( k_{\text{cat}} (s^{-1}) \)                      | \( 1.2 \pm 0.1 (k_{\text{cat}}^H) \) | \( 1.8 \pm 0.1 (k_{\text{cat}}^H = k_{\text{cat}}^0) \) | \( 1.9 \pm 0.4 (k_{\text{cat}}^0) \) |
| \( pK_a (pK_a^n) \)                                 | \( 7.4 \pm 0.2 \)           | \( 7.4 \pm 0.1 \)          | \( 7.8 \pm 0.2 \)          |
| \( pK_a^1 \)                                        | \( 5.8 \pm 0.2 (pK_a^b) \)  | \( 6.2 \pm 0.2 (pK_a^b) \) | \( 7.4 \pm 0.2 (pK_a^n) \) |
| \( pK_a^2 \)                                        | \( 7.6 \pm 0.1 (pK_a^n) \)  | \( 8.4 \pm 0.1 (pK_a^n) \) | \( 7.6 \pm 0.1 \)          |
Scheme 1.

\[ \begin{align*}
E\text{Kan} + \text{NTP} & \xrightleftharpoons[k_{-1}]{k_1} E\text{-NTP\textendash}Kan \\
E\text{-NTP\textendash}Kan & \xrightarrow[k_2]{k_2} E\text{-NDP\textendash}P\text{-Kan} \\
E\text{-NDP\textendash}P\text{-Kan} & \xrightarrow[k_3]{k_3} E + \text{NDP} + P\text{-Kan}
\end{align*} \]
Scheme 2

\[ \text{ESH}_3\text{NTP} \rightarrow X \rightarrow \]

\[ \text{H}^+ \quad K_a^b \]

\[ \text{ESH}_2 \quad K_m^H \quad \text{NTP} \quad k_{\text{cat}}^H \quad \text{EH}_2 + \text{NDP} + P \]

\[ \text{H}^+ \quad K_a^n \]

\[ \text{ESH} \quad \text{ESH}_2\text{NTP} \quad \text{ESHNTP} \quad k_{\text{cat}}^0 \quad \text{EH} + \text{NDP} + P \]

\[ \text{H}^+ \quad K_a^a \]

\[ \text{ESNTP} \quad X \rightarrow \]
Figure 1

kanamycin A

2'-phospho-kanamycin A
Figure 2

![Graph showing the relationship between relative viscosity and $k_{cat} / k_{cat}$]
Figure 3

R = 

ATP  
GTP  
ITP  
UTP  
CTP  
TTP
Figure 4

A

B

C
Figure 5

A

B

Asp208
Asp190
Asp208
Asp190

C

Asp208
Asp208

KAN
Asp190
KAN
Asp190
Aminoglycoside 2"-phosphotransferase type IIIa from enterococcus
Adriana Badarau, Qicun Shi, Joseph W. Chow, Jaroslav Zajicek, Shahriar Mobashery and Sergei Vakulenko

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