The COOH Terminus of Aminoglycoside Phosphotransferase (3')-IIIa Is Critical for Antibiotic Recognition and Resistance*

(Received for publication, May 19, 1999, and in revised form, August 5, 1999)

Paul R. Thompson‡§, Jeffrey Schwartzenhauer‡§, Donald W. Hughes**, Albert M. Berghuis§ ‡‡, and Gerard D. Wright‡§ §§

From the §Antimicrobial Research Centre, and Departments of §Biochemistry and **Chemistry, McMaster University, 1200 Main Street West, Hamilton, Ontario, L8N 3Z5 Canada

The aminoglycoside phosphotransferases (APHs) are widely distributed among pathogenic bacteria and are employed to covalently modify, and thereby detoxify, the clinically relevant aminoglycoside antibiotics. The crystal structure for one of these aminoglycoside kinases, APH(3')-IIIa, has been determined in complex with ADP and analysis of the electrostatic surface potential indicates that there is a large anionic depression present adjacent to the terminal phosphate group of the nucleotide. This region also includes a conserved COOH-terminal α-helix that contains the COOH-terminal residue Phe264. We report here mutagenesis and computer modeling studies aimed at examining the mode of aminoglycoside binding to APH(3')-IIIa. Specifically, seven site mutants were studied, five from the COOH-terminal helix (Asp261, Glu262, and Phe264), and two additional residues that line the wall of the anionic depression (Tyr225 and Arg211). Using a molecular modeling approach, six ternary complexes of APH(3')-IIIa-ATP with the antibiotics, kanamycin, amikacin, butirosin, and ribostamycin were independently constructed and these agree well with the mutagenesis data. The results obtained show that the COOH-terminal carboxylate of Phe264 is critical for proper function of the enzyme. Furthermore, these studies demonstrate that there exists multiple binding modes for the aminoglycosides, which provides a molecular basis for the broad substrate- and regiospecificity observed for this enzyme.

The aminoglycoside antibiotics constitute an important class of anti-infective agents which find use in the treatment of bacterial and some protozoan infections (1). These molecules are a significant component of our antimicrobial arsenal and, like all antibiotics, are under pressure from the spread of resistance mechanisms. Since the mode of antimicrobial action first requires interference with translational events mediated through binding of these polycationic molecules to the 16 S rRNA, ribosomal movement at both the RNA and protein levels has been associated with aminoglycoside resistance. However, this is a minor component of clinically relevant resistance, limited to slow growing bacteria such as mycobacteria. The major route of clinically important resistance is through the production of modifying enzymes. These include kinases, acetyltransferases, and nucleotidyltransferases (2).

We have studied the aminoglycoside kinase, APH(3')-IIIa, which is harbored by Gram-positive cocci such as the enterococci and staphylococci, both highly relevant human pathogens, especially in clinical institutions. This enzyme has a broad aminoglycoside substrate specificity with the demonstrated capacity to phosphorylate aminoglycosides at position 3', and for aminoglycosides with a pentose linked to position 6 of the 2-deoxyaminocyclitol ring, position 5' (see Fig. 1 for representative structures and ring numbering) (3, 4). In addition to naturally occurring aminoglycosides (and their semi-synthetic derivatives), APH(3')-IIIa has been shown to phosphorylate a series of de-aminated and deoxyaminoglycoside derivatives with higher tolerance than the related APH(3')-IIIa and APH(3')-IIa which are found in Gram-negative bacteria (5). APH(3')-IIIa thus has the broadest substrate specificity of the 3'-aminoglycoside kinases (6). The x-ray structure of the APH(3')-IIIa-ADP binary complex has been determined to 2.2 Å and demonstrates striking similarity with the Ser/Thr/Tyr protein kinase family (7). This observation is paralleled by work which indicates that APH(3')-IIIa has a similar chemical mechanism as protein kinases (8). Furthermore, aminoglycoside kinases, such as APH(3')-IIIa, have been demonstrated to be sensitive to inhibitors of protein kinases (9), and have been shown to have bona fide protein kinase activity with the potential to phosphorylate some proteins and peptides on Ser (10). Thus the aminoglycoside-binding pocket on APH(3')-IIIa is highly tolerant of many diverse structures.

In an effort to define the structural basis of substrate specificity, we have initiated site-directed mutagenesis studies in combination with molecular modeling. Our results demonstrate that the COOH-terminal residue of the enzyme is critical for efficient substrate binding and indicate that the bulk of the aminoglycoside specificity lies in the COOH-terminal domain, but that there are many binding modes for aminoglycoside substrates.

MATERIALS AND METHODS

Site-directed Mutagenesis of APH(3')-IIIa—The Phe264 deletion mutant (ΔPhe264) was generated by PCR amplification of the wild type gene, encoded on plasmid pETSACG1, using the mutagenic oligonucleotide, 5'-C CGAAGGCTTCTAATTCATCCAG-3', as the reverse

* This work was supported in part by Medical Research Council of Canada Grants MT-13536 (to G. D. W.) and MT-13107 (to A. M. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a Natural Sciences and Engineering Research Council of Canada graduate scholarship.

§§ Recipient of a Pharmaceutical Association of Canada Health Research Foundation Research Career Award.

** Recipient of a Medical Research Council of Canada Scholarship. For whom correspondence should be addressed. Tel.: 905-525-9140 (ext. 22943); Fax: 905-522-9033; E-mail: wrigghtg@hsu.ccus.mcmaster.ca.

††‡‡ Recipient of a Natural Sciences and Engineering Research Council of Canada graduate scholarship.

‡‡§§ Recipient of a Medical Research Council of Canada Scholarship.

††‡‡ Recipient of a Pharmaceutical Association of Canada Health Research Foundation Research Career Award.
primer. The forward PCR primer has been described previously (3). The D261A, E262A, F264A, and D261A/Phe264 double mutant were similarly generated using the mutagenic oligonucleotides, 5′-CCCGAAAGCTTCTAAAACATCTTGAAGATATA-3′, 5′-CCCGAAAGCTTCTAAAACATCTTGAAGATATA-3′, 5′-CAAGGACCGATGGATGTAAGGAGTGTGT-3′, and 5′-CCCGAAAGCTTCTAAAACATCTTGAAGATATA-3′, respectively, as the reverse primers. The PCR amplified products were digested with HindIII and NdeI and cloned into similarly digested pET22b(+).

The mutagenic oligonucleotides, 5′-AAAAAGCACCACGGTGTGCAGAAGGACCGATGGATGTAAGGAGTGTGT-3′, 5′-ATTGATCTTGGGCAACGGCGAGG-3′, and their reverse complements were used to generate Q-Sepharose column mutants by the QuikChange mutagenesis method (Stratagene, La Jolla, CA). Our APH(3’)-IIIa overexpression construct, pET5ACG1, was used as the template in PCR reactions. The resultant mutant overexpression constructs, pETY55A4 and pETR211A6, were cloned into Escherichia coli BL21(DE3) for protein overexpression and purification. All mutagenized genes were completely sequenced to ensure that the proper mutation had been incorporated and that no other mutations had been introduced during the PCR process.

Protein Purification—Mutant APH(3’)-IIIa proteins were overexpressed and purified by the method described previously (3) which consisted of two consecutive anion exchange chromatography steps. In this work, a Q-Sepharose column (Amersham Pharmacia Biotech) (2.5 × 10 cm) was used in place of the MacroPrep Q column (Qiagen) (3) to purify all of the mutants. Purification of certain mutant APH(3’)-IIIa proteins required modifications of the purification protocol. For example, during purification of the Y55A, E262A, and the F264A mutants, the second anion exchange step was replaced by chromatography over a smaller Q-Sepharose (Amersham Pharmacia Biotech) column (10 × 1 cm). Mutant proteins were eluted using a linear gradient (15–40%) of Buffer B over 45 ml. Similarly, purification of the D261A/Phe264 mutant required that the second anion exchange column be again replaced with the smaller Q-Sepharose column and the mutant proteins eluted as described above. In order to obtain pure protein, fractions of the small Q-Sepharose column were applied to an Superdex 200 column (HR 10/30) (Amersham Pharmacia Biotech). The column was equilibrated in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 200 mM NaCl and the proteins separated at a rate of 0.2 ml/min. The purification of the D61A and the D61A/Phe264 mutants required that the second anion exchange column be again replaced with the smaller Q-Sepharose column and the mutant proteins eluted as described above. In order to obtain pure protein, fractions of the D61A mutant were applied to a Mono Q (HR 5/5) (Amersham Pharmacia Biotech) analytical anion exchanger and proteins were eluted using a linear gradient (10–40%) of Buffer B over 45 ml. Similarly, the D61A/Phe264 mutant was applied to a 1 ml Resource Q column (Amersham Pharmacia Biotech) and proteins were eluted using a linear gradient (15–45%) of Buffer B over 50 ml.

Kinetic Analysis of Mutant APH(3’)-IIIa Proteins—The kinetic assay monitors the production of ADP and couples this to the oxidation of NADH by a pyruvate kinase/lactate dehydrogenase system. The assay is described in greater detail elsewhere (5). When the kinetic parameters for the determination of Phosphoaminoglycosides were determined for Neomycin we found that the concentration of protein was determined for each of the cleared lysates and included pulsed field gradients for coherence selection. The free induction decay (FID) was processed using Gaussian multiplication (line broadening: −1.5 Hz, Gaussian broadening: 0.2 Hz). The FID was also zero-filled to 64 K before Fourier transformation.

Proton COSY two-dimensional NMR spectra were recorded in the 90–180°−t − 180°−t′−180°−t′ window function. The slurry was washed with 1 liter of H2O and bound material was eluted with 5 × 50-ml fractions of 1.0% NH4OH. The presence of phosphorylated aminoglycoside in individual fractions was analyzed by TLC as described in McKay et al. (3). Fractions containing the phosphorylated aminoglycoside were pooled, neutralized with 6 M HCl, and lyophilized.

Typically, the lyophilized products were dissolved in 30 ml of H2O and assayed by the addition of 5-ml aliquots to a Sephadex G-25 column (50 × 2 cm). The mobile phase was water, the column run at a flow rate of 1.5 ml/min, and 1-ml fractions were collected. Fractions were analyzed by TLC and those fractions containing the phosphorylated aminoglycoside were pooled and applied to Mono S (HR 5/5), as described (4). Fractions containing pure phosphorylated aminoglycoside were pooled and lyophilized.

Determination of APH(3’)-IIIa Minimal Inhibitory Concentrations (MIC)—The MIC of E. coli BL21(DE3) harboring the various mutant aph(3’)-IIIa genes were estimated through the use of antibiotic gradient plates in the absence of isopropyl-1-thio-β-D-galactopyranoside (11). Gradient plates were prepared by the addition of aminoglycoside antibiotic and 25 µl of a 10% suspension of Antifoam B (Sigma) to 25 ml of molten Mueller Hinton Agar (Difco). Kanamycin A was added to a final concentration of 150 µg/ml and neomycin was added to 75 µg/ml. The resultant solution was then poured into a square sterile culture plate at an incline in order to create a wedge. The agar was allowed to solidify for approximately 1 h at which time an overlaying wedge, made up of molten Mueller Hinton Agar without antibiotic (25 ml) was poured while the culture dish laid flat. The overlaying wedge was allowed to solidify for a further 2 h. Overnight cell cultures of E. coli BL21(DE3) containing the Mating wild type or mutant APH(3’)-IIIa were diluted 100-fold in top agar (1% tryptone, 0.8% NaCl, 0.8% agar). The top agar was spread across a sterile microscope slide and a uniform distribution of cells was achieved by dipping the long edge of a second sterile microscope slide into the cell suspension. The edge of the slide was then applied to the surface of the plate parallel to the gradient of drug. The plates were inverted and grown for 24 h at which point 5 ml of a 0.2% solution of acridine orange in ethanol was poured over the top and allowed to evaporate. The stained plates were photographed under shortwave UV light. The antibiotic gradient was assumed to be linear and the fraction of the total distance of the gradient that the mutant cell strains were able to grow was estimated to be the MICs of the APH(3’)-IIIa mutants.

In order to ensure that protein expression for each mutant was comparable to the wild type enzyme, the volume of protein produced under noninducing conditions was qualitatively assessed by Western analysis. Overnight cultures of wild type and mutant APH(3’)-IIIa producing strains (50 ml) were harvested by centrifugation at 5000 × g and resuspended in 4 ml of 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 200 mM NaCl. Cells were then lysed by 2 passages through a French press at 18 000 × g. The concentration of protein was determined for each of the cleared lysates and included pulsed field gradients for coherence selection. The Western blot was developed using a donkey anti-rabbit IgG antibody coupled to alkaline phosphatase.

Characterization of Phosphoaminoglycosides—Mass spectra of neomycin and ribostamycin phosphorylated by the APH(3’)-IIIa ΔPhe264 deletion mutant were determined in the positive ion mode in the presence of 0.1 and 0.2% formic acid, respectively. Electrospray mass spectral analysis of ribostamycin phosphorylated by the Y55A and the E262A mutants was carried out in the positive ion mode in the presence of 0.2% formic acid. All mass spectra were recorded at the McMaster Regional Center for Mass Spectrometry. All NMR spectra were recorded on a Bruker Avance DRX-500 NMR spectrometer. Proton spectra were acquired at 500.13 MHz using a 5-mm broad band inverse probe with triple axis gradient capability. Spectra were obtained in 32 scans in 32 K data points over a 3.592 kHz spectral width (4.561 s acquisition time). Sample temperature was maintained at 30 °C by a Bruker Eurotherm variable temperature unit. Spectra were obtained in 32 scans in 32 K data points over a 3.592 kHz spectral width (4.561 s acquisition time). Sample temperature was maintained at 30 °C by a Bruker Eurotherm variable temperature unit. The free induction decay (FID) was processed using Gaussian multiplication (line broadening: −1.5 Hz, Gaussian broadening: 0.2 Hz). The FID was also zero-filled to 64 K before Fourier transformation.

Proton COSY two-dimensional NMR spectra were recorded in the absolute value mode using the pulse sequence 90°−t−45°−acquisition and included pulsed field gradients for coherence selection. The pulse sequence parameters were acquired in the 256 FIDs of the 2 K data points in the F2 dimension over a 3.592 kHz spectral width. The 2-90° pulse width was 6.6 µs. A 1.0-s relaxation delay was used between acquisitions. Zero-filling in the F1 dimension produced a 1 K × 1 K data matrix with a digital resolution of 3.508 Hz/point in both dimensions. During two-dimensional Fourier transformation a sine-bell squared window function was applied to both dimensions. The trans-
formed data were then symmetrized.

Selective one-dimensional TOCSY 1H NMR spectra were recorded over a 4.006 KHz spectral width in 32 K data points (4.089 s acquisition time). Gaussian shaped pulses were defined by 256 data points with the pulse being truncated at 1% of the maximum pulse amplitude. The 90° Gaussian pulse width was 25.5 μs. The pulse was further refocused and ultimately delayed by a 25.5 μs fixed delay and then by the standard TOCSY MLEV-17 spin lock pulse sequence. The 90° spin lock pulse width was 27.0 μs. A 1.0 s relaxation delay was used. The 20-ms spin lock period was followed by a z-filter which contained 5 variable delay times ranging from 4 to 18 ms. The transmitter offset was adjusted to the frequency of the 1H being selectively excited. Thirty-two scans were obtained with each of the 32 K data points resulting in a total of 160 scans. The FID's were processed using Gaussian multiplication (line broadening: 1.5 Hz; Gaussian broadening: 0.2) and was zero-filled to 64 K before Fourier transformation.

Phosphorus 31 NMR spectra were recorded at 202.456 MHz using the 5-mm triple axis gradient broad band inverse probe. The spectra were acquired over a 48.544 KHz spectral width in 32 K data points (0.353 s acquisition time). Spectra were obtained with a 31P pulse width of 3.0 μs (33° flip angle) and a relaxation delay of 0.5 s. The FID's were processed using exponential multiplication (line broadening: 5.0 Hz) and were zero-filled to 64 K before Fourier transformation.

The compounds were dissolved in 99.996% D2O (Isotec, Inc.) to a concentration of approximately 20.0 mg/ml. Chemical shifts are reported relative to external phosphoric acid in D2O at 0.0 ppm. 31P NMR spectra were referenced relative to external 85% phosphoric acid in D2O at 0.0 ppm.

Electrostatic Field Analysis and Docking Simulations with Aminoglycosides—The crystal structure of APH(3′)-IIa with bound ADP has been determined to a resolution of 2.2 Å (7). However, for examining the interaction of this enzyme with aminoglycoside substrates at an atomic level, analysis of the ATP bound form is more appropriate. Therefore, a model of APH(3′)-IIa with ATP bound in the nucleotide binding pocket was constructed in the following manner. One of the two crystallographically independent APH(3′)-IIa molecules was selected and the bound ADP molecule removed. Subsequently, an ATP molecule was modeled into the nucleotide binding pocket based on the conformation of ADP in the crystal structure of APH(3′)-IIa-ADP, and the conformation of ATP in crystal structures of two protein kinases (catalytic domain of cAMP-dependent protein kinase, Protein Data Bank code 1ATP (12), and catalytic domain of phosphorylase kinase, Protein Data Bank code 1PHK (13)). Note that the structure of APH(3′)-IIa shows extensive structural homology with Sar/Thr and Tyr protein kinases, especially around the nucleotide binding pocket (7), thus justifying the use of protein kinase crystal structures for modeling the ATP conformation. Furthermore, the conformation of ATP co-factors is essentially identical in the two protein kinase crystal structures, i.e. when overlaid, the γ-phosphate groups are within 0.3 Å of one another. Also, preliminary analysis of the crystal structure of APH(3′)-IIa with bound AMP-PNP (a non-hydrolyzable ATP analogue) validated the modeled conformation of ATP.

The electrostatic field surrounding the APH(3′)-IIa enzyme molecule with ATP was evaluated using the Poisson-Boltzmann equation as implemented in DELPHI (14, 15). For the calculation, a dielectric constant of 80 was used for the solvent and a value of 2 was assumed for the interior of the protein molecule. Partial charges for protein and co-factor atoms were adopted from the AMBER force field (16).

The molecular surface of the APH(3′)-III-ATP complex was calculated with the GRASP program (17) which incorporates the algorithm by Connolly (18). GRASP was also used to evaluate the electrostatic potential at the molecular surface, based on the DELPHI electrostatic field analysis.

Six models of the APH(3′)-III-ATP-aminoglycoside ternary complexes, with the aminoglycosides: kanamycin (3′ productive complex), amikacin (3′ productive complex), ribostamycin (3′ and 5′ productive complexes) and butirosin (3′ and 5′ productive complexes), were constructed using the following procedure. The molecular docking program DockVision (19) was employed to generate a large number (100-250) of possible models for each specific ternary complex, from which a 40-μl was subsequently selected that was consistent with functional studies. The precise process used by the DockVision software to arrive at possible models involved three stages. During stage one, 3000 conformations of an aminoglycoside were randomly generated and allowed to flexibly (i.e. torsion angles were allowed to change) dock in the APH(3′)-IIa aminoglycoside binding pocket. Approximately, 20% of these models were clustered resulting in 200–500 unique solutions. In stage two, the models were refined and again clustered, resulting in approximately 100–300 distinct models. And in the final stage, the remaining models were visually inspected. The graphical analysis allowed for the selection of one single model for each of the six ternary APH(3′)-IIa-ATP-aminoglycoside complexes.

RESULTS AND DISCUSSION

Examination of the electrostatic properties of APH(3′)-IIa reveals that adjacent to the modeled γ-phosphate group of ATP is a surface depression which is strikingly negatively charged under physiological conditions (Fig. 2). Considering that aminoglycosides, due to the preponderance of amine groups, invariably carry a positive charge at neutral pH, it is likely that the negatively charged surface depression is the site of antibiotic binding. The most prominent structural component which contributes to this putative aminoglycoside-binding site is the COOH-terminal helix.

The C-terminal helix of APH(3′)-IIa contains the amino acid sequence, Leu260–Asp-Glu-Leu-Phe-COOH, a sequence which is highly conserved among the 3′-aminoglycoside kinases (6). This COOH-terminal helix points "back" toward the nucleotide binding pocket, and in the APH(3′)-IIa structure, the COOH-terminal Phe ring is with 6 COOH of the modeled γ-phosphate of ATP. Thus, given the proximity to the active site, its participation in the creation of a negative surface patch, and the conservation of the amino acid sequence among aminoglycoside
Aminoglycoside Recognition by an Antibiotic Resistance Kinase

### Table I

| Substrate | Wild type | ΔPhc\(^{364}\) | D261A | E262A | D261A/ΔPhc\(^{364}\) | F264A |
|-----------|-----------|----------------|--------|--------|---------------------|--------|
|           | \(K_m\) \(\mu M\) | \(k_{cat}\) \(s^{-1}\) | \(K_i\) | \(K_m\) \(\mu M\) | \(k_{cat}\) \(s^{-1}\) | \(K_i\) | \(K_m\) \(\mu M\) | \(k_{cat}\) \(s^{-1}\) | \(K_i\) | \(K_m\) \(\mu M\) | \(k_{cat}\) \(s^{-1}\) | \(K_i\) |
| ATP       | 27.7 ± 3.7 | 1.76 ± 0.08 | NA    | 17.9 ± 1.8 | 0.26 ± 0.01 | NA    | 16.4 ± 0.7 | 0.23 ± 0.02 | NA    | 14.2 ± 0.4 | 0.18 ± 0.02 | NA    |
| Kanamycin A | 12.6 ± 2.6 | 1.79 ± 0.09 | 6.38 ± 1.67 | 11.5 ± 1.7 | 0.24 ± 0.01 | 1.9 ± 0.08 | 15.5 ± 1.4 | 0.23 ± 0.02 | 1.9 ± 0.08 | 14.2 ± 0.4 | 0.18 ± 0.02 | 1.9 ± 0.08 |
| Neomycin B | 7.72 ± 0.9  | 2.08 ± 0.07 | 2.65 ± 0.59 | 21.2 ± 5.22 | 0.24 ± 0.01 | NA    | 18.5 ± 0.7 | 0.27 ± 0.02 | NA    | 14.2 ± 0.4 | 0.18 ± 0.02 | NA    |
| Amikacin   | 245 ± 27    | 2.46 ± 0.11 | NA    | 226 ± 1.3 | 0.22 ± 0.01 | NA    | 195 ± 0.7 | 0.22 ± 0.02 | NA    | 14.2 ± 0.4 | 0.18 ± 0.02 | NA    |
| Butirosin  | 34.3 ± 1.1  | 0.07 ± 0.01 | 2.17 ± 0.44 | 17.1 ± 4.3 | 0.23 ± 0.01 | NA    | 14.2 ± 0.4 | 0.18 ± 0.02 | NA    | 14.2 ± 0.4 | 0.18 ± 0.02 | NA    |
| Ribostamycin | 9.3 ± 1.8  | 1.89 ± 0.10 | 1.73 ± 0.66 | 30.3 ± 4.3 | 0.23 ± 0.01 | NA    | 14.2 ± 0.4 | 0.18 ± 0.02 | NA    | 14.2 ± 0.4 | 0.18 ± 0.02 | NA    |
| Neamine    | 20.0 ± 2.8  | 2.00 ± 0.15 | 2.00 ± 0.4  | 363.2 ± 4.3 | 1.78 ± 0.18 | 4.0 ± 3.4 | 22.2 ± 0.9 | 2.03 ± 0.03 | NA    | 14.2 ± 0.4 | 0.18 ± 0.02 | NA    |
| Lividomycin A | 31.6 ± 5.1 | 3.97 ± 0.25 | 1.53 ± 0.42 | 42.1 ± 5.8 | 0.82 ± 0.03 | NA    | 14.2 ± 0.4 | 0.18 ± 0.02 | NA    | 14.2 ± 0.4 | 0.18 ± 0.02 | NA    |

### Table I—continued

| Substrate | Y55 A | ATP       | 18.6 ± 2.8 | 0.65 ± 0.02 | NA    |
|-----------|-------|-----------|-----------|-----------|--------|
| Kanamycin A | 15.5 ± 1.6 | 0.89 ± 0.09 | 1.9 ± 0.8  | 15.5 ± 1.6 | 0.89 ± 0.09 | 1.9 ± 0.8 |
| Neomycin B | 3.2 ± 1.1  | 0.33 ± 0.01 | NA        | 3.2 ± 1.1  | 0.33 ± 0.01 | NA        |
| Amikacin   | 588 ± 105 | 0.47 ± 0.03 | NA        | 588 ± 105 | 0.47 ± 0.03 | NA        |
| Isemapacin | 888 ± 111 | 0.63 ± 0.04 | NA        | 888 ± 111 | 0.63 ± 0.04 | NA        |
| Butirosin  | 138 ± 18   | 0.74 ± 0.03 | NA        | 138 ± 18   | 0.74 ± 0.03 | NA        |
| Ribostamycin | ND | ND ND | ND | ND ND | ND |
| Lividomycin A | 10.7 ± 1.6 | 0.30 ± 0.01 | NA | 10.7 ± 1.6 | 0.30 ± 0.01 | NA |
| ATP\(\gamma\) | 57.7 ± 5.3 | 0.28 ± 0.01 | NA | 57.7 ± 5.3 | 0.28 ± 0.01 | NA |

* NA, not applicable.
* ND, not determined.

### Note

In the crystal structure of APH(3’)-I\(\alpha\) complexed with ADP, Asp\(^{261}\) is pointing in toward the active site, but down and away from the predicted aminoglycoside-binding region. In docking studies with kanamycin A and amikacin, the backbone carbonyl oxygen of Asp\(^{261}\) is within hydrogen bonding distance (2.25–3.1 Å) of N-3 (Fig. 4). Similarly, the Asp\(^{261}\) carbonyl oxygen also is predicted to interact with the N-6’ of butirosin and ribostamycin positioned for 3’-phosphorylation and O-4’ kinases, we hypothesized that this helix plays a prominent role in aminoglycoside binding. We therefore made a series of point mutations in the most conserved residues in an effort to determine the contribution of this region to substrate binding and catalysis. Two additional amino acid residues, Tyr\(^{256}\) and Arg\(^{211}\), also line the presumed aminoglycoside binding pocket (Fig. 2) and these were also studied by site-directed mutagenesis.

Asp\(^{261}\)–Asp\(^{261}\) is completely conserved in the COOH-terminal helix of APH(3’) enzymes. Mutagenesis of this residue to Ala resulted in an enzyme that was significantly impaired in aminoglycoside phosphorylation capacity (Table I and Fig. 3 where the \(k_{cat}/K_m\) effects are illustrated graphically). This decrease in aminoglycoside affinity was largely the result of a drop in \(k_{cat}\) of 7.5–12-fold for the 4,6-disubstituted aminoglycosides kanamycin and amikacin, and 77–104-fold for the 4,5-disubstituted aminoglycosides neomycin, butirosin, and ribostamycin. While D261A phosphorylated the 4,5-disubstituted aminoglycosides quite poorly, there was in general, little effect on the \(K_m\) determined for these substrates. As an additional independent measure of aminoglycoside affinity, the poor substrate neomycin (approximately 100-fold decrease in \(k_{cat}\)) was examined as an inhibitor of kanamycin A phosphorylation. As expected, neomycin B was a competitive inhibitor of kanamycin phosphorylation with \(K_m\) of 3.21 ± 0.28 \(\mu\)M, in excellent agreement with the \(K_m\) determined for neomycin phosphorylation by the wild type enzyme (3).

The most striking effect of the D261A mutation is the magnitude of the decrease in the turnover number for this enzyme. For the 4,6-disubstituted aminoglycosides there is a 7.5-fold decrease in the \(k_{cat}\) for kanamycin A and an 11.7-fold decrease in the \(k_{cat}\) for amikacin. However, for the 4,5-disubstituted aminoglycosides the decrease in \(k_{cat}\) is more pronounced and is on the order of a 100-fold reduction. The reason for this reduction in turnover number is unclear.

In the crystal structure of APH(3’)-I\(\alpha\) complexed with ADP, Asp\(^{261}\) is pointing in toward the active site, but down and away from the predicted aminoglycoside-binding region. In docking studies with kanamycin A and amikacin, the backbone carbonyl oxygen of Asp\(^{261}\) is within hydrogen bonding distance (2.25–3.1 Å) of N-3 (Fig. 4). Similarly, the Asp\(^{261}\) carbonyl oxygen also is predicted to interact with the N-6’ of butirosin and ribostamycin positioned for 3’-phosphorylation and O-4’
for 5'-phosphorylation. The functional group of Asp^{261} could conceivably move as part of a conformational change upon aminoglycoside binding and act as a hydrogen bond acceptor or be involved in electrostatic interactions, thus providing a rationalization for the observed effects. However, in such a case, one would predict an increase in the $K_m$, which was not observed with this mutant enzyme. Alternatively, the change in the rate constant may reflect COOH-terminal helix stability issues rather than aminoglycoside binding. In the crystal structure of the APH(3')-IIIa-ADP binary complex, Asp^{261} appears to interact with Arg^{226} on an opposing helix. This contact could serve to orient and stabilize the COOH-terminal helix and consequently orient the main chain carbonyl group of Asp^{261} for optimal substrate binding. In addition to a potential role in aminoglycoside binding, the main chain carbonyl of Asp^{261} appears to form a hydrogen bond with the main chain amide hydrogen of Phe^{264}. The importance of this interaction likely reflects the requirement for the proper orientation of the COOH-terminal carboxylate of Phe^{264} for proper catalysis to occur (see below).

Glu^{262}—Glu^{262} is conserved among the 3'-aminoglycoside phosphotransferases, however, the function of this residue is not clear from the crystal structure of APH(3')-IIIa. In this structure, Glu^{262} is pointing away from the active site (Fig. 2) and therefore would not be expected to be involved in the binding of aminoglycoside substrates. Our analysis of the E262A enzyme demonstrates that this conserved COOH-terminal residue plays a minor role in aminoglycoside binding or catalysis. The E262A mutation had little effect upon the ability of APH(3')-IIIa to phosphorylate aminoglycoside antibiotics since the steady state kinetic parameters for ATP, neomycin B, amikacin, and lividomycin A are virtually unchanged from wild type (Table I, Fig. 4). There is, however, a significant 26-fold increase in the $K_m$ for kanamycin A and a smaller 8.6-fold
increase in the \( K_m \) for neamine which lacks ring C. This would suggest that this residue is potentially involved in the binding of ring A and/or the aminocyclitol rings. Docking studies indicate that the Glu262 carboxylate is within 3.30 Å of N-1 of kanamycin A (Fig. 4). This amine is acylated in amikacin and therefore does not have the capacity to interact with Glu262. This may be the molecular basis for the increased \( K_m \) for amikacin (19-fold over kanamycin (3)) in the wild type enzyme.

**Phe264**—The phenyl ring of the COOH-terminal Phe264 is unlikely to be directly involved in the binding or chemical events surrounding the reaction catalyzed by this enzyme, as aminoglycosides are highly polar substrates that are not expected to interact with the hydrophobic aromatic ring. Nevertheless, the position of this residue in the crystal structure and the absolute conservation of this residue among the 3'-aminoglycoside phosphotransferases indicated that the function of this residue needed to be more clearly defined.

In an effort to examine the role of Phe264, we initially generated an overexpression construct where the COOH-terminal phenyl ring was replaced by a hydrogen (F264A). This change did little to affect the ability of this enzyme to phosphorylate most aminoglycosides and yielded near wild type steady state kinetics (Table I, Fig. 3). Amikacin and butirosin both possess a hydroxybutyrate group and a modest increase in the \( K_m \) (3.6- and 3.7-fold respectively) was observed for these two substrates with the F264A mutant. The reason for such a decrease in the apparent affinity of this enzyme for these two substrates is not readily apparent from the crystal structure of APH(3'-IIIa)-IIIa complexed with ADP. In this structure, the phenyl ring of the COOH-terminal Phe is pointing away from the aminoglycoside binding pocket (Fig. 2) and is therefore unlikely to be involved in aminoglycoside binding. Furthermore, modeling studies sup-
Fig. 4. Ternary complex models of APH(3')-IIIa-ATP-aminoglycoside. Models were constructed as described under "Materials and Methods" and selected to be consistent with steady state kinetic data obtained from site-directed mutagenesis studies.
port such a conclusion (Fig. 4).

Since the F264A mutant yielded no additional dramatic changes in the substrate specificity of APH(3')-IIIa we next decided to determine the role of the COOH-terminal carboxylate which, in the crystal structure of APH(3')-IIIa-ADP, is pointing back toward the active site of this enzyme (Fig. 2). We therefore generated a Phe264 deletion construct and overexpressed the mutant protein in E. coli. Steady state kinetic analysis of $\Delta$Phe264 confirmed that the carboxylate of Phe264 plays an important role in the catalytic mechanism of this enzyme (Table I, Fig. 3). The $k_{cat}$ values for the 4,6-disubstituted deoxystreptamine aminoglycosides, kanamycin A and amikacin, dropped significantly: 7.2-fold for kanamycin A and 24.6-fold for amikacin. Furthermore, there was a corresponding 9.1- and 2.9-fold respective increase in the $K_m$ for kanamycin A and amikacin.

The effect of the deletion of this residue is more complex with respect to the 4,5-disubstituted deoxystreptamine aminoglycosides. For those 4,5-disubstituted aminoglycosides that possess ring D, neomycin B, and lividomycin A (Fig. 1), there was only a modest change in $K_m$ and $k_{cat}$ (Table I, Fig. 3). On the other hand, the 4,5-disubstituted aminoglycosides that lack ring D (ribostamycin, butirosin, and neamine) show intermediate changes in the steady state kinetic parameters (Table I, Fig. 3). Butirosin, which contains a 2-hydroxybutyrate group substituted at the N-1 position of the deoxystreptamine ring (Fig. 1), had a 12.6-fold increase in $K_m$ and a 14.4-fold decrease in $k_{cat}$. In contrast, there is only a minor (3.3-fold) decrease in the $K_m$ for ribostamycin, but there is an 8.2-fold decrease in the turnover rate of reaction. While it is difficult to estimate the effects of the new COOH-terminal carboxylate (Leu263) on aminoglycoside binding, these results likely reflect the manner in which the enzyme binds these different classes of aminoglycosides.

Docking studies with amikacin and kanamycin, and ribostamycin and butirosin in the 3'-OH phosphorylation binding mode, demonstrate that this residue can interact with the 4'-OH, positioning ring A for phosphoryl transfer to the 3'-OH (Fig. 4). Furthermore, there is the potential for interaction of the Phe264 carboxylate with N-3 of amikacin and kanamycin (Fig. 4). The models of butirosin and ribostamycin in the 3'-phosphorylation binding mode also reveal potential interactions with O-3'. An additional interaction with O-2' of butirosin can also be modeled and this is consistent with the more significant increase in $K_m$ for this substrate in the $\Delta$Phe264 mutant (Table I).

The fact that lividomycin A is a reasonably good substrate for the $\Delta$Phe264 enzyme indicates that it has little difficulty phosphorylating the 5” position of 4,5-disubstituted aminoglycosides. Furthermore, the fact that neamine displays near wild type kinetics as with this enzyme indicates that the COOH-terminal residue also has little effect on the ability of the enzyme to phosphorylate the 3’-hydroxyl of this smaller aminoglycoside consisting of only rings A and B (Fig. 1). The smaller size of neamine likely also explains the comparatively small increase in $K_m$ of 3.2-fold, for this substrate in comparison to kanamycin A which is increased 9.1-fold.

Since deletion of the COOH-terminal Phe264 predominantly affected the phosphorylation of 4,6-disubstituted aminoglycosides, and the D261A mutation has a more dramatic effect on the phosphorylation of the 4,5-disubstituted aminoglycosides, we prepared the D261A/$\Delta$Phe264 double mutant to determine if we could generate a mutant enzyme with poor activity toward both classes of aminoglycoside substrates. Determination of the steady state kinetic parameters validated this hypothesis and both the 4,5-disubstituted and 4,6-disubstituted aminoglycosides are poor substrates for the double mutant (Table I, Fig. 2). Surprisingly, the $k_{cat}$ for neomycin B has actually increased 3-fold from that obtained for the D261A single mutant. The structural or mechanistic basis of such a rate enhancement is not readily apparent. Those aminoglycosides containing the 2-hydroxybutyrate group are extremely poor substrates and the steady state kinetic parameters were not therefore determined for butirosin. The COOH-terminal peptide is therefore required for the phosphorylation of the diverse array of aminoglycosides which this enzyme modifies.

$Tyr^{25}$—While Tyr25 is poorly conserved among the 3'-APHs, the decision to prepare a Y55A mutant was influenced by the
observation that this residue could form part of the presumed aminoglycoside binding pocket. Furthermore, the phenolic ring of Tyr55 is relatively close (3.9–8.5 Å) to the COOH-terminal phenyl group. This proximity suggested the potential for a stacking interaction between the two aromatic rings should a conformational change occur upon aminoglycoside substrate binding. Mutation of Tyr55 residue to Ala, however, did not substantially perturb the catalytic efficiency of APH(3')-IIIa. The steady state kinetic parameters were determined for a variety of substrates and the only significant effect was seen with those aminoglycosides that contain the 2-hydroxybutyrate group at position N-1. For amikacin there was a 5.4-fold increase in the $K_m$ and a 3.5-fold decrease in $k_{cat}$ while for butirosin there is a 5.5-fold increase in $K_m$ but only a modest 1.3-fold decrease in $k_{cat}$. Thus despite the proximity of Tyr55 to the aminoglycoside-binding region, it plays little role in specificity for most aminoglycosides.

Arg211—The crystal structure of APH(3')-IIIa complexed with ADP revealed the close proximity of Arg211 to the active site where it is pointed toward the predicted aminoglycoside-binding site (Fig. 2). Despite this positioning, there was surprisingly little effect on the steady state kinetic parameters determined with this mutant enzyme (Table I, Fig. 3) with only modest decreases in $k_{cat}$ (e.g. 2.7-fold for ATP). However, the $K_m$ values increased for aminoglycosides that possess an N-1 substituent aminoglycoside antibiotics that possess both a 3'- and 5'-hydroxyl are diphosphorylated by APH(3')-IIIa (4). The second phosphorylation event is readily apparent in the steady state through the observation of an inflection point in the progress curves at the point where we expect complete monophosphorylation. We first noted the loss of this inflection point in the progress curves for the phosphorylation of neomycin B by the Phe264 deletion mutant (Fig. 5). We then examined the progress curves for the phosphorylation of neomycin B by all of the mutants described in this study (not shown). Only Tyr55, Glu262 and the Phe264 deletion mutant appeared to lack the second phosphorylation event as the inflection point normally observed in the progress curves for neomycin phosphorylation was absent in the progress curves for these mutants. To determine the significance of this observation we in vitro phosphorylated the 4,5-disubstituted-aminoglycoside ribostamycin using the purified Y55A, E262A, and Phe264 mutants. Ribostamycin was selected because of the relative ease of purification of this aminoglycoside once phosphorylated. Purification of the phosphorylated derivatives permitted the analysis of these compounds by electrospray mass spectrometry and two-dimensional NMR.

While the Y55A mutant did not appear to exhibit the biphasic progress curves for the phosphorylation of 4,5-disubstituted aminoglycosides, the purification of the phosphorylated derivatives of ribostamycin revealed that a diphosphorylated derivative could still be produced. The monophosphorylated derivative was purified in small quantity and found to be phosphorylated in a random fashion, analogous to the wild type enzyme. Thus it appears that this residue is involved in the second phosphorylation event, but is not involved in discriminating between the 3' and 5' sites of phosphorylation.

The E262A mutant is also impaired in the ability to diphosphorylate the 4,5-disubstituted class of aminoglycosides as measured by the apparent loss of biphasic kinetic progress curves for neomycin B. However, the in vitro phosphorylation of ribostamycin and the subsequent phosphorylation of the phosphorylated product indicated that this mutant enzyme could indeed diphosphorylate the 4,5-disubstituted aminoglycosides. The reaction products were purified as a mixture of mono- and diphosphorylated compounds. NMR analysis of the monophosphorylated compound revealed that monophosphorylated ribostamycin is phosphorylated exclusively at the 3'-hydroxyl. Using the wild type enzyme, monophosphorylation occurs in a random fashion. The predicted interaction between Glu262 and N-6' of butirosin and ribostamycin suggest that this residue may direct 5'-phosphorylation.

The deletion of the COOH-terminal Phe abrogates the ability of this enzyme to bisphosphorylate the 4,5-disubstituted aminoglycosides. This first became apparent by the loss of the biphasic progress curves (Fig. 5) and was confirmed by the in vitro phosphorylation and purification of both neomycin and ribostamycin. Electrospray mass spectrometry of the phosphorylated aminoglycosides demonstrated that this mutant enzyme no longer possesses the ability to bisphosphorylate antibiotics. Monophosphorylation was subsequently determined to exclusively occur on the 5'-oxygen by NMR. This likely reflects COOH-terminal carboxylate binding to ring A.

Comparison of Modeled Aminoglycoside Conformations with NMR-derived Conformations—The conformation of aminoglycosides in the ternary complex (APH(3')-IIIa-ATP-aminoglycoside) depicted in Fig. 4 was constructed through molecular modeling studies and the final models selected were biased by the biochemical data obtained through these mutagenesis studies. The structures of amikacin and butirosin bound to an APH(3')-IIIa-Cr$^{2+}$-ATP complex have been determined in solution by NOE NMR studies (25) and as a method of validation of the models prepared by our current docking studies, the NMR and docked models were overlaid using a least-squares superposition procedure and compared (Fig. 6). These structures were gratifyingly similar with rms differences of 2.3 Å for butirosin in both the 3' and 5' binding mode, and 3.4 Å for amikacin.
These structural similarities between the modeled and experimentally derived structures support the conformational implications drawn from the biochemical data.

**Effects of Mutations on Aminoglycoside Resistance**—The biological readout for APH(3′)-IIIa is antibiotic resistance, therefore we tested this capacity of the mutant enzymes through the estimation of MICs on antibiotic gradient agar plates. All mutant enzymes were produced in comparable amounts as assessed by Western analysis (Fig. 7), and showed lower MICs than wild type constructs with both kanamycin A and neomycin B in identical genetic backgrounds (Table II). Y55A, which demonstrated wild type steady state kinetic parameters for these aminoglycosides, nevertheless demonstrated significantly reduced MICs. R211A, which had $k_{cat}/K_m$ values approximately 50% of wild type, demonstrated MICs that were similar to the generally accepted view of stringent sub-site of APH(3′)-IIIa. While it may appear that this suggestion is antithetical to the generally accepted view of stringent sub-site of APH(3′)-IIIa, this results in a powerful effect and versatile antibiotic resistance element. The next challenge will be the exploitation of this broad specificity in the design of inhibitory molecules which could be used to reverse aminoglycoside resistance.

**Acknowledgment**—We thank Dr. Trevor N. Hart for assistance with docking studies.

**REFERENCES**

1. Wright, G. D., Berghuis, A. M., and Mobashery, S. (1999) in Resolving the Antibiotic Paradox: Progress in Drug Design and Resistance (Rosen, B. P., and Mobashery, S., eds) pp. 27–69, Plenum Press, New York
2. Shaw, K. J., Rather, P. N., Hare, R. S., and Miller, G. H. (1995) Microbiol. Rev. 57, 138–163
3. McKay, G. A., Thompson, P. R., and Wright, G. D. (1994) Biochemistry 33, 6936–6944
4. Thompson, P. R., Hughes, D. W., and Wright, G. D. (1996) Biochemistry 35, 8686–8695
5. McKay, G. A., Roestamadji, J., Mobashery, S., and Wright, G. D. (1996) Antimicrob. Agents Chemother. 40, 2645–2650
6. Wright, G. D., and Thompson, P. R. (1999) Front. Biosci. 4, D9–D21
7. Hon, W. C., McKay, G. A., Thompson, P. R., Sweet, R. M., Yang, D. S. C., Wright, G. D., and Berghuis, A. M. (1997) Cell 80, 887–895
8. Thompson, P. R., Hughes, D. W., and Wright, G. D. (1996) Chem. Biol. 3, 747–755
9. Daigle, D. M., McKay, G. A., and Wright, G. D. (1997) J. Biol. Chem. 272, 24755–24768
10. Daigle, D. M., McKay, G. A., Thompson, P. R., and Wright, G. D. (1998) Chem. Biol. 5, 11–18
11. Cunningham, R. P., Saporito, S. M., Spitzer, S. G., and Weiss, B. (1986) J. Bacteriol. 168, 1120–1127
12. Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991) Science 253, 407–414
13. Owen, D. J., Noble, M. E., Garman, E. F., Papageorgiou, A. C., and Johnson, L. N. (1995) Structure 3, 467–482
14. Nicholls, A., and Honig, B. (1991) J. Comp. Chem. 12, 435–445
15. Honig, B., and Nicholls, A. (1995) Science 268, 1144–1149
16. Weiner, S. J., Kollman, P. A., Case, D. A., Singh, U. C., Ghio, C., Alagona, G., Profeta, S., and Weiner, P. (1984) J. Am. Chem. Soc. 106, 765–784
17. Nicholls, A., Sharp, K. A., and Honig, B. (1991) Proteins Struct. Funct. Gen. 11, 281–296
18. Connolly, M. L. (1983) Science 219, 709–713
19. Hart, T. N., Ness, S. R., and Read, R. J. (1997) Proteins 1, (suppl.) 205–209
20. Hart, T., and Read, R. (1992) Proteins 13, 206–222
21. Halgren, T. A. (1996) J. Comp. Chem. 17, 490–614
22. Botto, R. E., and Coxon, B. (1983) J. Am. Chem. Soc. 105, 1021–1028
23. Cox, J. R., and Serperu, E. H. (1997) Biochemistry 36, 2353–2359
24. DiGiammarino, E. L., Draker, K.A., Wright, G. D., and Serperu, E. H. (1997) Biochemistry 37, 3638–3644
25. Cox, J. R., McKay, G. A., Wright, G. D., and Serperu, E. H. (1996) J. Am. Chem. Soc. 118, 1295–1301
26. Kraulis, P. J. (1991) J. Appl. Crystallogr. 24, 946–950
27. Merritt, E. A., and Murphy, M. E. P. (1994) Acta Crystallogr. Sect. D 40, 869–873