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ARTICLE *in* BIOCHEMISTRY · AUGUST 2006

Impact Factor: 3.02 · DOI: 10.1021/bi060723g · Source: PubMed

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Characterization of the Bifunctional Aminoglycoside-Modifying Enzyme ANT(3″)-Ii/AAC(6′)-IId from *Serratia marcescens*

Choonkeun Kim, Dusan Hesek, Jaroslav Zajíček, Sergei B. Vakulenko, and Shahriar Mobashery*

Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

Received April 14, 2006; Revised Manuscript Received May 15, 2006

ABSTRACT: A newly discovered bifunctional antibiotic resistance enzyme from *Serratia marcescens* catalyzes adenylation and acetylation of aminoglycoside antibiotics. The structure assignment of the enzymic products indicated that acetylation takes place on the 6′-amine of kanamycin A and the adenylation on 3″- and 9-hydroxyl groups of streptomycin and spectinomycin, respectively. The adenyltransferase domain appears to be highly specific to spectinomycin and streptomycin, while the acetyltransferase domain shows a broad substrate profile. Initial velocity patterns indicate that both domains follow a sequential kinetic mechanism. The use of dead-end and product inhibition, the solvent isotope effect, and the solvent viscosity effect reveals that the adenyltransferase domain catalyzes the reaction by a Theorell−Chance kinetic mechanism, where ATP binds to the enzyme prior to the aminoglycoside and the modified antibiotic is the last product to be released. The acetyltransferase domain follows an ordered bi-bi kinetic mechanism, in which the antibiotic is the first substrate that binds to the active site and CoASH is released prior to the modified aminoglycoside. The merging of two genes to create bifunctional resistance enzymes with expanded profiles has now been documented in four instances, including the subject of study in this report, which suggests a new trend in the emergence of resistance to aminoglycoside antibiotics among pathogens.

Polycationic aminoglycoside antibiotics consist of two or more amino sugars linked to a central aminocyclitol ring by glycosidic bonds. These are broad-spectrum antibiotics that are effective in the treatment of infections by both Gram-positive and Gram-negative bacteria. The most common mechanism for resistance to this class of antibiotics is the covalent modification of the drugs by aminoglycoside-modifying enzymes. Three different types of resistance enzymes have been described, termed aminoglycoside phosphotransferases (APHs), aminoglycoside acetyltransferases (AACs), and aminoglycoside nucleotidyltransferases (ANTs). These enzymes at times exhibit a broad range for their substrate preferences (*1*−*3*).

Whereas several dozens of these enzymes have been identified from clinical strains worldwide, a mere handful of bifunctional aminoglycoside-modifying enzymes are known to date. It is important to note that bifunctional/multifunctional enzymes are relatively rare in bacteria, in contrast to the eukaryotic cells. The emergence of the bifunctional antibiotic-resistance enzymes that exhibit two distinct activities presents a unique clinical challenge in that the activities often complement each other in broadening the resistance profile. The first such bifunctional resistance enzyme, AAC(6′)/APH(2″), was discovered in Gram-positive *Enterococcus faecalis* in 1986 (*4*) and *Staphylococcus aureus* in 1987 (*5*). This enzyme, detected in 25−40% of all *S. aureus* strains isolated globally (*6*), has been studied well (*7*−*11*). The organisms that harbor it are resistant to the older aminoglycosides as well as many newer ones such as gentamicin, tobramycin, dibekacin, amikacin, isepamicin, and fortimicin A. Recently, three additional genes encoding bifunctional enzymes, designated ANT(3″)-Ii/AAC(6′)-IId, AAC(3)-Ib/AAC(6′)-Ib′, and AAC(6′)-30/AAC(6′)-Ib′, have been isolated from Gram-negative bacteria *Serratia marcescens* (*12*) and *Pseudomonas aeruginosa* (*13*, *14*). Sequence analysis indicates that the *ant(3″)-Ii/aac(6′)-IId* gene encodes an enzyme that should possess both an N-terminal aminoglycoside nucleotidyltransferase domain and a C-terminal aminoglycoside acetyltransferase domain. According to the resistance profile (*12*), the product of the *aac* portion of this gene confers resistance to kanamycin, tobramycin, 5′-episisomicin, gentamicin, netilmicin, and 2′-*N*-ethylentilmicin, while that for the *ant* confers resistance to spectinomycin and streptomycin. In contrast to the case of the Gram-positive AAC(6′)/APH(2″), the new enzymes, ANT(3″)-Ii/AAC(6′)-IId, AAC(3)-Ib/AAC(6′)-Ib′, and AAC(6′)-30/AAC(6′)-Ib′, have never been purified or studied enzymologically. In light of the clinical significance of the bifunctional resistance enzymes and their insidious broadened resistance profiles, they have the potential to significantly complicate our ability to treat the organisms that harbor them, as is the case with *S. aureus*. A full knowledge of the properties of these enzymes will be important in appreciating the strategies used by nature in countering the use of antibiotics but also will help in devising strategies to circumvent them.

We report herein the cloning, purification to homogeneity, and investigations of the kinetic mechanisms of the two activities of ANT(3″)-Ii/AAC(6′)-IId using initial velocity patterns, dead-end and product inhibitions, solvent kinetic isotope effects, and solvent viscosity effects. We also address the nature of the modification reactions by isolating and characterizing the products of the enzymic reactions.

* To whom correspondence should be addressed. Telephone: (574) 631-2933. Fax: (574) 631-6652. E-mail: mobashery@nd.edu.

10.1021/bi060723g CCC: $33.50
Published on Web 06/20/2006

EXPERIMENTAL PROCEDURES

Construction of the ANT(3″)-Ii/AAC(6′)-IId Expression Plasmid. The pGM172 plasmid containing the fused gene *ant(3″)-Ii/aac(6′)-IId* was a generous gift from Dr. Daniela Centrón (*12*). To insert the gene into the pET22b(+) expression vector at sites of restriction enzymes *Nde*I and *Hin*dIII, it was amplified using PCR with the following primers: ANT-D, 5′-GTGTTAGACCATATGAGTAACGCAGTAC-CC-3′; and ANT-R, 5′-CCTGGAAAGCTTAGGCATCACT-GCGTGTTC-3′ (recognition sequences for *Nde*I and *Hin*dIII endonucleases are underlined, respectively). The PCR product was cleaned with a MiniElute PCR purification kit (QIAGEN Inc., Valencia, CA), digested with *Nde*I and *Hin*dIII, and ligated into the corresponding sites of the pET22b(+) vector. Ligated DNA was used to transform the *Escherichia coli* BL21(DE3) strain. The recombinant plasmids were isolated from several transformants, and DNA sequencing was performed by the CMMG Macromolecular Core Facility (Wayne State University, Detroit, MI) to verify the nucleotide sequences of the *ant(3″)-Ii/aac(6′)-IId* gene. The recombinant plasmid was named pET-ANTAAC.

Purification of ANT(3″)-Ii/AAC(6′)-IId. A 4 mL overnight culture of *E. coli* BL21(DE3) harboring the pET-ANTAAC plasmid was inoculated into 400 mL of Terrific Broth containing 100 μg/mL ampicillin and incubated at 37 °C with shaking at 140 rpm. Protein expression was induced with 0.4 mM IPTG when the OD_{600} of the culture reached 0.8, and bacteria were grown at either 15 or 25 °C (discussed in Results and Discussion) for an additional 18 h. Cells were harvested by centrifugation at 5500*g* for 20 min, washed with 50 mL of buffer A [25 mM HEPES (pH 7.5), 1 mM EDTA, and 0.2 mM DTT], and resuspended in 30 mL of buffer A. Cells were disrupted via sonication for 15 min using a Branson Sonifier 450 (VWR, West Chester, PA). After centrifugation at 21000*g* for 30 min, the supernatant was transferred to a fresh tube and nucleic acids were precipitated by centrifugation at 21000*g* for 1 h after the incubation with 1.5% streptomycin sulfate for 30 min. The pellet was discarded, and the supernatant was dialyzed against 4 L of 10 mM Tris (pH 7.0) at 4 °C overnight and loaded onto a DEAE anion-exchange column (2.5 cm × 20 cm; Bio-Rad, Hercules, CA). The column was washed at a flow rate of 4 mL/min with 400 mL of 10 mM Tris (pH 7.0), and the proteins were eluted with a linear gradient from 0 to 1.0 M NaCl (900 mL). Fractions were analyzed by SDS−PAGE, and those containing proteins of approximately 51 kDa were pooled and concentrated using an Amicon ultrafiltration system and dialyzed against 2 L of buffer A. The protein solution was loaded at a flow rate of 1.5 mL/min onto a gentamicin affinity column [2.5 cm × 10 cm; 50 mL of Affi-Gel 15 resin (Bio-Rad)], prepared according to published procedures (*15*). The column was washed with 200 mL of buffer A, followed by a linear gradient from 0 to 1.0 M NaCl in buffer A (600 mL). The desired protein was finally eluted with buffer A containing 1.5 M NaCl. Homogeneity of the purified protein was confirmed by SDS−polyacrylamide gel electrophoresis.

Enzymic Synthesis and Purification of the Adenylated Streptomycin. The product of the enzymic modification of streptomycin (**3**) was prepared by incubating the antibiotic with the purified enzyme. The reaction mixture consisted of 50 mM HEPES (pH 7.5), 1 mM dithiothreitol, 15 mM $MgCl_2$, 1.5 mM streptomycin, 3 mM ATP, 0.5 mg of ANT-(3″)-Ii/AAC(6′)-IId, and 250 units of inorganic pyrophosphatase in a total volume of 200 mL (*16*−*19*). The reaction proceeded at room temperature for 8 h with gentle agitation, and the extent of antibiotic modification was monitored by thin-layer chromatography (TLC) using a *n*-butanol/acetic acid/water/*p*-toluenesulfonic acid (3:1:1:0.7) mixture (*20*). Additional amounts of the enzyme (0.5 mg) were added to the reaction mixture every 2 h. Upon complete turnover of streptomycin, the mixture was filtered through the Amicon ultrafiltration system [molecular weight cutoff (MWCO) of 5000] to remove the enzymes, and the filtrate was concentrated under vacuum. The residue was then suspended in 100 mL of methanol and centrifuged at 4000*g* for 15 min. The pellet was subsequently dissolved in 3 mL of water. The product was separated from ATP on a TLC plate using a methanol/water (1:1) mixture and then eluted from the silica gel with the methanol/ethanol/acetic acid/water (5:5:4.5:4.5) mixture. The eluent was concentrated by rotary evaporation and dissolved in 2 mL of water. The solution was applied to an AG 1-X8 strong anion-exchange column (OH^- form, 1.5 cm × 12 cm, Bio-Rad), which had been pre-equilibrated with 1% NH_4OH. The column was washed with 5 bed volumes of distilled water, and the product was eluted using a stepwise gradient (0.005, 0.01, 0.02, 0.04, 0.08, and 0.2%) of an aqueous HCl solution. Fractions were analyzed by the absorbance at 254 nm on a TLC plate, and those containing modified streptomycin were pooled and lyophilized.

Enzymic Synthesis and Purification of the Adenylated Spectinomycin. Adenylation of spectinomycin (**2**) was performed by the procedure described above, replacing streptomycin (**3**) with spectinomycin. The product isolated from a preparative TLC plate was loaded on the Dowex 50WX8-200 strong cation-exchange column (H^+ form, 1.5 cm × 12 cm, Sigma-Aldrich) (*17*). The column was washed with 100 mL of water, followed by a stepwise gradient (0.06, 0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0%) of a NH_4OH solution.

Enzymic Synthesis and Purification of the Acetylated Kanamycin A. For the acetylation of kanamycin A (**1**) by ANT(3″)-Ii/AAC(6′)-IId, we used the reaction mixture containing 20 mM HEPES (pH 7.5), 3 mM acetyl-CoA, 2 mM kanamycin A, and 0.3 mg of ANT(3″)-Ii/AAC(6′)-IId in a total volume of 50 mL. The mixture was incubated at room temperature for 10 h with stirring. Additional portions of the enzyme (0.1 mg) were added into the mixture every 2 h. The reaction was monitored on a TLC plate using the 5:5:4.5:4.5 ethanol/methanol/ammonia/water mixture as the solvent system and *p*-anisaldehyde as the visualizing spray (*7*). The enzyme was separated from the reaction mixture by the Amicon ultrafiltration system (MWCO of 5000), and the filtrate was concentrated by rotary evaporation. The residue was suspended in 5 mL of water and loaded on silica gel packed into a 30 mL fritted glass filter (3 cm × 4 cm). The resin was washed with 100 mL of a methanol/water (1:1) mixture to remove acetyl-CoA and CoASH (coenzyme A). The product of the enzymic reaction was eluted from the resin with 30 mL of the methanol/water/ammonia (1:1:0.5) solution. The eluent was then concentrated and applied to an Amberlite CG-50 weak cation-exchange column (NH_4^+ form, 1.5 cm × 12 cm, Sigma-Aldrich). The column was washed with 150 mL of water, and a stepwise gradient (0.06,

0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0%) of a NH_4OH solution was utilized to elute acetylated kanamycin. The desired product was eluted at 0.5% NH_4OH, concentrated, and lyophilized.

NMR Analyses of Modified Aminoglycosides. The lyophilized samples were dissolved in D_2O. All ^1H, ^{13}C, and ^{31}P NMR experiments were performed at an ambient temperature of 21 °C using Varian Unity*Plus* and Inova spectrometers operating at ^1H resonance frequencies of 599.89 and 499.87 MHz, respectively. Various one (1D)- and two-dimensional (2D) homo- and heteronuclear NMR techniques, $^{13}C[^1H]$, $^{31}P[^1H]$, APT (attached proton test), $^1H-^1H$ COSY, $^1H-^1H$ TOCSY, $^1H-^1H$ ROESY, $^{13}C-^1H$ HETCOR, $^1H-^{13}C$ gHMQC, $^1H-^{13}C$ gHMQCTOCSY, and $^1H-^{13}C$ gHMBC, were employed to elucidate the structures of the compounds. Standard pulse sequences (*21*−*26*) were used in these experiments.

Time domain data (t_2 and t_1) for all ^1H-detected two-dimensional experiments were recorded as 2048 × 512 complex matrices with 16−80 scans per t_1 increment depending on the sample concentration. The relaxation delay between individual scans was 1.2 s. However, ROESY experiments (mixing time of 300 ms) were performed with the relaxation delay of 4 s. In homonuclear 2D experiments, zero filling was used in both t_2 and t_1 domains to obtain the final 4K × 2K complex time domain data. In heteronuclear 2D experiments, linear prediction to the 1024 complex data points was applied in the t_1 domain, which were zero filled to 4096 to obtain the final 2K × 4K complex time domain data. Shifted Gaussian weighting functions were applied in both domains prior to double Fourier transformation.

In the HETCOR experiments, 256 t_1 increments of 120−400 scans each (depending on the sample concentration) were sampled in 4096 complex data points and with a relaxation delay of 1.4 s. Linear prediction to the 512 and zero filling to 2048 complex data points were employed in the t_1 domain. Sine-bell weighting functions were applied in both domains to the final 4K × 2K complex time domain data matrices prior to double Fourier transformation. All spectra were processed using the Varian VNMR 6.1C software.

^1H spectra and the ^1H dimension in 2D heteronuclear spectra were referenced relative to the signal of d_4-TMSP (internal standard, $\delta = 0$ ppm). ^{13}C spectra and the ^{13}C dimension in the 2D heteronuclear spectra were referenced indirectly (*27*); 85% phosphoric acid was used (external standard, $\delta = 0$ ppm) for referencing $^{31}P[^1H]$ spectra.

6′-*N*-Acetylkanamycin A (**5**): ^1H NMR (600 MHz, D_2O) δ 5.24 (1H, H-1′), 5.05 (1H, H-1″), 3.93 (1H, H-5″), 3.83 (1H, H-5′), 3.79 (1H, H-6″), 3.69 (1H, H-3′), 3.66 (1H, H-5), 3.60 (1H, H-2′), 3.63−3.38 (2H, H-6′), 3.54 (1H, H-2″), 3.37 (1H, H-4″), 3.31 (2H, H-4, H-6), 3.28 (1H, H-4′), 3.06 (1H, H-3″), 2.98 (1H, H-3), 2.87 (1H, H-1), 2.02−1.27 (2H, H-2), 2.00 (3H, H-8′); ^{13}C NMR (151 MHz, D_2O) δ 174.3 (s, C-7′), 100.3 (d, C-1′), 99.9 (d, C-1″), 88.0 (d, C-6), 86.9 (d, C-4), 74.1 (d, C-5), 72.6 (d, C-3′), 72.0 (d, C-5″), 71.7 (d, C-2′), 71.3 (d, C-2″), 71.1 (d, C-5′), 70.9 (d, C-4′), 68.7 (d, C-4″), 60.1 (d, C-6″), 54.2 (d, C-3″), 50.3 (d, C-3), 49.1 (d, C-1), 40.0 (t, C-6′), 34.6 (t, C-2), 21.8 (q, C-8′); MS-FAB$^+$ 527 (M + H).

9-*O*-(Adenosine-5′-phosphoryl)spectinomycin (**6**): ^1H NMR (600 MHz, D_2O) δ 1.02 (d, $J = 4.0$ Hz, 3H, CH_3), 1.99 (m, 1H, H-3), 2.50 (s, 3H, NCH_3), 2.52 (s, 3H, NCH_3), 2.93 (m, 1H, H-8), 3.02 (s, 1H, H-6), 3.56 (m, 1H, H-2), 4.00−4.60 (m, 9H, H-5a, H-7, H-9, H-9a, H-10a, H-2′, H-3′, H-4′, H-5′), 6.03 (d, 1H, H-1′), 8.11 (s, 1H, H-2″), 8.37 (s, 1H, H-8″); ^{13}C NMR (151 MHz, D_2O) δ 155.2 (s, C-6″), 152.6 (d, C-2″), 148.5 (s, C-4″), 139.6 (d, C-8″), 118.3 (s, C-5″), 93.3 (s, C-4), 93.2 (d, C-10a), 91.4 (s, C-4a), 87.2 (d, C-1′), 83.5 (d, C-4′), 74.3 (d, C-2′), 73.0 (d, C-9), 70.2 (d, C-3′), 69.8 (d, C-9a), 67.9 (d, C-2), 66.6 (d, C-5a), 65.2 (t, C-5′), 62.2 (d, C-8), 61.2 (d, C-7), 58.9 (d, C-6), 40.8 (t, C-3), 32.0 (q, NCH_3), 30.6 (q, NCH_3), 19.4 (q, CH_3); ^{31}P NMR (in D_2O, reference H_3PO_4) δ 0.445; MS-FAB$^+$ 680 [M (hydrated) + H].

3″-*O*-(Adenosine-5′-phosphoryl)streptomycin (**7**): ^1H NMR (600 MHz, D_2O) δ 1.15 (s, 1H, H-5′$_{Strp}$), 2.78 (s, 1H, H-7″$_{Glc}$), 3.31 (m, 1H, H-2″$_{Glc}$), 3.33 (m, 1H, H-6$_{Strn}$), 3.38 (m, 1H, H-5″$_{Glc}$), 3.39 (m, 2H, H-1$_{Strn}$), 3.40 (m, 2H, H-8$_{Strn}$), 3.42 (m, 2H, H$_{Strn}$), 3.45 (m, 1H, H-2$_{Strn}$), 3.45 (m, 2H, H-5$_{Strn}$), 3.51 (m, 1H, H-4$_{Strn}$), 3.53 (m, 1H, H-3$_{Strn}$), 3.54 (m, 1H, H-7$_{Strn}$), 3.59 (m, 1H, H-4″$_{Glc}$), 3.61−3.75 (m, 1H, H-6″$_{Glc}$), 4.18 (m, 1H, H-5′$_{Amp}$), 4.31 (m, 1H, H-3″$_{Glc}$), 4.33 (m, 1H, H-2′$_{Strp}$), 4.34 (m, 1H, H-4′$_{Amp}$), 4.35 (m, 1H, H-4′$_{Strp}$), 4.42 (m, 1H, H-3′$_{Amp}$), 4.71 (m, 1H, H-2′$_{Amp}$), 4.97 (s, 1H, H-6′$_{Strp}$), 5.13 (s, 1H, H-1′$_{Strp}$), 5.53 (s, 1H, H-1″$_{Glc}$), 6.01 (s, 1H, H-1′$_{Amp}$), 7.98 (s, 1H, H-2″$_{Amp}$), 8.21 (s, 1H, H-8″$_{Amp}$); ^{13}C NMR (151 MHz, D_2O) δ 158.2 (s, C-8$_{Strn}$), 157.5 (s, C-7$_{Strn}$), 155.4 (s, C-6″$_{Amp}$), 152.9 (d, C-2″$_{Amp}$), 148.9 (s, C-4″$_{Amp}$), 139.9 (d, C-8″$_{Amp}$), 118.6 (s, C-5″$_{Amp}$), 105.7 (d, C-1′$_{Strp}$), 93.8 (d, C-1″$_{Glc}$), 89.1 (d, C-6′$_{Strp}$), 87.3 (d, C-1′$_{Amp}$), 83.6 (d, C-2′$_{Strp}$), 83.3 (d, C-4′$_{Amp}$), 82.2 (s, C-3′$_{Strp}$), 77.7 (d, C-4′$_{Strp}$), 77.0 (d, C-4$_{Strn}$), 74.7 (d, C$_{Strn}$), 74.4 (d, C-3″$_{Glc}$), 73.7 (d, C-2′$_{Amp}$), 73.1 (d, C-5$_{Strn}$), 72.3 (d, C-4″$_{Glc}$), 71.5 (d, C$_{Strn}$), 71.4 (d, C$_{Strn}$), 70.8 (d, C-6$_{Strn}$), 70.7 (d, C-2$_{Strn}$), 70.8 (d, C$_{Strn}$), 70.0 (d, C-3′$_{Amp}$), 68.3 (d, C-5″$_{Glc}$), 65.8 (t, C-5′$_{Amp}$), 61.6 (d, C-2″$_{Glc}$), 60.3 (t, C-6″$_{Glc}$), 59.0 (2d, C$_{Strn}$), 58.7 (d, C-1$_{Strn}$), 58.2 (d, C-3$_{Strn}$), 32.7 (q, C-7″$_{Glc}$), 12.3 (q, C-5′$_{Strp}$); MS-FAB$^+$ 956 (M + 2Na$^+$).

Kinetic Assays of ANT(3″)-Ii/AAC(6′)-IId. The catalytic activity of aminoglycoside nucleotidyltransferase was assayed by monitoring the accumulation of NADPH at 340 nm with a Cary 50 spectrophotometer (Varian Inc., Palo Alto, CA). The ANT(3″)-Ii activity was measured by coupling the enzymatic reaction to the reactions of UDP-glucose pyrophosphorylase, phosphoglucomutase, and glucose-6-phosphate dehydrogenase (*18*, *19*, *28*). The reaction mixtures contained 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.2 mM UDP-glucose, 0.2 mM glucose 1,6-bisphosphate, 0.2 mM NADP, 0.2 mM dithiothreitol, 2 units/mL UDP-glucose pyrophosphorylase, 20 units/mL phosphoglucomutase, 20 units/mL glucose-6-phosphate dehydrogenase, 50 nM ANT-(3″)-Ii/AAC(6′)-IId, and variable concentrations of ATP (10−800 μM) and aminoglycoside (0.5−100 μM) in a total volume of 0.5 mL. Reactions were initiated by the addition of the enzyme.

The activity of AAC(6′)-IId was measured spectrophotometrically by coupling the production of the free sulfhydryl group of CoASH, generated by the AAC(6′)-IId activity, to the chemical reaction with 4,4′-dithiodipyridine (*29*−*31*). The reaction was monitored continuously at 324 nm ($\Delta\epsilon$ = 19 800 M^{-1} cm^{-1} for free pyridine-4-thiolate). Assay mixtures contained 50 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM 4,4′-dithiodipyridine, 50 nM enzyme, and variable concentrations of aminoglycoside (1−100 μM) and acetyl-

CoA (10−200 μM) in a total volume of 0.5 mL.

Initial velocity patterns for the enzyme were obtained at variable concentrations of streptomycin (from 1 to 20 μM) with three different concentrations of ATP (20, 50, and 150 μM) for ANT(3″)-Ii and at variable concentrations of kanamycin A (from 2 to 20 μM) with several fixed concentrations of acetyl-CoA (15, 50, and 100 μM) for AAC(6′)-IId.

Inhibition Studies. ANT(3″)-Ii nucleotidyltransferase activity was measured in the presence of the dead-end inhibitors α,β-methyleneadenosine 5′-triphosphate (AMP-CPP) and kanamycin A or product **6** as an inhibitor. Inorganic pyrophosphate, the other product of the enzymic catalysis, could not be used for the inhibition studies because it was a component of the coupled reaction. The experiments were performed by varying the concentrations of one substrate at several different fixed concentrations of the inhibitor, while the other substrate was kept at a constant concentration. The inhibition patterns of AAC(6′)-IId acetyltransferase activity were also obtained with the dead-end inhibitors butyryl-CoA and paromomycin (**4**) or the product inhibitors CoASH and 6′-*N*-acetylated kanamycin A (**5**). For the product inhibition by CoASH, enzymic activity was measured by monitoring the decrease in absorbance at 232 nm ($\Delta\epsilon = 4500\ M^{-1}\ cm^{-1}$) due to breakage of the thioester bond of acetyl-CoA (*29*).

Solvent Isotope Effects. The solvent isotope effects on k_{cat} of ANT(3″)-Ii and AAC(6′)-IId were determined at varying concentrations of aminoglycosides and saturating concentrations of ATP (200 μM) and acetyl-CoA (100 μM), respectively. The reaction mixtures contained approximately 95% deuterium oxide. The pD value was measured with a pH meter by adding 0.4 unit (pD = pH + 0.4) (*32*, *33*).

Solvent Viscosity Effect. The effect of solvent viscosity was determined by adding a viscosogen to the assay mixtures. The reaction mixture for the analysis of the ANT(3″)-Ii domain contained 50 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 0.4 mM UDP-glucose, 0.4 mM glucose 1,6-bisphosphate, 0.4 mM NADP, 0.2 mM dithiothreitol, 10 units/mL UDP-glucose pyrophosphorylase, 80 units/mL phosphoglucomutase, 80 units/mL glucose-6-phosphate dehydrogenase, 100 nM ANT(3″)-Ii/AAC(6′)-IId, and variable concentrations of both ATP (20−400 μM) and streptomycin (2−40 μM). The assay mixture for the solvent viscosity effect of the AAC(6′)-IId domain was the same as the standard assay mixture. The relative viscosity of the glycerol solution was estimated by linear interpolation of published data from the *CRC Handbook of Chemistry and Physics* (*34*) as follows (w/v%, η_{rel}): 8, 1.2; 16, 1.5; 24, 2.0; and 32, 2.6. The effect of solvent viscosity of macromolecules such as enzymes was determined with 6.7% PEG 8000 ($\eta_{rel} = 3.6$), which does not influence diffusion of small molecules (*15*, *35*).

Data Analysis. Data for steady-state kinetics, in which the concentration of one substrate was varied and the other substrate was used at a fixed and saturating concentration, were fitted to eq 1 using Grafit 4.0 (Erithacus Software, Staines, U.K.). Equation 2 was used to fit the initial velocity patterns. All inhibition data were fitted to eqs 3−5 for competitive, uncompetitive, and noncompetitive/mixed inhibition, respectively (*36*).

$$v = V_m[A]/(K_a + [A]) \quad (1)$$

$$v = V_m[A][B]/(K_a[B] + K_b[A] + [A][B] + K_{ia}K_b) \quad (2)$$

$$v = V_m[A]/[[A] + K_a(1 + [I]/K_{is})] \quad (3)$$

$$v = V_m[A]/[K_a + [A]/(1 + [I]/K_{ii})] \quad (4)$$

$$v = V_m[A]/[K_a(1 + [I]/K_{is}) + [A](1 + [I]/K_{ii})] \quad (5)$$

In all equations, v is the initial velocity, V_m is the maximum velocity, [A] and [B] are the concentrations of substrates, [I] is the concentration of the inhibitor, K_a and K_b are the corresponding Michaelis−Menten constants, K_{ia} is the dissociation constant for A, and K_{is} and K_{ii} are the slope and intercept inhibition constants for inhibitors, respectively, and are evaluated by replotting the slope and the intercept from double-reciprocal plots, respectively, versus the concentration of inhibitor.

RESULTS AND DISCUSSION

The *ant(3″)-Ii/aac(6′)-IId* gene encoding the bifunctional enzyme was amplified by PCR from plasmid pGM172, and its nucleotide sequence was verified (three individual experiments). Comparison of the DNA sequence from our experiments with the sequence of the integron of *S. marcescens* harboring the *ant(3″)-Ii/aac(6′)-IId* gene (GenBank accession number AF453998) (*12*) revealed three differences: A345 → G (a silent mutation), G701 → A (Arg → Gln), and A1304 → G (Lys → Arg). To confirm the nucleotide sequence, we made a mutant gene possessing reverse mutations (both A701 → G and G1304 → A) and determined the MIC (minimum inhibitory concentration) values for *E. coli* BL21(DE3) strains harboring either the parental (containing nucleotides A701 and G1304) or the mutant gene. The MIC value of the strain carrying the mutant gene was 2 times lower than that of the strain harboring the parental gene. Since independent sequencing of each PCR product and the MIC determination were performed three times in our lab, we felt that it was likely that the original sequence report contained inadvertent errors. This was documented to be the case in a comparison of our gene sequence with those of both the gene and the protein sequences for other ANT or AAC enzymes from GenBank, which contain the very same three differences that we detected in the gene from our sequencing effort.

We prepared the pET-ANTAAC plasmid for overexpression of ANT(3″)-Ii/AAC(6′)-IId, as described in Experimental Procedures. Induction by IPTG resulted in high levels of expression of ANT(3″)-Ii/AAC(6′)-IId in *E. coli* BL21(DE3) cells. When bacteria harboring pET-ANTAAC were grown at 25 °C after induction, 90% of the expressed ANT(3″)-Ii/AAC(6′)-IId precipitated as inclusion bodies. By growing the microorganism at 15 °C, we were able to recover 50% of the soluble ANT(3″)-Ii/AAC(6′)-IId. Homogeneous enzyme was obtained by a two-step purification procedure (10 mg from 400 mL of cell culture).

The sequence analysis by Centrón and Roy had argued that the enzyme should have both aminoglycoside acetyltransferase (AAC) and aminoglycoside nucleotidyltransferase (ANT) activities. As will be discussed in this report, this prediction is borne out. Regardless, the protein sequence comparison with other known members of these groups of enzymes could suggest the kinds of enzymic reactions;

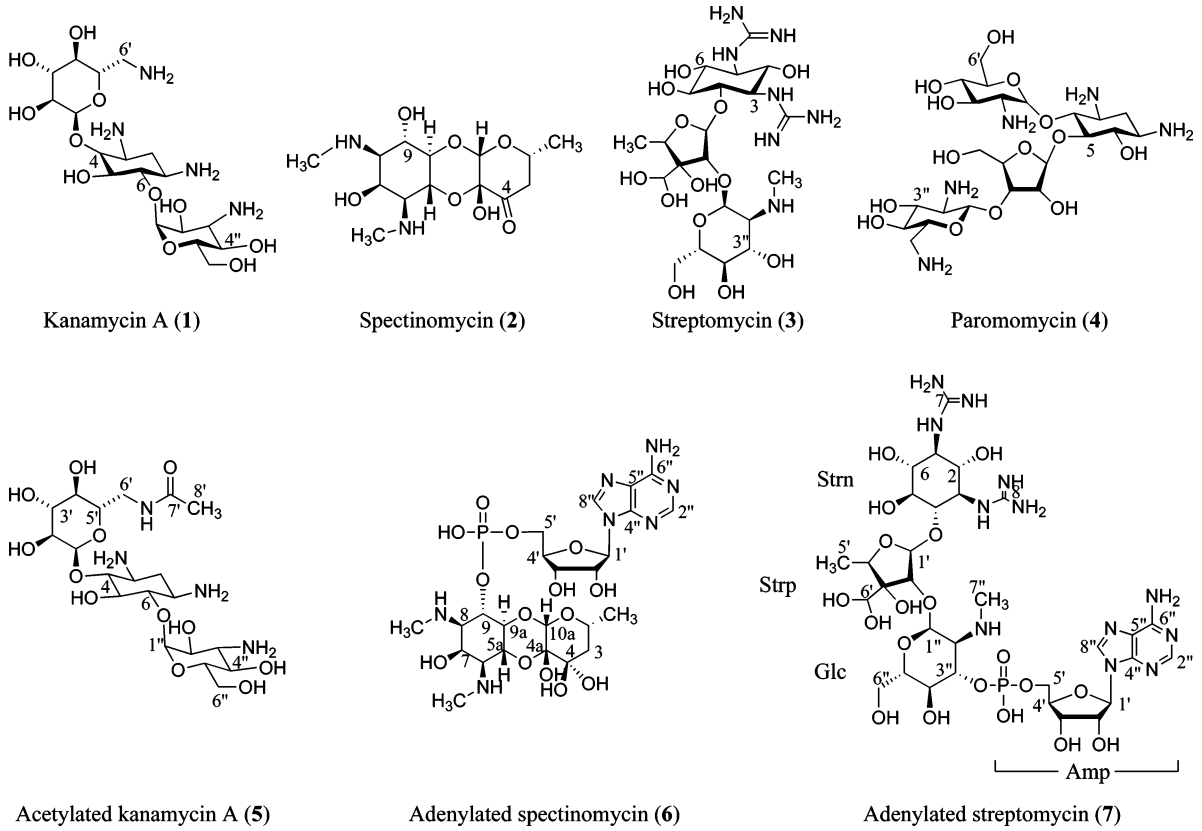

FIGURE 1: Structures of aminoglycoside antibiotics and the products of enzymic reactions.

however, such analyses should be followed by the actual determination of the structures of the products. We undertook such an effort with this bifunctional enzyme.

We were alerted to the antibiotics that were turned over by this bifunctional enzyme by the susceptibility investigations of Centrón and Roy (*12*). Their report indicated that bacteria harboring the gene for the enzyme showed resistance to several aminoglycoside antibiotics, of which we chose kanamycin A, spectinomycin, and streptomycin for our analyses (Figure 1). The three enzymic reactions were allowed to progress to completion, and then the products were purified by chromatographic procedures. The structures of the products were determined by analysis of 1D and 2D ^{1}H and ^{13}C NMR spectra. Proton connectivities were derived by examination of the COSY and TOCSY spectra. Signals of all carbons with direct proton attachments were assigned using HETCOR and gHMQC spectra. Finally, the gHMBC and gHMQC-TOCSY spectra were used to assign quaternary carbons and to check the correctness of the connectivities established by the interpretation of the other spectra. The details of the procedures for structure analyses are given as Supporting Information. The structures of the aminoglycosides modified by our enzyme are given in Figure 1. The AAC activity transfers the acetyl group to the 6′-amine of kanamycin A; hence, it is an AAC(6′), designated AAC(6′)-IId. Streptomycin and spectinomycin are adenylated at the 3″-hydroxyl and 9-hydroxyl, respectively, and the domain is designated ANT(3″)-Ii.

The steady-state kinetic parameters for ANT(3″)-Ii/AAC(6′)-IId-catalyzed reactions are summarized in Tables 1 and 2. The ANT(3″)-Ii domain carried out the adenylation of both spectinomycin and streptomycin with similar k_{cat}/K_m values of $(3.2 \pm 0.4) \times 10^5$ and $(4.8 \pm 0.5) \times 10^5$ M^{-1} s^{-1}, respectively, despite the fact that their chemical structures are quite different (Figure 1). This domain was highly specific for spectinomycin and streptomycin; that is, it did not adenylate kanamycin A, neomycin, gentamicin, or netilmicin. This finding is consistent with disk susceptibility testing reported earlier (*12*). We also discovered that these aminoglycosides competitively inhibit adenylation of spectinomycin and streptomycin. This was a surprising finding. Therefore, kanamycin A, gentamicin, and netilmicin bind both active sites, but they do not experience modification by the ANT(3″)-Ii active site. On the other hand, AAC(6′)-

Table 1: Steady-State Kinetic Parameters for the ANT(3″)-Ii Domain-Catalyzed Reaction of ANT(3″)-Ii/AAC(6′)-IId

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
spectinomcyin	0.5 ± 0.1	1.4 ± 0.2	$(3.2 \pm 0.4) \times 10^5$
streptomycin	0.6 ± 0.1	1.3 ± 0.1	$(4.8 \pm 0.5) \times 10^5$
ATP[a]	0.5 ± 0.1	14.2 ± 2.2	$(3.5 \pm 0.7) \times 10^4$

[a] Kinetic parameters for ATP were determined at the fixed concentration of streptomycin as described in Experimental Procedures.

Table 2: Steady-State Kinetic Parameters for the AAC(6′)-IId Domain-Catalyzed Reaction of ANT(3″)-Ii/AAC(6′)-IId

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})
kanamycin A	0.8 ± 0.1	12 ± 2	$(6.6 \pm 1.5) \times 10^4$
gentamicin[a]	0.5 ± 0.1	15 ± 1	$(3.3 \pm 0.3) \times 10^4$
netilmicin	0.2 ± 0.1	36 ± 4	$(5.6 \pm 0.7) \times 10^3$
amikacin	0.05 ± 0.01	313 ± 9	$(1.6 \pm 0.3) \times 10^2$
acetyl-CoA[b]	0.8 ± 0.1	44 ± 4	$(1.8 \pm 0.3) \times 10^4$

[a] Gentamicin is a mixture of gentamicin C1 (~45%), C1a (~35%), and C2 (~30%). [b] Kinetic parameters for acetyl-CoA were measured with the fixed concentration of kanamycin A (**1**) as described in Experimental Procedures.

IId activity did not catalyze acetylation of neomycin, isepamicin, paromomycin, or spectinomycin. Butyryl-CoA, used for the inhibition studies, was not a substrate for this domain.

Gentamicin and kanamycin A were acetylated favorably by the AAC(6′)-IId domain with k_{cat}/K_m values of (3.3 ± 0.2) × 10^4 and (6.6 ± 1.5) × 10^4 M^{-1} s^{-1}, respectively (Table 2). The range of the kinetic parameters is similar to those of other AAC(6′) enzymes (*7*, *10*, *37*−*39*). This domain catalyzes acetylation of both 4,5-disubstituted 2-deoxystreptamine and 4,6-disubstituted 2-deoxystreptamine aminoglycosides (Table 2). Interestingly, amikacin is a poor substrate with a 200-fold attenuated k_{cat}/K_m value [(1.6 ± 0.3) × 10^2 M^{-1} s^{-1}] compared to that of gentamicin, even though the nucleotide sequence of the *aac(6′)-IId* gene segment is nearly identical to that of other *aac(6′)-I* family members. Contrary to the case of the AAC(6′)-II family members, the AAC(6′)-I family members inactivate gentamicin but not amikacin (*1*−*3*). The chemical structure of amikacin is derived from that of kanamycin A by placing the hydroxybutyramide at N_1. This functionality affects the catalytic process (16-fold decrease in k_{cat} compared to that of kanamycin A) as well as the ability of the enzyme to achieve saturation (27-fold increase in K_m). Spectinomycin is not a substrate for the AAC(6′)-IId domain and does not inhibit the activity of this domain.

We characterized the kinetic mechanism of ANT(3″)-Ii/AAC(6′)-IId. First, initial velocity patterns of catalysis by each domain were examined for preliminary determination of the kinetic mechanism using various concentrations of streptomycin at different fixed concentrations of ATP for the ANT(3″)-Ii activity and variable concentrations of kanamycin A at various fixed levels of acetyl-CoA for the AAC(6′)-IId activity. The double-reciprocal plots exhibited intersecting lines for both reactions, indicative of a sequential kinetic mechanism (Figure 2). Both substrates would be bound to the enzyme before the formation and release of a product, in contrast to the ping-pong mechanism. The sequential kinetic mechanism, random or ordered, appears to be common for all classes of aminoglycoside-modifying enzymes (*18*, *37*, *38*, *40*−*42*).

The detailed kinetic mechanism of an enzymic reaction can be elucidated by determination of the order of substrate binding or product release, as well as the determination of the rate-limiting step in catalysis. Studies with dead-end and product inhibitors are methods for elucidating whether the kinetic mechanism was ordered or random for the enzymic modifications of aminoglycosides by ANT(3″)-Ii/AAC(6′)-IId. To discern between the random or ordered addition of the two substrates, dead-end inhibition studies were carried out (Table 3; all graphs of the inhibition patterns for the studies are available as Supporting Information.) For ANT(3″)-Id activity, substrate analogues kanamycin A and AMP-CPP were used against both substrates spectinomycin and ATP. Kanamycin A exhibited competitive inhibition (K_{is} = 4 ± 3 μM) versus spectinomycin and uncompetitive inhibition (K_{ii} = 10 ± 1 μM) versus ATP. This is compatible with an ordered binding of ATP to the ANT(3″)-Ii domain as a first substrate. The nonhydrolyzable ATP analogue AMP-CPP was a competitive inhibitor (K_{is} = 17 ± 2 μM) of ATP and gave a noncompetitive/mixed inhibition pattern (K_{is} = 21 ± 4 μM and K_{ii} = 31 ± 3 μM) with respect to

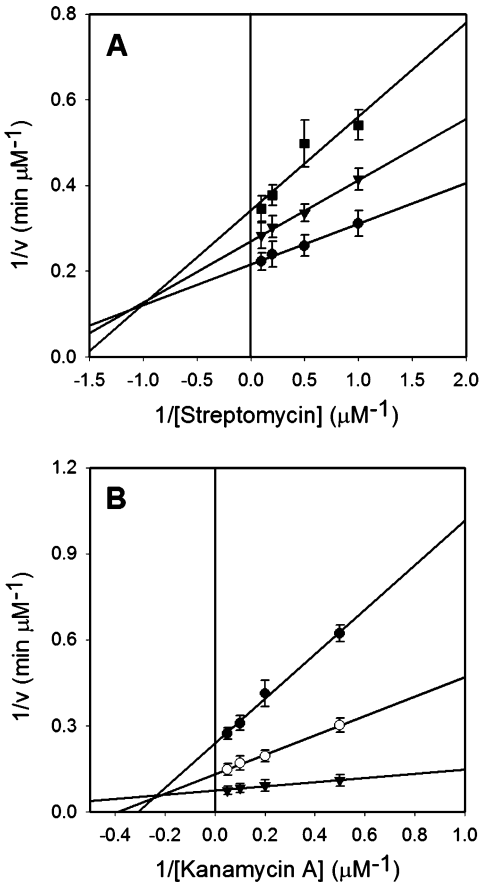

FIGURE 2: Initial velocity patterns for ANT(3″)-Ii/AAC(6′)-IId. (A) The initial velocity pattern for the ANT(3″)-Ii domain-catalyzed reaction was measured at variable concentrations of streptomycin (from 1 to 20 μM) with three different concentrations of ATP: 20 (■), 50 (▼), and 150 μM (●). (B) The initial velocity pattern for the reaction catalyzed by the AAC(6′)-IId domain was obtained at varied concentrations of kanamycin A (from 2 to 20 μM) with several fixed concentrations of acetyl-CoA: 15 (●), 50 (○), and 100 μM (▼). Initial velocity patterns for both domains intersect, indicative of a sequential kinetic mechanism.

spectinomycin. These results also support the possibility that ATP binds first to the active site followed by spectinomycin. Our results are consistent with the case of APH(3′)-IIIa (*41*), ANT(2″)-I (*43*), and ANT(3″) (isolated from *Corynebacterium acetoacidophilum*) (*42*) but are in contrast to ANT(4′) where kanamycin A is the first substrate followed by ATP (*18*). Product inhibition analysis is a useful tool in identifying the order of product release. Inorganic pyrophosphate could not be used as a product inhibitor because the release of this product was coupled to UDP-glucose pyrophosphorylase/phosphoglucomutase/glucose-6-phosphate dehydrogenase for the enzymic assay. Enzymic product **6** was used as a product inhibitor versus both spectinomycin and ATP. A competitive inhibition (K_{is} = 93 ± 40 μM) and a noncompetitive/mixed inhibition (K_{is} = 76 ± 27 μM and K_{ii} = 89 ± 37 μM) versus ATP and spectinomycin, respectively, indicate that inorganic pyrophosphate must leave from the active site prior to the other product **6** (Table 3). The ANT(3″)-Ii domain therefore follows an ordered bi-bi substrate addition and product release, where ATP binds to the enzyme prior to the aminoglycoside and the modified aminoglycoside is the last product to be released. The inhibition pattern of the first product (inorganic pyrophosphate), critical for distinguishing a Theorell−Chance kinetic mechanism from a simple ordered

Table 3: Patterns of Dead-End and Product Inhibitions of ANT(3″)-Ii/AAC(6′)-IId

variable substrate[a]	inhibitor[b]	pattern of inhibition	K_{is} (μM)[c]	K_{ii} (μM)[c]
ANT(3″)-Ii domain				
spectinomycin (**2**)	kanamycin A (**1**)	competitive	4 ± 3	
spectinomycin	AMP-CPP	noncompetitive/mixed	21 ± 4	31 ± 3
ATP	kanamycin A	uncompetitive		10 ± 1
ATP	AMP-CPP	competitive	17 ± 2	
spectinomycin	**6**	noncompetitive/mixed	76 ± 27	89 ± 37
ATP	**6**	competitive	93 ± 40	
AAC(6′)-IId domain				
kanamycin A	paromomycin (**4**)	competitive	60 ± 4	
kanamycin A	butyryl-CoA	uncompetitive		188 ± 37
acetyl-CoA	paromomycin	noncompetitive/mixed	117 ± 7	145 ± 11
acetyl-CoA	butyryl-CoA	competitive	25 ± 4	
kanamycin A	**5**	competitive	40 ± 5	
kanamycin A	CoASH	uncompetitive		69 ± 14
acetyl-CoA	**5**	noncompetitive/mixed	150 ± 66	385 ± 89
acetyl-CoA	CoASH	noncompetitive/mixed	42 ± 3	43 ± 6

[a] These substrates were also used as the fixed substrates with the following concentrations: 20 μM spectinomycin (**2**), 100 μM ATP, 100 μM kanamycin A (**1**), and 200 μM acetyl-CoA. [b] AMP-CPP, α,β-methyleneadenosine 5′-triphosphate; **6**, 9-*O*-(adenosine-5′-phosphoryl)spectinomycin; **5**, 6′-*N*-acetylkanamycin A; CoASH, coenzyme A. [c] K_{is} and K_{ii} were evaluated by replotting the slope and the intercept, respectively, from double-reciprocal plots vs the concentration of inhibitor.

Table 4: Solvent Kinetic Isotope Effects for ANT(3″)-Ii/AAC(6′)-IId

	ANT(3″)-Ii domain[a]				AAC(6′)-IId domain[b]			
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}^H/k_{cat}^D	$(k_{cat}/K_m)^H/(k_{cat}/K_m)^D$	K_m (μM)	k_{cat} (s^{-1})	k_{cat}^H/k_{cat}^D	$(k_{cat}/K_m)^H/(k_{cat}/K_m)^D$
pH 7.5	1.3 ± 0.1	0.6 ± 0.1			11.8 ± 2.3	0.8 ± 0.1		
pD 7.5	1.5 ± 0.2	0.5 ± 0.1	1.2 ± 0.2	1.5 ± 0.3	14.9 ± 1.0	0.3 ± 0.1	3.3 ± 0.3	4.2 ± 1.0
pH 6.5	5.7 ± 0.7	0.4 ± 0.1			15.6 ± 2.1	0.5 ± 0.1		
pD 6.5	6.0 ± 0.9	0.3 ± 0.1	1.3 ± 0.1	1.7 ± 0.3	25.3 ± 3.6	0.1 ± 0.1	4.2 ± 0.4	6.8 ± 1.5

[a] Spectinomycin was used as a variable substrate at a fixed concentration (200 μM) of ATP. [b] Solvent isotope effects were determined at varied concentrations of kanamycin A with a fixed acetyl-CoA concentration (100 μM).

bi-bi mechanism, could not be studied. Despite this lack of ability, an ordered Theorell−Chance mechanism of the activity of the ANT(3″)-Ii domain is supported by both the lack of solvent isotope effect and the maximal solvent viscosity effect on the enzymic catalysis.

Analyses of solvent kinetic isotope effects can be a powerful tool for elucidating if proton transfer step(s) would limit the overall rate of the reaction. The measurement of solvent isotope effects at two pD values is necessary because pK_a values of potential catalytic residues may be perturbed by deuterium oxide (*41*). The solvent isotope effects were determined at pD 7.5 and 6.5 for ANT(3″)-Id/AAC(6′)-IId. The assays were carried out at fixed, saturating concentrations of ATP and acetyl-CoA with variable concentrations of spectinomycin and kanamycin A for ANT(3″)-Ii and AAC-(6′)-IId domains, respectively. Solvent isotope effects on spectinomycin at two different pH (or pD) values were insignificant with k_{cat}^H/k_{cat}^D values of 1.2 ± 0.2 at pD 7.5 and 1.3 ± 0.1 at pD 6.5 (Table 4). The lack of the solvent isotope effect suggests that deprotonation of the hydroxyl group of spectinomycin by the ANT(3″)-Ii domain, a necessity for the chemical step, does not contribute to the overall rate of the reaction. Hence, the diffusional step of product release would be rate-limiting according to the Theorell−Chance kinetic mechanism. According to this mechanism, the immediate release of the first product occurs after the second substrate binding (Figure 3). This observation is further supported by the result of the solvent viscosity effect, described below.

Glycerol and PEG 8000 were used as the microviscosogen and macroviscosogen, respectively, in investigations of

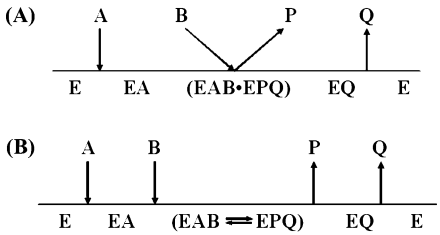

FIGURE 3: Schematic presentations of the proposed kinetic mechanisms of ANT(3″)-Ii/AAC(6′)-IId. (A) The ANT(3″)-Ii domain follows a Theorell−Chance kinetic mechanism: A, ATP; B, aminoglycoside; P, inorganic pyrophosphate; Q, adenylated aminoglycoside. (B) The AAC(6′)-IId domain follows an ordered bi-bi kinetic mechanism: A, aminoglycoside; B, acetyl-CoA; P, CoASH; Q, acetylated aminoglycoside.

viscosity effects on catalysis. The viscosity effect on k_{cat} for both ATP and aminoglycosides with the ANT(3″)-Id domain is close to a theoretical maximal value of 1.0 (*33*, *35*) with 1.33 ± 0.45 for ATP and 1.02 ± 0.23 and 0.96 ± 0.25 for spectinomycin and streptomycin, respectively (Table 5). This large effect on k_{cat} indicates that the physical step, the release of product **6** in this study, in the enzymic reaction of the ANT(3″)-Ii domain should be only rate-limiting. Since the reaction step contributing to the k_{cat}/K_m of the second substrate is the release of inorganic pyrophosphate in a Theorell−Chance kinetic mechanism, the viscosity effect on k_{cat}/K_m of the aminoglycoside substrates would be expected to be small if the release of the pyrophosphate is much more rapid than that of the adenylated aminoglycoside. Solvent viscosity effects on k_{cat}/K_m for aminoglycosides are relatively small (0.31 ± 0.04 for spectinomycin and 0.26 ± 0.06 for

Table 5: Solvent Viscosity Effects for ANT(3″)-Ii/AAC(6′)-IId

viscosogen	variable substrate	fixed substrate[a]	$k_{cat}°/k_{cat}^{\eta}$ [b]	$(k_{cat}/K_m)°/(k_{cat}/K_m)^{\eta}$ [b]
ANT(3″)-Ii domain				
glycerol	spectinomycin	ATP	1.02 ± 0.23	0.31 ± 0.04
glycerol	streptomycin	ATP	0.96 ± 0.25	0.26 ± 0.06
glycerol	ATP	streptomycin	1.33 ± 0.45	0.92 ± 0.21
PEG 8000	streptomycin	ATP	0.02 ± 0.01	0.05 ± 0.06
PEG 8000	ATP	streptomycin	0.01 ± 0.01	−0.06 ± 0.04
AAC(6′)-IId domain				
glycerol	kanamycin A	acetyl-CoA	0.46 ± 0.11	0.73 ± 0.16
glycerol	acetyl-CoA	kanamycin A	0.35 ± 0.09	0.33 ± 0.13
PEG 8000	kanamycin A	acetyl-CoA	−0.01 ± 0.02	0.02 ± 0.03
PEG 8000	acetyl-CoA	kanamycin A	0.03 ± 0.02	0.06 ± 0.03

[a] The fixed concentrations of substrates were as follows: 400 μM ATP, 100 μM streptomycin, 100 μM acetyl-CoA, and 100 μM kanamycin A. [b] $k_{cat}°$ and $(k_{cat}/K_m)°$ are the rate constants without viscosogen. The values of $(k_{cat}°/k_{cat})^{\eta}$ and $[(k_{cat}/K_m)°/(k_{cat}/K_m)]^{\eta}$ are the slopes of plots of $k_{cat}°/k_{cat}$ and $(k_{cat}/K_m)°/(k_{cat}/K_m)$ vs the relative viscosity of the solution. The value of 1 indicates the maximal viscosity effect.

streptomycin) in comparison with that for ATP (0.9 ± 0.2), indicative of the insensitivity of the second substrate binding to the solvent viscosity and rapid dissociation of the first product (inorganic pyrophosphate) as the characteristics in the Theorell−Chance mechanism (*33*). These findings are consistent with the result of dead-end inhibition studies that the aminoglycoside would be the second substrate binding to the domain. This mechanism is consistent with that of APH(3′)-IIIa (*33*, *41*) and the other ANTs studied to date (*18*, *42*, *43*).

The kinetic mechanism of the AAC(6′)-IId domain was elucidated in the same manner as that for the ANT(3″)-Ii domain. Paromomycin was chosen as a dead-end inhibitor due to the lack of a 6′-amino group. Paromomycin provided competitive (K_{is} = 60 ± 4 μM) and noncompetitive/mixed inhibitions (K_{is} = 117 ± 7 μM and K_{ii} = 145 ± 11 μM) versus kanamycin A and acetyl-CoA, respectively. An uncompetitive inhibition pattern (K_{ii} = 188 ± 37 μM) was observed with butyryl-CoA against kanamycin A. As mentioned above, uncompetitive inhibition and noncompetitive/mixed inhibition with respect to kanamycin A and acetyl-CoA, respectively, suggest that kanamycin A is the first substrate binding to the enzyme followed by acetyl-CoA. This is supported by the CoASH inhibition pattern versus kanamycin A in which only the intercept inhibition constant was obtained, that is, uncompetitive inhibition as described below. Product inhibition studies were performed with two products, 6′-*N*-acetylated kanamycin A (**5**) and CoASH. Competitive inhibition (K_{is} = 40 ± 5 μM) versus kanamycin A and noncompetitive/mixed inhibition (K_{is} = 150 ± 66 μM and K_{ii} = 385 ± 89 μM) versus acetyl-CoA were observed with **5**, and CoASH inhibited the enzymic activity uncompetitively (K_{ii} = 69 ± 14 μM) and noncompetitively (K_{is} = 42 ± 3 μM and K_{ii} = 43 ± 6 μM) with respect to kanamycin A and acetyl-CoA, respectively. These data indicate that **5** would be released last from the active site. The orders of substrate binding and product release on the AAC(6′)-IId domain are consistent with those of histone acetyltransferase (*44*) but opposite to those of AAC(6′)-Ii from *E. faecium*, which exhibits an ordered sequential mechanism where acetyl-CoA is the first substrate and CoASH is the last product to be released (*38*). Furthermore, uncompetitive inhibition of CoASH (the first product) with respect to kanamycin A (the first substrate) at the saturating concentration of acetyl-CoA indicates that the catalytic reaction of the AAC(6′)-Ii domain follows a simple ordered bi-bi kinetic mechanism (Figure 3B). This proposed mechanism is supported by the results of both the solvent isotope effect analyses and the solvent viscosity effect experiments on the AAC(6′)-IId domain.

In contrast to the solvent isotope effects on ANT(3″)-Ii, the effects on the AAC(6′)-IId domain were relatively substantial with k_{cat}^{H}/k_{cat}^{D} values of 3.3 ± 0.3 at pD 7.5 and 4.2 ± 0.4 at pD 6.5. Theses results indicate that proton transfer might be partially rate-limiting in the AAC(6′)-IId-catalyzed reaction and are reasonably close to the solvent isotope effect of 10 for AAC(3)-I, which follows an ordered bi-bi kinetic mechanism (*38*). Small solvent viscosity effects on k_{cat} for both acetyl-CoA (0.35 ± 0.09) and aminoglycoside (0.46 ± 0.11) are supportive and also imply that substrate binding or product release would partially limit catalysis. Therefore, the AAC(6′)-IId domain catalyzes the reaction through an ordered bi-bi kinetic mechanism (Figure 3B), where kanamycin A binds to the active site prior to acetyl-CoA and the modified aminoglycoside is the last product.

Concluding Remarks. Resistance to antibiotics is widespread. D'Costa et al. (*45*) recently described what they called the soil resistome, that is, the resistance determinants present in the soil. Every strain in the resistome appears to be resistant to seven or eight antibiotics generally. Although resistance elements of the resistome might not directly be related to those of pathogenic bacteria, understanding of the soil resistome can provide information about frequencies and unidentified mechanisms of antibiotic resistance that might become clinically problematic. Six antibiotics of 11 appeared to be modified by the isolates. These observations argue for the prevalence of resistance genes in nature, which contribute to a gene pool that at times is shared by pathogenic bacteria.

Over the past 60 years, the advent of antibacterial chemotherapy and the subsequent use of antibiotics in animal husbandry and agriculture have placed a strong selection pressure on the emergence, maintenance, and dissemination of antibiotic resistance genes. Various mechanisms of resistance to all known antibiotics classes now exist (*46*−*51*). The facility by which resistance to new types of antibiotic emerges is disconcerting, and this is not limited merely to natural product antibiotics. As an example, resistance to the oxazolidinone class of antibiotics (e.g., linezolid), man-made molecules, emerged within 1 month of the introduction of the first member to clinical use (*52*).

An important trend in the resistance to antibiotics is broadening of the existing phenotypic spectra. Literally

hundreds of β-lactam antibiotics have been developed over the past few decades. The exposure of pathogens to the various classes of β-lactams has given rise to resistance to all. β-Lactamases, enzymes that hydrolytically inactivate these antibiotics, have been important in Gram-negative organisms. Of the four classes of β-lactamases, class A enzymes are the most prevalent (*53*−*55*). These enzymes have undergone random mutation and selection in the face of the challenge by the antibiotics. The TEM-1 β-lactamase from *E. coli* has given rise to ∼150 mutant variants,[1] many of which broaden the spectrum for antibiotic resistance by the organisms that harbor them (*55*, *56*).

In Gram-positive bacteria, the trend has been in alteration of penicillin-binding proteins (PBPs). These targets for β-lactams have been altered such that they resist inhibition by the antibiotics (*55*). In an intriguing example, acquisition of a novel PBP, designated PBP 2a, has given rise to the clinical scourge methicillin-resistant *S. aureus* (MRSA) (*49*). PBP 2a of MRSA resists inhibition by all commercially available β-lactam antibiotics.

The emergence of resistance to aminoglycosides has been less frequent that that for β-lactams, in part due to the restrictions on the clinical use of these antibiotics. Regardless, several dozen enzymes resistant to aminoglycosides have been identified (*1*, *3*). The identification of the first bifunctional resistance enzyme, AAC(6′)/APH(2″), in *E. faecalis* (*4*) and in *S. aureus* (*5*) in the mid to late 1980s was a curiosity. This enzyme clearly demonstrated a broadened spectrum of resistance against aminoglycosides. The two activities came together due to the merger of two disparate genes, reconstituting a two-domain protein with two active sites.

The recent discoveries of three additional bifunctional aminoglycoside resistance enzymes, ANT(3″)-Ii/AAC(6′)-IId (the subject of this study), AAC(3)-Ib/AAC(6′)-Ib′, and AAC(6′)-30/AAC(6′)-Ib′, might imply a trend in this direction. We cannot be definitive about the advantage to the organism for selection of these fused genes. The larger size for the resultant gene would exact its own cost on the organism for replication, transcription, and ultimately retention. A pertinent question becomes why the two genes are not kept separate but retained. The answer is not obvious. These genes are often found as a cassette within integrons that would allow easy dissemination of both genes among various organisms, but one wonders if regulation of independent genes might be a player. For example, gene cassettes with multiple antibiotic genes have been identified (*57*, *58*). It is also known that not all genes are transcribed as efficiently. The farther along the genes are in an integron under the control of one promoter, the less efficient they are transcribed (*59*), and this might be the basis for the gene mergers.

The broadening of the substrate profile documented for ANT(3″)-Ii/AAC(6′)-IId is remarkable in light of disparities in the various structural classes of the antibiotics that it takes as a substrate. Whereas this enzyme is not exceptional in its catalytic competence for the two activities as documented herein, it is sufficiently so to confer the antibiotic resistance phenotype. The emergence of this type of resistance enzyme poses a special challenge clinically.

[1] http://www.lahey.org/studies/temtable.asp.

ACKNOWLEDGMENT

We gratefully acknowledge the generous gift of the pGM172 plasmid from Dr. Daniela Centrón.

SUPPORTING INFORMATION AVAILABLE

Graphs of inhibition patterns for ANT(3″)-Ii/AAC(6′)-IId and structural characterizations of the enzymic products. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI060723G