Domain−Domain Interactions in the Aminoglycoside Antibiotic Resistance Enzyme AAC(6′)-APH(2″)†

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ABSTRACT: The most common determinant of aminoglycoside antibiotic resistance in Gram positive bacterial pathogens, such as *Staphylococcus aureus*, is a modifying enzyme, AAC(6′)-APH(2″), capable of acetylating and phosphorylating a wide range of antibiotics. This enzyme is unique in that it is composed of two separable modification domains, and although a number of studies have been conducted on the acetyltransferase and phosphotransferase activities in isolation, little is known about the role and impact of domain interactions on antibiotic resistance. Kinetic analysis and in vivo assessment of a number of N- and C-terminal truncated proteins have demonstrated that the two domains operate independently and do not accentuate one another's resistance activity. However, the two domains are structurally integrated, and mutational analysis has demonstrated that a predicted connecting α-helix is especially critical for maintaining proper structure and function of both activities. AAC(6′)-APH(2″) detoxifies a staggering array of aminoglycosides, where one or both activities make important contributions depending on the antibiotic. Thus, to overcome antibiotic resistance associated with AAC(6′)-APH(2″), aminoglycosides resistant to modification and/or inhibitors against both activities must be employed. Domain−domain interactions in AAC(6′)-APH(2″) offer a unique target for inhibitor strategies, as we show that their disruption simultaneously inhibits both activities >90%.

Aminoglycoside aminocyclitols are polycationic, broad-spectrum, bactericidal antibiotics that are used to treat a number of bacterial infections. The primary target of aminoglycosides is the prokaryotic 30S small ribosomal subunit. Specifically, they have been shown to bind to the 16S ribosomal RNA of the small ribosomal subunit (*1*−*4*). The 16S rRNA has been suggested to be involved in the proof-reading mechanism of protein translation, where upon a correct codon−anticodon match, the bases A1492 and A1493 "flip-out" to stabilize the interactions between the codon of the mRNA and anticodon of the tRNA (*5*). The ability of aminoglycosides to also displace these adenines that interact with codon−anticodon pairing is thought to increase the protein translation infidelity (*5*, *6*); the first step that eventually leads to cell death.

Bacteria may protect themselves in a variety of ways from the action of the aminoglycosides, including through the expression of efflux pumps and target modification by methylation of important ribosomal RNA bases (*7*). However, the most clinically important mode of resistance is through the modification of the antibiotics themselves, by acetylation, phosphorylation, or adenylation, catalyzed by aminoglycoside acetyltransferases (AAC),[1] phosphotransferases (APH), and adenyltransferases, respectively (*8*, *9*). Indeed, modification of aminoglycosides through acetylation and/or phosphorylation has been shown to substantially decrease the affinity of the antibiotic for 16S rRNA (*10*).

There are representative 3-D structures for all three classes of aminoglycoside resistance enzymes (*11*−*16*). These structural studies have demonstrated that the aminoglycoside acetyltransferases are similar to members of the large GCN-5 histone *N*-acetyltransferase (GNAT) related superfamily (*13*, *17*), and aminoglycoside phosphotransferases are structurally similar to Ser/Thr/Tyr protein kinases (*11*). The aminoglycoside modifying enzymes are also functionally similar to other family members, where both aminoglycoside acetyltransferases and phosphotransferases have been demonstrated to have protein/peptide modification capabilities (*14*, *18*). Enzyme kinetic and chemical mechanism studies have further validated the similarities between resistance enzymes and other family members (e.g., *9*, *18*−*24*, and references therein). These studies have been integral in both the design of aminoglycosides resistant to modification (e.g., *25*−*28*) and the identification of inhibitors of aminoglycoside resistance enzymes (*22*, *29*−*36*), which could overcome resistance and return efficacy to these clinically proven antibiotics.

The most important, yet most difficult, aminoglycoside resistance protein to overcome is arguably the bifunctional enzyme AAC(6′)-APH(2″), which possesses both acetyl CoA-dependent acetylation and ATP-dependent phosphorylation activities (*37*). The protein is the most common

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[1] Abbreviations: AAC, aminoglycoside antibiotic acetyltransferase; APH, aminoglycoside antibiotic phosphotransferase; GNAT, GCN-5 histone *N*-acetyltransferase related superfamily of proteins; MIC, minimal inhibitory concentration.

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determinant in Gram positive pathogens such as *Enterococcus* and *Staphylococcus*, where it confers incredibly high protection with minimum inhibitory concentrations over 2000 μg/mL, and has shown a growing importance in the resistance profiles of Gram negative organisms (*38*).

Initial sequence alignments first suggested, and gene truncation experiments later confirmed, that there are two distinct active sites, where the acetyltransferase and phosphotransferase activities reside in the N- and C-terminal portions of the protein, respectively (*39*, *40*). It has been suggested that this bifunctional enzyme arose from a gene fusion between an *aac* and an *aph* (*39*, *41*). Sequence analyses have shown that both the acetyltransferase and phosphotransferase domains have closely related monofunctional enzymes (*41*, *42*). Moreover, a closely related aminoglycoside acetyltransferase and a phosphotransferase have been detected together in both *Escherichia coli* and *Enterococcus*, where their genes were found to be only 40 nucleotides apart (*43*).

AAC(6′)-APH(2″) has been cloned and sequenced from isolates of *Enterococcus faecalis* (*39*) and *Staphylococcus aureus* (*40*), purified from a number of sources and characterized (*44*−*47*). Both catalytic domains have unique properties. The APH domain, for instance, has an unusually broad regiospecificity, being able to catalyze the phosphorylation of hydroxyls on four different aminoglycoside ring systems, and the AAC domain, although it has much stricter regiospecificity, has been shown to catalyze *O*-acetyltransfer, and is the only member of the large AAC(6′) subclass known to have this functionality (*46*, *47*). The protein is also capable of doubly modifying (6′-acetyl, 2″-phosphoryl) aminoglycosides (*47*). Thus, both the AAC and APH domains have broad substrate profiles for their class, and together, they detoxify nearly all known clinically relevant aminoglycosides.

Early kinetic studies have suggested that both activities operate through random BiBi sequential mechanisms, where the substrates of one activity do not impact the kinetics of the other activity (*44*). The details of each of the chemical mechanisms have also begun to be revealed: the active site base Asp99 is responsible for the unique abilities of the AAC domain (*22*), and the APH domain likely operates through a "dissociative" phosphoryl transfer mechanism similar to the well-studied aminoglycoside phosphotransferase APH(3′)-IIIa (*18*, *20*, *36*). From these studies and others, there are a number of inhibitors, including irreversible (*22*, *36*) and reversible modulators (*33*−*35*), that have been identified for both activities, although none of these simultaneously impact both activities and so, are insufficient to completely overcome resistance by themselves.

To complement the studies on the individual domains, it is imperative to also understand how the domains interact functionally and/or structurally. This may aid in the identification of compounds that can negatively affect both domains, and hence, simplify treatment of this resistance determinant. In the absence of a high-resolution structure of this enzyme, we have explored the domain−domain interactions in AAC(6′)-APH(2″) by further refining and characterizing the minimal domain sequences of the acetyltransferase and phosphotransferase activities in terms of both in vitro and in vivo activities. Our studies have suggested that although the domains do not functionally interact, they have intimate structural linkages that are important for conformation and stability, and disruption of these domain−domain contacts negatively impacts both activities.

EXPERIMENTAL PROCEDURES

Reagents. Kanamycin A and gentamicin were from Bioshop (Burlington, ON, Canada). Fortimicin and neamine were the gifts of Drs. J. Davies (University of British Columbia) and S. Mobashery (Wayne State University), respectively. 4,4′- Dithiodipyridine was from Amersham Pharmacia (Baie d'Urfe, PQ, Canada). DNA polymerases (Taq, Vent, T4), together with *Hind* III and *Pst* I restriction endonucleases were purchased from MBI Fermentas (Burlington, ON, Canada). Remaining restriction endonucleases were purchased from New England Biolabs (Mississauga, ON, Canada). Ni NTA agarose was purchased from Qiagen (Mississauga, ON, Canada), and all other chromatography resins were purchased from Amersham Pharmacia. All other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. All oligonucleotide primers were synthesized at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, ON, Canada.

Construction of Plasmids Expressing N-Terminal and C-Terminal Truncated Proteins. A list of plasmids used in this study can be found in Supporting Information, Table 1. The construction of pBF9 (*46*), pBF16, and pET22BFAPH (*36*) has been previously described. The construction of the other pKK223−3** (*46*) based plasmids, followed similar procedures, where sequences were amplified using Vent or Taq DNA polymerase from plasmid pBF9 using appropriate primers (Table 1) that incorporate unique *Nde* I and *Hind* III restriction sites and ligated into pK223−3**, following digestion of both DNA fragment and plasmid with *Nde* I and *Hind* III. pET15BFAAC and pET22BFAAC were constructed by ligating the *Nde* I-*Hind* III digested insert from pKKBFAAC into similarly digested pET15b(+) or pET22b(+) (Novagen, Madison, WI), respectively. The pKK223−3** and pET based plasmids were used to transform $CaCl_2$-competent *E. coli* XL1 Blue and BL21(DE3) cells, respectively.

Purification of Proteins. The purification of N-terminal hexahistidine tagged AAC(6′)-APH(2″) has been previously described (*36*), and mutant enzymes were purified similarly. N- and C-terminal truncated proteins were expressed using the pET15b(+) or pET22b(+) system, where 1−2 L of Luria Bertani (LB) broth supplemented with ampicillin (100 μg/mL) was inoculated with 10−20 mL of overnight culture and grown to an OD_{600} of 0.6 at 37 °C, prior to inducing with 10 mL of 100 mM isopropyl-β-D-thiogalactopyranoside. For non-His tagged N-terminal truncated protein, cells were grown for an additional 6 h at 30 °C following induction, whereas for the remaining truncated proteins, cells were grown for an additional 3 h at 37 °C. Cells were harvested by centrifugation at 10000*g*, washed with 0.85% NaCl, and stored at −20 °C. The N-terminal truncated His tagged protein was purified in a similar fashion as the full-length hexahistidine tagged protein (*36*).

The non-His tagged proteins were purified following a similar method used for *B. subtilis*-expressed, non-His-tagged

Table 1: Steady-State Kinetic Characterization of N- and C-Terminal Truncated Versions of AAC(6′)-APH(2″)

activity	substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	$(k_{cat})^{Full}/(k_{cat})^{Frag}$	$(k_{cat}/K_M)^{Full}/(k_{cat}/K_M)^{Frag}$
		N-Terminal 6-His Tagged AAC(6′)-APH(2″)				
acetyltransferase	amikacin	97.1 ± 14.9	0.27 ± 0.01	2.8×10^3		
	butirosin	60.9 ± 19.0	0.13 ± 0.01	2.1×10^3		
	fortimicin A[a]	25.4 ± 5.5	0.28 ± 0.04	1.1×10^4		
	kanamycin A[b]	31 ± 3	1.7 ± 0.2	5.6×10^4		
	ribostamycin	131 ± 25	0.37 ± 0.04	2.8×10^3		
	acetyl CoA[b]	38 ± 5	1.2 ± 0.3	3.1×10^4		
phosphotransferase	kanamycin A[b]	4.7 ± 0.5	0.32 ± 0.03	6.8×10^4		
	GTP	3.74 ± 0.47	0.13 ± 0.01	3.6×10^4		
		N-Terminal 6-His Tagged AAC[1−194]				
	amikacin	110 ± 29	0.01 ± 0.00	9.4×10^1	26	30
	butirosin	29.2 ± 12.0	0.002 ± 0.000	6.4×10^1	69	33
	fortimicin A	94.0 ± 5.7	0.20 ± 0.07	2.1×10^3	1.4	5.2
	kanamycin A	233 ± 45	0.15 ± 0.01	6.5×10^2	11	86
	ribostamycin	28.7 ± 7.7	0.01 ± 0.00	2.1×10^2	61	13
	acetyl CoA	247 ± 52	0.19 ± 0.02	7.6×10^2	6.4	41
		AAC[1−194]				
	amikacin	214 ± 21	0.06 ± 0.00	2.8×10^2	4.5	10
	butirosin	383 ± 47	0.01 ± 0.00	3.5×10^1	10	61
	fortimicin A	105 ± 39	1.5 ± 0.3	1.4×10^4	0.19	0.78
	kanamycin A	146 ± 16	0.38 ± 0.05	2.6×10^3	4.5	21
	ribostamycin	120 ± 30	0.04 ± 0.00	3.0×10^2	10	9.3
	acetyl CoA	74.4 ± 18.3	0.34 ± 0.04	4.6×10^3	3.5	6.7
APH[175−479]	kanamycin A[b]	6.0 ± 1.1	0.24 ± 0.01	4.0×10^4	1.3	1.7
	GTP	7.87 ± 1.32	0.08 ± 0.00	1.0×10^4	1.7	3.6

[a] Values taken from ref *22*. [b] Values taken from ref *36*.

AAC(6′)-APH(2″) according to ref *46*. After resuspending the cells in lysis buffer (50 mM HEPES-NaOH pH 7.5, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride), cells were lysed by three passes through a French pressure cell at 20 000 psi. The cell debris was removed by centrifugation at 10000*g* for 20 min. The lysate was applied to a Q sepharose FF anion exchange column (50 mL bed volume), washed with three column volumes of buffer A (50 mM HEPES-NaOH pH 7.5, 1 mM EDTA) and the proteins eluted with a gradient of buffer B (50 mM HEPES-NaOH pH 7.5, 1 mM EDTA, 1 M NaCl) which included the following steps: linear gradient from 0 to 20% buffer B over one column volume, held at 20% buffer B for one column, linear gradient of 20 to 50% buffer B over 4 column volumes, held at 50% buffer B for one column volume and a linear gradient of 50 to 100% buffer B over 1 column volume. Fractions were analyzed using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), where the proteins generally eluted between 200 and 500 mM NaCl. Fractions were pooled and concentrated to a final volume of 5 mL over an Amicon PM10 ultrafiltration membrane, before being applied to a Sepharose S200 gel filtration column (4 × 110 cm) and eluted with buffer A + 200 mM NaCl at a flow rate of 0.5 mL/min. Following analysis by SDS−PAGE, the proteins were concentrated over PM10 membrane and dialyzed against 25 mM HEPES-NaOH pH 7.5.

Enzyme Kinetic Assays. The phosphotransferase assay employed has previously been described (*36*, *48*). The assay measures the production of ADP/GDP, generated upon aminoglycoside phosphorylation, and couples this to the oxidation of β-NADH using the enzymes pyruvate kinase and lactate dehydrogenase. The rate of ADP/GDP production was determined by monitoring the decrease in absorbance at 340 nm. Aminoglycoside acetylation was monitored by the in situ titration of free coenzyme A product with 4,4′-dithiodipyridine as previously described (*36*, *46*). Initial rates were fit to the Michaelis−Menten eq 1 using Grafit 4.0 (*49*).

$$v = (k_{cat}/E_t)[S]/(K_m + [S]) \quad (1)$$

For measuring kinetic parameters for APH activity, kanamycin and GTP were fixed at 100 μM when measuring the steady-state kinetic parameters for GTP and aminoglycoside substrate, respectively. For the acetyltransferase assays, acetyl CoA and kanamycin A were held at 300 μM when measuring the steady-state kinetic parameters for aminoglycoside substrate and acetyl CoA, respectively.

Circular Dichroism. The CD spectra of 2−10 μM protein solutions in 10 mM Na_2HPO_4/NaH_2PO_4 pH 8.0 were measured using an AVIV 215 spectrophotometer at 25 °C and a 1-mm path length quartz cell. Secondary structure of the constructs was predicted using the program CDNN ver. 2.1 (*50*, *51*). The neural network was trained to an appropriate level with the following proteins: cytochrome C, hemoglobin, lactate dehydrogenase, myoglobin, ribonuclease, flavodoxin, subtilisin, triosephosphate isomerase, thermolysin, and hemerythrin.

Site Directed Mutagenesis. Site-directed mutagenesis was performed using the Quick-Change method (Stratagene, La Jolla, CA). The appropriate mutagenic primers and their reverse complements were used in combination with 20 ng of template DNA (pET15AACAPH (*36*)) in Pfu DNA polymerase (Stratagene, La Jolla, CA) catalyzed PCR reactions. After parental DNA was digested with *Dpn* I, mutant plasmid DNA was transformed into $CaCl_2$-competent *E. coli* XL-1 Blue. Positive clones were sequenced in their entirety, and then used to transform into *E. coli* BL21(DE3) for subsequent protein purification.

Thermal Inactivation Assays. Proteins (2−3 mg/mL) were incubated at 50 °C, in the presence or absence of substrate,

for various time intervals before being transferred to appropriate, thermally equilibrated (37 °C) assay buffer and assayed for acetyltransferase and/or phosphotransferase activities, according to the previously described procedures (*36*, *46*, *48*). Assays typically contained 2−10 ng of protein. Assays were initiated with the addition of both substrates. For AAC assays, kanamycin A and acetyl CoA had final concentrations of 300 μM, and for APH assays, kanamycin A and ATP had final concentrations of 100 and 2000 μM, respectively. Thermal inactivation of enzyme activity follows first-order kinetics and rates were analyzed accordingly using Grafit 4.0 (*49*).

Analytical Gel Filtration. Analytical gel filtration experiments were performed on an AKTA FPLC (Amersham Pharmacia) using a Superdex 200 column (10 × 30 mm, 24 mL bed volume) with 50 mM Tris-HCl pH 7.5, 100 mM KCl as elution buffer. The column was standardized using β-amylase (M_r = 200 000), alcohol dehydrogenase (150 000), bovine albumin (66 000), carbonic anhydrase (29 000), cytochrome C (12 400), and blue dextran for calculation of void volume, using appropriate concentrations (2−10 mg/mL, 100 μL injections) according to supplier instructions (Sigma MW-GF-200). Experimental protein samples (2−6 mg/mL, 100 μL injections) were run similarly, and the linear relationship between the logarithm of M_r and elution volume for the standard proteins was used to calculate the apparent M_r for the various AAC(6′)-APH(2″) constructs.

Construction of B. subtilis Integrants. Sequences were amplified using *Taq* DNA polymerase from plasmid pBF9 using appropriate primers (Table 1). The fragments were purified, treated with T4 DNA polymerase to ensure blunt ends, and cloned into pCR4-Blunt, according to the instructions supplied with the Zero Blunt TOPO PCR cloning kit (Invitrogen, Carlsbad, CA). The original primers generated *Pac* I and *Bam* HI restriction sites flanking the desired sequences, and so, were subsequently used to subclone the fragments into *Pac* I-*Bam* HI treated pSWEET-bgaB (*52*). Following transformation and selection into $CaCl_2$-competent *E. coli* XL1 Blue, the plasmids were purified, linearized by restriction digest with *Pst* I, and used to transform *B. subtilis* 1A717, according to previous procedures (*52*). *B. subtilis* 1A717 contains an erythromycin resistant cassette in place of the α-amylase gene locus, and so, positive integrants were initially selected by growth on LB agar containing chloramphenicol (10 μg/mL) and the absence of growth with erythromycin (1 μg/mL). Integrants were confirmed by PCR amplification of the appropriately sized inserts, and protein expression was determined through Western blot analysis as described below.

Minimum Inhibitory Concentration Determinations. Minimum inhibitory concentration (MIC) determinations for the *E. coli* constructs were performed as described in ref *53*. For the *B. subtilis* constructs, the procedure was modified to account for the more tightly regulated *xyl* promoter of the pSWEET system (*52*). A single colony from a LB agar plate supplemented with 10 μg/mL chloramphenicol was used to inoculate a culture of LB broth supplemented with 10 μg/mL chloramphenicol and 2% (w/v) xylose. This culture (1 μL) was added to a 96-well polypropylene microtiter plate containing LB broth supplemented with 2% (w/v) xylose along with increasing concentrations of antibiotic (99 μL) and incubated at 30 °C for 20−24 h. MICs were determined by visual inspection for bacterial growth. All of the MIC determinations were performed minimally in duplicate.

Quantification of Protein Expression Copy Number Using Western Blot Analyses. For *E. coli* constructs, LB broth (100 mL) supplemented with 100 μg/mL ampicillin was inoculated with 1 mL overnight culture and grown at 37 °C until OD_{600} ∼ 0.8. *B. subtilis* constructs were treated similarly, except that the LB broth was supplemented instead with 10 μg/mL chloramphenicol and 2% (w/v) xylose. For *E. faecalis* ATCC 49383, 100 mL of Bacto tryptic soy broth (Becton, Dickinson and Company, Reference Number 211825) supplemented with 200 or 2000 μg/mL gentamicin was inoculated with 2 mL overnight culture and grown at 37 °C until OD_{600} ∼ 0.8. Serial dilutions were plated onto LB agar plates supplemented with ampicillin (100 μg/mL), chloramphenicol (10 μg/mL), and kanamycin (50 μg/mL) for *E. coli*, *B. subtilis*, and *E. faecalis*, respectively, and grown at 37 °C for 16−24 h to determine colony-forming units (CFUs) in the original 100 mL cultures. Cells were collected at 10000*g* for 20 min and stored at −20 °C.

Cells were resuspended in 3−5 mL of lysis buffer and treated with mutanolysin (0.017 mg/mL) and lysozyme (1.7 mg/mL) for 2 h, prior to the addition of DNAase (1.7 mg/mL) and RNAase (1.7 mg/mL) and an additional hour of incubation at 22 °C. Cell lysis was completed by passing the solutions 4−5 times through a French pressure cell at 20 000 psi. Cellular debris was pelleted by centrifugation at 10 000*g* for 20 min, and the supernatant was dialyzed against 50 mM HEPES−NaOH pH 7.5 and stored at −20 °C.

Aliquots of supernatant and standard purified proteins (5−1000 ng) were analyzed by 15% SDS−PAGE and electroblotted onto polyvinylidene fluoride membrane (Millipore Immobilon-P transfer membrane) using standard methods (*54*). The Western blot was developed using rabbit polyclonal antibodies directed against AAC(6′)-APH(2″) and goat anti-rabbit antibodies conjugated with horseradish peroxidase (PerkinElmer) according to the protocol outlined by the supplier of the chemiluminescent detection system (Western Lightning Chemiluminesce Reagent Plus, PerkinElmer Life Sciences, Boston, MA). Blots were scanned into digital format with a Typhoon 9200 variable mode imager (Molecular Dynamics, Amersham Pharmacia Biotech) and the visualized bands were quantified using the computer program ImageQuant, supplied with the imager. A plot of pixel volume vs logarithm of protein amount for the known standard proteins gave a linear slope that was used to determine the amount of protein in the original sample solutions.

RESULTS

Construction of Minimum Acetyltransferase and Phosphotransferase Domains. Sequence analysis and initial truncation experiments of AAC(6′)-APH(2″) situated the acetyltransferase and phosphotransferase activities in the N- and C-terminal portions of the protein (*39*). Previously, in our attempts to purify AAC(6′)-APH(2″) from an *E. coli* overexpression host, we noted the synthesis of both full-length AAC(6′)-APH(2″) and a C-terminal truncated protein, starting at Met175, that possessed only aminoglycoside phosphotransferase activity (*46*).

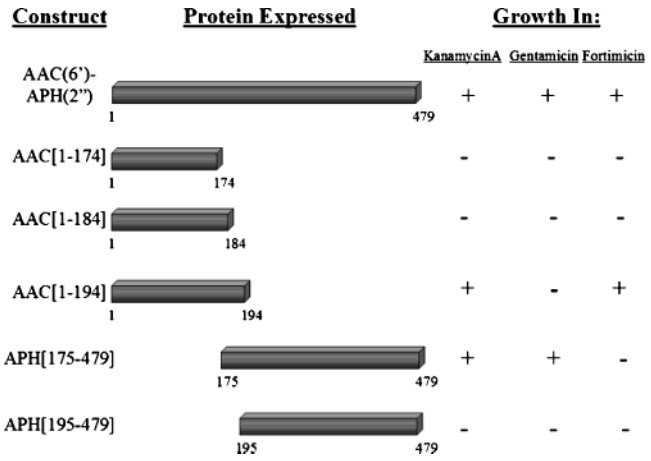

FIGURE 1: Antibiotic screening of *E. coli* XL1 Blue expressing N- and C-terminal truncated versions of AAC(6′)-APH(2″). Growth was assayed by inoculating 2 mL of Luria Bertani (LB) broth containing 10 μg/mL aminoglycoside antibiotic from a single colony on LB agar (100 μg/mL ampicillin) and incubating for 16−20 h at 37 °C.

To localize the minimum regions of both the acetyltransferase and phosphotransferase domains, we constructed additional plasmids expressing both N- and C-terminal truncated proteins (Figure 1). Following transformation into *E. coli* XL1 Blue, constructs were screened for growth in media containing aminoglycoside antibiotics, including fortimicin that is only inactivated by acetylation (Table 1). Only two constructs provided resistance to aminoglycoside, corresponding to the expression of amino acid residues 1−194 (hereafter designated as AAC[1−194]) and the originally identified C-terminal truncated protein, expressing amino acid residues 175−479 (designated as APH[175−479]). The AAC[1−194] fragment provided resistance to kanamycin A and fortimicin A but not to gentamicin, consistent with aminoglycoside 6′-acetyltransferase activity, whereas the APH[175−479] fragment provided resistance to kanamycin A and gentamicin but not to fortimicin A, consistent with aminoglycoside phosphotransferase activity. Constructs expressing amino acid residues 1−174 (designated as AAC[1−174]), 1−184, and 195−479 (designated as APH[195−479]) did not provide resistance to any of the aminoglycosides tested. This was not the result of a lack of protein expression, as SDS−PAGE analysis demonstrated similar protein levels as the resistance-providing constructs (data not shown).

Purification and Steady-State Kinetic Characterization of N- and C-Terminal Truncated Versions of AAC(6′)-APH(2″). To characterize the in vitro activity of the truncated gene constructs, the fragments were cloned into pET15b(+) and/or pET22b(+), expressed, and purified (Figure 2) according to Experimental Procedures. APH[175−479] has previously been shown to have similar steady-state kinetic parameters as the phosphotransferase activity of full-length AAC(6′)-APH(2″) (*36*) (Table 1). AAC[1−194], while still maintaining in vivo antibiotic resistance activity, is catalytically impaired compared to full-length enzyme (Table 1). There are significant steady-state effects for a number of aminoglycoside substrates and acetyl CoA, where the N-terminal hexahistidine tagged AAC[1−194] (hereafter referred to as His-AAC[1−194]) is more severely affected than nontagged AAC[1−194]. Neither addition of APH[175−479] and/or GTP into the acetyltransferase assay buffer had any affect on the kinetic parameters for AAC[1−174], His-AAC[1−194], or AAC[1−194] (data not shown).

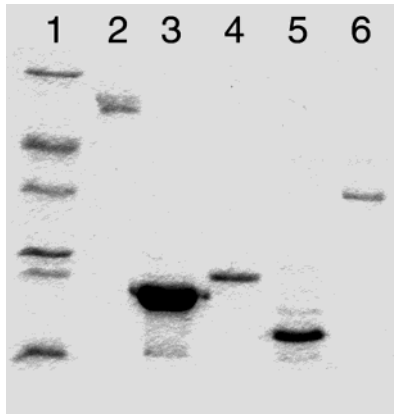

FIGURE 2: Purity of full-length and truncated versions of AAC(6′)-APH(2″) assessed by 15% SDS−PAGE. Proteins were visualized using Coomassie brilliant blue stain. Lanes are as follows: 1, standard molecular weight markers (66, 45, 36, 29, 24, 14.2 kDa), 2, N-terminal 6-His tagged AAC(6′)-APH(2″), 3, AAC[1−194], 4, N-terminal 6-His tagged AAC[1−194], 5, AAC[1−174], 6, APH[175−479].

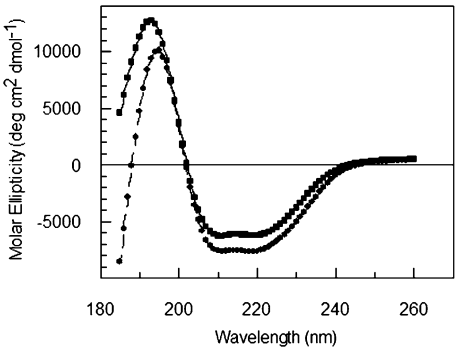

FIGURE 3: Secondary structure determinations for AAC[1−174] (●) and AAC[1−194] (■) using an AVIV 215 spectrophotometer at 25 °C and a 1-mm path length quartz cell with 10 μM solutions in 10 mM Na_2HPO_4/NaH_2PO_4 pH 8.0.

Secondary Structure of Full-Length and Truncated Versions of AAC(6′)-APH(2″). Circular dichroism spectra of purified full-length and truncated versions of AAC(6′)-APH(2″) were determined to assess their secondary structure and folded state. Although AAC[1−174] did not possess any measurable in vitro activity and it could not confer aminoglycoside resistance when expressed in *E. coli*, it still retained significant secondary structure (Figure 3). In particular, the CD spectrum displayed strong α-helical content, similar to AAC[1−194]. These results suggest that the lack of acetyltransferase activity in AAC[1−174] is not due to unfolding of the protein.

Secondary Structure Predictions for Region Spanning 175−204. Truncation experiments were inconsistent with a flexible loop adjoining the acetyltransferase and phosphotransferase domains of AAC(6′)-APH(2″). To determine if the region between the two domains may possess a regular secondary structure, the sequence between residues 175 and 204 was analyzed for potential α-helical or β-structure using in silico predictive software. All four programs used to analyze the sequence, PSIPRED (*55*, *56*), Prof (*57*), nnPredict (*58*), and jpred (*59*), predicted α-helical structure spanning residues 186−196.

Table 2: Steady-State Kinetic Parameters for His-AAC(6′)-APH(2″) "Helix" Mutants

mutation	substrate	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}s^{-1}$)	$(k_{cat})^{WT}/(k_{cat})^{Mut}$	$(k_{cat}/K_M)^{WT}/(k_{cat}/K_M)^{Mut}$
			Lys190Pro			
acetyltransferase	kanamycin A	46.1 ± 11.5	0.21 ± 0.07	4.7×10^3	7.9	12
	acetyl CoA	73.8 ± 18.4	0.13 ± 0.01	1.7×10^3	9.6	18
phosphotransferase	kanamycin A	3.28 ± 0.20	0.03 ± 0.00	8.4×10^3	5.9	1.1
	GTP	6.38 ± 2.82	0.03 ± 0.00	5.0×10^3	4.2	7.2
			Leu192Pro			
acetyltransferase	kanamycin A	36.8 ± 8.9	0.03 ± 0.00	6.7×10^2	68	83
	acetyl CoA	21.3 ± 5.5	0.01 ± 0.00	6.4×10^2	86	49
phosphotransferase	kanamycin A	0.4 ± 0.1	0.03 ± 0.00	7.4×10^4	5.3	0.12
	GTP	3.14 ± 1.31	0.03 ± 0.00	1.0×10^4	4.1	3.6
			Lys190Pro, Leu192Pro			
acetyltransferase	kanamycin A	36.5 ± 11.3	0.01 ± 0.00	3.4×10^2	142	166
	acetyl CoA	45.1 ± 4.8	0.01 ± 0.00	2.4×10^2	109	130
phosphotransferase	kanamycin A	2.66 ± 0.55	0.01 ± 0.00	4.5×10^3	13	2.0
	GTP	3.55 ± 0.69	0.01 ± 0.00	3.3×10^3	11	11

Mutational Analysis of Predicted α-Helix Connecting Acetyltransferase and Phosphotransferase Domains of AAC(6′)-APH(2″). To address the role of the predicted connecting α-helix in the structure and function of AAC(6′)-APH(2″), Lys190 and Leu192 in the middle of the predicted helix were mutated to Pro individually and together. These mutations had significant impacts on both the acetyltransferase and phosphotransferase activities of AAC(6′)-APH(2″) (Table 2). The acetyltransferase activity was more affected than the phosphotransferase activity, where mutation of Leu192 was more deleterious than mutation of Lys190. The kinetic effects were primarily on k_{cat} values, whereas the Michaelis constants, K_M, were very similar between WT and mutant proteins.

These results are not due to gross secondary structure changes (i.e., unfolding of the proteins) as the CD spectra of the mutant proteins were similar to wild-type His-AAC(6′)-APH(2″) (data not shown). Furthermore, analysis of the CD spectra by CDNN ver. 2.1 (*50*, *51*) yielded similar results between wild-type and "helix mutant" proteins (data not shown).

Effects of GTP on the Thermal Stability of the AAC and APH Domains. It has been previously noted that GTP can protect both the acetyltransferase and phosphotransferase activities of AAC(6′)-APH(2″) from thermal inactivation (*44*). We conducted additional thermal inactivation studies on full-length and truncated versions of His-AAC(6′)-APH(2″) to assess the stability of the individual domains in the absence and presence of the other domain and nucleotide (Figure 4A). Our results confirmed that GTP has a protective effect on both the AAC and APH activities of His-AAC(6′)-APH(2″) (Figure 4B). GTP can also protect APH[175−479] from thermal inactivation but not AAC[1−194] (Figure 4B). Addition of APH[175−479] into the thermal inactivation broth with AAC[1−194] did not confer any protection to the acetyltransferase activity in the absence or presence of GTP (data not shown). Moreover, the protection provided by GTP to the acetyltransferase activity of His-AAC(6′)-APH(2″) Lys190Pro, Leu192Pro decreased substantially compared to wild-type protein (Figure 4B).

Analytical Gel Filtration of Full-Length and Truncated Versions of AAC(6′)-APH(2″). To assess the oligomeric nature of full-length and truncated versions of AAC(6′)-APH(2″) and to determine whether the separated domains may interact with one another in vitro, the constructs alone and together were subjected to analytical gel filtration. His-AAC(6′)-APH(2″) and APH[175−479] elute as monomers, whereas AAC[1−174] and AAC[1−194] elute partially (10−30%) in dimeric form. After incubation of APH[175−479] with either AAC[1−174] or AAC[1−194], subsequent analysis failed to show any evidence of an association

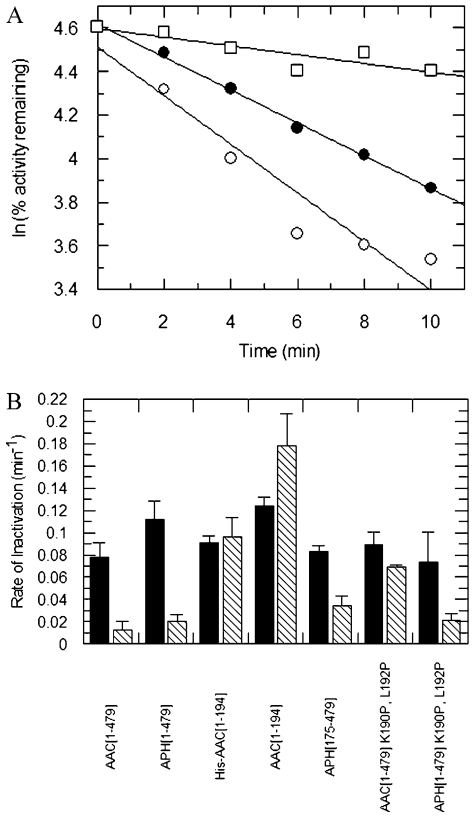

FIGURE 4: Thermal inactivation of acetyltransferase and phosphotransferase activities of full-length and truncated versions of AAC(6′)-APH(2″). Proteins were incubated at 50 °C for various time intervals before aliquots were transferred to thermally equilibrated 37 °C assay buffer to assess remaining acetyltransferase or phosphotransferase activity. (A) Thermal inactivation of the phosphotransferase activity of His-AAC(6′)-APH(2″) incubated with no nucleotide (○), 5 mM ATP (●), or 5 mM GTP (□). (B) The first-order decays in enzyme activity were used to determine rate constants of inactivation (solid, no nucleotide; hatched, with 5 mM GTP).

Table 3: Minimum Inhibitory Concentration Determinations for *E. coli* and *B. subtilis* Constructs Expressing Full-Length and Truncated Versions of AAC(6′)-APH(2″)

	minimum inhibitory concentration (μg/mL)							
	E. coli XL1 Blue				*B. subtilis*			
	AAC(6′)-APH(2″)	AAC [1−194]	APH [175−479]	control[a]	AAC(6′)-APH(2″)	AAC [1−194]	APH [175−479]	control[a]
amikacin	32	16	16	1	8	4	8	<0.25
butirosin	4	4	<2	2	1	1	1	<0.25
fortimicin A	160	80	<4	2	64	64	1	<0.25
gentamicin C	64	1	64	<0.25	16	0.5	16	<0.25
kanamycin A	500	125	250	2	64	16	32	<0.25
ribostamycin	128	128	4	4	8	8	2	0.5
tobramycin	128	8	64	0.5	16	2	8	<0.25

[a] Control for *E. coli* carried the plasmid p*K*K233−3**, and control for *B. subtilis* was *B. subtilis* 1A717.

Table 4: Quantification of Protein Expression in *B. subtilis* and *E. faecalis*

construct	protein copy/cell
B. subtilis	
w/ AAC(6′)-APH(2″)	1×10^4
w/ AAC[1−194]	2×10^4
w/ APH[175−479]	2×10^4
E. faecalis	
grown in 200 μg/mL gentamicin	3×10^4
grown in 2000 μg/mL gentamicin	3×10^4

between AAC and APH, although the dimers of AAC could no longer be detected.

Biological Assessment of Domain Interactions in Providing Protection to E. coli and B. subtilis. To assess the biological role that domain interactions in AAC(6′)-APH(2″) may play in antibiotic resistance, *B. subtilis* chromosomal integrants were constructed expressing AAC(6′)-APH(2″), AAC[1−194], APH[175−479], and AAC[1−174]+APH[175−479] using either the pSWEET (*52*) or the pDR67 system. The resistance level of the *B. subtilis* integrants and previous *E. coli* constructs were assessed through minimum inhibitory concentration (MIC) determinations (Table 3). Expression of both AAC[1−174] and APH[175−479] simultaneously in *B. subtilis* did not result in resistance to fortimicin A, an antibiotic exclusively detoxified by the acetyltransferase activity, suggesting that APH[175−479] did not have any biological impact on the activity of AAC[1−174] (data not shown). The MIC results also demonstrate that for some aminoglycosides, the acetyltransferase activity is most important for resistance (e.g., fortimicin), while in other cases, the phosphotransferase activity is most important (e.g., gentamicin), paralleling in vitro kinetic results. Finally, for the remaining antibiotics, both activities make significant contributions toward resistance (e.g., kanamycin A; Table 3) and these contributions appear to be additive.

Quantitative Western Analysis to Determine Expression Levels in B. subtilis and E. faecalis. The protein copy numbers of the resistance constructs were evaluated by quantitative Western analysis according to Experimental Procedures. The three *B. subtilis* constructs, expressing AAC(6′)-APH(2″), AAC[1−194], and APH[175−479], expressed protein to similar levels (Table 4), indicating that the MIC results are not biased by different expression levels.

The growth of *E. faecalis* in different amounts of aminoglycoside did not have an impact on protein expression (Table 4), demonstrating the constitutive nature of AAC(6′)-APH(2″) expression. This study also represents the first report of protein expression levels for an aminoglycoside resistance protein, an important consideration when developing strategies to overcome antibiotic resistance.

DISCUSSION

The enzyme AAC(6′)-APH(2″) is a critical aminoglycoside resistance determinant in Gram positive pathogens, including *Staphylococcus* and *Enterococcus*, where it is able to inactivate nearly all clinically relevant aminoglycoside antibiotics (*37*). It is a bifunctional enzyme, consisting of a N-terminal acetyltransferase and C-terminal phosphotransferase domains, and is thought to have arisen from a gene fusion event between an *aac* and an *aph* (*39*, *40*). The juxtaposition of two different aminoglycoside modifying activities may have important consequences in terms of antibiotic resistance. One hypothesis is that the ability of AAC(6′)-APH(2″) to doubly modify (6′-acetylation, 2″-phosphorylation) aminoglycosides leads to greater detoxification of the antibiotic and further decreases the host's susceptibility to the harmful effects of the antibiotic. Thus, a thorough understanding of the protein and its resistance patterns requires a determination of the importance of domain−domain interactions in enzyme function and how this relates to the phenotypic read-out of antibiotic resistance.

To begin analysis, we generated minimal protein segments that expressed either acetyltransferase or phosphotransferase activity. These protein fragments, AAC[1−194] and APH[175−479], were able to modify aminoglycosides in vitro and consequently, provide resistance to aminoglycoside substrates appropriate for each activity when expressed in *E. coli* and *B. subtilis*. The active fragments share a short peptide linkage in common, encompassing residues 175−194 that is predicted to be α-helical by in silico secondary structure prediction software. This region is not predicted to form part of either the acetyltransferase or the phosphotransferase active site (*39*, *40*) and is likely required for proper structure for both domains, involved in mediating and/or potentiating structural interactions between the two domains of AAC(6′)-APH(2″).

The AAC domain requires the APH domain for full activity, as it is catalytically impaired in vitro with a number of aminoglycosides when expressed as a N-terminal truncated protein. The sole exception is the aminoglycoside fortimicin A, where AAC[1−194] has a higher k_{cat}/K_M than does full-length protein. Fortimicin A has a significantly different

structure than do the other aminoglycosides tested and may bind in a different conformation so that it is more efficiently detoxified by the N-terminal truncated protein.

The thermostabilities of each domain are impacted by the presence of the other domain, especially in the presence of GTP. GTP protects both the AAC and APH activities from thermal inactivation, despite the fact that GTP is not a ligand for the AAC domain (*44*). The effect of GTP must be mediated through the APH to the AAC domain, considering that GTP does not protect AAC[1−194]. Moreover, when the predicted "linking helix" between the AAC and APH domains is disturbed, by mutation of Lys190 and Leu192 to Pro, GTP protection is diminished substantially.

The mutation of Lys190 and Leu192 to Pro also negatively affects the activities of both AAC and APH catalytic domains. In fact, these mutations had an even more drastic impact on the steady-state kinetic characteristics than the N- and C-terminal truncations of the proteins, further highlighting the importance of this "linking helix" to the structure and function of both domains. The side chain of Lys190 is not critical to APH function considering that mutation of this residue to Ala, in a previous study, had no effect on the activity of APH[175−479] (*36*). This residue is likely solvent exposed in the C-terminal truncated protein since it is susceptible to modification by wortmannin (*36*). In this case, the absence of the AAC domain would unmask the "linking helix" to solvent and potential covalent modifiers.

Thus, the AAC and APH domains make important interactions that are required for proper structure and thermostability; however, there is no evidence that there are functional interactions between the two domains (e.g., substrate channeling). The cofactors of either domain do not affect the kinetics of the other domain, and there is no absolute requirement for phosphorylation or acetylation of aminoglycoside prior to the modification catalyzed by the other activity (*44*, *46*, *47*). Furthermore, when expressed in either *E. coli* or *B. subtilis*, the fragments only give additive resistance, in terms of MIC, with respect to the full-length enzyme. This is consistent with the observation that 2″-phosphorylated, 6′-acetylated kanamycin A has similar affinity to a model 16S rRNA as does 2″-phosphorylated kanamycin A (*10*); that is, there is no added benefit in acetylation following phosphorylation of the antibiotic at least in terms of decreasing the thermodynamic interaction between drug and target. There may be other "kinetic" considerations such as the presence of bacterial sugar deacetylases and phosphatases that would inadvertently "retoxify" aminoglycosides and make dual modification of antibiotic advantageous for the bacteria.

The results with AAC(6′)-APH(2″) parallel the results seen with AAC(6′)-Im and APH(2″)-Ib, two monofunctional enzymes found expressed together in *E. faecalis* and *E. coli* (*43*). In this case, resistance was also additive when AAC(6′)-Im and APH(2″)-Ib were expressed together in *E. coli* (*43*), and not synergistic as one would expect if there were functional interactions between the two resistance enzymes. Instead, the presence of two different classes of modifying enzyme broadens the range of aminoglycosides that can be detoxified, and the absolute level of resistance to individual antibiotics is only impacted slightly.

These results do have important implications in inhibitor design strategies for AAC(6′)-APH(2″). The most commonly used aminoglycoside in the clinic is gentamicin, which is detoxified primarily through the APH activity, and thus, inhibitors against APH(2″) will be more critical for returning aminoglycoside efficacy. However, other clinically important aminoglycosides, such as amikacin and kanamycin A, are inactivated by both AAC and APH activities, and overcoming resistance to these antibiotics will require the design of inhibitors for both domains of AAC(6′)-APH(2″).

The best targets for inhibitors will be the active sites of the domains, but there is also a unique opportunity to screen for compounds that can disrupt the linking helix and negatively impact both activities simultaneously. Compounds are known that can interfere with the folded nature of proteins and/or affect the activity of an enzyme by binding to a distant site (i.e., allosteric inhibitors), including recent inhibitors of β-lactamase that bind 16 Å from the active site and act by forcing apart two structurally important helices (*60*). In general, prediction and design of these types of inhibitors are difficult; nonetheless, a compound that can interfere with the AAC−APH domain interactions would be able to overcome both resistance mechanisms simultaneously. From our studies, it can be predicted that disruption of the linking helix would inhibit the AAC and APH activities by over 99 and 92% respectively. Furthermore, targeting multiple sites on AAC(6′)-APH(2″) would make it more difficult for newer versions to evolve that do not respond to the inhibitors, where domain−domain contacts is a logical choice considering their importance to the structure and function of AAC(6′)-APH(2″). A crystal structure of AAC(6′)-APH(2″) will reveal the validity of this approach and further bring to light the intimate linkages between the domains of this important antibiotic resistance enzyme.

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SUPPORTING INFORMATION AVAILABLE

Table 1 contains a list of plasmids and primers used in the study. This information is available free of charge via the Internet at http://pubs.acs.org.

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